

# Nitrogen removal capacity of wetlands: sediment versus epiphytic biofilms

S. Bourgues \* and B. T. H. Hart.\*\*

\* Melbourne Water Corporation, GPO Box 4342, Melbourne Victoria 3001, Australia

(E-mail: [sophie.bourgues@melbournewater.com.au](mailto:sophie.bourgues@melbournewater.com.au))

\*\* Water Studies Centre, Monash University, Clayton Campus, Wellington Road, Clayton Victoria 3168, Australia

(E-mail: [barry.hart@sci.monash.edu.au](mailto:barry.hart@sci.monash.edu.au))

## Abstract

Wetlands are important sinks for nutrients and constructed wetlands are current practice for stormwater treatment. For nitrogen, the main removal process is denitrification (microbial reduction of nitrate to nitrogen gas). The bacteria responsible for this process are mostly found in the sediments and in epiphytic biofilms growing on wetland macrophytes. This paper reports on a project which aimed at measuring denitrification potential in sediments and epiphyton in urban wetlands. This study showed that wetland sediments could support high rates of denitrification. Interestingly, the most polluted of the wetlands studied had the highest denitrification potential. The management implication from this result is that indicators of pollution, such as hydrocarbon levels, will not necessarily reflect the ability of a wetland to denitrify. Two of the wetlands were studied in more detail. Here the denitrification potential of the epiphyton on dominant macrophytes and sediments were measured. The results indicated that the potential denitrification activity of the epiphyton was comparable to those measured in the sediments. Hence, biofilms could play a significant part in removing nitrogen loads. This work contributes to a better knowledge of the functioning of wetlands. This will lead to improved design and management of wetlands used for treating stormwater.

## Keywords

Wetland; denitrification; sediment; macrophyte; epiphytic biofilm

## INTRODUCTION

Wetlands are known to be very important nutrients sinks. Often, the efficiency of wetlands at removing nutrients is assessed by monitoring the quality of influent and effluent water. This “black box” approach is limited and does not provide information on the processes assumed to take place in these systems. For nitrogen, an important removal process is denitrification. Denitrification is the loss of nitrogen gas through the microbial reduction of nitrate to nitrogen. This process is controlled by the levels of oxygen, nitrate and organic matter (Seitzinger, 1990).

Denitrifying bacteria can be found in sedimentary environments, where their activity is strongly depending on nitrate supply. They can also be found in epiphytic biofilms among other bacterial populations and microalgae, embedded in a matrix of mucus and polysaccharides (Sand-Jensen & Revsbech, 1987; Freeman & Lock, 1995). Macrophytes, usually abundant in wetlands, offer ideal surfaces for the attachment and development of such biofilms (Wetzel, 1990).

The aim of the study was to survey urban wetlands around Melbourne to assess their potential denitrification activity. During the second part of the project, denitrifying potential activities measured in sediments as well as in epiphytic microbial communities attached to macrophytes were compared.

## METHODS

### Study Sites

Thirteen wetlands were selected within the Melbourne metropolitan area (**Figure 1**). The first part of the study was conducted on all wetlands but Hampton Park wetland. The second part was carried out on Ruffey and Hampton Park wetlands only. This project was funded by the NSW Environmental Trust and Melbourne Water Corporation.



**Figure 1:** Map showing the locations of the 13 wetlands selected in the Melbourne metropolitan area (source Melbourne Water Corporation).

### Survey of the denitrification ability of 12 urban wetlands

A survey of water and sediment quality was conducted on 14 November 2002 and 11 December 2002. For each wetland, one water sample was collected, filtered on-site through a 0.2 $\mu$ m pore size polycarbonate filter and stored frozen until concentrations of ammonium ( $\text{NH}_4^+$ ),  $\text{NO}_x$  ( $\text{NO}_3^-$  and  $\text{NO}_2^-$ ) and filterable reactive phosphorus (FRP) were determined using standard methods (APHA-AWWA-WPCF, 1998). Two sediment samples (top 5cm) were also collected at each wetland. Both were stored at 4°C prior to analysis. One sample was centrifuged to extract pore water, and the heavy metals concentrations (Cu, Ni, Pb, Zn, Fe, Mn) were determined according to standard methods (APHA-AWWA-WPCF, 1998). An aliquot of the pore water was filtered through a 0.45 $\mu$ m pore size filter to determine  $\text{NH}_4^+$  and FRP sediment pore water concentrations. The remaining fraction of the sediment was air dried and then analysed for TKN, TP and heavy metals (As, Cd, Cr, Cu, Ni, Pb, Zn, Fe). A second sediment sample was air dried and analysed for Total Petroleum Hydrocarbons (US EPA Methods) and Total Organic Carbon. All analyses were performed in NATA accredited laboratories.

A survey of denitrification potential activity was carried out using sediment sampled from all wetlands but Hampton Park between 5 February 2003 and 2 April 2003. Sediment samples were collected from each wetland using acrylic tubes (internal diameter 10.5cm). The top 2cm of each core (3 replicates) was sliced off on site, and returned to the laboratory where slurries (wet sediment to water ratio = 1:1; final volume of 60ml) were prepared with sediment and water collected from the same site. Potential denitrification activity was measured on the freshly made slurries using the acetylene blockage technique (Sorensen, 1978). This method is based on the inhibition of nitrous oxide reductase, the enzyme responsible for the conversion of  $\text{N}_2\text{O}$  to  $\text{N}_2$ .

Additions of nitrate were made to produce selected final concentrations. Vials with no addition of nitrate contained endogenous nitrate from the wetland water used in the preparation of the slurry. The headspace of each vial was flushed for a few minutes under  $\text{N}_2$  to remove oxygen. Experiments

were initiated by replacing 10% of the headspace gas with acetylene. Treatments were performed in triplicate. The slurries in the blanks were spiked with a solution of trichloroacetic acid (final concentration 5%) in order to kill bacterial populations. A single blank vial was prepared for each treatment. All vials were placed on a rotating table and incubated at room temperature in the dark for 4 hours. The headspace of each vial was sampled after 30, 60 and 120 minutes incubation. Prior being sampled, the vials were vigorously shaken in order to equilibrate the N<sub>2</sub>O concentrations in the slurry and gas phases. Gas samples were analysed for N<sub>2</sub>O using a gas chromatograph (Hewlett Packard 5710A) equipped with a <sup>63</sup>Ni electron capture detector (nitrogen as a carrier gas at 30ml min<sup>-1</sup> flow rate, oven temperature 50°C, detector temperature 300°C, components separated on a 80/100 Poropak Q column). Aqueous N<sub>2</sub>O concentrations were back calculated from the headspace concentrations using Henry's law. Rates of potential denitrification were calculated from the regression equation determined by the plot [N<sub>2</sub>O concentration versus incubation time].

### **Comparison of epiphytic biofilms and sediments ability to denitrify**

Macrophyte samples were collected on 8 August 2004 for Hampton Park and 15 August 2004 for Ruffey wetland. The core collection occurred on 11 August for Hampton Park and on 23 August 2004 for Ruffey wetland. A total of 5 colonised macrophytes were selected on each occasion, with 3 samples per plant tested for denitrification potential activity. The sampling consisted of filling a hollow plastic tube (2.5cm internal diameter) with colonized shoots. The entrapped shoots were then cut to obtain a final length of shoot of approximately 6cm. This method was chosen in order to get comparable volume samples (approx. 30ml) between sediment and macrophyte shoot for the latter assessment of their respective denitrification capacities. For comparison, five sediment cores (internal diameter 10.5cm) were sampled in the vicinity of the macrophytes, and 3 samples were retrieved from each core, using the same hollow plastic tube described previously as a mini core.

Potential denitrification activity was measured as already described above on the freshly made mixtures of (1) macrophyte shoots (30ml) + water from the site (30ml) and (2) sediment (30ml) + water from the site (30ml).

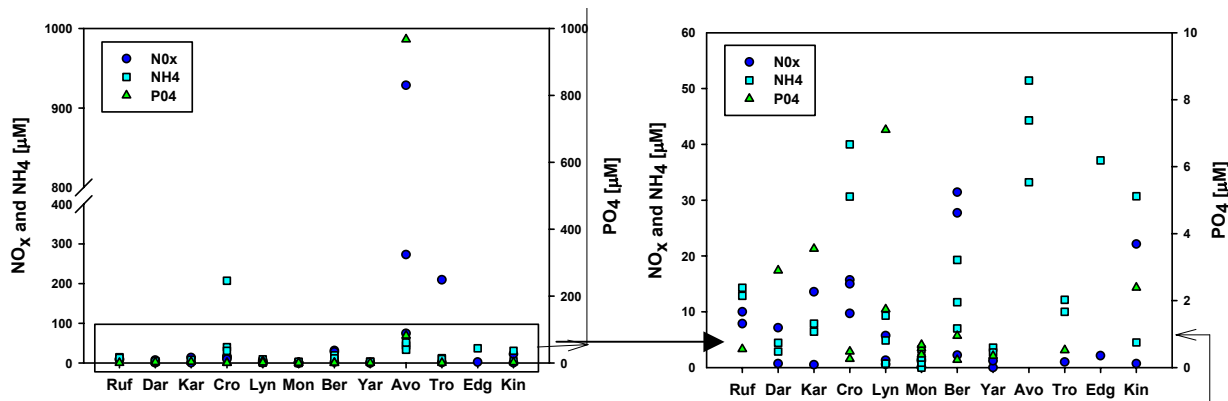
Data were analysed using the statistical package SYSTAT. Normal distribution of the data was visually checked using density histograms. For all analyses of variance (ANOVA), significance was accepted at  $p < 0.05$ . The differences in denitrification potential of epiphytic biofilm and sediment in both wetlands were tested on transformed data ( $\sqrt{\text{data}}$ ) using a two-way nested ANOVA, with Wetland (2 levels) as a fixed main factor, Substrate (sediment or macrophyte) as a fixed main factor (2 levels) and Sample (core of sediment or shoots) as nested within the combination of Wetland and Substrate. Because of the significant difference in denitrification potential rates, one-way ANOVAs were subsequently run on data for each wetland separately to test the effect of substrate.

## **RESULTS AND DISCUSSION**

### **Survey of the denitrification ability of 12 urban wetlands**

Water column NH<sub>4</sub><sup>+</sup> and NO<sub>x</sub> concentrations measured in the 12 wetlands are reported in **Figure 2**. Nutrient concentrations were quite variable over the study period. Generally, they ranged from not detectable to 51µM for NH<sub>4</sub><sup>+</sup> and 31µM for NO<sub>x</sub>, and to not detectable to approximately 7µM for filterable reactive phosphorus (FRP). Avoca retention basin was the exception, with very high NO<sub>x</sub> concentrations that ranged from 74 to 929µM. This wetland also exhibited very high concentrations of FRP. This site also had the lowest recorded oxygen concentration (1.85mg.l<sup>-1</sup> – data not shown).

Ammonium and FRP were also measured in the pore water of sediment samples from each wetland (**Table 1**). Highest concentrations of FRP were recorded for Avoca wetland, similar to the water column results. The pore water  $\text{NH}_4^+$  concentration for this wetland was very high as well ( $2230\mu\text{M}$ ) compared to the other sites, but the highest concentration was recorded in the sediment of Croydon wetland ( $2320\mu\text{M}$ ). Edgars wetland also exhibited very high levels of  $\text{NH}_4^+$  and FRP.



**Figure 2.** Nutrient concentrations [ $\mu\text{M}$ ] measured in the water column. A few outliers were recorded and have been plotted on the second graph for clarity (note the different scales).

Total metal concentrations were also measured in the same sediment pore water samples (**Table 1**). Nickel concentrations were in general lower than  $0.01\text{mg/l}$ , except for Troups wetland ( $0.012\text{mg/l}$ ) and Avoca retention basin where a concentration of  $0.081\text{mg/l}$  was measured. Lead concentrations ranged from  $0.004\text{mg/l}$  (Monteray wetland) to  $0.25\text{mg/l}$  in the sediment pore waters of Avoca. Avoca wetland also exhibited the highest concentrations of copper ( $0.169\text{mg/l}$ ) and zinc ( $6.18\text{mg/l}$ ). Manganese concentrations ranged from  $0.054\text{mg/l}$  (Monteray) to  $1.27\text{mg/l}$  (Berwick). Finally, iron concentrations ranged from  $8.31\text{mg/l}$  for Ruffey to  $101\text{mg/l}$  at Croydon basin.

**Table 1.** Nutrient [ $\mu\text{M}$ ] and total metal [ $\text{mg/l}$ ] concentrations in the sediment pore water samples.

Site	$\text{NH}_4^+$	FRP	Fe	Cu	Mn	Ni	Pb	Zn
Ruf	552	34	8.31	0.010	0.107	0.002	0.006	0.190
Dar	161	5.5	23.9	0.014	0.076	0.007	0.011	2.000
Kar	219	2.3	9.56	0.016	0.196	0.004	0.024	0.318
Cro	2320	25	101	0.016	0.401	0.003	0.025	0.700
Lyn	104	0.3	18.8	<0.001	0.958	0.004	0.006	0.010
Mon	286	45	11.7	0.006	0.054	<0.001	0.004	0.160
Ber	179	2.3	10.6	0.005	1.270	0.007	0.008	0.021
Yar	268	1.0	86.6	0.017	0.277	0.007	0.021	0.170
Avo	2230	231	20.7	0.169	0.582	0.081	0.25	6.180
Tro	189	28	36.4	0.022	0.452	0.012	0.014	0.164
Edg	1110	173	25.3	0.016	0.275	0.004	0.013	0.730
Kin	256	111	55.6	0.008	1.240	0.004	0.005	0.180

Metals concentrations (As, Cd, Cr, Cu, Ni, Pb, Zn, Fe) and TKN and TP measured in the same sediment samples are shown **Table 2**. Sediments represent a significant reservoir of nitrogen and phosphorus. Highest TKN concentration was recorded for Monterey sediment with a value of  $5,810\text{mg/kg}$  and the lowest was measured at Lynbrook ( $925\text{mg/kg}$ ). TP concentrations ranged from 164 to  $2,510\text{mg/kg}$ , for Troups and Avoca sediment, respectively.

Total metal concentrations (**Table 2**) can be compared with the range of values given by ANZECC guidelines (2000). For arsenic, all concentrations measured were below the lowest published trigger value, with a maximum of  $9.1\text{mg/kg}$  recorded at Monterey. It was a similar situation for cadmium and chromium, with all concentrations measured below the range of trigger values. Concentrations

of nickel for Ruffey, Darebin, Edgars and Kinterbury were within the range given by ANZECC guidelines, they were lower than 21mg/kg for all other wetlands. Copper concentrations were all lower than 270mg/kg, with the highest measured at Ruffey (224mg/kg). Some very high concentrations of zinc were measured in several wetland sediments compared to the range given by ANZECC guidelines. For instance, Ruffey exhibited a concentration of 1,480mg/kg, Edgars sediments exhibited a concentration of 1,870mg/kg, which represented the maximum recorded. Finally, iron concentrations ranged from 2,930mg/kg at Karkarook to 40,300mg/kg at Ruffey.

**Table 2.** Total metal concentrations [mg/kg], TKN and TP measured in the wetland sediments.

Site	As	Cd	Cr	Cu	Fe	Ni	Pb	Zn	TKN [mg/kg]	TP [mg/kg]
Ruf	6.4	1.2	36.1	224	40300	25.3	188	1480	3950	1120
Dar	1	0.7	38.4	30.3	10200	28.8	27.4	794	3850	971
Kar	1.7	<0.5	8	17.3	2930	5.2	34.4	367	2060	432
Cro	4.5	0.5	13.6	41	6850	7.3	57.1	396	5570	1270
Lyn	1.2	<0.5	8.9	2	7340	4	5.0	5.4	925	172
Mon	9.1	0.8	24.9	81.2	9780	12.7	79.7	933	5810	1650
Ber	<0.5	<0.5	7.5	3	4140	2.9	5.6	5.2	1110	200
Yar	1	0.2	12.8	8.1	6880	4.8	18.9	40.3	2460	518
Avo	2.8	0.8	25	75.8	7290	13.5	105	921	2880	2510
Tro	0.6	<0.5	9.1	8.1	4850	4.8	7.6	46.8	1040	164
Edg	2.5	4.3	36.2	150	11000	35.2	147	1870	4440	1270
Kin	3.9	0.7	33.9	52.4	35500	29.3	66.3	548	2300	1120

Very high levels of hydrocarbons were measured in Avoca, Edgars and Monterey sediments (**Table 3**). The heavier fractions (C<sub>15</sub>-C<sub>28</sub> and C<sub>29</sub>-C<sub>36</sub>) were the dominant forms detected as they are more refractory to degradation and persist longer in the environment. Berwick, Darebin, Karkarook, Lynbrook and Troups wetlands did not show any hydrocarbons pollution, with all fractions measured below the method detection limits. Total Organic Carbon (TOC) measured in the sediments ranged from 1% for Berwick wetland to 8.1% at Avoca wetland (**Table 3**).

**Table 3.** Total Petroleum Hydrocarbons [mg/kg], TOC [%] and Moisture content [%] measured in the wetland sediments.

Site	Total Petroleum Hydrocarbons [mg/kg] Air dried samples					
	TOC [%]	Moist. [%]	Fraction C6-C9	Fraction C10-C14	Fraction C15-C28	Fraction C29-C36
Ruf	3.8	56.1	<4	30	250	562
Dar	1.8	41.6	<2	<25	<50	<50
Kar	5.9	75.8	<5	<25	<50	<50
Cro	5.3	56.9	<4	32	286	492
Lyn	1.5	47.1	<2	<25	<50	<50
Mon	4.6	67.2	<4	40	1050	1460
Ber	1.0	30.5	<2	<25	<50	<50
Yar	3.6	70.9	<4	<25	54	224
Avo	8.1	71.9	14	344	2120	1250
Tro	3.5	65.8	<4	<25	<50	<50
Edg	4.2	63.4	<4	68	1290	1440
Kin	4.1	70	<4	<25	398	698

Avoca wetland was very different from the other wetlands studied. It contained the highest nutrient concentrations and lowest level of oxygen. In addition, Avoca exhibited high levels of nutrients in the pore water of its sediments. Finally, this wetland revealed a high level of hydrocarbon pollution. It is difficult to draw any general trend for the remaining wetlands. Sediment analyses revealed high

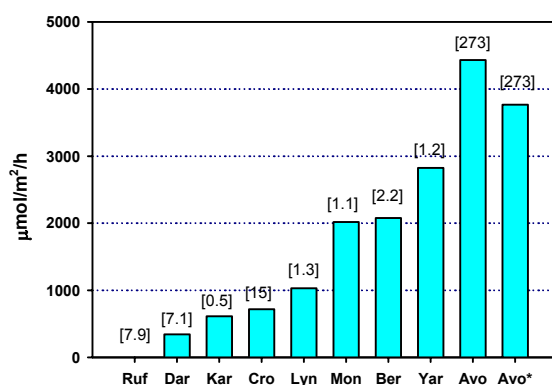
levels of hydrocarbons for Monterey and Edgars wetland. This latter site, as well as Ruffey, exhibited a high level in zinc. Otherwise the wetlands were relatively unpolluted.

The potential denitrification activities for the studied wetlands are reported in **Figure 3**. Figures in brackets are the  $\text{NO}_x$  concentrations measured in the water column of each wetland at the time of the sampling. These concentrations were in general lower than  $10\mu\text{M}$ , except in Avoca wetland where  $\text{NO}_x$  concentration was  $273\mu\text{M}$ . To avoid any nitrate limitation for the denitrifying bacteria, all vials were spiked with a solution of nitrate to obtain a final concentration of  $50\mu\text{M}$  at the beginning of all incubations. Rates are therefore normalised for all wetland sediments with regard to nitrate availability, with the exception of Avoca where  $\text{NO}_x$  was already much higher than  $50\mu\text{M}$ .

Denitrification activity recorded for Avoca wetland with and without the spike are reported in the same figure; the second rate (Avo\*) corresponds to the activity measured in the vials which did not receive any extra nitrate. The rate with additional nitrate was  $4,430\mu\text{mol N/m}^2/\text{h}$  against  $3,770\mu\text{mol N/m}^2/\text{h}$  measured in the vials without any additional nitrate. For all other wetland sediments, no activity was recorded without the nitrate spike of  $50\mu\text{M}$ .

Potential denitrification activity measured for the sediment from Troups wetland is reported separately because it was determined with  $500\mu\text{M}$  spike of nitrate. The water quality survey undertaken earlier showed that  $\text{NO}_x$  concentration in the water column of this wetland could be very high ( $209\mu\text{M}$ , **Figure 2**). The potential activity measured was very high ( $27,700\mu\text{mol N/m}^2/\text{h}$ ), but cannot be compared directly with the other measurements for which all sediments received the same nitrate supply. Unfortunately, it was impossible to repeat the experiment because Troups wetland remained dry during the study period. Also, no activity was measured for Kinterbury and Edgars wetlands because of technical problems.

Overall, there was a wide range of potential denitrification. No activity was detected in Ruffey sediments (samples taken from the inlet cell) even when supplied with nitrate, while Avoca sediments had the highest activity ( $4,430\mu\text{mol N/m}^2/\text{h}$ ). This latter result was at first a surprise because the general survey showed that Avoca pore waters and sediments contained high metal and hydrocarbon levels (**Tables 1, 2 & 3**). However, numerous denitrifiers are able to metabolise a range of aromatic compounds, including toluene, xylene, phenols, cresols, phthalate, cyclohexanol, benzoate and other aromatic acids, alcohols and aldehydes (Zumft, 1997), and for this reason, despite the high levels of pollution, this wetland obviously still had viable denitrifier populations. As a comparison, potential denitrification rates in sediments from the Yarra and Goulburn Rivers, both located in Victoria, ranged from 100 to  $500\mu\text{mol N/m}^2/\text{h}$  (Bourgues, unpublished data), and in Port Phillip Bay rates of approx.  $110\mu\text{mol N/m}^2/\text{h}^{-1}$  have been reported (Heggie *et al.*, 1999).



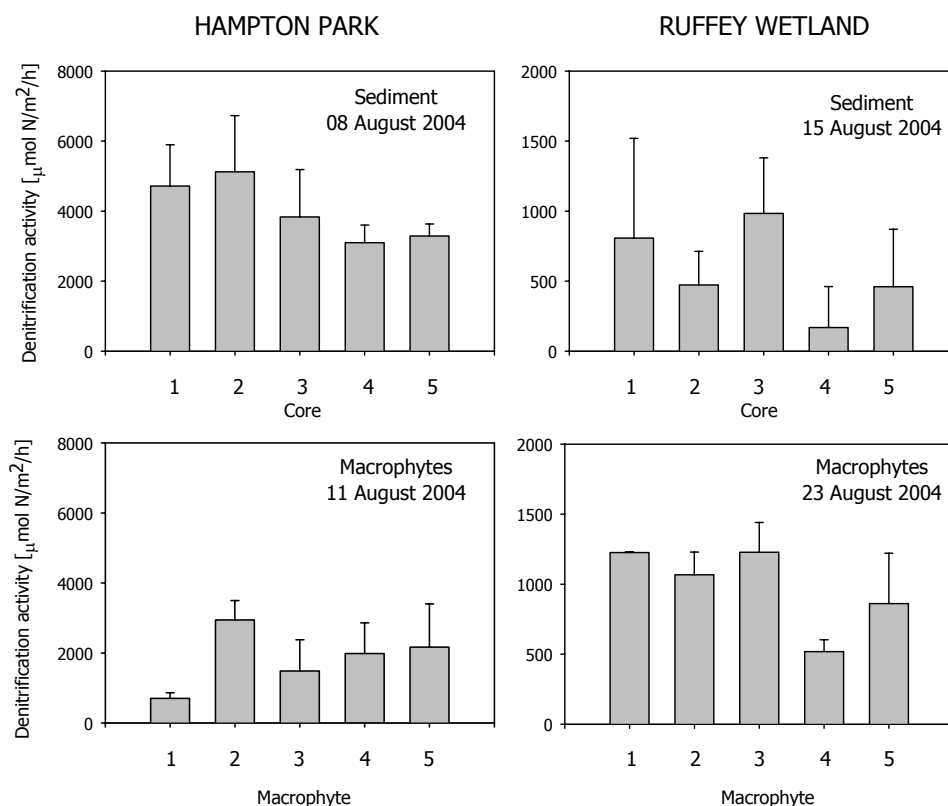
**Figure 3.** Denitrification potential expressed in  $\mu\text{mol N denitrified/m}^2/\text{h}$ . Figures in brackets give the ambient  $\text{NO}_x$  concentration in  $\mu\text{M}$  at the sampling time.

### Comparison of epiphytic biofilms and sediments ability to denitrify

Denitrification potential activities measured for sediment samples and biofilm samples in both wetlands are given in **Figure 4**. It is interesting to note that potential activity was measurable in the sediment sampled from the “macrophyte cell” of Ruffey wetland. The first part of the study revealed that no activity was measured in the inlet cell of the same wetland.

There was a significant difference in denitrification activities among wetlands ( $p < 0.001$ ; **Table 4**) and among substrate ( $p < 0.05$ ; **Table 4**). However, the interaction between these two factors was also significant, which implies that the differences among wetlands were not constant at all levels of the second factor considered. The significant variation among samples within a substrate in a given wetland translates the high degree of field heterogeneity ( $p < 0.05$ ; **Table 4**). Overall, activities for the sediments ranged from  $4010 \pm 880$  to  $580 \pm 320 \mu\text{mol N/m}^2/\text{h}$  for Hampton Park and Ruffey respectively. Potential activities for the macrophyte biofilms ranged from  $1860 \pm 830$  to  $980 \pm 300 \mu\text{mol N/m}^2/\text{h}$  for Hampton and Ruffey wetlands, respectively. The total activity (sediment + macrophyte biofilm) was higher in Hampton Park wetland.

The one-way ANOVA performed on Hampton Park data alone revealed that denitrification potential activities were higher for the sediments than for the macrophyte samples ( $p < 0.005$ ). On the contrary, denitrification potentials were higher for the biofilms growing on the macrophytes than for the sediments in Ruffey wetland (one-way ANOVA;  $p < 0.05$ ).



**Figure 4.** Denitrification potential activities ( $\mu\text{mol N/m}^2/\text{h}$ ) measured for the sediment (5 replicate cores with 3 samples per core) and the biofilm growing on macrophytes (5 macrophytes with 3 samples per macrophyte). Sampling dates are indicated in the graphs.

**Table 4.** Two-way nested ANOVA, with Wetland (2 levels) as a fixed main factor, Substrate (sediment or macrophyte) as a fixed main factor (2 levels) and Sample (core of sediment or macrophyte) as nested within the combination of Wetland and Substrate.

Factor	df	MS	p
Wetland	1	10065.837	***
Substrate	1	403.198	*
Wetland X Substrate	1	3691.191	***
Sample(Substrate(Wetland))	16	185.045	*
Error	38	93.090	

## CONCLUSIONS

With a supply of nitrate, low oxygen levels and appropriate redox conditions, high rates of denitrification were measured in urban wetland sediments. The exception was Ruffey wetland where no activity was observed in the inlet zone. However, denitrifying activity was measured in sediments sampled from the macrophyte cell of the same wetland. This would indicate that cells comprising constructed wetlands are not identical in terms of denitrification ability. Interestingly, despite the fact that Avoca was the most polluted wetland, it had the highest potential to denitrify. This finding suggests that traditional pollution indicators do not necessarily reflect the ability of the wetland to remove nitrogen. In addition, this study has shown that the biofilms growing on the surfaces of macrophytes shelter bacterial populations able to potentially carry denitrification at comparable rates than those measured in adjacent sediments, emphasizing the importance of plants in designing wetland to treat nutrient rich stormwater.

## REFERENCES

- ANZECC & ARMCANZ. (2000). Australian guidelines for water quality monitoring and reporting. National Water Quality Management Strategy, Australian and New Zealand Environment and Conservation Council & Agriculture and Resource Management Council of Australia and New Zealand, Canberra
- APHA-AWWA-WPCF. (1998). Standard methods for the examination of water and wastewater, 20<sup>th</sup> edition, American Public Health Association, Washington
- Freeman C. & Lock M. A. (1995). The biofilm polysaccharide matrix: a buffer against changing organic supply. *Limnology and Oceanography* **40**: 273-278
- Heggie, D. T., Skyring, G. W., Orchard, J., Longmore, A. R., Nicholson, G. J., and Berelson, W. M. (1999). Denitrification and denitrifying efficiencies in sediments of Port Phillip Bay: direct determinations of biogenic N<sub>2</sub> and N-metabolite fluxes with implications for water quality. *Marine and Freshwater Research* **50**: 589-596
- Sand-Jensen K. & Revsbech N. P. (1987). Photosynthesis and light adaptation in epiphytic-macrophyte associations measured by oxygen microelectrodes. *Limnology and Oceanography* **32**: 452-457
- Seitzinger S. P. (1990). Denitrification in aquatic sediments. In: Revsbech N. P. and Sorensen J. (Ed.), *Denitrification in soil and sediment*, pp301-322
- Sorensen, J. (1978). Denitrification rates in a marine sediment as measured by the acetylene inhibition technique. *Applied and Environmental Microbiology* **36**: 139-143
- Wetzel R. G. (1990). Land-water interfaces: metabolic and limnological regulators. *Verhandlungen der Internationale Vereinigung fur Theoretische und Angewandte Limnologie* **24**: 6-24
- Zumft W. G. (1997). Cell biology and molecular basis of denitrification. *Microbiology and Molecular Biology Reviews* **61**: 533-616