Aquat Ecol (2008) 42:95–101
DOI 10.1007/s10452-007-9085-3

ORIGINAL PAPER

# Analysis of denitrifying communities in streams from an urban and non-urban catchment

**Shane E. Perryman · Gavin N. Rees · Christopher J. Walsh**

Received: 13 April 2006 / Accepted: 19 February 2007 / Published online: 13 March 2007


**Abstract** Urbanization leads to degradation in water quality and has a major effect on the biota of streams, but its effect on microbial communities is not as well understood. DNA-techniques that target functional genes are being used to examine microbial communities, but less frequently applied to freshwater aquatic systems. Our aim was to determine whether terminal restriction fragment length polymorphism and sequence analysis of polymerase chain reaction-amplified (PCR) *nosZ* gene sequences could be used to show if there were measurable differences in the denitrifying community in two urban streams in catchments with contrasting degrees of catchment urbanization. Community structure in the sediments and associated riparian zones were studied at the contrasting sites. We showed that the denitrifying community in the sediments and riparian soils of the two streams were significantly different. There were also significant differences between the sediment and riparian zone communities within each of the sites. Terminal restriction fragment length polymorphism analysis proved to be a valuable technique that could resolve patterns of the denitrifying community in streams of contrasting degrees of urbanization, but sequence analysis was required to confirm the identity of the amplified products.

**Keywords** Community structure · Denitrification · *nosZ* · Sediment bacteria · T-RFLP

S. E. Perryman · G. N. Rees (✉)
Murray-Darling Freshwater Research Centre,
P.O. Box 991, Wodonga, VIC 3689, Australia
e-mail: Gavin.Rees@csiro.au

S. E. Perryman
Water Studies Centre and School of Chemistry,
Monash University, Clayton, VIC 3800, Australia

C. J. Walsh
Water Studies Centre and School of Biological
Sciences, Monash University, Clayton, VIC 3800,
Australia

## Introduction

Urbanization has had a major impact on ecosystem structures and functions (Grimm et al. 2000). Large areas of impervious surfaces and high levels of hydraulic connection of impervious surfaces to streams, through stormwater pipes or drains, are two characteristics of urban environments that can lead to major changes in urban streams (Walsh et al. 2005b). These characteristics cause changes in streams through the combined effects of decreased levels of evapotranspiration and infiltration and rapid delivery of waters to streams.

Increased delivery of nitrogen to rivers and streams is a major consequence of human

activities in landscapes (Seitzinger 1988; Peierls et al. 1991), however, aspects of nitrogen delivery and metabolism in urban systems have only recently been examined (Zhu et al. 2004; Groffman et al. 2005). Nitrate is a key nutrient in urban aquatic systems (Groffman et al. 2005; Grimm et al. 2005), introduced either through direct input of nitrate to streams, or produced within streams through nitrification. Subsequent denitrification can occur in sediments or biofilms, where anoxic conditions result in a switch from aerobic respiration to denitrification, thus removing nitrogen from the system. The critical role played by riparian zones in nitrogen cycling is well recognized (Hedin et al. 1998; Martin et al. 1999; Bardgett et al. 2001) and similarly, urban riparian zones may also be an important site for the transformation of nitrogen compounds.

The advent of DNA-based techniques has lead to significant advances in our understanding of nitrogen transformations. Terminal restriction fragment length polymorphism (T-RFLP) is one such DNA-based technique (Avaniss-Aghajani et al. 1994) and has been used to examine community structure by targetting genes coding for denitrifying enzymes (Braker et al. 1998; Scala and Kerkhof 1998, 2000; Prieme et al. 2002; Rösch et al. 2002). Relatively few studies have examined denitrifying communities in freshwater aquatic ecosystems, particularly urban streams, leading to our limited understanding of the microbial dynamics in such systems.

To address this knowledge gap, we targeted a segment of the *nosZ* gene and tested whether two different DNA-techniques would be able to resolve any potential differences in the denitrifying community of two urban streams. To this end, we selected two streams identified in earlier studies as representing the extremities of an urban gradient (Hatt et al. 2004; Taylor et al. 2004; Walsh et al. 2005a). We tested whether T-RFLP and sequence analysis of cloned DNA fragments could demonstrate whether denitrifying communities differed between the urban and non-urbanized streams. In addition, we compared the communities between sediments and riparian zones within the sites.

## Materials and methods

Brushy Creek is located in the outer suburbs of Melbourne, South Eastern Australia (37°46.90′, 145°18.30′), approximately 30 km east of the city centre. Brushy Creek is a highly modified incised stream with a clay bed, over which patchy deposits of gravel, sand, silt and debris collect. The 1,479 ha catchment upstream of the sampling location was 22% impervious, dominated by low to medium density housing (Hatt et al. 2004; Taylor et al. 2004). Of the total impervious surface, 89% was directly connected to Brushy Creek by piped municipal drains.

Lyrebird Creek rises in the Dandenong Ranges 37 km to the east of Melbourne (37°49.82′, 145°23.82′). The sampling site was primarily within a messmate (*Eucalyptus obliqua*) forest, with some Manna Gums (*E. viminalis*) and an understorey of ferns (*Blechnum* sp.) and tree ferns (*Dicksonia* sp.) close to the stream. The stream sediment consisted of sand and silt deposits over a stream bed dominated by cobbles. The 724 ha catchment upstream of the sampling location has 0.1% impervious area not directly connected to the stream (Hatt et al. 2004; Taylor et al. 2004).

The average water quality in each stream was derived from a data set of water quality variables, obtained on a fortnightly basis from the year prior, up to and including the day that samples were collected for microbial community analysis (Table 1). These data represent a subset of an extensive dataset published elsewhere and reflect the degree of urbanization in the two study sites (Hatt et al. 2004; Taylor et al. 2004).

Eight samples from the top 5–10 cm of sediment were collected along an ~20 m section of each stream during September 2002 and transferred to pre-weighed 50 ml centrifuge tubes. Eight samples from the adjacent riparian areas were collected from the top 5–10 cm of the soil profile. For our purpose, we defined riparian soil as un-inundated soil or sediment at the time of sampling (at base flow) at distances ranging from ~30 cm to ~10 m from the stream edge. Samples were frozen for later DNA analysis.

Detailed descriptions of the general methods for DNA extraction and T-RFLP procedures

**Table 1** Average annual water nutrient concentrations and benthic chlorophyll-*a* concentrations in Brushy and Lyrebird Creeks in the year preceding microbial community analysis

| Parameter[a] | Brushy Creek | | | Lyrebird Creek | | |
|---|---|---|---|---|---|---|
| | Mean | SD | *n* | Mean | SD | *n* |
| TN (mg $l^{-1}$) | 2.5 | 1.5 | 190 | 1.0 | 0.4 | 66 |
| $NO_x$ (mg $l^{-1}$) | 0.65 | 0.36 | 198 | 0.37 | 0.12 | 64 |
| $NH_4^+$ (mg $l^{-1}$) | 0.08 | 0.10 | 198 | 0.02 | 0.02 | 63 |
| TP (mg $l^{-1}$) | 0.33 | 0.27 | 190 | 0.05 | 0.03 | 66 |
| FRP (mg $l^{-1}$) | 0.03 | 0.02 | 198 | 0.004 | 0.002 | 64 |
| TOC (mg $l^{-1}$) | 11 | 4 | 5 | 11 | 4 | 14 |
| DOC (mg $l^{-1}$) | 7.2 | 2.9 | 23 | 4.2 | 2.1 | 50 |
| EC (μS $cm^{-1}$) | 408 | 238 | 160 | 84.7 | 7.7 | 71 |
| TSS (mg $l^{-1}$) | 208 | 237 | 188 | 55 | 32 | 72 |
| Benthic chlorophyll-*a* (mg $m^{-2}$) | 92 | 200 | 145 | 8 | 13 | 147 |

[a]TN—Total Nitrogen, $NO_x$—Nitrogen Oxides, $NH_4^+$—Ammonium, TP—Total Phosphorus, FRP—Free Reactive Phosphorus, TOC—Total Organic Carbon, DOC—Dissolved Organic Carbon, EC—Electrical Conductivity, TSS—Total Suspended Solids

have been described previously (Rees et al. 2004, 2006). Polymerase chain reaction (PCR) amplification of an approximately 780 base pair fragment of the *nosZ* gene was carried out in a final volume of 50 μl, using the HotStar *Taq*™ DNA polymerase kit and master mix described by the manufacturer (Qiagen, Clifton Hill, Australia), with the exception that 2 units of *Taq* polymerase were used per reaction. The forward primer *nosZ*f (5′-CGYTGTTCMTCGACAGCCAG-3′) and reverse primer *nosZ*-R (5′-CATGTGCA GNGCRTGGCAGAA-3′) were used for the PCR (Rösch et al. 2002). For T-RFLP analysis, the forward primer was labelled at the 5′ terminus with 6-carboxyfluorescein. Unlabelled forward primer was used for PCRs destined for sequence analysis.

PCR was carried out on a BioRad iCycler thermal cycler (Regents Park, Australia) with the following conditions: 95°C for 15 min to activate the HotStar*Taq*, denaturation at 94°C for 30 s, 35 cycles starting with a touchdown procedure beginning at 58°C for 40 s, decreasing in 0.5°C steps for 9 cycles until a final annealing temperature of 54°C, and extension at 72°C for 1 min 20 s—final extension was 72°C for 7 min. DNA extracted from *Pseudomonas denitrificans* (NCTC 10688) was used as the positive control and amplicon size was confirmed by agarose gel electrophoresis. Amplified samples were purified using the MoBio UltraClean™ PCR clean-up kit following the manufacturer's instructions (MoBio Laboratories, Solena, CA, USA).

A TOPO TA cloning kit (Invitrogen Life Technologies, CA, USA) was used to clone PCR products from *nosZ* PCRs into chemically competent, One Shot TOP10 *Escherichia coli* cells, using the manufacturer's instructions. Colonies that grew well on LB agar plate containing 100 μg $ml^{-1}$ ampicillin were picked and PCR was used to screen clones for the correct sized insert. Clones containing an insert of the correct size were re-amplified using the primers supplied with the TOPO TA kit, purified and sequenced at the Australian Genomic Research Facility (Queensland, Australia).

Terminal restriction fragment length polymorphism data sets were analyzed as described previously (Rees et al. 2006). Raw data sets require a standardization procedure prior to statistical analysis (Dunbar et al. 2001; Sait et al. 2003) and accordingly, we adopted a constant percentage standardization (Sait et al. 2003; Rees et al. 2004), rejecting peaks that made up less than 0.25% of the total fluorescence in each sample. Similarity matrices were calculated using the Bray–Curtis coefficient based on the presence or absence of terminal fragment lengths. Differences between streams (Brushy and Lyrebird) and sediment/soil types (sediment and riparian) were tested using a 2-way crossed analysis of similarity (ANOSIM) (Clarke and Warwick 2001). Non-metric multi-dimensional scaling (MDS) plots with 20 random restarts were used for visual interpretation of community patterns (Clarke and Warwick 2001).

Sequences from the clone library were analyzed using the freeware program *MEGA* version 3.0 (Kumar et al. 2004). Sequences were submitted to the National Centre for Biotechnology Information online BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm sequence identity. Sequences confirmed to be

*nosZ* were aligned with sequences from named organisms retrieved from the NCBI database and a ~760 bp segment common to all sequences was used for phylogenetic analysis, using the neighbour joining algorithm within the default settings of the *MEGA* software. The *nosZ* sequences obtained in this study have been submitted to GenBank under the accession numbers DQ324382 to DQ 324421.

## Results

Cloned PCR products, ~780 base pairs (bp), were obtained from all the soil and sediments samples of the two study sites. Of the 50 cloned fragments that were sequenced, 40 were recognized as *nosZ* gene sequences and were used for phylogenetic analysis (Fig. 1). The *nosZ* clones were distributed across three broad clusters; 11 clones grouped

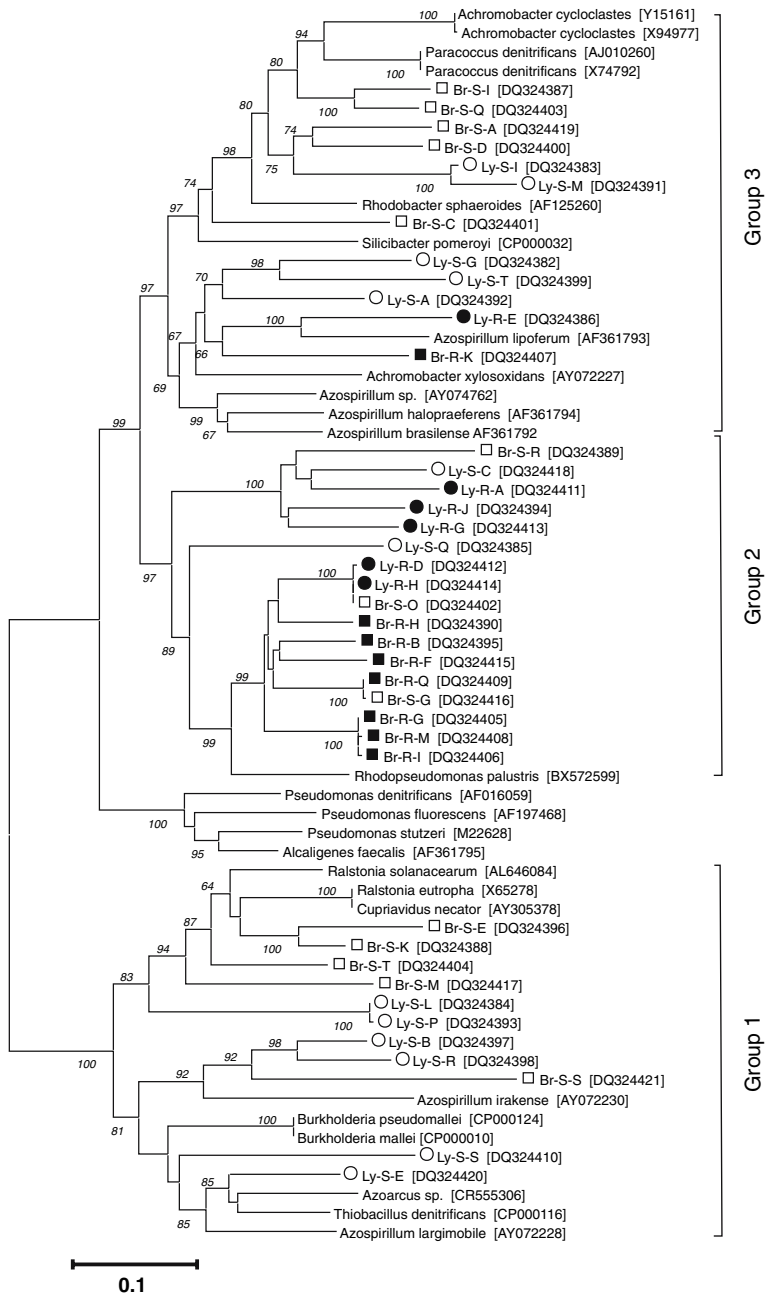


**Fig. 1** Neighbour-joining tree for *nosZ*. Clones are indicated by their site (Br = Brushy Creek, Ly = Lyrebird Creek) and the sample type (S = sediment, R = riparian soil. The scale represents 10% nucleotide change and numbers at the nodes indicate bootstrap re-sampling based on 750 replicates

within cluster 1, 17 clones in cluster 2 and 12 clones in cluster 3. Clones from both sites were present in cluster 1, but all the clones were derived from sediment samples. Six of the clones in cluster 1 aligned most closely to the *Cupriavidus* group. Of the 17 clones in cluster 2, 12 were derived from riparian soil and only 5 from sediments. Cluster 2 clones were also most dissimilar from other known (i.e. named) *nosZ* sequences available in databanks. In cluster 3, 10 clones were derived from sediment samples and only 2 from riparian soil. Five of the clones in cluster 3 could be assigned to the *Azospirillum/Achromobacter xylosoxidans* group and the reminder clustered within the taxonomically diverse group represented by *Paracoccus denitrificans* and *Silicibacter pomeroyi*.

A total of 114 terminal restriction fragments (T-RFs) were derived over all but one Lyrebird Creek sediment sample. Our normalizing manipulations of the T-RFLP raw data gave approximately 15–20 T-RFs for each sample. The MDS ordination (Fig. 2) shows good separation of the denitrifying community groups from the two streams and the two zones within each site (sediment and riparian soil). Notably, Brushy Creek samples appear on the right-hand side of the ordination, with sediments generally in the upper half and riparian soils in the lower half. Lyrebird Creek samples are predominantly on the left-hand side of the ordination, and like Brushy samples, the sediments generally are in the upper half of the ordination and riparian soils in the lower half of the ordination.

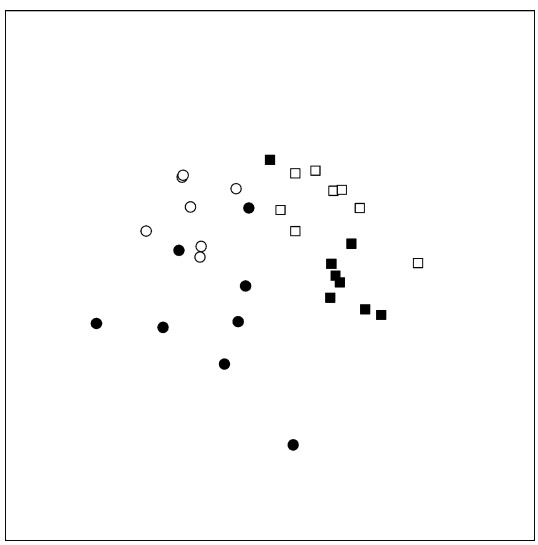

**Fig. 2** Non-metric multi-dimensional scaling (MDS) plot of *nosZ* gene community structure, derived by terminal restriction fragment length polymorphisms, in Brushy Creek (*squares*), Lyrebird Creek (*circles*), sediments (*open symbols*) and riparian soil (*closed symbols*). Two Lyrebird sediment points are superimposed. The stress for this plot is 0.21

The denitrifying communities at the Brushy Creek and Lyrebird Creek sites were significantly different (ANOSIM, $R = 0.516$, $P < 0.001$). There were also significant differences between the riparian and sediment habitat types ($R = 0.754$, $P < 0.001$). Within sites, the difference between the sediment and riparian soil habitats was greater at Brushy Creek ($R = 0.648$, $P < 0.005$) than at Lyrebird Creek ($R = 0.378$, $P < 0.005$). Interestingly, the difference between riparian soil samples ($R = 0.625$, $P < 0.005$) was not as large as the difference between sediment samples ($R = 0.931$, $P < 0.005$).

The average percent (±SD) of sediment greater than 1 mm at Brushy and Lyrebird creeks were 23 (10)% and 6 (5)%, respectively. The average (±SD) of the sediment less than 355 μm were 17 (18)% and 58 (10)% at Brushy and Lyrebird, respectively. Mean (±SD) loss on ignition at Brushy and Lyrebird were 3(1)% and 8(2)%, respectively.

## Discussion

Terminal restriction fragment length polymorphism and sequence analysis of clone fragments showed that the denitrifying community structure was measurably different in two urban streams with contrasting degrees of urban impact, at the time of sampling. Differences were also shown between the sediment and adjacent riparian areas within a given site. Both clone and T-RFLP analyses provided different insights into the denitrifying communities. First, sequence analysis of *nosZ* clones provided an important validation of our PCR reaction. It also showed that a diverse range of organisms with *nosZ* genes was distributed across the two sediments and soils. Although clone analysis is proving to be a very

useful approach to examining denitrifying microbes, the limited number of sequences from known, or described organisms, reduces our capacity to make inferences on the phylogenetic aspects or distribution of *nosZ* genes in nature. T-RFLP analysis provided a rapid and powerful approach to examining *nosZ* community structure, showing separation of communities from different sites, and between sediments and riparian soils within each site. Importantly, the T-RFLP analysis in this study was not free from experimental problems, as a small percentage of the amplified products were not from the *nosZ* gene. Further method development and validation will be required before T-RFLP analysis with *nosZ* fragments can be carried out in the absence of clone library and sequence analysis.

Although this study showed differences between sites (urban and non-urban) and within sites (stream sediment and riparian soil), the scope was limited to a single sampling time and as such, only limited interpretation into the ecological implications can be made on the effects of urbanization on streams. Urbanized streams do have markedly modified inundation patterns and constant ''resetting'' of the sediment due to hydrologic disturbance (flash flooding) in urban streams will have a significant influence on sediments (Walsh et al. 2005b). The difference in particle size classes in the two creeks suggest that Brushy Creek experiences such disturbance, with larger heavier particles remaining in the channel and smaller material transported downstream. Organic material may also be removed from the sediment during higher flows. These factors are likely to impinge on the sediment microbe communities.

The difference in denitrifying communities between sites paralleled a range of water quality variables previously shown to be significantly correlated with measures or indices of urbanization (Hatt et al. 2004; Taylor et al. 2004). The average concentrations of DOC were significantly different at the two sites. Community compositions of aquatic microbes have been shown to change in response to increasing DOC concentrations (Eiler et al. 2003) and enrichment with selected carbon sources has a strong structuring effect on soil bacterial communities (Wawrik et al. 2005). However, relationships between water column DOC and sediment denitrifying communities are not presently known.

Distinct differences in the *nosZ* communities of sediment, riparian and agricultural soils have also been noted previoulsy (Rich and Myrold 2004). However, it is not known whether the factors driving such differences in agricultural soil environments are the same as in urban environments. Vegetation density and type will certainly play a role and should be investigated in future studies.

Evidence is now gathering on the importance of denitrification in urban streams (Groffman et al. 2005). We have shown that T-RFLP, following further refinement, could be a powerful tool in the study of the effects of urbanization on denitrifying communities. Sequence analysis is still required to confirm the identities of the PCR-amplified *nosZ* gene fragments.

**Acknowledgements** The Cooperative Research Centre for Freshwater Ecology funded this project and also postgraduate funding to SP. We thank Dr M. Grace for useful discussions on the project, S. Hanafi for assistance with field work and the Australian Genomic Research Facility (Melbourne, Australia) for carrying out the final T-RFLP analysis.

## References

Avaniss-Aghajani E, Jones K, Chapman D, Brunk C (1994) A molecular technique for identification of bacteria using small subunit ribosomal RNA sequences. BioTechniques 17:143–149

Bardgett RD, Anderson JM, Behan-Pelletier V, Brussaard L, Coleman DC, Ettema C, Moldenke A, Schimel JP, Wall DH (2001) The influence of soil biodiversity on hydrological pathways and the transfer of materials between terrestrial and aquatic ecosystems. Ecosystems 4:421–429

Braker G, Fesefeldt A, Witzel K-P (1998) Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. Appl Environ Microbiol 64:3769–3775

Clarke KR, Warwick RM (2001) Change in marine communities: an approach to statistical analysis and interpretation. Primer-E Ltd, UK

Dunbar J, Ticknor LO, Kuske CR (2001) Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. Appl Environ Microbiol 67:190–197

Eiler A, Langenheder S, Bertilsson S, Tranvik LJ (2003) Heterotrophic bacterial growth efficiency and com-

munity structure at different natural organic carbon concentrations. Appl Environ Microbiol 69:3701–3709
Grimm NB, Grove JM, Pickett STA, Redman CL (2000) Integrated approaches to long-term studies of urban ecological systems. BioScience 50:571–584
Grimm NB, Sheibley RW, Crenshaw CL, Dahm CN, Roach WJ, Zeglin LH (2005) N retention and transformation in urban streams. J N Am Benthol Soc 24:626–642
Groffman PM, Dorsey AM, Mayer PM (2005) N processing within geomorphic structures in urban streams. J N Am Benthol Soc 24:613–625
Hatt BE, Fletcher TD, Walsh CJ, Taylor SL (2004) The influence of urban density and drainage infrastructure on the concentrations and loads of pollutants in small streams. Environ Manage 34:112–124
Hedin LO, von Fischer JC, Ostrom NE, Kennedy BP, Brown MG, Robertson GP (1998) Thermodynamic constraints on nitrogen transformations and other biogeochemical processes at soil-stream interfaces. Ecology 79:684–703
Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetic analysis and sequence alignment. Brief Bioinformatics 5:150–163
Martin TL, Kaushik NK, Trevors JT, Whiteley HR (1999) Review: denitrification in temperate climate riparian zones. Water Air Soil Pollut 111:171–186
Peierls BL, Caraco NF, Pace ML, Cole JJ (1991) Human influence on river nitrogen. Nature 350:386–387
Prieme A, Braker G, Tiedje JM (2002) Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. Appl Environ Microbiol 68:1893–1900
Rees GN, Baldwin DS, Watson GO, Perryman S, Nielsen DL (2004) Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics. Antonie van Leeuwenhoek 86:339–347
Rees GN, Watson GO, Baldwin DS, Mitchell AM (2006) Variability in sediment microbial communities in a semipermanent stream: impact of drought. J N Am Benthol Soc 25:370–378
Rich JJ, Myrold DD (2004) Community composition and activities of denitrifying bacteria from adjacent agricultural soil, riparian soil, and creek sediment in Oregon, USA. Soil Biol Biochem 36:1431–1441
Rösch C, Mergel A, Bothe H (2002) Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. Appl Environ Microbiol 68:3818–3829
Sait L, Galic M, Strugnell RA, Janssen PH (2003) Secretory antibodies do not affect the composition of the bacterial microbiota in the terminal ileum of 10-week-old mice. Appl Environ Microbiol 69:2100–2109
Scala DJ, Kerkhof LJ (1998) Nitrous oxide reductase (*nosZ*) gene-specific PCR primers for detection of denitrifiers and three *nosZ* genes from marine sediments. FEMS Microbiol Lett 162:61–68
Scala DJ, Kerkhof LJ (2000) Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis. Appl Environ Microbiol 66:1980–1986
Seitzinger SP (1988) Denitrification in freshwater and coastal marine systems: ecological and geochemical significance. Limnol Oceanogr 33:702–724
Taylor SL, Roberts SC, Walsh CJ, Hatt BE (2004) Catchment urbanisation and increased benthic algal biomass in streams: linking mechanisms to management. Freshw Biol 49:835–851
Walsh CJ, Fletcher TD, Ladson AR (2005a) Stream restoration in urban catchments through redesigning stormwater systems: looking to the catchment to save the stream. J N Am Benthol Soc 24:690–705
Walsh CJ, Roy AH, Feminella JW, Cottingham PD, Groffman PM, Morgan RP III (2005b) The urban stream syndrome; current knowledge and the search for a cure. J N Am Benthol Soc 24:706–723
Wawrik B, Kerkhof L, Kukor J, Zylstra G (2005) Effect of different carbon sources on community composition of bacterial enrichments from soil. Appl Environ Microbiol 71:6776–6783
Zhu W-X, Dillard N, Grimm NB (2004) Urban nitrogen biogeochemistry: status and processes in green retention basins. Biogeochemistry 71:177–196