Assessing the Effect of Wastewater Treatment Plant Effluent on Microbial Function and Community Structure in the Sediment of a Freshwater Stream with Variable Seasonal Flow

Steven A. Wakelin,1* Matt J. Colloff, 2 and Rai S. Kookana1

CSIRO Land and Water, PMB 2, Glen Osmond, SA 5064, Adelaide,1 and
CSIRO Entomology, GPO BOX 1700, Canberra, ACT 2601, Australia2

*Corresponding author. Mailing address: CSIRO Land and Water, Environmental Biogeochemistry, PMB 2, Glen Osmond, SA 5064, Australia. Phone 61 8 83038708.

E-mail: Steven.Wakelin@csiro.au.
ABSTRACT

We investigated the effects of wastewater treatment plant (WWTP) discharge on the ecology of bacterial communities in the sediment of a small, low gradient stream in South Australia. Quantification of genes involved in the biogeochemical cycling of carbon and nitrogen were used to assess potential impacts on ecosystem functions. Effects of disturbance on bacterial community structure was assessed by PCR-DGGE of 16S rRNA genes, and clone library analysis used to phylogenetically characterise significant shifts. Significant ($P<0.05$) shifts in bacterial community structures were associated with alteration of sediment physicochemical properties, in particular nutrient loading from the WWTP discharge. Effects were greatest at a sampling location 400m downstream of the outfall where the stream flow is reduced. This highly-affected stretch of sediment contained representatives of γ-proteobacteria absent from less disturbed sites, including Oceanospirales and Methyllococcaceae. 16S rRNA gene sequences from less disturbed sites had representatives of Caulobacteraceae, Sphingomonadaceae and Nitrospirae which were not represented in samples from disturbed sediment. Diversity was lowest at the reference site, increased with proximity to the WWTP outfall and declined towards highly disturbed (400m downstream) sites ($P<0.05$). The potential for biological transformations of N significantly varied with stream sediment location ($P<0.05$). The abundance of amoA, $nairG$, and $nifH$ genes increased with distance downstream of the outfall. These processes are driven by N and C availability as well as redox conditions. Together these data suggest cause-and-effect between nutrient loading into the creek, shift in bacterial communities through habitat change, and alteration of capacity for biogeochemical cycling of N.
INTRODUCTION

Few rivers in Australia are considered close to being in ‘pristine’ condition and the trend over recent years has been towards increasing degradation (1, 30). Factors that have contributed towards declining ecological condition include chemical pollution, salinity, acid mine drainage, sediment loading, alteration of flow (input, extraction or regulation), loss or alteration of riparian zones, and nutrient enrichment (eutrophication). Such stresses in combination can have synergistic negative impacts on ecological communities and functions.

Discharge of effluent from wastewater treatment plants (WWTP) has major detrimental effects on the health of aquatic ecosystems. WWTP outfall can deposit large amounts of organic matter (OM) and nutrients into receiving waterways. Increased nutrient loading can lead to eutrophication (20) and temporary oxygen deficits (35). Increased OM can alter energy relationships in the stream, disrupting biotic community structure and function (17, 20, 40). Effluent discharge can also deposit sand and grit into aquatic systems, affecting sediment physical characteristics. The discharge itself can perturb the natural flow regime, particularly when it enters waterways during periods of low natural flow. In this regard, Brooks et al. (3) highlighted the considerable biomonitoring challenges to scientists and water resource managers of effluent-receiving streams with ephemeral or seasonally-variable flow. Assessing the effects of effluent discharge on the health of receiving waterways is of considerable environmental consequence, especially in catchments with variable stream flows and where population pressure through urbanisation and peri-urbanisation is placing increasing pressure upon WWTP infrastructure and the health of freshwater ecosystems (15, 39, 43).

Although assessments of river health are widely based on the use of macroinvertebrates as indicators (6, 51), bacteria and other microorganisms may also be informative of the condition of aquatic ecosystems. Important attributes not only include their sessile habit in sediments and biofilms and continued exposure to the water column, but also their ubiquitous presence and high abundance in aquatic systems (27). Most importantly, bacteria are responsible for biogeochemical transformations such as nitrification and denitrification, and thus the impacts of stress and disturbance upon microbial communities can have implications for ecosystem functions and processes as well as biodiversity and aquatic community structure. However, much research on microbial bioindicators in aquatic systems has been
limited to heterotrophic bacteria in relation to the decomposition of dissolved organic matter (47) and as a measure of sewage pollution (19, 29).

Previous limitations towards using microorganisms as indicators of river health have largely been superseded by recent advances in molecular biology and the development of a new suite of tools and techniques that are revolutionising environmental microbiology and microbial ecology (22, 38, 53). For example, culture-independent techniques are providing new insights into the phylogenetic structure of microbiological communities (23, 46). Similarly, methods for the detection of microorganisms based on conserved functional genes, i.e. those that code for enzymes that mediate a specific biogeochemical transformation, are being used to investigate the ecology of functionally-significant biological communities (45, 46).

Wastewater contains significant amounts of ammonium, of which only a small proportion is oxidised by conventional treatment plants (42). Ammonium oxidation and decomposition of organic matter within receiving waters can have a significant draw-down effect on dissolved oxygen (14), with potentially detrimental consequences for aerobic biota. Much of the research on nitrification and nitrifying bacteria associated with wastewater effluent has concentrated on the microbiota within the water column (8) rather than in sediments. However, it is in sediments that highest concentrations of organic matter and microbial biomass are likely to be found (36). Furthermore, oxygen gradients and concentration interfaces, such as those between sediments and the overlying water are important sites for coupling between microbially-mediated biogeochemical processes, such as nitrification and denitrification (37) and methylotrophic cycling of C₁ compounds (25).

In this study we used a combination of phylogenetic and functional-gene based molecular approaches to investigate the diversity and functional ecology of sediment microbial communities in a stream that receives WWTP discharge. The creek is relatively small, has seasonally-variable flow and, as such, the magnitude of ecological disturbance from the WWTP discharge is likely to have relatively large effects (20) including alteration of stream energy sources, flow regime and sediment habitat. The impacts of these factors on gross changes in bacterial community structure were assessed by PCR-DGGE, and phylogenetic shifts at two stream locations characterised by clone-library analysis. Due to the importance of nitrification in reducing the environmental impact of ammonia in effluent (8, 21) we assessed, using real-time PCR (qPCR), the abundance of functional genes associated
with nitrogen cycling, including those involved in nitrogen fixation (\textit{nifH}), nitrification (\textit{amoA}) and denitrification (\textit{narG}). Also, the abundance of the formyltransferase/hydrolase gene, \textit{fchD}, was used as an indicator of C\textsubscript{1} metabolism by methylotrophs.

**MATERIALS AND METHODS**

**Study Area**
Hahndorf Creek is located in a rural area, approximately 30 km south east from Adelaide, South Australia in the western Mt Lofty Ranges (Fig. 1). The creek forms part of the Upper Onkaparinga Catchment and discharges into the Onkaparinga River. Mean annual rainfall for Hahndorf is 860 mm per year, predominantly between May and October. As such, the base stream flow is highly variable between seasons. Mean annual stream flow (modelled) from the Hahndorf sub-catchment (area 1468 ha) is 2225 ML, consisting of base flow (groundwater) of 355 ML and run-off of 1870 ML (54). The WWTP services a population of ca. 3,000 people from the towns of Balhannah, Hahndorf, Oakbank and Verdun. The plant, built in two phases in 1977 and 1992/3, emits approximately 1 ML day\textsuperscript{-1} of tertiary-level-treated (chlorinated) discharge. Downstream of the outfall, the stream flow is continuous but variable throughout the year. Hahndorf Creek flows through an area largely cleared of native vegetation and used mainly for grazing and horticulture. The landscape is gently undulating and averages 350 to 430 m altitude. The riparian zone consists of an overstorey of \textit{Eucalyptus} spp. with a shrubby, sclerophyllous understory interspersed with weeds such as blackberry (\textit{Rubus fruticosus}). The geomorphology of the area is described in detail elsewhere (54) and consists of Adelaidean sediments, including Woolshed Flat Shale.

**Sediment Sampling**
Samples of in-stream sediments were taken from 6 locations along the Hahndorf Creek (Fig. 1) during November 2005. The locations were 640m upstream of the WWTP outfall (inferred as an undisturbed reference site with respect to the outfall), immediately above and below the outfall, and then progressively downstream 150m, 400m (where the stream slows and enters a small farm dam) and 1040m below the outfall.
At each location, four replicate sediment samples were taken from 0 to 5 cm depth using a hand-held coring device. Upon sampling, the sediments were transferred into sealable plastic bags and placed on ice until delivery to the laboratory within 4 h of sampling. DNA extractions were made from each of the 4 replicate samples, but for physicochemical analysis, sub-samples of the replicates were pooled to a single sample representative of the sampling location.

**Sediment Physicochemical Properties**

The percentage water content of sediment samples was determined by mass difference after oven drying at 105°C to constant mass. Dried samples were coarse ground to <2 mm and physicochemical properties determined by the analytical services unit of CSIRO Land and Water, Adelaide. Total carbon (TC) and total nitrogen (TN) were determined by direct combustion using a LECO CNS 2000 automated dry combustion analyser. Inorganic nitrogen was determined from a 2M KCl extraction followed by colorimetric determination of NH$_4^+$ and NO$_3^-$ (33) using an AlpKen Flow Solution III colorimeter (O.I Analytical, OR, USA). A suspension of each sample (1:5 sample:water, w/v) was used to determine pH and electrical conductivity (E.C.). P, Cu, Zn, and Cd were measured by US-EPA method 3051A (a nitric/hydrochloric acid microwave digest followed by ICP-OES analysis). Na, K, Ca and Mg as exchangeable cations were analysed according to method 15B2 and 15C1 (1M NH$_4$Cl adjusted to pH 7 for sediments extracted at pH 7 and 1M NH$_4$Cl in 60% ethanol adjusted to pH 8.5 for sediments extracted at pH 8.5) (33). In both cases the equipment described in method 15D2 was used to mechanically extract the samples under controlled conditions. Particle size analysis was done according to McKenzie et al. (28). The sediments were dispersed and the organic matter removed with H$_2$O$_2$ and carbonate removed using acetic acid. Size fractions were determined by sedimentation using the pipette method and sieving to separate <2 µm clay, 2-20 µm silt, 20-200 µm fine sand and 200-2000 µm coarse sand.

**Microbial Biomass**

Microbial biomass at each sampling location was measured using the chloroform-fumigation extraction technique (24). From 10g of fresh sediment samples, the amount of C held in the microbial biomass was determined by comparing dissolved C
before and after 7 day chloroform fumigation. Dissolved C was extracted from the sediment using 30mL of 0.5M K$_2$SO$_4$ (pH 6.3), shaking for 1 h and filtering through 2 layers of Whatman No. 42 filter paper. Total C was determined by infrared detection following combustion at 950°C on a Formacs series combustion TOC/TN analyser (Skalar Analytical Ltd; Breda, The Netherlands). To account for extraction efficiency, microbial biomass carbon (MBC) values were multiplied by a constant of 2.22 (52).

**DNA Extraction and Quantification**

DNA was extracted in duplicate from 0.8 g of each of the four replicate sediment samples from each location using the UltraClean Soil DNA extraction kit (MoBio Laboratories, CA). A FastPrep cell disruptor (Bio101) was used to enhance extraction efficiency. DNA from the duplicate samples was pooled into a final volume of 50µL in TE buffer. The concentration of DNA in the samples was quantified using PicoGreen dsDNA quantitation reagent (Invitrogen) against a standard curve of Lambda-phage DNA on a Stratagene MX3000P qPCR system.

**PCR-DGGE**

The community structures of bacteria in the stream sediment were analysed by PCR-DGGE profiling of a variable portion of the 16S rRNA gene. Bacterial-specific PCR primers F968-GC and R1401 were used as described by Duineveld et al. (16). Each PCR was conducted in total reaction volume of 25µL, and used: primers at 20 pmol each, dNTP at 10mM each, 1 U of Qiagen HotStar Taq DNA polymerase, 2.5 µL of PCR buffer, and 2 µL of DNA. The PCR cycle used a touchdown profile with a reduction in annealing temperature from 67°C to 57°C over the first 20 cycles and at 57°C for 20 cycles thereafter. Each cycle involved denaturation at 94°C for 1 min, annealing for 1 min and extension at 72° for 1 min. Confirmation of PCR was achieved by agarose gel electrophoresis of 2 µL of each PCR mixture, staining with ethidium bromide and visualising under UV light. The remaining reaction mix was used for DGGE analysis of amplified bacterial 16S rRNA genes, which was performed on the Ingeny PhorU system (Ingeny International, The Netherlands). The polyacrylamide gels (7% w/v of acrylamide:bis-acrylamide at 37.5:1) contained a linear formamide/urea gradient ranging from 45% to 55% and were overlayed with a non-denaturing stacking gel. Each well contained 10 µL of PCR product and 4 µL of
2 × loading buffer. Electrophoresis was done at 110V for 17h, after which gels were stained with SYBR Gold (Molecular Probes) for 40 min, rinsed in water and visualised on a Dark Reader (Clare Chemical Inc). An Olympus E500 SLR digital camera was used to photograph the gels. Position and intensity of bands on DGGE gels were measured using Gel-Quant™ software (Multiplexed Biotechnologies Inc.). Band position (ribosomal genotype) and intensity (abundance) data were collected.

**Cloning and Sequencing of Bacterial rRNA genes**

DNA from locations 1-4 and 5-6 were pooled to create 2 samples: upstream and downstream of the 400m site. Partial-length 16S rRNA gene sequences were generated by PCR from environmental DNA samples as described above, except that primer F986 was used without a GC clamp. PCR reactions were purified using the Promega PCR cleanup kit, ligated into pGEM-T plasmids and transformed into competent *E. coli* JM109 cells (Promega Inc.). DNA sequencing was conducted by the Australian Genome Research Facility (Adelaide) using the M13F primer location. Plasmid and primer sequences were identified and removed. Each sequence was checked against those on Genbank using a BlastN search. From the upstream sample, 39 16S rRNA gene sequences were obtained, and from the downstream sample of DNA, 25.

**Quantification of Functional Genes**

Real-time PCR (qPCR) was used to quantify the abundance of four genes known to have a role in the biochemical cycling of N and C. The target functional genes, their enzymes and processes are shown in Table 1. All reactions were conducted on a Stratagene MX3000P real-time PCR instrument (Integrated Sciences Inc.) in 25μL reaction volumes. PCR chemistry was based on QuantiTect SYBR-Green Taq and buffer (Qiagen). Quantification of the *amoA* gene was based on the *amoA*-1F and *amoA*-2R* primer set (41). Primers were added to give 0.4μM in the PCR master mix and 5μL of DNA was used in each reaction. Following hot-start activation, PCR thermocycling conditions involved 40 cycles of 92°C for 2min, 50°C for 1 min, and 72°C for 45 sec. Quantification of the *nifH* gene was based on the *nifH*-F and *nifH*-R primer set (34). Primers were added to give 0.8μM in the PCR master mix and 5μL
of DNA was pipetted into reactions. Following hot-start activation, PCR thermocycle conditions involved 40 cycles of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 45 sec. Quantification of the narG gene was based on the narG1960f and narG2650R primer set (32). Primers and DNA were used at concentrations given for nifH. Thermocycle conditions were based on touch-down PCR. In the first 8 cycles, the annealing temperature was decreased from 60°C to 55°C, and then maintained at 55°C for a further 30 cycles. Denaturation was conducted at 94°C for 30 sec, primer annealing for 30 sec and extension at 72°C for 45 sec. Quantification of fchD gene copies was based on the fchD105 and fchD947 primer set (26). Primers were used at 0.8 µM in the PCR master mix and 5 µL of DNA was pipetted into reactions. Following hot-start activation, PCR thermocycle conditions involved 35 cycles of 94°C for 40 sec, 58°C for 40 sec, and 72°C for 60 sec.

Standard curves of known copy numbers of each gene were generated. PCR product generated using each primer pair was cloned into pGEMT cloning vector. Plasmid containing the correct insert (determined by sequencing) was harvested from recombinant E. coli host using the MoBio Inc. Midi-Plasmid Prep Kit and quantified using PicoGreen, as described above. Ten-fold serial dilutions of plasmid DNA were prepared to obtain a standard curve for each gene. The standard curves were found to be linear over at least 8 orders of magnitude during qPCR. The copy numbers of functional gene per ng plasmid DNA were calculated. For all PCRs, the threshold cycle (C_T) in which all reactions samples were in exponential phase amplification was determined and compared with C_T values of the standard curve to give copies of gene per reaction. Finally, this was adjusted to DNA loaded per reaction volume (gene copies / ng of DNA) and corrected for the size of the microbial biomass at each site (multiplied by MBC). To ensure amplification of specific product (correct amplicon size), 4 µL aliquots of PCR reaction mixes were separated by agarose gel electrophoresis against a known DNA fragment size ladder, stained using ethidium bromide and visualised under UV trans-illumination.

**Statistical Analysis**

Band intensity data from the DGGE gels were 4th-root transformed and a resemblance matrix generated using the Bray-Curtis method. Clustering, with the group average method, was used to group the samples by similarity. SIMPROF (α=0.05) was used
to test the statistical validity of the clustering (10). Simpson’s index (1-\(\lambda\)) was used to quantify diversity of the bacterial communities at each sampling location.

Sediment physicochemical data was normalised and the samples compared using principal components analysis (PCA). Changes in bacterial community structure at locations along the creek (DGGE banding pattern) were related to the sediment physicochemical properties using the BIO-ENV routine with Spearman Rank Correlation (9,10). The physicochemical and biotic data were tested for seriation; i.e. gradual change along the stream, indicative of a natural gradient. Spearman Rank Correlation was employed and 999 permutations of the data used to test for the level of significance. The analysis of DGGE and physicochemical data was performed using the Primer6 software package (PrimerE Ltd) using methods described by Clarke and Warwick (11).

16S rRNA gene sequences from the 2 aggregated stream locations were assembled into FastA format. The two data sets were compared phylogenetically using the Library Comparison tool (Naive Bayesian rRNA Classifier) available via the Ribosomal Database Project (http://rdp.cme.msu.edu/index.jsp).

Functional genes, determined using real-time PCR, were quantified per \(\eta\)g of template DNA extracted from the sediment. The data, adjusted for microbial biomass (cf. above), were log_{10} -transformed and this data, and the MBC measurements, were tested for significance using one way-ANOVA. Tukey’s test at \(\alpha=0.05\) was used to compare significance of the values between the sediment samples.

RESULTS

Physicochemical Properties of the Sediments

The physicochemical properties of the sediment varied considerably along the stream bed. When compared using PCA, 54% of the variation was described in PC1 which effectively discriminated the site 400m below from the outfall from the rest of the sites (Fig 2). This location has the highest values of E.C., C, total N, \(\text{NH}_4^+\)-N, \(\text{NO}_3^-\)-N, P, Zn, Ca, Mg and Na, as well as high Cu (Table 2). PC2, accounting for 28% of the variation in physicochemical properties discriminated between the other sediment sampling locations (Fig 2). Eigenvectors strongly associated with PC2 were clay (-0.416) coarse sand (0.406) fine sand (0.375), K (-0.383) and Na (-0.374). This axis
primarily discriminates samples based on clay content, thereby affecting exchangeable cations, with percentage clay decreasing down the stream and percentage sand increasing.

When tested for seriation, the physicochemical properties of the sediment showed no pattern of natural or gradual change with distance along the creek (Rho 0.221; $P = 0.253$).

**Bacterial Community: Abundance, Diversity and Phylogenetic Structure**

The microbial biomass, assessed by measuring the release of C from the sediment after chloroform fumigation, increased with distance down the stream (Fig. 3A; $P = 0.009$). Lowest microbial biomass carbon (MBC) values were found immediately below the WWTP outfall. Further downstream, MBC was at, or higher than, levels recorded above the outfall. Highest MBC - nearly 2-fold greater than other values - was found 1040 m below the WWTP outfall.

The diversity of the dominant members of the bacterial community (Simpson’s Diversity Index, based on DGGE 16S rRNA gene banding profiles) differed significantly between sites ($P = 0.009$; Fig 3B). The highest diversity was recorded from samples taken 150m below the outfall. Beyond this point, diversity declined. Diversity at the sites immediately below the outfall, 150m and 400m below were significantly greater than in the sediment taken from the ‘undisturbed’ site 640m above the outfall (Fig. 3B).

There were significant differences in the structure, or composition, of the bacterial communities according to location ($P_i 4.068; P < 0.001$). The largest differences were between sites ≥400 m downstream of the outfall and those upstream of this location (13% similarity; Figure 4). The community structure of bacteria from the two sites denoted ‘≥400m’ was not significantly different. These results were reinforced by sequence analysis and phylogenetic assignment of bacterial communities upstream and downstream of the 400m site. Significant differences in phylogenetic structure were detected at phylum level ($P = 0.007$). The 16S rRNA gene sequences originating from sediment above the 400m site (site 5, Fig 1) were mostly of α- and β-proteobacterial origin whereas sequences originating from the sediment downstream of 400m were identified as α-, β-, γ-, and δ-proteobacterial origin (Table 3).
Abundance of Bacteria Involved in N and C cycling

The relative abundance of bacteria involved in N and C biotransformation was measured by PCR quantification of N- and C-functional genes. The abundance of the amoA gene varied significantly with stream location ($P = 0.001$; Fig 5A). After dipping immediately below the WWTP outfall, the abundance of ammonia oxidising bacteria (i.e. amoA gene copies) increased with distance downstream. The samples from sites at 150 m, 400 m and 1040 m below the outfall contained significantly higher amounts of amoA than those from the pool below the outfall and from 640 m above the outfall. Similarly, the abundance of the nifH gene varied significantly ($P=0.001$; Fig 5B), with more copies in sediments from the pool above the outfall and 1040 m below the outfall, than elsewhere. Abundance of narG also varied significantly with site ($P=0.042$; Fig 5C), with lower abundance immediately below the outfall and highest abundance at 400 and 1040 m downstream. Abundance of the fchD gene did not vary with stream location ($P>0.05$; Fig 5D).

DISCUSSION

Using a suite of molecular tools, we evaluated the impact of wastewater discharge on the ecology of bacterial communities in the sediment of Hahndorf Creek. Differences in community structure were strongly linked with physicochemical characteristics of the sediment. The discharge from the WWTP gradually increased the percentage of sand in the sediment downstream of the outfall, whereas chemical alteration occurred more abruptly at 400 m downstream of the outfall, where stream-flow slows as the creek enters an artificial dam. As the stream enters the dam, 400 m below the outfall, the flow is very slow, the nutrient concentration high, and the stream bed porous and full of organic material. At the most downstream site, the stream bed is very compact, and receives a constant flow of water with dissolved nutrients. The value of nearly every chemical property we measured was highest at the 400 m site, indicating that the reduction in flow resulted in deposition of organic matter and other material. This site was also characterised by a marked difference in bacterial community structure, based on a similarity index of 16S-rRNA gene sequences and on the phylogenetic identification of those sequences. Overall, differences in chemical properties of the sediment appeared to have a greater influence on bacterial community structure than changes in physical attributes.
During analysis of the data, we were initially surprised to find that the effects of the WWTP discharge were not strongly evident in the pools immediately above and below the outfall. However, analysis of the sediment physicochemical properties (showing loading of C, N, P and changes in other variables) showed that the strongest environmental changes were present where the stream entered the dam. The alteration of the stream flow regime appeared to be the trigger for the manifestation of ecological effects which impacted on bacterial community structure. In the absence of the farm dam, it is possible that ecological effects from the WWTP discharge would be less pronounced, but possibly expressed over a longer distance. In this regard, Birch et al. (2) found that Lake Wallace, on the Coxs River (NSW, Australia), was a significant sink for heavy metals from coal-mining activities and municipal wastewater. Containment of the metals was important as the water ultimately enters a drinking water reservoir for Sydney. Clearly, the accumulation of nutrients and metals in aquatic sediments can be desirable or undesirable, depending on the end-use of the water and the points of abstraction. In the Hahndorf Creek, it is likely that ecological impacts of the WWTP will be much reduced downstream of point of deposition due to the presence of the dam. However, there may be potential for greater environmental impact downstream if an increase in seasonal stream-flow, due to increased runoff and stream recharge from the catchment resulted in perturbation and re-suspension of the nutrients in the sediment.

Microbial biomass was highest at the most downstream sampling location (1040m). This may have been due to eutrophication. Although WWTP effluents are known to increase microbial activity (35), it is not immediately apparent why population size was not higher at the location 400m below the outfall (Fig 3). Differences in MBC may have been due to variation in flow rates between the locations. Such conditions are conducive to the development of a surface biofilm resulting in higher total microbial biomass.

Bacterial community structure formed two main clusters based on distance from the WWTP outfall, with sites at 400m and 1040 m being markedly different from those upstream. Again, this was associated with changes in chemical properties of the stream sediment at the sampling locations. Analysis of ribosomal gene sequence data from the two community types (i.e. from a mix of samples from the two clusters identified in Fig 4) showed that the differences in community structure were evident at the level of phyla, indicating a very large phylogenetic shift. In particular, the
down-stream community had representatives of γ-proteobacteria which were not detected in the up-stream community, including *Oceanospirillales* and *Methylococcaceae*. Similar spatial partitioning in microbial community structure as estimated using DNA sequence analyses has been reported for ammonia-oxidisers by (7) and for total bacterial communities in reservoir sediments (50). In both cases, chemical characteristics were the major determinants of microbial community structure.

Little is known of the ecology of *Oceanospirillales*, a relatively newly-described order (18). However, the *Methylococcaceae* are aerobic and derive energy from C-1 metabolism; particularly methane. The source of methane is likely to be from anaerobic decomposition of organic material deeper in the sediment. Sequences from the upstream community included those belonging to representatives of the *Caulobacteraceae* and *Sphingomonadaceae*. These families are common in water, and are considered to be oligotrophic - adapted to conditions of low availability of metabolic substrate (13, 31). Sphingomonads are capable of a wide range of transformations of metals and nutrients (44, 49), indicating they have physiological attributes of functional relevance to the maintenance of ecosystem health. Their presence in this section of the stream may be indicative of the higher habitat quality of the up-stream sites. *Verrucomicrobia* sequences were detected from both up-stream and down-stream locations. This domain of prosthecate bacteria has been detected in many ecosystems, including freshwater, but their ecological role remains unknown and few have been cultivated (5, 23). *Nitrospirae* sequences were also detected in the upstream samples. These bacteria are chemolithoautotrophic, gaining energy from the oxidation of nitrite to nitrate. Nitrate concentrations, however, were highest in the downstream sediments (Table 2), and it would be expected that these bacteria would be more abundant in these samples. The total abundance of *Nitrospirae* may be much higher in the lower region of the stream, but in relative terms represent only a small fraction of the total bacterial community. This concept is supported by the increase in total microbial community size (MBC) at the site furthest downstream, and higher abundance of N-cycling bacteria, as measured by N functional gene quantification. However, a much larger library of rDNA clones would need to be examined in order to identify the bacteria more precisely.

Community diversity, based on PCR-DGGE banding profiles and Simpson’s Index, was lowest at the reference site, highest in the region of the WWTP outfall, and
then lower $\geq 400$m downstream at the sites where strong impacts from the outflow were found. This pattern of diversity can be explained by the intermediate disturbance hypothesis (12), which proposes diversity is highest where disturbance is neither too rare nor frequent. The disturbance caused to sediments immediately downstream of the WWTP outfall may create conditions of greater habitat heterogeneity than elsewhere and allow the development of greater community diversity.

Differences in microbial community structure provide useful information on the effects of disturbance and stress on the biological integrity of ecosystems, but generally convey little information regarding ecosystem function. Increasing focus is therefore being placed on understanding environmental impacts on specific groups of microorganisms known to be involved in ecological processes (48) such as processing of heavy metals, degradation of pesticides, and biogeochemical cycling of nutrients.

Given the potential for eutrophication as a source of disturbance from the WWTP outfall, we measured the abundance of genes involved in geochemical transformation of C and N, and found the biological capacity within the sediment to mediate three key steps in the cycling of N varied greatly along the Hahndorf creek although capacity for C-1 cycling did not. The abundance of each of the N functional genes was greater in the pool immediately upstream from the outfall than the pool downstream. At the time of sampling, the natural stream flow was very low, enabling some of the discharge to diffuse upstream. Nevertheless, the upstream pool had lower total amounts of C and N in the sediments, indicating that there may have been a secondary effect from the WWTP affecting N-functional capacity of this site, such as chlorination treatment (42). Importantly, no significant shift in bacterial community structure was detected in the pools either side of the outfall, yet N-functional capacity was different. Below the WWTP outfall, the size of the N-cycling bacterial populations increased with distance along the stream. The values at the most downstream location need to be viewed with caution. As discussed previously, MBC measured at this site was by far the highest and functional gene abundances were adjusted to MBC. The limitations of using MBC as a measure of the microbial population size were detailed (4). With this caveat in mind, the abundance of *amo*A and *nar*G increased in accord with the availability of their enzyme substrates, $\text{NH}_4^+$ and $\text{NO}_3^-$ respectively.
The strong correlation between increased abundance of amoA and narG in the pool above the outflow and with increasing distance downstream suggests a degree of spatial coupling of nitrification and denitrification activity. Nitrification occurs under aerobic conditions and requires a source of ammonium, whereas denitrification requires suboxic conditions and a source of nitrate (from external inputs or nitrification of ammonium). Spatial coupling in aquatic systems is likely to occur at oxic/suboxic interfaces, such as those between bottom waters and water-saturated sediments of differing oxygen concentrations (37). The percentage of effluent that contributes to the streamflow of Hahndorf Creek (estimated from the sub-catchment water balance model (54)) indicates strong seasonal variation, with ca. 8-14% between April and October, the period of heaviest rainfall, to 20-29% between November and March when rainfall is lowest. Thus sediments are likely to be more oxic, and nitrification-denitrification more closely linked during the austral winter than during summer, when sub-oxic conditions would be enhanced by higher water temperatures as well as higher inputs of N and organic matter. Increased C, N and P from WWTP effluent have been shown to alter OM assimilation and secondary productivity, impacting negatively on invertebrate community structure and nutrient fluxes (39). Seasonal increases in such stress events are likely to exacerbate effects on ecosystem function.

Given the large amount of inorganic N in the sediment, the response of N2 fixing communities (nifH gene) to the disturbance was surprising. The abundance of nifH is generally considered to increase with widening C:N ratios, particularly in anoxic environments. C:N ratios were widest at the two most downstream sites (approximately 16:1 to 19:1; from Table 2), which correlated with high nifH abundance. However, in the pool immediately below the outfall the C:N ratio was approximately 15:1, yet the abundance of nifH was much lower than at other sites. Again, this may have been a secondary effect associated with the WWTP discharge, such as chlorination.

Our data indicates strong cause-and-effect relationship between loading of carbon and nitrogen into the creek from the WWTP outfall and major responses in bacterial abundance, community structure and function. The effect of the outfall on microbiological characteristics was evident for more than 1 km downstream, showing extensive influence of the treatment plant on the ecology of the stream sediment. The effect of this disturbance on the longer-term ecological function of the stream is not
clear but is likely to impact upon decomposition and nutrient cycling, and the provision of balanced C:N:P to aquatic organisms.

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38. Sharma, S., V. Radl, B. Hai, K. Kloos, M. M. Fuka, M. Engel, K. Schauss,


List of Figure Captions:

Figure 1. Map of Hahndorf Creek, the wastewater treatment plant (WWTP) and the sampling locations. Location 1 is ‘undisturbed’ with respect to the location of the WWTP outfall, locations 2 and 3 are immediately above and below the outfall, location 4 is 150 m downstream of the outfall, location 5 is 400 m downstream of the outfall (where the creek slows and enters a lagoon) and location 6 is 1040 m downstream of the outfall. SA = South Australia.

Figure 2. Principal Components Analysis (PCA) plot showing resemblance of physicochemical characteristics of the sediments in Hahndorf Creek (Table 2) to the location of the sampling sites illustrated in Fig. 1.

Figure 3. Abundance and diversity of bacteria in sediment samples from different locations in Hahndorf Creek. Error bars = standard error. A) Microbial biomass carbon, as a measure of abundance, B) Simpson’s Diversity Index based on the DGGE banding profiles of 6S rRNA gene fragments.

Figure 4. Cluster plot grouping of (group average) similarity between bacterial community structures from the Hahndorf Creek sediment samples. Community structures were assessed by PCR-DGGE of the 16S-rRNA gene. The resemblance matrix was generated using the Bray-Curtis similarity algorithm on 4th-root transformed abundance (band intensity) data.

Figure 5A-D. Log. copies of nitrogen- and carbon-cycling functional genes involved in the geochemical cycling of N and C at locations down the Hahndorf creek. Units on the Y-axis for each graph are ‘log gene copy numbers / ng of sediment- extracted DNA × MBC’. n.s. = not significant. Error bars = SEM.
A) amoA

- log copies amoA/ ng DNA
- P=0.001

B) nifH

- log copies nifH/ ng DNA
- P=0.001

C) narG

- log copies narG/ ng DNA
- P=0.042

D) fchD

- log copies fchD/ ng DNA
- n.s.
TABLE 1. Nitrogen and carbon functional genes detected, enzymes encoded for and associated processes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Enzyme</th>
<th>Process / function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>amoA</em></td>
<td>α-subunit of ammonia mono-oxygenase</td>
<td>Oxidation of ammonia to hydroxylamine: ( \text{NH}_3 + 0.5\text{O}_2 \rightarrow \text{NH}_2\text{OH} )</td>
</tr>
<tr>
<td><em>narG</em></td>
<td>Nitrate reductase</td>
<td>Reduction of nitrate to nitrite: ( \text{NO}_3^- \rightarrow \text{NO}_2^- )</td>
</tr>
<tr>
<td><em>nifH</em></td>
<td>Nitrogenase reductase Fe-protein</td>
<td>Nitrogen fixation</td>
</tr>
<tr>
<td><em>fhcD</em></td>
<td>δ-subunit of formyltransferase / hydrolase complex</td>
<td>C1 cycling: bacteria possessing ( \text{H}_2\text{MPT} )-linked C1 transfer pathway</td>
</tr>
</tbody>
</table>

\( \text{C1} \) transfer pathway.
TABLE 2. Sediment physicochemical properties at sampling sites in Hahndorf Creek, in relation to the position of a wastewater treatment plant outfall.

<table>
<thead>
<tr>
<th>Property</th>
<th>640 m above outfall</th>
<th>pool above outfall</th>
<th>pool below outfall</th>
<th>150 m below outfall</th>
<th>300 m below outfall (above the dam)</th>
<th>1040 m below outfall</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.C. dS/m</td>
<td>0.30</td>
<td>0.30</td>
<td>0.32</td>
<td>0.20</td>
<td>1.06</td>
<td>0.38</td>
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<tr>
<td>pH</td>
<td>7.24</td>
<td>7.03</td>
<td>6.23</td>
<td>7.10</td>
<td>6.43</td>
<td>6.88</td>
</tr>
<tr>
<td>C Total %</td>
<td>0.43</td>
<td>0.71</td>
<td>1.34</td>
<td>0.14</td>
<td>7.39</td>
<td>2.07</td>
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<tr>
<td>N Total %</td>
<td>0.04</td>
<td>0.06</td>
<td>0.09</td>
<td>0.04</td>
<td>0.47</td>
<td>0.11</td>
</tr>
<tr>
<td>NH₄-N KCl ext mg/kg</td>
<td>7.77</td>
<td>9.96</td>
<td>15.22</td>
<td>20.82</td>
<td>81.75</td>
<td>10.44</td>
</tr>
<tr>
<td>NO₃-N KCl ext mg/kg</td>
<td>0.27</td>
<td>0.28</td>
<td>0.30</td>
<td>0.24</td>
<td>1.10</td>
<td>0.35</td>
</tr>
<tr>
<td>P Total mg/kg</td>
<td>339.53</td>
<td>184.20</td>
<td>299.26</td>
<td>454.80</td>
<td>973.25</td>
<td>830.16</td>
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<tr>
<td>Cu Total mg/kg</td>
<td>22.39</td>
<td>24.72</td>
<td>17.01</td>
<td>31.69</td>
<td>30.43</td>
<td>19.28</td>
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<tr>
<td>Zn Total mg/kg</td>
<td>46.40</td>
<td>41.18</td>
<td>42.05</td>
<td>64.90</td>
<td>191.32</td>
<td>98.42</td>
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<tr>
<td>Ca exch cmol(+)/kg</td>
<td>5.08</td>
<td>8.21</td>
<td>7.66</td>
<td>4.83</td>
<td>16.87</td>
<td>4.14</td>
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<tr>
<td>Mg exch cmol(+)/kg</td>
<td>4.66</td>
<td>7.49</td>
<td>7.71</td>
<td>4.12</td>
<td>9.15</td>
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<tr>
<td>Na exch cmol(+)/kg</td>
<td>1.23</td>
<td>1.24</td>
<td>1.12</td>
<td>1.00</td>
<td>1.37</td>
<td>0.48</td>
</tr>
<tr>
<td>K exch cmol(+)/kg</td>
<td>0.44</td>
<td>0.70</td>
<td>0.54</td>
<td>0.33</td>
<td>0.66</td>
<td>0.22</td>
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<tr>
<td>Clay %</td>
<td>35.77</td>
<td>57.28</td>
<td>25.38</td>
<td>19.66</td>
<td>18.39</td>
<td>10.74</td>
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<tr>
<td>Silt %</td>
<td>28.71</td>
<td>14.51</td>
<td>18.85</td>
<td>27.72</td>
<td>18.95</td>
<td>19.68</td>
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<tr>
<td>Fine sand %</td>
<td>28.70</td>
<td>26.26</td>
<td>37.77</td>
<td>39.57</td>
<td>40.62</td>
<td>41.04</td>
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<tr>
<td>Coarse sand %</td>
<td>3.04</td>
<td>2.38</td>
<td>15.94</td>
<td>12.51</td>
<td>7.34</td>
<td>24.99</td>
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</table>
TABLE 3. Phylogenetic assignment of bacterial 16S rRNA gene sequences generated from sediment from above and below sampling position 5 (Fig. 1), 400m downstream of the WWTP outfall. The number of 16S rRNA gene sequences matched to this family or class of bacteria, according to the classification of the Ribosomal Database Project.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Class</th>
<th>Order or Family</th>
<th>Sequences upstream of 400 m</th>
<th>Sequences downstream of 400 m</th>
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<tbody>
<tr>
<td>Proteobacteria</td>
<td>α-proteobacteria</td>
<td>Caulobacteraceae</td>
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<td>Sphingomonadaceae</td>
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<td>β-proteobacteria</td>
<td>Burkholderiales</td>
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<td></td>
<td>2</td>
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<td></td>
<td></td>
<td>Hydrogenophilales</td>
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<tr>
<td></td>
<td></td>
<td>Rhodocyclusles</td>
<td>6</td>
<td>2</td>
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<td>Unclassified</td>
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<td>2</td>
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<tr>
<td>γ-proteobacteria</td>
<td>Oceanospirillales</td>
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<td>Methylcocccaeae</td>
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<td>δ-proteobacteria</td>
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<td>Bacteroidetes</td>
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<td>Sphingobacteria</td>
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<td>Gemmatimonadetes</td>
<td>Actinobacteriales</td>
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<td>Verrucomicrobia</td>
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