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

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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. The quantification of genes involved in the biogeochemical cycling of carbon and nitrogen was used to assess potential impacts on ecosystem functions. The effects of disturbance on bacterial community structure were assessed by PCR-denaturing gradient gel electrophoresis of 16S rRNA genes, and clone library analysis was used to phylogenetically characterize significant shifts. Significant ($P < 0.05$) shifts in bacterial community structures were associated with alteration of the sediment's physicochemical properties, particularly nutrient loading from the WWTP discharge. The effects were greatest at the sampling location 400 m downstream of the outfall where the stream flow is reduced. This highly affected stretch of sediment contained representatives of the gammaproteobacteria that were absent from less-disturbed sites, including *Oceanospirillales* and *Methylococcaceae*. 16S rRNA gene sequences from less-disturbed sites had representatives of the *Caulobacteraceae*, *Sphingomonadaceae*, and *Nitrospirae* which were not represented in samples from disturbed sediment. The diversity was lowest at the reference site; it increased with proximity to the WWTP outfall and declined toward highly disturbed (400 m downstream) sites ($P < 0.05$). The potential for biological transformations of N varied significantly with the stream sediment location ($P < 0.05$). The abundance of *amoA*, *narG*, and *nifH* genes increased with the 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.

Few rivers in Australia are considered close to being in "pristine" condition, and the trend over recent years has been toward increasing degradation (1, 30). Factors that have contributed toward 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.

The discharge of effluent from wastewater treatment plants (WWTPs) has major detrimental effects on the health of aquatic ecosystems. WWTP outfall can deposit large amounts of organic matter and nutrients into receiving waterways. Increased nutrient loading can lead to eutrophication (20) and temporary oxygen deficits (35). Increased organic matter 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 the physical characteristics of sediment. The discharge itself can perturb the natural flow regimen, 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 flows. 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 urbanization and periurbanization 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 include not only their sessile habits 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 on using microorganisms as indicators

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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 revolutionizing 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, 45). 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 oxidized by conventional treatment plants (42). Ammonium oxidation and the 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 the 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 the methylotrophic cycling of C_1 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 and 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 regimen, and sediment habitat. The impacts of these factors on gross changes in bacterial community structure were assessed by PCR-denaturing gradient gel electrophoresis (DGGE), and phylogenetic shifts at two stream locations were characterized 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 quantitative PCR (qPCR), the abundance of functional genes associated with nitrogen cycling, including those involved in nitrogen fixation (*nifH*), nitrification (*amoA*), and denitrification (*narG*). Also, the abundance of the formyltransferase/hydrolase gene, *fchD*, was used as an indicator of C_1 metabolism by methylotrophs.

MATERIALS AND METHODS

Study area. Hahndorf Creek is located in a rural area, approximately 30 km southeast of 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. The 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. The mean annual stream flow (modeled) from the Hahndorf subcatchment (1,468 ha in area) is 2,225 Ml, consisting of a base flow (groundwater) of 355 Ml and runoff of 1,870 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^{-1} of tertiary-level-treated (chlorinated) discharge.

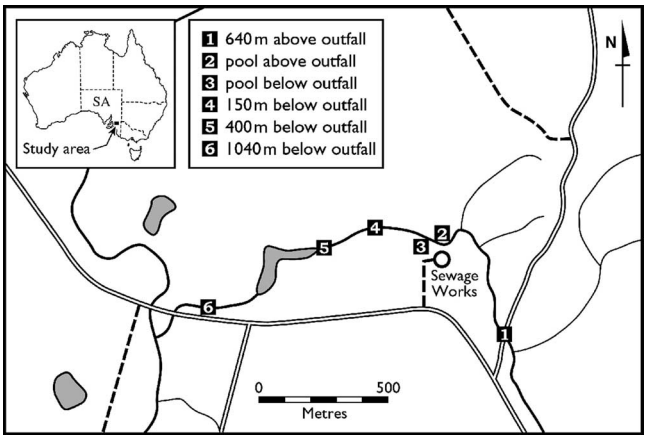

FIG. 1. Map of Hahndorf Creek, the WWTP, and the sampling locations. Location 1 is "undisturbed" with respect to the location of the WWTP outfall, locations 2 and 3 are immediately above and below the outfall, location 4 is 150 m downstream of the outfall, location 5 is 400 m downstream of the outfall (where the creek slows and enters a lagoon), and location 6 is 1,040 m downstream of the outfall. SA, South Australia.

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 in altitude. The riparian zone consists of an overstory of *Eucalyptus* spp. with a shrubby, sclerophyllous understory interspersed with weeds, such as blackberry (*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 six locations along the Hahndorf Creek (Fig. 1) during November 2005. The locations were 640 m 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 at 150 m, 400 m (where the stream slows and enters a small farm dam), and 1,040 m 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 four replicate samples, but for physicochemical analysis, subsamples of the replicates were pooled to a single sample representative of the sampling location.

Sediment physicochemical properties. The percent water content of the sediment samples was determined by the difference in mass after oven drying at 105°C to constant mass. Dried samples were coarse ground to <2 mm, and the physicochemical properties determined by the analytical services unit of CSIRO Land and Water, Adelaide. The total carbon and total nitrogen were determined by direct combustion using a LECO CNS 2000 automated dry-combustion analyzer. The inorganic nitrogen was determined from a 2 M KCl extraction followed by colorimetric determination of NH_4^+ and NO_3^- content (33) using an AlpKen Flow Solution III colorimeter (O.I. Analytical, OR). A suspension of each sample (1:5 sample:water, wt/vol) was used to determine the pH and electrical conductivity (E.C.). P, Cu, Zn, and Cd were measured by United States Environmental Protection Agency (U.S. EPA) method 3051A (a nitric/hydrochloric acid microwave digest followed by inductively coupled plasma-atomic emission spectrometry analysis). Na, K, Ca, and Mg as exchangeable cations were analyzed according to methods 15B2 and 15C1 from reference 33 (1 M NH_4Cl adjusted to pH 7 for sediments extracted at pH 7 and 1 M NH_4Cl in 60% ethanol adjusted to pH 8.5 for sediments extracted at pH 8.5). 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 the method of McKenzie et al. (28). The sediments were dispersed, the organic matter removed with H_2O_2, and carbonate removed using acetic acid. Size fractions were determined by sedimentation using the pipette method and sieving to separate clay (<2 μm), silt (2 to 20 μm), fine sand (20 to 200 μm), and coarse sand (200 to 2,000 μm).

TABLE 1. Nitrogen- and carbon-functional genes detected, enzymes encoded, and associated processes

Gene	Enzyme	Process/function
amoA	α-Subunit of ammonia monooxygenase	Oxidation of ammonia to hydroxylamine: $NH_3 + 0.5O_2 \rightarrow NH_2OH$
narG	Nitrate reductase	Reduction of nitrate to nitrite: $NO_3^- \rightarrow NO_2^-$
nifH	Nitrogenase reductase Fe protein	Nitrogen fixation
fhcD	δ-Subunit of formyltransferase/hydrolase complex	C_1 cycling: bacteria possessing tetrahydromethanopterin-linked C_1 transfer pathway

Microbial biomass. The microbial biomass at each sampling location was measured by using the chloroform fumigation extraction technique (24). From 10 g of fresh sediment samples, the amount of C held in the microbial biomass was determined by comparing the dissolved C before and after 7 days of chloroform fumigation. Dissolved C was extracted from the sediment by using 30 ml of 0.5 M K_2SO_4 (pH 6.3), shaking for 1 h, and filtering through two layers of Whatman no. 42 filter paper. The total C was determined by infrared detection following combustion at 950°C on a Formacs series combustion total organic carbon/total nitrogen analyzer (Skalar Analytical Ltd; Breda, The Netherlands). To account for the extraction efficiency, the 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 by using an UltraClean soil DNA extraction kit (MoBio Laboratories, CA). A FastPrep cell disruptor (Bio101) was used to enhance the extraction efficiency. DNA from the duplicate samples was pooled into a final volume of 50 μl in Tris-EDTA buffer. The concentration of DNA in the samples was quantified by using PicoGreen double-stranded DNA quantitation reagent (Invitrogen) against a standard curve of Lambda-phage DNA on a Stratagene MX3000*P* qPCR system.

PCR-DGGE. The community structures of bacteria in the stream sediment were analyzed by PCR-DGGE profiling of a variable portion of the 16S rRNA gene. *Bacteria*-specific PCR primers F968-GC and R1401 were used as described by Duineveld et al. (16). Each PCR was conducted in a total reaction mixture volume of 25 μl, with primers at 20 pmol each, deoxynucleoside triphosphates at 10 mM 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 touch-down 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 visualization under UV light. The remaining reaction mix was used for DGGE analysis of amplified bacterial 16S rRNA genes, which was performed on an Ingeny PhorU system (Ingeny International, The Netherlands). The polyacrylamide gels (7% [wt/vol] of acrylamide:bis-acrylamide at 37.5:1) contained a linear formamide/urea gradient ranging from 45% to 55% and were overlaid with a nondenaturing stacking gel. Each well contained 10 μl of PCR product and 4 μl of 2× loading buffer. Electrophoresis was done at 110 V for 17 h, after which gels were stained with Sybr gold (Molecular Probes) for 40 min, rinsed in water, and visualized on a Dark Reader (Clare Chemical Inc.). An Olympus E500 SLR digital camera was used to photograph the gels. The positions and intensities of the bands on the DGGE gels were measured by using GelQuant 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 to 4 and 5 and 6 were pooled to create two samples from upstream and downstream of the 400-m 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. PCRs were purified by using a Promega PCR cleanup kit, ligated into pGEM-T plasmids, and transformed into competent *Escherichia coli* JM109 cells (Promega, Inc.). The 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 by using a BlastN search. From the upstream sample, 39 16S rRNA gene sequences were obtained, and from the downstream sample of DNA, 25 were obtained.

Quantification of functional genes. 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 and their enzymes and processes are shown in Table 1. All reactions were conducted on a Stratagene MX3000*P* real-time PCR instrument (Integrated Sciences, Inc.) in 25-μl reaction mixture volumes. The PCR chemistry was based on QuantiTect Sybr green *Taq* and buffer (Qiagen). The quantification of the *amoA* gene was based on the *amoA*-1F and *amoA*-2R* primer set (41). Primers were added to give 0.4 μM in the PCR master mix, and 5 μl of DNA was used in each reaction. Following hot-start activation, the PCR thermocycling conditions involved 40 cycles of 92°C for 2 min, 50°C for 1 min, and 72°C for 45 s. The quantification of the *nifH* gene was based on the *nifH*-F and *nifH*-R primer set (34). Primers were added to give 0.8 μM in the PCR master mix, and 5 μl of DNA was pipetted into the reaction mixtures. Following hot-start activation, the PCR thermocycle conditions involved 40 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. The quantification of the *narG* gene was based on the *narG*1960f and *narG*2650R primer set (32). Primers and DNA were used at the concentrations given for *nifH*. The themocycle conditions were based on touch-down PCR. In the first 8 cycles, the annealing temperature was decreased from 60°C to 55°C, and it was then maintained at 55°C for a further 30 cycles. Denaturation was conducted at 94°C for 30 s, primer annealing for 30 s, and extension at 72°C for 45 s. The quantification of *fchD* gene copies was based on the *fchD*105 and *fchD*947 primer set (26). Primers were used at 0.8 μM in the PCR master mix, and 5 μl of DNA was pipetted into the reaction mixtures. Following hot-start activation, the PCR thermocycle conditions involved 35 cycles of 94°C for 40 s, 58°C for 40 s, and 72°C for 60 s.

Standard curves of known copy numbers of each gene were generated. The PCR product generated using each primer pair was cloned into the pGEM-T cloning vector. Plasmid containing the correct insert (determined by sequencing) was harvested from the recombinant *E. coli* host using a MoBio, Inc., Midi-Plasmid prep kit and quantified by using PicoGreen, as described above. Tenfold 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 each functional gene per ng plasmid DNA were calculated. For all PCRs, the threshold cycle in which all reaction samples were in exponential-phase amplification was determined and compared with the threshold cycle values of the standard curve to give the number of copies of the gene per reaction. Finally, this was adjusted to the volume of DNA loaded per reaction (gene copies/ng of DNA) and corrected for the size of the microbial biomass at each site (multiplied by the MBC). To ensure the amplification of specific products (correct amplicon size), 4-μl aliquots of PCR mixes were separated by agarose gel electrophoresis against a known DNA fragment size ladder, stained by using ethidium bromide, and visualized under UV transillumination.

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 ($\alpha = 0.05$) was used to test the statistical validity of the clustering (10). Simpson's index (1-λ) was used to quantify the diversity of the bacterial communities at each sampling location.

The sediment physicochemical data were normalized, and the results for the samples compared using principal component analysis (PCA). Changes in bacterial community structure at locations along the creek (DGGE banding pattern) were related to the sediment physicochemical properties by 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 the DGGE and physicochemical data was performed by using the Primer6 software package (Primer-E, Ltd.), using methods described by Clarke and Warwick (11).

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

The functional genes, determined by using real-time PCR, were quantified per ng of template DNA extracted from the sediment. The data, adjusted for mi-

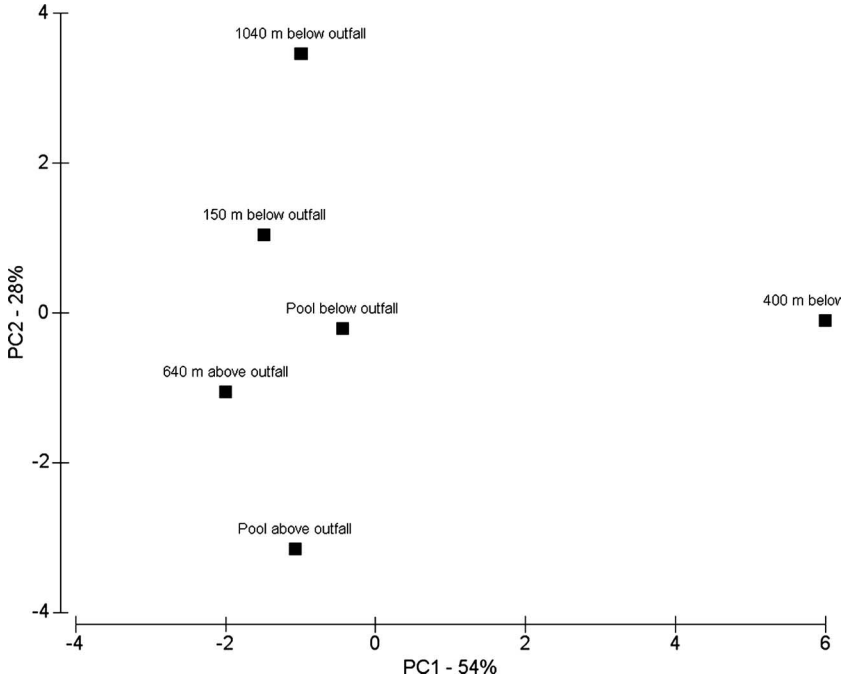

FIG. 2. PCA plot showing the resemblance of physicochemical characteristics of sediments in Hahndorf Creek (Table 2) to the location of the sampling sites illustrated in Fig. 1. PC1, principal component axis 1; PC2, principal component axis 2.

crobial biomass (see above), were $\log_{10}$ transformed, and these data and the MBC measurements were tested for significance using one-way analysis of variance. Tukey's test at $\alpha = 0.05$ was used to compare the 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 by using PCA, 54% of the variation was described in principal component one, which effectively discriminated the site 400 m below from the outfall from the rest of the sites (Fig. 2). This location had the highest values for E.C., C, total N, NH_4^+-N, NO_3^--N, P, Zn, Ca, Mg, and Na, as well as high Cu (Table 2). Axis 2, accounting for 28% of the variation in physicochemical properties, discriminated between the other sediment sampling locations (Fig. 2). The 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,

TABLE 2. Sediment physicochemical properties at sampling sites in Hahndorf Creek in relation to the position of a WWTP outfall

Property (unit of measure)	Value at:					
	640 m above outfall	Pool above outfall	Pool below outfall	150 m below outfall	400 m below outfall (above the dam)	1,040 m below outfall
E.C. (dS/m)	0.30	0.30	0.32	0.20	1.06	0.38
pH	7.24	7.03	6.23	7.10	6.43	6.88
Total C (%)	0.43	0.71	1.34	0.14	7.39	2.07
Total N (%)	0.04	0.06	0.09	0.04	0.47	0.11
NH_4-N KCl ext (mg/kg)	7.77	9.96	15.22	20.82	81.75	10.44
NO_3-N KCl ext (mg/kg)	0.27	0.28	0.30	0.24	1.10	0.35
Total P (mg/kg)	339.53	184.20	299.26	454.80	973.25	830.16
Total Cu (mg/kg)	22.39	24.72	17.01	31.69	30.43	19.28
Total Zn (mg/kg)	46.40	41.18	42.05	64.90	191.32	98.42
Ca exch [cmol($^+$)/kg]	5.08	8.21	7.66	4.83	16.87	4.14
Mg exch [cmol($^+$)/kg]	4.66	7.49	7.71	4.12	9.15	2.41
Na exch [cmol($^+$)/kg]	1.23	1.24	1.12	1.00	1.37	0.48
K exch [cmol($^+$)/kg]	0.44	0.70	0.54	0.33	0.66	0.22
Clay (%)	35.77	57.28	25.38	19.66	18.39	10.74
Silt (%)	28.71	14.51	18.85	27.72	18.95	19.68
Fine sand (%)	28.70	26.26	37.77	39.57	40.62	41.04
Coarse sand (%)	3.04	2.38	15.94	12.51	7.34	24.99

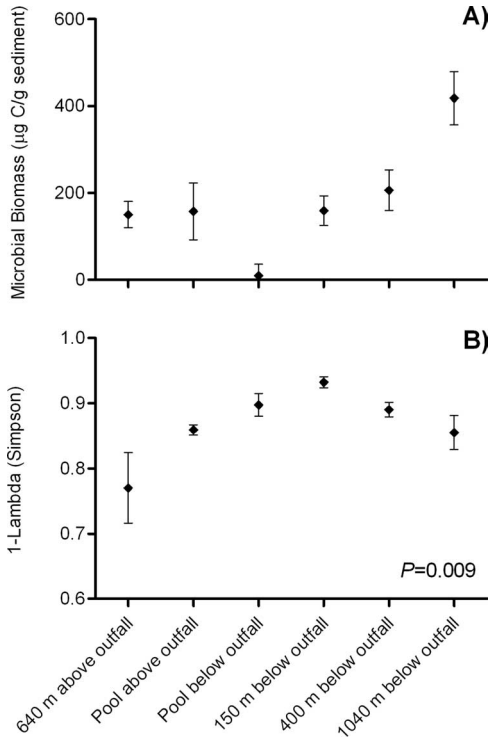

FIG. 3. Abundance and diversity of bacteria in sediment samples from different locations in Hahndorf Creek. Error bars show standard errors. (A) MBC is used as a measure of abundance. (B) Simpson's diversity index, based on the DGGE banding profiles of 16S rRNA gene fragments, was used to quantify diversity.

with the percentage of clay decreasing down the stream and the percentage of 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; $P = 0.253$).

Abundance, diversity, and phylogenetic structure of bacterial community. The microbial biomass, assessed by measuring the release of C from the sediment after chloroform fumigation, increased with distance down the stream (Fig. 3A) ($P = 0.009$). The lowest MBC values were found immediately below the WWTP outfall. Further downstream, the MBC was at or higher than the levels recorded above the outfall. The highest MBC, nearly twofold higher than the other values, was found 1,040 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 150 m below the outfall. Beyond this point, diversity declined. The diversities at the sites immediately below, 150 m below, and 400 m below the outfall were significantly greater than in the sediment taken from the "undisturbed" site 640 m above the outfall (Fig. 3B).

There were significant differences in the structure or composition of the bacterial communities according to location (R, 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) (Fig. 4). The community structures of bacteria from the two sites denoted "≥400 m" were not significantly different. These results were reinforced by sequence analysis and phylogenetic assignment of the bacterial commu-

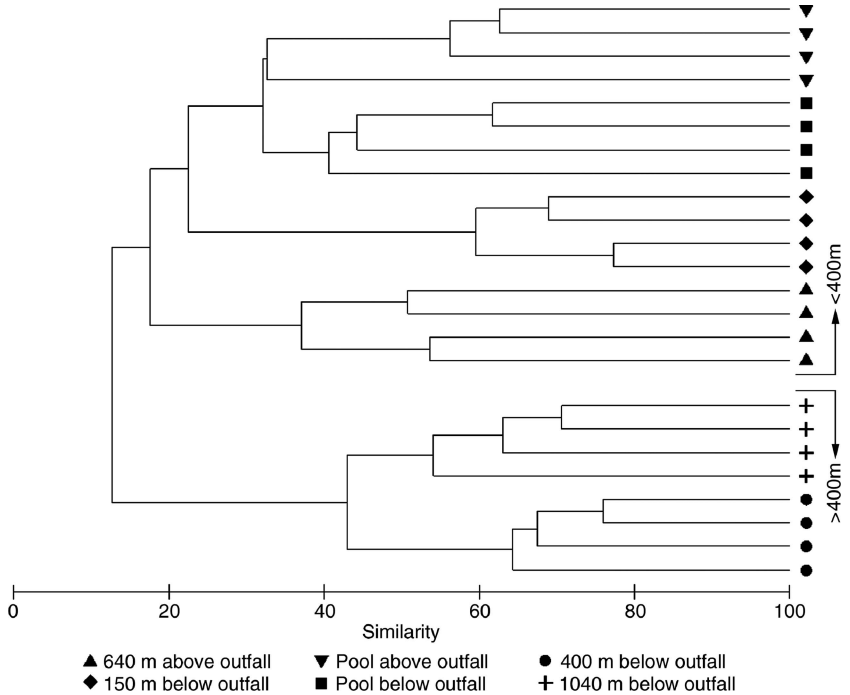

FIG. 4. Cluster plot grouping of (group average) similarity between bacterial community structures from the Hahndorf Creek sediment samples. Community structures were assessed by PCR-DGGE of the 16S rRNA gene. The resemblance matrix was generated by using the Bray-Curtis similarity algorithm on 4th-root-transformed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

TABLE 3. Phylogenetic assignment of bacterial 16S rRNA gene sequences generated from sediment downstream of the WWTP outfall[a]

Phylum	Class	Order or family	No. of sequences found[b]	
			Upstream of 400 m	Downstream of 400 m
Proteobacteria	*Alphaproteobacteria*	*Caulobacteraceae*	2	
		Sphingomonadaceae	2	
		Unclassified	1	3
	Betaproteobacteria	*Burkholderiales*	9	2
		Hydrogenophilales		2
		Rhodocyclales	6	2
		Unclassified	10	2
	Gammaproteobacteria	*Oceanospirillales*		2
		Methylococcaceae		3
		Unclassified		2
	Deltaproteobacteria	*Desulfobulbaceae*	1	
		Cystobacterineae		3
Nitrospirae	*Nitrospirales*	*Nitrospiraceae*	2	
Bacteroidetes	*Sphingobacteria*	Unclassified		2
Actinobacteria		*Actinobacteridae*	2	
Gemmatimonadetes		*Gemmatimonadetes*	2	
Verrucomicrobia	*Verrucomicrobiales*	*Verrucomicrobiaceae*	1	2

[a] Sediment from above and below sampling position 5 (see Fig. 1), 400 m downstream of the WWTP outfall, was sampled.
[b] 16S rRNA gene sequences were matched to the family or class of bacteria according to the classification of the Ribosomal Database Project.

nities upstream and downstream of the 400-m site. Significant differences in phylogenetic structure were detected at the phylum level ($P = 0.007$). The 16S rRNA gene sequences originating from sediment obtained above the 400-m site (site 5) (Fig. 1) were mostly of alpha- and betaproteobacterial origins, whereas sequences originating from the sediment downstream of 400 m were identified as being of alpha-, beta-, gamma-, and deltaproteobacterial origins (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 the stream location ($P = 0.001$) (Fig. 5A). After dipping immediately below the WWTP outfall, the abundance of ammonia-oxidizing bacteria (i.e., *amoA* gene copies) increased with distance downstream. The samples from sites at 150 m, 400 m, and 1,040 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 1,040 m below the outfall than elsewhere. The abundance of *narG* also varied significantly with site ($P = 0.042$) (Fig. 5C), with lower abundance immediately below the outfall and the highest abundances at 400 and 1,040 m downstream. The 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 structures were strongly linked with the 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 the 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 the deposition of organic matter and other material. This site was also characterized 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 the chemical properties of the sediment appeared to have a greater influence on bacterial community structure than changes in physical attributes.

During the 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, and 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 regimen appeared to be the trigger for the manifestation of ecological effects which had impacts on the bacterial community structure. In the absence of the farm dam, it is possible that the ecological effects from the WWTP discharge would be less pronounced but possibly expressed over a

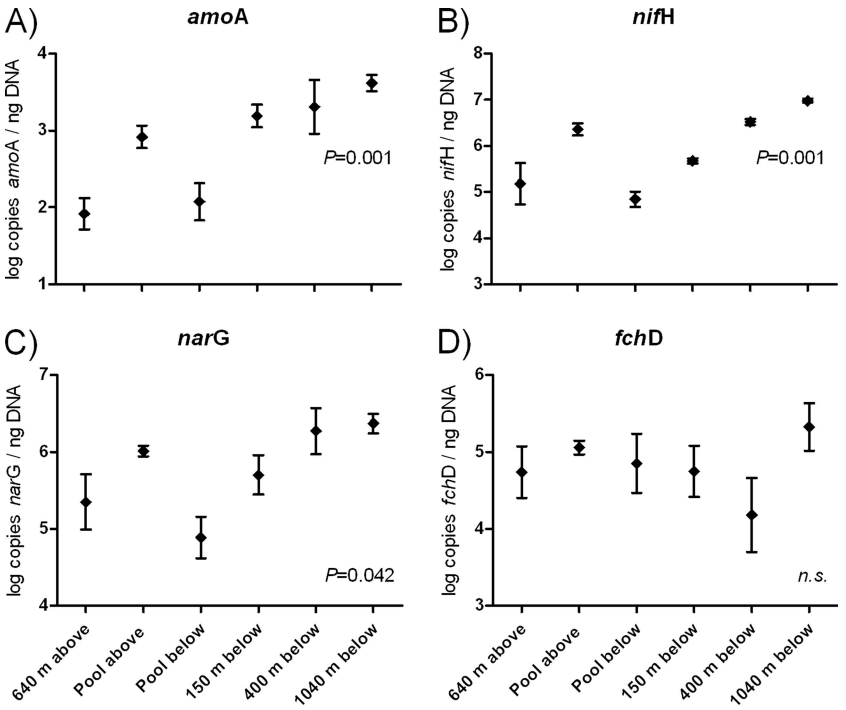

FIG. 5. Log copies of nitrogen- and carbon-cycling functional genes involved in the geochemical cycling of N and C at locations down the Hahndorf Creek. Units on the *y* axis for each graph are log gene copy numbers/ng of sediment-extracted DNA × MBC. *n.s.*, not significant. Error bars show standard errors of the means.

longer distance. In this regard, Birch et al. (2) found that Lake Wallace, on the Coxs River (New South Wales, 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 the ecological impacts of the WWTP will be much reduced downstream of the 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 the perturbation and resuspension of the nutrients in the sediment.

The microbial biomass was highest at the most-downstream sampling location (1,040 m). This may have been due to eutrophication. Although WWTP effluents are known to increase microbial activity (35), it is not immediately apparent why the population size was not higher at the location 400 m below the outfall (Fig. 3). The 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.

The bacterial community structure formed two main clusters, based on distance from the WWTP outfall, with the bacterial community structures at the sites at 400 m and 1,040 m being markedly different from those upstream. Again, this was associated with changes in chemical properties of the stream sediment at the sampling locations. The 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 downstream community had representatives of the gammaproteobacteria which were not detected in the upstream community, including members of *Oceanospirillales* and *Methylococcaceae*. Similar spatial partitioning in microbial community structure as estimated by using DNA sequence analyses has been reported for ammonia oxidizers (7) and for total bacterial communities in reservoir sediments (50). In both cases, the 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 the 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 that 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 upstream sites. *Verrucomicrobia* sequences were detected from both upstream and downstream 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. The 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 they 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 the 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.

The 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 ≥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 that 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 structures 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 the environmental impacts on specific groups of microorganisms known to be involved in ecological processes (48) such as the 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 the geochemical transformation of C and N and found that the biological capacity within the sediment to mediate three key steps in the cycling of N varied greatly along Hahndorf Creek, although the 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 in 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 the N-functional capacity of this site, such as chlorination treatment (42). Importantly, no significant shift in the bacterial community structure was detected in the pools on either side of the outfall, yet the N-functional capacities were different. Below the WWTP outfall, the sizes 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, the MBC measured at this site was by far the highest, and the functional gene abundances were adjusted to the MBC. The limitations of using the MBC as a measure of the microbial population size were detailed in reference 4. With this caveat in mind, the abundance of *amoA* and *narG* increased in accordance with the availability of their enzyme substrates, NH_4^+ and NO_3^-, respectively.

The strong correlation between the 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 activities. 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 stream flow of Hahndorf Creek (estimated from the subcatchment water balance model) (54) indicates strong seasonal variation, from ca. 8 to 14% between April and October, the period of heaviest rainfall, to 20 to 29% between November and March, when rainfall is lowest. Thus, the sediments are likely to be more oxic and nitrification-denitrification more closely linked during the austral winter than during summer, when suboxic 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 organic matter 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 N_2-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. The C:N ratios were widest at the two most-downstream sites (approximately 16:1 to 19:1; 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 indicate a strong cause-and-effect relationship between the loading of carbon and nitrogen into the creek from the WWTP outfall and the 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 an 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 have an impact on decomposition, nutrient cycling, and the provision of balanced C:N:P to aquatic organisms.

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