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Subsurface landfill leachate contamination affects microbial metabolic potential and their expression in the Banisveld aquifer

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Abstract

Microbial communities in groundwater ecosystems can develop the capacity to degrade complex mixtures of chemicals resulting from pollution by landfill leachate. Monitoring this natural attenuation requires insight into the metabolic potential and activity of microbial communities. We contrasted the metagenomes and metatranscriptomes from a leachate-polluted aquifer downstream of the Banisveld (the Netherlands) landfill with uncontaminated groundwater, which revealed changes in microbial genomic content and activity. Banisveld landfill leachate contains mono-aromatic hydrocarbons and the assessment of natural attenuation of these compounds in the aquifer had been a focal point of research. In the contaminated groundwater, active microbial functions were the ones involved in degradation of complex carbon substrates and organic pollutants. We found that benzylsuccinate synthase genes –involved in the catabolism of toluene– were highly expressed close to the source of contamination, confirming the ongoing natural attenuation of organic mono-aromatic hydrocarbon pollution in this aquifer. Additionally, metatranscriptomes were indicative of phosphorus limitation that can constrain total microbial activity and agree with the low phosphate concentrations (< 0.4 μmol/L) in this aquifer. Through the application of metagenomics and metatranscriptomics, we were able to determine functional potential and expression patterns to assess the natural attenuation processes and constraints on microbial communities.

Introduction

The major potential environmental impact related to landfill leachate is the pollution of surrounding groundwater and surface water resources. Historically, landfills were operated without any lining preventing percolation of leachate into the subsurface and these unlined landfills provide a major threat to groundwater resources (Christensen 2001, Kjeldsen 2002). Rainwater facilitates the transport of landfill leachate, containing complex mixtures of pollutants, into the underlying water bodies, which creates anoxic contaminant plumes (Christensen 2001; Bjerg 2003; Brad 2008). The most common type of landfill receives a mixture of municipal, commercial, and mixed industrial waste, where landfill leachate may be characterized as a water-based solution of salts, dissolved organic matter, ammonium, heavy metals, and xenobiotic organic compounds (Christensen 1994, Kjeldsen 2002). Natural attenuation has been shown to be a strategy for remediating landfill-leachate contaminated groundwater resources (Christensen 2001). Indigenous groundwater microbial communities can degrade contaminants depending on presence of favorable geochemical conditions (Christensen 1994; Christensen 2001; Richnow 2003; van Breukelen 2003; Botton 2007). Thus, understanding the microbial metabolic potential and the environmental factors shaping microbial community functioning are important to understand the drivers of biodegradation of contaminants and to develop advanced strategies for bioremediation and management of old landfills.

The aquifer downstream of the Banisveld landfill in the Netherlands is one of the best studied landfill-leachate contaminated aquifers in terms of hydrochemistry and microbiology (Röling 2000; van Breukelen 2003; van Breukelen 2004; Lin 2005; Mouser 2005; Lin 2007; Brad 2008; Staats 2011; Brad 2013; Bjerg 2014). Its leachate contains high amounts of ammonium (19.8 mmol/L), carbon (8.2-10.3 mmol/L dissolved organic carbon) and a broad

range of xenobiotic compounds (Röling 2001, van Breukelen 2003). Despite the lack of any active bioremediation effort, concentrations of mono-aromatic hydrocarbons [i.e. benzene, toluene, ethylbenzene and xylene (BTEX)] in the leachate plume decreased significantly over time, where BTEX oxidation coupled to iron reduction was proposed to be the major biodegradation pathway (van Breukelen 2003; Botton 2007). Downstream of the pollution source, fermentative and iron-reducing conditions and members of the *Clostridiaceae* and *Geobacteraceae* were encountered. 16S rRNA gene-based detection revealed that not yet cultured *Geobacteraceae* were abundant in this contaminated aquifer (Lin 2005), however, further functional screening of groundwater samples for genes involved in BTEX degradation suggested that *Georgfuchsia* species (*Deltaproteobacteria*) were more likely the key organisms in BTEX degradation under iron-reducing conditions at Banisveld (Staats 2011).

Even though the aquifer polluted by the Banisveld landfill is one of the most extensively investigated aquifers, our knowledge about the metabolic functioning of this aquifer is limited to few functional marker genes relating to anaerobic BTEX degradation (Botton 2007; Staats 2011). BTEX compounds constitute only 0.1% of the available dissolved organic carbon (DOC) in this system where DOC from the landfill leachate may support a wide range of biochemical reactions. Lu *et al.* (2012) employed functional gene arrays to study microbial functional potentials in a leachate contaminated aquifer in a broader functional perspective (Norman landfill, OK, USA). While such studies unequivocally provide novel insights into potential microbial metabolism, they are limited to selected genes and functions that they are designed for, and employed DNA as target. The advent of high-throughput sequencing technologies provides a direct access to environmental genomes. Metagenomics offers novel information on functional metabolic potential of microbial communities (Shi 2010), whereas metatranscriptomics

facilitates insights into the potential expression of genes at the time of the sampling (Moran 2012). An important payoff of combining the power of these two meta-omics technologies is the knowledge gained in both microbial metabolic potential and functioning as response to changing environmental conditions. Recently, Jewell *et al.* applied metagenomics and metatranscriptomics to resolve microbial community responses to nitrate injection at a field study site in Rifle, Colorado (Jewell 2016). The authors demonstrated that the use of metatranscriptomics was pivotal to detect functioning of diverse chemolithoautotrophic bacteria in the Rifle aquifer and provided evidence for nitrate-dependent iron and sulfur oxidation.

We employed pyrosequencing of environmental genomic DNA and mRNA on groundwater samples collected from three wells positioned along the central flow path of the leachate plume and a reference well upstream of the Banisveld landfill (Fig. 1A) representing pristine conditions, 1) to gain knowledge on the overall genomic potential and metabolic activity in this system, 2) to test whether the functional gene composition and expression are indicative for intrinsic bioremediation of pollutants through anaerobic processes.

Materials and Methods

Groundwater Sampling

The study location, the Banisveld aquifer, is located in the southeast of the Netherlands (Fig. 1A, Fig. S1). The Banisveld landfill was used for the deposition of household and industrial wastes between 1965-1977. This sandy area lacks artificial or natural liners. Additionally, most of the waste is located below the groundwater table (van Breukelen 2004). As a result, the groundwater in this location is polluted with landfill leachate, where water flow is directed towards a nature reserve. Groundwater flow velocity is estimated to be around 4 meters per year (van Breukelen

2004). Groundwater samples were taken in May 2009 (Fig. 1A). All sampling wells were flushed for 15 – 20 min (>3 well volumes pumped out) before sample retrieval. One reference and three contaminated wells were sampled at a depth of 4-5 meters below surface level, corresponding to the core of the plume. Sampling from the core of the plume enabled us to collect samples representative of anaerobic conditions in the plume. Contaminated wells are located 6, 21, 39 meters downstream of the source of contamination. The reference well is approximately 200 meters upstream of the point of contamination. A total volume of 8-10 L groundwater for each well was filtered through 0.22 μm Sterivex filter (Milipore, Bedford, MA) (<12 minutes per sample, 0.4-0.6 L groundwater per filter). Filters were immediately frozen in liquid nitrogen and stored at -80°C until extraction. The hydrochemistry of groundwater samples was characterized as described previously (van Breukelen 2003). Samples used in this study characterize microbes that were free-living and as a result, the degree to which they also represent microbes attached to sediment matrix in aquifer is uncertain.

Metagenome and metatranscriptome sequencing

Metagenome libraries were created from DNA that was extracted from the individual filters with the MoBio PowerSoil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). Several DNA extracts were pooled together (approximately 2.1-5.0 L groundwater) and 5 μg of genomic DNA per sample was sequenced using 454 Roche GS-FLX Titanium technology (Macrogen, Korea). Four metagenomes were multiplexed in a single GS-FLX run that generated a total of ~480Mbp data (Table 2), where 91-96% of the bases/base calls had quality scores of 40 or above. Metatransciptome libraries were created from RNA extracted from approximately 3.0-5.0 L filtered groundwater with an acid phenol-based method (Zoetendal 2006). Extracted RNA was purified with Qiagen RNeasy mini kit (Qiagen, Valencia, CA) and treated with DNaseI (Roche,

Mannheim, Germany) to remove possible DNA contamination. Integrity and concentration of purified total RNA was checked with the BioRad Experion RNA StdSens kit (Bio-Rad, Hercules, CA); 0.54-1.20 μg total RNA was obtained. rRNA was depleted from the total RNA by enzymatic treatment with the mRNA-ONLY Prokaryotic mRNA Isolation Kit (Epicentre Biotechnologies, Madison, WI) followed by subtractive hybridization with the MICROBExpress kit (Ambion, Austin, TX), prior to mRNA amplification with the MessageAmp-II Bacteria Kit (Ambion, Austin, TX) using the T7-GsuI-$(dT)_{16}$VN (Frias-Lopez 2008) primer. cDNA was synthesized from this amplified mRNA with Superscript Double-Stranded cDNA synthesis kit (Invitrogen, Carlsbad, CA) and the polyA tail in the cDNA was removed with GsuI (Fermentas, St. Leon-Rot, Germany) digestion for 16 h at 30 ºC. Subsequently, approximately 5.5 μg cDNA per metatrascriptome was sequenced with 454 Roche GS-FLX instrument (Macrogen, Korea) (Table 2).

Sequence annotation

454-Titanium sequencing reads were assembled into contiguous sequences (contigs) using Newbler (454 Life Sciences, Roche Applied Sciences, Branford, CT, USA). After quality filtering (average quality score value of 21, minimum read length of 50 bp reads, removing reads containing more than 5 ambiguous bases and/or homopolymers longer than 10 bases), the data set was analyzed to detect replicated sequences (Gomez-Alvarez 2009). This analysis resulted in removal of 0.01-0.03% of the sequences. The remaining sequences from mRNA sequencing were screened for remaining rRNA (that couldn't be removed via depletion) by BLASTN against the SILVA database (Quast 2013) and were removed from the metatranscriptomics dataset (not used in this study). Non-replicate, non-rRNA sequences were characterized by BLASTX searches against NCBI's non-redundant Protein Database (nr) using BLAST 2.2.22 (Altschul

1997) with default settings, except an e-value threshold of 10^{-5} and reporting a maximum of five hits per query. KEGG orthology (KO) annotations were obtained via BLASTX against the KEGG database (max e-value = 10^{-5} and minimum alignment length was 100 bps) and assignments were mapped onto KEGG pathways. The original/raw sequence reads were deposited in the Short Read Archive of the NCBI (BioProject ID: PRJNA217310).

Statistical Analyses

BLASTX output files were binned according to the NCBI taxonomy in MEGAN 5 (Huson 2012) with default LCA-parameters (min score: 35, top percent: 10.0 and min support: 5). All taxa were enabled. The species richness was estimated via rarefaction analysis implemented in MEGAN. The program randomly chooses a total number of reads as subsets in percent increments. For each of these random subsets the number of leaves (hit with at least 5 reads (MinSupport) is determined. The analysis was performed at the most resolved level of the NCBI taxonomy to capture the highest possible richness. A *de novo* comparative metagenomic approach, Compareads was used to compute the pairwise similarity measures between metagenomics and metatranscriptomic reads (Maillet 2012). Two reads were assumed to be similar if they share at least 2 k-mers of 33 nucleotides. The Compareads approach is sensitive to the number of reads used in the analysis, where imbalance between number of sequences in the samples results in false similarity estimates. Therefore, at first metagenomes and metatranscriptomes were analyzed separately as to use as much reads as possible. All metagenomic reads were compared by using a minimum of $2x10^5$ reads for each metagenome and similarly metatranscriptomes were compared separately by using $1.5x10^5$ reads for each metatranscriptome. Additionally, to relate the metatranscriptomes to their corresponding metagenomes, pairwise comparisons were made for metagenomes and metatranscriptomes

originating from the same well, based on a minimum of $1.5x10^5$ reads. Changes in the relative abundance of genes and transcripts were presented as relative abundance of a gene or a gene/functional category in contaminated groundwater with respect to the reference, and were calculated as:

$$\text{Gene enrichment (Relative enrichment)} = \frac{\text{Reads of gene (category)A in contaminated well}}{\text{Reads of gene (catogory) A in the reference well}} \quad \text{[Eq.1]}$$

The relative transcriptional activity (expression level) of each expressed gene was normalized to account for variations in gene abundance in (DNA):

$$\text{Expression level of gene (category) A} = \frac{\text{RNA reads of gene (category) A} / \text{Total RNA reads matching NCBI}-\text{nr}}{\text{DNA reads of gene (category)A} / \text{Total DNA reads matching NCBI}-\text{nr}} \text{[Eq. 2]}$$

All statistical analyses were performed with R (3.2) software using the vegan (Oksanen 2007) and ade4 (Chessel 2012) packages. Statistically significant differences ($p<0.05$) in the NCBI nr-designated relative abundances of DNA and mRNA reads or derived taxonomic assignments were determined in pairwise comparisons (99% confidence level by two-tailed Fisher exact test) between reference and contaminated wells. p-values were adjusted using a false discovery rate correction (Benjamin-Hochberg) where appropriate.

Results

Site hydrochemical properties.

Groundwater hydrochemical parameters are detailed in Table 1. The positioning of the wells and the leachate plume is shown in Fig.1A. Compared to the upstream reference well (uncontaminated), all downstream samples in the core of the leachate plume were much higher in electrical conductivity (EC), alkalinity, methane, ammonium (NH_4^+), total iron (Fe), and higher

in pH. Dissolved organic carbon (DOC) concentrations in the plume wells were similar as measured earlier (van Breukelen 2003). The DOC concentration in the reference well was much higher than measured before both in pristine groundwater at the site (0.25-1.90 mmol/L) (van Breukelen 2003). The low levels of leachate indicators (EC, Alkalinity, CH_4) show the groundwater is clean and the DOC is not from leachate. Possibly the elevated DOC concentration is caused from land-use changes in relation to nature development around the landfill site, which started in 1998. SO_4^{2-} concentrations were on average 100 times lower in leachate-contaminated groundwater in comparison to the reference well and when compared to previous measurements in 1999 and 2004 (van Breukelen 2003; Brad 2008). Moreover, absence of oxygen, low nitrate (NO_3^-; 1.8-10.7 μmol/L) and high Fe (0.8-0.9 mmol/L) concentration further supports the earlier studies on this aquifer, identifying Fe-reduction as the driving redox process. Phosphate levels were low both in the leachate plume (PO_4^{3-}; 0.2-0.4 μmol/L) and in the reference well (<0.1 μmol/L). This is in agreement with earlier measurements in the plume where PO_4^{3-} was typically below detection limit (<0.1 μmol/L). In this region of the Netherlands, phosphate concentrations in aquifers are shown to be significantly lower (<0.15 mmol PO_4^{3-}/L) than the rest of the country (0.40–3.04 mmol PO_4^{3-}/L) (Griffioen 2013). BTEX concentrations in leachate-contaminated wells were now below the detection limit, although they were present before at low levels (van Breukelen 2003). Overall, these findings are in-line with previous hydrochemistry surveys (van Breukelen 2003; Brad 2013) conducted on this aquifer, and confirmed that the downstream wells still sampled from the core of the contaminant plume.

Metagenome and metatranscriptome characteristics

Quality filtering and removal of (artificially) replicated reads from our sequences resulted in 205,763-437,913 (average length 431-648 bp) reads in metagenome (MG) and 145,200-381,787 (average length 343-408 bp) reads in metatranscriptome (MT) libraries (Table 2). MG libraries had a GC content of 48-54%, whereas the GC content of MT libraries was lower and within the range of 43-47%. The mRNA amplification and cDNA synthesis methods applied here are not known to be biased towards low GC microorganisms (Francois 2007). Analysis by Compareads (a *de novo* comparative metagenomic approach not requiring annotation; Maillet 2012) showed that the pollution had a clear impact on genomic content where the reference was different from the other samples, in both DNA- and mRNA-based analysis (Fig. 1B). Both MGs and MTs from contaminated groundwater were highly dissimilar to the reference well indicating an ongoing influence of leachate plume on microbial functions. The MG of the upstream reference had 0.2% similarity to MGs from the plume of leachate. Likewise, its MT was 0.4-4.4% similar to contaminated groundwater. MGs from polluted samples had a higher resemblance and were 27-34% similar to each other. Moreover, their MTs were also highly similar to each other (28-62%). Sample-wise comparison of the MT to the MG revealed a 1.1% similarity for the reference well, whereas it was higher for samples from the plume of pollution (2.2-13.1% similarity) (Fig. 1B).

Taxonomic profiling of metagenomes and metatranscriptomes

The majority of the DNA (91-92%) and cDNA reads (79-90%) that were annotated via BLASTX and MEGAN, originated from *Bacteria*, revealing the presence of 17 phyla (Fig. 1C). *Archaea* comprised 5-8% of sequences of the MG and 7-20% of the MT reads. *Eukarya* and viruses contributed less than 1% of the sequences in each MG. The low amounts of eukaryotic reads in the MTs are likely to be result of the prokaryotic mRNA enrichment step applied during sequencing library preparation. However, it should be noted that eukaryotes are known to

comprise only a small part of the microbial community (<1% of microbial cells) in the investigated aquifer (Brad 2008). Rarefaction analysis showed that a sizeable coverage of species richness was achieved in all eight libraries, with the richness based on the MT libraries always being lower than the corresponding MG library. Only clear difference in richness between the reference and landfill-leachate impacted groundwater was observed at 21 meters (Fig. S2).

In all MG libraries, 16S rRNA genes were low in abundance (0.03-0.09%) and either of unclassified or proteobacterial origin. Pair-wise comparisons ($p<0.001$, Fisher's exact test) and cluster analysis conducted between reference and contaminated groundwater showed that all plume samples were different from the reference, both for the MG and the MT libraries (Fig. 1C). Moreover, MG and MT libraries cluster separately, supporting the low similarities observed between raw MG and MT reads in Compareads analysis (Fig. 1B). MTs of plume samples revealed more variation than MGs.

MEGAN analysis of MG sequences showed that reads affiliated with *Alpha-*, *Beta-*, *and Deltaproteobacteria* and *Firmicutes* were abundant in all samples. We detected phylum and order level changes in the taxonomic distribution of the reads between reference and plume samples. In contaminated groundwater, *Euryarchaeota*, *Betaproteobacteria*, *Deltaproteobacteria, Bacteroidetes* and *Actinobacteria* had higher relative abundances compared to the reference. Especially genes from *Rhizobiales*, *Burkholderiales* (*Betaproteobacteria*), *Desulfuromonadales* (in particular members of *Geobacteraceae*) and *Syntrophobacterales* (*Deltaproteobacteria*) were significantly more abundant ($p<0.001$). In MTs, reads belonging to *Alpha-*, *Beta-*, and *Deltaproteobacteria* were significantly less (within a range of 21-96%) abundant in contaminated groundwater when compared to the reference. In the reference MT, sequences of *Euryarchaeota*, *Bacteroidetes* and *Firmicutes* were more abundant than in the MG.

In comparison to their MGs, MTs of plume samples contained significantly less *Actinobacteria* (27-58%), *Betaproteobacteria* (12−80%), *Deltaproteobacteria* (40-68%) and more *Firmicutes* (9-57%), *Gammaproteobacteria* (13-81%) and *Archaea* (34-79%) reads. There were no large differences in the phylogenetic distribution in the MTs of the contaminated groundwater (Fig. S3) besides the high relative abundance of *Betaproteobacteria* and *Thaumarchaeota* reads in the plume 39 meters downstream of the landfill. Sequences annotated to *Thaumarchaeota* were mostly belonging to an uncultured marine *Crenarchaeota*.

Metabolic functional categories and their expression

Despite the observed taxonomic differences (Fig. 1C) and differences observed through Compareads analysis, annotated sequences in both MGs and MTs revealed a rather similar distribution amongst the KEGG metabolic function categories (Fig. S4). The majority of these genes, 22.1±0.3% in MGs and 21.3±0.6% in MTs, belonged to the category Carbohydrate Metabolism. Metabolism of Cofactors and Vitamins (17.3±0.9% in MG and 16.3±0.9% in MT), Amino Acid Metabolism (15.0±1.1% in MG and 16.2±0.7% in MT) and Energy Metabolism (11.4±0.1% in MG and 12.1±1.1% in MT) showed subtle differences between MGs and MTs. Genes involved in Nucleotide Metabolism comprised on average 10.5±1.6% of the MG and 13.0±1.2% of the MT reads. Xenobiotics Biodegradation and Metabolism genes were detected in 5.8±0.9% of MG and 3.6±0.2% MT reads.

To better reveal the impact of leachate pollution on functional properties of microbial communities, we first compared the relative enrichment of each KEGG category within MGs and MTs of each plume sample to that of the reference (according to Eq. 1) (Fig. 2, Fig. S5). For MGs, there were no large differences between the reference and contaminated groundwater. In

contrast, genes belonging to the Carbohydrate and Xenobiotics Metabolism categories were significantly more abundant in MTs derived from contaminated groundwater, while the opposite was observed for the Nucleotide and Amino Acid Metabolism categories ($p<0.001$, Fisher's exact test). Next, we calculated the relative transcriptional activity (as expression level) in each sample by normalizing the transcript counts to gene abundance in the metagenome of the same sample (Eq. 2). Nucleotide metabolism category had its highest expression in the reference well (Fig. 2). Expression levels for Amino Acid, Energy, Carbohydrate, Lipid, Co-Factor and Vitamin Metabolism categories in contaminated groundwater were not largely different from the reference (Fig. 2). Conversely, genes belonging to the Xenobiotics Metabolism category had lower expression levels than other categories. However, the Xenobiotics Metabolism expression level in plume samples was significantly higher than in the reference ($p<0.05$, Fisher's exact test) (Fig. 2).

Genes involved in organic contaminant degradation and their expression

The KEGG pathway "Benzoate degradation via CoA ligation" was the most abundant pathway in MGs amongst all detected organic contaminant degradation pathways (Fig. S6). Benzoyl-CoA is a central intermediate in the anaerobic metabolism of a range of aromatic compounds (Fuchs 2011). The key genes encoding benzylsuccinate synthase (*bss*) in the catabolism of toluene were detected only in contaminated groundwater MGs, corresponding to 0.026-0.047% of all detected genes. Detected genes were closest related (BLASTX max identity 68-74%, e-value < 10^{-50}) to those from toluene degrading anaerobic isolates belonging to *Beta-* (*Georgfuchsia toluolica*, *Azoarcus aromaticum* EbN1) and *Deltaproteobacteria* (*Desulfobacula toluolica* Tol2, *Geobacter* sp. TMJ1 and *Geobacter* sp. FRC-32). However, expression of the genes coding for *bssA* was detected only at 6 meters and 21 meters downstream of the landfill (Fig. 3) and these genes were

closest related (BLASTX max. identity 72-86%, e-value < 10^{-50}) to those of *Betaproteobacteria*. Metabolic pathways leading to the production of benzoyl-CoA were present and expressed both in reference and contaminated groundwater but their expression was lower compared to *bssA*. Benzoyl-CoA reductase initiating the degradation of benzoyl-CoA to cyclohex-1,5-diene-1-carboxyl-CoA were found in all samples and had relatively high expression levels in contaminated wells (Fig. 3). However, although all MGs contained genes belonging to the 4-hydroxybenzyl-CoA degradation pathway (from phenol degradation), they were only expressed in contaminated groundwater. Furthermore, both MGs and MTs contained genes involved in cyclohex-1,5-diene-1-carboxyl-CoA conversion to acetyl-CoA via 6-hydroxycyclohex-1-ene-1-carboxyl-CoA, suggesting this pathway to be the most prominent in aromatic ring opening. Benzoate degradation can also be achieved via hydroxylation (Chakraborty 2005). However, this pathway was not detected (Fig. 3)

Besides anaerobic toluene degradation pathway genes, transcripts of several genes from intermediary pathways were found in high relative abundance (Table S2). 4-oxalocrotonate tautomerase (EC:5.3.2.6), 4-hydroxybenzoate decarboxylase (EC:4.1.1.61), 2-haloacid dehalogenase (EC:3.8.1.2) and phenol 2-monooxygenase (EC:1.14.13.7) are examples for those highly abundant transcripts. Genes belonging to degradation of complex compounds such as haloalkane dehalogenase (EC:3.8.1.5) for the initial transformation of β-hexachlorocyclohexane and cyclohexanone monooxygenase (EC:1.14.13.22) were found to be expressed 6m downstream of the landfill.

Other catabolic processes and their expressions

We aimed at revealing those genes with higher mRNA transcript abundances in the plume in comparison to the reference. Most of these genes belonged to Carbohydrate and Energy metabolism categories and involved in gluconeogenesis, TCA cycle, galactose, fructose, starch, pyruvate and propanoate metabolism (Fig. S7). Several genes involved in the oxidation of sugar monomers (fructose, sucrose and mannose) were detected, however, their expression was not significantly different between reference and contaminated groundwater. The Banisveld landfill mainly received municipal waste. Among the genes involved in the release of monomers from the type of complex organic polymers one would expect in household waste, chitinases and lipases showed high expression levels in the plume of pollution, whereas expression of xylosidases, cellobiosidases and amylases were lower (Fig. 4).

We detected high expression levels in pathways involved in production and consumption of intermediate metabolites, such as pyruvate and acetate (Table. S1). These include the expression of genes encoding pyruvate-oxidoreductase complex (EC:1.2.7.1), aldehyde dehydrogenase (EC:1.2.1.3) and acetate kinase (EC:2.7.2.1). Expression of genes involved in metabolic pathways for fermentative growth with sugars [*i.e.* reversible lactate dehydrogenase (EC:1.1.1.28), pyruvate formate lyase (EC:1.97.1.4), and acetaldehyde CoA dehydrogenase/alcohol dehydrogenase (EC:1.2.1.10)] were not detected, although these genes were present in MGs. Malate dehydrogenase (oxaloacetate-decarboxylating, NADP+) (EC:1.1.1.40) that could facilitate growth with fumarate had high expression levels in the leachate plume (Table S1), whereas the expression of the genes involved in citrate metabolism were not significantly different between reference and contaminated groundwater. Succinate (EC:1.3.99.1) and NADH dehydrogenase (EC:1.6.5.3, EC:1.6.99.3) had relatively higher mRNA

transcript abundances in contaminated groundwater than formate dehydrogenase (EC:1.2.1.2) and hydrogenases (EC:1.12.99.6, EC:1.12.7.2, EC:1.12.98.1) (Fig. S7).

Genomic potential for terminal electron accepting processes and their expression

Microbial oxidation of organic matter coupled to the reduction of Fe(III) by in particular *Geobacteraceae* was implicated as the major pathway for the degradation of contaminants and natural organic substrates in the Banisveld aquifer (Röling 2001; van Breukelen 2003). Consequently, we have attempted to investigate the expression of several c-type cytochromes that were shown to be involved in Fe(III) reduction by *Geobacteraceae* (Chin 2004; Lovley 2011). Neither *ppcA* and related periplasmic low-molecular-weight cytochromes nor *omcB*, an outer membrane c-type cytochrome (Tremblay 2011), were detected in both MGs and MTs. However, *mtrAB* that encodes an outer membrane protein required for Fe(III) reduction in *Shewanella spp.* (Beliaev 1998) was found in low abundance (0.006-0.009%) and had low expression levels (1.5-1.8) in contaminated groundwater.

Within the nitrogen and methane metabolism categories, which constituted a small fraction of the expressed genes (Fig. S9; resp. 0.05-0.28% and 0.03-0.11%), several genes related to electron accepting processes were expressed. Nitrate (EC:1.7.99.4) and nitrite reductases (*nirK*, , EC:1.7.2.1) had highest relative abundances in MTs of the contaminated groundwater (Fig. S4). Dissimilatory ammonia-forming nitrate reductase (*nrfA*, EC:1.7.2.2) was only expressed at 6m downstream of the landfill but was detected in all MGs. Methane production potential and the expression of the key enzyme methyl-coenzyme M-reductase (*mcr*) were only found in contaminated groundwater (Fig. S9). Genes coding for the sulfite reductase complex (*dsrAB*, EC:1.8.99.3 1.8.99.1) were detected in all MGs but were not found in MTs.

Indicators for nutrient limitation

Investigation of genes involved in phosphorus (P) acquisition showed that in the reference well, the alkaline phosphatase regulon (*phoP*) had a high expression level, whereas expression of alkaline phosphatase (*phoD*, [EC:3.1.3.1]) was not detected (Fig. 5). In contrast, alkaline *phoD* gene expression was higher in the plume (Fig. 5). Inorganic pyrophosphatase (*ppa*, [EC:3.6.1.1]) expressions levels were high in all samples but the reference had the highest expression (Fig. 5). Acid phosphatase (*phoN*, [EC:3.1.3.2]) expression was not detected (Fig. 5). The *nifH* and *nifD* genes relating to nitrogen fixation were detected in all MGs. Even though NH_4^+ concentrations were high in the plume of pollution (5.9-10.5 mmol/L), and low in the reference (<0.1 mmol/L; Table 1), *nifH* transcripts were found only in contaminated groundwater (0.026% at 21 m and 0.006% at 39 m), whereas *nifD* transcripts were detected in all samples (0.012-0.013% of total transcripts).

Discussion

Genomic potential and metabolic functions in Banisveld groundwater

The aquifer polluted by the Banisveld landfill has been a focal point of research on natural attenuation of landfill leachate, with emphasis on mono-aromatic hydrocarbons, in the past decade (Röling 2000, Röling 2001, van Breukelen 2003, van Breukelen 2004, van Breukelen & Griffioen, 2004, Lin 2007, Staats 2011, Brad 2013). Long exposure time has been proposed to promote stability and adaptation of microbial communities to contaminants (Staats 2011), however, information on relevant metabolic potentials and their expression is limited. In this light, we employed pyrosequencing of DNA and mRNA on groundwater samples retrieved from an upstream reference and three wells within the leachate plume. Our results showed that both

genetic potential and microbial activity, as indicated by gene expression, were highly variable throughout the contaminant plume (Fig. 1). Even though based on annotated reads amongst MG and MT libraries reference and contaminated wells were taxonomically different (Fig. 1C), the differences observed between functional gene categories were marginal (Fig. S4). The functional redundancy in microbial ecosystems indeed show parallels to macroecosystems. For example, even though the gut microbiota across different individuals is divergent, the functional gene profiles show similarities (Lozupone 2012). In contrast, our sampling approach focused on the core of the plume assumes a longitudinal sequence of redox processes and is likely to miss fringe processes and related variance in microbial populations. Plume fringes are hypothesized to control the mass transfer of electron acceptors and microbial activities creating hot spots for degradation (*e.g.,* Meckenstock 2015). Van Breukelen and Griffioen (2004) showed, however, that at this specific site contribution of the plume fringe to natural attenuation processes was limited due to absence of oxygen and nitrate in groundwater above the plume.

The detection of functional activity via mRNA from environmental samples presents several challenges, such as low abundance, rapid turnover (Evguenieva-Hackenberg & Klug, 2011) and instability (Moran 2012). While functional gene abundances calculated from mRNA expression are not a reliable proxy for metabolic rates in naturally fluctuating environments (Moran 2012), they can be especially informative for ongoing ecologically relevant processes in stable or low activity environments (Moran 2012) like groundwater. Moreover, MTs can help to resolve important functions and biochemical pathways for contaminant degradation in high diversity ecosystems (Jewell 2016, Men 2017, Guan 2018). Indeed in our case, MTs were more informative than MGs to differentiate significant variations in metabolic functions, where Carbohydrate, Energy and Xenobiotics Metabolism categories were significantly more

frequently expressed in contaminated wells (Fig. 2). MTs were indicative of carbon degradation from complex substances in DOC to pyruvate and acetate. It should also be noted that cells attached to surfaces can account for 90% to 99.99% of the microbial biomass in porous aquifers (Griebler & Lueders, 2009). As our sampling approach only access free-living microbes or the ones associated with colloidal particles, we are unable to provide comprehensive assessment of the all microbial processes. Even though free-living microbes and those attached to the sediment surfaces can share a considerable core community (Zhou 2012, Flynn 2013, Griebler 2014), previously no structural relationship was found between sedimentary and free-living microbial populations in Banisveld (Röling 2001). Likewise, the microbial community structure of sediments collected from the reference and contaminated (6, 21 and 48 m) wells was not impacted by the leachate pollution (Röling 2001). On a geological scale, relatively short time has passed since the beginning of the landfill activities in 1965. Thus, we expect minimal impact on microbial populations associated with the 10,000-100,000 year-old sediments (Röling 2001). Additionally, a large portion of the sediment-bound microorganisms could be protected from the leachate: physically in pores and aggregates or biologically in biofilm form.

Natural attenuation of organic pollutants

Aromatic hydrocarbons such as benzene, ethylbenzene, xylenes, and naphthalene were previously detected and contributed only 0.1% of DOC in leachate from the Banisveld landfill (van Breukelen 2003). Therefore, their impact on overall community composition and metabolic potential is likely to be low. Several other studies also showed evidence for the degradation of organic compounds along the flow path of contaminated aquifers (Cozzarelli 2011) and the presence of a large number of functional genes that are indicative of potential for contaminant degradation (Lu 2012, Abbai & Pillay, 2013). In the Banisveld aquifer, genes involved in

anaerobic BTEX degradation (as a part of "Benzoate degradation via CoA ligation" category) were the most abundant in MGs and highly expressed in the MTs. We were able to detect significant differences in KEGG pathway for "Benzoate degradation via CoA ligation" and relatively high expression levels for genes coding for specialized functions, such as benzylsuccinate synthase-*bss* enzyme complex. In concurrence with previous work presenting the impact of toluene and xylene contamination on functional gene diversity in the Banisveld aquifer (Staats 2011), we provide evidence for the expression of a key functional gene *bbsA* and the pathway leading to 6-Hydroxycyclohex-1-ene-1-carboxyl-CoA production via benzoate Co-ligation in groundwater close to the source of contamination (Fig. 3). Besides BTEX compounds, anaerobic degradation of a diverse group of substrates, such as phenol, vanillin, aniline and phenylalanine, results in production of benzoyl-CoA as an intermediate (Head 2006). Our results suggest that indigenous microbial communities are actively involved in bioremediation of organic contaminants in the core of the plume. Natural attenuation of BTEX compounds by indigenous anaerobic microbial populations was previously studied in detail for the aquifer investigated here (Lin 2005, Botton, 2007, Lin 2007, Staats 2011) and led to the isolation of a previously unknown iron-reducing toluene degrading *Betaproteobacterium*, *Georgfuchsia toluolica* (Weelink 2009). A large diversity of genes involved in anaerobic BTEX degradation was detected via PCR-based screening across the aquifer at different depths and distances from the landfill (Staats 2011). The authors proposed the *Georgfuchsia* species to be the dominant alkylbenzene degraders in the Banisveld aquifer due to the widespread presence of their benzylsuccinate synthase (*bssA*) gene and specialized substrate utilization. *Georgfuchsia toluolica* grows only with few aromatic compounds such as toluene, ethylbenzene and phenol, but not with benzoate, xylenes or benzene (Weelink 2009). We also detected *bss* gene clusters

from *Beta*- and *Deltaproteobacteria* in the MGs derived from contaminated groundwater, however expression of the genes coding for *bssA* were of betaproteobacterial origin, closest related to *Azoarcus aromaticum* EbN1. In line with our findings in the MGs, Staats *et al.* (2011) already showed that *bssA*-containing microbes represented only a small fraction of the total microbial community. Relatively low expression levels observed in MTs were indicative of low metabolic activity.

Other microbial processes in the Banisveld aquifer

In the Banisveld aquifer, indigenous microorganisms are exposed to mixtures of carbon sources originating from the landfill leachate. Accurate regulation of the uptake and metabolism of these carbon compounds is of importance for the functioning and survival of microorganisms (Görke & Stülke, 2008). We detected expression of variety of chitinases, lipases, xylosidases, cellobiosidases and amylases (Fig. 4B) in reference and contaminated wells, however, DOC concentrations remained stable throughout the plume (Table 1). In line with these findings, we hypothesize that carbon processing in this aquifer was constrained by other factors than availability of carbon, such as nutrient limitation. The concept of P limitation in polluted aquifers is generally overlooked and P levels are not reported. Total P concentrations can range between 0.1-23 mg/L (Christensen 2001) in landfill leachates but Christensen *et al*. do not report on P levels in the leachate plumes. A low (<0.1 μmol/L) P concentration was reported in a petroleum-contaminated aquifer (Bennett 2000) in Bemidji, Minnesota, USA, and considered to reflect P limitation as microorganisms colonized feldspars containing trace levels of P. P addition stimulated degradation of oil components in another aquifer in France (Ponsin 2014). Recently, through a series of incubation studies, Hofmann and Griebler (2018) showed that prokaryotic productivity in oxic oligotrophic groundwater can be constrained by the availability of

biodegradable organic carbon and a co-limitation by P. In Banisveld, PO_4^{3-} was below detection limit (<0.1 μmol/L) in the reference well and only slightly above this limit at the three contaminated wells in the core of the plume (ranging 0.2-0.4 μmol/L or 0.02-0.04 mg/L PO_4^{3-}, Table.1). These findings are in line with previous measurements from the same wells suggesting that PO_4^{3-} concentrations were consistently low in this plume. In contrast, levels of NH_4^+ were high (3-19 mmol/L) resulting in high N:P ratios. Even though decomposition of organic waste in the landfill can produce PO_4^{3-}, sorption to iron-oxides during transport through the aquifer is a likely mechanism limiting PO_4^{3-} availability. Consequently, we hypothesize that high N:P ratios in the plume core together with low PO_4^{3-} concentrations result in P limitation for microbial growth. Exploration of the expression of P acquisition genes, as indicators for nutrient limitation, supports this hypothesis. Phosphatases are produced when the available P content falls to critical levels for microbial growth (Acosta-Martínez & Tabatabai, 2011). Previous studies conducted in P starved bacterial isolates showed elevated expression of *phoD* (Adams 2008; Karl, 2014). Expression analysis and metatranscriptomics data from *Proteobacteria* and *Actinobacteria* suggest that expression of *phoD* is highly regulated by phosphate concertation and induced under phosphate limitation (Apel 2007, Santos-Beneit 2015, Oyserman 2016). This gene had a relatively high expression in leachate contaminated groundwater. Another P acquisition gene, *phoP*, is induced at low phosphate concentration, although its expression is not strictly tied to P limitation (Yao 2016). We detected high expression of this regulon only in the reference well. P limitation might be an important constrain on microbial growth and activity in the Banisveld aquifer and contribute to the persistence of contaminants.

Fe(III) was identified as the major redox process in the aquifer polluted by the Banisveld landfill (van Breukelen 2003) with members of the iron-reducing *Geobacteraceae* dominating

microbial communities and widespread throughout the plume of pollution (Röling 2001, Lin 2005). Both MGs and MTs contained anaerobic species that could potentially perform Fe(III) reduction. Based on the low expression of important genes for Fe(III) reduction such as *Geobacter spp.* genes *ppcA* and *omcB,* it is tempting to speculate that besides *Geobacter spp.* other microbial populations are of importance to Fe(III) reduction in this aquifer. For example, we also detected the expression *mtrAB* gene from *Shewanella spp.* However, it should be noted that *Geobacter spp.* contain hundreds of genetically different cytochromes (Lovley 2011) that are potentially involved in Fe(III) reduction.

We detected genes involved in methanogenesis only in contaminated groundwater. It is likely that methanogenic populations originate from the landfill itself and are not part of the indigenous populations (Röling 2001). Banisveld landfill leachate contains high concentrations of methane, whereas $\delta^{13}C–CH_4$ studies indicated that methanogenesis did not occur in the plume (van Breukelen 2003). Genes involved in methanogenesis, especially the key *mcr* gene, were not highly expressed. It should be noted that the *mcr* gene can also be employed in methane oxidation (Knittel & Boetius, 2009). Therefore, its expression cannot be directly interpreted as a source for methane production. Moreover, it had been previously shown that nitrite-dependent anaerobic methane oxidation potential is present in the Banisveld aquifer (Luesken 2011) and $\delta^{13}C–CH_4$ studies suggested that anaerobic methane oxidation occurred (van Breukelen & Griffioen, 2004). As a result, methane is likely to be a carbon source rather than end product in this system. In addition to methanogenesis, sulfate-reduction potential was present, however, its expression was not detected. Previously, $\delta^{34}S$ measurements suggested that sulfate reduction is not occurring within the contaminant plume (van Breukelen 2003).

Conclusions

Application of two omics approaches enabled us to investigate the impact of landfill leachate contamination both on microbial functional diversity and activity in detail. Genes encoding mono-aromatic hydrocarbon degradation were active and indicative of ongoing natural attenuation of organic contaminants in this system; however, they constitute a small fraction of ongoing microbial processes. Strikingly, MGs did not resolve the functional differences between reference and contaminated wells. Even though metagenomics is an increasingly popular tool to access microbial metabolism, it is likely to fall short in detecting critical processes in slowly evolving systems such as aquifers. MTs, however, did provide new insights into ongoing microbial processes in Banisveld. We found evidence for the expression of genes that are potentially involved in DOC degradation in all wells and identified nutrient limitation – specifically P limitation– as a potential constraint for microbial activity in contaminated wells. These findings brings further concerns on management of contaminated groundwater sites where besides impact of the contamination lack of necessary nutrients might limit the success of natural attention of pollutants.

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Figure Legends

Figure 1. A) Sampling locations along the aquifer polluted by the Banisveld landfill. Grey represents the shape of the plume and sampling wells are demonstrated in red. Groundwater sampling wells are indicated by their corresponding distance from the landfill. B) Similarity matrix resulting from the comparison of MG and MT reads with Compareads between different samples from the Banisveld aquifer. Color levels correspond to similarity levels; red-high similarity, blue-low similarity (see scale, 0 dissimilar – 1 similar). Based on both DNA and mRNA analyses, the reference was highly dissimilar to the contaminated wells. The table shows the similarity calculated between DNA and mRNA reads in each well. C) Cluster analysis based on Euclidean distance and generated with the MEGAN software with the UPGMA algorithm for Banisveld MG and MT read annotations from BLASTX results. Pie charts show the phylogenetic distributions of the reads.

Figure 2. Differences in expression levels (relative transcriptional activity) of KEGG metabolism classifications among sampling wells. Expression levels were calculated in Eq.2 for each well.

Figure 3. Expression levels (relative transcriptional activity; y-axis) of genes belonging to toluene, benzoate and 4-hydroxybenzyl-CoA degradation pathways. Expression of the key genes converting toluene to benzylsuccinate (benzylsuccinate synthase, *bss*) was detected only at 6 meters and 21 meters downstream of the landfill. Benzoyl-CoA reductase involved in the degradation of benzoyl-CoA to cyclohex-1,5-diene-1-carboxyl-CoA were found in all MGs and had relatively high expression levels in contaminated wells.

Figure 4. Expression levels (relative transcriptional activity) of several genes encoding carbon degradation such as chitinases and xylanases were higher in contaminated groundwater wells. Expression levels were calculated in Eq.2 for each well.

Figure 5. Expression levels (relative transcriptional activity) of several genes encoding for phosphate acquisition in Banisveld aquifer.

Table.1 Hydrochemistry of the Banisveld aquifer. EC: Electrical conductivity; DOC: Dissolved organic carbon

	Reference well	6 meters	21 meters	39 meters
pH	5.5	6.5	6.1	6.5
Temperature (°C)	9.1	12.2	11.0	11.0
EC (μS/cm)	129	2150	2046	2640
Alkalinity (mmol/L)	0.1	24.7	26.3	27.0
O_2 (mmol/L)	<0.1	0.0	0.0	0.0
NH_4^+(mmol/L)	<0.1	5.9	7.0	10.5
NO_3^- (μmol/L)	31.9	10.7	7.7	1.8
Total Fe (mmol/L)	0.1	0.8	0.9	0.8
SO_4^{2-} (μmol/L)	230	1.5	1.1	2.3
PO_4^{3-} (μmol/L)	<0.1	0.2	0.4	0.2
CH_4 (mmol/L)	<0.1	1.4	1.6	1.7
DOC (mg C/L)	54.8	52.0	52.0	70.1
Total cations (meq/L)	0.9	25.3	26.9	28.5
Total anions (meq/L)	0.7	25.7	27.5	29.1

Table.2 Sequencing output

	Metagenome				Metatranscriptome			
	Reference	6 meters	21 meters	39 meters	Reference	6 meters	21 meters	39 meters
Number of sequences	205,763	222,781	437,913	236,215	373,596	282,244	145,200	381,787
Average length (bp)	431	438	648	425	397	405	343	408
GC content	0.54	0.54	0.48	0.55	0.46	0.47	0.46	0.43
% 16S rRNA genes	0.03	0.04	0.09	0.04	20.2	13.5	4.5	15.5