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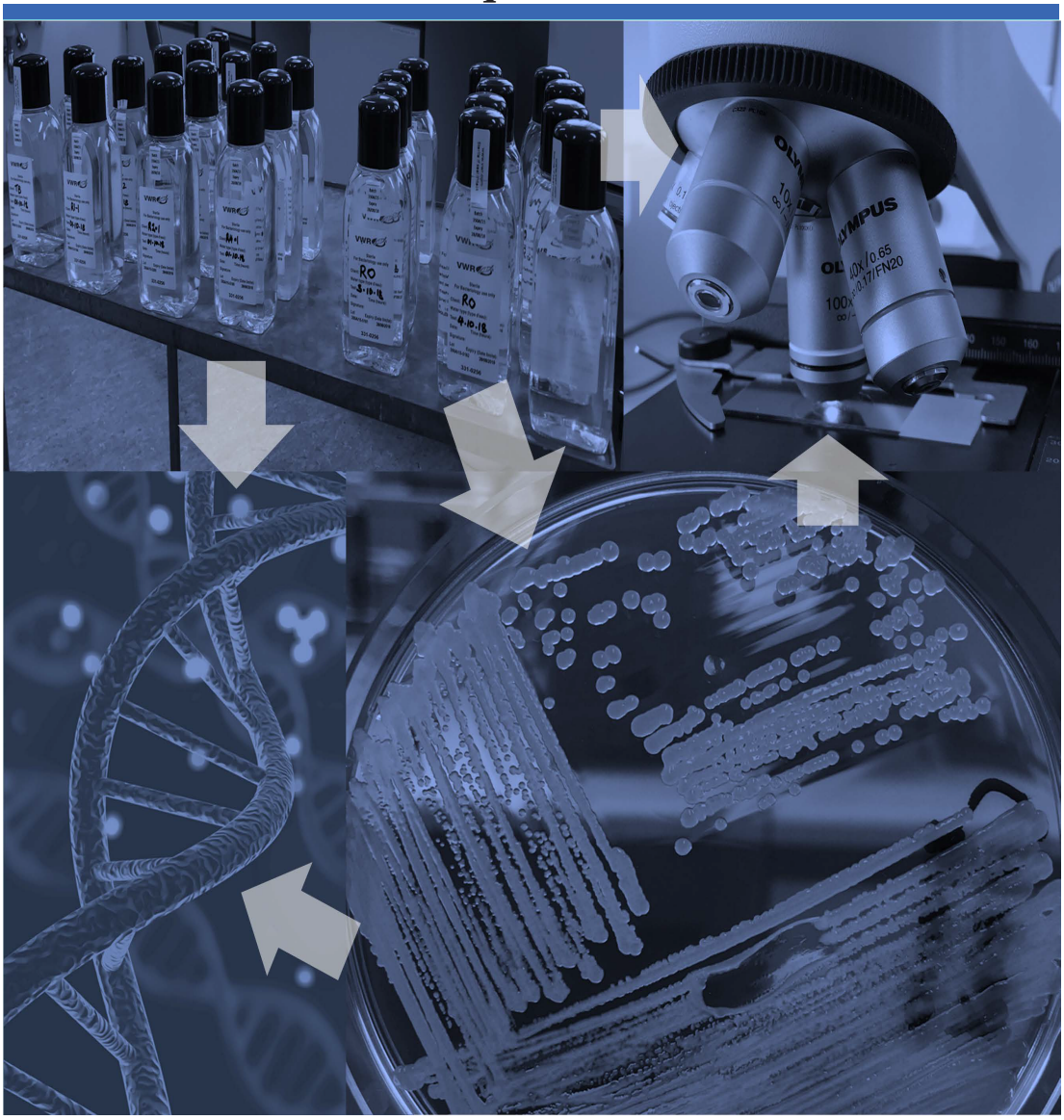
Daniel Abiriga

The microbiology and geochemistry of a landfill-contaminated aquifer



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Ecology

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Preface

This dissertation is based on results from a PhD research project "the microbiology and geochemistry of a landfill-contaminated aquifer". The project was initiated to address knowledge gaps identified during a master research project on the same aquifer. It was found necessary to understand how the operation of the landfill affected the microbiome of the aquifer and whether the resident microorganisms have the potential to bioremediate the pollution; and that more monitoring of geochemical parameters needed to be carried out in order to be able to conclude on the prospect of the natural remediation process. The present study is, therefore, twofold: microbial ecology, and groundwater geochemistry. The project was led by Associate Professor Harald Klempe, and co-supervised by Professor Andrew Jenkins and Associate Professor Live Semb Vestgarden. The project was funded internally by University of South-Eastern Norway. Findings from the study are presented and knowledge on microbiology, bacteriology, geochemistry, and hydrogeology is necessary, but not required to understand the contents presented herein.

To the readers, I hope you find this piece of work enjoyable!

Bø, June 2021

Daniel Abiriga

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Abstract

Thousands of aquifers worldwide have been polluted by landfill leachates and many more remain threatened. However, the ecology of these perturbed aquifers remained understudied. The current study incorporates aspects of both the microbiology and geochemistry of a leachate-receiving aquifer. The microbiological component includes comparing the results of different microbiological techniques; assessing the effect of season, water chemistry, distance, and time on the composition of the aquifer microbial communities; determining the overall microbial assembly and co-occurrence network; and comparison of planktonic and sediment-attached microbial communities. The groundwater geochemistry was used to evaluate the significance of the natural attenuation processes in the landfill-aquifer system.

Comparisons of results from culture-based approach, direct fluorescence microscopy, and 16S rRNA metabarcoding show a good concordance. Results from variation partitioning analyses show that the microbial community composition of the groundwater was influenced by the groundwater geochemistry, distance, season, and time, although both season and time seems to have played a minor role. The microbial co-occurrence network analysis results indicate that the microbial communities in the aquifer assemble deterministically. A key contributor to the deterministic assembly is the landfill leachate, which caused an ecological gradient to develop along the groundwater flow path as reflected by the groundwater geochemistry. Comparison of the microbiota of groundwater and sediment indicate a strong difference in the microbial community composition between the groundwater and the sediment. While the planktonic communities were dominated by *Proteobacteria*, the sediment-attached communities were enriched in *Acidobacteria*. Thus, for a more complete characterisation of an aquifer microbiome, it is important to sample both groundwater and aquifer sediment.

These findings shed light into the microbiology of an understudied ecosystem and has clearly demonstrated that the operation of the landfill has altered the microbial composition of the aquifer. The long-term analysis of the groundwater geochemistry

suggests that the landfill has attained its stabilised stage, as indicated by the tailing-off of contaminants, which hints on the possibility of the aquifer recovery. This underscores the significance of non-invasive natural attenuations and has significant consequences for future pollution intervention strategies. This thesis provides a good resource to researchers and environmental practitioners both in the government and private sectors, particularly for strategic planning, designing, implementation and management of site remediation. It is of interest to microbiologists, microbial ecologists, geochemists, hydrogeologists, and environmental scientists.

Keywords: microbial ecology; groundwater microbiology; aquifer sediment microbiology; groundwater chemistry; natural attenuation

List of papers

Article 1

Daniel Abiriga, Andrew Jenkins, Kristian Alfsnes, Live S. Vestgarden and Harald Klempe (2021). Characterisation of the bacterial microbiota of a landfill-contaminated confined aquifer undergoing intrinsic remediation. Science of the Total Environment, 785, Article 147349. DOI: https://doi.org/10.1016/j.scitotenv.2021.147349.

Article 2

Daniel Abiriga, Andrew Jenkins, Kristian Alfsnes, Live Semb Vestgarden and Harald Klempe (2021). Spatiotemporal and seasonal dynamics in the microbial communities of a landfill-leachate contaminated aquifer. FEMS Microbiology Ecology, fiab086, DOI: htt://doi.org/10.1093/femsec/fiab086.

Article 3

Daniel Abiriga, Andrew Jenkins, Kristian Alfsnes and Harald Klempe (2021). Microbial deterministic assembly and co-occurrence network in an aquifer under press perturbation. Submitted to Frontiers in Microbiology.

Article 4

Andrew Jenkins, Daniel Abiriga, Kristian Alfsnes, Live Semb Vestgarden and Harald Klempe (2021). A comparison of sediment and groundwater microbiomes in a landfill leachate-contaminated aquifer undergoing intrinsic remediation. Manuscript.

Article 5

Daniel Abiriga, Andrew Jenkins, Live S. Vestgarden and Harald Klempe (2021). A nature-based solution to a landfill-leachate contamination of a confined aquifer. Under second review in Scientific Reports.

Abbreviations

CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
MSW	Municipal Solid Waste
NMDS	Nonmetric multidimensional scaling
OTU	Operational Taxonomic Unit
rRNA	ribosomal Ribonucleic Acid
TOC	Total Organic Carbon
PERMANOVA	Permutational multivariate analysis of variance
RDA	Redundancy analysis

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1 Introduction

Freshwater is an essential natural resource. Among the freshwater reservoirs, groundwater is an important component and the main source of potable water for drinking, agriculture and industry in many countries globally (Mays and Scheibe, 2018; O'Connor et al., 2018; Zaporozec and Miller, 2000). In addition, groundwater ecosystems harbour the largest terrestrial biome, which accounts for up to 40% of the earth's prokaryotic biomass (Griebler and Lueders, 2009; Griebler et al., 2014). Unfortunately, the ever-increasing human population exerts pressure on the finite water resources available to each country. The past decades have been characterised by a tremendous increase in human activities that negatively influence groundwater quality (Brad et al., 2013; Chapman et al., 1996; Pous et al., 2018; Röling et al., 2001), with a concomitant decline in access to clean freshwater (Appelo and Postma, 2005).

One of the leading causes of groundwater contamination is landfill operation. All over the world, landfills have served as the ultimate destination for municipal wastes (Reinhard et al., 1984), and continue to do so in many countries across the globe (Chen et al., 2017; Eggen et al., 2010; Mouser et al., 2005). In Norway and as was elsewhere, there was little recycling of wastes until the 1990s and most of the wastes from households and industries were deposited in municipal solid waste (MSW) landfills with no provision for treatment or containment of the resultant leachate. The term leachate refers to the liquid formed from precipitation or moisture that drains through waste bodies and contains degradative inorganic and organic products (Lema et al., 1988). The siting for historic landfills hereafter 'old landfills' was based on convenience – the need to fill man-made abandoned or natural pits than on geological criteria of suitability (Hamer, 2003). Although newer sanitary landfills are equipped with liners that help minimise leakage of leachate, old landfills, thus, represent a major source of groundwater contamination (Brad et al., 2013; Kjeldsen et al., 2002). Revdalen Landfill (the present study site) represents one such historic old landfills. It was active from 1974 to 1996 and its operation led to the contamination of an aquifer situated underneath the waste body. Pollutants of environmental concern such as heavy metals and toxic

organic compounds e.g., polycyclic aromatic hydrocarbons have been detected in the groundwater samples (Abiriga et al., 2020).

Old landfills operated before waste segregation was adopted require special attention, because they were filled with a mixture of nearly anything (Christensen et al., 2000) and the leachate they generate is often highly variable and complex in nature, consisting of a cocktail of contaminants (Baun et al., 2003; Christensen et al., 2000; Eggen et al., 2010; Moody and Townsend, 2017; Mouser et al., 2005). This makes the landfills a potential public health concern as they may contain both legacy and emerging pollutants (Eggen et al., 2010; Lapworth et al., 2012), as well as acting as hotspots for antibiotic resistance selection in the environment (Chen et al., 2017).

The complex composition of the leachate makes remediation of landfill-polluted aquifers a more costly and demanding operation than the remediation of hydrocarbon-polluted aquifers (Christensen et al., 2000). This is exacerbated by the characteristically long leaching patterns experienced with landfills, which may last for e.g., centuries (Bjerg et al., 2011). Thus, non-invasive passive remediation options, which utilise naturally occurring degradation, dilution and retardation processes, are preferred over expensive conventional active remedial options such as the pump and treat techniques (Azadpour-Keeley et al., 2001; Majone et al., 2015; Pleasant et al., 2014; Rügner et al., 2006). Other benefits of natural remediation include efficiency and lack of secondary wastes that would require additional disposal stage (Azadpour-Keeley et al., 2001; Logeshwaran et al., 2018; Majone et al., 2015; Rügner et al., 2006). The major disadvantage of natural remediation as applied in landfills is the long time required to achieve remediation targets. Thus, pollutants have to be monitored for a long period, which is costly (Sizirici and Tansel, 2015) and this has led to few literature from such interventions because projects end prematurely before substantial recovery is achieved. Revdalen Aquifer in its present status is considered to be partially remediated after nearly thirty years of monitoring and the data should contribute towards enhancing our understanding of the potentials provided by natural attenuation.

Traditionally, groundwater remediation has been demonstrated empirically by measuring geochemical parameters, with little use of microbial data (Mouser et al., 2005). This explains why the literature from landfill leachate plumes is dominated by studies involving groundwater geochemistry. Over the years, however, it has become apparent that studying microbial community composition in addition to geochemical measurements offers a more complete picture of remediation (Lu et al., 2012; Pilloni et al., 2019; Röling et al., 2001). This is particularly true because microbially-catalysed reactions dominate processes that drive natural attenuation of both organic and inorganic contaminants in the environment (Smets and Pritchard, 2003), which underscores the importance of the intrinsic microorganisms.

Studies on the microbiology of such environments may involve use of metabolic functional analysis, stable isotope probing, metagenomics, and identifying resident microbes to unravel their ecological characteristics such as pollutant transformation capabilities, abundance and distribution (Lueders, 2017; Majone et al., 2015; Mouser et al., 2005; Scow and Hicks, 2005; Smets and Pritchard, 2003). Previous studies from landfill-impacted aquifers have been reported (Albrechtsen et al., 1995; Chen et al., 2017; Holm et al., 1992; Lin et al., 2007; Ludvigsen et al., 1999; Mouser et al., 2005; Taş et al., 2018), but the impact of landfill leachate on the microbial ecology of leachate-receiving aquifers requires more elucidation. The prolonged discharge of leachate into groundwater by landfills is likely to leave an ecological footprint on aquifers, including permanently eliminating the native microbial species (Herzyk et al., 2017; Song et al., 2015; Zhou et al., 2014) while allowing for incursion of new species in the aquifer. The global challenge associated with the operation of landfills thus threatens the rich biodiversity in aquifers. The study of landfill-leachate-polluted aquifers is, therefore, important to assess changes in the aquifer ecosystem.

Aspects of microbial ecology of leachate plumes that the present study addressed include spatiotemporal changes in the microbial community composition. Studying the microbial ecology of landfill leachate plumes not only informs on the effect of the leachate on the microbial communities, but also informs on the population of microbes

that may be involved in the degradation of contaminants in the plumes. Thus, focusing on the abundance, diversity and dynamics of these microbes provides a better understanding of the affected groundwater ecosystems (Pilloni et al., 2019). In addition, the study performed microbial co-occurrence network and assessed the relative contribution of deterministic versus random microbial community assembly. Network analysis has been successfully applied to study microbial co-occurrence across multitudes of habitats (Barberán et al., 2012; de Vries et al., 2018; Horner-Devine et al., 2007; Ju et al., 2014; Lupatini et al., 2014; Williams et al., 2014) and has helped in resolving ecological questions that cannot be addressed by use of other community metrics such as alpha and beta diversity (Lupatini et al., 2014). Analysing co-occurrence patterns can decipher complex microbial systems such as providing information on the ecological traits of uncharacterised microbes that co-occur with well characterised microbes (Barberán et al., 2012; Fuhrman, 2009; Williams et al., 2014). Lastly, the comparison between planktonic and sediment-attached microbial communities was conducted. It has been reported that the two habitats harbour different microbial communities (Flynn et al., 2008; Scow and Hicks, 2005; Smith et al., 2018a). However, most of the literature comparing planktonic communities with sediment-attached communities relied on surrogate sediment samples. In the present study, comparisons were made on fresh aquifer sediment samples taken a few centimetres away from the well from which corresponding water samples were obtained.

2 Objectives

The main objective was to assess the impact of Revdalen Landfill on the microbiology and geochemistry of the leachate-receiving aquifer and to evaluate the significance of the natural remediation in the aquifer.

Specific objectives were:

1) compare the results of different microbiological techniques, **paper I**.

It is well acknowledged in the scientific community that each of the different microbiological techniques has its own strengths and limitations. For example, the culture method is biased to only quantify culturable bacteria and is unable to account for nonculturable ones, including microbes requiring special growth conditions such as co-culture growth medium. On the other hand, both fluorescence microscopy and 16S rRNA gene metabarcoding give no distinction between culturable and nonculturable microbes. However, direct fluorescence microscopy suffers from lack of discriminative power to exclude other cell types than bacteria, while the 16S rRNA metabarcoding sequences may include reads from dead microbes, including naked DNA from the environment. Thus, comparing data from the different techniques helps validate the results. Finally, the putative degradative capabilities of both the pure isolates and the 16S rRNA operational taxonomic units (OTUs) were described to associate their occurrence to degradation and groundwater geochemistry.

2) examine the effects of season, groundwater chemistry, distance, and time on the composition of the aquifer microbiology, **paper II**.

This paper examined the diversity, abundance, and changes in microbial community composition, as a function of the groundwater chemistry, distance, time, and season to give an insight into the microbiome of Revdalen Aquifer. The groundwater geochemistry used in the analysis included 15 physicochemical variables: pH, electrical conductivity, dissolved oxygen, sodium, potassium, ammonium, calcium, magnesium, iron, manganese, sulphate, chloride, nitrate, total nitrogen, and total organic carbon (TOC).

In terms of distance, both lateral and vertical distance were considered. The seasons considered were spring and autumn. These are the seasons for which groundwater recharge is expected to be highest (Kløve et al., 2017) and it was of interest to see if this will affect the microbial composition. The effect of time was examined to ascertain changes in the microbial communities over the two years of sampling.

3) determine microbial co-occurrence and the relative contribution of deterministic versus random microbial assembly in the aquifer, **paper III**.

Here, microbial co-occurrence network of OTUs from the 16S metabarcoding was generated to identify taxa coexistence and the keystone taxa (Banerjee et al., 2018; Lupatini et al., 2014) in the aquifer. In addition, the relative importance of deterministic versus random microbial community assembly was assessed and the overall contribution of measured variables in explaining the microbial composition was quantified. In this analysis, samples obtained from a single well level in each of the wells located in the contaminated aquifer were used.

4) compare planktonic and sediment-attached microbial communities, **paper IV**.

This paper contrasts the microbial communities in the groundwater (planktonic) with those in the aquifer sediment (biofilm). Both samples were subjected to 16S rRNA metabarcoding. In addition, heterotrophic plate counts for the two sample types were compared.

5) evaluate the significance of natural remediation in the aquifer, **paper V**.

Here, the paper evaluated the significance of the natural attenuation in remediating Revdalen Aquifer. Groundwater geochemistry data from 1992 to 2019 was used in the analysis. The analysis was performed to ascertain how the groundwater quality changed with distance along groundwater flow path, and with landfill stabilisation phase.

3 Materials and methods

3.1 Study site

Revdalen Landfill and Revdalen Aquifer are located within the township of Bø in Vestfold and Telemark County, Norway, at coordinates 59°25'58.26"N and 9°06'1.53"E. The landfill is located near the aquifer outcrop region. It was established to hold municipal solid wastes from Bø and Sauherad Municipalities (now merged to form Mid-Telemark Municipality) in 1974-1997. Being an old landfill, it received all kinds of wastes ranging from household waste to industrial waste and wastewater treatment sludge. The landfill neither has liners nor leachate collection system, but the original plan was that the leachate would penetrate the sand deposit which should act as filters to treat the leachate. However, over the years, the landfill leachate managed to break through the sand/gravel layer without sufficient treatment, which has let to contamination of the groundwater reservoir in an aquifer underneath.

Revdalen Aquifer is a confined aquifer in a complex of quaternary deposits consisting of moraines of hard-packed till, subglacial glaciofluvial deposit, and glaciofluvial delta deposits (Klempe, 2004). The till acts as both the top aquitard and aquifer bottom. The aquifer matrix comprises of deposits of medium to high permeability sand and gravel. It is a small aquifer of 5-8 m thick and 70-100 m wide constrained by bedrock walls and covers a distance of about 1.7 km (Abiriga et al., 2020; 2021; Klempe, 2015). The aquifer is recharged by a small watershed at the upper end near the landfill area. Due to the location of the landfill in the recharge zone, the recharge water transports pollutants along as it infiltrates the aquifer. A plume of the leachate was found to reach up to 324 m away from the landfill. A monitoring programme has shown variation of groundwater quality in space and time, and indicate that the aquifer acted as a good treatment plant to the leachate (Abiriga et al., 2020; 2021, Klempe, 1992; 1996; 2001). The major current land use types in the area are quarrying of the Precambrian bedrocks, and gravel and sand mining of the esker of glaciofluvial deposit.

3.2 Sampling procedure

3.2.1 Groundwater

Three multilevel monitoring wells (R1, R2 and R4) were established along the groundwater flow direction to monitor the groundwater quality (Figure 1). Wells R1 and R2 were constructed using the Waterloo Groundwater Monitoring System. R1 has five levels: R101-R105 (at 126, 125, 124, 123 and 122 m.a.s.l); R2 has four: R201-R204 (at 122, 121, 119 and 118 m.a.s.l); and R4 has three: R401-R403 (at 118, 117 and 114 m.a.s.l). In addition, a background well (R0 having a single screen) was established in an adjacent aquifer for benchmarking the local groundwater quality. The well codes R1, R2, R4 and R0 may be used synonymously with terms proximal, intermediate, distal, and background, respectively.

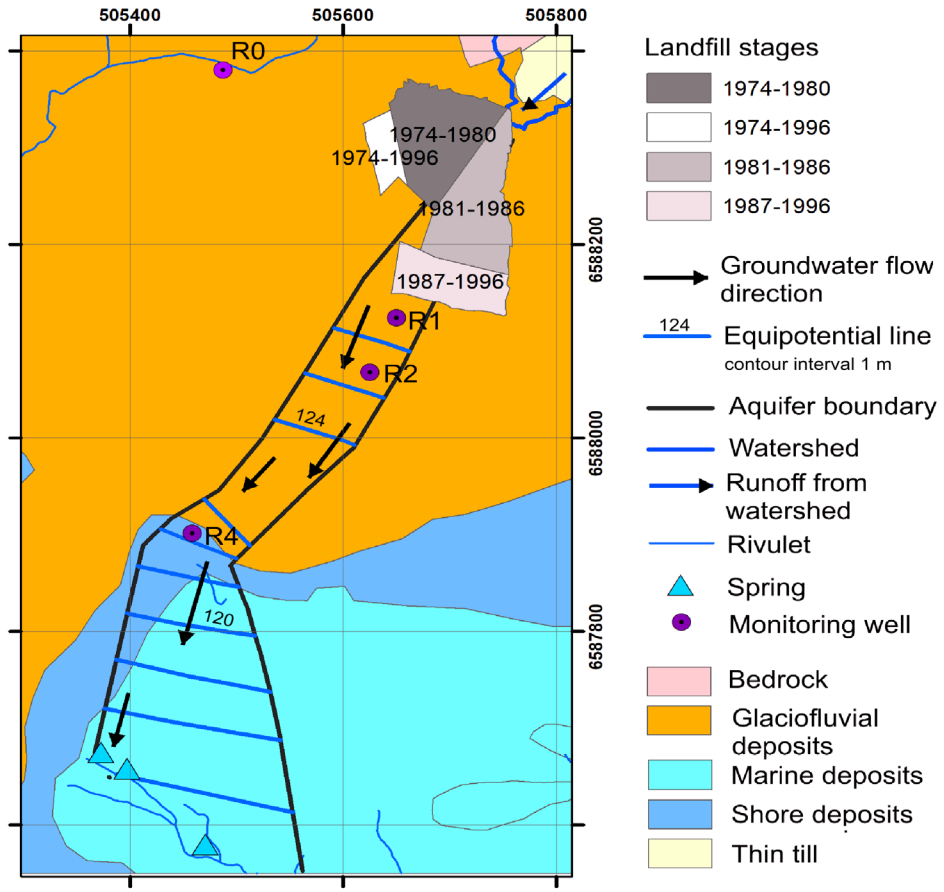

Figure 1. An overview map of the study site showing the landfill and the sampling wells R0, R1, R2 and R4; the hydrogeological properties, and the geology. For clarity of readability, the location for the aquifer sediment samples is not shown; it is only ~30 cm downgradient of R4.

Groundwater sampling in R1 and R2 were performed by a repeated cycle of applying nitrogen pressure through drive valves and venting, until groundwater samples emerge from the sample Teflon tubes with a gentle pulsating flow into the sample bottle. Samples from R4 were taken by using a hand pump, while samples from R0 were taken using a submersible pump. In all cases, samples were collected after purging the well volume (R0 and R4) and after micro-purging (R1 and R2) in accordance with ISO 5667-11 (2009). Samples for groundwater chemistry were collected in 500 ml PETE bottles, while samples for microbiology were collected in sterile 350 ml PETE bottle without headspace. pH and electrical conductivity were measured onsite, while dissolved oxygen was fixed onsite and later determined in the laboratory using the Winkler method. The samples were maintained at ≤4 °C using icepacks and a cooler box and transported to the laboratory at University of South-Eastern Norway. The samples were collected twice a year in spring and autumn in 2018-2019. In addition, during each sampling campaign, a timepoint samples for two days were collected from one level in R1 (R104), R2 (R203), R4 (R402) and R0 (not a multilevel well).

3.2.2 Aquifer sediment

The aquifer sediment samples were obtained from only a single location in the R4 area, because it is both destructive and expensive to perform drilling near the aquifer outcrop (R1/R2) area. Three saturated aquifer sediment samples were obtained at depths of 6-7, 8-9 and 9-10 m 30 cm downstream of R4 using the piston method. The sediment samples were dispensed into sterile 500 ml glass bottles, kept at 4 °C and transported to the laboratory. In the laboratory, approximately 25 g of the saturated sediment subsamples were transferred to a 50 ml Falcon tubes and stored at -70 °C prior to DNA extraction. The remaining samples were used for heterotrophic plate counts.

3.3 Laboratory analyses

3.3.1 Geochemistry (paper I-III, V)

In the laboratory, samples for iron and manganese were filtered through 0.45µm and acidified with nitric acid to pH ~2. Samples for total nitrogen were preserved directly by acidifying using sulphuric acid. Samples for determination of TOC were kept frozen at -20 °C until analysis, while those for the rest of the parameters were kept at 4 °C. Norwegian Standards were followed for determination of dissolved oxygen (NS-ISO 5813), alkalinity (NS-EN ISO 9963-2), iron (NS 4773), and manganese (NS 4773). Major ions (ammonium, sodium, potassium, calcium, magnesium, chloride, nitrate, and sulphate) were determined using Ion Chromatography DIONX ICS-1100 (Thermo Scientific, USA). Total nitrogen and TOC were determined using FIAlyzer-1000 (FIAlab, USA) and TOC Fusion (Teledyne Tekmar, USA), respectively. In addition, included in the analysis was a historical data on the groundwater geochemistry for period 1992 to 2017. Both the field and laboratory analytical procedures for the parameters have been described previously (Abiriga et al., 2020; 2021). How the dataset was used in the various papers is described in the table (Table 1).

Table 1. Sample source for characterisation of the groundwater geochemistry for the different papers.

Sample source	Sampling year	Article
R101, R102, R103, R104, R105; R201, R202, R203, R204; R401, R402, R403; R0 (n = 26)	2018 [a]	Paper I
R101, R102, R103, R104, R105; R201, R202, R203, R204; R401, R402, R403; R0 (n = 52)	2018-2019 [a]	Paper II
R104; R203; R402; R0 (n = 48)	2018-2019 [a]	Paper III
R101, R102, R103, R104, R105; R201, R202, R203, R204; R401, R402, R403 (n = 632)	1992-2017[b] and 2018-2019 [a]	Paper V

[a] Samples were collected twice a year in spring and autumn. In addition, samples for paper III had timepoint samples (see section 3.2.1, pg. 9).

[b] The monitoring programme underwent changes over the years in which both the frequency of sampling and the well levels sampled were adjusted (see references Abiriga et al., 2020; 2021).

3.3.2 Microbiology

3.3.2.1 Fluorescence microscopy (paper I)

Samples for fluorescence microscopy were fixed with 2.5% (final concentration) phosphate-buffered glutaraldehyde and stained with 5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Kepner and Pratt, 1994). Cells were enumerated under ×100 oil objective using Olympus IX70 fluorescence microscope (Tokyo, Japan). Ten fields were counted, and the average count was used to estimate bacterial density per sample. Counting was conducted on all the samples collected in the sampling campaign 2018-2019, except the timepoint samples.

3.3.2.2 Cultivation and characterisation of heterotrophic bacteria (paper I)

To count the heterotrophic groundwater bacteria, 1 ml of water was serially diluted and 100 µl of three dilutions were inoculated on half-strength tryptic soy agar in triplicates. Colonies were counted after incubation at 15 °C for at least 5 days under both aerobic and anaerobic conditions. The heterotrophic plate count was performed for all the samples (2018-2019). Again, the timepoint samples were excluded in this microbial analysis.

Based on observable colony morphologies such as shape, elevation, margin, size, and colour, colonies were purified by repeated streaking and incubation. The pure isolates were subjected to oxidase test, catalase test and Gram staining, and were eventually identified by sequencing the V3-V5 16S rRNA gene region using Sanger sequencer. Purified Cycle Sequencing products were analysed using Genetic Analyser 3130xl from Applied Biosystems. Chromatogram files were processed using ChromasPro version 2.1.8 and species identification was conducted by Blastn search in NCBI database. The isolation, characterisation and identification of pure isolates was conducted on samples collected in 2018.

3.3.2.3 Heterotrophic plate count of sediment-attached microbes (paper IV)

The sediment samples were mixed thoroughly, and 1 ml of the supernatant was used to make serial dilutions of up to 10^{-6}. The diluted samples were treated as above (section 3.3.2.2), but no anaerobic growth was carried out nor were the isolates subjected to the 16S rRNA identification.

3.3.2.4 16S rRNA metabarcoding (paper I, II, III, and IV)

For 16S rRNA gene metabarcoding, 300 ml of water samples was filtered through 0.2 µm filter paper which were preserved at -70 °C prior to DNA extraction. DNA was extracted from one half filter paper using DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturer's instructions. DNA from the aquifer sediment samples (250 mg) was also extracted using DNeasy PowerSoil Kit following the manufacturer's instructions. PCR amplification and the 16S rRNA gene library preparation (Fadrosh et al., 2014) for the samples were conducted at Norwegian Sequencing Centre (https://www.sequencing.uio.no). The V3-V4 hypervariable 16S rRNA gene region was sequenced using the primer set 319F (5'-ACTCCTACGGGAGGCAGCAG-3') and 805R (5'-GGACTACNVGGGTWTCTAAT-3'). The sequencing was performed on Illumina MiSeq (600 cycles) by applying the 300 bp paired-end protocol with 10% PhiX. The sequencing was performed on the full-scale sampling, including both the dense and discrete timepoint samples. How the different samples were used in the different papers is summarised below (Table 2).

Table 2. The source of samples for the different papers in which the 16S rRNA data was used.

Sample source	Sampling year [a]	Article
R101, R102, R103, R104, R105; R201, R202, R203, R204; R401, R402, R403; R0 (n = 26)	2018	Paper I
R101, R102, R103, R104, R105; R201, R202, R203, R204; R401, R402, R403; R0 (n = 52)	2018 and 2019	Paper II
R104, R203, R402; R0 (n = 48)	2018 and 2019	Paper III
R401, R402, R403; 3 aquifer sediments (n = 6)	2019	Paper IV

[a] Samples were collected twice a year except for the aquifer sediment which was collected once in 2019. In addition, samples for paper III had timepoint samples (see section 3.2.1, pg. 9).

3.4 Data analysis

3.4.1 Geochemistry (paper I, II, V)

Statistical analyses were performed using the R environment for statistical computing version 4.0.2 (R Core Team, 2020). To compare the groundwater chemistry between the background sample (R0) and the contaminated water samples (R1-R2-R4) (**paper I**), one-tailed Wilcox rank test was used. Comparison of groundwater quality across the wells in the contaminated aquifer (**paper I, II, V**) was performed using Kruskal-Wallis rank sum test. Difference in groundwater chemistry between spring and autumn (**paper II**) was tested using Mann-Whitney test. The hydrochemical facies (Piper diagrams) (**paper V**) were generated using package hydrogeo (Myles, 2017), and the groups considered in the hydrochemical facies were tested for significant difference using the nonparametric Kruskal-Wallis test. Trend analysis (**paper V**) on selected parameters was performed using Mann-Kendall trend test from package Kendall (McLeod, 2011).

3.4.2 Heterotrophic plate count and microscopic count (paper I).

One-tailed paired *t*-test was used for both within-sample and overall comparison between plate counts and microscopic counts. Similarly, comparison between aerobic and anaerobic counts was also done using one-tailed paired *t*-test. One-way ANOVA was conducted to test for differences in aerobic and microscopic counts across the wells and a post-hoc Tukey's Honest Significant Difference test for the pairwise comparisons. All statistical tests were considered significant at *P*-value ≤ 0.05.

3.4.3 16S rRNA metabarcoding (paper I, II, III, IV)

The 16S rRNA demultiplexed sequences were denoised and grouped into amplicon sequencing variants using DADA2 algorithm (Callahan et al., 2016) plug-in for QIIME2 version 2019.1.0 (Bolyen et al., 2019). Default settings were applied except for primer length (set to 20 bp) and minimum sequence length of reads (set to 280 bp). The

amplicon sequencing variants were subjected to taxonomic assignment using Naïve Bayes classifier algorithm trained on data from SILVA version 138 using QIIME2 version 2020.2.0. The OTU data was subjected to multivariate analyses using package vegan in R (Oksanen et al., 2019). Beta diversity based on Bray-Curtis dissimilarity metric was visualised using nonmetric multidimensional scaling (NMDS) (**paper II and III**) or principal coordinate analysis (PCoA) (**paper IV**). Difference in the microbial community composition among the sampling wells (**paper I-III**), between autumn and spring, and between 2018 and 2019 (**paper II**) were tested using permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) on 999 permutations. The assumption of homogeneity of group dispersion (**paper I-III**) was checked using the function *betadisper* (Anderson, 2006). Redundancy analysis (RDA) (**paper I and II**) was conducted to find out how much variation in the microbial community composition was explained by the measured explanatory variables: water chemistry, well distance, season, and time (year). The overall explained variance (**paper II and III**) was partitioned among the explanatory variables using variation partitioning (Borcard et al., 1992). Co-occurrence network of the microbial OTUs in the contaminated aquifer (**paper III**) was determined using the top 25 OTUs that were present at least 5 times in at least 50% of the samples from the contaminated aquifer (R104, R203 and R402; n = 36). The filtering left only 79 OTUs (out of 1870) and were considered as the core members of the aquifer community (generalist). In addition, the filtering reduced the network complexity and allowed for better resolution of the interactions between OTU nodes. To ascertain if the OTU co-occur randomly or deterministically, a null community co-occurrence was simulated using the checkerboard-score (C-score) (Stone and Roberts, 1990) in package EcoSimR (Gotelli et al., 2015). Finally, the keystone taxa among the OTUs were identified by using the network topologies.

4 Summary of the main results

4.1 Paper I

Comparing the results of different microbiological techniques

Bacterial cell density in the groundwater samples were estimated by plate count and direct fluorescence microscopic count. Overall, the microscopic count was higher than the plate count (t = 6.94, df = 51, $P < 0.05$). The plate count was significantly different across the wells (F = 3.09, df = 3, P = 0.0357), but the difference was not significant for direct microscopic count. Although the cell density estimates cannot be directly related to species composition, beta diversity analysis through PERMANOVA utilising the 16S rRNA metabarcoding data showed that the microbial compositions across the wells were significantly different (F = 4.58, df = 3, P = 0.001, 999 permutations). Like the culture-based approach where bacterial cell counts in the wells were in the order R4 < R0 < R1 < R2, the alpha diversity calculated using the metabarcoding data followed the same pattern. In addition, a number of the bacterial isolates were among the most abundant taxa detected by the 16S rRNA metabarcoding technique. Functional analysis based on both the culture-based (bibliographic review) and 16S rRNA metabarcoding data (METAGENassist; Arndt et al., 2012) revealed the presence of sulphur transformers, nitrogen transformers, carbon transformers, and iron and manganese transformers.

4.2 Paper II

Factors influencing the microbial community composition of in the aquifer

Paper II is an investigation of how the groundwater geochemistry, distance, season, and year influence the aquifer microbiome as inferred from the metabarcoding data (for 2018-2019). Before partitioning the explained variation in microbial community composition into independent components (Figure 2), difference in beta diversity between and among the factor levels were assessed. PERMANOVA results showed a significant difference in beta diversity in only the well levels of the proximal well (F =

1.47, *df* = 4, *P* = 0.002), while both the intermediate and distal well levels showed nonsignificant differences. However, in moving from the top to the bottom along the depths in the proximal and intermediate wells, larger dispersion was observed in the topmost levels R101 (R1) and R201 (R2). A global comparison of microbial communities across the wells indicated a significant difference, and variation partitioning (Figure 2) indicates that well (as a categorical with four levels) accounts for 23% of the explained variance (33.2%). This is the second largest variation, only exceeded by groundwater geochemistry (25%). It was also observed that, both well (due to their location they have spatial [distance] attributes) and groundwater geochemistry are intercorrelated, as they jointly account for most of the explained variance (18.5%).

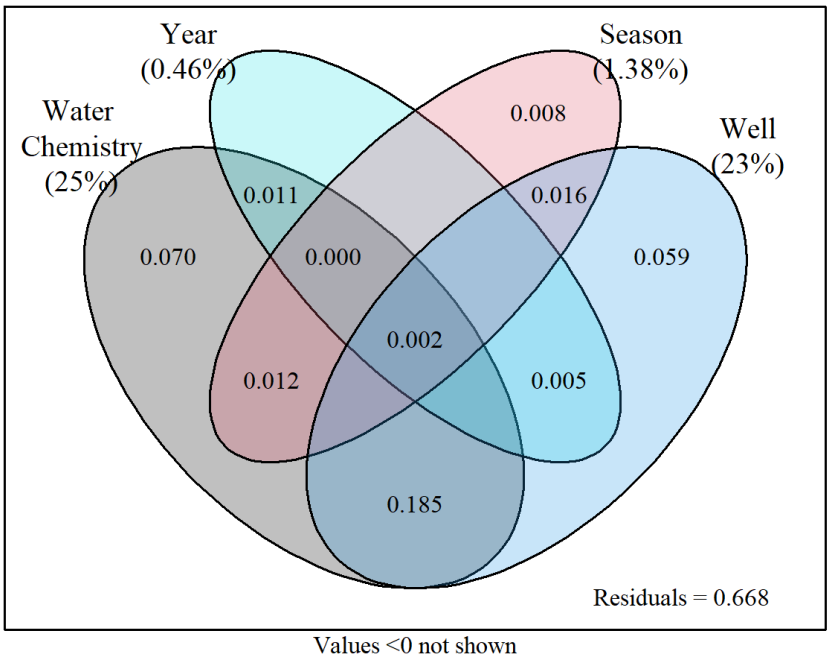

Figure 2. Variation partitioning of proportions of variation in microbial community composition explained by water chemistry, year, season, and distance (well). Values in parenthesis indicate variations explained by the variables without removing the effects of covariables.

To find out if microbial community composition varied seasonally, PERMANOVA was conducted, and the result showed significant differences only in the proximal (*P* = 0.007) and intermediate (*P* = 0.001) wells. This result can be compared with the seasonal groundwater geochemical changes, where a few of the parameters showing significant

differences between spring and autumn were in the proximal (sulphate, TOC, sodium, potassium, and calcium) and intermediate (conductivity, ammonium, and TOC) wells.

Beta diversity analysis to assess whether the microbial community composition between 2018 and 2019 were different showed a significant result only for the intermediate well (F = 2.29, df = 1, P = 0.024), but a closer look into the data revealed this variation was tied to the seasonal fluctuation. The less variability in the microbial community was further substantiated by the output from variation partitioning (Figure 2), which indicates that the overall contribution of time in explaining the microbial composition was only 0.46% and was statistically nonsignificant (F = 0.97, P = 0.539).

4.3 Paper III

Microbial network and deterministic assembly in the aquifer

We employed the C-score (Stone and Roberts, 1990) to simulate null community models and the metrics generated were used to identify the pattern of community assembly. A community is said to co-occur non-randomly if the observed metric is greater or smaller than the expected metric under null model (Horner-Devine et al., 2007). Both the aquifer-wide (contaminated aquifer; R104-R402) and well-by-well (R0-R402; Table 3) community co-occurrence indicate non-random patterns. Higher effect size (Table 3) and microbial diversity (Figure 3) were observed from the intermediate well.

Table 3. Results of null model simulations for the four different communities.

	N [a]	OTUs [b]	C-score$_{observed}$	C-score$_{random}$	SES	*P*-value
R104	12	830	2.72	2.68	11.9	<0.001
R203	12	709	2.56	2.50	21.9	<0.001
R402	12	558	2.39	2.31	15.3	<0.001
R0	12	473	2.933	2.930	1.35	0.07

[a] Number of samples in each well

[b] OTUs were filtered to only include taxa present in abundances of greater or equal to 10.

SES: standardised effect size.

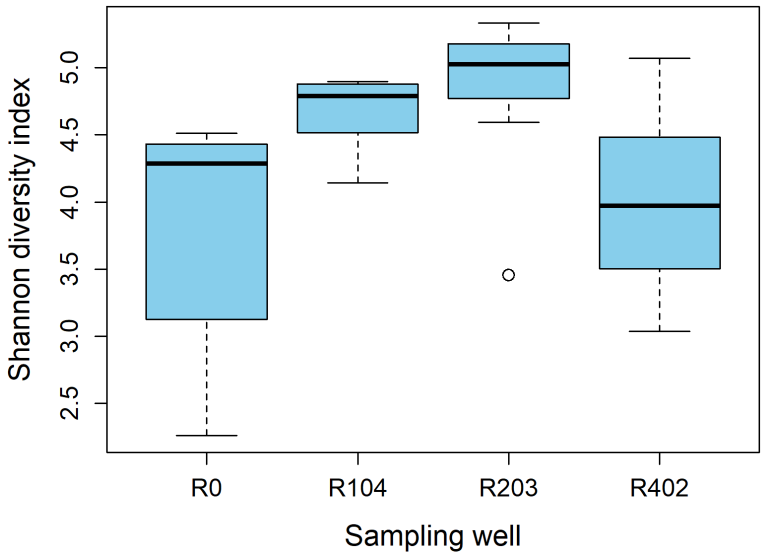

Figure 3. The Shannon diversity index for the samples from R0, R104, R203 and R402.

Since the network analysis indicate that the microbial communities assemble deterministically, the amount of variation in the microbial community explained by the measured explanatory variables was quantified using variation partitioning. Four composite factors were considered: groundwater chemistry (15 variables), distance (as a factor with four levels), season (spring and autumn), and year (2018 and 2019). The amount of variation explained by the explanatory variables was 55.3%. This is an increase from 33.2% when all the well multilevels were included in the analysis (**paper II**).

4.4 Paper IV

Planktonic versus sediment-attached microbial communities

In this paper, the microbial community composition between free-flowing water (planktonic) and sediment-bound (biofilm) were compared. Both alpha species diversity (Shannon diversity metric) and heterotrophic plate counts showed higher values in the aquifer sediment sample compared to the groundwater sample (Figure 4).

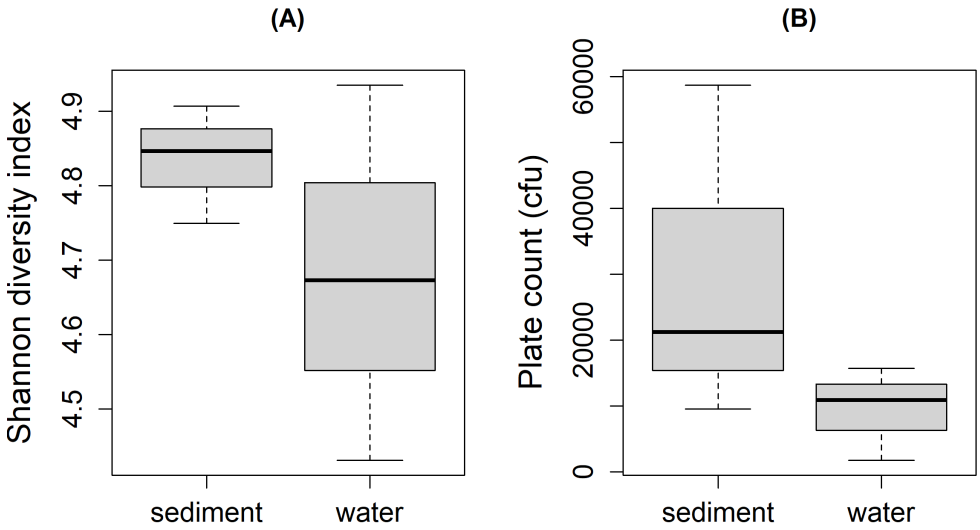

Figure 4. Alpha species diversity (Shannon diversity index) **(A)**, and heterotrophic plate count **(B)** in sediment (CFU/g dry weight) and water (CFU/ml).

The result from beta diversity analysis (Figure 5) shows that the microbial composition is different between the free-flowing groundwater and the aquifer sediment which are separated by PCoA1. The second axis (PCoA2) separated the samples by depth at which the samples were obtained. However, it shows that there is a concordance among the sampling depths regardless of the sample type.

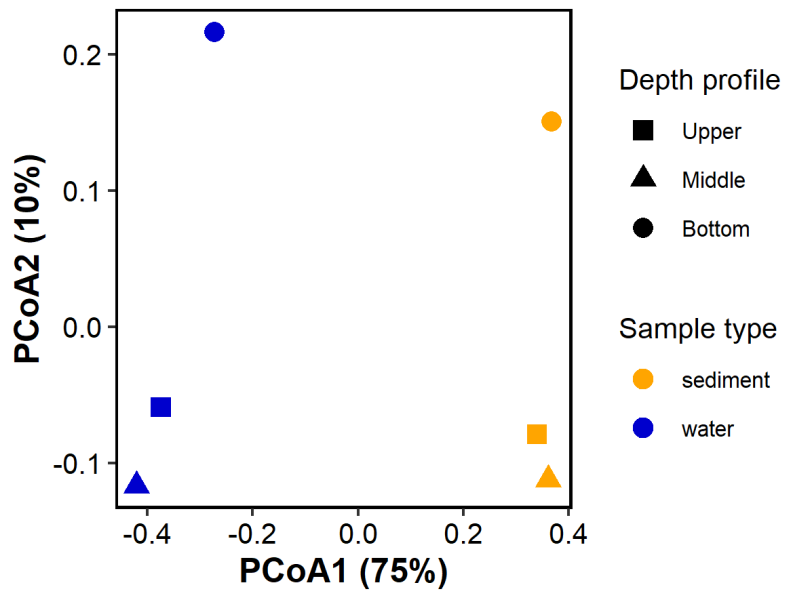

Figure 5. Beta diversity between groundwater samples and the aquifer sediment samples based on the Bray-Curtis dissimilarity distance.

Compositionally, the groundwater column was dominated by phylum *Proteobacteria*, while the aquifer sediment was dominated by phylum *Acidobacteria*. In addition, phyla *Actinobacteria* and *Chloroflexi* were more abundant in the aquifer sediment, while phyla *Patescibacteria* and *Bacteroidetes* were more abundant in the groundwater column.

4.5 Paper V

Significance of natural attenuation in Revdalen Aquifer

The groundwater geochemistry used in **paper I and paper II** were combined and compared with historical data on the aquifer geochemistry. This was to put to perspective, the relevance of the natural attenuation. It was found that concentrations of contaminants decreased with both the age of the landfill and the distance from the landfill (Figure 6). Both sodium and chloride decreased and tailed off earlier than other parameters. The decreasing trend prevailed over the study period and particularly for most chemical species, while oxidised species sulphate and nitrate showed an upward trend. However, although the leaching pattern for nitrate was observed to be complex, levels of sulphate have at present attained a pseudo-stationary phase.

It was found that the effect of distance mirrored that of landfill status in the type of water identified from the hydrochemical facies. Both the proximal/intermediate wells and active/closed landfill status were characterised by three predominant water types: Ca-$(HCO_3)_2$ type, Ca-Na-HCO_3 type and Ca-Na-Cl type, in decreasing order. However, Na-HCO_3, Na-Cl and Ca-Cl_2 type waters were occasionally observed. Similarly, it was observed that both the distal well and the stabilised landfill stage were characterised by one water type, Ca-$(HCO_3)_2$.

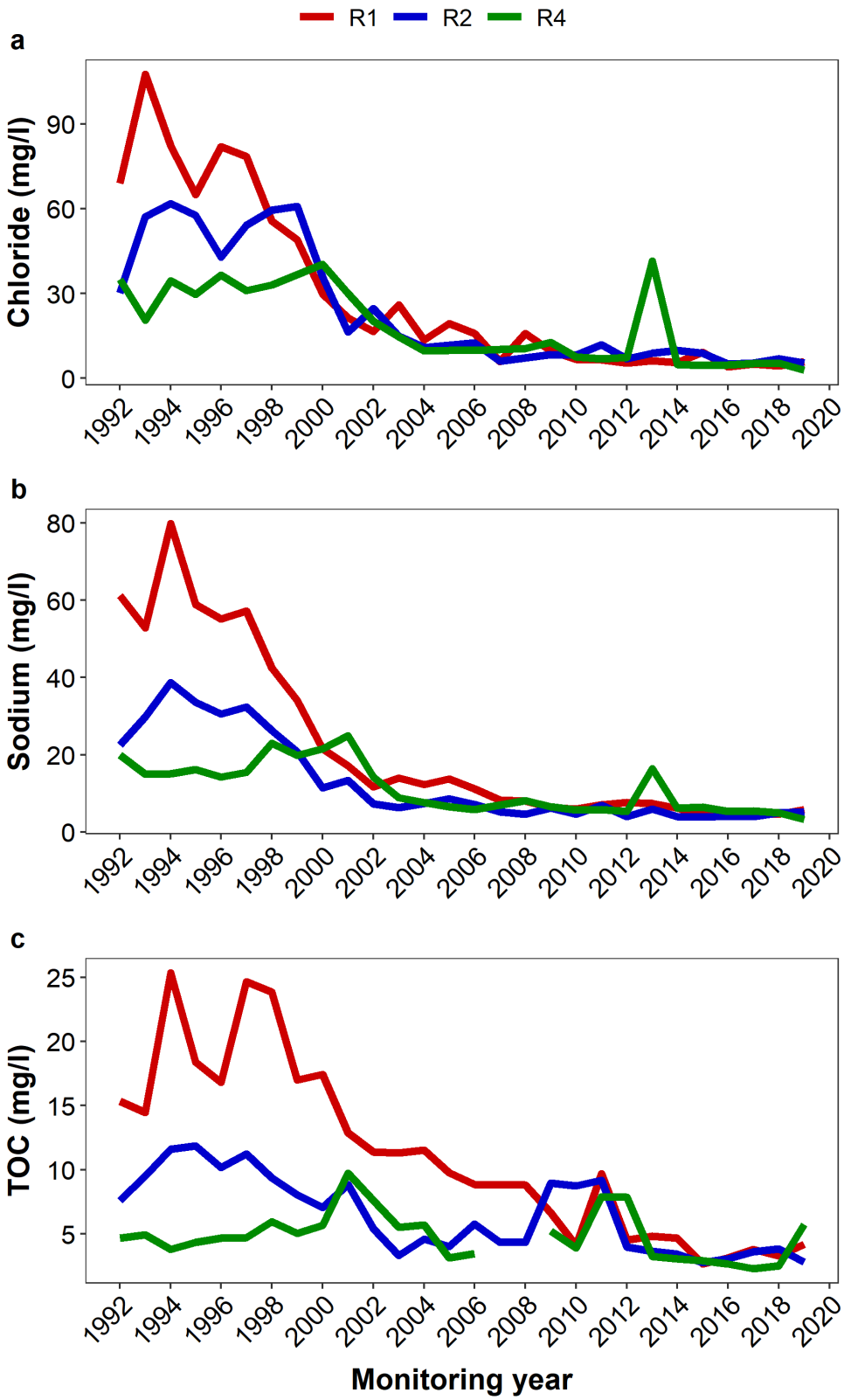

Figure 6. Long-term changes in annual mean values of chloride (**a**), sodium (**b**), and TOC (**c**) across the sampling wells R1, R2 and R4 in the period 1992 to 2019.

5 Discussion

5.1 Groundwater microbiology

The observation that microscopic cell count was significantly greater than plate count (**paper I**) agrees with the literature (Gregorich and Carter, 2007; Muyzer and Smalla, 1998). However, a detailed analysis showed that microscopic counts were all but one higher than plate counts. Moreover, only four samples showed statistically significantly higher microscopic counts than plate counts. The higher microscopic cell count may suggest the presence of nonculturable microbes in the water samples, although it should be noted that the plate count may not represent all the culturable microbes given that the culture medium may not provide all the nutrients required by some microbes. On the other hand, the observation that only a few samples registered statistically significant difference between plate count and microscopic count suggests that the cell density estimates from the two methods were not remarkably different.

Results further showed that cell counts (**paper I**), and microbial diversity and composition (**paper I-III**) vary considerably between the contaminated aquifer and uncontaminated aquifer, an observation which agrees with the literature (Brad et al., 2013; Brad et al., 2008; Mouser et al., 2005). The discrepancy is likely due to the different nutritional status of the two aquifers; the landfill-contaminated aquifer is expected to contain e.g., more carbon source (Röling et al., 2000). However, within the contaminated aquifer, microbial diversity and composition varied spatially, both vertically and horizontally, although the horizontal variation was stronger than the vertical variation. Similarity in microbial composition decreased with increasing distance from the landfill, indicating the existence of an ecological gradient that developed due to the landfill leachate.

The landfill leachate likely imposed a deterministic selection process on the microbiota of the aquifer, as the microbial co-occurrence network analysis (**paper III**) indicates idiosyncratic non-random microbial community assembly pattern in the wells along the flow path. This confirms that the microbial communities in the aquifer assemble

deterministically, although the approach does not identify the casual mechanistic processes (Horner-Devine et al., 2007). The proximal well, which is located closest to the landfill, is expected to have the greatest influence from the landfill leachate. This makes the microorganisms to co-occur more than those in the intermediate and distal wells, which is consistent with the lower effect size recorded in the proximal well (Table 3). The intermediate well is expected to experience an intermediate level of impact which is characterised by enhanced ecological processes. Thus, the higher effect size and microbial diversity (Figure 3) observed from the intermediate well fits with the intermediate disturbance hypothesis which states that the highest diversity occurs at an intermediate level of disturbance (Miller et al., 2011; Svensson et al., 2012). The distal well, which can be compared to a recovery stage of perturbation, recorded lower diversity (Figure 3) and effect size (Table 3). This implies that there is a decrease in the significance of the ecological processes as the influence of the leachate become attenuated along the groundwater flow path. Since the co-occurrence network analysis could not identify causal processes, an additional study needs to be performed to identify the causal mechanistic processes shaping the microbial community assembly in the aquifer. Such a mechanistic identification of the underlying causal factors has been studied for other ecosystems (Danczak et al., 2018; Stegen et al., 2013; Stegen et al., 2012), but not for landfill leachate plumes.

Significant vertical variation (**paper II**) in microbial composition was observed only in the proximal well. The significant difference across the depth profiles of the proximal well could be attributed to the differential overrepresentation of some taxa particularly between the topmost (*Aquabacterium*, *Janthinobacterium*, *Oxalobacteraceae* and *Pedobacter*) and deepmost (*Sulfurifustis* and *Sulfuritalea*) levels. The same line of reasoning could explain the large dispersion observed in R101 and R201, with the latter being influenced by taxa of *Pseudomonas*, *Rhodoferax*, and *Saccharimonadales* which were in higher abundance in R201 than the rest of the levels in R2. Due to considerable variation in geological formations, contradicting results regarding vertical variation in microbial composition have been reported and thus depth-resolved investigations from different subsurface strata are urgently needed (Smith et al., 2018a). The most

important determinant of vertical variability is the inherent variation in the geological layering of the aquifer lithologies. For example Lin et al. (2012) observed significant vertical variation in microbial communities because the aquifer matrix is well stratified and in addition, it is a phreatic aquifer with a river-body aquifer boundary type. In the present study, the significant difference in microbial communities across depth profiles was more of a seasonal fluctuation than due to inherent vertical stratification. Since these aquifers have peculiar formation and hydrogeological conditions, the interpretation of this discrepancy needs to be done with caution. Perhaps a valid comparison to understand the effect of vertical variation would be to use data from aquifers which have similar formations and hydrological conditions.

Microbial composition was found to be influenced by season. Such seasonal variation in composition and diversity of microbes have been observed in pristine aquifers (Farnleitner et al., 2005; Lin et al., 2012; Zhou et al., 2012) and industry-impacted urban aquifers (Smith et al., 2018b). Beta diversity indicated that larger variability was registered in spring, which implies that the microbial communities were less variable in autumn than in spring, presumably due to calmer groundwater flow conditions in autumn. In Norway, higher groundwater recharge occur in spring after snowmelt (Kløve et al., 2017) and this may be followed by groundwater mixing, which can cause instability in the subsurface environment (Smith et al., 2018a). This may be exacerbated by the inherent variation in the aquifer matrix across the depths. This makes the different layers to respond differently to changes in hydraulic regimes (Smith et al., 2018a), although Revdalen Aquifer does not constitute distinctively stratified geologic layering as the deposits are not well-sorted sediments. The seasonal dynamics cause shifts in microbial communities (Pilloni et al., 2019) such as the variations observed with the depth profiles in the proximal and intermediate wells. However, it seems that season has only a marginal effect as it accounted for only 1.3% (P = 0.034) of the variation in the microbial community composition (Figure 2) (**paper II**). On the other hand, considering only a single well level (**paper III**) minimised the influence of the confounding factors and has greatly improved the overall amount of variation that was explained by all the explanatory factors; 33.2% (**paper II**) versus 55.3% (**paper III**). This

also resulted in nonsignificant ($P > 0.05$) and numerically small variance (0.4%) explained by season, implying that seasonal variation greatly affected the microbial composition when all the multilevels were considered than when just one level was surveyed. This is an important finding for designing future studies involving subsurface microbiology.

Changes in the microbial composition between 2018 and 2019 was significant only for the intermediate well (**paper II**). The result suggests that the microbial community composition in the proximal and distal wells were less variable over the study period. Variation partitioning indicates time has small (0.4-0.46%) (**paper II and III**) and nonsignificant effect on the microbial community composition, which further strengthens that the microbial communities were less variable. A similar observation of microbial community stability over a one-year period have been made from pristine aquifers (Farnleitner et al., 2005). How long such implied community stability will prevail in aquifers is a matter of future studies, as apparently, no data exists for field observation >2 years.

Results from the three different microbiological methods, i.e., culturing, microscopy and 16S metabarcoding (**paper I**) showed good agreement. The approach can thus be used to validate results from different methods. It also means that, although low throughput, data generated using the traditional culture method can provide useful information about a system. Further, unlike metabarcoding, use of the culture technique assures that the data comes from live bacterial cells. Moreover, with culture method, taxa can readily be identified to species level. The disadvantages of culture-based approach include being time-consuming, labour-intensive, and bias to favour taxa that are easy to grow in the laboratory (Chen et al., 2014). This has put advances on microbiology at low speed but the development of culture-independent method, particularly the 16S rRNA gene sequencing has precipitated numerous studies on microbial ecology, including surveys of habitats that were originally thought to be sterile (Fadrosh et al., 2014). However, the method is not free of limitation. Known limitations include low taxonomic resolution which at the best gives genus-level identification, recovering sequences from dead cells and naked DNA in the environment (Carini et al., 2016), and the high cost of

sequencing service. Thus, operating a combination of methods ensures the limitations of the individual techniques are minimised. The concordance achieved from the use of the three methods means that even with the use of least-advanced techniques, a fair amount of information can be obtained from an ecosystem. This is useful, especially for research institutions from low-income countries where access to advanced instruments is a big challenge.

5.2 Groundwater microbiota versus the sediment microbiota

The finding that both heterotrophic plate count and microbial diversity are higher in the aquifer sediment than in the groundwater (**paper IV**) agrees with the literature that higher cell counts and species diversity are to be expected in sediments (Balkwill and Ghiorse, 1985; Holm et al., 1992; Smith et al., 2018a). As with the vertical variation in microbial community composition of the groundwater (**paper II**), the aquifer sediment communities also clustered according to the depth from which samples were obtained. This indicates vertical variability in the aquifer sediment communities. This might be due to variation in the aquifer sediment grain sizes across the aquifer depth, which influences hydrogeological conditions and thus the distribution of microbes. The result further suggests that the most dissimilar microbial community composition is to be found in samples obtained from the deepest level of the aquifer, while the topmost and middle samples clustered close to each other.

Although most of the literature comparing planktonic and sediment attached microbiota have used surrogate sediment samples, it is well-acknowledged that different microbial communities may form in groundwaters and aquifer sediments (Röling et al., 2000; Scow and Hicks, 2005; Smith et al., 2018a). Thus, groundwater samples alone cannot capture the whole aquifer diversity (Flynn et al., 2008; Röling et al., 2000), but the ease of obtaining groundwater samples favours its routine applicability (Flynn et al., 2008). On the other hand, it is both technically challenging and economically costly to obtain aquifer sediments and this has resulted in literature on subsurface microbiology being dominated by groundwater microbiology, leaving a

dearth of information on microbiology of different aquifer lithologies (Smith et al., 2018a). Moreover, the destructive nature of obtaining aquifer sediments means that repetitive sampling such as in long term monitoring is impracticable. While the ease of groundwater movement in porous medium makes it ideal as a representative medium for a larger research area (Brad et al., 2013), aquifer sediment microbial communities show high spatial heterogeneities (Brad et al., 2008), but they offer better degradation potentials than planktonic communities (Holm et al., 1992).

A major limitation in the interpretation of the observed difference in the microbial composition between the groundwater and sediment is the delay in taking the aquifer sediment samples, which due to technical challenges, were taken nearly 1½ months later. There should, nonetheless, be minimum or no change at all within this time gap, as previous studies have shown that aquifer sediment microbial communities remained stable over a period of one year (Farnleitner et al., 2005; Zhou et al., 2012). In addition, both beta diversity analysis and compositional analyses (RDA) on the groundwater samples (**paper II and III**) indicate non-significant changes between 2018 and 2019, implying that the microbial compositions are less variable if not stable, thus, the comparison between planktonic and sediment-attached communities is valid. However, the high heterogeneity inherent to sediments over a scale of metres to kilometres needs to be remembered and the aquifer sediment analysed in the present study may possibly not represent the full characteristics of the sediment-attached community in the whole aquifer system. Another limitation is the lack of statistical power as there were only three samples per sample type included in the analysis.

5.3 Relating groundwater microbiology to the geochemistry

The earlier tailing-off observed with sodium and chloride (Figure 6) (**paper V**) is probably due to their less reactivity (Schwartz and Zhang, 2003), which makes them to be easily released from the landfill. The decrease in levels of contaminants with age is believed to be due to continuous leaching of inorganic ions, and attenuation of organic compounds through biodegradation, volatilisation and sorption, which depletes their

reserves in the landfill (Bhalla et al., 2013; Reinhart and Grosh, 1998). On the other hand, the decrease in contaminant loads with distance from the landfill is due to dilution and attenuation processes (Abiriga et al., 2020; 2021; Bjerg et al., 2011; Christensen et al., 2001). The apparent stationary phase suggests that the condition in the landfill is predominantly aerobic. This agrees with the suggestion that the landfill has attained its stabilised phase. It is during this phase that the amount of oxygen entering a landfill surpasses that depleted during microbial degradation (Kjeldsen et al., 2002). The excess oxygen may cause additional oxidation in the landfill (Mårtensson et al., 1999), leading to the generation of oxidised chemical species such as sulphate and nitrate.

The recent rise in the levels of sulphate and nitrate (**paper V**) is followed by nonsignificant differences in nitrate and sulphate across the wells and between 2018 and 2019 (**paper II**). This suggests a balance between reductive and oxidative metabolism of sulphate and nitrate. Among the microorganisms capable of transforming sulphur compounds included *Sulfuritalea*, *Sulfurifustis*, *Sulfuricella*, and *Rhodanobacter*. Those capable of nitrogen metabolism included nitrate reducers (29 isolates and four genera from the metabarcoding data: *Cavicella*, *Sterolibacterium*, *Aquabacterium* and *Novosphingobium*), ammonia oxidisers *Nitrosospira*, and nitrite oxidisers *Nitrospinacea* (**paper I**). The involvement of these microorganisms in nitrogen and sulphur transformations would be strengthened if sulphide and nitrite were measured. The presence of various putative carbon degraders: *Phenylobacterium*, *Parvibaculum*, *Alkanindiges*, and *Patulibacter*, to mention a few, suggests that the resident microorganisms may be involved in the natural remediation processes. Unequivocal evidence of degradation particularly by the isolates, however, requires benchtop experiments of pollutant transformation, which can only be addressed in the future.

No substantial difference in the groundwater composition was observed between R1/R2 and active/closed (**paper V**). This implies that the water composition did not change much between R1 and R2, and between active and closed landfill stages, although the contaminant loads changed (perhaps strongly) between the two wells and landfill stages. This result indicates a concordance between time and distance in attenuating

contaminants. In other words, the contaminant loads at R4, which have been persistently low, are comparable to values recorded after the landfill attained the stabilised stage. This further illustrates the significance of natural attenuation processes in the aquifer. While it is difficult to relate the geochemical changes due to landfill status to the microbiology, the effect of change in the landfill status is likely to be strongest within the landfill itself. On the other hand, the microbiological data (**paper I-III**) indicate that R1 and R2 are more related as indicated by the geochemistry composition (**paper V**). The groundwater composition and low contaminant loads of R4 suggests a recovered quality. The microbial communities in this well may thus be viewed as those that represent the initial/native communities. However, the lack of data on the native community composition is a hinderance to this conclusion. Moreover, although the geochemistry in R4 may truly represent the recovered quality, it has been shown that physicochemical parameters revert to original status more quickly than the microbiota (Herzyk et al., 2017), thus, equating geochemical recovery to microbial recovery is not straightforward. However, multivariate statistical analyses (**paper II and III**) indicate that the groundwater geochemical variables explained most of the variation in the microbial composition.

The nature-based processes have operated efficiently and prevented a potential ecosystem degradation due to the leachate. Concentrations of nearly all parameters have decreased to levels acceptable as per the Norwegian drinking water standards, except iron and manganese. Although iron and manganese were above the Norwegian drinking water standards, the values were under 1 mg/l (but have decreased from 99 and 16 mg/l, respectively) (**paper V**). Interestingly, the well (R4) with detectable iron and manganese was enriched with iron/manganese metabolisers *Ferribacterium* and *Rhodoferax* (**paper I**), which may suggest an ongoing biogeochemical cycling of iron and manganese in the aquifer.

6 Conclusion and perspectives

6.1 Conclusion

This study investigated the geochemistry, microbial diversity and composition of a landfill-leachate-contaminated confined aquifer. Based on the study, the following conclusions were drawn:

- A fair amount of details can be obtained about a system whether the most advanced or least advanced methodological techniques are applied, and the results may show good agreement if compared.
- The study found microbial cell density and diversity to vary depending on whether the aquifer system is polluted or not. The polluted aquifer recorded higher cell counts and microbial diversity than the unpolluted aquifer.
- Both vertical and horizontal distances may affect the microbial composition, but the horizontal variation was found to be stronger than the vertical variation. In addition, the groundwater geochemistry had a profound influence on the microbial community composition; an influence that was greater than the spatial/distance effect. Further, both season and time were also found to influence the microbial composition, although season had a stronger effect than time. The order in a decreasing influence follows the trend geochemistry > distance > season > time.
- Microbial communities were found to assemble deterministically, illustrating the existence of a selection pressure on the microbial composition.
- The groundwater and the aquifer sediment harbour different microbial communities. Planktonic communities were dominated by phyla *Proteobacteria*, *Patescibacteria* and *Bacteroidetes*, while the aquifer sediment was dominated by phyla *Acidobacteria*, *Actinobacteria* and *Chloroflexi*.
- The natural attenuation of the contaminated groundwater was found to be substantial and prevented ecosystem degradation due to the leachate. Several of the aquifer microbes are members of lineage capable of degradation and biogeochemical cycling, which hints on their involvement in the remediation.

6.2 Perspectives

A clear difference in the microbial communities between the contaminated and uncontaminated aquifer was observed, which highlights the impact of the operation of the landfill on the ecosystem of the leachate-receiving aquifer. Thus, bacterial isolates recovered from the contaminated aquifer may compose strains that have been primed by the contaminants in the landfill leachate. This could have switched on certain degradative genes in the bacteria. Subjecting the isolates to metagenomic shotgun sequencing to determine whether they harbour specialised genes can provide information on the genetic repertoires of isolates. This can further be complemented by benchtop experiments where the isolates are allowed to grow on specific compounds of interest e.g., the isolates are currently being tried on degradation of polyethylene.

In addition, through controlled experiments/microcosms, the unexplainable observation such as the seasonal dynamics exhibited by taxa such as *Duganella*, which due the field investigation as presented here, could not be unambiguously interpreted (Pilloni et al., 2019) are investigated. Moreover, in the present study when analysing for seasonality, only spring and autumn were considered. It is recommended that future studies and particularly those in the northern hemisphere should ensure coverage of the four seasons in order to completely understand the dynamics caused by seasonal fluctuations. Similarly, the issue of microbial community stability/variability for >2 years is a matter that needs to be considered in future studies.

The finding that microbial diversity and composition vary both longitudinally and vertically has consequences for designing future sampling network in perturbed aquifers. Samples should be taken from more than one location along the groundwater flow path, especially if the goal is to capture the entre aquifer microbiology. Similarly, the vertical variation in the community diversity highlights another important implication in study design; whether samples should be obtained from a single level or more. The biggest limiting factor in this case is that most of the studies from perturbed subsurface environments utilise already existing monitoring wells, so it becomes a question of whether the wells were equipped with multilevel systems (discrete samples)

or with one long screens (composite samples). Otherwise, multilevel sampling design is encouraged and, in some instances, such as for demonstrating intrinsic bioremediation, it becomes an extremely important experiment design.

Another important aspect to consider in studying the microbial ecology of subsurface environments is if a study aims to characterise the whole microbial composition. In such a case, samples should be taken from both the groundwater and the aquifer sediment. As demonstrated in the present study and in the literature, these matrices support different microbial communities. However, the challenges of obtaining aquifer sediment samples are more compelling than for sampling groundwater. This has skewed the available literature towards groundwater microbial composition and most of the literature from attached communities come from surrogate sediment samples. While the present study aimed to make a direct time-bound comparison between planktonic and sediment-attached communities, technical challenges were encountered, and the sediment samples were taken after one and half months. Moreover, the present study did not consider the lateral spatial variation for the aquifer sediment. If resource allow, future studies aiming to study full microbial composition should ensure good spatial and temporal coverage of sampling sites for both groundwater and aquifer sediment.

Also, not all geochemical parameters were analysed in the present study, and this affected the models used to explain variations in the microbial community compositions. Although it is not practicable to measure all variables in a single investigation, including as many variables as possible can help resolve the issue with unexplained variance. In a similar but a different dimension, the incomprehensive variable coverage precludes performing water quality index as an indicator of potential human health risk commonly done in impact risk assessments. Moreover, should there be a future environmental impact assessment for the site, including toxicity test in the list of parameters is highly recommended.

There is a growing concern on the occurrence of antibiotics/antimicrobial agents as well as compounds in personal care products in the environment due to operation of landfills. Future studies from landfill-impacted environments should include

quantification of antibiotic resistance gene number using qPCR and/or quantification of the actual antimicrobial agents of concern, as well as compounds of concern from personal care products, especially in landfills that hold wastewater treatment sludge.

The natural attenuation in the aquifer have been substantial, and this resulted in the groundwater chemistry from the distal well being predominantly of one water type, Ca-$(HCO_3)_2$. The same water type was recorded at the stabilised landfill stage, indicating that the same groundwater composition, which may be viewed as the recovered water quality, could be achieved either through time (landfill aging) or natural attenuation in the aquifer. However, a major limitation in ascertaining if full recovery has been attained or can be attained is the lack of data on the aquifer collected before the actual contamination occurred and/or absence of a reference well in the same setting. Since each pollution case is unique, it is difficult to make a generalised recommendation that is universally applicable. If the situation allows, however, it is valuable to have a reference water quality (usually upstream from a contamination source) so that groundwater quality from the same aquifer is compared. This should always be borne in mind whenever designing a monitoring scheme for managing pollution cases.

It is difficult to follow natural attenuation project using microbial data, because it is expensive to perform a long-term microbial analysis in general. Similarly, ascertaining whether the microbial communities have reverted to the initial status is even more challenging than for geochemical characteristics, because a benchtop experiment indicates that the groundwater qualities return to normal more quickly than do the microbial communities (Herzyk et al., 2017). Thus, this aspect is completely open to future research. Following a contaminated aquifer e.g., every after three or five years can shed light into the microbial succession processes in landfill-contaminated aquifers.

This study highlights the impact of operation of a landfill on the microbiology and geochemistry of the affected aquifer. A similar approach can be employed elsewhere to assess the impacts of other land use types on the ecology of threatened aquifers. These impacts may be due to forestry logging, mining, pulse perturbation from use of agrochemicals and aircraft de-icing chemicals in airports.

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Article 1

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Characterisation of the bacterial microbiota of a landfill-contaminated confined aquifer undergoing intrinsic remediation

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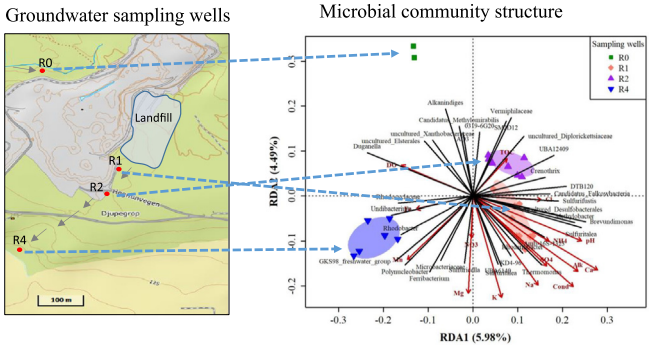
HIGHLIGHTS

- The microbiome of a contaminated aquifer in southeast Norway was studied.
- Culture-dependent and culture-independent techniques were employed.
- Microbial composition were different between polluted and unpolluted aquifers.
- Community composition was different across the wells in the polluted aquifer.
- Functional prediction suggests the presence of organic and inorganic metabolisers.

GRAPHICAL ABSTRACT



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Contaminated groundwater

ABSTRACT

Literature on microbiomes of landfill leachate-contaminated aquifers is scarce despite groundwater contaminations from landfills being common globally. In this study, a combination of microbiological techniques was applied to groundwater samples from an aquifer contaminated by a municipal landfill and undergoing intrinsic remediation. Groundwater samples were obtained from three multilevel sampling wells placed along the groundwater flow path in the contaminated aquifer, and additionally from a background well located in a nearby uncontaminated aquifer. The samples were subjected to chemical analysis, microbial culturing and characterisation, cell counting by fluorescence microscopy, and 16S rRNA metabarcoding. Good concordance was realised with the results from the different microbiological techniques. Samples from the uncontaminated aquifer had both lower cell density and lower microbial diversity compared to samples from the contaminated aquifer. Among the wells located in the contaminated aquifer, microbial diversity increased between the well closest to the landfill and the intermediate well but was lower at the most distant well. The majority of the cultured microbes represent taxa frequently recovered from contaminated environments, with 47% belonging to taxa with previously documented bioremediation potential. Multivariate redundancy analysis showed that the microbial composition was most similar in wells located closer to the landfill, although beta diversity analysis indicated a significant difference in microbial composition across the wells. Taken together with the results of cell counting, culture, and metabarcoding, these findings illustrate the effect of landfill leachates on microbial communities.

1. Introduction

Groundwater is the main source of freshwater for drinking, agriculture and industry in many places globally (Mays and Scheibe, 2018;

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O'Connor et al., 2018), but faces serious pollution challenges (Brad et al., 2013; Pous et al., 2018; Röling et al., 2001) of which leachate from landfills is one. All over the world, landfills have served as the ultimate destination for municipal solid wastes (Reinhard et al., 1984), and continue to do so (Eggen et al., 2010). In Norway, there was little recycling of wastes until the late 1990s and most of the wastes from households and industries were deposited in municipal solid waste landfills with no provision for treatment or containment of the resultant leachate. Revdalen Landfill represents one such historic site. It was active from 1974 to 1996, leading to the contamination of Revdalen Aquifer. Pollutants of environmental concern such as heavy metals and polycyclic aromatic hydrocarbons have been detected in Revdalen Aquifer (Abiriga et al., 2020).

Several strategies exist to reclaim contaminated aquifers. They are broadly categorised as artificial and natural. The former, which includes the conventional pump and treat are faster but require a major economic input for operation and maintenance (Hyldegaard et al., 2019). Natural attenuation such as *in situ* bioremediation, on the other hand, offers inexpensive, eco-friendly yet efficient remedies (Logeshwaran et al., 2018; Nunes et al., 2013). In addition, unlike pump and treat, *in situ* bioremediation does not generate secondary wastes. It is therefore the most widely preferred option in *in situ* remediation of groundwater (O'Connor et al., 2018). However, *in situ* remediation, particularly intrinsic bioremediation is slow, and the groundwater remains polluted for a long time, although recovery can be accelerated by amended bioremediation (Logeshwaran et al., 2018). Revdalen Aquifer is undergoing intrinsic remediation and the relevance of natural attenuation processes have been discussed previously (Abiriga et al., 2020; Abiriga et al., 2021).

Traditionally, groundwater bioremediation has been demonstrated empirically by measuring geochemical parameters, with little use of microbial data (Mouser et al., 2005). Over the years, however, it has become apparent that studying microbial community composition in addition to geochemical measurements offers a more complete picture of bioremediation (Lu et al., 2012; Pilloni et al., 2019; Röling et al., 2001). In order to make inferences about bioremediation and effectively manage the processes, an understanding of the microorganisms responsible is necessary (Alfreider et al., 2002; Dlugonski, 2016; Köchling et al., 2015; Röling et al., 2000). A number of studies have been conducted on the microbiology of contaminated aquifers (Anantharaman et al., 2016; Cozzarelli et al., 2000; Harvey et al., 1984; Hug et al., 2015; Kleikemper et al., 2005; Ludvigsen et al., 1999; Röling et al., 2000; Tischer et al., 2012; Watanabe et al., 2002). Nonetheless, this area still requires more elucidation (He et al., 2018; Meckenstock et al., 2015). Bioremediation of hydrocarbon-polluted aquifers is well documented in the literature (Dojka et al., 1998; Harvey et al., 1984; Kleikemper et al., 2005; Pickup et al., 2001; Rooney-Varga et al., 1999; Tischer et al., 2012; Watanabe et al., 2002), but there is a dearth of studies on landfill leachate contaminations. The bias may reflect high-profile cases of hydrocarbon pollutions and the potential health hazard presented by the concomitant xenobiotics, which are often toxic, mutagenic and carcinogenic (Logeshwaran et al., 2018). Moreover, hydrocarbons, at least, are easily degraded in the environment and their compositions are less complex than effluents emanating from landfills. The complicated attenuation processes in landfill-leachate-impacted groundwater makes assessments of bioremediation processes more difficult (Christensen et al., 2000) and less attractive.

Studies on the microbiology of landfill-impacted aquifers have been reported (Albrechtsen et al., 1995; Lin et al., 2007; Ludvigsen et al., 1999; Mouser et al., 2005; Röling et al., 2000). These studies have provided insights into the microbial profiles of leachate-impacted aquifers, but they employed low throughput methods that cannot capture the full microbial diversity. Fewer studies have applied high throughput sequence-based methods (Chen et al., 2017; Lu et al., 2012; Taş et al., 2018) and they have not specifically focused on microbial diversity. Thus, there is insufficient literature on the microbial diversity of landfill-perturbed aquifers. The present study aimed to delineate the microbial diversity and composition of a landfill-leachate-contaminated confined aquifer using a combination of three microbiological techniques: culture and characterisation, cell counting by fluorescence microscopy, and Illumina MiSeq 16S rRNA metabarcoding. To the best of our knowledge, no such multi-methodological study of landfill-perturbed subsurface microbiology has been reported.

2. Materials and methods

2.1. Study area and groundwater sampling wells

Revdalen Aquifer is a glaciofluvial deposit located in Vestfold and Telemark County in southeast Norway at coordinate 59°25′58.26″N and 9°06′1.53″E (Fig. 1A). In 1958, a kettle hole was adopted as a waste disposal site and through 1958–1974, the hole was directly filled up with waste. Four successive landfill cells were later opened in the area and were filled up between 1974 and 1996. Due to the leachate leaking from the landfill, the aquifer, which was serving as a water source for the surrounding communities, became contaminated and water extraction was halted. Since the closure of the landfill in February 1997, the aquifer has been allowed to undergo monitored natural attenuation. Three multilevel monitoring wells (Fig. 1B) were established along the groundwater flow direction to monitor the groundwater quality: R1, with five levels (R101–105); R2, with four levels (R201–204) and R4, with three levels (R401–403). The increasing number in the multilevel (#101–105) depicts an increasing depth in the respective wells. Details on the multilevel systems are described elsewhere (Abiriga et al., 2020). The wells are hereafter referred to as proximal, intermediate and distal wells, respectively. In addition, a background well (R0) was established in a nearby aquifer for benchmarking the water quality. Additional information on the study site can be found elsewhere (Abiriga et al., 2020, 2021; Klempe, 2004, 2015).

2.2. Groundwater sampling and chemical analyses

Groundwater sampling procedure for proximal-distal wells has been described previously (Abiriga et al., 2020) while samples from the background well were taken using a submersible pump after purging the well volume. Sampling was conducted as per ISO 5667-11(2009). Samples for this study were collected in spring and autumn 2018 (water chemistry and 16S rRNA metabarcoding), and spring and autumn 2018–2019 (plate count and microscopic count); with one sampling campaign per season. For dissolved oxygen analysis, samples were collected in Winkler bottles, fixed onsite and protected from direct light. For other chemical analyses, samples were collected in 500 ml polyethylene bottles. pH and electrical conductivity were determined in the field using the pH meter pH-110 (VWR International) and conductivity meter Elite CTS Tester (Thermo Scientific, Singapore), respectively. The samples were maintained at ≤4 °C using icepacks and a cooler box and transported to the laboratory at University of South-Eastern Norway. Samples for iron and manganese were filtered through 0.45 μm and acidified with nitric acid to pH ~2, while samples for total nitrogen were preserved by acidifying using sulphuric acid. All samples were stored at 4 °C until analysis. Norwegian Standards were followed for determination of dissolved oxygen (NS-ISO 5813), alkalinity (NS-EN ISO 9963-2), iron (NS 4773), and manganese (NS 4773). Major ions (ammonium, sodium, potassium, calcium, magnesium, chloride, nitrate and sulphate) were determined using Ion Chromatography DIONX ICS-1100 (Thermo Scientific, USA). Total nitrogen and total organic carbon (TOC) were determined using FIAlyzer-1000 (FIAlab, USA) and TOC Fusion (Teledyne Tekmar, USA), respectively.

2.3. Microbial analyses

2.3.1. Sample handling

Groundwater samples were collected in sterile 350 ml PETE bottles (VWR, UK) without headspace and transported as described above.

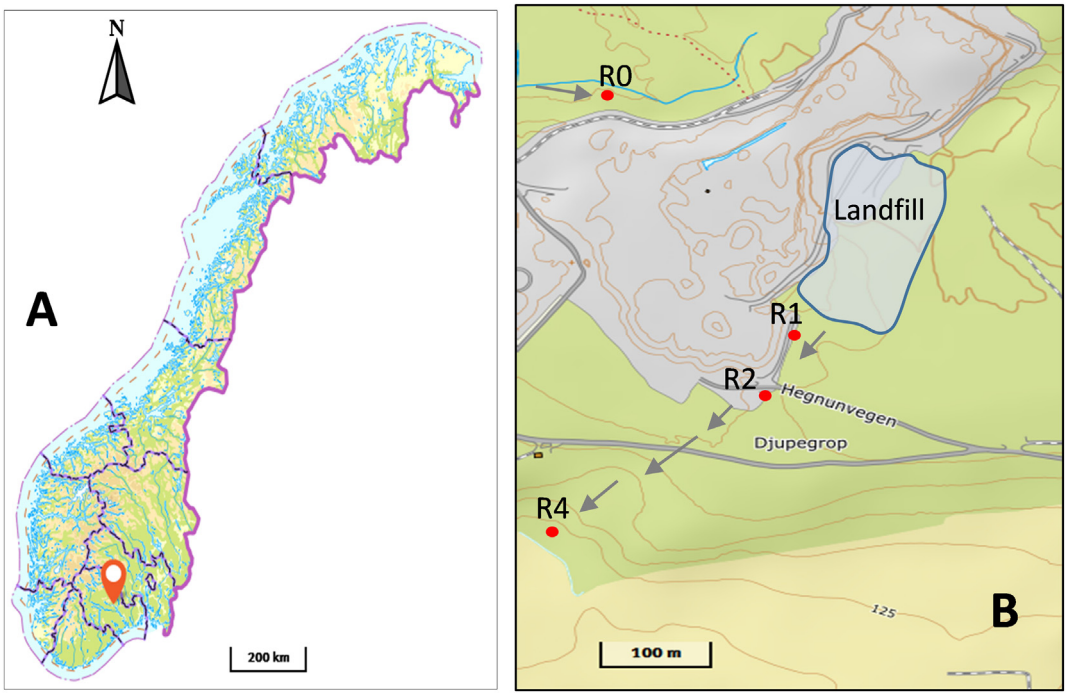

Fig. 1. Location of the study site on a map of Norway (A) and the location of the landfill and sampling wells R0, R1, R2 and R4 (B) [Map source: Norwegian Geographical Survey, **www.norgeskart.no**, with permission]. R0 is the background well located in an uncontaminated aquifer, while wells R1, R2 and R4 are located in the contaminated aquifer placed at the proximal, intermediate and distal positions, respectively. Arrows indicate the groundwater flow direction. Green shading indicates woodland, yellow indicates farmland, and grey indicates industrial land, including the landfill and an adjacent active quarry/gravel pit. Contour interval 5 m.

Upon arrival at the microbiology laboratory, 4.5 ml portions were fixed with 2.5% (final concentration) phosphate-buffered glutaraldehyde for microscopy. For 16S rRNA metabarcoding, 300 ml of water was filtered through 0.2 μm poresize 47 mm diameter polycarbonate membrane filters. The filters were stored at −70 °C prior to DNA extraction. The remaining water was used for culturing heterotrophic bacteria. All the different sample handlings were conducted within 48 h.

2.3.2. Direct microscopic count

1.9 ml of fixed groundwater samples were stained with 5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Kepner and Pratt, 1994). The stained cells were filtered onto 0.2 μm black polycarbonate Nuclepore membrane filters (Sigma-Aldrich, Germany), transferred onto microscope slides and overlaid with antifade mountant oil (Citifluor AF87, EMS, PA, USA). Cells were enumerated under ×100 oil objective using Olympus IX70 fluorescence microscope (Tokyo, Japan). Ten fields were counted and the average count was used to estimate bacterial density using the formula: Bacteria (Cells/ml) $= (N \times A_t)/(V_f \times A_g \times d)$, where N = average number of cells, A_t = effective area of the filter paper, V_f = volume of water sample filtered, A_g = area of the counting grid, and d = dilution factor (Kepner and Pratt, 1994). No observable cells were found in our blanks and therefore correcting for background noise due to contamination was not necessary.

2.3.3. Culturing and sequencing of heterotrophic bacteria

Preliminary assessment (data not shown) indicated dilutions ≥10^2 could generate countable colonies and best growth occurred on half-strength tryptic soy agar. Thus, serial dilutions of 10^2, 10^3, and 10^4 were prepared for each water sample and triplicates of 100 μl aliquots were spread on half-strength tryptic soy agar (20 g/l TSA and 7 g/l agar). Plates were incubated at 15 °C for ≥5 days. Aerobes were counted following incubation under aerobic condition. Anaerobes were enumerated in a parallel setup following incubation under anaerobic condition using GasPak with EZ Anaerobe Container System (BD, USA). Plates from two dilutions of each sample were counted and the average was reported as colony forming units (cfu) per ml.

Based on observable colony morphologies such as shape, elevation, margin, size, and colour, colonies covering the full diversity of colony morphologies were picked and purified by repeated streaking and incubation until pure cultures were obtained. These cultures were then observed by wet field microscopy at ×1000 (Olympus, CX22LEDRFS1, China) to observe motility and cell shape, followed by Gram staining and determination of oxidase and catalase activity (Csuros et al., 1999). Strains were stored at −70 °C in nutrient broth (Sigma, Switzerland) supplemented with 25% glycerol. The laboratory procedure for DNA extraction and sequencing of the V3–V5 16S rRNA gene region of the pure isolates is provided in the supplementary information (Method S1).

2.3.4. 16S rRNA metabarcoding

The frozen filters were retrieved and cut into two halves using sterile surgical blades and DNA was extracted from one half using DNeasy PowerSoil Kit (Qiagen, Germany) following the manufacturer's instructions. The DNA quantity and quality were checked using Qubit® Flourometer 3.0 (Life Technologies, Malaysia) and 2% agarose gel electrophoresis, respectively. PCR and 16S rRNA gene library preparation (Fadrosh et al., 2014) for the samples were conducted at Norwegian Sequencing Centre (https://www.sequencing.uio.no). Both forward and reverse oligos included Illumina-specific adaptor sequence, a 12-nucleotide barcode sequence, a heterogeneity spacer and respectively the primers 319F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 805R (5′-GGACTACNVGGGTWTCTAAT-3′) for the V3–V4 hypervariable 16S rRNA gene region. The pooled libraries were sequenced using Illumina MiSeq (600 cycles), applying the 300 bp paired-end protocol with 10% PhiX.

Sequence demultiplexing was conducted using a demultiplexer available at https://github.com/nsc-norway/triple_index-demultiplexing/tree/master/src. Barcodes and heterogeneity spacers were removed in

the process with no mismatches allowed in the barcode. Denoising (primer trimming, and removal of shorter sequences and chimeras), dereplication, merging, and clustering of sequences into amplicon sequencing variants were performed using DADA2 algorithm (Callahan et al., 2016) plug-in for QIIME2 version 2019.1.0 (Bolyen et al., 2019). Default parameters were implemented, apart from primer length (adjusted to 20 bp) and minimum sequence length of reads (adjusted to 280 bp). Taxonomic assignment was conducted using Naïve Bayes classifier algorithm trained on data from SILVA v.138 using QIIME2 version 2020.2.0.

2.3.5. Quality control

Quality control samples included in the sequencing run consisted of an elution buffer, extraction blanks and mock communities, which are described in more detail in the supplementary information (Method S2).

2.4. Data availability statement

The 87 16S rRNA gene sequences of the cultured isolates have been deposited in GenBank under Accession Nos. MT348616-MT348702. The raw sequence reads from the metabarcoding have been deposited in Sequence Read Archive under BioProject PRJNA677875 (BioSamples used in this study: SAMN16775936-SAMN16775961).

2.5. Data analysis and statistics

All data analyses were conducted in R version 4.0.2 (R Core Team, 2020). To compare water chemistry between the background sample and the contaminated water samples, one-tailed Wilcox rank test was used. Comparison of water quality across the wells in the contaminated aquifer was performed using Kruskal-Wallis rank sum test. These tests were chosen as most of the variables showed non-normal distribution. One-tailed paired *t*-test was used for both within-sample and overall comparison between plate and microscopic counts. Similarly, comparison between aerobic and anaerobic counts was also done using one-tailed paired *t*-test. One-way ANOVA was conducted to test for differences in aerobic and microscopic counts across the wells and a post-hoc Tukey's Honest Significant Difference for the pairwise comparisons. All the parametric tests were performed on log-transformed data. Normality of data distribution was assessed graphically using histograms and boxplots, and by Shapiro-Wilk normality test. Statistical significance was inferred at alpha = 0.05.

Multivariate analyses were performed using package vegan in R (Oksanen et al., 2019). Prior to redundancy analysis (RDA), the OTU abundance data was pre-transformed using fourth-root transformation to reduce asymmetry of the data distribution before standardising it using Hellinger standardisation (Legendre and Gallagher, 2001). Also, because groundwater physicochemical variables are dimensionally heterogeneous, the chemistry data was standardised (centred and scaled by standard deviation) prior to RDA ordination. The difference in the community profiles of the sample groups in the RDA triplot was tested using permutational multivariate analysis of variance (PERMANOVA) on Euclidean distance. The homogeneity of group dispersion was assessed beforehand using the function *betadisper* (Anderson, 2006). For the RDA, the full dataset was used without any merging ($N = 26$: R0 = 2, R1 = 10, R2 = 8 and R4 = 6). Principal component analysis (PCA) was applied on square-root transformed and Hellinger-standardised culture-based microbial data and the physicochemical data was fitted onto the PCA using the command *envfit* in package vegan. Taxonomic-to-phenotypic mapping was implemented using METAGENassist (Arndt et al., 2012) and to reduce the number of categories, abundances were merged by summing across the well levels and presented as the wells instead.

3. Results

3.1. Groundwater chemistry

Most of the solutes in the groundwater were present at levels significantly above that of the background well (Table 1). Kruskal-Wallis test showed significant differences across the wells in the contaminated aquifer for eight of the fifteen variables measured (Table S1). While most of the variables showed a decrease in concentration along the groundwater flow path, a few *i.e.*, dissolved oxygen, magnesium, manganese and iron showed an increase in concentration downgradient.

3.2. Groundwater microbiology

3.2.1. Viable plate count

The cell density estimate from the plate count was in the range 1×10^2 - 3.2×10^5 cfu/ml (aerobic) and 0–2.4×10^5 cfu/ml (anaerobic). Despite the comparable maximum counts from the two growth conditions, the aerobic plate count was significantly higher than the anaerobic plate counts ($t = 3.63$, $df = 37$, $p = 0.0004$). The same strains dominated under both aerobic and anaerobic conditions and isolates recovered under anaerobic condition were all facultative anaerobes. Nonetheless, some strains were isolated only under anaerobic condition. Wells in the contamination plume had higher plate counts than the background well, and the distal well had lower plate counts compared to the intermediate and proximal wells (Fig. 2). The count was significantly different across the wells ($F = 3.09$, $df = 3$, $p = 0.0357$), but pairwise comparisons between the wells did not give significant results.

3.2.2. Direct fluorescence microscopy

Microscopic counts were in the range 7×10^3–3.5×10^5 cells/ml. Cell morphologies observed under fluorescence microscopy included small rods, long large rods, coccobacilli and vibrios. Small rods were most frequently encountered. Water samples from the proximal and intermediate wells were dominated by short rods, vibrios and elongated narrow rods, some occurring in chains. The distal well on the other hand, did not exhibit predominance of any specific cell types, an observation

Table 1
Characteristics of the groundwater chemistry measured in spring and autumn 2018. Values from the background well (R0) were used as a benchmark against which those in the proximal (R1), intermediate (R2) and distal (R4) wells were compared. All units are in mg/l, except for pH (pH units), conductivity (μS/cm) and alkalinity (mM).

	Mean	Range (min - max)		
	R0 (N = 2)	R1 (N = 10)	R2 (N = 8)	R4 (N = 6)
pH	5.1	**6.3–7.7**	**6.6–7.1**	**5.9–6.2**
Conductivity	33	**196–251**	**190–220**	**107–190**
Dissolved oxygen[a]	4.5	**1.3–2.9**	**1.3–4.8**	1.1–9.1[b]
Sodium	1.7	**4.5–5.9**	**5.4–5.6**	**4.0–5.5**
Potassium	0.32	**5.0–7.5**	**6.7–7.5**	**6.4–7.5**
Calcium	2.1	**25–34**	**23–26**	**10.7–20**
Magnesium	0.48	**2.4–2.8**	**2.9–3.2**	**2.7–3.6**
Ammonium[c]	0.0	**0.13–1.9**	**0.32–0.57**	**0.0–0.41**
Iron[d]	0.02	0.02	0.02	0.02–0.09
Manganese	0.04	**0.05–0.45**	**0.17–0.31**	**0.01–1.4**
Alkalinity	0.06	**1.2–1.9**	**1.2–1.7**	**0.77–1.3**
Sulphate	2.9	**8.4–25**	**7.4–9.7**	**5.7–10**
Nitrate (as Nitrogen)	1.1	**1.6–4.8**	0.48–3.7[b]	**1.3–4.0**
Chloride	1.8	**3.4–4.7**	**5.6–6.9**	**3.6–4.1**
Total nitrogen	0.97	**2.9–4.5**	3.4–3.5[b]	2.9–3.4[b]
Total organic carbon	4.1	2.7–5.0[b]	2.9–5.9[b]	1.8–6.3[b]

Values significantly higher than the background value ($p < 0.05$) are indicated in bold face. N = number of samples.
[a] Dissolved oxygen was lower than the background value, but only significantly so in R1 and R2.
[b] Non-significant difference from the background value ($p > 0.05$).
[c] Values below the limit of detection were treated as zero.
[d] Values below the limit of detection were reported as half the limit of detection (0.02).

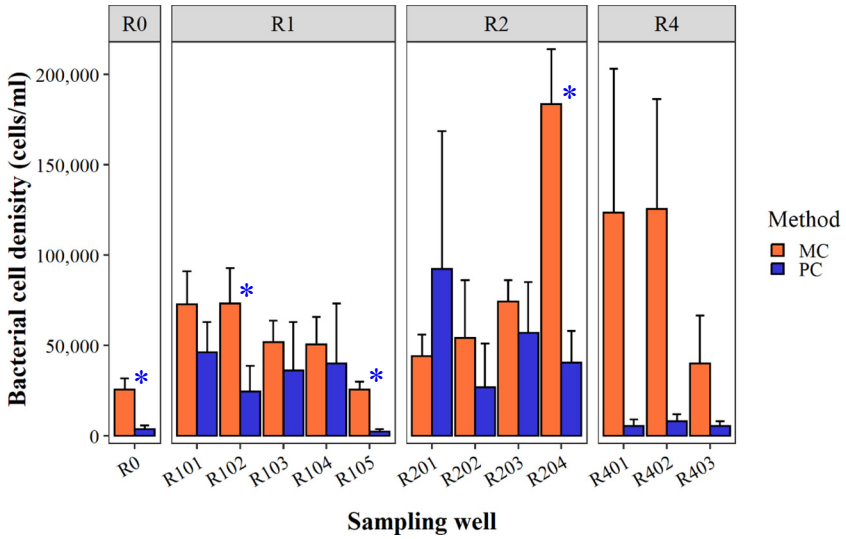

Fig. 2. Bacterial cell density in the groundwater samples estimated from direct microscopic count (MC) and aerobic plate count (PC); error bars are mean + standard error ($n = 4$). R0 is the background well located in an aquifer upstream of the landfill. R101–R105, R201–R204 and R401–R403 are the multilevels in R1, R2 and R4 respectively, which are located in the contaminated aquifer placed along the groundwater flow direction at the proximal, intermediate and distal positions, respectively. Asterisks indicate a significant difference.

that agrees with the more diverse colony morphology (higher richness) observed in samples from this well (see below). The total cell count was not significantly different across the four wells ($F = 1.44$, $df = 3$, $p = 0.243$), nor were the pairwise comparisons.

The distal well, which showed very low plate counts, gave a relatively higher microscopic counts. Within-sample comparisons indicate that microscopic counts were, in all but one sample, higher than plate counts, but only significantly so in four of the thirteen samples: R0, R102, R105 and R204 (Fig. 2 and Table S2). Overall, the microscopic count was higher than the plate count ($t = 6.94$, $df = 51$, $p < 0.05$) (Fig. S1).

3.2.3. Identification of isolates

Small subunit rRNA gene sequences of the pure isolates revealed higher species richness in the wells located in the contamination plume than in the background well (Fig. S2). The distal well had the highest species richness. Eighty-seven taxa belonging to forty-six genera were identified (Fig. S2, Table S3), *Pseudomonas* being the most frequently isolated taxon (Fig. S4). Of the eighty-seven taxa, seven were found only in the background aquifer, seventy-seven found only in the contaminated aquifer and three were found in both. These three were *Rhodoferax ferrireducens*, *Pseudomonas* sp. (2) and *Rugamonas* sp. detected in both the background and distal wells (Fig. 3, Table S3). As shown in Fig. 3, twenty-eight taxa were unique to the distal well. Not a single species was found in all the four wells, although two genera (*Pseudomonas* and *Janthinobacterium*) were common. Among the wells located in the contamination plume, only two species: *Pseudomonas veronii* and *Rhodococcus degradans* were found in all of them. The proximal-intermediate was the most similar pair of wells, with ten species in common. PCA ordination further depicts this similarity (Fig. S5).

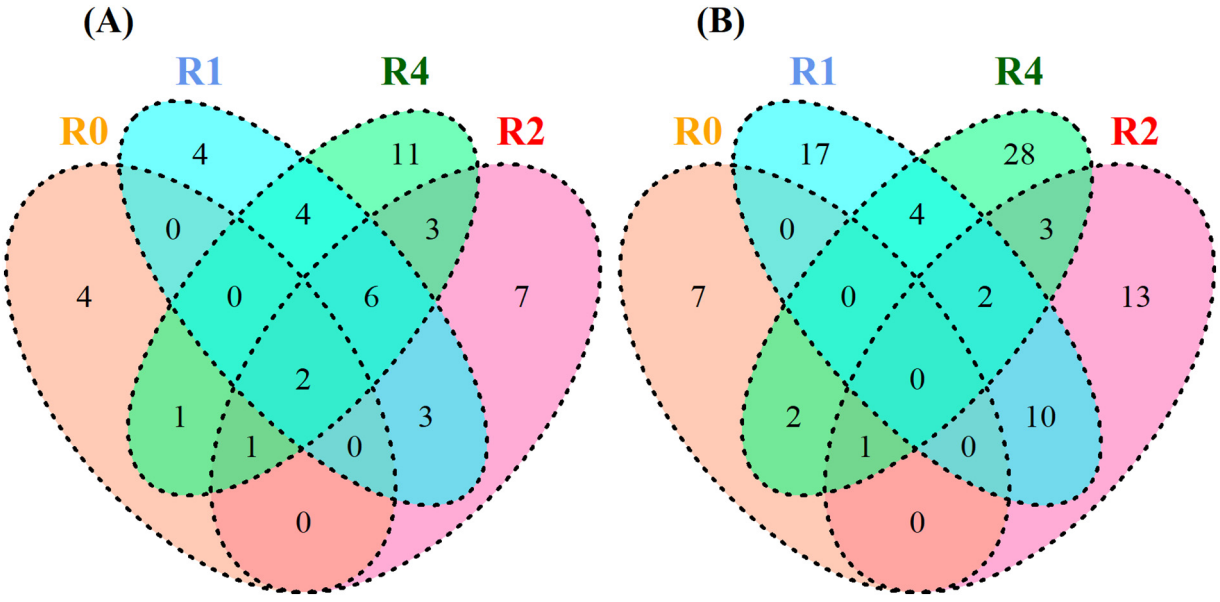

Fig. 3. Venn diagram showing relationships among the groundwater sampling wells at genus-level classification **(A)** and species-level classification **(B)**. R0 is the background well located in an aquifer upstream of the landfill. R1, R2 and R4 are wells located in the contaminated aquifer placed along the groundwater flow direction at the proximal, intermediate and distal positions, respectively.

3.2.4. 16S rRNA metabarcoding

A total of 1496 OTUs were identified by metabarcoding. 98.73% were bacteria; the rest were archaea (1.2%) or unclassified (0.07%). These were classified to 57 microbial phyla, with *Proteobacteria* (31%), *Patescibacteria* (9.2%), *Bacteroidetes* (7.8%), *Actinobacteria* (6.5%), *Chloroflexi* (6.1%), *Acidobacteria* (5.5%), *Verrucomicrobia* (5.4%) and *Firmicutes* (3.5%) as the top eight phyla in the entire OTU table.

For the sake of simplicity and in order to target taxa likely to be stable members of the microbial community, only OTUs comprising at least 2% in at least one sample are considered here. This resulted in only 68 genera being represented (Fig. 4). Of these, 15 had unresolved taxonomic classifications and 17 were OTUs of uncultured microbes. Twelve of the thirty-six (33%) phylogenetically well-resolved genera were among those isolated through the culture-based approach. These were *Brevundimonas*, *Flavobacterium*, *Janthinobacterium*, *Pedobacter*, *Phyllobacterium*, *Polaromonas*, *Pseudomonas*, *Rhodanobacter*, *Rhodoferax*, *Sphingomonas*, *Stenotrophomonas* and *Undibacterium*. These genera also showed greater frequency in the PCA ordination for the cultured taxa (Fig. S5). The 12 genera accounted for 38 of the total cultures isolated (Fig. S4).

Shannon diversity measure was applied to the OTU data to infer species richness and diversity. Microbial diversity was greatest in the intermediate well, followed by the proximal well and was lowest in the distal well (Fig. 5B and Table S4). Similarly, the evenness estimate followed the same trend, indicating that the distal well was dominated by few taxa, while the intermediate well had fairly even representation of microbial taxa present at the site. However, both the highest (692) and lowest (136) OTU richness was recorded at the different levels in the intermediate well. This resulted in a larger spread of OTU richness in the intermediate well (Fig. 5A). A count of species (at genus level) (Fig. 5C) indicates that a core of 137 genera were common to all the sampling wells, while a further 153 genera were common to wells of the contaminated aquifer. The number of unique genera varied between

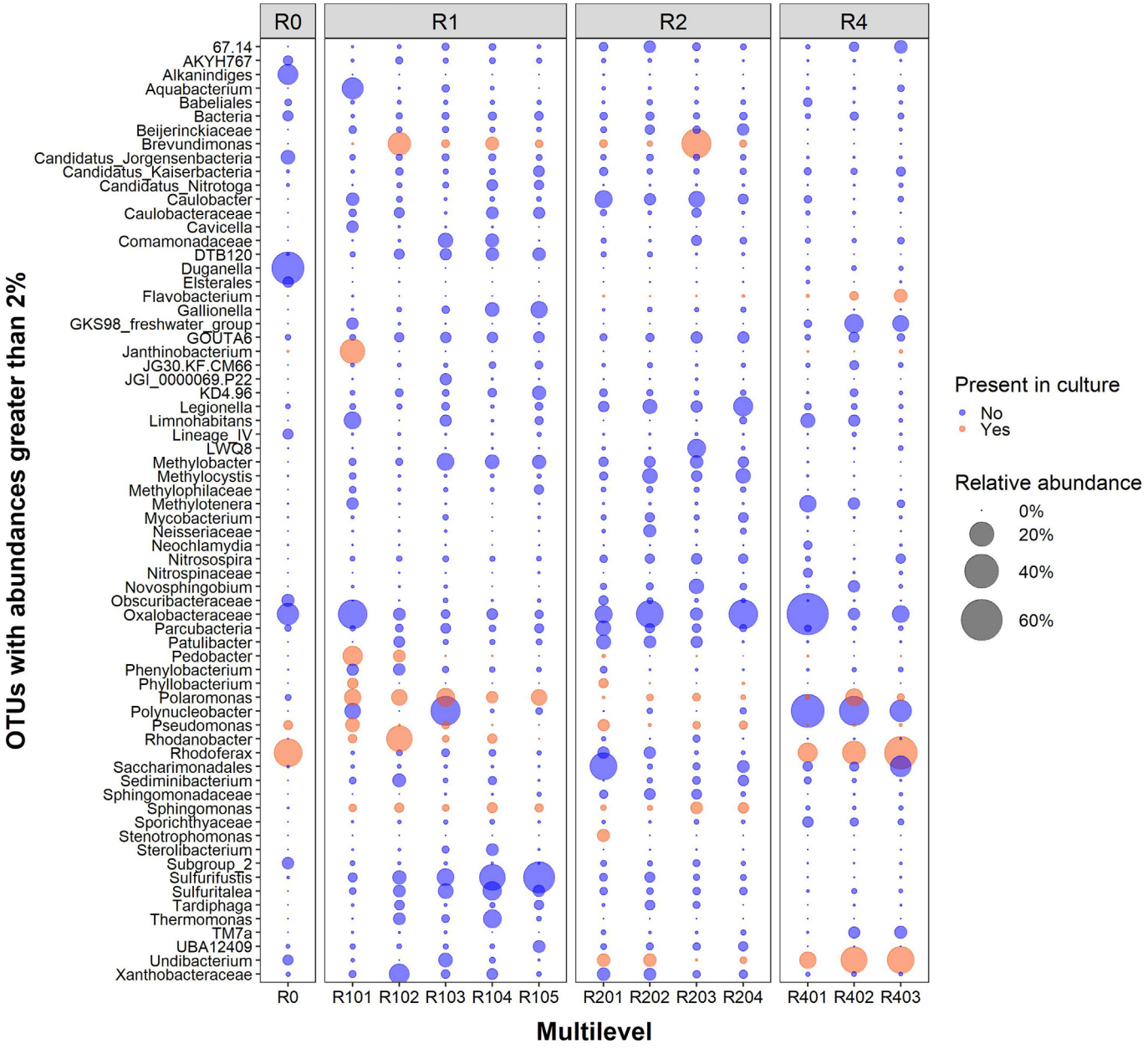

Fig. 4. OTUs greater than 2% of the total reads in at least one of the samples. R0 is the background well located in an aquifer upstream of the landfill. R101–R105, R201–R204 and R401–R403 are the multilevels in R1, R2 and R4 respectively, which are located in the contaminated aquifer placed along the groundwater flow direction at the proximal, intermediate and distal positions, respectively. For clarity, the relative abundances for spring and autumn were merged by summing so the total per sample would be >100% but <200% since only OTUs greater than 2% are shown.

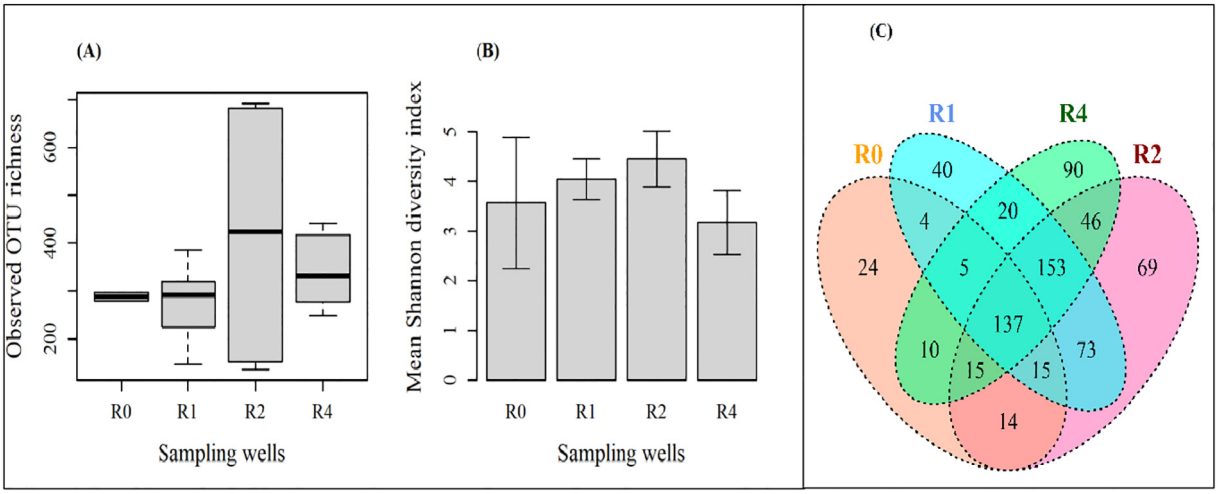

Fig. 5. OTU richness **(A)**, Shannon diversity index **(B)** (error bars are ±SD) and species count at genus level for the full dataset **(C)** recorded in the sampling wells R0, R1, R2 and R4. For figs. **A–C**, $N = 2$ (R0), 10 (R1), 8 (R2) and 6 (R0) (Table S4). R0 is the background well located in an aquifer upstream of the landfill. R1, R2 and R4 are the wells located in the contaminated aquifer placed along the groundwater flow direction at the proximal, intermediate and distal positions, respectively.

24 in the background well and 90 in the distal well. The number of genera shared between just two wells varied from 4 (background/proximal) to 73 (distal/intermediate).

Multivariate analysis using RDA showed that microbial composition varied spatially, with the intermediate and proximal wells clustering next to each other as was observed with the culture-based method (Fig. 6 and Fig. S5). PERMANOVA showed that the microbial composition across the wells was significantly different ($F = 4.58$, $df = 3$, $p = 0.001$). Three canonical axes were significant ($p = 0.001$, 999 permutations) and the model explained 34.9% (adjusted R^2) of the variation in microbial composition. The proximal and the intermediate wells correlated positively with RDA1, while the distal and background wells correlated negatively with RDA1. The second axis (RDA2) separated mainly the background well from the rest of the wells located in the contaminated aquifer, but also separated the proximal well from the distal well.

The groundwater physicochemical parameters showed two major gradients: those that showed higher levels towards the proximal well

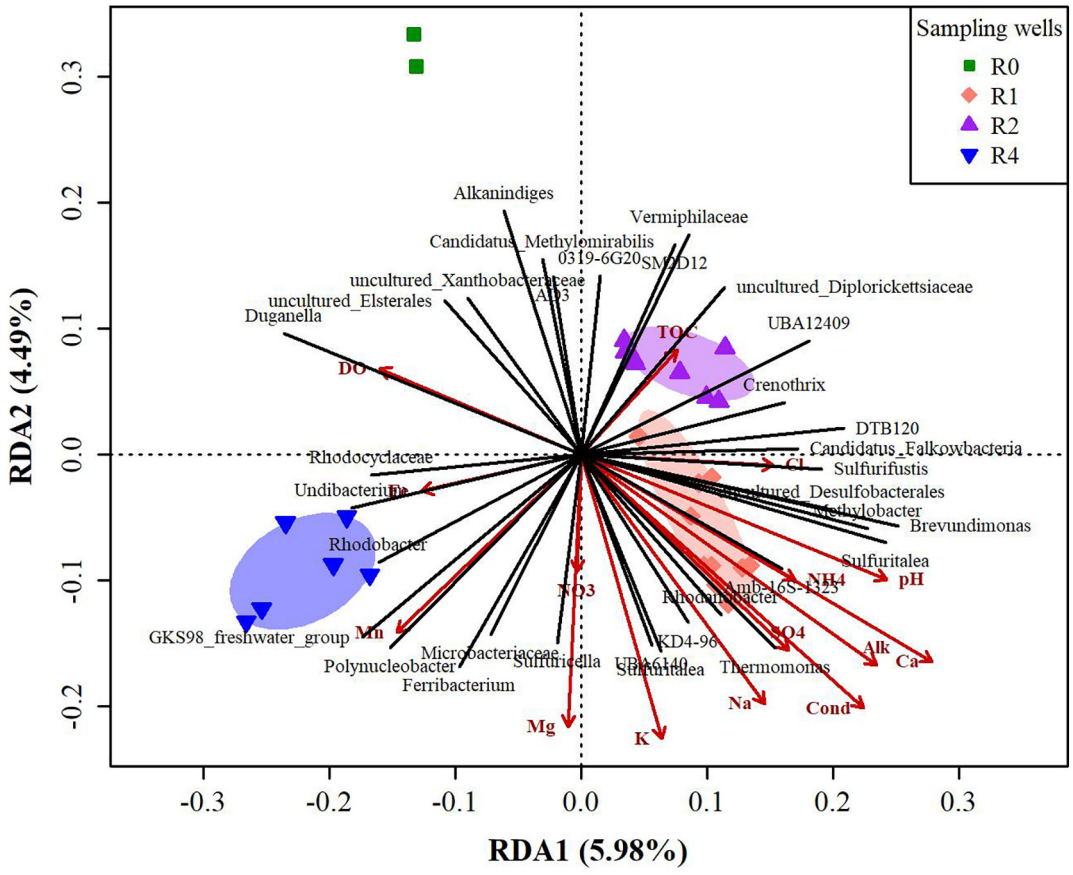

Fig. 6. RDA of microbial composition using the full OTU dataset among the sampling wells: R0 (background well), R1 (proximal well), R2 (intermediate well) and R4 (distal well). For clarity of readability, only OTUs with prominent vector length are shown.

and those that showed higher levels towards the distal well. The former included pH, ammonium, calcium, alkalinity, sulphate, conductivity, sodium, chloride and potassium, which have correlated negatively with RDA2. The second gradient was driven by dissolved oxygen, magnesium, iron and manganese, which have likewise correlated negatively with RDA2, except dissolved oxygen that correlated positively with RDA2. Nitrate and magnesium have shown moderate gradient between the proximal and distal wells, while TOC showed higher levels towards the intermediate well.

The prominent OTUs in the intermediate and background wells were dominated by uncultured lineages. On the other hand, the abundant OTUs in the distal and proximal wells composed mainly culturable taxa, although those in the proximal well seem to constitute those requiring special growth conditions. Some of the OTUs that showed prominent vectors in the RDA analysis were also among the abundant (>2%) OTUs. They include *Alkanindiges*, *Duganella*, *Undibacterium*, GKS98 (uncultured *Alcaligenaceae*), *Sulfuritalea*, *Sulfurifustis*, *Thermomonas*, *Rhodanobacter*, *Brevundimonas*, *Methylobacter*, DTB120 and UBA12409 (uncultured *Babeliales*).

3.2.5. Predicted phenotypic functions

Taxonomic-to-phenotypic mapping using METAGENassist (Fig. 7) predicted 15 potential energy source phenotypes, predominantly phototroph, heterotroph, autotroph and organotroph, methanotroph, methylotroph and oligotroph, in varying abundances. Five groups were present at a relative abundance >0.2. These were phototroph in the background and distal wells, heterotroph in the background and proximal wells, organotroph in the proximal well, and autotroph and methanotroph in the intermediate well. Heterotroph and phototroph accounted for >50% of the total abundance in the background and distal wells, respectively. Overall, the background well consisted of two major energy source-types, the proximal and intermediate wells seven and six, respectively, and the distal well four.

Twenty-five different potential metabolic profiles were predicted (Fig. 8). Degraders of both inorganic and organic compounds were predicted. Organic compound transformers were, however, in lower abundances than inorganic compound metabolisers. Microbes involved in sulphur and nitrogen transformations formed the dominant groups. Generally, the abundance of metabolic profiles varied from well to well.

4. Discussion

4.1. Cell density and microbial diversity

Overall, cell density estimate from microscopic count was significantly higher than plate count, which is consistent with the literature (Gregorich and Carter, 2007; Muyzer and Smalla, 1998) and expected from theoretical considerations. However, within the wells located in the contaminated aquifer, this difference was seldom greater than a factor of two in most cases, and only in three of the twelve multilevel samples was the difference significant. This suggests that in most samples, a large proportion of the microbial population is culturable. The compounds in the landfill leachate likely favoured the growth of culturable heterotrophic microorganisms. The physiological profiles of microbial communities in aquifers receiving a landfill leachate are expected to be due to culturable bacteria (Röling et al., 2000). However, the metabarcoding data conflict with these findings, the highest proportion of uncultured OTUs being found in the intermediate well, rather than in the distal well as indicated by the comparison of microscopic and plate counts.

The background aquifer had low solute levels, indicating that the aquifer is nutrient-poor. Based on the culture method, both cell density and the microbial diversity were low, although a comparison of microscopic and plate counts suggests that a fairly large proportion of the population is non-culturable. This is consistent with the metabarcoding data, in which the microbiome of the background aquifer was dominated by uncultured taxa and only a few culturable taxa such as *Duganella*, *Rhodoferax* and *Alkanindiges* were abundant. Similarly, the Shannon diversity index (3.57 ± 1.32) was lower than the overall diversity index recorded in the contaminated aquifer (3.96 ± 0.71). Further, the RDA ordination (Fig. 6) indicates that the background well is compositionally very different from the contaminated aquifer. Similar differences in microbial communities between contaminated and uncontaminated groundwater have been reported (Brad et al., 2013; Brad et al., 2008; Mouser et al., 2005).

The contaminated aquifer, on the other hand, is relatively solute-rich and supports a denser and more diverse microbial population; a scenario likely to occur where a landfill leaches easily degradable organic matter (Röling et al., 2000). Although the concentrations of solutes

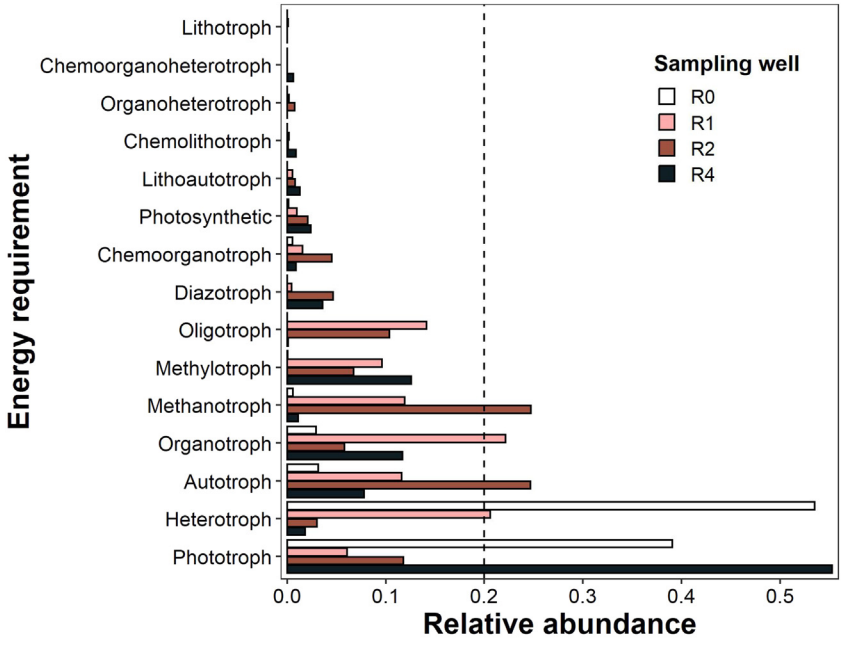

Fig. 7. Predicted energy source requirements of the OTUs in R0, R1, R2 and R4. R0 is the background well located in a nearby uncontaminated aquifer, while R1, R2 and R4 are the wells located in the contaminated aquifer placed at the proximal, intermediate and distal positions from the landfill. The vertical dashed line depicts 20% abundance.

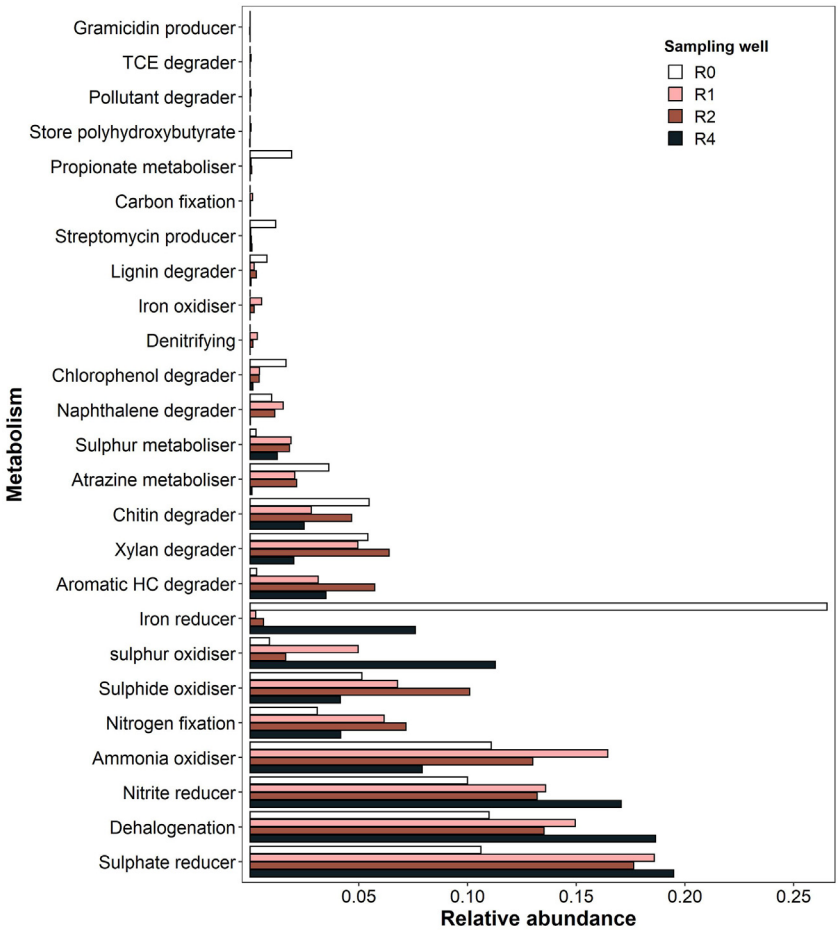

Fig. 8. Predicted metabolic potentials of the OTUs in R0, R1, R2 and R4. R0 is the background well located in a nearby uncontaminated aquifer, while R1, R2 and R4 are the wells located in the contaminated aquifer placed at the proximal, intermediate and distal positions from the landfill.

have decreased greatly compared to previous data (Abiriga et al., 2020), they are evidently still sufficient to maintain a distinctive bacterial community; a previous study (Herzyk et al., 2017) indicates that physicochemical parameters return to normal more quickly than the microbial community. In line with this, data from culturing, fluorescence microscopy and metabarcoding showed higher species and cell density, diversity and species richness in the contaminated aquifer. The highest species richness and Shannon entropy were recorded in the intermediate well (Fig. 5A-B and Table S4), an observation that agrees with the culture and microscopic methods, where the highest cell and plate counts were recorded in the intermediate well (Fig. 2). On the other hand, moderate and lowest species richness and diversity were recorded in the proximal and distal well, respectively (Fig. 5A-B). This suggests the existence of an ecological gradient along the groundwater flow path, in which the proximal and intermediate wells are expected to have a high resemblance, as they are spatially close to each other. This is consistent with both the culture and metabarcoding data (Fig. 3 and Fig. 5C), where the highest shared species and genera was recorded between the proximal and intermediate wells. Further, these wells clustered next to one another (Fig. 6), suggesting that they are relatively similar and are thus made up of fairly similar microbial communities. The distal well is therefore considered to be ecologically more different from the upstream wells, which is consistent with the observations that the highest unique species (28) (Fig. 3B) and genera (90) (Fig. 5C) were found in the distal well. This is further supported by the beta diversity (Fig. 6) which indicates that the microbial composition of the distal well is different from the upstream wells.

The concentration of some of the groundwater physicochemical variables was found to decrease with distance from the landfill (Table 1), and this was accompanied by an increase in microbial diversity (Fig. 5 and Table S4) at least from the proximal to intermediate wells. This may be analogous to increase in diversity and community stability as leachate becomes less contaminated over time (Köchling et al., 2015). Thus, close to the landfill, only species resistant to the toxic effects of the leachate are probably able to survive and grow, while as toxic pollutants become attenuated through biotic and abiotic processes, it allows the growth of more sensitive species. A previous study from Norman Landfill in the United States has shown that microbial gene diversity varied with distance from the landfill (Lu et al., 2012). In either study (both the previous and the present), the observed changes in diversity as a function of distance indicates the significance of attenuation mechanisms *in situ* that shape the microbial composition and function. Such spatial variation may enhance biodegradation as pollutants migrate from one region with a given microbial composition to another of a different microbial composition (Brad et al., 2013; Brad et al., 2008; Mouser et al., 2005; Röling et al., 2000).

4.2. Microbial compositions and environmental significance

Four microbial phyla were identified by the culture method (Fig. S3) compare to fifty-seven identified by the metabarcoding of which only eight occurred in greater (>3.5%) abundances. Of these eight phyla, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Firmicutes* were represented among the cultured isolates, while *Patescibacteria*, *Chloroflexi*, *Acidobacteria* and *Verrucomicrobia* were only present in the metabarcoding data. *Proteobacteria* was the most abundant phylum and accounted for 31% and 55.2% of the total microbiome in the metabarcoding and culture data, respectively. *Bacteroidetes* (16.1%)

and *Actinobacteria* (25.3%) were present in comparable proportions, while *Firmicutes* (3.4%) was the least abundant of the eight phyla (based on culture data).

Putative hydrocarbon degraders among the isolates are presented in Table S5. Those from the metabarcoding data include the antipyrine/chloridazon-degrader *Phenylobacterium* (Lingens et al., 1985; Oh and Roh, 2012), alkane/alkyl-degraders *Parvibaculum* and *Alkanindiges* (Bogan et al., 2003; Lai et al., 2011; Schleheck et al., 2004), ibuprofen-degrader *Patulibacter medicamentivorans* (Almeida et al., 2013) and oil-degrader *Aquabacterium* (*A. olei*) (Jeong and Kim, 2015). Others include the single-carbon metabolisers *Methylobacter*, *Methylotenera* and *Methylocystis*. Energy source prediction showed the presence of methylotrophs and methanotrophs in high proportions in the contaminated wells (R1-R4) (Fig. 7), which may suggest the presence of methane in the aquifer. The landfill has now matured (manuscript under consideration) and is supposedly in transition from methanogenic to aerobic stage, *i.e.*, from phase III to phase IV of landfill stabilisation (Kjeldsen et al., 2002), but there could still be active methanogenesis. A wide range of hydrocarbon metabolisms were also predicted through the phenotypic mapping (Fig. 8). Although the TOC in the groundwater was low with a weak gradient along the groundwater flow path, it could still provide carbon source to the aquifer microbiome. Considering the age of the landfill, the TOC should be predominantly of recalcitrant fractions (Kulikowska and Klimiuk, 2008) and this makes it difficult to degrade (Appelo and Postma, 2005), leading to a non-significant difference across the wells.

From the metabarcoding data, sulphur cyclers were detected and included the sulphur/sulphide oxidisers *Sulfuritalea* and *Sulfurifustis* (Kojima et al., 2015; Kojima and Fukui, 2011) (Fig. 4), and *Sulfuricella* (Kojima and Fukui, 2010) and *Rhodobacter* (Imhoff et al., 1984) (Fig. 6). However, sulphate showed a weak gradient with a non-significant difference across the wells (Table S1), suggesting that the aquifer contains a significant sulphate sink, such as re-reduction in local anaerobic pockets.

Both nitrate reducers and ammonia oxidisers were identified. Nitrate reducers included 29 cultured isolates (Table S6), and genera *Cavicella*, *Sterolibacterium*, *Aquabacterium* and *Novosphingobium* from the metabarcoding data. *Sulfuricella*, which at present is monotypic (*S. denitrificans*), correlated positively with nitrate and could therefore be involved in denitrification. Among the nitrogen oxidisers were the ammonia oxidisers *Nitrosospira* (Watson, 1971) and nitrite oxidisers *Nitrospinaceae* (Lücker and Daims, 2014). Taxonomic-to-phenotypic mapping (Fig. 8) indicates the presence of ammonia oxidisers and nitrate/nitrite reducers. Both ammonium and nitrate were detectable in the groundwater, although nitrate was present in higher levels than ammonium. The presence of both ions may suggest complete cycling of nitrogen, although the reduction reaction might be limited due to low organic matter. The lack of a significant difference in nitrate across the wells suggests a balance between reductive and oxidative nitrate metabolism.

Iron metabolisers include *Gallionella*, a genus known for centuries to clog well screens (Chapelle, 2001) by oxidising ferrous iron at an ecotone between reducing and oxidising environments. Genus *Rhodoferax*, which the culture-based analysis showed to be represented by *R. ferrireducens*, together with genus *Ferribacterium*, are both iron-reducing bacteria; a role that counteracts that played by *Gallionella*. In addition, *R. ferrireducens* is known to carry out metabolism of manganese and oxygen (Finneran et al., 2003). Although the level of iron was low across the wells, the distal well where *Rhodoferax* and *Ferribacterium* were abundant was slightly enriched in iron, manganese and oxygen (Table 1 and Fig. 6). The greater abundance of iron-reducers in the background well (Fig. 8) can be attributed to *Rhodoferax*, although iron in this well was below the limit of detection. Like sulphate reduction, iron reduction must be limited due to the low organic matter. We propose that the high proportion of phototrophs (Fig. 7) is due to the high abundance of *Rhodoferax* found in the respective wells since two species of this genus are phototrophs (Finneran et al., 2003). Although phototrophy does not mean being obligately photosynthetic, the result here is likely due to misclassification as the programme (METAGENassist) uses OTU information which in metabarcoding are seldom resolved to species-level and unambigous functional assignment is, therefore, currently a methodological limitation.

Genus *Polynucleobacter* was one of the abundant taxa (Fig. 4). Members of the genus are obligate endosymbionts of ciliates (Heckmann and Schmidt, 1987), and thus the presence of a substantial protistan population is indicated, which is further supported by the presence of *Legionella*, a parasite of amoebae. In addition, *Polynucleobacter*, and other genera such as *Aquabacterium*, *Rhodoferax*, *Duganella* and *Limnohabitans*, were disproportionately more abundant in spring (Fig. S6). The seasonal variation in the microbial community composition will be addressed in a future manuscript (under consideration). *Duganella* showed higher abundance in the background and distal wells (Fig. 4). The genus showed stronger correlation with dissolved oxygen (Fig. 6) and given that dissolved oxygen was higher in these wells, it might be a relevant selection factor for the genus.

5. Conclusion

This study reports the microbial diversity of a landfill-contaminated confined aquifer. Both culture-dependent and culture-independent microbial techniques were applied. Comparisons of microscopic cell counts and plate counts as well as metabarcoding data suggest that the microbes in the most contaminated part of the aquifer were mostly culturable heterotrophs. In the contaminated aquifer, microbial diversity was moderate in the proximal well, highest in the intermediate well and lowest in the distal well. The lower diversity in the distal well indicates dominance of the microbial community by a few taxa. Each well had a distinctive microbial flora, but the proximal and intermediate wells seemed to be ecologically related as they had more similar microbial community composition. Comparison between the uncontaminated and contaminated aquifers showed higher microbial diversity in the contaminated aquifer. Compositionally, there was a clear difference between the flora of the contaminated wells and the uncontaminated well. The data suggests that the microbiome of the contaminated aquifer has been impacted by the landfill leachate. Functional analysis indicates the presence of microbes capable of hydrocarbon, sulphur, nitrogen, iron and manganese metabolism.

CRediT authorship contribution statement

Daniel Abiriga: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Andrew Jenkins:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Kristian Alfsnes:** Formal analysis, Software, Validation, Writing – review & editing. **Live S. Vestgarden:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing. **Harald Klempe:** Conceptualization, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147349.

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Article 2

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RESEARCH ARTICLE

Spatiotemporal and seasonal dynamics in the microbial communities of a landfill-leachate contaminated aquifer

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One sentence summary: Spatiotemporal dynamics of microbial communities were discovered in a landfill-leachate contaminated aquifer.

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ABSTRACT

The microbiome of an aquifer contaminated by landfill leachate and undergoing intrinsic remediation was characterised using 16S rRNA metabarcoding. The archaeal/bacterial V3–V4 hypervariable region of the 16S rRNA gene was sequenced using Illumina MiSeq, and multivariate statistics were applied to make inferences. Results indicate that the aquifer recharge and aquifer sediment samples harbour different microbial communities compared to the groundwater samples. While *Proteobacteria* dominated both the recharge and groundwater samples, *Acidobacteria* dominated the aquifer sediment. The most abundant genera detected from the contaminated aquifer were *Polynucleobacter*, *Rhodoferax*, *Pedobacter*, *Brevundimonas*, *Pseudomonas*, *Undibacterium*, *Sulfurifustis*, *Janthinobacterium*, *Rhodanobacter*, *Methylobacter* and *Aquabacterium*. The result also shows that the microbial communities of the groundwater varied spatially, seasonally and interannually, although the interannual variation was significant for only one of the wells. Variation partitioning analysis indicates that water chemistry and well distance are intercorrelated and they jointly accounted for most of the variation in microbial composition. This implies that the species composition and water chemistry characteristics have a similar spatial structuring, presumably caused by the landfill leachate plume. The study improves our understanding of the dynamics in subsurface microbial communities in space and time.

Keywords: microbial ecology; multivariate analysis; contaminated groundwater; aquifer sediment; aquifer recharge; municipal landfill

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INTRODUCTION

Much of the global freshwater used as potable drinking water, in agriculture and in industries comes from groundwater. In Norway, groundwater supports 15% of the total drinking water supply (Kløve *et al.* 2017). From biodiversity point of view, groundwater forms the largest terrestrial freshwater biome, harbouring up to 40% of the earth's freshwater prokaryotic biomass (Griebler and Lueders 2009; Griebler, Malard and Lefébure 2014), but studies on groundwater ecosystems are still scarce (Griebler *et al.* 2016). Despite these benefits, groundwater is subjected to frequent contamination from anthropogenic activities globally (Chapman 1996; Zaporozec and Miller 2000). In Norway, groundwater contamination from agricultural activities and landfill operations have been frequently reported (Basberg, Banks and Sæther 1998; Haarstad and Ludvigsen 2007; Haarstad and Mæhlum 2008; Kværner *et al.* 2014; Abiriga, Vestgarden and Klempe 2020), but industries and forestry management such as logging practices are other perturbations (Kløve *et al.* 2017). Landfill is still the primary municipal solid waste (MSW) disposal strategy practiced in both developing and developed countries (Mouser *et al.* 2005; Eggen, Moeder and Arukwe 2010; Zhang *et al.* 2016; Chen *et al.* 2017). Most of the pollution issues associated with MSW landfills relate to the leachate. Whereas newer sanitary landfills are equipped with liners, old MSW landfills represent a major source of groundwater contamination (Kjeldsen *et al.* 2002; Brad *et al.* 2013) due to lack of leachate containment systems. This makes them a potential public health concern as they may contain both legacy and emerging pollutants (Eggen, Moeder and Arukwe 2010; Lapworth *et al.* 2012), as well as being hotspot for antibiotic resistance (Chen *et al.* 2017).

The chemical composition of MSW landfill leachate can be categorised into four major components: organics, inorganics, heavy metals and xenobiotics (Kjeldsen *et al.* 2002), but the actual load of the individual components depends on the waste material landfilled. MSW landfills are predominantly composed of organic waste, but the story is far different in and before the 1990s, when MSW would essentially contain any waste (Christensen, Bjerg and Kjeldsen 2000). At Revdalen (the present study site), a wide range of contaminants have been detected in the groundwater (Abiriga, Vestgarden and Klempe 2020). It is widely believed that groundwater contamination from landfills can persist for decades or centuries (Bjerg *et al.* 2011). Such prolonged discharge of leachate into the groundwater is likely to leave an ecological footprint on the aquifer. Longer perturbations are thought to cause permanent elimination of native microbial species and allow incursion of new species which may come to dominate the microbial community (Herzyk *et al.* 2017).

Since landfills leach a complex mixture of pollutants, a combination of technologies is required to achieve better treatment results (Remmas *et al.* 2017; Ye *et al.* 2019). However, the versatile metabolic capabilities of microorganisms make them suitable for remediation of a wide range of contamination cases (Majone *et al.* 2015), and are frequently applied in monitored natural attenuation of landfill-impacted environments (Mouser *et al.* 2005). Microbially-catalysed reactions dominate the processes that drive natural attenuation of both organic and inorganic contaminants in the environment (Smets and Pritchard 2003). Monitored natural attenuation is by far the cheapest, but also an efficient and eco-friendly remediation strategy compared to other remediation techniques such as the conventional pump and treat remedial option (Majone *et al.* 2015; Logeshwaran *et al.* 2018).

Providing unequivocal evidence of intrinsic bioremediation involves use of metabolic functional analysis, stable isotope probing, reactive transport modelling, recording decrease in contaminant concentrations and identifying resident microbes to unravel their ecological characteristics such as pollutant transformation capabilities, abundance and distribution (Smets and Pritchard 2003; Mouser *et al.* 2005; Scow and Hicks 2005; Majone *et al.* 2015; Zhang *et al.* 2016; Lueders 2017). Notwithstanding, data from landfill-impacted groundwater microbial ecology utilising the latest available molecular techniques are scarce. Despite contaminations from landfills being numerous globally, attention has mostly been given to characterising the groundwater geochemistry or the leachate chemistry, although microbiological studies on leachate microbiology using the latest molecular techniques are now gaining momentum. Several previous studies have investigated the microbiology of landfill-impacted aquifers (Albrechtsen, Heron and Christensen 1995; Ludvigsen *et al.* 1999; Mouser *et al.* 2005; Lin *et al.* 2007; Chen *et al.* 2017; Taş *et al.* 2018). However, the spatiotemporal and seasonal microbial community dynamics in general remains understudied (Smith *et al.* 2018a), which highlights an important knowledge gap. Focusing not only on the abundance of degrading microbes but also on the diversity and dynamics provides a better understanding of contaminated groundwater ecosystems (Pilloni *et al.* 2019). The present study gives an insight into the microbiome of groundwater contaminated by a municipal landfill, by examining the diversity, abundance and changes in microbial community composition, as a function of the groundwater chemistry, distance, time and season over a period of 2 years.

MATERIALS AND METHODS

Study area

The study site is in a complex of quaternary deposits consisting of moraines, till, subglacial glaciofluvial deposit and glaciofluvial delta deposits (Klempe 2004). The presence of a few kettle holes in the delta deposits attracted dumping of waste and in the period 1958–1974, one of the largest kettle holes was directly filled up with MSW. Due to the isolation of the site from the town centre and the demand for a landfill for Bø and Sauherad Municipalities (now merged to form Mid-Telemark Municipality), four cells were opened to establish the Revdalen Landfill, which was operational from 1974 to 1996 (Abiriga, Vestgarden and Klempe 2020). Due to lack of liners and leachate collection system (less stringent regulation at the time), the leachate from the 1974 to 1996 cells have migrated and contaminated the confined aquifer underneath which is inside a submoraine glaciofluvial deposit (Klempe 2004). Additional information on the study site is available elsewhere (Klempe 2004, 2015; Abiriga, Vestgarden and Klempe 2020, 2021b).

Experimental procedure

Groundwater and aquifer sediment sampling

Groundwater samples were collected from four wells: R0, located in a nearby uncontaminated aquifer; R1, R2 and R4, located downgradient of the landfill in the contaminated aquifer (Fig. 1). R1 has five levels (R101–R105) at 126, 125, 124, 123 and 122 m above sea level; R2 has four levels (R201–R204) at 122, 121, 119 and 118 m above sea level; R4 has three levels (R401–R403) at 118, 117 and 114 m above sea level. R1, R2 and R4 were placed along the groundwater flow direction at the proximal, intermediate

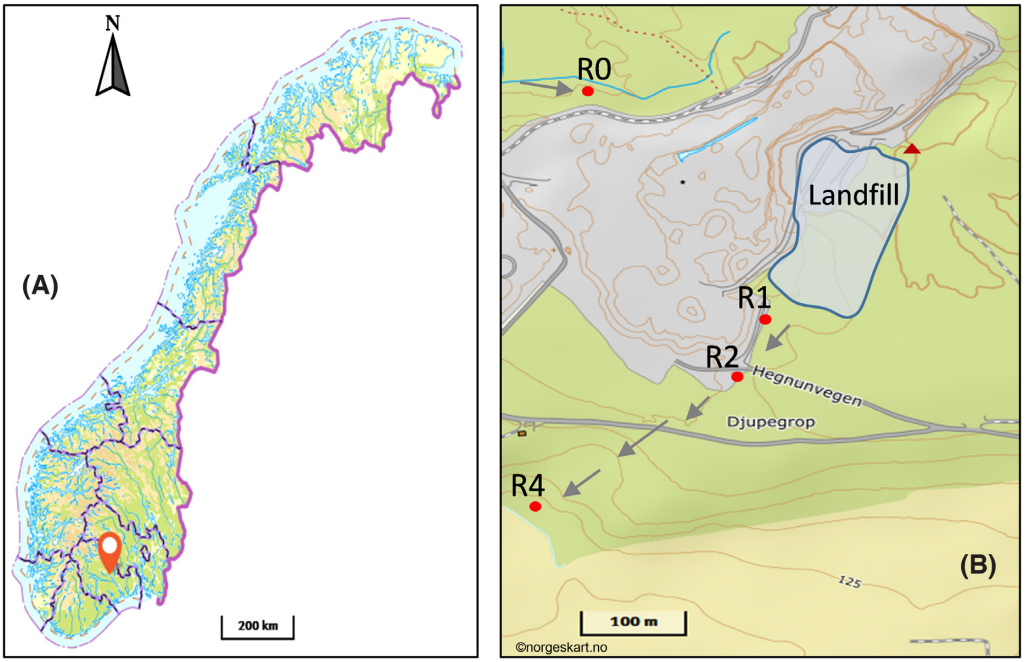

Figure 1. Location of Revdalen Landfill on a map of Norway (A) and a detailed map showing the location of the landfill and the sampling wells R0, R1, R2 and R4 (B). R0 is the background well located in a nearby uncontaminated aquifer, while R1, R2 and R4 are located in the contaminated aquifer, placed at the proximal, intermediate and distal positions from the landfill. The arrows indicate the groundwater flow direction, and the red triangle depicts the location of a seasonal rivulet which recharges the confined aquifer. For clarity of readability, the location for the aquifer sediment samples is not shown; it is only ~30 cm downgradient of R4. Green shading indicates woodland, yellow indicates farmland and grey indicates industrial land, including the landfill and an adjacent active gravel pit/quarry. [Mapping source: Norwegian Mapping Authority, www.norgeskart.no, with permission].

and distal positions, respectively, and are hereafter referred to as such. Groundwater samples were collected twice a year in spring and autumn in 2018 and 2019 from all the levels in the four wells. Groundwater sampling procedures have been described elsewhere (Abiriga, Vestgarden and Klempe 2020), as well as groundwater chemical analysis (Abiriga *et al.* 2021a). Samples for analysis of the diversity of the 16S rRNA genes were collected in sterile 350 mL PETE bottles. A total of 300 mL of the water was filtered through 0.2 μm polycarbonate membrane filters within 48 h and the filters were stored at −70°C prior to DNA extraction. A total of 52 samples were collected from the four monitoring wells. In addition, one sample was taken from a rivulet which feeds the aquifer at the outcrop area. Further, three aquifer sediment samples were obtained at depths of 6–7, 8–9 and 9–10 m using the piston method. 25 g saturated sediment subsamples were taken and stored at −70°C prior to DNA extraction.

DNA extraction, sequencing and bioinformatics

The procedure for the extraction of DNA from the filters has been described previously (Abiriga *et al.* 2021a). DNA extraction from the aquifer sediment was done using DNeasy PowerSoil Kit following the manufacturer's instructions. The amount of genomic DNA in the samples was quantified using Qubit Flourometer 3.0 (Life Technologies, Malaysia) and the quality evaluated by gel electrophoresis on 2% agarose. All the samples were subjected to 16S rRNA gene metabarcoding of the V3–V4 hypervariable region using the universal primer set 319F (5′-ACTCCTACGGGAGGCAG CAG-3′) and 805R (5′-GGACTACNVGGGTWTCTAAT-3′). Samples were sequenced using Illumina MiSeq (600 cycles) by applying the 300 bp paired-end protocol. PCR amplifications and library preparation, as well as the bioinformatics pipeline implemented in sequence data analysis are described in Abiriga *et al.* (2021a). The library statistics for the samples are provided in the supplementary material (see supplementary data).

Statistical data analysis

All statistical analyses were conducted in R version 4.0.2 (R Core Team 2020). Water chemistry data was subjected to principal component analysis. Due to the dimensionally heterogeneous physicochemical data, the data was standardised (centred and normalised) prior to principal component analysis to ensure all parameters are given equal weight. Difference in groundwater chemistry across sampling wells and between spring and autumn was tested using the nonparametric Kruskal–Wallis and Mann–Whitney tests respectively, as the data showed nonnormal distribution. Because the levels of solutes in the background well were different from the other wells (assessed during data exploration), data from the background well was excluded in the Kruskal–Wallis analysis to minimise type-I error. In all the above analyses, iron has been excluded because it was below the limit of detection in all the wells except R4, while temperature was excluded due to many missing observations.

Nonmetric Multidimensional Scaling (NMDS) was used to visualise spatial group clustering based on Bray–Curtis dissimilarity distance. The operational taxonomic unit (OTU) data was transformed using fourth-root transformation (except for intermediate well which was subjected to square root transformation as fourth-root transformation was too strong for it) and standardised using Wisconsin double standardisation. Differences in the microbial community composition among sampling wells, between 2018 and 2019, and between autumn and spring were tested using permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) by applying the function *adonis* from package vegan (Oksanen *et al.* 2019) on 999 permutations.

The analysis was performed on Bray–Curtis dissimilarity measure calculated from fourth/square root-transformed and Wisconsin double-standardised community data. The assumption of homogeneity of group dispersion was checked using the function *betadisper* (Anderson 2006).

Canonical ordination i.e. redundancy analysis (RDA) was conducted on fourth-root-transformed and Hellinger standardised (Legendre and Gallagher 2001) OTU abundance data and standardised water chemistry data. Water chemistry variables that were significantly associated with variation in microbial species composition were selected using forward selection based on 999 permutations at 0.05 significance level (Akaike Information Criterion if there are ties) by implementing the function *ordistep* from package vegan (Oksanen *et al.* 2019). To quantify the contributions of water chemistry, season, year and distance (well) to the variation in microbial composition, variation partitioning (Borcard, Legendre and Drapeau 1992) was conducted and significance was tested by permutation (999 permutations). In total, two parameters: total nitrogen and temperature were removed from the data during the constrained ordination as they contain numerous missing values.

RESULTS

Groundwater chemistry

The groundwater chemistry was characteristically different across the four wells (Figures S1 and S2, Supporting Information). Overall, the wells in the contaminated aquifer were more related to each other than to the background well. Within the contaminated aquifer, similarity in water characteristics was greatest between the proximal and intermediate wells (Figure S2, Supporting Information). Nonparametric Kruskal–Wallis test showed significant differences across the wells in the contaminated aquifer for most of the groundwater parameters, except nitrate, total nitrogen and manganese (Table S1, Supporting Information). None of the parameters showed statistically significant differences across the depth profiles in the proximal, intermediate or distal wells (not shown). Seasonal variation was strongest in the proximal well, followed by the intermediate well (Table S2, Supporting Information). A total of eight groundwater variables showed significant differences between spring and autumn, however, none of the 15 variables showed significant seasonal changes in the distal and background wells. Similarly, interannual change in the groundwater chemistry was greatest in the proximal well (nine variables), followed by the intermediate well (six variables; Table S2, Supporting Information). The distal and background wells had only moderate (four variables) and marginal (one variable) significant interannual changes.

Groundwater and aquifer sediment microbiology

Microbial composition and abundance

There were 1763 OTUs detected from the contaminated groundwater samples, which were classified to 62 phyla, constituting 98.7% bacteria, 1.24% archaea and 0.06% unclassified. The background groundwater samples had 485 OTUs classified to 39 phyla, consisting of 97.73% bacteria, 2.06% archaea and 0.21% unclassified. The eight most abundant phyla from both the background and contaminated aquifer were *Proteobacteria*, *Patescibacteria*, *Actinobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Chloroflexi* and *Nitrospirota* (Fig. 2). The aquifer recharge sample had 599 OTUs, all from domain bacteria. These were classified to 29 phyla, of which, *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Verrucomicrobia*, *Acidobacteria*, *Actinobacteria*, *Patescibacteria* and *Cyanobacteria* were the top eight phyla. From the aquifer sediments, 652 OTUs were detected, comprising 39 phyla. Of these, *Acidobacteria*, *Proteobacteria*, *Chloroflexi*, *Actinobacteria*, *Firmicutes*, *Methylomirabilota*, *Planctomycetota* and WPS.2 (candidate phylum) formed the top eight phyla (Fig. 2).

In order to simplify comparison, the top ten most abundant OTUs in each sample were identified. In the background well, these included genus *Duganella*, which displayed a higher abundance in spring. Other OTUs were *Collimonas*, *Pseudomonas*, *Polaromonas*, *Alkanindiges* and *Rhodoferax*; with *Duganella*, *Alkanindiges* and *Rhodoferax* as the most abundant OTUs in the spring samples of the first sampling campaign (Figure S5, Supporting Information). The proximal well showed that *Aquabacterium*, *Janthinobacterium*, *Oxalobacteraceae* and *Pedobacter* were highly abundant in the uppermost level of the well, while *Sulfurifustis* and *Sulfuritalea* were more abundant in the deeper levels (Figure S6, Supporting Information). Taxa *Methylobacter* and *Polaromonas* were ubiquitous throughout the levels in this well. Other taxa include *Gallionella*, *Polynucleobacter*, *Pseudomonas*, *Rhodanobacter* and *Rhodoferax*. A medically relevant genus among the top 10 taxa in this well was *Enterococcus*. In the intermediate well, the top 10 taxa showed modest abundances, except *Oxalobacteraceae*, *Saccharimonadales*, *Brevundimonas*, *Rhodoferax*, *Pseudomonas*, *Pedobacter*, *Caulobacter*, LWQ8 (uncultured family of *Saccharimonadales*) and *Undibacterium* (Figure S7, Supporting Information). Taxa of medical relevance in the top 10 OTUs were *Legionella* and *Stenotrophomonas*. Genera *Caulobacter* and *Methylobacter* were ubiquitous in the intermediate well. The most abundant taxa in the distal well were *Oxalobacteraceae*, *Rhodoferax*, *Polynucleobacter* and *Undibacterium* (Figure S8, Supporting Information), but *Saccharimonadales* was also moderately abundant. Genus *Duganella* appeared in the second year of the sampling campaign, but only occurred in high abundance in spring. A similar seasonal trend was observed for *Polynucleobacter* and GKS98 (uncultured *Alcaligenaceae*).

Spatial and seasonal variation in microbial community composition

The microbial community compositions of the water samples collected from the four sampling wells clustered separately from each other, although the proximal and intermediate wells showed some slight overlap (Fig. 3). The aquifer sediment samples also clustered separately and well away from the nearby distal well, and the recharge sample was also well isolated from the other groups. PERMANOVA results from a global comparison ($F = 7.14$, $df = 4$ and $P = 0.001$) and pairwise comparisons (Table 1) revealed significant differences in microbial community composition across and between the wells, respectively. The overall quantitative contribution of distance to the microbial community composition is provided under RDA (Fig. 7).

In the proximal well, an overall analysis of variance (PERMANOVA) showed a statistically significant difference among the levels ($F = 1.47$, $df = 4$ and $P = 0.002$). However, performing pairwise comparisons between the levels did not give significant differences after Bonferroni correction (Table S3, Supporting Information). In moving from top to bottom within the proximal well, a higher dispersion was observed among the samples from the topmost level (Fig. 4 and Figure S4, Supporting Information). No significant overall difference was observed among the depth profiles in the intermediate or distal wells ($F = 0.89$, $df = 3$ and $P = 0.651$ and $F = 1.08$, $df = 2$ and $P = 0.322$, respectively). Similarly, no significant differences in the pairwise comparisons between the levels in each well were observed (Table S3, Supporting Information). As in the proximal well, samples from the

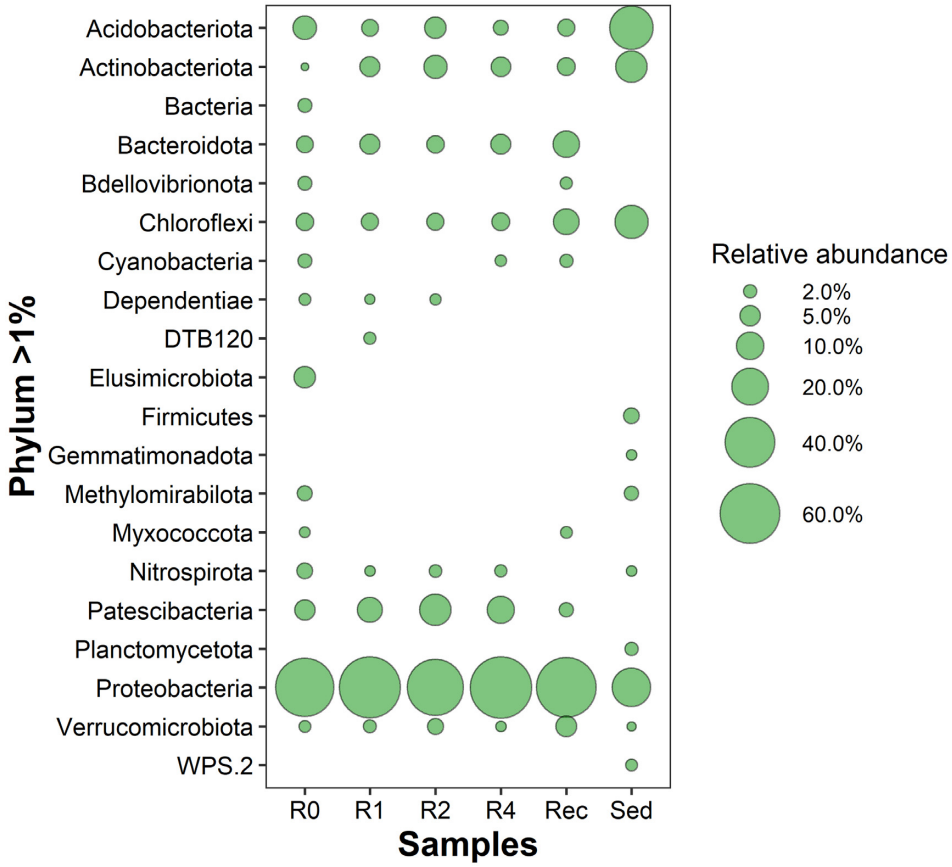

Figure 2. Abundant microbial phyla in the nearby uncontaminated aquifer (R0), in the contaminated aquifer (R1, R2 and R4), recharge water (Rec) and aquifer sediment (Sed) near R4. R1, R2 and R4 were placed along the groundwater flow direction at the proximal, intermediate and distal position from the landfill, respectively.

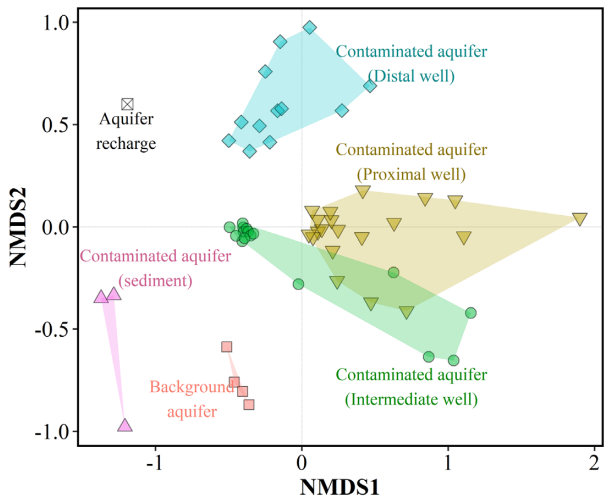

Figure 3. Nonmetric multidimensional scaling (NMDS) plot showing the beta diversity (Bray–Curtis) among the samples: the background aquifer (R0), the contaminated aquifer (proximal R1, intermediate R2, distal R4 and aquifer sediment), and the aquifer recharge.

Table 1. PERMANOVA result of pairwise comparisons of the microbial community composition between the samples. R0 is the background well located in a nearby uncontaminated aquifer, while R1, R2 and R4 are the wells located in the contaminated aquifer at the proximal, intermediate and distal positions from the landfill. Sed is the aquifer sediment sample.

Pairs	F. Model	R^2	P. adjusted[a]
R0 vs. R1	6.621	0.231	0.01
R0 vs. R2	7.022	0.281	0.01
R0 vs. R4	7.791	0.358	0.02
R0 vs. Sed	3.655	0.422	0.23
R1 vs. R2	7.379	0.178	0.01
R1 vs. R4	8.373	0.218	0.01
R1 vs. Sed	4.525	0.177	0.02
R2 vs. R4	8.191	0.240	0.01
R2 vs. Sed	3.680	0.178	0.01
R4 vs. Sed	3.822	0.227	0.03

[a]P-value adjusted using Bonferroni correction.
The recharge sample was removed during the permutation testing as it has only one observation.

topmost level in the intermediate well (R201) showed the highest dispersion (Fig. 4 and Figure S4, Supporting Information). In the distal well, the dispersion among samples was comparable, although the dispersion seemed higher in samples of the middle level (R402; Fig. 4 and Figure S4, Supporting Information). Among the levels in the proximal and intermediate wells, a lower microbial diversity was observed in the uppermost levels, while no clear trend was observed among the levels in the distal well (Figure S3, Supporting Information).

PERMANOVA conducted on seasonality showed significant differences in the microbial community composition between spring and autumn only in the proximal and intermediate wells

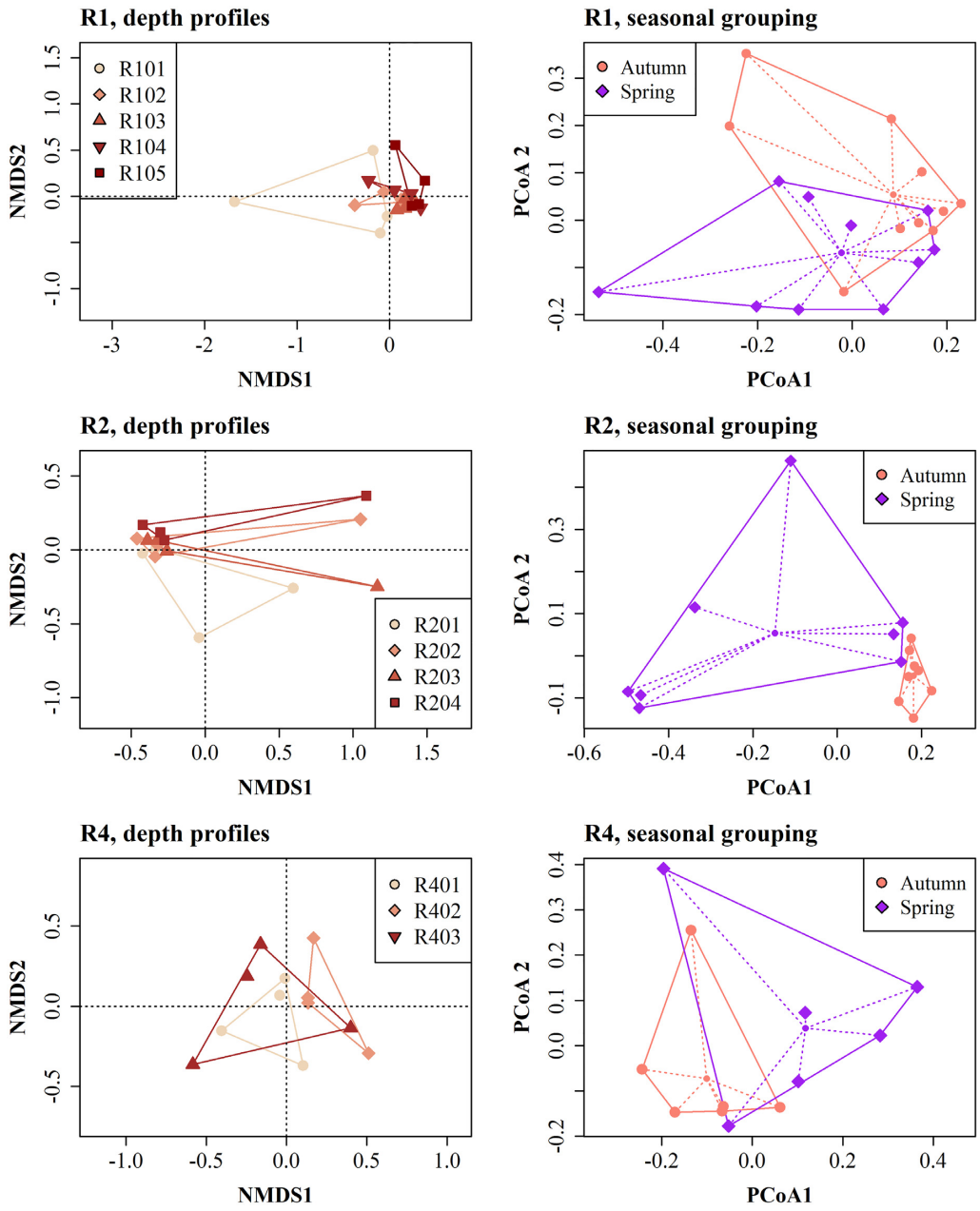

Figure 4. Nonmetric Multidimensional Scaling (NMDS) plots of the depth profiles and Principal Coordinate Analysis plots of seasonal grouping (the centres to which the samples are connected are the centroids) from the depth profiles. Colour schemes of light to dark indicate increasing depth. R1, R2 and R4 are the wells located in the contaminated aquifer at the proximal, intermediate and distal positions from the landfill.

(Table 2); this was accompanied by a large increase in dispersion in the intermediate well (Fig. 4 right hand panel). The overall seasonal effect on the microbial community is presented under RDA (Fig. 7).

Interannual variation (2018 and 2019)

Beta dispersion (Fig. 5) suggests difference in microbial community composition between 2018 and 2019. However, this was significant only for the intermediate well ($F = 2.29$, $df = 1$ and $P = 0.024$). No statistically significant change in the microbial community between 2018 and 2019 was observed for the proximal ($F = 1.34$, $df = 1$ and $P = 0.104$), distal ($F = 1.09$, $df = 1$ and $P = 0.355$) or background ($F = 1.22$, $df = 1$ and $P = 0.148$) wells.

Redundancy analysis

The first two axes of the RDA (Fig. 6) account for 12.1% of the total constrained variance (31.4%). Note that the inferred variance (explained proportion) is the adjusted R^2. A total of five canonical axes were statistically significant (RDA1-RDA3, $P = 0.001$; RDA4, $P = 0.016$ and RDA5, $P = 0.024$; after 999 permutations). Groundwater chemical parameters that significantly influenced the microbial composition were pH, sodium, calcium, magnesium, manganese, alkalinity, nitrate and total organic carbon (TOC). These are the variables which have also demonstrated stronger gradients in the studied ecosystem (Figure S1, Supporting Information). Notwithstanding the output from the statistical model selection, all the variables in the global model were retained in

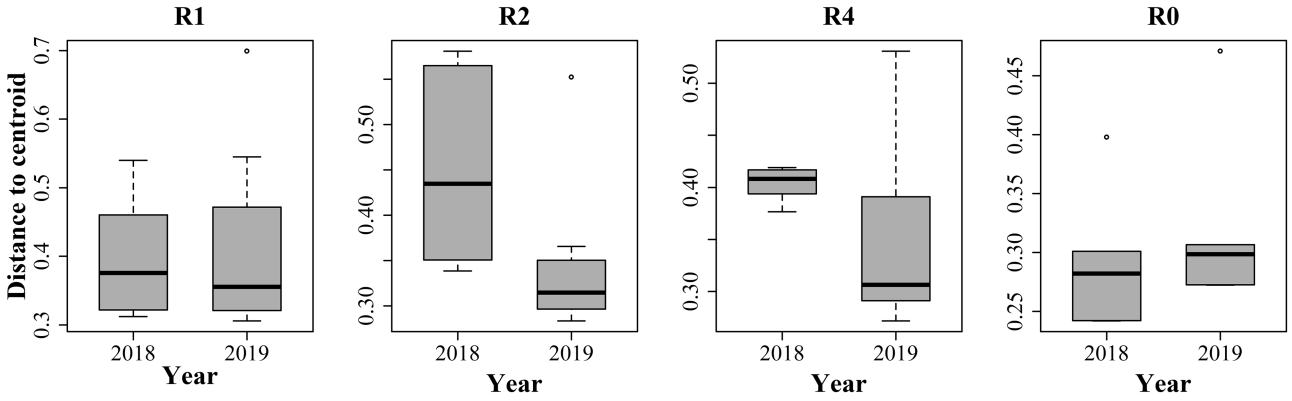

Figure 5. Beta dispersion between 2018 and 2019 for R1, R2, R4 and R0. R0 is the background well located in a nearby uncontaminated aquifer, while R1, R2 and R4 are the wells located in the contaminated aquifer at the proximal, intermediate and distal positions from the landfill.

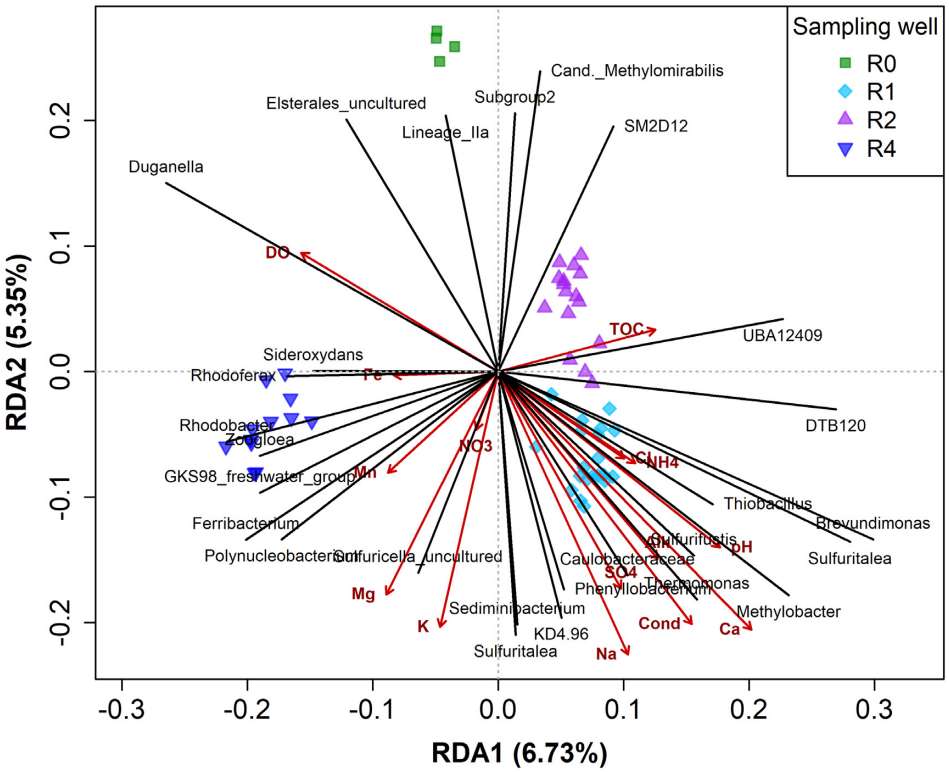

Figure 6. Redundancy analysis (RDA) performed on fourth-root transformed and Hellinger-standardised OTU abundance data. To distinguish water chemistry variables from OTUs, OTU scores are plotted without arrows. Only the prominent species vectors are shown. R0 is the background well located in a nearby uncontaminated aquifer, while R1, R2 and R4 are the wells located in the contaminated aquifer at the proximal, intermediate and distal positions from the landfill.

the final model to explore potential biogeochemical processes in the contaminated aquifer, moreover such automatic variable selection strategies would sometimes choose ecologically irrelevant models (Ramette 2007). In the RDA triplot (Fig. 6), dissolved oxygen showed a strong but opposite gradient to pH, ammonium and chloride along RDA1. Conductivity, sodium, potassium, calcium, magnesium and sulphate, on the other hand, showed stronger gradients along RDA2. The geochemical and microbial data have structured the sampling sites first along RDA1, separating the background and the distal wells which correlated negatively with the axis, from the proximal and intermediate wells which correlated positively with the axis. The second axis (RDA2) separated mainly the uncontaminated groundwater from the contaminated groundwater. The proximal and intermediate wells clustered close to each other.

Output from variation partitioning (Fig. 7) shows that all the four explanatory variables (water chemistry, year, season and well) collectively accounted for 33.2% of the total variance, while 66.8% variance remained unexplained. Much of the explained variance was shared by water chemistry and well (18.5%), while only 0.2% of the variation in microbial composition was jointly explained by all the four variables. The result also showed that the unshared variation in microbial composition (excluding the effects of covariables) explained individually by the variables was higher for water chemistry (7%) and well (5.9%), but lower for season (0.8%) and year (<0%). The overall contribution with-

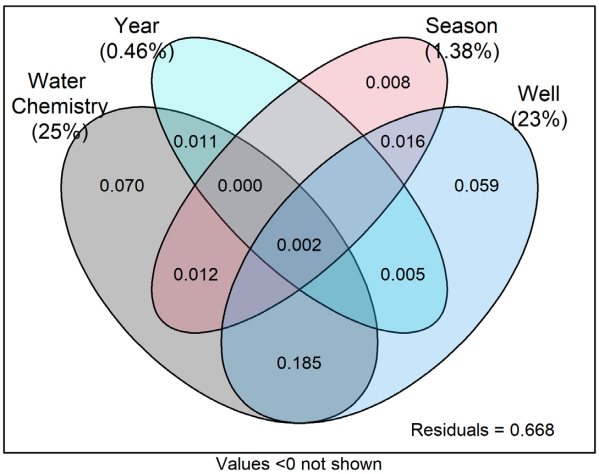

Figure 7. Variation partitioning of proportions of variation in microbial community composition explained by water chemistry, year, season and distance (well). Values in parenthesis indicate variations explained by the variables without removing the effects of covariables.

Table 2. PERMANOVA results of seasonal microbial community variation in the sampling wells R0, R1, R2 and R4. R0 is the background well located in a nearby uncontaminated aquifer, while R1, R2 and R4 are the wells located in the contaminated aquifer at the proximal, intermediate and distal positions from the landfill.

Well	Number of sample	Number of OTU	F. Model	R^2	*P*-value
R1	20	927	1.71	0.087	0.007
R2[a]	16	1342	3.54	0.202	0.001
R4	12	1027	1.36	0.12	0.086
R0	12	616[b]	1.43	0.125	0.064

[a]OTU data was square-root transformed, as fourth-root transformation was too strong in this case.
[b]OTUs based on 12 samples, but only 4 samples were used in all the other analysis.

out removing the effects of covariables was 25% for water chemistry, 0.46% for year, 1.3% for season and 23% for well. Testing for these proportions based on 999 permutations yielded significant results for water chemistry ($F = 1.83$ and $P = 0.001$), well ($F = 2.00$ and $P = 0.001$), and season ($F = 1.37$ and $P = 0.035$), but not for year ($F = 0.97$ and $P = 0.539$). Thus, the variables can be ordered based on their importance in explaining the variation in the microbial composition as water chemistry > distance > season > year.

DISCUSSION

Spatial variation

Microbial community composition showed a significant spatial variation. The background well, which is an unperturbed environment, was most dissimilar to the other wells. This is a clear manifestation of the impact of the landfill leachate on the aquifer ecosystem. However, as the background and contaminated aquifers are not connected, their original communities may have been different. In the contaminated aquifer, there was clear evidence of variation of the microbial community along the line of flow, with the two wells nearest the landfill and each other being more similar. Similar findings have been reported for a landfill leachate plume in Banisveld landfill using denaturing gradient gel electrophoresis community profiling (Brad *et al.* 2013). Such spatial differentiation of microbial communities has been suggested to contribute to better degradation of contaminants (Brad *et al.* 2013) and, in addition, provides useful information, as it gives insight into differential adaptation of microbes to leachate (Mouser *et al.* 2005). The ecological gradient in Revdalen is short, where samples primarily differ in species abundances (Ramette 2007). The water chemistry demonstrated two maximum directions (Fig. 6): towards the proximal well (for eight variables) and towards the distal well (for six variables). On a similar pattern of structuring, the abundant OTUs in the proximal and distal wells were mostly culturable, while those in the intermediate and background wells were predominantly uncultured taxa.

Depth-resolved variation in microbial composition indicated a significant difference across the levels in the proximal well, which could be attributed to the disproportionately high abundance of certain OTUs, e.g. *Aquabacterium*, *Janthinobacterium*, *Oxalobacteraceae* and *Pedobacter* in the uppermost level, and *Sulfurifustis* and *Sulfuritalea* in the deeper levels (Figure S6, Supporting Information). In addition, beta diversity analysis (Fig. 4) indicates that samples from the uppermost level have larger dispersion. Both the intermediate and distal wells showed non-significant differences across the discrete depth profiles and in pairwise comparisons, which suggest that the microbial floras of the intermediate and distal wells were compositionally similar across the depths. This agrees with the observation that only three most abundant OTUs in the intermediate well (*Brevundimonas*, *Pseudomonas* and *Saccharimonadales*) and one in the distal well (*Oxalobacteraceae*) showed disproportionately higher abundance in one level (Figures S7 and S8, Supporting Information). Similar lack of vertical variation within sampling wells has been reported in a crude-oil contaminant plume (Fahrenfeld *et al.* 2014). However, a study on microbial communities from wells at depths 10–17 m but having different hydraulic conductivities recorded significant vertical variation (Lin *et al.* 2012). In Revdalen, the aquifer matrix comprises sand and gravel (~5 m thick), which can be considered to have relatively less varied hydraulic conductivities compared to matrices made up of completely different geological layering materials that can influence microbial and nutrient distribution (Smith *et al.* 2018a). In addition, Revdalen has a different hydrological regime than the unconfined aquifer studied by Lin *et al.* (2012). Depth-resolved microbial community variation in various types of geological strata remains poorly understood (Smith *et al.* 2018a), which calls for more elucidation.

Variation partitioning showed that both water chemistry and distance (wells) were intercorrelated and they jointly accounted for most of the variation in the microbial composition. This implies that the microbial composition and water chemistry characteristics have a similar spatial structuring, presumably caused by the landfill leachate plume. A gradient of an increase in concentrations of dissolved oxygen, magnesium, potassium, manganese and sulphate, and a decrease in pH, conductivity, calcium and alkalinity were observed along the proximal–intermediate–distal path (Figures S1 and S2, Supporting Information). This highlights the importance of distance on attenuation of pollutants which has been described previously (Abiriga, Vestgarden and Klempe 2020; 2021b). This likely influenced the microbial composition and structure. Thus, homogenisation force from the leachate plume should be strongest at the proximal and intermediate wells, which makes the microbial composition and structure more similar in these wells,

although the close proximity would also imply microbial propagation from proximal to intermediate well. At the distal well, such homogenisation force is diminished, and other processes become increasingly important in driving the microbial composition and structure.

Seasonal and Interannual variations

By the virtue of their formation, aquifers are viewed as being environmentally stable (Zhou, Kellermann and Griebler 2012; Pilloni *et al.* 2019). In Snowbelt countries such as Norway, there can be considerable aquifer recharge after snowmelt, accounting for 60–80% of the yearly recharge in some regions (Kløve *et al.* 2017). This is likely to cause shifts in the geochemical processes within an aquifer and is particularly important in unconfined aquifers, where seasonal groundwater table fluctuations can lead to redistribution of contaminants (Fretwell *et al.* 2005). This causes shifts in plume movement, which can exert control over microbial community assemblages (Pilloni *et al.* 2019). In Revdalen, a significant difference in water chemistry between spring and autumn was observed, but only in the proximal and intermediate wells. The variables with significant changes were sulphate, TOC, sodium, potassium and calcium in the proximal well, and conductivity, ammonium and TOC in the intermediate well (Table S2, Supporting Information).

Considering aquifers as microbial habitats, their intrinsic features such as changes in geochemical and hydraulic regimes are now acknowledged as key influencers of activity and population of microorganisms (Griebler and Lueders 2009; Brad *et al.* 2013; Lueders 2017). Results suggest that microbial composition in Revdalen is influenced by season, but it seems that although statistically significant, season has only a marginal effect, as it accounted for only 1.3% of the variation. Previous studies have reported seasonal variation in composition and diversity of microbes in pristine aquifers (Farnleitner *et al.* 2005; Lin *et al.* 2012; Zhou, Kellermann and Griebler 2012) and in industry-impacted urban aquifers (Smith *et al.* 2018b). Beta diversity analysis indicated significant seasonal variations in the proximal and intermediate wells. Given the locations of these wells relative to the landfill, these observations suggest that the microbial communities in them are more susceptible to seasonal changes. This agrees with the observation that more geochemical parameters have shown significant seasonal fluctuations in the proximal and intermediate wells than in the distal and background wells (Table S2, Supporting Information). However, seasonal variation was also evidenced in the distal and background well, where taxa *Duganella*, *Polynucleobacter* and GKS98 (uncultured *Alcaligenaceae*), showed higher abundances in spring. It is unclear why these taxa showed higher abundances only in spring. It can be speculated that the recharge water brings along nutrients that specifically favoured these microorganisms. *Duganella* for instance, might be responding to dissolved oxygen, as dissolved oxygen was found to be replenished in spring (Abiriga, Vestgarden and Klempe 2020) and the genus showed a positive correlation with dissolved oxygen (Fig. 6). In a batch experiment (Griebler *et al.* 2016), growth of *Duganella* was strongly stimulated when nutrient source (R2A) was amended in a reactor. Our study is based on field observation and unambiguous interpretation of such dynamics is difficult (Pilloni *et al.* 2019). More study is required to understand this dynamism, but the results suggest that the microbial communities were less variable in autumn than in spring, probably due to calmer groundwater flow conditions in autumn. The higher recharge occurring in spring (Kløve *et al.* 2017) may be followed by mixing and this causes instability in the subsurface (Smith *et al.* 2018a).

Results from beta diversity analysis and variation partitioning suggest that the microbial communities were less variable over the study period. A similar observation of less community variability over a one-year period have been made from pristine aquifers (Farnleitner *et al.* 2005). However, a closer look into the data indicated that the microbial community in the intermediate well was more variable, as the beta diversity analysis gave a significant difference between 2018 and 2019 ($P = 0.024$). Even so, the temporal variation was related to season, especially with samples collected in spring 2018 (not shown). The meteorological conditions in 2018 were exceptional, with unusually high winter snowfall, leading to a higher aquifer recharge, followed by very dry weather in the summer. This likely lowered the water table, creating zones of unsaturation around the proximal and intermediate wells that favoured oxidation reactions. Consequently, concentrations of sulphate, dissolved oxygen and nitrate were highest in autumn (Figure S2, Supporting Information). How long such implied community stability will prevail in aquifers is a matter of future studies as apparently no data exists for field observation >2 years.

CONCLUSION

Literature on landfill-leachate-impacted aquifers is scarce. Here, we examined key influencers of the microbial community in an aquifer contaminated by a municipal landfill leachate in southeast Norway to understand the interplay between microbial community composition and environmental factors: groundwater geochemistry, distance, season and time. The explanatory variables explained 33.2% of the variation in microbial composition, thus a bigger proportion (66.8%) of the variation remained unexplained. The unexplained proportion likely represents both deterministic but unmeasured variables (that the present data was unable to capture) and stochastic processes. The explained variation was largely jointly accounted for by the groundwater chemistry and distance, which were intercorrelated. Season had only a marginal effect on the microbial communities, as it explained only 1.3% of the variation. Interannual variation was negligible, which suggests that the microbial communities were less variable over the study timeframe. The findings of the study are important in understanding how environmental factors influence microbial composition of anthropogenically impacted aquifers, which is very useful in ensuring proper management of remediation sites.

DATA AVAILABILITY

The raw sequence data supporting the study have been deposited in Sequence Read Archive under BioProjects PRJNA677875 (groundwater; biosamples SAMN16775936–SAMN16775995 and recharge SAMN16776020) and PRJNA677889 (aquifer sediment). The groundwater biosamples consisted of samples collected in 2018 and 2019.

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SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

Conflict of interest. None declared.

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Article 3

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Microbial deterministic assembly and co-occurrence network in an aquifer under press perturbation

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Abstract

Thousands of aquifers worldwide have been polluted by leachate from landfills and many more remained threatened. Microbial communities from these environments play a crucial role in mediating biodegradation and maintaining the biogeochemical cycles, but their co-occurrence and assembly mechanism have not been investigated. Here, we coupled network analysis with multivariate statistics to determine the relative importance of deterministic versus stochastic microbial assembly in an aquifer undergoing intrinsic remediation. Both the aquifer-wide generalists and localised communities were found to co-occur less than expected under null model, indicating the predominance of deterministic processes in shaping microbial communities. The amount of variation in the microbial community explained by the measured deterministic variables was 55.3%, which corroborates the dominance of deterministic processes in the aquifer. By using the network topology (betweenness centrality), Eleven putative keystone taxa among the generalists were identified: *Saccharimonadales*, KD4.96 (uncharacterised *Chloroflexi*), *Saprospiraceae*, *Vicinamibacteriales*, *Rhodoferax*, *Neisseriaceae*, *Gemmatimonadaceae*, *Gaiellales*, *Holosporaceae*, *Rhizobiales* and *Alphaproteobacteria*. The keystone taxa are in principle those that provide crucial functions that stabilise the microbial community. The findings provide answers to fundamental ecological questions which improve our understanding of the microbial ecology of landfill leachate plumes, an ecosystem that remains understudied.

Keywords: contaminated aquifer; pristine aquifer; landfill leachate plumes; co-occurrence network; variation partitioning; ecological processes

Introduction

Groundwater ecosystems harbour the largest terrestrial biome, accounting for up to 40% of the earth's freshwater prokaryotic biomass (Griebler and Lueders, 2009; Griebler et al., 2014). This rich biodiversity is threatened on a global scale by groundwater contamination. There are thousands of cases of groundwater contamination globally due to landfill operations. A good amount of research has focused on characterising landfill leachate chemistry (Kjeldsen et al., 2002; Masoner et al., 2020; Zhao et al., 2018) and groundwater leachate plumes (Abiriga et al., 2020; 2021c; Bjerg et al., 2011; Christensen et al., 2001). The literature on these issues is extensive. While study focusing on the leachate microbiology is now gaining momentum (Rajasekar et al., 2018; Song et al., 2015b; Staley et al., 2018; Zainun and Simarani, 2018), our understanding of the microbial ecology of landfill leachate-impacted aquifers remains scant. Due to the complex composition of landfill leachate (Christensen et al., 2000; Eggen et al., 2010; Moody and Townsend, 2017; Mouser et al., 2005), chemicals inherent to the landfill leachate may have deleterious effect on the microbiology of the receiving groundwater, including permanently eliminating the native species due to chronic disturbances (Herzyk et al., 2017; Song et al., 2015a; Zhou et al., 2014) which may last for decades to centuries (Bjerg et al., 2011).

Microorganisms found in these contaminated groundwaters play an important role in degradation of contaminants. The complex mix of contaminants in landfills limits the applicability of conventional treatments and necessitates more robust approaches. Natural attenuation is considered superior in this respect (Mouser et al., 2005). This emphasises the importance of the intrinsic microorganisms because biodegradation, which is a core process in natural attenuation, is mediated by microbes. Studying the microbial ecology of landfill leachate plumes not only informs on the effect of the leachate on the microbial communities, but also

68 informs on the population of microbes that may be involved in the degradation of contaminants
69 in the plumes. Previous studies from landfill leachate plumes (Abiriga et al., 2021a; Lu et al.,
70 2012; Mouser et al., 2005; Taş et al., 2018) have almost exclusively focused on a single aspect
71 of microbial communities such as alpha diversity, beta diversity and microbial functions. We
72 previously showed how multiple factors influenced the microbial community composition of
73 the current study aquifer (Abiriga et al., 2021b). While these studies have given insights into
74 the microbiology of leachate plumes, microbial associations and the relative importance of
75 deterministic versus random community assembly have not been addressed. This presents an
76 important gap in understanding the microbial ecology of landfill-perturbed aquifers.

77 To address this, we applied network analysis to identify microbial co-occurrence in an aquifer
78 which was contaminated by a municipal landfill in southeast Norway. Network analysis has
79 been successfully applied to study microbial co-occurrence across multitudes of habitats
80 (Barberán et al., 2012; de Vries et al., 2018; Horner-Devine et al., 2007; Ju et al., 2014; Lupatini
81 et al., 2014; Williams et al., 2014) and has helped resolved aspects of microbial ecology that
82 cannot be addressed by other community metrics such as alpha and beta diversity (Lupatini et
83 al., 2014). Analysis of co-occurrence patterns can decipher complex microbial systems such as
84 providing information on the ecological traits of uncharacterised microbes that co-occur with
85 well characterised microbes (Barberán et al., 2012; Fuhrman, 2009; Williams et al., 2014). This
86 may allow such taxa, which are very difficult to cultivate in the laboratory, to be grown in a co-
87 culture with the well characterised species (Lupatini et al., 2014). Network topologies can be
88 used to identify important microbial community members known as keystone taxa (Banerjee et
89 al., 2018; Lupatini et al., 2014). The relative importance of deterministic versus random
90 assembly can be determined by comparing the checkerboard score (C-score) (Stone and
91 Roberts, 1990) between a simulated and observed community. Non-random pattern is inferred
92 if the observed index is greater or less than the expectation under the null model (Horner-Devine

et al., 2007). The overall contribution of deterministic processes in shaping the aquifer microbiology was quantified by applying a multivariate statistic by leveraging on the environmental data collected during the study. Coupling network analysis to multivariate statistics offers a better interpretation of microbial community data (Williams et al., 2014).

The main objectives of the study were to answer the following questions: (i) Which taxa show strong and significant interactions and which are the keystone taxa in the aquifer? (ii) Are the microbial taxa in the aquifer assembled randomly or deterministically? (iii) How much variation in the microbial community is explained by the measured variables? A caveat to minimise inflating the unexplained variance was to consider only one level per well, because inherent vertical variability in the aquifer material is likely to introduce immeasurable variation in microbial communities across the aquifer depth. Answering these fundamental ecological questions should give insight into the microbial ecology of understudied landfill-perturbed environments.

Materials and methods

Study site and field procedure

The study aquifer is a confined aquifer of quaternary glaciofluvial delta deposit located in southeast Norway. The aquifer matrix is characterised by medium to high permeability sand and gravel. It is a small aquifer fed by a small watershed (Klempe, 2015). In the period 1974-1996, a municipal landfill was operated in the area and because no leachate containment system was in place, the leachate from the landfill contaminated the aquifer. Additional information on the study site is accessible elsewhere (Abiriga et al., 2020; 2021c; Klempe, 2004; 2015).

Groundwater samples were collected twice a year in spring and autumn in 2018 and 2019 from a single level from four monitoring wells: R1, R2 and R4 located in the contaminated aquifer,

and R0 located in a nearby uncontaminated aquifer. The three wells R1, R2 and R4 have been placed downstream of the landfill at 26 m, 88 m and 324 m, respectively. The well levels sampled were R104 (R1), R203 (R2), and R402 (R4). The location of monitoring wells and field sampling procedures are described elsewhere (Abiriga et al., 2021a; Abiriga et al., 2020). Groundwater chemical analyses have been described previously (Abiriga et al., 2021a). The samples were analysed for 16 physicochemical parameters: pH, dissolved oxygen, specific conductance, sodium, potassium, ammonium, calcium, magnesium, iron, manganese, chloride, nitrate, alkalinity, sulphate, total nitrogen. All analyses were conducted in accordance with the Norwegian Standards and/or ISO (Abiriga et al., 2021a). Groundwater samples for 16S rRNA metabarcoding of V3-V4 hypervariable region were collected in sterile 350 ml PETE bottles (VWR, UK). Sample processing, DNA extraction, PCR amplification, 16S library preparation and sequencing procedures, and the bioinformatics pipeline used for the sequence data analysis have been described previously (Abiriga et al., 2021a).

Data analysis

Statistical analyses were performed using the R environment for statistical computing v4.0.2 (R-Core-Team, 2020). Multivariate analysis: nonmetric multidimensional scaling (NMDS), permutational analysis of variance (PERMANOVA) (Anderson, 2001) and variation partitioning (Borcard et al., 1992) were performed using package vegan in R (Oksanen et al., 2019). As water chemistry datasets are dimensionally heterogeneous (measured in different metrics), the data was standardised prior to variation partitioning and so was the microbial community dataset which was square-root transformed and Hellinger standardised (Legendre and Gallagher, 2001) prior to multivariate analysis. NMDS was used to visualise beta diversity based on Bray-Curtis dissimilarity measure. The sample clusters in the NMDS were tested for significant difference using PERMANOVA on 999 permutations. Group homogeneity was assessed using function *betadisper* (Anderson, 2006). Likewise, the change in the beta diversity

between the two years (2018 and 2019) and seasons (spring and autumn) were analysed for significance using PERMANOVA on 999 permutations. The contribution of the measured variables to explaining the variation in the microbial community composition was analysed using variation partitioning. Total nitrogen and ammonium were removed from the dataset during variation partitioning, due to missing observations.

Co-occurrence network analysis was performed using R script provided by Ju et al. (2014) on Github[1]. Prior to the co-occurrence network, the OTUs data was filtered by selecting the top 25 OTUs that were present at least 5 times in at least 50% of the samples from the contaminated aquifer (R101, R203 and R402; n = 36). This filtering left only 79 OTUs (out of 1870) and were considered to be the core members of the aquifer community (generalist) and in addition, allowed for better resolution of the interactions between OTU nodes by reducing the network complexity. This way, OTUs that are rare or with too many zeros, which have to be avoided (Banerjee et al., 2018), were excluded. Co-occurrence network was performed for OTUs with significant ($P < 0.01$ after Benjamini-Hochberg correction) Spearman's rank correlations with coefficients (ρ) greater than 0.6. The co-occurrence network was generated in package igraph (Csardi and Nepusz, 2006) and was visualised in Gephi (Bastian et al., 2009). Only positive correlations, which may indicate common preference to conditions or cooperative associations (Fuhrman, 2009) were considered. The network topologies of the final model were compared with those generated from a random network according to Erdős and Rényi (1960). Prior to the network analysis, the OTU co-occurrence was evaluated for randomness by simulating a null community co-occurrence using the checkerboard-score (C-score) in package EcoSimR (Gotelli et al., 2015) for both the whole 1870 OTUs and the quality-filtered 79 OTUs.

Results

Alpha and beta diversity

Alpha diversity was highest in R203 and lowest in R402 (Figure S1). Beta diversity analysis based on Bray-Curtis dissimilarity metric showed distinct microbial community composition across the wells, although a slight overlap between R104 and R203 exists (Figure 1). The first axis (NMDS1) separated the samples by aquifer; samples from the contaminated aquifer correlated positively with the axis while samples from the uncontaminated aquifer (R0) correlated negatively. The second axis (NMDS2) separated the samples by the degree of contamination; R0 is uncontaminated, R402 is the least contaminated well in the contaminated aquifer; they correlate negatively with this axis. R104 and R203 are the most contaminated wells, and they correlate positively with NMDS2.

The groups in the NMDS were tested for significant difference using PERMANOVA. Both global and pairwise analyses showed statistically significant differences across ($F_{3.0}$ = 11.8, P = 0.001) and between the wells (Table 1). Similarly, differences in microbial community composition between spring and autumn and between 2018 and 2019 were tested. Results indicated nonsignificant difference in the microbial community composition between spring and autumn ($F_{1.0}$ = 1.13, P = 0.275) and 2018 and 2019 ($F_{1.0}$ = 1.15, P = 0.293).

Co-occurrence network

Comparing the OTU co-occurrence of the whole dataset with expectations from the null model that assumes patterns arise by chance revealed non-random pattern as the observed C-score for the observed community was significantly higher than the mean C-score expected under the null model ($C\text{-score}_{observed}$ = 16.2, $C\text{-score}_{random}$ = 15.8; $P < 0.001$; standardised effect size [SES] = 9.17). When only the OTUs displayed in the co-occurrence network (Figure 2) were

187 considered, a non-random co-occurrence was again recorded but this time with a much larger
188 SES (C-score$_{observed}$ = 15.1, C-score$_{random}$ = 13.3; $P < 0.001$; SES = 18.9). The arbitrary quality
189 filtering implemented both pre-analysis and during the network generation resulted in 72 OTU
190 nodes and 452 edges. Some of the network topological properties include the average degree
191 (12.6), graph density (0.18), average path length (2.8), node diameter (9), clustering coefficient
192 (0.63) and modularity (0.23). The last four topological properties were all above that calculated
193 from a random network for the same number of nodes and edges: average path length (1.91),
194 node diameter (3.0), clustering coefficient (0.18) and modularity (0.18). The network was based
195 on a strong positive ($\rho > 0.6$) and significant ($P < 0.01$) correlations. OTU nodes of *Rhizobiales*
196 and *Gemmatimonadaceae* have the highest number of connections (32) to other nodes. OTUs
197 from phylum *Patescibacteria* (Cand. Kaiserbacteria *Saccharimonadales* and *Parcubacteria*)
198 occurred prominently in the network and they have connections to multiple other OTU nodes.
199 However, nodes corresponding to its members were dispersed to four of the five different
200 network modules (Figure S2). Phylum *Chloroflexi* represented by phylotypes JG30 and
201 *Anaerolineaceae* showed fewer connections to other OTU nodes and they nearly all fell into a
202 single network module (Figure S2).
203
204 Based on betweenness centrality, OTU nodes with values greater than a 100 were considered
205 as putative keystone taxa in the aquifer. Eleven OTUs were identified as the keystone taxa
206 (Figure 3): *Saccharimonadales*, KD4.96 (uncharacterised *Chloroflexi*), *Saprospiraceae*,
207 *Vicinamibacteriales*, *Rhodoferax*, *Neisseriaceae*, *Gemmatimonadaceae*, *Gaiellales*,
208 *Holosporaceae*, *Rhizobiales* and *Alphaproteobacteria*, in the order of decreasing betweenness
209 centrality. Half of the OTUs identified as keystone taxa have a smaller (<10) number of
210 connections (node degree) and the remaining half have a larger (≥20) number of connections.
211 Of the keystone taxa, only *Rhodoferax* was present in high (13%) abundance (Figure S3).

A well-by-well basis simulation of null communities showed significant non-random taxa co-occurrence in R104, R203 and R402 (Table 2). Among these wells, the SES was highest in R203, moderate in R402 and lowest in R104. R0 by contrast, showed a non-significant marginally higher C-score (2.9333) than expected under random null model (2.9301), with 70/1000 simulations that occurred greater than the observed C-score.

Variation partitioning

The variation in the microbial community composition (Figure 4) was partitioned among the variables water chemistry (47.6%, F = 4.29, $P = 0.001$), distance (well) (44.8%, F = 13.7, $P = 0.001$), and both season and time (year) (0.4%, $P > 0.05$). Removing the effects of covariables resulted in explained variances of 7.5%, 4.5%, 1.1%, and 0.7% for water chemistry, distance, season and time, respectively. Of the explained variance (55.3%), 42.5% of this was accounted for by an interaction term between the groundwater chemistry and space, leaving only 12.8% of the variance attributable to the other terms in the model. The collective variance explained by all the variables was only 0.4%, and the unexplained variance was 44.7%.

Discussion

The higher observed C-scores than expected under null models suggest OTU segregation (Horner-Devine et al., 2007), which indicates the presence of asymmetrically large OTU pairs that are highly segregated (Gotelli et al., 2015). Non-random co-occurrence pattern for the aquifer-wide community was inferred due to the larger observed C-scores (Horner-Devine et al., 2007). Such non-random patterns imply ecological interactions (Lupatini et al., 2014). The connection of *Rhizobiales* and *Gemmatimonadaceae* to various OTU nodes may highlight how important these bacteria are in the community function. While *Rhizobiales* may include nitrogen-fixing bacteria that make nitrogen bioavailable for other microorganisms, two of the

three species described so far for phylum *Gemmatimonadetes* are capable of bioaccumulating polyphosphate (Pascual et al., 2018; Zhang et al., 2003). Phosphate and nitrogen are essential nutrients, and their accumulation can be viewed as provision of 'public goods' of the microbial community that increase its stability diversity (Konopka et al., 2015). Both *Rhizobiales* and *Gemmatimonadaceae* were identified as keystone taxa in the contaminated aquifer, which further strengthens their importance in the community. *Rhizobiales* have been frequently identified as a keystone taxon from various environments (Banerjee et al., 2018).

The *Patescibacteria* are endosymbiotic bacteria that have compromised metabolic capabilities (Brown et al., 2015) and rely on other microbes to meet their physiological needs. The strong correlations between *Patescibacteria* and the other OTUs in the co-occurrence network may therefore suggest host-symbiont relationship. The wide distribution of members of the *Patescibacteria* (*Parcubacteria*, *Saccharimonadales* and Cand. Kaiserbacteria) in the co-occurrence network modules (Figure S2) may highlight the underlining niche selection occurring in the aquifer, which limits intra-phylum co-occurrence patterns (Ju et al., 2014). This further demonstrates that ecological relatedness among the taxa in the network may not necessarily follow phylogenetic relatedness (Fuhrman, 2009). *Saccharimonadales* was among the keystone taxa identified, which suggests that the taxon is an important member of the aquifer microbiome. Another endosymbiont, which showed numerous connections in the network, is taxon *Diplorickettsiaceae*. The strong correlations to multiple potential hosts in a real world would be advantageous to the symbiont since it will have multiple alternative hosts to explore. Since networks are good for identifying ecological traits (Williams et al., 2014), the interaction patterns displayed here may be further investigated in control laboratory experiments to decipher roles of less studied taxa such as *Patescibacteria* and *Gemmatimonadaceae*, especially because network analysis provides a starting point for empirical observation and hypothesis testing (Banerjee et al., 2018; Fuhrman, 2009).

Well-by-well null models for all the three wells showed non-random community co-occurrences across the wells located in the contaminated aquifer. Non-random co-occurrence microbes implies that deterministic factors operate to shape the microbial communities (Horner-Devine et al., 2007). In the present study, the main driving factor is the landfill leachate and because this varies from well to well due to natural attenuation (Abiriga et al., 2020), a gradient exists and the communities from the wells showed idiosyncratic co-occurrence patterns. Thus, the proximal and distal wells represent opposing ends of a spectrum, with the proximal being highly influenced while the distal well being least impacted comparable to a recovery phase. From an ecological point of view, this presents differences in niche-based processes that may be characterised by successions similar to those observed in perturbation experiments (Herzyk et al., 2017; Zhou et al., 2014). The low SES recorded in the proximal well indicates that the microbial taxa in the proximal well co-occur more than in the intermediate (R203) and distal wells. This attests to our assertion that environmental filtering due to the leachate is strongest in the proximal well due to its proximity to the landfill (Abiriga et al., 2021a). The strong disturbance in the proximal well like other perturbations, will increase cell mortality and niche selection, and decrease microbial diversity and ecological drift (Zhou et al., 2014), causing the OTUs to coexist more than in the intermediate and distal wells.

In the intermediate well, disturbance is expected to be of an intermediate strength. The higher SES and alpha diversity in the intermediate well agree with the intermediate disturbance hypothesis that the highest diversity occurs at an intermediate level of disturbance (Miller et al., 2011; Svensson et al., 2012). Probable mechanisms shaping the microbial community in the intermediate well are niche-based processes such as predation and symbiosis. Evidence suggesting predation from protozoans is the presence of *Legionella*, *Polynucleobacter* and *Holosporaceae*, which are endosymbionts of amoeba, ciliates and paramecium, respectively. *Holosporaceae* was identified as a keystone taxon which further stresses the importance of the

taxon in maintaining community diversity. Other endosymbionts (*Patescibacteria* and *Diplorickettsiaceae*) were discussed above. All these endosymbionts except *Polynucleobacter*, were more abundant in the intermediate well (Figure S4).

In the distal well where the influence of leachate is expected to be minimal due to leachate attenuation (Abiriga et al., 2020) gives room to other ecological processes to drive the microbial community. This may include variable selection, competition, predation and phylogenetic history. The same endosymbionts were also present here (but *Polynucleobacter* was more abundant) and may provide evidence for predation. The difference in the observed metrics across the wells highlights the variation in the extent of these ecological processes among the communities.

In contrast to the contaminated aquifer, the null model community analysis of the background well showed only a marginally larger but non-significant observed C-score than expected due to chance. This suggests that the microbial community in the background well exhibits some degree of aggregation. An evidence of putative aggregation is the co-occurrence of 7% of the 1000 simulations more than the observed. Possible explanation for species aggregation are mutualistic and syntrophic interactions (Horner-Devine et al., 2007). The predominance of the background well by uncultured microbial taxa (Abiriga et al., 2021a) tilts the reasoning in favour of syntrophy and mutualism, since most of the cultivation conditions for these taxa are difficult to meet in laboratories, including syntrophic and mutualistic behaviours implicit of co-culture growth requirements.

Identifying the ecological processes shaping community compositions in any system is predicted by determining whether it is deterministic or stochastic. While our analysis does not identify the causal mechanistic processes, the non-random community assembly patterns do indicate the dominance of deterministic processes (Horner-Devine et al., 2007). Using

310 measured environmental variables, variation partitioning was employed to quantify the overall
311 contribution of deterministic factors in shaping the microbial community compositions across
312 the four wells sampled. The result showed that the deterministic variables collectively explained
313 55.3% of the variation in the microbial community compositions. Of the explained variance,
314 the water chemistry and distance jointly accounted for most of the variance (42.5%), indicating
315 that both the microbial community composition and the groundwater chemistry have similar
316 spatial structuring (Borcard et al., 1992), which is attributed to the influence of the landfill
317 leachate (Abiriga et al., 2021b). Given that not all environmental variables are measurable in
318 any single study, the unexplained variance (44.7%) may represent both the unmeasured
319 deterministic, and stochastic factors, although stochastic processes may play a partial role in
320 shaping microbial community compositions (Stegen et al., 2012; Williams et al., 2014). We
321 posit that in systems such as Revdalen Aquifer which is under press perturbation (Zhou et al.,
322 2014), deterministic processes are more important than stochastic processes.

323 We previously reported a lower explained variance (33.2%) (Abiriga et al., 2021b) than found
324 in the present study (55.3%). In the previous study, the samples analysed covered the entire
325 aquifer depth compared to a single depth considered in the present study. The big difference
326 between the two results could be attributed to the inherent variation in the aquifer layering
327 across the depth, which are expected to respond differently to changes in hydraulic regimes
328 (Smith et al., 2018). Although the aquifer matrix is not discernibly stratified, the aquifer matrix
329 is heterogeneous (Abiriga et al., 2020) and vertical variation is, therefore, expected. This has
330 resulted in differences in the microbial community compositions across the depths in the aquifer
331 (Abiriga et al., 2021b). The inherent random variations in subsurface environments are among
332 the variables that magnify the unexplained proportion in microbial community compositions.
333 Sampling groundwater from only one level avoided this bottleneck and improved the amount
334 of explained variance. The non-significant seasonal difference in beta diversity and the small

variance (0.4%) explained by season further indicates that seasonal variability due to differential response of aquifer layers to hydrologic regimes, which causes shift in microbial communities (Pilloni et al., 2019), was minimal. This finding has a serious implication for future studies on subsurface microbiology, where a great deal of attention needs to be given in designing a study for heterogeneous systems.

Our study shows the co-occurrence of important microbial taxa that are frequently recovered from subsurface and because some of these group to-dates are not well characterised, the co-existence as suggested by the network analysis provides valuable information about possible ecological interactions that can be investigated in the feature. The null model analysis identified deterministic processes as the driving force shaping the microbial community assembly in the landfill-leachate-impacted aquifer. The dominance of deterministic community assembly was substantiated by employing variation partitioning, which indicated that the measured environmental variables explained most of the variation in the microbial community composition. The novelty of this research is the application of a combination of network analysis, ecological null model analysis and multivariate statistics to microbial data from an environment which has not been previously studied for the ecological processes. Findings from this study should therefore advance our understanding of microbial community assembly in ecosystems subjected to press perturbation.

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Data availability statement

The raw sequence data supporting the study have been deposited in Sequence Read Archive under BioProjects PRJNA677875 (Biosamples SAMN16775936-37, SAM16775944-45, SAM16775952-53, SAM16775958-59 and SAMN16775996-6019).

Footnote

[1] https://github.com/RichieJu520/Co-occurrence_Network_Analysis

[2] https://www.sequencing.uio.no

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Figures

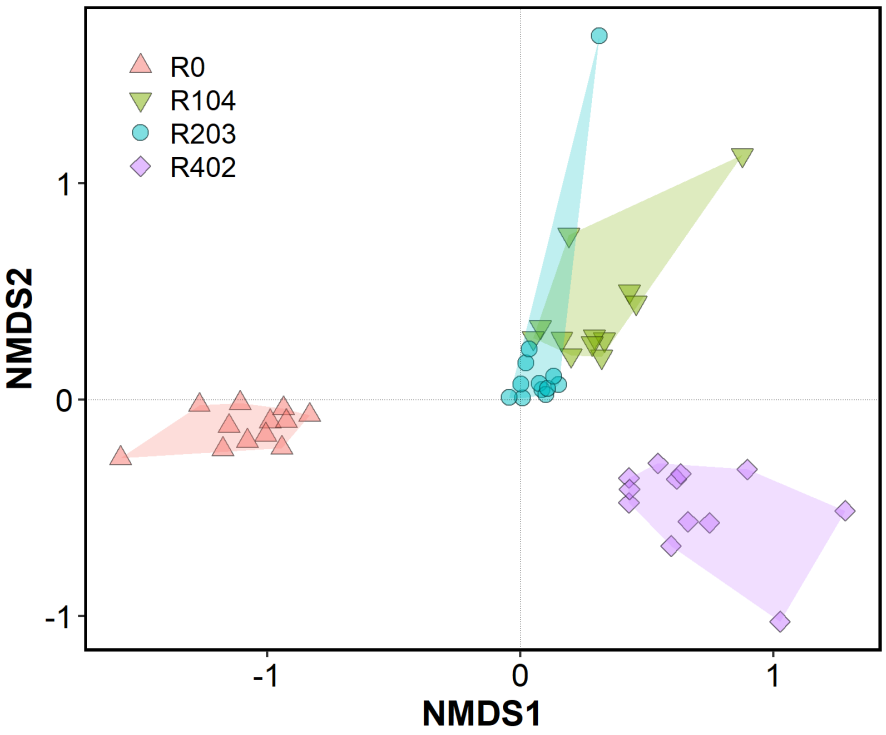
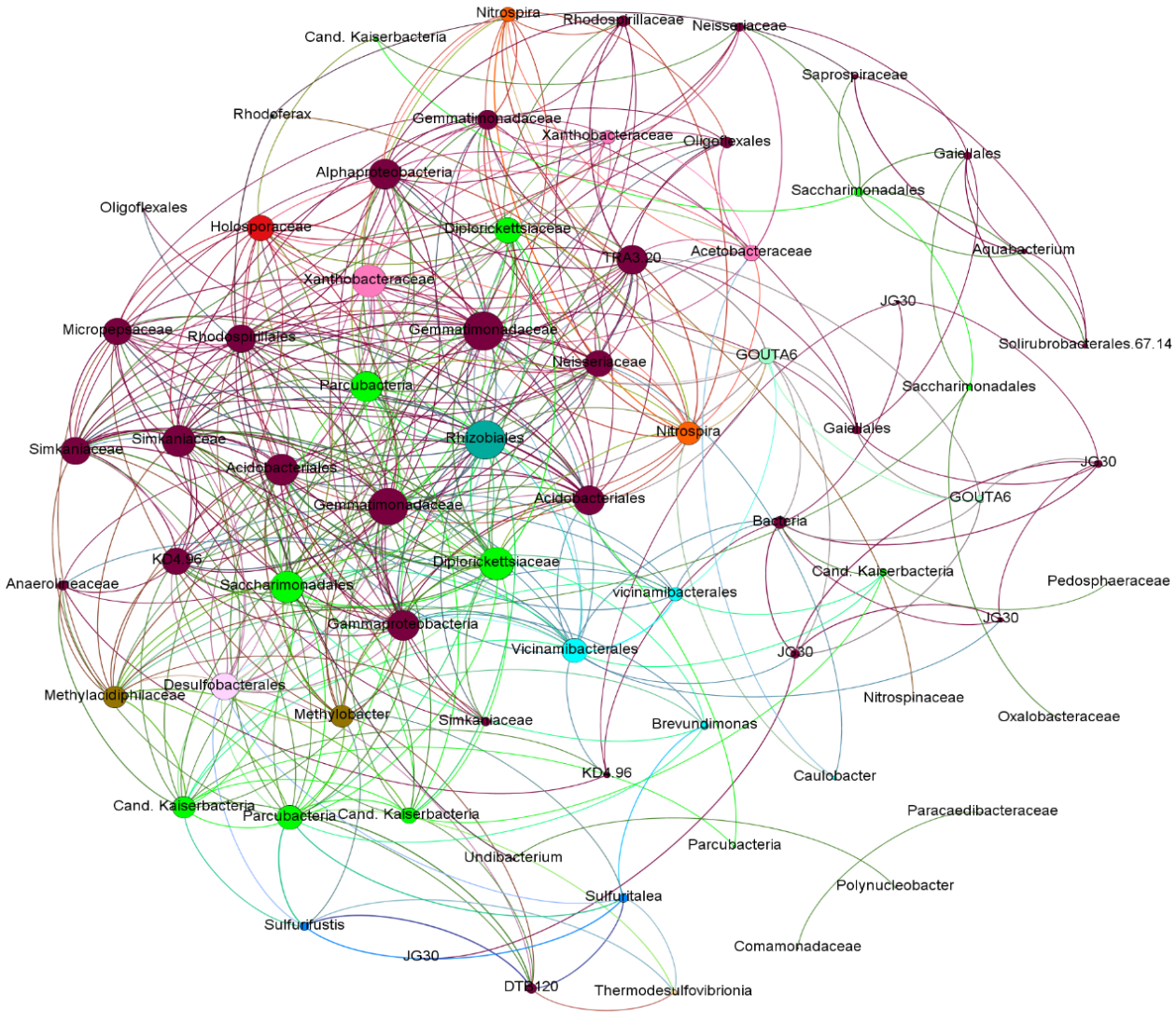


Figure 1. Nonmetric multidimensional scaling (NMDS) plot of sites. R0 is the background well located in a nearby uncontaminated aquifer, while R104, R203 and R402 are the wells located in the contaminated aquifer.

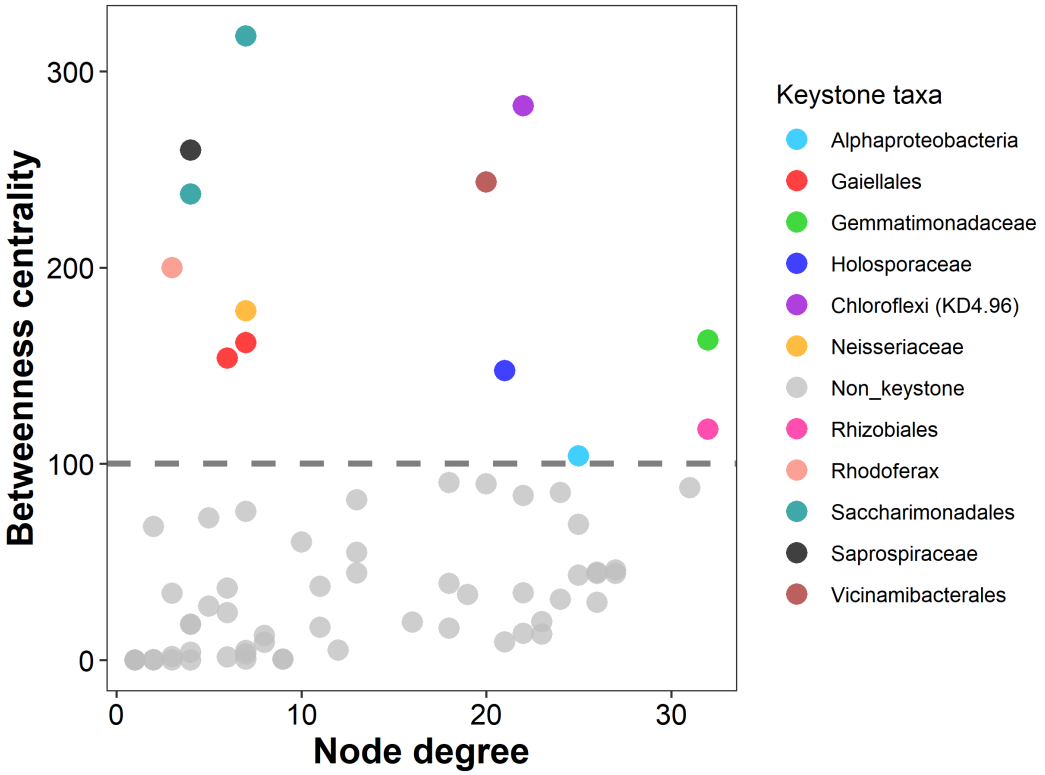


516

517 Symbionts
Heterotrophs
518 Sulphate reducing
Parasitic
Nitrogen fixation
Methylotrophs
Ammonia oxidising
Others
Nitrite oxidising
Sulphur oxidising
Degrades hydrocarbons

519 **Figure 2.** Co-occurrence network of generalists in the contaminated aquifer (n = 36). Each connection represents
520 a strong and significant Spearman's correlation ($\rho > 0.6$, $P < 0.01$). The size of the nodes is proportional to the
521 node degree (number of connections).

522

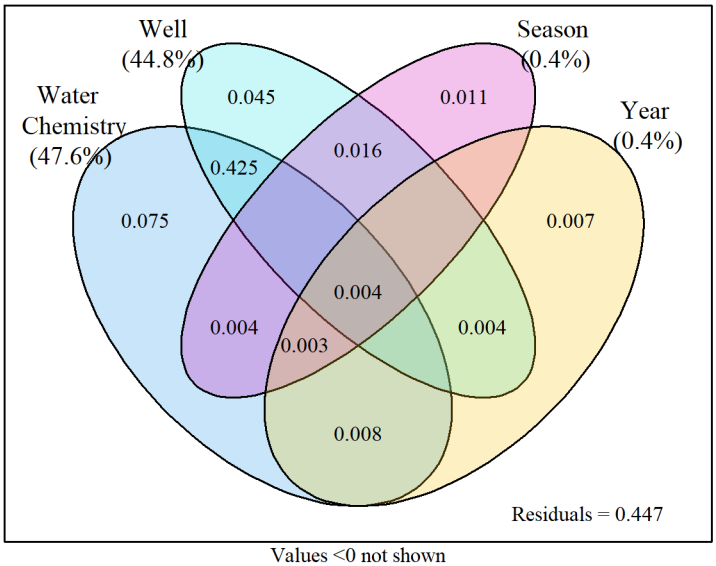


523

524 **Figure 3.** Keystone taxa identified among the generalist taxa in the network. The dashed line depicts an arbitrary

525 cut-off based on betweenness centrality value (100) above which OTUs were considered to be a keystone taxon.

526



Values <0 not shown

527

528 **Figure 4.** Variation partitioning of proportions of variation in microbial community composition explained by

529 water chemistry, year, season and distance (well). Values in parenthesis indicate the variance explained by the

530 respective variables but without removing contribution from covariables.

Tables

Table 1. PERMANOVA pairwise comparison of microbial community composition between the wells. R0: background well; R104: proximal well; R203: intermediate well; R402: distal well.

Pairs	F	R^2	*P*-value [a]
R0 vs. R104	14.99	0.405	0.006
R0 vs. R203	16.32	0.426	0.006
R0 vs. R402	15.54	0.414	0.006
R104 vs. R203	6.66	0.232	0.006
R104 vs. R402	8.34	0.275	0.006
R203 vs. R402	9.85	0.309	0.006

[a] *P*-value adjusted using Bonferroni correction.

Table 2. Results of null model simulations for the four different communities.

	N [a]	OTUs [b]	$C\text{-score}_{observed}$	$C\text{-score}_{random}$	SES	*P*-value
R104	12	830	2.72	2.68	11.9	<0.001
R203	12	709	2.56	2.50	21.9	<0.001
R402	12	558	2.39	2.31	15.3	<0.001
R0	12	473	2.933	2.930	1.35	0.07

[a] Number of samples in each well

[b] OTUs were filtered to only include taxa present in abundances of greater or equal to 10.

Microbial deterministic assembly and co-occurrence network in an aquifer under press perturbation

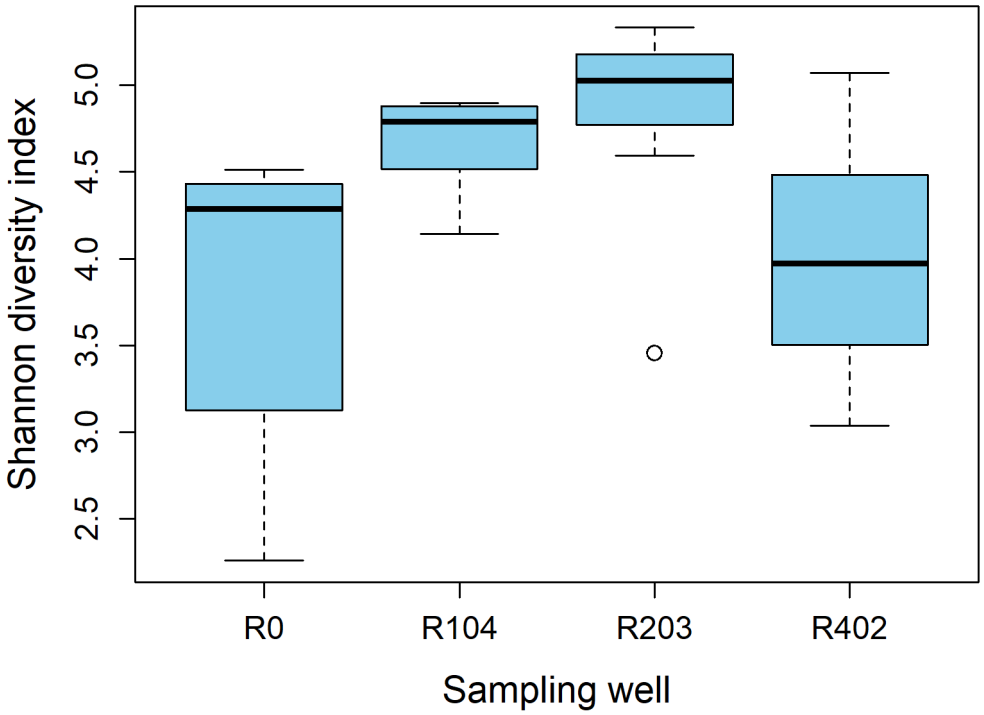


Figure S1. Shannon diversity index across the sampling wells.

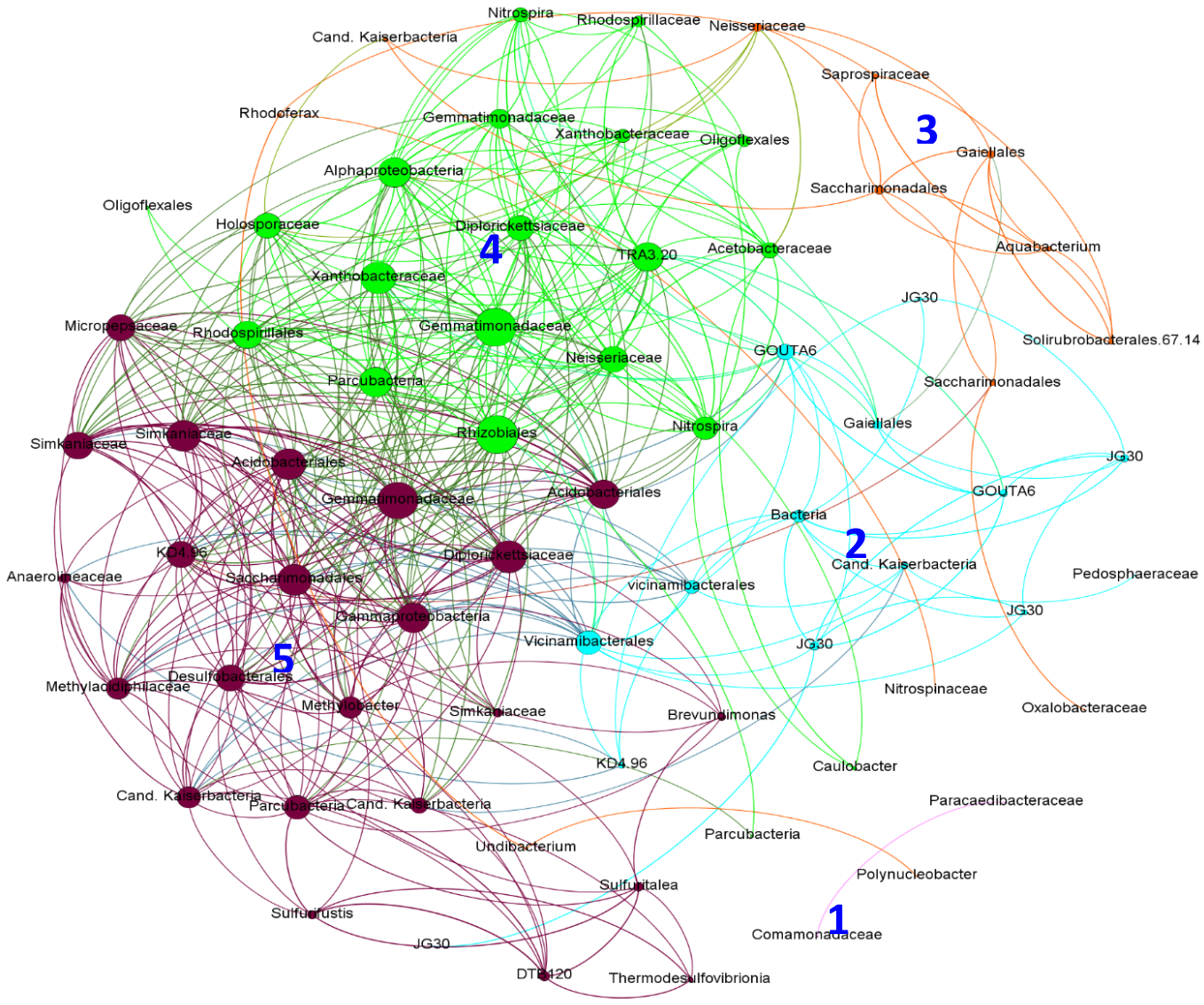


Figure S2. Co-occurrence network modules. Five community modules were identified.

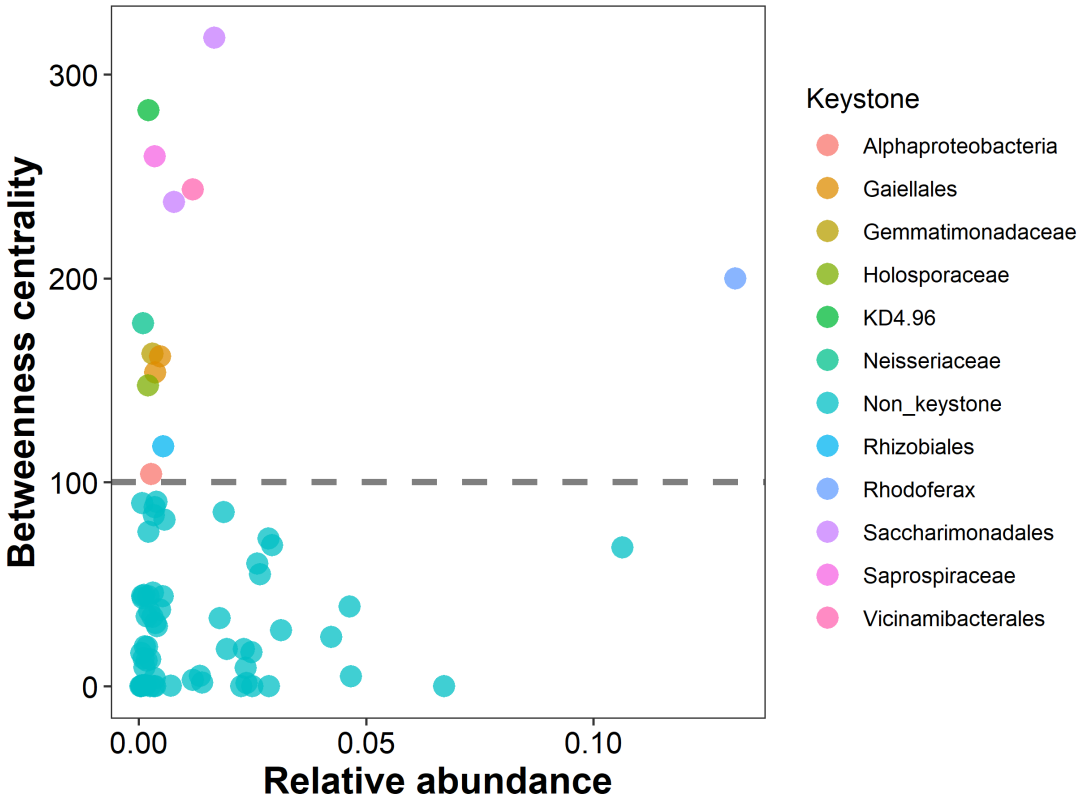


Figure S3. A plot of betweenness centrality against the relative abundance of the 72 taxa in the network.

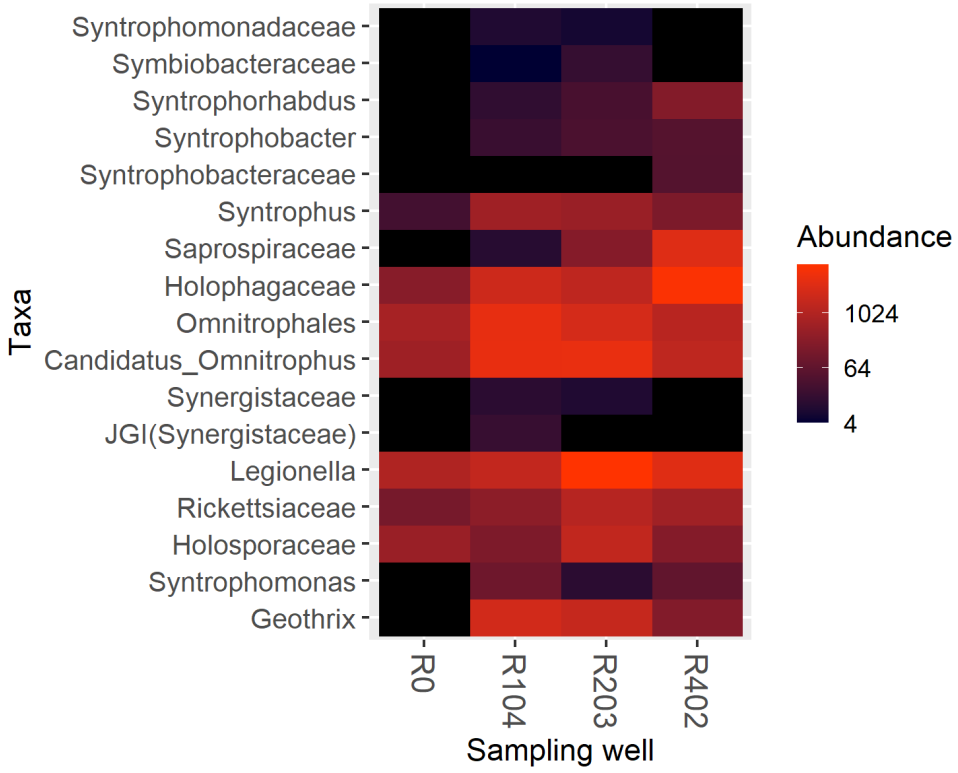


Figure S4. Taxa that may be of special interest in microbial species interactions.

Article 4

Andrew Jenkins, Daniel Abiriga, Kristian Alfsnes, Live Semb Vestgarden and Harald Klempe (2021). A comparison of sediment and groundwater microbiomes in a landfill leachate-contaminated aquifer undergoing intrinsic remediation. Manuscript.

A comparison of sediment and groundwater microbiomes in a landfill leachate-contaminated aquifer undergoing intrinsic remediation

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Abstract

Sediment and the groundwater are very different habitats. However, most of the literature comparing planktonic communities with sediment-attached communities relied on surrogate sediment samples. Here, we compare the microbial community composition between free-flowing water (planktonic) and sediment-bound (biofilm). Comparisons were made on fresh aquifer sediment samples taken a few centimetres away from the well from which corresponding water samples were obtained. Result shows that both the alpha diversity and heterotrophic plate counts were higher in the aquifer sediment sample compared to the groundwater sample. Beta diversity (Bray-Curtis) shows that the microbial composition is different between the free-flowing groundwater and the aquifer sediment. Moreover, the samples clustered by depth at which they were obtained. Compositionally, the groundwater column was dominated by phyla *Proteobacteria*, *Patescibacteria* and *Bacteroidetes*, while the aquifer sediment was dominated by phyla *Acidobacteria*, *Actinobacteria* and *Chloroflexi*. The finding has practical implication for studying the microbial ecology of subsurface environments. Studies aiming at characterising the whole microbial composition, it is recommended that both groundwater and aquifer sediment be sampled.

Keywords: aquifer sediment microbiology; groundwater microbiology; microbial composition; planktonic bacteria; biofilm

Introduction

One of the leading causes of groundwater contamination is landfill operation. Landfills continue to be a destination to municipal solid wastes across the globe (Chen et al., 2017; Eggen et al., 2010; Mouser et al., 2005). The past century was characterised by unregulated rudimentary waste disposal practices in many places globally, which gave rise to landfills operated with little regard to environmental contamination (Reinhard et al., 1984), where the resultant leachates are not collected, contained nor treated. These landfills are often referred to as 'old landfills'. They represent significant environmental concern because they hold all kinds of waste (Christensen et al., 2000) and the leachate they generate usually contain complex pollutants contaminants (Baun et al., 2003; Christensen et al., 2000; Eggen et al., 2010; Moody and Townsend, 2017; Mouser et al., 2005).

The Revdalen aquifer (the present study aquifer) is an aquifer contaminated by an old landfill in Mid-Telemark Municipality, Southeastern Norway. The aquifer is some 5–8 m thick, 70–100 m wide and 1700 m long. It is housed in a glaciofluvial deposit and confined by bedrock at its base and sides and by an overlying layer of till. Unregulated dumping of waste near its upper reaches from 1958 to 1974, followed by establishment of a municipal landfill which operated from 1974–1996 resulted in contamination of the aquifer with landfill leachate. Since closure of the landfill in 1997 the landfill-aquifer system has been allowed to undergo monitored intrinsic remediation and the purity of the water has steadily improved.

In previous articles (Abiriga et al., 2021a; Abiriga et al., 2021b; Abiriga et al., 2020; 2021c), we report chemical and microbiology conditions in the groundwater at different depths and distances from the landfill. In the present study, we compare the microbiomes of the groundwater and the host sediment. It has been reported that the two habitats harbour different microbial communities (Flynn et al., 2008; Scow and Hicks, 2005; Smith et al., 2018). Thus, groundwater samples alone cannot capture the whole aquifer diversity (Flynn et al., 2008; Röling et al., 2000), but the ease of obtaining groundwater samples favours its routine applicability (Flynn et al., 2008). Most of the literature comparing planktonic communities with sediment-attached communities relied on surrogate sediment samples. On the other hand, it is both technically challenging and economically costly to obtain aquifer sediments and this has resulted in literature on subsurface microbiology being dominated by

groundwater microbiology, leaving a dearth of information on microbiology of different aquifer lithologies (Smith et al., 2018). Moreover, the destructive nature of obtaining aquifer sediments means that repetitive sampling such as in long term monitoring is impracticable. While the ease of groundwater movement in porous medium makes it ideal as a representative medium for a larger research area (Brad et al., 2013), aquifer sediment microbial communities show high spatial heterogeneities (Brad et al., 2008), but they offer better degradation potentials than planktonic communities (Holm et al., 1992). In the present study, comparisons were made on fresh aquifer sediment samples taken a few centimetres away from the well from which corresponding water samples were obtained.

Materials and Methods

The Revdalen landfill and aquifer (grid position) have been described elsewhere (Abiriga et al., 2020; 2021c; Klempe, 2004; 2015). Groundwater was collected from a multilevel sampling well (three levels: R401, R402, R403) 324 m downstream of the landfill at depths 118, 117 and 114 m.a.s.l (Mid-October). Elsewhere, this well is referred to as 'the distal well' or R4, but here it is referred simply as 'the well'. Sample collection, treatment and analysis are described elsewhere (Abiriga et al, 2021). Sediment samples at depths of 6-7, 8-9 and 9-10 m were extracted from a core sample extracted 0.3 m downstream of the well (early-December) using the piston method. Moisture content was estimated as loss of weight on drying.

Viable counts for sediment and water were performed by colony counts on nutrient media. This procedures is described elsewhere (Abiriga et al., 2021a).

DNA was extracted from filter retentates corresponding to 150 ml of water as described previously (Abiriga et al, 2021a) or from 250 mg of sediment using the DNeasy Powersoil Kit (Qiagen, Germany). Metabarcoding targeting the V3-V4 region of the 16s rRNA gene and bioinformatical analysis have been described previously (Abiriga et al, 2021a). Abundant OTUs were identified to the lowest taxonomic level available in the SILVA v.138 database; identifications were checked for taxonomic consistency at the phylum, class, order, family and genus levels by reference to the NCBI taxonomy browser, and, where discrepancies were observed, to the primary literature.

Metabarcoding was conducted on three water samples and three sediment samples separately, but in some analyses, the results were pooled, while in some they are kept separate.

Results

Overview of sequencing results

The three groundwater and three sediment samples yielded a total of 220 459 bacterial sequence reads, 111 888 (50.8%) from the groundwater and 108 571 (49.2%) from the sediment. These reads clustered into 1070 OTUs at the 97.5% similarity level, which, broadly speaking, corresponds to species level. 420 OTUs, corresponding to 43 653 reads were unique to the groundwater; 366 OTUs corresponding to 43 835 reads were unique to the sediment and 284 OTUs, corresponding to 132971 reads were common to both sets of samples.

Cell counts and alpha diversity

Mean microscopic cell count for the water samples was 2.4×10^4/ml, (mean viable count was 9.4×10^3/ml). Mean sediment moisture content was 322 mg/g. This implies that each 250 mg sediment samples contained 0.04 ml of groundwater which would have contributed approximately 1000 cells to each of the three sediment samples, and 3000 cells altogether. Given that >100 000 reads were obtained, this suggests that the contribution of microbiota to the sediment sequences is slight.

Both alpha species diversity (Shannon diversity metric) and heterotrophic plate counts showed higher values in the aquifer sediment sample compared to the groundwater sample (Figure 1).

Comparison of groundwater and sediment microbiota.

Comparisons of groundwater and sediment microbiota are shown in tables 1, 2 and 3. Table 1 shows taxa preferentially found in the groundwater; table 2 shows taxa preferentially found in the sediment and table 3 shows taxa roughly equally distributed between the two.

The results show that the groundwater and sediment microbiota are clearly distinct. Only a few OTUs exhibit less than threefold difference in abundance. The result from beta diversity analysis (Figure 2) shows that the microbial composition is different between the free-flowing groundwater and the aquifer sediment which are separated by PCoA1. The second axis (PCoA2) separated the samples by depth at which the samples were obtained. However, it shows that there is a concordance among the sampling depths regardless of the sample type.

Groundwater Taxa

Nitrite-oxidising members of the *Nitrospirota*, notably *Nitrospira*, and *Nitrospinota* (Family Nitrospinaceae) showed a strong preference (310x and 32x, respectively) for the groundwater. The GOUTA6 clade of the Nitrosomonadaceae also shows a preference for the groundwater, although the difference is much less.

A second feature of the groundwater microbiota was the abundance of taxa that are metabolically dependent on or strongly associated with other organisms. These include the *Patescibacteria* (53x overabundant and one of the most abundant taxa in the groundwater), *Legionella*, a parasite of amoebae, *Polynucleobacter*, which includes endosymbiont of ciliates, and *Reyranella*, which is associated with amoebae.

Also, prominent among the groundwater microbiota were aerobic (*Caulobacter*, *Edaphobaculum*, *Ferruginibacter*, *Polaromonas*, *Sediminibacterium*, *Sphingomonas*, *Terrimonas*, *Undibacterium*, or facultative anaerobic heterotrophs. *Rhodoferax,* one of the most abundant taxa may also belong among these, but the genus is so metabolically diverse that a firm conclusion cannot be drawn without a species-level identification.

Other activities associated with the groundwater microbiota were chemoautotrophic oxidation of reduced sulphur species (*Sulfuricella*) and methylotrophy (*Methylotenera*).

However, not all the taxa in the groundwater were aerobic. Microaerophilic taxa, such as *Aquabacterium* and *Reyranella* were also found, as well as the anaerobic iron (III) reducer, *Ferribacterium* and the fermentative anaerobe *Obscuribacter*.

Sediment Taxa

Few of the sediment-associated OTUs could be classified at the genus level and most mapped to non-cultivated and poorly characterised taxa, which limits ascertainment of their properties.

With the exception of the methylotrophic *Methyloligellaceae*, all taxa were heterotrophic, with a wide range of preferred substrates. Most were aerobic, although microaerophilic (*Roseiarcus*) taxa also occurred. Three acid tolerant taxa (*Bryobacter, Acidothermus, Roseiarcus*) were identified. Apart from the *Xanthobacteriaceae*, which includes both motile and non-motile taxa, none of the taxa are capable of flagellar motility, although gliding motility

is common in members of the abundant *Chloroflexi*. Several psychrotolerant taxa (*Bryobacter, Vicinamibacterales, Solirubrobacteraceae, Roseiarcus*) were found, but surprisingly, a thermophilic genus, *Acidothermus* (Bergey's Manual; Kulichevskaya et al, 2014; Kulichevskaya et al, 2010; Huber & Overmann,2018; Ward et al, 2009; (Solibacter); Sekiguchi et al, 2003; Doronina et al, 2013) was also found.

Shared taxa

A minority of taxa were shared approximately equally between the sediment and the groundwater. These included species of class *Dehalococcoidia*, all of whose members are anaerobic and dehalogenate halogenated hydrocarbons, and the chemolithotrophic genus *Nitrospira*.

Discussion

Sediment and the groundwater are very different habitats. Sediment particles provide surfaces for the growth of biofilms and mineral grains, which though insoluble, may be attacked by the biofilm microbes and/or used as electron sinks or sources. The presence of biofilm and microscopic pockets in the particles will increase the environmental diversity, with strong gradients of pH, nutrient and oxygen concentration potentially developing. Biofilm microhabitats are typically spatially diverse but temporally stable; motility is suppressed. This makes aquifer sediments to high spatial heterogeneities (Brad et al., 2008). On the other hand, a liquid environment like the groundwater cannot support such microheterogeneity, due to higher mixing rates, and gradients will thus be less steep. However, the ease of groundwater movement makes it an ideal for a larger research area (Brad et al., 2013). Temporal variation, driven by variations in leachate input, driven in turn by variation in precipitation, is expected to be larger. Nutrients will be predominantly soluble and readily assimilated. Motility will be favoured, as it will allow bacteria to seek optimal conditions along these large-scale gradients and avoid washout.

Our results support this picture. Both multivariate analysis of the microbiomes and comparison of major taxa, as well was cell counts show a clear difference between the microbiota of the groundwater and the sediment, which agrees with the literature (Balkwill and Ghiorse, 1985; Holm et al., 1992; Röling et al., 2000; Scow and Hicks, 2005; Smith et al., 2018), with very few taxa being evenly shared between the two environments.

The groundwater supported many motile taxa; heterotrophic aerobes and facultative anaerobes were abundant, but microaerophiles and anaerobes were present in significant numbers. Methylotrophs and chemoautotrophic oxidisers of nitrite and reduced sulphur compounds were also prevalent. Parasites and symbionts of protists (*Legionella*, *Polynucleobacter* and *Reyrenella*) or other bacteria (*Patescibacteria*) were plentiful, with the *Patescibacteria* being the most abundant bacterial group. These results imply the presence of a significant protistan community in the groundwater.

The sediment community was less easy to characterise as it was dominated by poorly characterised, non-cultivable taxa. Taxa that were non-motile or had only gliding motility dominated the sediment microflora and filamentous forms were frequent. Methylotrophs

were abundant, but chemoautotrophic oxidisers of nitrite and reduced sulphur compounds were not. Parasitic and symbiotic taxa were scarce. The absence of parasites and symbionts of protists probably reflect the fact that protists are excluded from biofilm.

One surprising finding was the abundance of the thermophilic genus *Acidothermus* in the sediment samples. This is difficult to explain, to the best of our knowledge, there is no local source of industrial or domestic waste heat, the observed levels of organic matter are insufficient to support significant biogenic heating, and the site is not geothermally active. At the depths at which the sediment samples were taken (6 – 10 m), temperatures should not deviate greatly from the local mean annual temperature, while thermophiles typically have minimum growth temperatures around 37°C and optimum growth temperatures around 55°C. While we cannot entirely exclude the possibility that thermophilic conditions are achieved in highly bioactive pockets in the sediment, we think it is most likely that the result is due to a hitherto unidentified non-thermophilic members of *Acidothermus*.

Although our results indicate a clear distinction between the microflora of the sediment and groundwater with plausible biological explanations, certain caveats apply. Metabarcoding is subject to biases in all of its stages, particularly the extraction stage; it was not possible to apply the same extraction method to sediment and groundwater samples and any extraction biases may have had knock-on effects in later stages. Furthermore, there was a 6-week time lapse between groundwater sampling and extraction of the sediment cores. However, since spring and autumn the groundwater samples from the well over a two-year period formed a distinct cluster in multivariate analysis, that was well-separated from the sediment samples, this seems unlikely to be a major issue.

Although we are confident of our findings, certain caveats apply and must be acknowledged. In this study, we treat number of sequences reads as equivalent to species (OTU) abundance. This, however, is an oversimplification. A sequence read corresponds to a single copy of the 16s rRNA gene in the input DNA, but not necessarily to a single cell, as some bacterial species have multiple copies of the gene. Secondly, metabarcoding is subject to biases, particularly at the stages of DNA extraction and amplification. I should be noted here that the DNA extraction methods used for groundwater and sediment were not the same. Thus, although abundant OTUs are undoubtedly truly abundant, they may appear more abundant than they truly are, while other abundant OTUs may fail to appear so because they are recalcitrant to DNA

extraction and/or amplification. Nonetheless, the scale of the difference between the sediment and groundwater microflora seems too great to be explained by artefact alone.

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Author Contributions: HK is the initiator and manager of the Revdalen monitoring project, devised the methods for sample recovery and was principal supervisor for DA. DA conducted sampling and all subsequent laboratory procedures and statistical analysis of the data. KA advised the group on metabarcoding, performed bioinformatic analyses and provided advice on their interpretation. AJ performed descriptive analyses of the data and drafted the manuscript. AJ and LSV served as co-supervisors for DA and were active in discussions throughout the project. All authors reviewed and approved the manuscript.

Figures

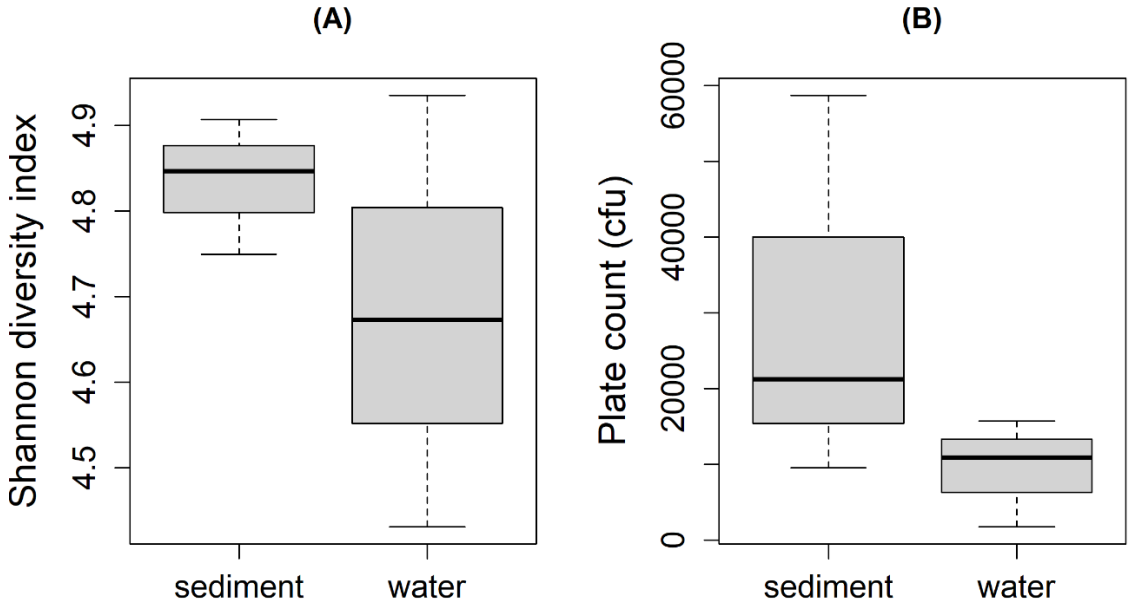

Figure 1. Alpha species diversity (Shannon diversity index) **(A)**, and heterotrophic plate count **(B)** in sediment (CFU/g dry weight) and water (CFU/ml).

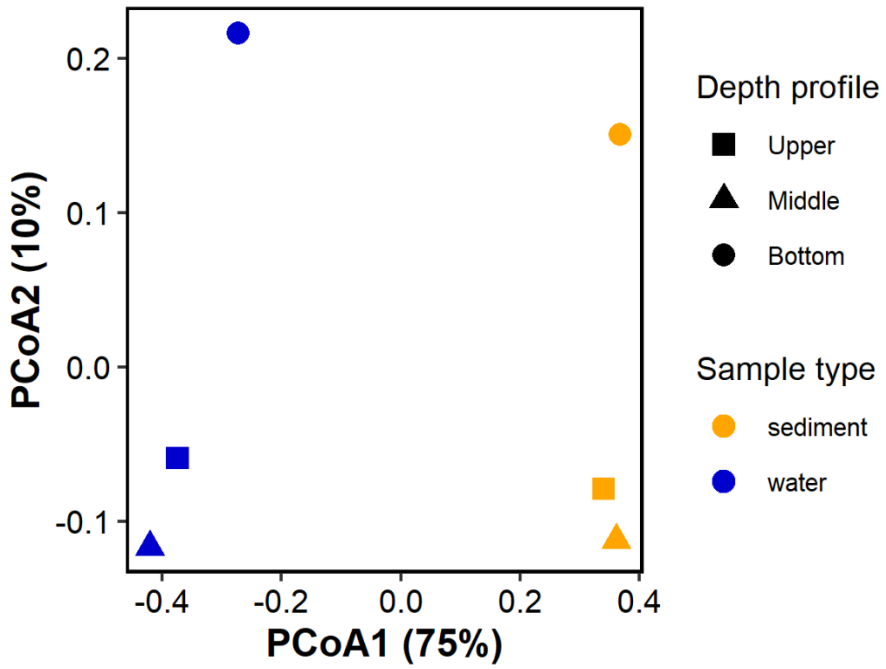

Figure 2. Beta diversity between groundwater samples and the aquifer sediment samples based on the Bray-Curtis dissimilarity distance.

Tables

Table 1. Planktonic Taxa. Phyla and OTUs strongly associated with the groundwater.

Phylum	Genus	Groundwater	Sediment	Ratio[1]
Acetothermia		177	18	10:1
Bacteroidota		3234	447	7:1
Campylobacterota		1001	0	-
Hydrogenedentes		10	0	-
Nitrospinota		227	7	32:1
Nitrospirota		2794	9	310:1
Patescibacteria		14889	282	53:1
Verrucomicrobiota		1744	448	3.9:1
Acidobacteriota	Holophagaceae sp.	1438	122	12:1
	Solirubrobacterales 67.14	1457	0	-
Bacteroidota	Edaphobaculum sp.	741	0	-
	Ferruginicater sp.	966	0	-
	Kapabacteriales sp.	550	0	-
	Sediminibacterium sp.	611	0	-
	Terrimonas sp.	968	0	-
Campylobacteria	Campylobacterales sp.	883	0	-
Cyanobacteria	Ca. Obscuribacter	4934	0	-
Patescibacteria	Ca. Kaiserbacteria	3332	0	-

	Ca. Kerfeldbacteria	535	0	-
	Berkelbacteria	630	0	-
Chloroflexi	Kdenobacteria Armatimonadetes sp	1104	133	8:1
Nitrospirota	Nitrospira sp.	1898	406	4.6:1
Patescibacteria	Parcubacteria sp	3178	57	56:1
	Saccharimondales sp.	3305	42	79:1
	Ca. Peribacteria sp.	2859	11	260:1
	Parcubacteria sp.	1614	35	46:1
	Ca. Adlerbacteria sp	1376	32	43:1
Proteobacteria	Aquabacterium	875	0	
	Burkholderiales, TRA3.20 sp.	1800	584	3.1:1
	Caulobacter	581	0	
	Comomonadaceae sp.	1465	9	163:1
	Ferribacterium	741	0	-
	Legionella sp	934	89	10:1
	Methylotenera	835	0	
	Nitrosomonadaceae GOUTA6 sp.	1241	394	3.3:1
	Polaromonas	674	0	-
	Polynucleobacter	804	0	-
	Reyranella sp.	1372	114	12:1
	Rhodoferax	9521	15	635:1
	Sphingomonas	1609	0	-
	Sulfuricella	605	0	-
	Undibacterium	701	0	-
Unspecified	Anonymous OTU	3115	371	8:1

1. Rounded to nearest whole number for ratios > 5 or to first decimal place for ratios < 5.

The table lists phylum-level and species-level taxa that are >3x more abundant in groundwater. Phyla represented by less than 10 reads are excluded. Species-level OTUs are confined to those showing at least 1000 reads if present in both sediment and groundwater or 500 reads if present only in groundwater.

353 Table 2. Characteristics of Phyla and OTUs strongly associated with the sediment.

	Aerobic[1]	Acidotolerant	Motile	Form[2]	Cryotolerant	Thermophilic	Heterotrophic[3]
Entotheonellota							
Spirochetota							
Sumerlaeota							
Acidobacteriales sp.	+						+
Bryobacter sp.	+	+	-	c/r	+		+
Ca. Solibacter sp	+						+
Vicinamibacterales sp.	+	-	-		+/-		+
Acidimicrobiia sp.							
Acidothermus sp	+	+	-	F-r	-	+	+
Solirubrobacteraceae sp.	+		-	R	+/-		+
Chloroflexi AD3 sp.			-	F			
Ktdenobacteraceae sp.	+			F			+
Xanthobacteraceae sp.	+		+/-	R			+
Elsterales sp.							
Roseiarcus	m	+	-	v*	+	-	+
Methyloligellaceae	+		-	R			Me

354 (1) m: microaeophilic (2) c: cocci; F: filamentous; R: rods; v: variable (3) Me: methylotroph

355

356 Table 3. Shared Taxa. Abundant OTUs not strongly segregated between the sediment or the
357 groundwater.

Phylum		Sediment	Groundwater	Ratio
Chloroflexi	KD4.96	639	395	1.6:1
	Dehalococoidia SO85	1118	544	2:1
Methylomirabilota	Rokubacteriales sp.	1331	682	2:1
Nitrospirota	Nitrospira sp.	308	853	1:2.8

358

359 1. Rounded to nearest whole number for ratios > 5 or to first decimal place for ratios < 5.

360 The table lists taxa whose abundance differs by <3x in sediment and groundwater. Only OTUs
361 showing at least 1000 reads are shown.

Article 5

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A nature-based solution to a landfill-leachate contamination of a confined aquifer

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Abstract

Remediation of groundwater from landfill contamination presents a serious challenge due to the complex mixture of contaminants discharged from landfills. Here, we show the significance of a nature-based solution to a landfill-contaminated aquifer in southeast Norway. Groundwater physicochemical parameters monitored for twenty-eight years were used as a proxy to infer natural remediation. Results show that concentrations of the major chemical variables decreased with time and distance until they tailed-off. An exception to this was sulphate, which showed an increase, but apparently, exhibits a stationary phase. The water types were found to be most similar between samples from active landfill and post-closure stages, while samples from the stabilised stage showed a different water type. All the parameters for samples from the stabilised stage were found to be within the Norwegian drinking water standards, except iron and manganese, which were only marginally above the limits, an indication of a possible recovery of this aquifer. The findings highlight the significance of natural attenuation processes in remediating contaminated aquifers and have significant consequences for future contamination managements, where natural remediation can be viewed as an alternative worth exploring. This is promising in the wake of calls for sustainable remediation management strategies.

Introduction

Contamination of groundwater due to human activities remains a global challenge. Of particular concern is leachate from landfills, which can pollute both surface water and groundwater[1,2]. This may negatively affect an ecosystem functioning, as leachates contain both legacy and emerging contaminants[2-5]. Several factors influence the composition and concentration of contaminants discharged by landfills[6-8] and the eventual degree of groundwater contamination which in turn shapes the resident microbial composition responsible for degradation[9]. These factors include waste composition, landfill age, moisture content of the waste, amount of rainfall received, the nature of the underlying geological material, and the waste composition[6-8,10].

Landfills operated in the seventies and earlier before waste segregation was adopted were fill-up with a mix of nearly anything[11]. Leachate composition from such landfills is highly variable and complex in nature, consisting of a mixture of contaminants[3,11-14]. This makes remediation of groundwater polluted by landfills even more costly and demanding than the remediation of hydrocarbon-polluted groundwater[11]. Therefore, passive remediation options, which utilise naturally occurring degradation, dilution and retardation processes (natural attenuation), are preferred over expensive conventional active remedial options such as the pump and treat techniques[15-18]. The impetus for natural attenuation besides cost, lies in other merits of the technique such as its being efficient and nonintrusive to the environment, its wide applicability, and the absence of secondary wastes that would otherwise require an additional disposal stage[15-17,19]. Thus, natural attenuation has gained popularity in remediation of groundwater pollution from landfill leachate[12,20,21]. Newer remediation technologies are being developed, but are still in their infancy, with the majority being at laboratory scale[22] and few field investigations[23].

65 The main drawback of monitored natural attenuation is the time required to achieve remedial
66 goals for the polluted environment[16],[20]. The time frame is set by the regulatory body for
67 pollution control and varies from country to country, with 30 years being common for non-
68 hazardous wastes[15],[24]. Such long-term remediation perspectives have significant financial
69 implications. The highest expense being related to water quality monitoring, which may
70 represent over 90% of the total cost[24]. Despite the cost, long-term monitoring helps to ensure
71 that the attenuation capacity of the aquifer is not exceeded by the contaminant loads[14].
72 Providing unequivocal evidence of recovery of a contaminated aquifer requires that the
73 contaminant loads be monitored in time and space for a period determined by the attainment of
74 the minimum concentration of contaminants. This is evidenced by tailing-off of the
75 concentration of the monitored contaminants, which should approximate that quantified before
76 the contamination occurred and/or that from a reference well located upstream of the
77 contamination source. By this time, the landfill leachate may be considered to pose a minimal
78 health risk[21]. Despite the tailing-off, a potential risk may ensue if the contaminants tail-off at
79 levels above that of the reference value, in such scenarios tailing-off cannot be equated to
80 recovery. Also, the presence of chemical containers buried in landfills may undermine the
81 perceived low risk[11], due to a delayed leaching of contaminants as a result of corrosion of
82 containers.

83 The high resource requirement associated with the implementation of long-term monitoring
84 schemes remains a major limiting factor. This has hampered many monitored natural
85 attenuation projects and led to few literature on successful natural remediation operations. In
86 the present study, we report a study from a long-term monitoring of an aquifer in southeast
87 Norway which was contaminated by a municipal landfill. The monitoring programme was
88 followed for 28 years, starting from 1992 during active leaching of contaminants to 2019 when
89 the landfill was considered stabilised. The study aimed to 1) evaluate the long-term patterns in

the groundwater quality, and 2) examine the changes in the groundwater chemistry as a function of the landfill stage and distance from the pollution source. Studies that have spanned so large a part of the lifetime of a landfill, and that have assessed the effect of different landfill stages on the receiving groundwater are scarce.

Materials and methods

Study area

The area is a glaciofluvial deposit at an elevation of 150 m above sea level at coordinates 59°25′58.26″N and 9°06′1.53″E. The mean air temperature of the study area varies from 15 °C in summer to -2 °C in winter, with moderate temperatures of 6 °C and 7 °C occurring in spring and autumn, respectively (https://seklima.met.no). The annual precipitation received in the area is 500-800 mm. Due to the nature of the area such as occurrence of kettle holes and the distance from the urban centre was viewed as ideal for a landfill site to the municipality, and between 1974 and 1987, four landfill cells were opened and filled through 1974-1996 and has covered a total area of 30137 square meters[25]. As an old landfill operated before waste segregation came into force, it received all kinds of waste typically generated from households but wastewater treatment sludge, construction and demolition waste, and industrial waste were also deposited. The solid wastes came from Bø and Sauherad Municipalities (know merged to form Midtelemark Municipality). Neither liners for containment of leachate nor leachate collection system were in place. The landfill was closed in February 1997 and the cover consisted of 40-50 cm compacted clay[26]. The leachate from the landfill contaminated the underlying aquifer, and as required by regulation, the groundwater quality was monitored from the time of detection of pollution to the landfill post-closure. The aquifer is a subglacial river deposit with a matrix consisting of sand and gravel. It is confined by till (both as aquifer top and aquifer bottom) which is overlaid by a hard-packed moraine complex[27]. Both the bottom of the aquifer

(bedrock) over which the till was deposited, and the aquifer walls are composed of Precambrian crystalline rock[26,28]. The mean transmittivity and the hydraulic conductivity from a pumping test at R4 were 3.9×10^{-3} m^2/s and 7×10^{-4} m/s, respectively, and a calculated groundwater flow velocity of 0.88 m/d[25]. The estimated mean aquifer recharge is 92 m^3/d[28]. The aquifer was a source of drinking water to several surrounding farms but due to the contamination, the use of the groundwater from the aquifer was discontinued.

A monitoring scheme was developed to monitor the contamination and included three multilevel wells R1 (five levels at 126, 125, 124, 123 and 122 m.a.s.l), R2 (four levels at 122, 121, 119 and 118 m.a.s.l) and R4 (three levels at 118, 117 and 114 m.a.s.l) located in the contaminated aquifer downstream of the landfill (Fig. 1). The wells are respectively located at 26, 88 and 324 m from the edge of the landfill. In addition, well R0, which is located in the same geological formation but in a different (phreatic) aquifer, was used as a background well. The groundwater level in this well is ~3 m below the ground surface. Additional information on the site description including the hydrogeology is accessible elsewhere[25-28].

Experimental procedure

Groundwater sampling and chemical analysis

Groundwater samples were taken from the monitoring wells for the period 1992-2019. In the period 1992-2002, groundwater samples were collected quarterly, but during the period 2003-2019, samples were collected biannually. In addition, samples were collected from all the levels whenever possible in 1992-2006, but due to the associated cost of monitoring, the monitoring programme was revised to cover only two levels in of each the wells except in 2018-2019 (again full-scale sampling was performed). The groundwater samples from R0 included in this study were collected in 2018 and 2019. Sampling was conducted in accordance with the ISO 5667-11 guideline, as described previously[25].

Laboratory analyses were conducted following Norwegian Standards (NS) and/or ISO guidelines. The analyses were conducted for pH (NS 4720), conductivity (NS-ISO 7888), dissolve oxygen (NS 5813), nitrate (NS 4745), sulphate (ISO 10304), ammonium (NS 4746), chloride (ISO 10304), bicarbonate (NS-EN ISO 9963-2), total organic carbon (TOC) (NS 1484), calcium (NS-EN ISO 7980), potassium (NS-EN ISO 14911), magnesium (NS-EN ISO 7980) and manganese (NS 4773). Iron, sodium, zinc and copper were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES), while lead, chromium and cadmium were detected using inductively coupled plasma mass spectrometry (ICP-MS). Mercury was determined using cold vapour atomic fluorescence spectrometry (CV-AFS).

Data analysis

Statistical analyses were conducted in R version 4.0.2[29]. The groundwater hydrochemical compositions among the sampling wells and the different landfill status were analysed using package hydrogeo[30] and visualised using the R code available in github (https://gist.github.com/johnDorian/5561272). The groups were tested for significant difference using the nonparametric Kruskal-Wallis test. The datasets used in this analysis were for the periods 1992-2003 and 2018-2019, because the major ions (calcium, magnesium, potassium, and bicarbonate), which form part of the input parameters to the hydrochemical model, were measured only during these periods. Two-dimensional contaminant profiles were constructed using package akima[31] and were performed using a linear interpolation method. In this analysis, only chloride, sulphate, nitrate and bicarbonate for 2001 and 2019 were considered. These were selected as they tend to be more mobile than their cationic counterparts[32], while the years 2001 and 2019 were chosen because they had samples from all the levels in R1-R4. The groundwater parameters that were measured for the entire duration of monitoring were analysed for trends using Mann-Kendall trend test from package Kendall[33], performed on the annual mean values.

Results

Changes in groundwater chemistry with time and distance

The concentrations of all the major chemical variable decreased over time. Sodium and chloride tailed-off as early as by 2010 (Fig. 2a-b). On the other hand, TOC (Fig. 2c), iron and ammonium (Fig. 3a-b) have tailed-off much later; 2013 (iron), 2015 (TOC) and 2017 (ammonium). The oxidised chemical species and in particular, sulphate, showed a decrease immediately after the landfill closure in 1997, but only in R1 and R2 (Fig. 3d). From 2008, however, sulphate concentration increased gradually across the wells until it reached a plateau in 2012, when its concentrations varied somewhat narrowly. Nitrate showed some complexity in its long-term leaching pattern, although recent records indicate an increase in its levels particularly in R1 (Fig. 3c). The concentrations of most of the solutes decreased in moving along the flow path i.e., R1-R2-R4 (Supplementary Fig. S1 online). Sulphate and nitrate concentrations, however, increased along the groundwater flow direction. All the parameters showed a statistically significant difference across the wells (Supplementary Fig. S1 online).

Trend analysis as in Table 1 shows that both sulphate and nitrate are increasing in R1 and R2, while only sulphate shows an increasing trend in R4. However, the increase was significant only for nitrate and in R1. The rest of the parameters indicate decreasing trends in all the three wells. The decrease was strongest in R1, moderate in R2, and weak to moderate in R4.

Based on the levels of the major ions (calcium, magnesium, potassium, sodium, chloride, sulphate and bicarbonate), the groundwater chemical composition was most similar between R1 and R2 (Fig. 4). Water from these wells were characterised by higher sodium+potassium and calcium, whereas R4 was richer in calcium. While the groundwater across the monitoring wells was enriched in bicarbonate, chloride was on few occasions quantified in higher levels in R2. R1 and R2 were predominantly characterised by three water types: Ca-$(HCO_3)_2$ type, Ca-

187 Na-HCO_3 type and Ca-Na-Cl type, in decreasing order. Occasionally, however, Na-HCO_3, Na-
188 Cl and Ca-Cl_2 type waters were also measured. In R4, by contrast, Ca-$(HCO_3)_2$ type water
189 predominated, while a few samples exhibited Ca-Na-Cl type water.

190 Changes in groundwater chemistry relative to the landfill status

191 Changes in groundwater chemistry as a function of the different landfill status (Fig. 5) showed
192 that the water chemistry was most similar between active and closed landfill phases. The
193 samples were mostly characterised by high sodium+potassium and bicarbonate, but
194 occasionally higher levels of chloride were observed. These samples composed three
195 predominant water types: Ca-$(HCO_3)_2$ type, Ca-Na-HCO_3 type and Ca-Na-Cl type. However,
196 Na-HCO_3, Na-Cl and Ca-Cl_2 type waters were occasionally encountered. By contrast,
197 groundwater samples taken in 2018-2019 clustered separately from the pre- and post-closure
198 samples. These samples were characterised by higher levels of calcium and bicarbonate and
199 were all Ca-$(HCO_3)_2$ type water.

200 Depth-resolved profiles in Figure 6 show changes in levels of chloride, sulphate, nitrate and
201 bicarbonate in the aquifer in November 2001 and October 2019. In November 2001, a plume
202 of chloride was moving out of the monitoring area, as evidenced by the higher measurements
203 recorded in R4. By October 2019, however, chloride demonstrated a weaker gradient along the
204 groundwater flow line and has decreased remarkably, with the highest measurement being ~5X
205 lower than that measured in 2001. Both sulphate and nitrate registered higher concentrations in
206 R4 in 2001, but in 2019, higher levels of these anions were detected in R1. The maximum
207 concentration of both anions was similar between the two sampling campaigns, except that the
208 patterns of distribution were reversed during the recent sampling campaign. Bicarbonate
209 exhibited a similar pattern of distribution in 2001 and 2019. However, the actual concentrations

between the two timepoints differ greatly, with the highest measurement recorded in the most recent sampling being lower by a factor of 4.6 (240/52).

The groundwater samples collected in 2018 and 2019, which represent samples under the stabilised landfill phase, were compared to the Norwegian drinking water standards and the background water quality from a nearby uncontaminated aquifer (R0). All the parameters except iron in R1, manganese in R1-R4, were below the limit for Norwegian drinking water standards (Table 2). Compared to the local groundwater quality, only pH, conductivity, dissolved oxygen, manganese and TOC in the contaminated aquifer occurred in levels greater or equal to that in R0.

Discussion

Levels of the measured parameters varied differently throughout the monitoring period. Sodium and chloride declined and tailed-off relatively earlier than the other ions. They also showed the strongest downward trends of -0.86 and -0.82, respectively (Table 1). Given that sodium is only slightly retarded while chloride is both nonreactive and almost not retarded at all[7],[32], the early tailing-off of these two ions is suggestive of the depletion of leachable salts of sodium and chloride from the waste mass with age. The age of the landfill was found to be the most influential factor in explaining the levels of chloride measured in the groundwater[25].

TOC and the reactive inorganic species such as iron and ammonium showed a delayed tailing-off. While iron might be leached directly from the waste mass, it could also originate from the dissolution of minerals in the underlying strata under the influence of leachate. The source of the pollutant would be identified easily if there was data on the raw leachate, which could provide information on the source loading. The TOC and ammonium on the other hand, are believed to originate from the landfill as degradative products of the resident microorganisms. Regardless of the source and type, these solutes are more reactive than the monovalent ions[32]

234 and depending on the prevailing redox conditions, their mobility can be strongly retarded[26,34].
235 Therefore, this may cause delayed release of these solutes into the groundwater. It is also
236 possible that recalcitrant nitrogenous and organic compounds might sustain the prolonged
237 leaching of these pollutants, although such sources may only be minor, given the overall low
238 concentrations of the contaminants. Sulphate and nitrate are the other reactive chemical species
239 and unlike the other solutes, they were found to have upward trends in the proximal-distal wells
240 (sulphate) and proximal-intermediate wells (nitrate). This may be attributed to oxidation of
241 metal sulphide and reduced nitrogen species in the landfill, due to a transition from reducing to
242 oxidising condition[26] as a result of the landfill stabilisation[8],[35]. The microbiome of the aquifer
243 has been found to harbour a wide range of biogeochemical cyclers, including iron oxidisers,
244 sulphide oxidisers and ammonia oxidisers etcetera[36]. These microbes may be viewed as being
245 involved in various oxidation and reduction processes. It is believed that the decrease in
246 concentration of inorganic ions is primarily due to leaching of salts from the waste body, while
247 the decline in levels of organic matter is due to attenuation mechanisms such as biodegradation,
248 volatilisation and sorption[37],[38].

249 R1 (the proximal well), which is 26 m from the edge of the landfill, was the most polluted. In
250 moving from the proximal well through R2 (the intermediate well) to R4 (the distal well), the
251 concentrations of most of the ions decreased. An exception to this pattern was observed with
252 nitrate and sulphate. The decrease in levels of the solutes with distance along the groundwater
253 flow direction may be ascribed to dilution, sorption, complexation, precipitation and
254 biodegradation[25,26]. The pattern observed with nitrate and sulphate could be attributed to
255 oxidation of reduced compounds of the two elements along the groundwater flow path as
256 dissolved oxygen increased along the groundwater flow path (Table 2 and Supplementary Fig.
257 S6 online).

The piper diagram (Fig. 4) indicates that the water chemistry composition in both the proximal and intermediate wells were variable, consisting of six different water types. This suggests that the groundwater was under constant influence from the landfill leachate, causing the hydrochemistry to be transient. The episodic occurrence of chloride-rich water type observed in these wells might be attributed to the uneven leaching pattern which characterises landfill leachates[34]. Samples from the proximal and intermediate wells co-clustered, suggesting that they have similar water chemistry. However, the parameters indicated significant differences across the wells (Supplementary Fig. 1S online). The water type in the distal well was predominantly Ca-$(HCO_3)_2$. A previous study using piezometric analysis has reported a similar finding[21]. Natural attenuation in the aquifer is so substantial that the contaminant loads at the distal well have always been low. As evidenced from the trend analysis, the strength of downward trends decreased from the proximal to the distal wells, implying that the decay of contaminants has been minimal at the distal well. It is therefore not unlikely that the hydrochemistry of the distal well is dominated by a single hydrochemical facies. Because the composition of leachates may be complex[3],[11-14], the inferred water type may not reflect the true water-rock interaction and unambiguous interpretation is therefore, difficult.

Samples collected when the landfill was active (1992-1996) and after closure (1997-2003) clustered together, suggesting they have similar water chemistry, although the individual parameters showed significant difference across the three stages of the landfill (Supplementary Fig. S4 online). Up to six different water types, both single and mixed types characterised the active/closure stages (Fig. 5). Based on chloride concentration, it was previously observed that there was a change in the leaching pattern from random prior to landfill closure to less random after the landfill closure, and that closing the landfill significantly affected the groundwater quality[25]. However, no clear pattern between active and closed could be discerned from the major ions collectively (Fig. 5), suggesting that closing the landfill did not alter the relative

283 compositions but rather altered the contaminant loads. The stabilised stage was, however,
284 clearly distinct and composed only a single water type, Ca-$(HCO_3)_2$, illustrating that the
285 leachable salts in the waste have been markedly depleted. Clearly, the hydrochemical facies
286 recorded on landfill status mirrored that of distance (wells), which indicates recovery of the
287 groundwater quality either as a function of landfill status or distance along flow path.

288 The most recent measurements indicate that nitrate and sulphate have increased in concentration
289 across the wells. This reflects a transition to oxidising condition since 2009 and the fact that
290 sulphate has reached a plateau is an indication of a stable oxidising environment in the landfill.
291 This suggests that the landfill has attained its stabilised phase, during which more oxygen enters
292 into the landfill than is depleted by microorganisms[8]. The oxygen would support additional
293 oxidation of metal compounds within the landfill[35], leading to the mobility of oxidised chemical
294 species such as nitrate and sulphate. Both nitrate and sulphate have become mobile and less
295 retarded in the aquifer, as evidenced by a weak gradient between the proximal and distal wells
296 (Fig. 6). Because the landfill now leaches little reduced chemical species, there are insufficient
297 electron donors to cause reduction of nitrate and sulphate to the extent comparable to the early
298 years of monitoring. For example, iron and ammonium have since 2013 and 2017, respectively,
299 become so low that the difference across the wells is virtually indiscernible (Fig. 3). Similarly,
300 TOC has decreased remarkably at about the same period (2015) and fluctuated around 3 mg/l.
301 Although detectable, the TOC may be inadequate to cause substantial reduction of nitrate and
302 sulphate, or the TOC could be predominantly of recalcitrant compounds, as is expected of aged
303 landfills[39].

304 Heavy metals were unfortunately, not monitored consistently as the major chemical variables.
305 Except for a few instances, the concentrations of most of the heavy metals were low
306 (Supplementary Fig. S5 online). Previous studies have reported low concentrations of heavy
307 metals in landfill leachates[1,25,37,40] and it is now widely viewed that heavy metals do not

308 constitute a serious pollution problem in municipal landfills[7,8,37,40,41]. On a long-term basis,
309 heavy metals are thought to be precipitated in landfills under reducing condition, but at old age,
310 oxygen is expected to intrude the landfill and cause an ecosystem shift from reducing to
311 oxidising condition[8,35]. The transition leads to oxidation of previously precipitated heavy metals
312 and results in a delayed release of trace elements into the environment[35]. So far, no excessive
313 heavy metals were measured during the stabilised phase, but it remains to be seen if the
314 anticipated delayed release will eventuate.

315 While there was lack of a clear trend in moving from the proximal through intermediate to the
316 distal well, the maximum measured concentrations of chromium and copper were recorded
317 from the distal well. A probable explanation could be the low pH in the distal well. The pH of
318 the proximal and intermediate wells fluctuated within a narrow range at near-neutral
319 (Supplementary Fig. S2 online), while measurements from the distal well remained relatively
320 stable at ~5 over the monitoring period. pH is known to be one of the key influencers of metal
321 speciation, with circum-neutral to high pH promoting metal precipitation and sorption, and
322 lower pH triggering mobilisation[42].

323 The goal of any remediation effort is full recovery of a contaminated environment. However,
324 no data for the investigated aquifer before the contamination occurred exists and the tailing-off
325 could, therefore, not be unequivocally equated to full recovery. Instead, groundwater quality
326 values were compared to the most stringent national quality standards, the Norwegian drinking
327 water standards, and secondarily to a nearby well which should represent the local groundwater
328 quality. Only manganese in the proximal-distal wells and iron in the intermediate well exceeded
329 the Norwegian drinking water norms. Despite breaching the norms, these values were only
330 marginally above the limits (under 1 mg/l) but have recovered from 99 and 16 mg/l for iron and
331 manganese, respectively[26]. Clearly the groundwater is under the course of recovery, although it
332 is arguable if the manganese will decrease lower than the current values attained in the

respective wells, since a comparable concentration was recorded from the background well. Although within the drinking water limits, the highest pH value at present is 2 units above the local background value (Table 2), suggesting discharge of an alkaline leachate from the landfill. Likewise, the electrical conductivity, which is an indicator of the quantity of soluble salts in the groundwater, was within the guideline and decreased from >1000 to <250 µS/cm over the years (Supplementary Fig. S3 online), but apparently, the most recent measurement from the proximal well is about sixfold higher than that in the background well. This implies that there is still some active leaching from the landfill taking place. Since there will always be input coming from the buried waste, it is likely that neither the pH nor the conductivity will attain the natural background level. Landfills present continuous source loading[32] that may take decades to centuries before substantial decay in concentration can be achieved[43].

Conclusion

Long-term groundwater chemical data for a period of 28 years was used to analyse for changes in water quality as a function of time, distance, and the stage of landfill stabilisation. Distance in this case was used to ascertain the significance of natural attenuations, which was found to be substantial. The results also showed that contaminant loads declined with time and reached a minimum stationary value, indicating depletion of leachable salts and suggests the attainment of a stabilised phase. The depletion of leachable salts is supported by the stabilised stage showing only a single hydrochemical facies as opposed to prior to stabilisation where six hydrochemical facies were found, suggesting discharge of a mixture of pollutants prior to the landfill stabilisation. Different pollutants attained the stabilised phase over different durations e.g., sodium and chloride after 19 years, iron 22 years, TOC 24 years and ammonium 26 years. The attainment of a stabilised phase with a concomitant substantial reduction in pollutant

loading leading to tailing-off of contaminants informs on the aquifer recovery. This highlights the significance of a non-invasive nature-based solution to the aquifer.

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Author contributions

Conceptualisation, Methodology, Writing review & editing: **DA, AJ, LSV, HK**; Project administration; Resources; Supervision; Funding acquisition: **AJ, LSV, HK**; Data curation, Software, Formal analysis, Visualisation, Writing original draft: **DA**; Investigation: **HK**

Competing interests

The authors declare no competing interests.

Figure captions

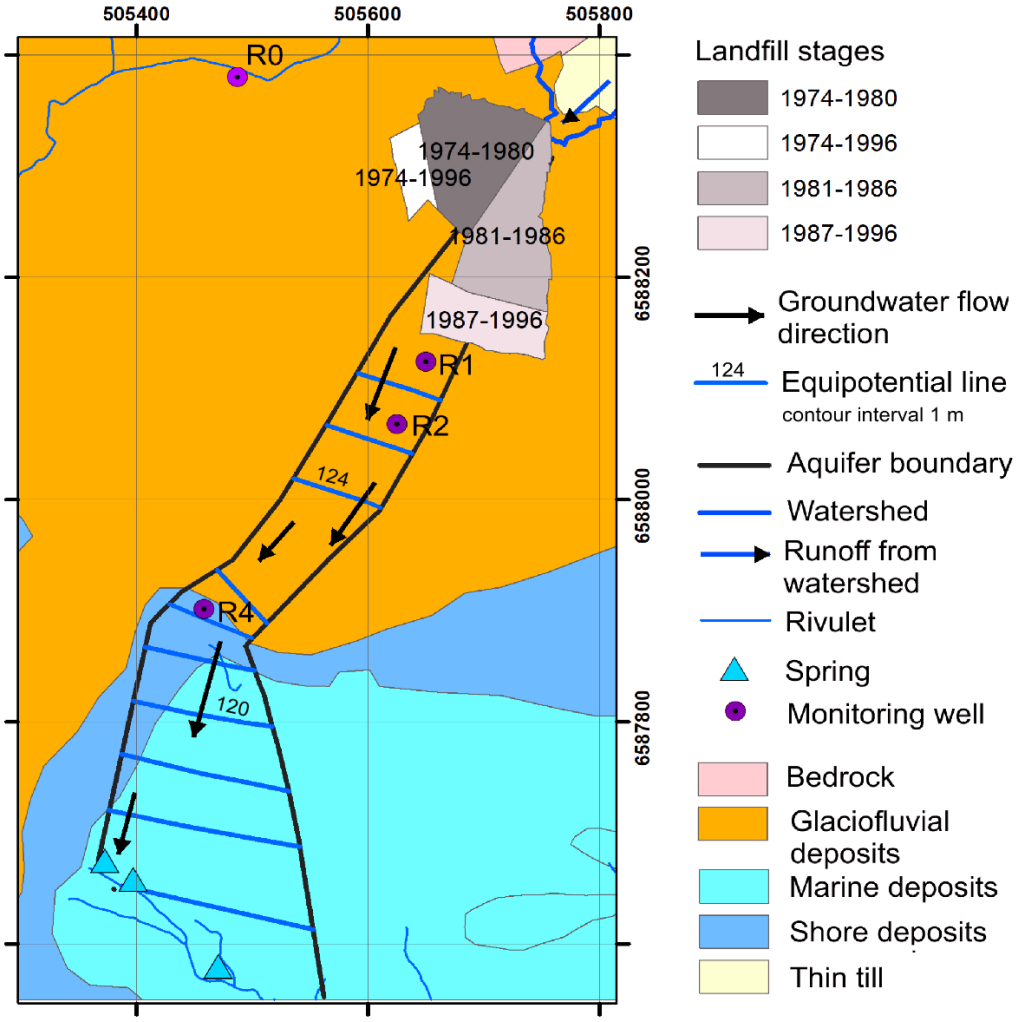
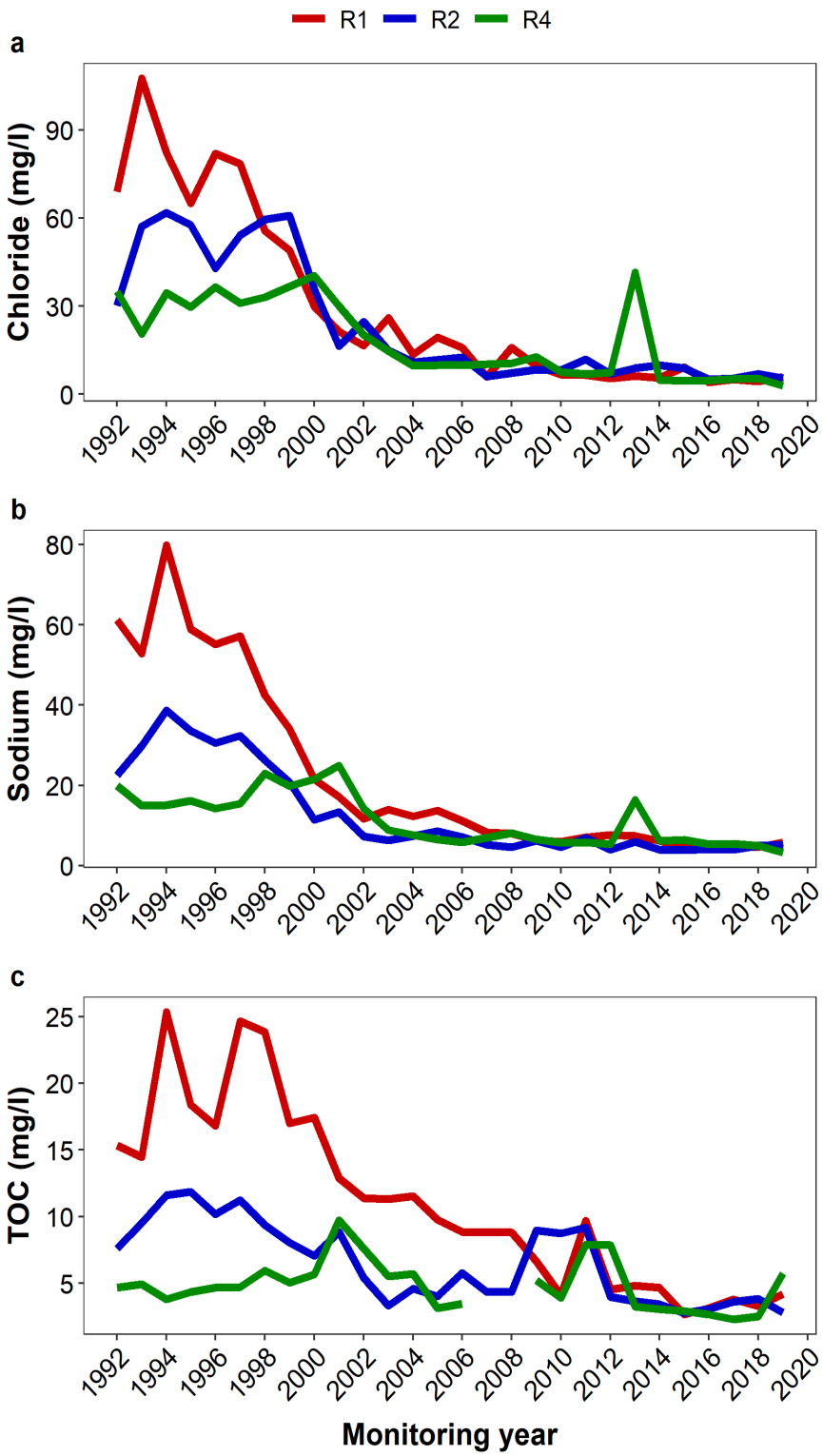


Figure 1. The study area showing the landfill, the site hydrogeology, and the location of the monitoring wells R0, R1, R2 and R4.



493

494 **Figure 2.** Long-term changes in annual mean values of chloride (**a**), sodium (**b**) and TOC (**c**) across the sampling
495 wells R1, R2 and R4 from 1992 to 2019. The wells have been placed along the groundwater flow path in an
496 increasing distance from the edge of the landfill.

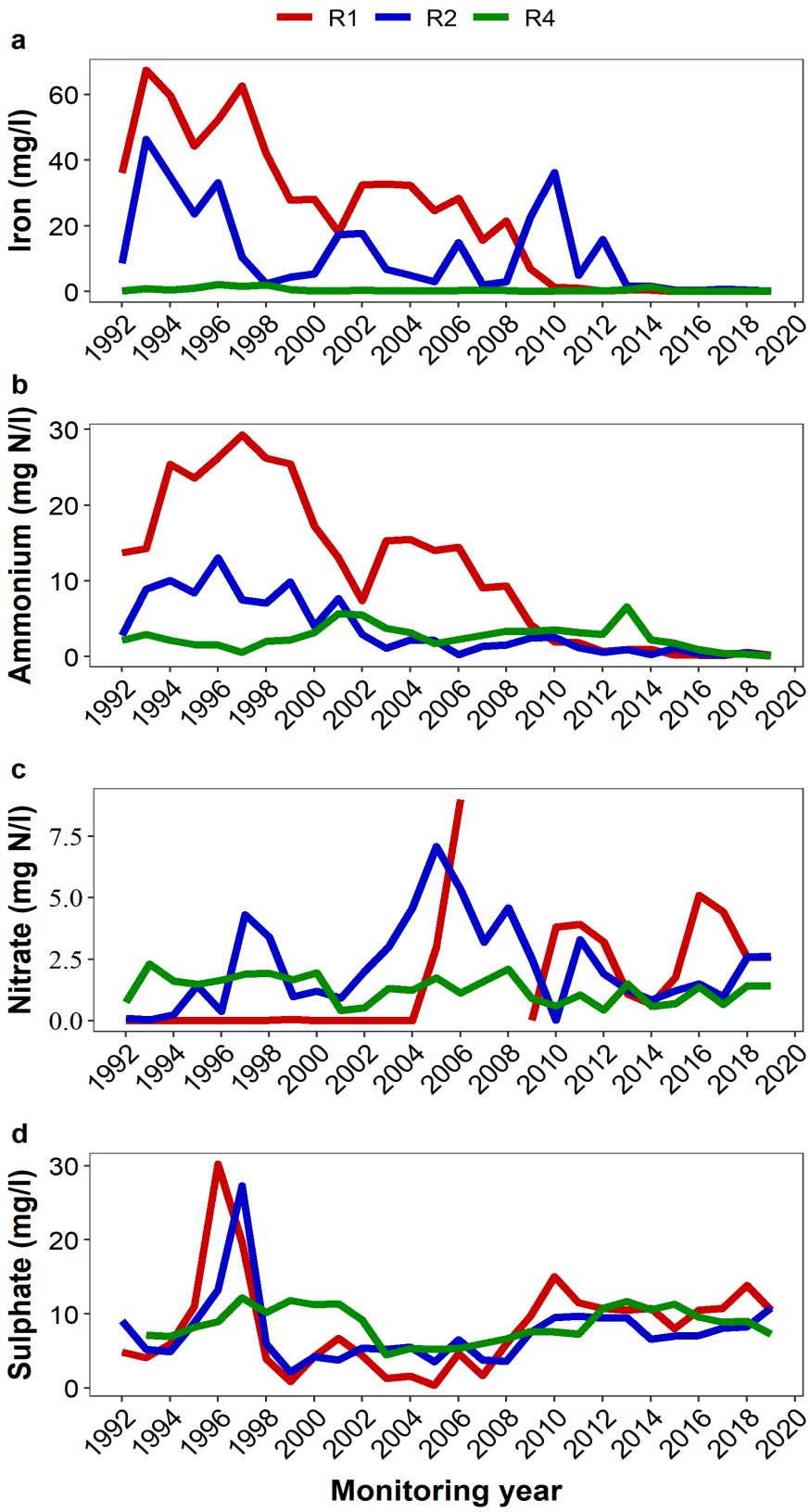
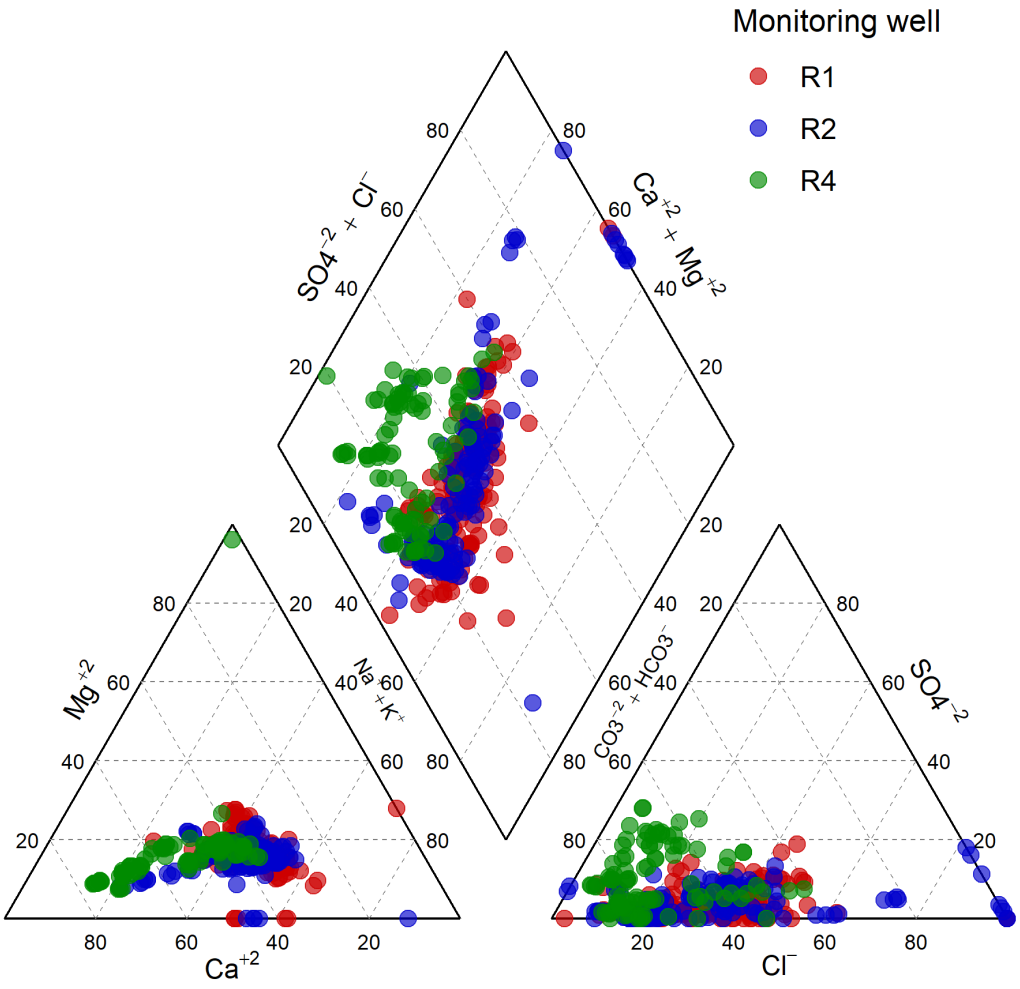
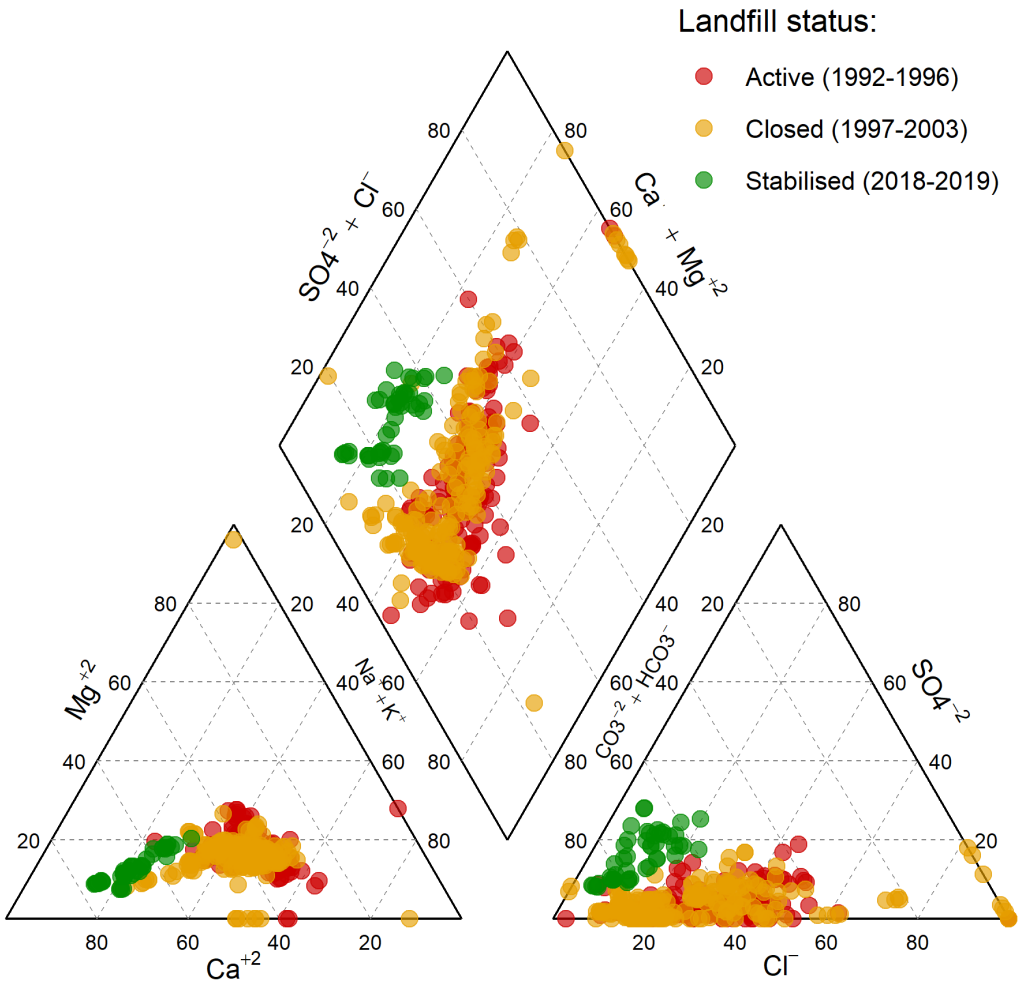


Figure 3. Long-term changes in annual mean values of iron (**a**), ammonium (**b**), nitrate (**c**) and sulphate (**d**) across the sampling wells R1, R2 and R4 from 1992 to 2019. The wells have been placed along the groundwater flow path in an increasing distance from the edge of the landfill.



501

502 **Figure 4.** Characteristics of groundwater chemistry based on levels (% meq/l) of calcium, magnesium, potassium,
503 sodium, chloride, sulphate and bicarbonate from the monitoring wells R1, R2 and R4. The wells were placed along
504 the groundwater flow direction in an increasing distance from the edge of the landfill.



505

506 **Figure 5.** Characteristics of groundwater chemistry based on levels (% meq/l) of calcium, magnesium, potassium,
507 sodium, chloride, sulphate and bicarbonate categorised by the landfill status (active, closed and stabilised). The
508 years in parenthesis denote the periods for which groundwater chemistry data for classifying water types were
509 available; otherwise, "closed" should span the period 1997-2016, while "stabilised" should cover 2017-2019.

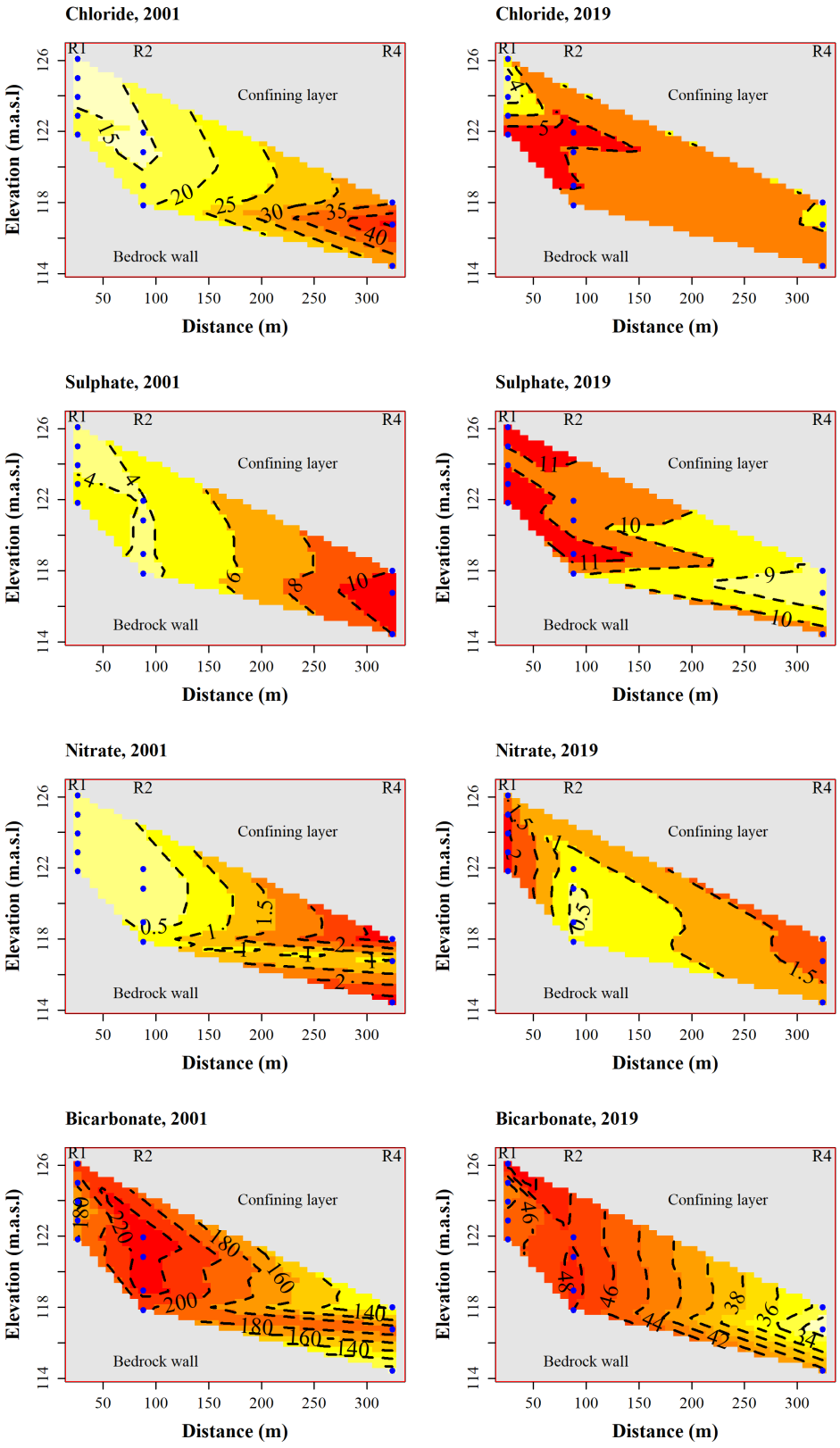


Figure 6. Depth-profiles of a few selected ions at two timepoints: November 2001 and October 2019, for chloride, sulphate, nitrate and bicarbonate. The blue dots depict the multilevel samplers in R1, R2 and R4. The wells have been placed along the groundwater flow path in an increasing distance from the edge of the landfill.

Tables

Table 1. Mann-Kendall trend test results for parameters measured from 1992 to 2019. Significant results are indicated in bold face. R1, R2 and R4 are the monitoring wells located along the groundwater flow path in an increasing distance from the edge of the landfill. τ is the Kendall's test statistic.

	R1		**R2**		**R4**	
	τ	*p*-value	τ	*p*-value	τ	*p*-value
Sulphate	0.24	0.08	0.21	0.12	0.04	0.79
Chloride	-0.82	**<0.001**	-0.69	**<0.001**	-0.62	**<0.001**
Sodium	-0.86	**<0.001**	-0.72	**<0.001**	-0.62	**<0.001**
Iron	-0.81	**<0.001**	-0.54	**<0.001**	-0.33	**0.02**
Nitrate	0.51	**<0.001**	0.15	0.28	-0.24	0.09
Ammonium	-0.72	**<0.001**	-0.69	**<0.001**	-0.12	0.39
TOC	-0.77	**<0.001**	-0.60	**<0.001**	-0.21	0.15

Table 2. Comparison between the current groundwater quality under the stabilised landfill phase versus the Norwegian drinking water standards and the local background groundwater quality. Values are the means for samples collected in 2018 and 2019. All units are in mg/l, otherwise indicated. ND: not detected (below limit of detection); NQ: not quantified. All values for mercury were below limit of detection but reported as zero. Values for nitrate and ammonium in the Norwegian drinking water standards are in mg/l.

	R1	**R2**	**R4**	**R0**	**Norwegian drinking water standards**
pH	6.9	6.8	6.2	4.9	6.5-9.5
Conductivity (µS/cm)	223	136	160	35	2500
Dissolved oxygen	1.2	0.96	2.0	4.33	
Sulphate	12.2	9.5	8.1	3.0	250
Chloride	5.1	6.1	4.1	2.1	250
Nitrate (as N)	2.6	2.6	1.4	1.6	50
Bicarbonate	67	61	62	2.9	
Ammonium (as N)	0.37	0.29	0.2	ND	0.5
Sodium	5.2	5.1	4.1	1.8	200
Potassium	6.7	6.6	6.6	0.44	
Calcium	25	24	23	2.2	
Magnesium	2.9	2.9	3.0	0.51	
Manganese	0.21	0.24	0.5	0.51	0.05
Iron	0.03	0.27	0.15	ND	0.2
Zinc (µg/l)	NQ	NQ	NQ	NQ	
Copper (µg/l)	NQ	NQ	NQ	NQ	2000
Cadmium (µg/l)	0.09	0.03	0.07	NQ	5
Chromium (µg/l)	0.08	0.17	0.14	NQ	50
Lead (µg/l)	0.12	0.07	0.83	NQ	10
Mercury (ng/l)	0	0	0	NQ	1000
TOC	3.7	3.3	4.1	4.1	

A nature-based solution to a landfill-leachate contamination of a confined aquifer

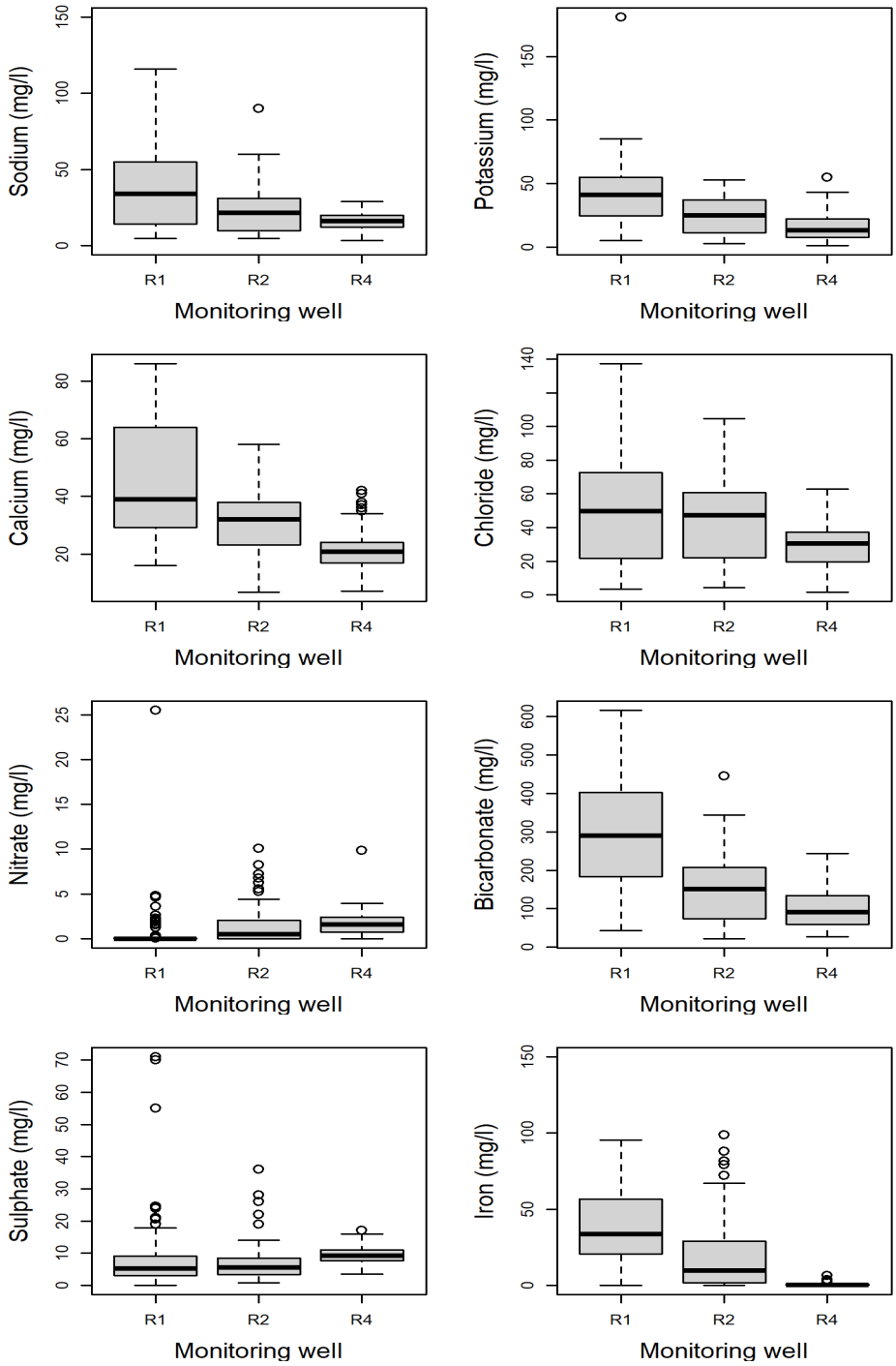


Figure S1. Changes in the groundwater quality across the sampling wells. The wells have been placed along the groundwater flow direction in an increasing distance from the edge of the landfill. All the parameters indicate significant differences. Sodium χ^2 = 54.3, df = 2, p = 1.652e-12; potassium χ^2 = 95.3, df = 2, p < 2.2e-16; calcium χ^2 = 137, df = 2, p < 2.2e-16; chloride χ^2 = 36.7, df = 2, p = 1.045e-

08; nitrate $\chi^2 = 110$, df = 2, p < 2.2e-16; bicarbonate $\chi^2 = 122$, df = 2, p < 2.2e-16; sulphate $\chi^2 = 52.8$, df = 2, p = 3.451e-12; iron $\chi^2 = 144$, df = 2, p < 2.2e-16.

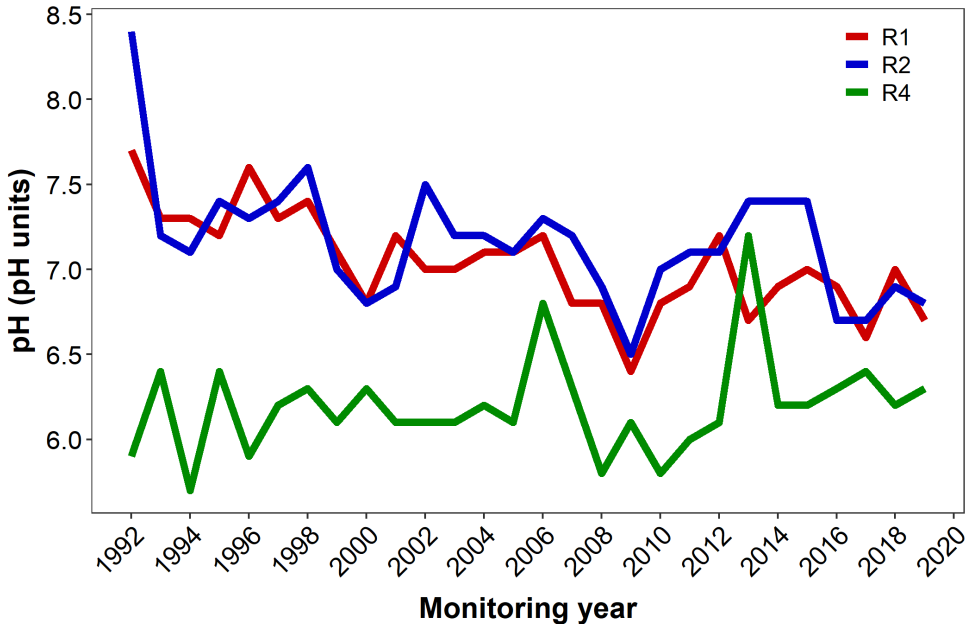


Figure S2. Long-term changes in annual mean values of pH across the sampling wells R1, R2 and R4 from 1992 to 2019. The wells have been placed along the groundwater flow direction in an increasing distance from the edge of the landfill.

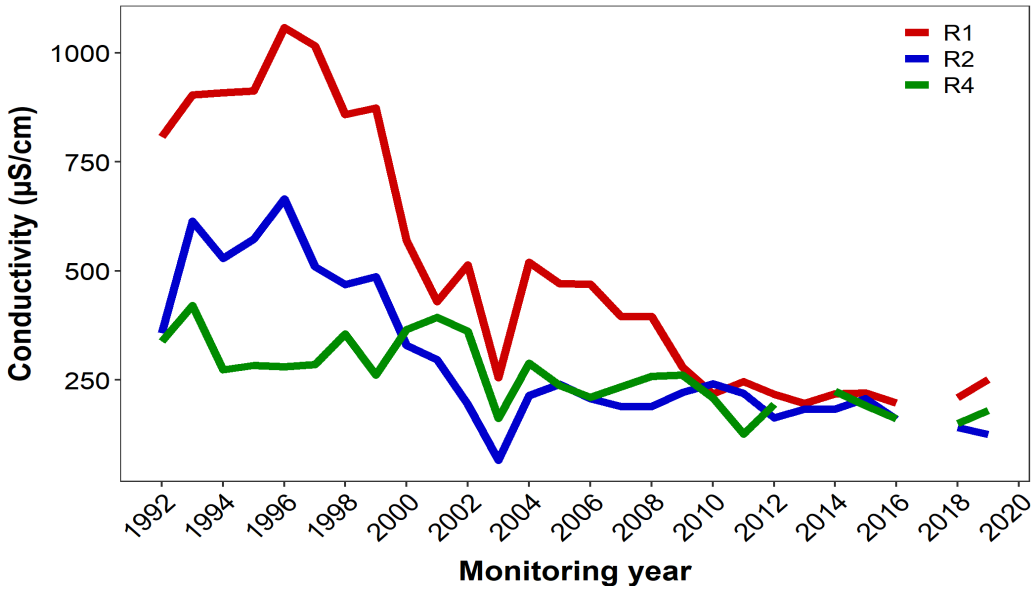


Figure S3. Long-term changes in annual mean values of conductivity across the sampling wells R1, R2 and R4 from 1992 to 2019. The wells have been placed along the groundwater flow direction in an increasing distance from the edge of the landfill.

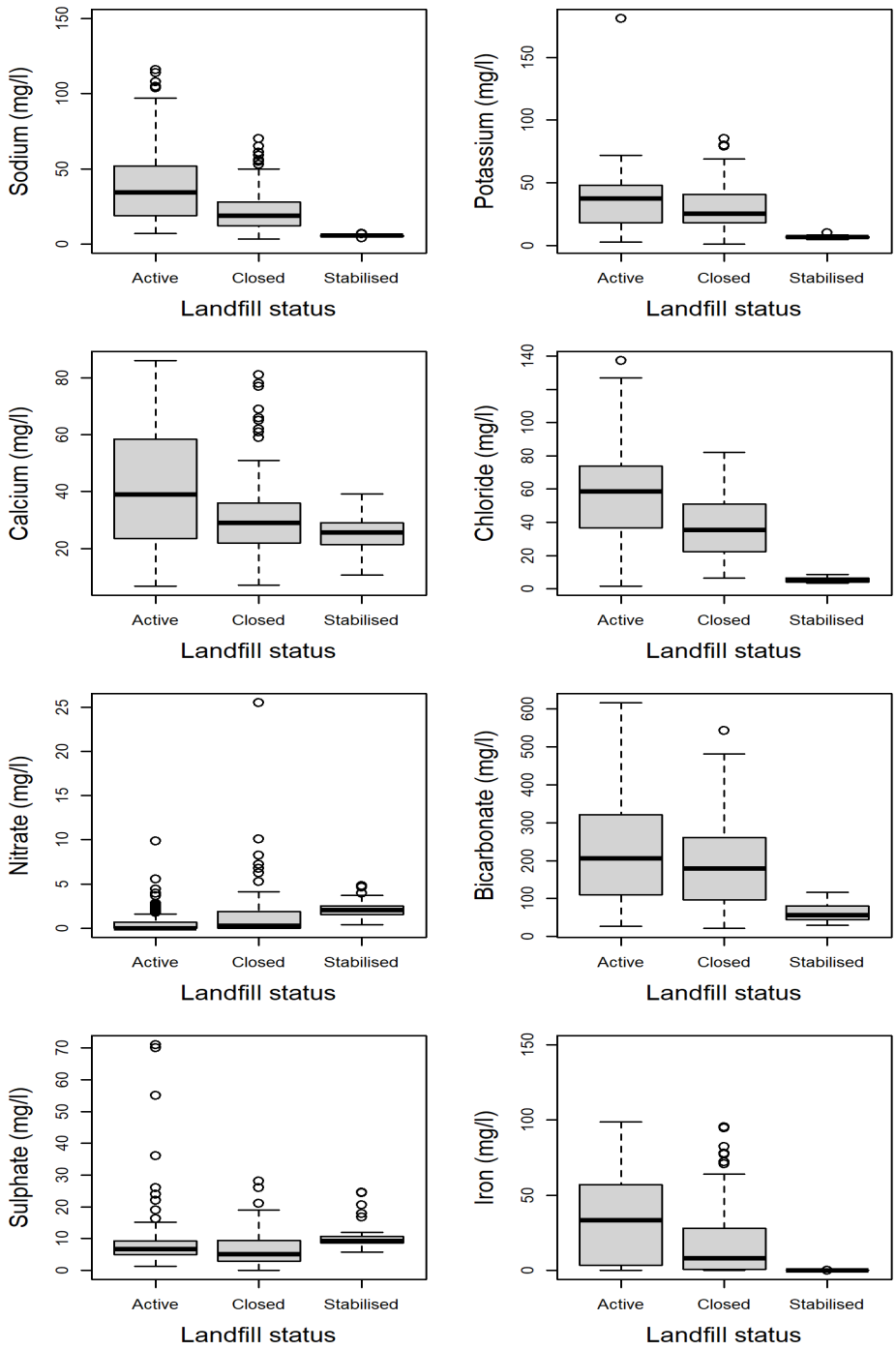


Figure S4. Changes in the groundwater quality as a function of the stages of the landfill. All the parameters indicate significant differences across the different stage. Sodium $\chi^2 = 167.86$, df = 2, p < 2.2e-16; potassium $\chi^2 = 95.9$, df = 2, p < 2.2e-16; calcium $\chi^2 = 41.3$, df = 2, p = 1.093e-09; chloride $\chi^2 = 164$, df = 2, p < 2.2e-16; nitrate $\chi^2 = 65.6$, df = 2, p = 5.541e-15; bicarbonate $\chi^2 = 80.5$, df = 2, p < 2.2e-16; sulphate $\chi^2 = 45.3$, df = 2, p = 1.446e-10; iron $\chi^2 = 146$, df = 2, p < 2.2e-16.

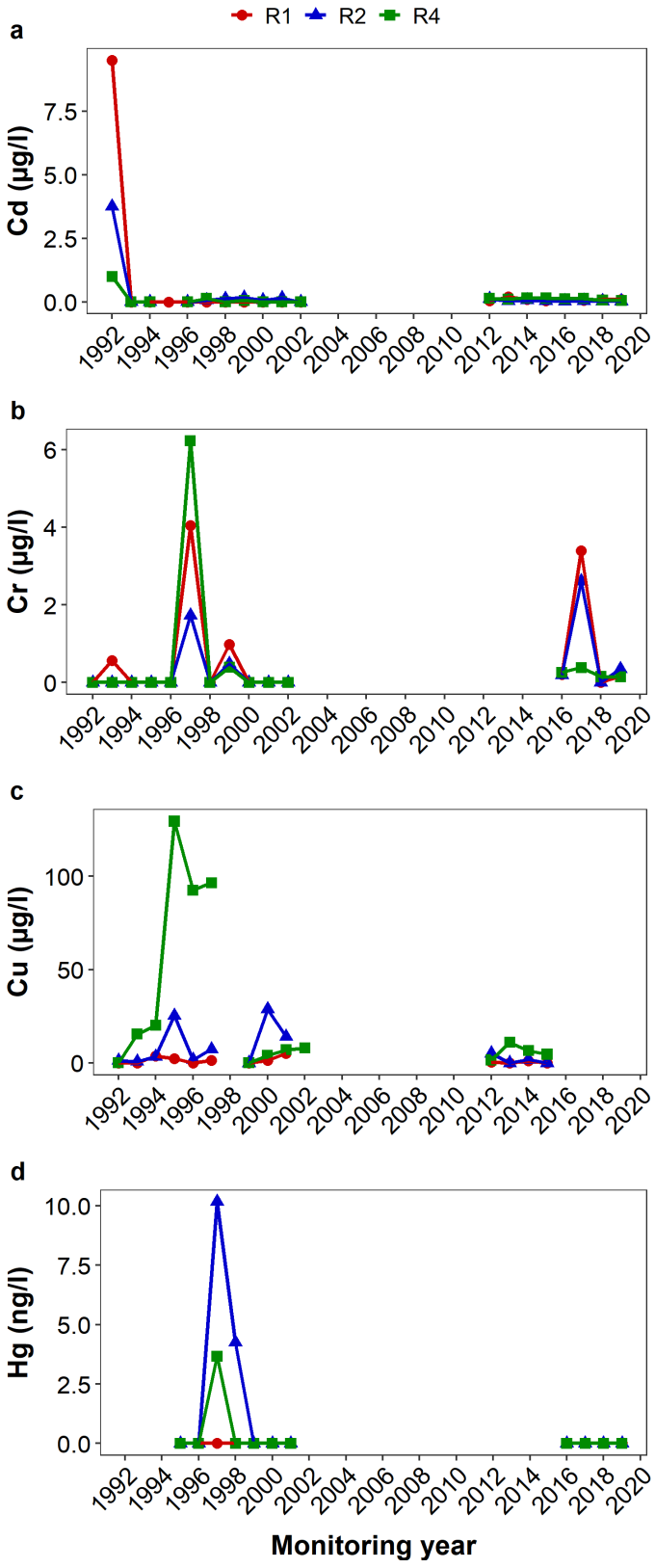


Figure S5. Long-term changes in annual mean values of cadmium (a), chromium (b), copper (c) and mercury (d) across the sampling wells R1, R2 and R4 from 1992 to 2019. The wells have been placed along the groundwater flow direction in an increasing distance from the edge of the landfill. all measurements below the limit of detection were treated as zero.

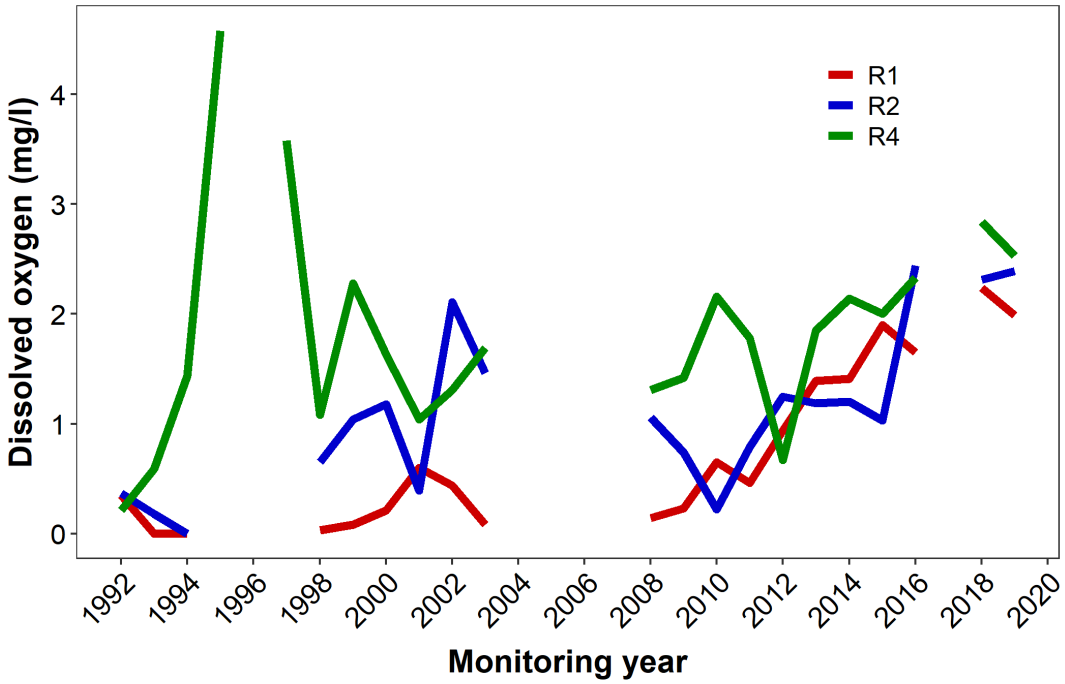


Figure S6. Long-term changes in annual mean values of dissolved oxygen across the sampling wells R1, R2 and R4 from 1992 to 2019. The wells have been placed along the groundwater flow direction in an increasing distance from the edge of the landfill.

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