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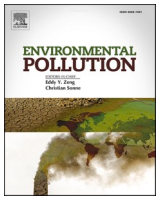

Selective pressure on microbial communities in a drinking water aquifer – Geochemical parameters vs. micropollutants☆

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ABSTRACT

Groundwater quality is crucial for drinking water production, but groundwater resources are increasingly threatened by contamination with pesticides. As pesticides often occur at micropollutant concentrations, they are unattractive carbon sources for microorganisms and typically remain recalcitrant. Exploring microbial communities in aquifers used for drinking water production is an essential first step towards understanding the fate of micropollutants in groundwater. In this study, we investigated the interaction between groundwater geochemistry, pesticide presence, and microbial communities in an aquifer used for drinking water production. Two groundwater monitoring wells in The Netherlands were sampled in 2014, 2015, and 2016. In both wells, water was sampled from five discrete depths ranging from 13 to 54 m and was analyzed for geochemical parameters, pesticide concentrations and microbial community composition using 16S rRNA gene sequencing and qPCR. Groundwater geochemistry was stable throughout the study period and pesticides were heterogeneously distributed at low concentrations (μg L^{-1} range). Microbial community composition was also stable throughout the sampling period. Integration of a unique dataset of chemical and microbial data showed that geochemical parameters and to a lesser extent pesticides exerted selective pressure on microbial communities. Microbial communities in both wells showed similar composition in the deeper aquifer, where pumping results in horizontal flow. This study provides insight into groundwater parameters that shape microbial community composition. This information can contribute to the future implementation of remediation technologies to guarantee safe drinking water production.

1. Introduction

Groundwater is a key water resource, with 45.7% of all drinking water abstracted from groundwater globally (United Nations World Water Assessment Programme, 2009). Therefore, maintaining good groundwater quality is crucial to maintain safe drinking water production and as such of great interest to the public and research communities (Zhang et al., 2017). Micropollutants (MP, i.e. pollutants at μg L^{-1} concentration), including pesticides, threaten groundwater quality and consequently drinking water quality. In 2016, more than four billion kilograms of pesticides were used for agricultural purposes worldwide (FAO, 2018). Pesticides may leach into groundwater, resulting in pesticide concentrations in groundwater that often exceed regional legislations for drinking water production (Helbling, 2015). Post extraction treatment by advanced water treatment technologies, such as adsorption to activated carbon or advanced oxidation, can effectively remove MPs, including pesticides, in some cases, but are neither cost-effective nor always suitable (Benner et al., 2013).

As an alternative, in situ biodegradation and natural attenuation of MPs, mediated by indigenous microorganisms, are regarded as a more sustainable approach to safeguarding drinking water resources (Fenner et al., 2013). However, harnessing the biodegradation capacity of aquifers is especially challenging in oligotrophic and anoxic groundwater. Within aquifers, microorganisms usually rely on electron acceptors other than oxygen, such as nitrate, iron (III), and sulfate. Groundwater is also typically depleted in nutrients and DOC, because

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easily assimilable carbon substrates are consumed by microbes in mineral soil and vadose zones (Francois et al., 2016).

The effects of pesticides at micropollutant concentrations on the groundwater microbial communities remain relatively unexplored. Pesticides at micropollutant concentrations make an unattractive carbon source, which makes the ability to metabolize them a negligible selective advantage for microorganisms (Helbling, 2015). However, pesticides have been linked to altered microbial community structures in groundwater even at low concentrations. Bacteria taken from subsurface aquifers that were exposed to herbicides at micropollutant concentrations (40 μg L^{-1}) showed to have more similar community structure compared to communities that were not exposed (Lipthay et al., 2004). However, bacterial community adaptation was only investigated in the context of pesticide exposure. To understand the selective pressures exerted by pesticide MPs on groundwater microbial communities, the effects of pesticides need to be explored in context with other environmental variables, such as electron acceptor availability, rather than separately. Recent studies have tried to describe microbial communities in groundwater ecosystems in situ, or in laboratory experiments that simulated environmental conditions (Erdogan et al., 2019; Gülay et al., 2016; He et al., 2018; Hedegaard et al., 2018; Hemme et al., 2016; Lee et al., 2018; Miao et al., 2019; Posman et al., 2017; Santos et al., 2018; Sonthiphand et al., 2019). Whereas some studies examined the correlations among microbial community, contaminant abundance, and geochemical parameters (Braun et al., 2016; He et al., 2018; Hemme et al., 2016; Posman et al., 2017; Sonthiphand et al., 2019; Yan et al., 2019), these datasets did not consider spatial and temporal trends in groundwater composition that exert a selective pressure on the microbial community. Considering the diversity of electron acceptors, DOC as electron donor, and MPs in groundwater, it is important to understand how these conditions affect spatial and temporal variation in microbial community composition.

This study aimed to elucidate the selective pressures exerted by MPs compared to groundwater geochemistry on aquifer microbial communities. We performed long-term monitoring at different discrete depths of a drinking water aquifer to understand spatial and temporal variability in groundwater and microbial community composition. The results presented here provide insight into microbial community composition and distribution in the aquifer, and the selective pressure of groundwater parameters including MPs on microbial community composition.

2. Materials and methods

2.1. Site description

The groundwater monitoring wells analyzed in this study are located in an agricultural area in the northeast of The Netherlands, in the vicinity of a drinking water production location. The monitoring wells are used to analyze groundwater quality prior to extraction for drinking water production. A map displaying the wells' locations is provided in Supplementary Information (Figure S1). The first well (designated as well 22) is further upstream from the extraction site and adjacent to a canal, while the second one (designated as well 23) is closer to the extraction site. The soil profile from both wells shows two sandy aquifers are present, divided by a heterogeneous clay layer between approximately 30-35 m below ground level (Tables S1 and S2). Specifically, well 22 contains coarse to fine sand from 0 to 32 m, with clay layers at 25 m and 32 m. The deeper aquifer from 34 to 66 m contains coarse sand. Well 23 contains coarse to fine sand from 0 to 41.5 m, followed by a 1.5 m thick clay layer. The deeper aquifer from 43 to 66 m is mainly very coarse sand. Drinking water is extracted from this deeper aquifer. The distance between well 22 and 23 is around 500 m, and both wells are filtered at five discrete depths from 13 to 55 m (Table 1).

Table 1
Discrete depths of the groundwater samples per well measured in meters.

Sample name	Depth (m)	Sample name	Depth (m)
22–1	12–14	**23–1**	12.5–14.5
22–2	20.5–22.5	**23–2**	26–28
22–3	27–29	**23–3**	36–38
22–4	39–41	**23–4**	46–48
22–5	45–47	**23–5**	53–55

2.2. Groundwater sampling

Groundwater was sampled from the above-mentioned monitoring wells at each discrete depth. Samples were collected in duplicates for the years 2014, 2015 and 2016 (2014: a,b; 2015: c,d; 2016: e,f). Before sample collection, each well was flushed by extracting and discarding three times the volume of the well. Turbidity was continuously measured, and the samples were taken after turbidity stabilized below 1 NTU (Nephelometric Turbidity Units). Samples were collected in 10L jerry cans pre-washed with ethanol and stored at 4 °C before further analysis.

2.3. Geochemical and micropollutant quantification

Geochemical parameters and MPs had been monitored starting in 2000 by an accredited Dutch Governmental Laboratory. Samples for these analyses were taken according to specific requirements of the different analytical techniques. DOC concentration was determined by combustion in accordance with NEN-EN 1484:1997 (Nederlands Normalisatie, 1997). Iron (II) concentration was analyzed using an inductively coupled plasma and mass spectrometry (ICP-MS) in accordance with NEN-EN-ISO 17294-2: 2004 (International Organization for Standarization and Normalisatie Nederlands, 2004). Ammonium (N) concentration was determined with a discrete analyzer in accordance with NEN-ISO 15923-1: 2013 (International Organization for Standarization and Normalisatie Nederlands, 2013) and nitrate (N) concentration by ion chromatography (IC) (Waterlaboratorium voor waterkwaliteit onderzoek en behandeling, 2019). Sulfate concentration was determined in accordance with NEN-EN-ISO 10304-1:1995 (Normalisatie Nederlands, 1995).

The pesticides bentazone, mecoprop (MCPP), chloridazon and the metabolites chloridazon-desphenyl (CLZD) and chloridazon-methyl-desphenyl (CLZMD) were quantified by liquid chromatography coupled with a mass spectrometer (LC-MS). After acidification and addition of labeled internal standards, the samples were injected and analyzed (Waterlaboratorium voor waterkwaliteit onderzoek en behandeling, 2019). 1,4-dioxane (henceforth dioxane) concentration was determined using purge and trap gas chromatography coupled with a mass spectrometer (GC-MS), and the metabolite 2,6-dichlorobenzamide (henceforth BAM) was measured in a GC-MS triple quad (QQQ) (Waterlaboratorium voor waterkwaliteit onderzoek en behandeling, 2019).

2.4. Groundwater filtration

In order to concentrate biomass, groundwater samples were filtered through Isopore membrane filters with a pore size of 0.2 μm and 47 mm diameter (Merck Group, Darmstadt, Germany). The exact volume of water filtered per sample is given in Tables S3 and S4. Filters were snap frozen in liquid nitrogen and stored at −20 °C prior to DNA extraction. The glassware from the filtration equipment was cleaned with absolute ethanol between each filtration.

2.5. DNA extraction and quantification, PCR amplification and sequencing of the 16S rRNA gene, and quantitative PCR (qPCR)

Microbial DNA was extracted from each filter using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To this end, filters were thawed and cut into smaller pieces. Several filter pieces were used per extraction tube. DNA quality (average molecular size) was checked with 1% (w/v) agarose gels stained with 1x SYBR® Safe (Invitrogen, Grand Island, NY) and quantified using the dsDNA HS Assay kit for Qubit fluorometer (Invitrogen).

The V1 – V2 region of the 16S rRNA gene was amplified using DNA extracted from samples as template, and using primers 27F (GTTYGATYMTGGCTCAG) and 338R (GCWGCCWCCCGTAGGWGT) (Daims et al., 1999).

A two-step PCR protocol was used. With this approach, tags and adapters were added in a second round of PCR amplification (Tables S5 and S6).

The initial PCR mix was prepared as described in Tables S7 and the second PCR mix as in Tables S8. The amplification program for both PCR mixes is detailed in Tables S9. The PCR products were cleaned with the MagBio Beads Cleanup Kit (MagBio, MD, USA) according to the manufacturer's instructions. Quality and concentration of the PCR products were checked with a 1% (w/v) agarose gels stained with 1x SYBR® Safe (Invitrogen) and using the dsDNA HS Assay kit for Qubit fluorometer (Invitrogen). The barcoded samples were pooled in equimolar concentrations and sent for Illumina sequencing in HiSeq Rapid Run 300bp PE mode (GATC-Biotech, Konstanz, Germany; now part of Eurofins Genomics Germany GmbH). Sequence data were submitted to the European Bioinformatics Institute under study accession No PRJEB34986. The barcode sequences used for each sample are detailed in Tables S10.

qPCR analyses were used to quantify total bacteria and archaea based on the 16S rRNA gene, and functional genes involved in nitrate reduction (*nirS*, *nirK*, and Clade I of *nosZ*) (Throbäck et al., 2004) and sulfate reduction (*dsrB*) (Dar et al., 2007). Analyses were performed on an iQ SYBR Green using Bio-Rad super mix using a CFX384 Touch™ Real-Time PCR Detection System. All qPCR assays were performed in triplicate with a total volume of 10 μL reactions. Gene copy numbers were calculated per mL groundwater. Detailed information of the qPCR primers and amplification protocols can be found in Tables S11.

2.6. Data processing and analysis

Processing of the raw data was performed with the NG-Tax pipeline using default settings (Poncheewin et al., 2020). In short, paired-end libraries were demultiplexed using read pairs with perfectly matching barcodes. Amplicon sequence variants (ASV) were picked as follows: for each sample, sequences were ordered by abundance and a sequence was considered valid when its cumulative abundance was $\geq$0.1%. Taxonomy was assigned using the SILVA reference database version 128 (Quast et al., 2013). ASVs are defined as individual sequence variants rather than a cluster of sequence variants with a shared similarity above a pre-specified threshold such as operational taxonomic units (OTUs).

Analyses of microbial communities were performed in R version 3.5 (R Core Team, 2014). Alpha diversity (diversity within a sample) was calculated using Faith's phylogenetic diversity (Faith, 1992) from the picante package (Kembel et al., 2019). Beta-diversity (diversity between samples) was calculated using unweighted UniFrac with the phyloseq package (Mcmurdie and Holmes, 2013). To determine the multivariate effects of the geochemical environment and MPs on the microbial composition, redundancy analysis (RDA) was performed on center log ratio (clr) transformed relative abundance data using the rda function from vegan (Oksanen et al., 2019). The clr-transformation of relative abundances allows for the application of statistical methods that have been developed for real random variables, such as RDA (Gloor et al., 2017; Jones and Aitchison, 1987). RDA is a gradient analysis technique which summarizes linear relationships between multiple components of response variables (microbes) explained by a set of explanatory variables (geochemical components and pollutants) by multiple linear regression of multiple response variables on multiple explanatory variables. To determine which of the electron acceptors and MPs significantly explained the most variance in the composition of microbial communities and which combination of variables generated the most parsimonious model, forward and backward automatic stepwise model selection were performed using the ordistep function, which bases the term choice on Akaike's information criterion. For robustness we performed the same automatic model selection using the ordiR2step function which only performs forward model building and bases term choice solely on adjusted R2 and *p*-value. To determine the relation and overlap in modulation of the microbiota by the electron acceptors and selected MP, variation partitioning was performed using the varpart function from vegan.

3. Results and discussion

3.1. Microbial community composition and dynamics in the aquifer

Two groundwater monitoring wells in a drinking water aquifer were sampled in 2014–2016 for 16S rRNA gene sequencing. We calculated alpha diversity for all samples using Faith's phylogenetic diversity (PD, Faith, 1992). PD varied from 2.6 (23-46m-b) to 29.2 (23-46m-d, Tables S12). PD was similar between wells 22 (20.3 $\pm$ 5) and 23 (20.2 $\pm$ 5.8), but increased with depth in both wells (Tables S12). A portion of ASVs (7–55%) could not be classified even at phylum level (Fig. 1A), partially due to technical limitations of the primer (lack of coverage in the reference database) but also due to microbial communities in groundwater being barely described (Luef et al., 2015). Overall, the most abundant phyla in well 22 were *Proteobacteria* (26.8 $\pm$ 14.9%), *Chloroflexi* (11.8 $\pm$ 5.7%), *Candidatus Omnitrophica* (8.0 $\pm$ 3.2%), *Nitrospirae* (7.5 $\pm$ 12.1%), *Bacteroidetes* (4.0 $\pm$ 3.3%), *Firmicutes* (3.3 $\pm$ 1.5%), *Microgenomates* (2.2 $\pm$ 2.2%), *Nitrospinae* (2.4 $\pm$ 3.8%), *Parcubacteria* (1.7 $\pm$ 1.3%), and *Candidatus Berkelbacteria* (0.5 $\pm$ 0.9%, Fig. 1A). The most abundant phyla of well 23 were classified as *Proteobacteria* (25.6 $\pm$ 8.2%), *Omnitrophica* (16.9 $\pm$ 9.2%), *Microgenomates* (8.4 $\pm$ 4.0%), *Nitrospirae* (7.9 $\pm$ 9.2%), *Chloroflexi* (6.6 $\pm$ 4.8%), *Ignavibacteriae* (4.6 $\pm$ 6.6%), *Parcubacteria* (1.5 $\pm$ 1.4%), *Bacteroidetes* (1.1 $\pm$ 1.3%), *Acidobacteria* (0.9 $\pm$ 1.5%), and *Actinobacteria* (0.6 $\pm$ 0.9%, Fig. 1B). In a similar project, microbial communities from groundwater with changing geochemical conditions from monitoring sites in New Zealand were studied. The V5–V7 region of the bacterial 16S rRNA gene was sequenced, and *Proteobacteria* was the most abundant phylum across the examined samples (Sirisena et al., 2018). The oligotrophic groundwater environment favors small bacterial cells, some of them also with reduced genomes such as *Microgenomates* (OP11) and *Parcubacteria* (OD1) (Atashgahi et al., 2017; Castelle and Banfield, 2018; Luef et al., 2015).

The fact that groundwater microorganisms can only be classified at a high taxonomic level (i.e. phylum), provides limited information about the role of the microorganisms; although, they had been acknowledged as key players of different element biogeochemical cycles in clean as well as polluted environments (Guo et al., 2019; Sheng et al., 2016). Furthermore, the limitations of sequencing the region V1–V2 must be acknowledged. V1–V2 had showed consistent taxonomic assignment for a wide range of genera back in 2014 when the initial samples were collected (Guo et al., 2013). Although later studies showed that the region V4 was more suitable for environmental samples like groundwater (Lee and Lee, 2018; Ma et al., 2019; Safonov et al., 2018; Sonthiphand et al., 2019), to be able to compare samples from 2015 to 2016, the region V1–V2 was consistently used.

Besides understanding community composition, we further aimed to understand the spatial and temporal variability of microbial communities in the aquifer. Samples from the same well and depth group closely together, implying temporally stable microbial communities

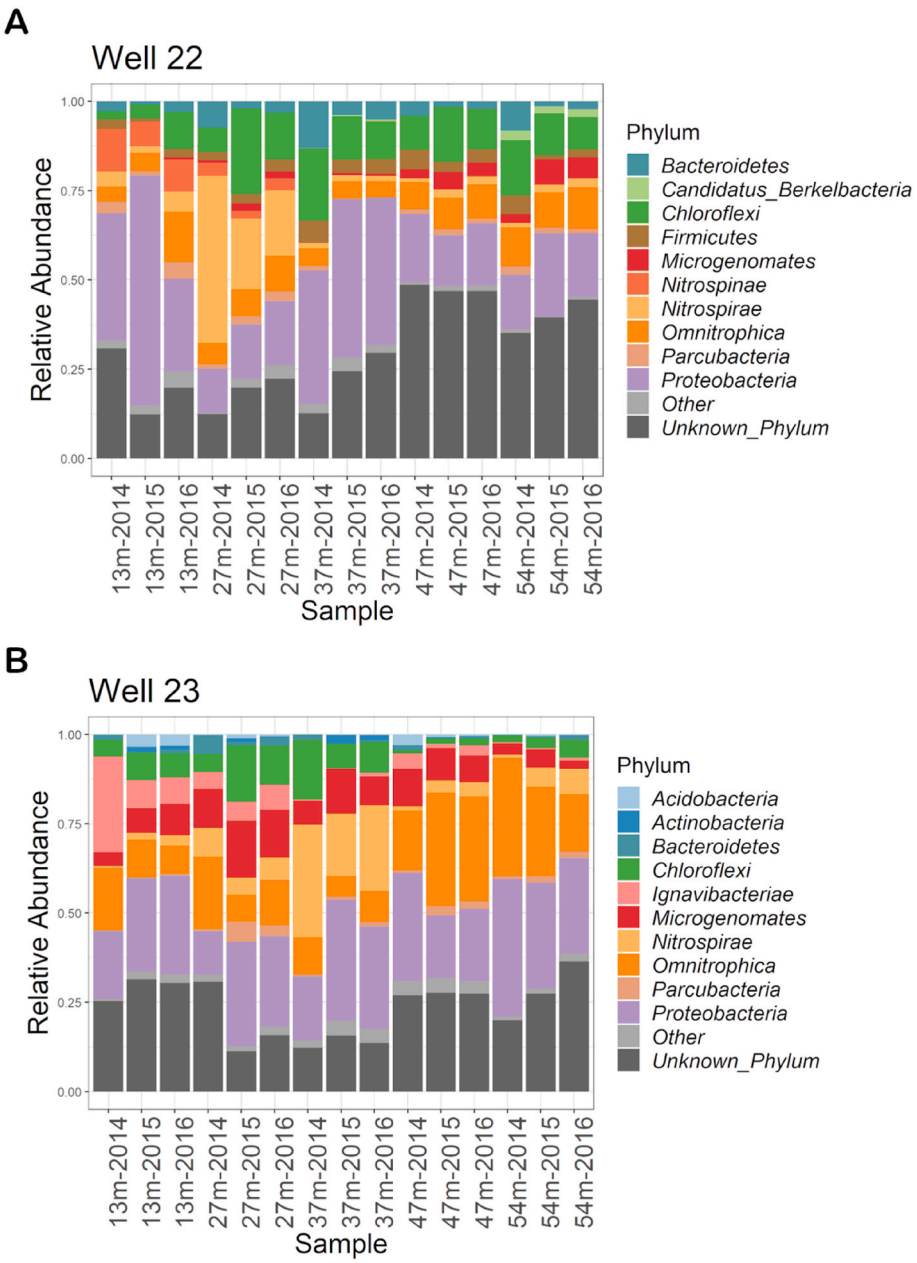

Fig. 1. Relative abundance of the top 10 microbial phyla of well 22 (A) and well 23 (B). The labels on the X-axis refer to the depth and year the sample was taken, the value is the average of the duplicates taken on the same day. "Unknown_Phylum" stands for all sequences that could not be classified at phylum level. All other phyla are summarised into the "Other" category. The abundance and ASV tables as well as the genus rank table can be found in Supplementary Tables S17-S19.

within single sampling points (Fig. 2). Samples formed three groups, separated according to depth (samples >40 m from both wells grouped together) and the well (samples from <40 m split into two distinct groups – one per well). This observation seems to imply a hydrological connection between the wells in depths >40 m or a hydrogeological disconnection between shallow (<40 m) and deep (>40 m) parts of the wells. The observed hydrological connection between different depths of a single monitoring well or between different monitoring wells might facilitate the distribution of microbial communities to different parts of the aquifer. Accordingly, microbial communities from the depths >40 m had a higher phylogenetic diversity, and were more similar across the two wells at 500 m distance than microbial communities from shallow and deep parts within a single well (Fig. 2).

The sediment profile from both wells was studied and showed that sand is the main material present in the aquifer (Tables S1 and S2). However, clay lenses were also found at 25 and 32 m in well 22 and 42 m in well 23, which partially separate the shallow and deep part of the aquifer. These compressed clay layers can enclose part of the aquifer (Custodio, 2010) and therefore reduce vertical movements of the microbial community as well as electron donors and acceptors. This phenomenon is exacerbated by the fact that in the deeper aquifer, large volumes of groundwater are extracted for drinking water production. The sand was coarse in the deeper aquifer, indicative of higher hydraulic conductivity, allowing groundwater to easily flow laterally towards the extraction well. Therefore, it is likely that there is a hydrological connection between the deep sections of wells 22 and 23 resulting in more similar groundwater and microbial composition than the less connected shallow sections of these wells.

3.2. Electron acceptor availability

Groundwater geochemical composition at each measured depth

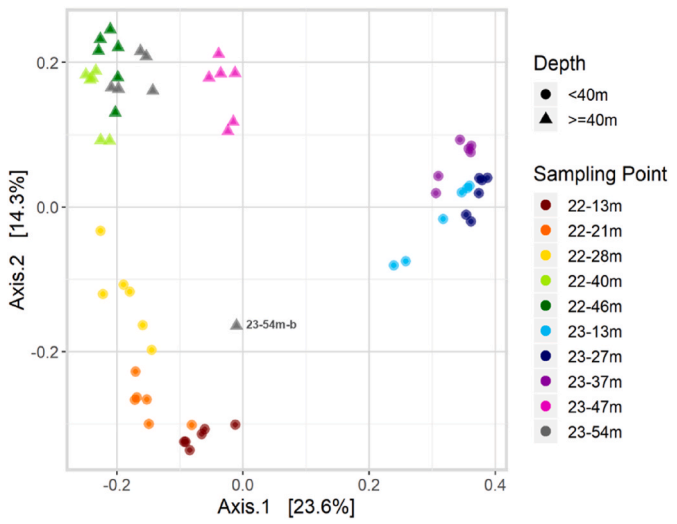

Fig. 2. Principle coordinate analysis of microbial communities in wells 22 & 23 based on unweighted Unifrac of samples taken in 2014–2016, sequenced with region V1–V2 of the 16S rRNA gene. Sample b of well 23 at 54m had a very low concentration of DNA after extraction leading to a low number of reads after sequencing, which could explain its position due to bias. The label "deep" refers to sampled depths of 40m and below, where there is lateral movement of the groundwater due to drinking water extraction. Samples from depths <40m are displayed as circles, samples from depths≥40m as triangles.

remained stable during the 16 years of monitoring data (Fig. 3A and B). Electron acceptors showed vertical gradation in the aquifer, indicating sequential consumption. This was further confirmed by quantification of genes specific for respiration on each electron acceptor. The clear zonation of the different available electron acceptors is presented in detail for each well in the Fig. 3AB. The groundwater geochemical data showed zonation in electron acceptor availability and DOC as the key electron donor, which was found in both wells 22 and 23 at concentrations between 2.4 and 19.7 mg C L^{-1} (Fig. 3A and B). In both wells, DOC concentrations decreased with depth, with the larger decrease observed in well 22.

Well 22 was characterized by stronger anaerobic conditions, with no nitrate measured in the available 16 years of monitoring. Consequently, well 22 had less clear zonation of electron acceptors than well 23 (Fig. 3A), with a mixture of iron- and sulfate-reducing conditions. Sulfate reduction appeared to be most active in depths between 13 and 22 m of well 22 as indicated by the low concentration of sulfate compared to 40–46 m. In a previous study in a pristine aquifer, sulfate was the main electron acceptor which resulted in high abundance of sulfate-reducing bacteria (Detmers et al., 2004). In the present study, a high abundance of the *dsrB* gene (Fig. 3C), which is involved in sulfate reduction, provided further evidence for sulfate as the dominating electron acceptor in depths 13–22 m. This was accompanied by a change in microbial composition (Fig. 1A). *Proteobacteria* was the most dominant group overall, however its presence was more predominant at 13 and 37 m deep. At 27 m, *Nitrospirae* increased in relative abundance, corresponding with the decrease of ammonia concentration (Figs. 1 and 3A), which matches with the recent discovery of Nitrospira being able of complete ammonia oxidation under low dissolved oxygen and substrate conditions (Mehrani et al., 2020). In the deeper sections of the well (47–54 m) the phylum *Omnitrophica* increased in abundance. The phylum *Omnithrophica* has been also associated with groundwater samples in tropical island aquifers, and it is mainly present in anoxic environmental conditions, although further phyla descriptions are lacking (Kirs et al., 2020; Rivas-Marín and Devos, 2018).

Well 23 showed a clear zone of nitrate-reduction at depths <40 m, with average concentrations of 18, 15, and 12 mg L^{-1} $N–NO_3$ at depths 13.5, 27 and 37 m, respectively (Fig. 3B). A low concentration of iron (II) was observed, suggesting that nitrate is most likely the dominating electron acceptor. This was further supported by qPCR data (Fig. 3D), which showed a higher abundance for the genes *nirS* and *nosZ*, involved in nitrite and nitrous oxide reduction, respectively, in depths <40 m of well 23 compared to the other samples. At 47 and 54 m below the surface in well 23, iron (II) was observed at average concentrations of 11.4 and 14 mg L^{-1}, indicating active iron reduction in the deeper zones. This was accompanied by a 1-2 order of magnitude drop in *nirS* and *nosZ* abundance compared to depths between 13.5 and 37 m in well 23, indicating a shift in community composition in response to electron

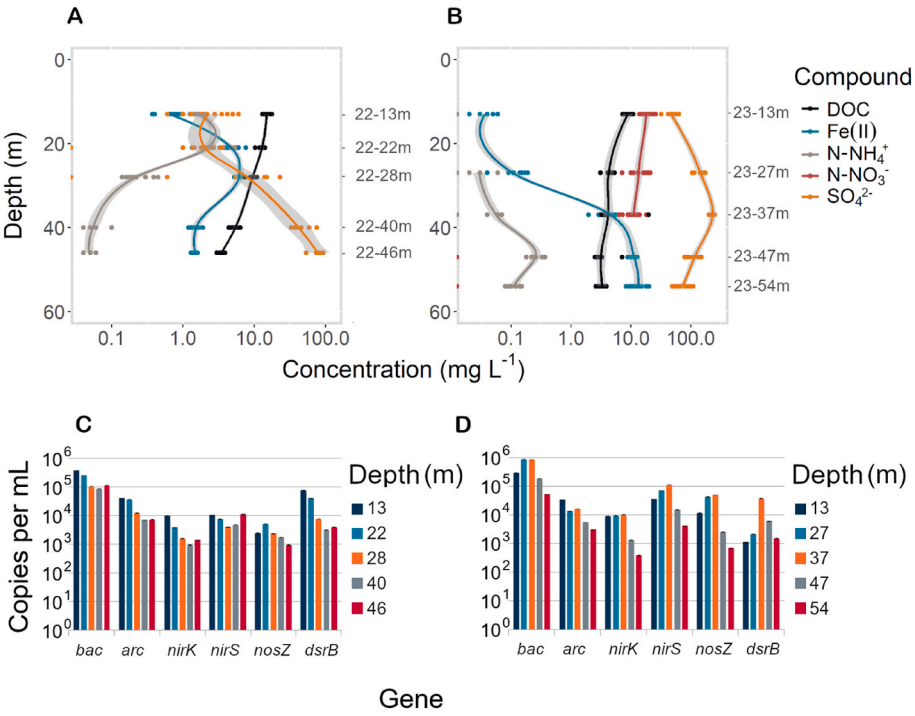

Fig. 3. Top: Geochemistry of wells 22 (A) and 23 (B). Each point of the same color represents one measurement in one year between 2000 and 2016. The colored lines represent the average of all measurements between 2000 and 2016, whereas the grey area on both sides of the lines represent Loess 95% confidence intervals that were calculated and visualized using R. Measurements were taken at least one year apart. All values can be found in Supplementary table S14. Note that ammonium and nitrate was measured in mg L^{-1} nitrogen belonging to ammonium/nitrate. Bottom: qPCR measurements of bacteria (bac), archaea (arc) 16S rRNA genes and functional genes of denitrification (*niK*, *nirS*, and clade I *nosZ*) and sulfate reduction (*dsrB*) in wells 22 (C) and 23 (D). The values represent the averages of 6 samples per depth: duplicates taken in three consecutive years (2014–2016), with error bars corresponding to standard deviation between measurements. The colors represent the different depths the samples were taken from. The qPCR data can be found in Supplementary table S15. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

acceptor availability. The relative abundance of *Proteobacteria* was fairly constant in the aquifer. *Ignavibacteriae* was more abundant in the shallow (13.5–271 m) than in the deeper section of the aquifer. In the middle of the aquifer, the relative abundance of *Nitrospirae* increased. *Nitrospira* genus that belongs to the phyla *Nitrospirae* is the most widespread group of nitrite oxidizing bacteria, both in aerobic and microaerophilic environments (Bayer et al., 2020), which corresponds with high nitrate concentration. In the deeper section (>40 m), *Omnitrophica* increased in relative abundance, similar to well 22, which is in agreement with both the similar geochemical composition and proposed hydraulic connectivity in the deeper aquifer.

3.3. Organic MPs in groundwater

Regular monitoring of hundreds of organic pollutants over 16 years revealed that seven compounds were consistently encountered in wells 22 and 23 (Fig. 4) at micropollutant concentrations (i.e. μg L^{-1}). However, in contrast to the stability of geochemical components, the concentrations of the MPs varied greatly both spatially (between depths) but also temporally (between years at a specific depth; Fig. 4). Three pesticides (bentazone, chloridazon, MCPP), three pesticide transformation products (BAM, CLZD, CLZMD), and one solvent (dioxane) exceeded the threshold (0.1 μg L^{-1}) of the European framework for groundwater quality (European Union, 2006) on at least one occasion. BAM, bentazone and MCPP are accordingly among the most frequently found compounds in drinking water abstractions as reported by the National Institute for Public Health and the Environment of The Netherlands (RIVM) (Swartjes and Van der Aa, 2020). Overall, we found concentrations ranging from below the detection limit to 8.4 μg L^{-1}. All measured MPs were detected in both wells in at least one measurement except chloridazon, which was only detected in well 22. The most abundant MP was dioxane with an average concentration of 0.2 μg L^{-1} in well 22 and 1.0 μg L^{-1} in well 23. CLZD, a recalcitrant metabolite of chloridazon, was particularly abundant in well 22 and observed at concentrations of between 0.3 and 1.6 μg L^{-1}.

Since MPs may percolate from the surface, different pesticide distribution patterns in the wells could correspond to different application patterns and sources in the surrounding fields, but also to different permeability of the subsurface (Postigo and Barceló, 2015). Well 22, for instance, is adjacent to a canal which carries agricultural runoff and WWTP effluent, so there could be infiltration of larger range MPs from the canal into well 22. In the upper part of well 22 (<25 m) bentazone, dioxane and chloridazon are the predominant MPs, whereas only CLZMD was observed in the upper part of well 23 (<25 m). MP presence in the deeper aquifer indicates that the clay layers are not fully restricting vertical migration of the compounds. Well 22 for instance, has two partial clay layers, one at 25 m and another one at 32 m deep. Nonetheless, chloridazon, CLZMD and dioxane are observed in the deeper aquifer of well 22 (>40 m). In well 23, BAM, MCPP, dioxane and bentazone were found in the deeper aquifer (>40 m), showing once again that the clay layer is not impermeable or fully sealing.

Contrary to correlations that were explained in Section 3.2, where some microbial groups could be associated with different electron acceptors, determining such associations is rather challenging with organic MPs. This is mainly because: (1) anaerobic MPs degraders are not well-described in literature, (2) taxonomic classification at low taxonomic levels is challenging in groundwater, and (3) MPs presence do not necessarily imply MPs-microbe interactions. The third point will be further explained in Section 3.4."3.4 Impact of geochemical parameters and MPs on microbial communities.

The central goal of the present study was to characterize correlations of groundwater geochemical composition on the spatial and temporal variability of microbial community composition, potentially indicating a selective pressure exerted by these factors. To this end, we performed redundancy analysis (RDA) focusing on geochemical parameters such as electron acceptors, DOC, and ammonium, as well as MPs. As mentioned

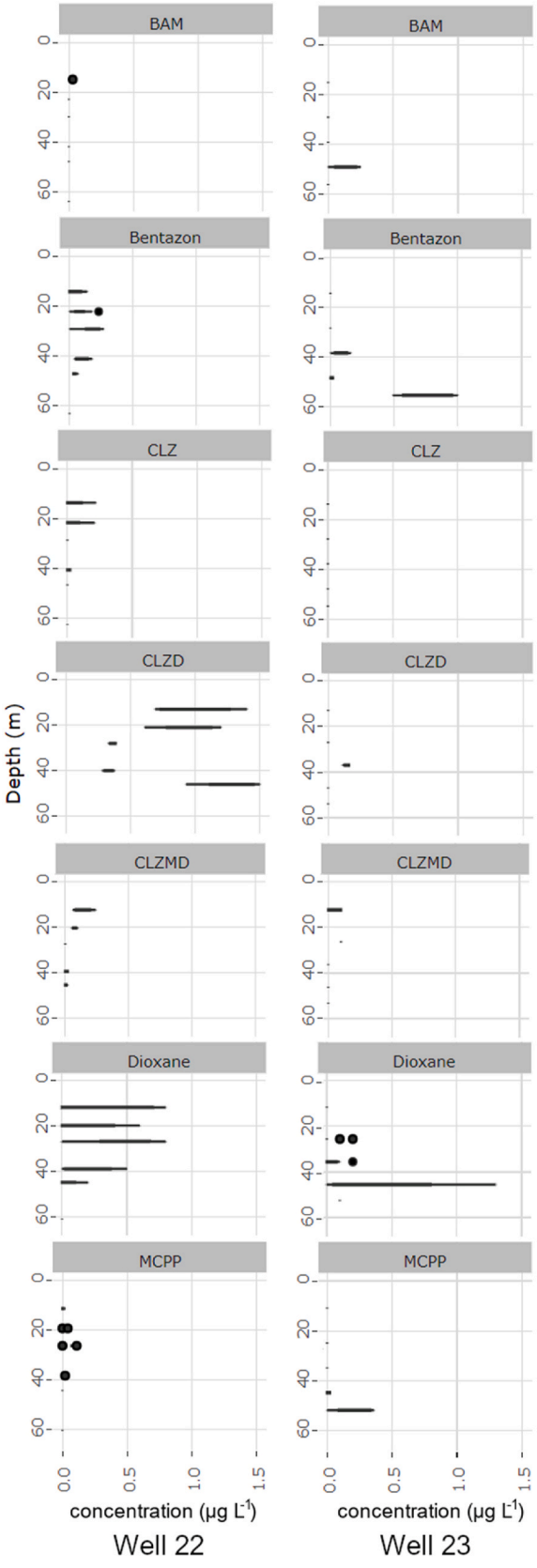

Fig. 4. Variable concentrations of micropollutants in wells 22 and 23 from 2000 to 2016. Each box plots represent series of 4–16 measurements. The measurements were taken at least one year apart. Yearly trends could not be observed, because the available data (measured depths, measured dates) varies for each pesticide. The complete micropollutant data can be found in Supplementary Table S16.

above, geochemical composition remained relatively stable during the 16 years monitored, while the MPs showed spatial and temporal heterogeneity. RDA indicated that nitrate, sulfate, iron (II), DOC, ammonium, dioxane, and CLZD most strongly (p-value = 0.005) contributed to explaining the observed variation in o microbial community composition (Fig. 5A). Variation partitioning for the four variables with the strongest effect showed that the microbial communities in the samples were shaped especially by abundance of DOC, nitrate, iron (II), and sulfate. The strongest independent shaping effect was exhibited by DOC, which alone explained 7.7% of the total variation, followed by nitrate (6.9%), iron (II) (6.2%), and sulfate (5.1%, Fig. 5B). Strong joint variation was explained by nitrate with sulfate (3.9%), and DOC with sulfate (2.1%). Altogether, the joint and separate effects of the four geochemical parameters explained 33.3% of the total variation in the microbial community composition.

Electron acceptor availability exerted a selective pressure on the microbial community, and was reflected in key microbial groups present at different depths. The microbial groups most associated with nitrate were family FW13 from the phylum *Nitrospirae* and two members of the *Omnitrophica* phylum that could not be classified to lower taxonomic levels. These microbial groups can be participating in different steps of the nitrogen cycle such as nitrite-oxidation (Daims et al., 2016; Daims

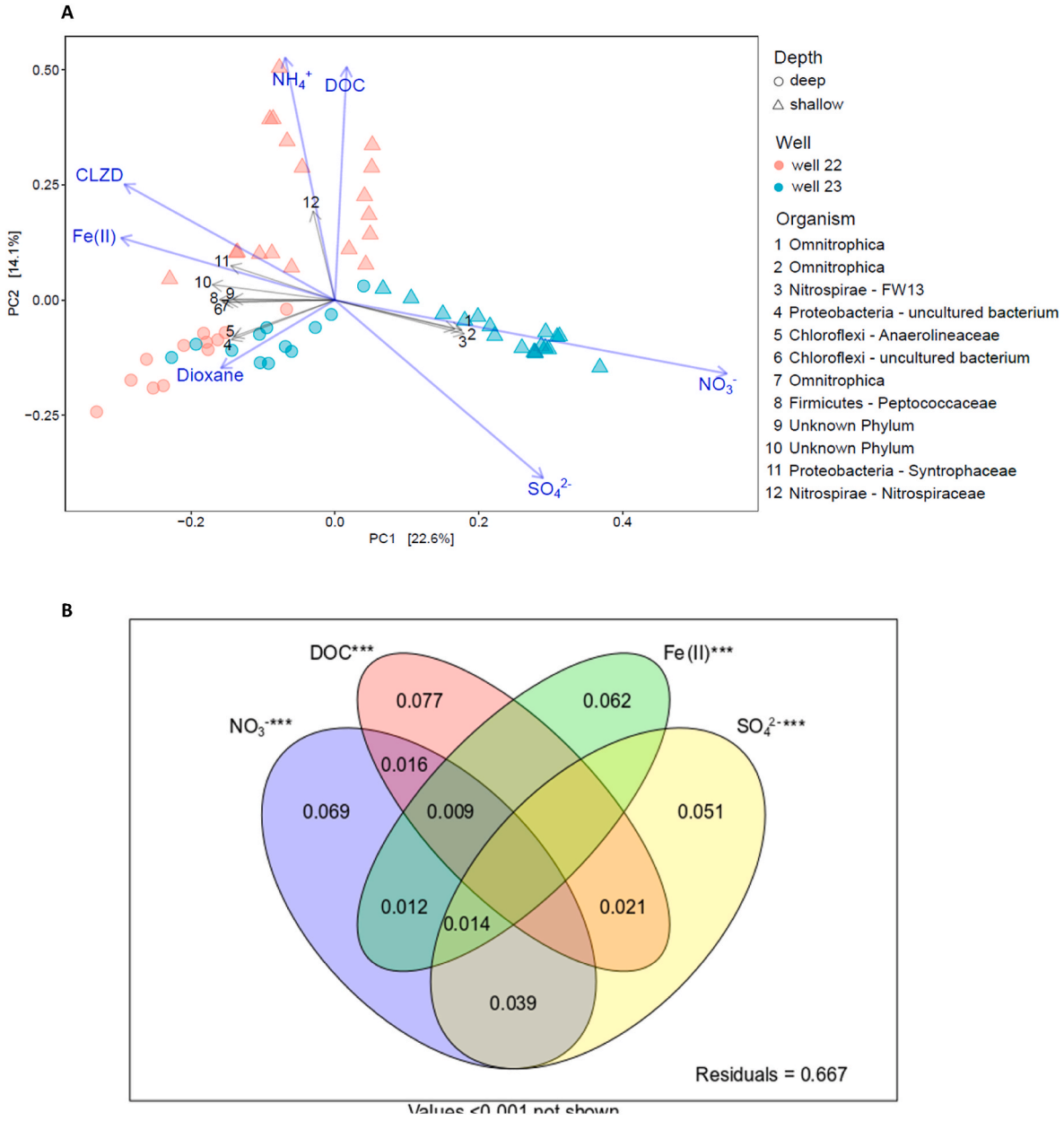

Fig. 5. Redundancy analysis (RDA) tri-plot of microbial communities in wells 22 & 23 with NO_3^-, SO_4^{2-}, Fe(II), DOC, Chloridazon-desphenyl, and 1,4-dioxane as environmental variables. A. RDA visualizing microbial community composition of samples from all 10 sampled depths (n = 60) colored by well and shaped according to sampled depth. The ordinations were done based on centered log ration transformed relative abundance data to account for the inherent compositionality of the 16S data. Complete metadata can be found in Supplementary table S20. The label "deep" refers to sampled depths of 40m and below, where there is lateral movement of the groundwater due to drinking water extraction. depths < 40m: triangles, depths≥40m: circles. Redundancy analysis (RDA) visualises the effect of geochemical compounds and pollutants on the microbial communities. Blue arrows indicate geochemical compounds, grey arrows indicate microbial groups. Length of the arrows is directly proportional to the variance in the microbial community the variable would explain alone. The ordinations for the RDA were done with raw data. b. Venn diagram visualizing the partitioning of the variation and shared variance explained by the significant predictors. ***P < 0.001, **P < 0.01, *P < 0.05. Values smaller than 0.1% are not shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and Wagner, 2018; Mehrani et al., 2020). Furthermore, two members of *Proteobacteria*, one belonging to the order *Methylococcales* and one to the genus *Synthrophus,* correlated with both iron (II) and ammonium concentration. The influence of electron acceptor availability on shaping aquifer microbial communities was previously observed under denitrifying (Bellini et al., 2018) and sulfate-reducing conditions (Boyd et al., 2007; Detmers et al., 2004).

Our analyses indicate that MPs shape groundwater microbial communities, however, to a lesser extent than groundwater geochemistry. Results do indicate that dioxane and CLZD could exert similar selective pressure on the sampled microbial communities as geochemical parameters, despite being present at much lower concentration than the measured geochemical parameters (Fig. 5A). However, it should be noted that RDA analyses are sensitive to confounding effects. RDA compares the distribution of environmental variables to the distribution of the present microbial groups. Meaningful correlation coefficients depend on co-occurrence/co-absence of a chemical with a microbial group, as well as on similar abundance ratios in samples where both are present. If a chemical and a microbial group are only present in a minority of the samples, can be challenging to differentiate the effect of each. In our study, it should be noted that CLZD and dioxane were not evenly distributed across the depths of well 22 and well 23, meaning that these MPs were always observed in conjunction with one or other electron acceptor. For example, while dioxane appeared in both wells since 2000, often at high concentration; between 2014 and 2016 when our samples were taken (Fig. 5), dioxane was only present in well 23 at depths >40 m. Dioxane was consistently present in two depths of well 23 between 2014 and 2016 at concentrations between 1.8 and 2.7 μg L^{-1}. Thus, other parameters, that are less heterogeneously distributed in the groundwater, for instance sulfate, consistently correlated with dioxane presence, thus possibly influencing the observations in the RDA.

3.4. Implications for future research

We discovered that different electron acceptors and DOC are the main factors influencing microbial communities in a system where also MPs are present. While our results did show an influence of MPs on bacterial communities in groundwater, further research is needed to unravel this observation. The heterogeneous distribution of MPs can lead to cofounding effects in our analyses. Therefore we recommend expanding this study to include a larger number of sampling points. Furthermore, we observed many relatively unexplored groups in our 16S rRNA sequencing data (Fig. 1). We recommend further exploration of oligotrophic, anaerobic groundwater microbial ecology and especially of candidate pesticide degrading groups in order to better understand pesticide transformation in drinking water aquifers.

The main factors exerting selective pressure on microbial communities need to be considered when assessing natural attenuation of MPs in groundwater and when developing new remediation technologies to eliminate MPs from aquifers. We discovered that microbial community composition is very stable with time, most likely due to the temporal stability of groundwater geochemistry. Our results show that different microbial communities develop per depth due to different redox conditions, and that DOC, as the main carbon and energy source, also shapes the community. These are important conclusions when assessing natural attenuation of MPs in groundwater. Previous research has demonstrated that MP biodegradation is to a large extent determined by electron acceptor availability, with different MPs being degraded under different redox conditions (Egli, 2010; Luo et al., 2019; Meckenstock et al., 2015). Furthermore, previous research shows that DOC can either stimulate or inhibit MP biodegradation under oligotrophic conditions, depending on the relative growth rates of degraders on DOC vs MPs (Helbling, 2015; Helbling et al., 2014; Kundu et al., 2019). Based on our results, we can infer that our drinking water aquifer contains a large number of distinct niches for microbial activity, based on electron acceptor and DOC availability, which thus represents different niches for biotransformation of MPs. When assessing natural attenuation, we must consider these niches and the unique biotransformation processes that can occur there. When possible, we can design bioremediation technologies that exploit or enhance this existing biodegradation activity for the removal of MPs from drinking water aquifers.

4. Conclusions

In the present study, we assessed key factors shaping microbial communities in an oligotrophic groundwater system used for drinking water production. Composition of groundwater geochemical parameters and, possibly, recurrent MPs exerted selective pressure on microbial community composition, despite MP concentrations being orders of magnitude lower than geochemical parameters. The microbial community composition of both monitoring wells was more similar in the deep aquifer layers compared to the shallow aquifer layers. This finding is consistent with the hydrogeology of the location, where clay layers create a partial hydrological separation between the deep and shallow aquifer. Our observations that electron acceptor and DOC availability exert a strong selective pressure on the microbial community composition indicates that our oligotrophic, anoxic aquifers has distinct niches from microbial activity and pesticide biodegradation. These niches must be considered both when assessing natural attenuation and when designing pesticide bioremediation technologies.

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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

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