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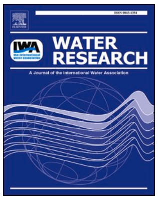

Seasonally recurring patterns of dominant *Crenothrix* spp. in a European alluvial drinking water well: Significance and potential indicator role

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ABSTRACT

Iron and manganese (Fe/Mn) often lead to aesthetic quality issues in water supply. Strong and problematic black-brown particle formation was persistently observed in an alluvial drinking water well, even though oxygen enrichment probes, intended for *in situ i.e.,* subsurface iron/manganese removal, were installed. To investigate the cause of the problem, a comparative and multiparametric approach was undertaken at the problematic well, seven additional wells (with 0.3 to 70 km distance to the affected well) and all the adjacent surface waters. Via a time-series investigation of up to 2.5 years, microbiological analysis (high-throughput 16S rRNA gene amplicon sequencing, total cell count) and chemical analysis (high-resolution elemental analysis using inductively coupled mass spectrometry and others) of the water samples were performed. Results revealed previously unreported, extremely dynamic, and seasonally recurring patterns of genus *Crenothrix* (a sheathed, filamentous bacterial population) in water samples obtained from the particle-affected well. *Crenothrix* spp. dominated the microbial community in summer months (up to 82 % relative abundance), being virtually absent in winter. Explanatory models for the high dynamics and association with bio-geochemical processes were established. These included methane formation and manganese mobilization in relation to riverbank filtration in the summer months, as well as changing aerobic and anaerobic conditions in the aquifer. Dominance of *Crenothrix* spp. in the affected well, low abundance in weak particle-affected wells, and total absence in non-affected wells was observed. This led to the suggestion of *Crenothrix* spp. as a technical indicator for Fe/Mn treatment failure for alluvial groundwater (*e.g.*, genetic marker quantification by q/dPCR), to be evaluated in future studies regarding their applicability across a broader geographic context. Despite being first described in association with drinking water deterioration 150 years ago, this is the first study reporting seasonally recurring dominant patterns of *Crenothrix* spp. in association with operational/aesthetic issues for drinking water production.

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1. Introduction

Groundwater is one of the main sources of freshwater used for drinking water production, with an estimated total volume of up to 22 million km^3 worldwide (Gleeson et al., 2016). Currently, various anthropogenic pressures (*e.g.*, urbanization and population growth), changing climate patterns, as well as imbalances between water withdrawal and aquifer recharge, are leading to groundwater depletion (Bierkens and Wada, 2019). Alluvial aquifers, located adjacent to rivers, are highly efficient groundwater sources for drinking water production because of the high permeability of floodplain sediments. These aquifers play crucial roles in managed aquifer recharge, helping mitigate water scarcity issues (Dimkic, 2021; Jannis et al., 2023). Besides being very productive, these shallow aquifers may also be affected by changes in chemical or microbiological water quality due to the infiltration of chemicals of natural or anthropogenic origin, microorganisms or the inflow of organic matter and inorganic nutrients from surface water (Baillieux et al., 2014; Oudega et al., 2022). Organic matter introduced into an aquifer may lead to the additional dissolution of manganese and iron as the redox potential changes towards reducing conditions as a result of oxygen depletion (Bourg and Berlin, 1994; Dimkic, 2021; Yang et al., 2023). Iron and manganese are commonly present in groundwater and can cause problems associated with drinking water production because of the formation of black–brown oxides leading to water quality deterioration and technical problems such as fouling and clogging. Both iron and manganese are therefore incorporated into drinking water regulations worldwide as technical indicator parameters (The European Parliament and the Council of the European Union, 2020; U.S. Environmental Protection Agency, 1994; World Health Organization, 2022). According to the World Health Organization (WHO), health relevant concentrations of iron and manganese are rarely exceeded in drinking water (World Health Organization, 2022).

Several technical treatment methods for iron and manganese removal in raw water have been developed in recent decades. Most technical approaches incorporate aeration (*i.e.*, the formation of particulate oxides) and the subsequent removal of precipitates, either after groundwater extraction, or *in situ* (Wilhelm, 2007; Worch, 2019). In the context of drinking water production, *in situ* refers to a subsurface removal (also known as the Vyredox process*)*, in which oxygen-enriched water is pumped into the aquifer through probes in close proximity to the water extraction well (Hallberg and Martinell, 1976; Van Halem et al., 2011). Injected oxygen changes the redox conditions, thus leading to the oxidation of dissolved iron and manganese ions, which in turn precipitate and can then be removed via the passage of water through the subsurface (Gounot, 1994; Paufler and Grischek, 2018). Manganese oxidation, in particular, is frequently promoted by manganese-oxidizing bacteria (MnOB) which are known to accelerate these kinetically slow chemical reactions, and can facilitate the process in environments with low redox potentials and pH (Tuhela et al., 1997; Worch, 2019). Recent investigations on the microbial community composition of the sand filter material in *ex situ* filtration facilities for manganese removal from groundwater have provided insight into the species/taxa that tend to co-occur. Among others, *Pseudomonas* spp*., Gallionella* spp*., Leptothrix* spp*., Crenothrix* spp. and *Nitrospira* spp. have been reported (Cai et al., 2015; Cheng et al., 2023, 2017; Haukelidsaeter et al., 2024; Zuo et al., 2021). The current study found only one publication analyzing the microbial community of water from wells with *in situ*/subsurface aeration for Mn/Fe removal at a single point in time. In their report, Braun and colleagues observed the presence of *Gallionella* spp*., Rhodoferax* spp., *Crenothrix* spp. and *Methylotenera* spp. (Braun et al., 2016). In general, only a limited number of studies have investigated changes in community composition over time (Haukelidsaeter et al., 2023).

The present study was triggered by the persistent formation of visible black–brown particles associated with traces of microbiological contaminants in an alluvial groundwater well in Central Europe (subsequently referred to as well M4). Associated water quality deterioration posed a significant optical and aesthetic problem for drinking water production, thereby resulting in increased operational challenges and additional costs for post hoc removal and cleaning of the system (see black particles on filters in Supplementary Fig. S1).

The goal of the study was to thoroughly investigate the cause of the problem during drinking water production at the alluvial groundwater source. In order to achieve this, an innovative three-tiered methodology was realised. Firstly, to understand the complex biogeochemical situation, a multiparametric investigation employing comprehensive chemical and microbiological analyzes (*e.g.*, high-resolution multi-elemental inductively coupled mass spectrometry (ICP–MS), high-throughput 16S rRNA gene amplicon sequencing, flow cytometric cell analysis, as well as EDX scanning electron and epifluorescence microscope particle analysis) was conducted. Secondly, to allow a comparative and systematic analysis of different field sites, several alluvial groundwater wells along the Danube River in addition to the affected well M4, with comparable background situations and/or technical installations were selected (see Fig. 1, Table 1). Thirdly, the multiparametric investigation was conducted over a time span of up to 2.5 years to support appropriate spatial and temporal resolutions. In addition, a groundwater flow model was created for the affected well M4 (including the proximal wells) to facilitate data interpretation.

2. Methods and materials

2.1. Study areas

Water samples were obtained from five distinct well fields, comprising eight individual wells in alluvial aquifers in Austria, Central Europe. The focus was on well field M, with the affected well M4 exhibiting substantial black–brown particle formation and the other wells in the same well field (proximal wells M1, M2 and M3), where only minor precipitates were observed. Additionally, more distant wells (distal wells D, G, A, and E) with distances of 20 to 70 km to M4, were investigated to allow systematic biogeographical comparisons with alluvial wells other than those in well field M. All the wells under investigation were located along the same large transnational river (Danube River) and its alluvial backwater systems (see Fig. 1 and Fig. 2). All the wells under investigation were located in an alluvial sandy gravel aquifer underlaid by an impermeable clay layer (approximate thickness is 7 m; determined for well field M). Further information on the porosity and hydraulic conductivity at the well field M are given in the Supplementary S2.9. The wells are actively used for drinking water production and equipped with various subsurface iron/manganese removal installations via *in situ* aeration/oxygen enrichment (Van Halem et al., 2011; Hallberg and Martinell, 1976). At the affected well M4, water is withdrawn from the aquifer, enriched with oxygen by simple aeration devices and pumped back into the aquifer via probes positioned in a semicircle around the well (see Fig. 2A.1). For wells G and D, the same principle of constant oxygen enrichment is employed, although additional probes forming a fully closed circle around each well were installed (see Fig. 2B). Wells A and E had periodic oxygen enrichment installations, a process which involved temporarily using the wells for extraction or injection of aerated water, with samples taken before the injection phase. The proximal wells M1, M2 and M3 had no technical oxygen enrichment. A numerical groundwater model for well field M was conducted with the model parameters and set-up described in Supplementary S2.9.

2.2. Water sampling

Sampling of well M4 and proximal wells in well field M (M1, M2 and M3), as well as the adjacent surface waters (m1 and m2), was performed monthly from May 2017 to May 2019, resulting in a total of 135 water samples. Between May 2019 and October 2019, the four distal wells (D, G, A, and E) and well M4 plus their adjacent surface waters (d, g, a, e1, e2, m1, and m2) were sampled monthly ($n = 72$). Detailed information on the individual sampling days and analyzed parameters of the water from the individual wells can be found in Supplementary Tables S1 and S2 and Fig. S4.

2.3. *Physicochemical parameters*

The temperature and dissolved oxygen content were measured on site via fixed probes employed by the well operator for remote monitoring. Other parameters, such as pH, electrical conductivity (EC), nitrate, nitrite, sulfate, phosphate, hydrogen carbonate, dissolved organic carbon (DOC) and faecal indicator bacteria (*E. coli* and Enterococci, data not shown), were determined by an ISO 17043 accredited laboratory on behalf of the well operator.

2.4. *High-resolution analysis of geochemical elements*

From September 2018 onwards, concentrations of 35 chemical elements were analyzed via high-resolution inductively coupled (sector field) mass spectrometry (HR–ICP–MS) with a Finnigan Element 2 mass spectrometer (Thermo Fisher Scientific, Germany). The list of elements included calcium, magnesium, sodium, potassium, chlorine, and sulfur (given as sulfate), expressed in mg/L, and aluminium, silver, arsenic, boron, barium, cadmium, cerium, cobalt, chromium, copper, iron, gadolinium, mercury, lanthanum, lithium, manganese, molybdenum, nickel, phosphorus, lead, antimony, selenium, silicon, tin, strontium, uranium, vanadium, zinc and beryllium, expressed in μg/L. All samples were acidified with 1 % ultrapure HNO_3 to reach a pH of 1–2 for stabilization. Scandium, indium, and thallium were added as internal standards for quality control at concentrations of 20 μg/L, 10 μg/L, and 10 μg/L, respectively. A more detailed explanation of the method can be found in Schachner-Groehs et al., (2024).

2.5. *Microscopy and flow cytometry*

Following SYBR Gold staining, epifluorescence microscopy images of the black−brown matter (precipitates/biofilm) and water samples were taken with a NIKON Eclipse Ni with Nis-Element software equipped with a 50 HBO AC-L1 mercury lamp following a protocol described by van Driezum et al., (2017). Scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) were performed on a FLEXSEM-1000 (Hitachi, Japan). Total cell counts (TCCs) for groundwater and surface water samples were determined with an Attune NxT flow cytometer (Thermo Fisher Scientific, Germany) after staining with SYBR Green following the protocol published by Schrammel et al. (2018).

2.6. *Microbial community analysis*

For biomass accumulation, approximately 2 L of groundwater from the wells or approximately 500 mL of surface water was filtered through a polycarbonate filter (pore size 0.22 μm, GTTP04700, Merck Millipore, USA) and stored at –80 °C. DNA extraction was performed following a bead-beating, CTAB (cetyltrimethyl ammonium bromide) and phenol/chloroform protocol, originally described by Griffiths et al., (2000). This method was previously adapted and optimized by our group (Reischer et al., 2006) and is described in detail elsewhere (Linke et al., 2021; Mayer et al., 2018). DNA was eluted in 50 μL of 10 mM TRIS at pH 8. All samples were evaluated for the presence of PCR inhibition according to Savio et al., (2019). Amplicon sequencing of the V3-V4 region of the 16S rRNA gene using primers 341F (CCTACGGGNGGCWGCAG; S-D-Bact-0341-b-S-17; (Herlemann et al., 2011)) and 805R, also referred to as 785R (GACTACHVGGGTATCTAATCC; S-D-Bact-0785-a-A-21; (Herlemann et al., 2011)) evaluated by Klindworth et al., (2013), was performed using the Illumina MiSeq platform (Illumina, USA). Amplicon libraries were prepared following the manufacturer's protocol (Illumina, 2019). Additional specific procedures, cycling conditions, and quality controls, such as additional analysis of a mock community, are given in Supplementary S2.6. Sequencing was performed using the MiSeq Reagent Kit v3 in three different runs, with some samples being repeated as technical replicates or re-sequenced for inter-run comparison (see Supplementary Fig. S6).

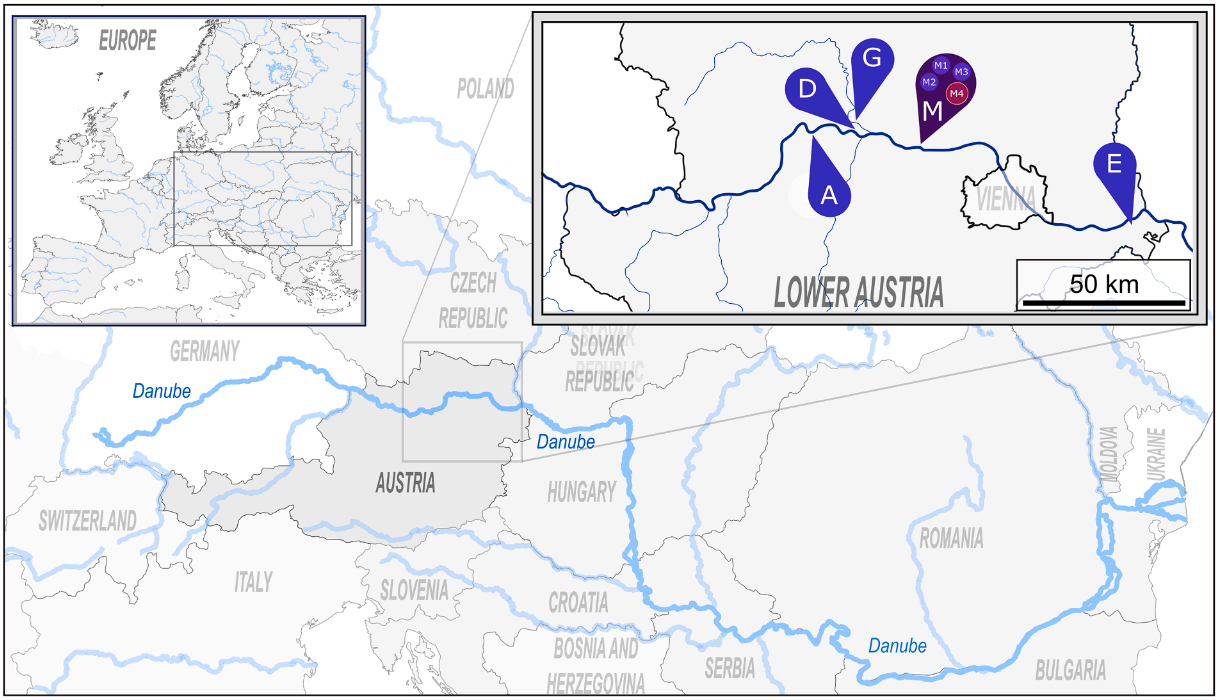

Fig. 1. Map of the study areas, located in the province of Lower Austria, next to the transnational Danube River; upper right: well field M (purple marker) with the affected well M4 (highlighted in red); proximal wells M1, M2, and M3; and distal wells A, G, D and E in blue.

2.7. Nucleic acid sequence data processing and analysis

For all sequence runs, raw sequence data processing was performed in R and RStudio (R Core Team, 2022; RStudio Team, 2021) via the DADA2 pipeline according to the workflow proposed by Callahan et al., (2016). Further information on sequence data processing can be found in Supplementary S2.7. A total of 204 samples from the eight distinct groundwater wells and the seven surface water sampling sites were incorporated into the analysis (182,347 ASVs in total). For adjustment of different sequence depths, rarefaction was performed using the function *phyloseq::rarefy_even_depth(sample.size = 5000, rngseed = 312)* from the *phyloseq* package, resulting in 50,404 ASVs and 195 samples. The cutoff for rarefaction was assessed by analyzing the smallest number of reads not being defined as outliers, which was found to be similar and close to 5000 reads for both surface and groundwater samples; therefore, this cut-off was selected. Archea were not excluded because of their possible relevance in environmental nutrient cycling, *e.g.*, methanotrophy and methanogenesis.

2.8. Statistical analysis and data visualization

Statistical data analysis and visualization were performed in R and RStudio (R Core Team, 2022; RStudio Team, 2021). Alpha diversity analysis was performed using the *vegan* package, with *diversity* (index = "invsimpson"), *kruskal_test()* and *dunn_test (p.adjust.method= "BH")* from the rstatix package (Kassambara, 2023). Beta diversity analysis was performed via the *microViz* package using Bray–Curtis dissimilarity with tax_transform (rank = "unique", trans = "identity") and non-metric multidimensional scaling (NMDS) for display (Barnett et al., 2021). For correlation analysis, robust centred log-ratio transformation (rclr) of the *Crenothrix* abundance (microViz package tax_transform(trans = "rclr", rank = "Genus") was performed. Spearman rank correlation was calculated using the *cor()* function from the stats package using the false discovery rate ("fdr") for p value adjustment and a set level of significance of $p \leq 0.05$ (Benjamini and Hochberg, 1995). Other RStudio packages used for data analysis and visualization are described in Supplementary S2.7.

3. Results

3.1. Overview of well characteristics and basic microscopic analysis

The affected well M4 was located near a backwater of the Danube River and was equipped with *in situ* oxygen enrichment installations, positioned in a semicircle around the well, intended to remove Mn/Fe in the subsurface. Nevertheless, strong black–brown particle formation was persistently observed, with the causing issues unknown. Therefore, a comprehensive investigation, including other alluvial wells with similar technical installations, but without (wells D and G) or with only minor (wells M1, M2, M3, A and E) particle formation was done.

In the first basic comparison, the general characteristics of well M4 and the other proximal and distal wells revealed no obvious differences (see Table 1). Compared to the proximal wells M1, M2, and M3, the setback distance of M4 to the adjacent backwater was shorter, and the temperature was higher and more variable, whereas the well depths were slightly lower than those of the distal wells. Compared with distal wells A and E (with only very minor particle formation), similar high temperatures and distances to surface waters were observed. Water from the distal wells D and G, was free of particles. At wells D and G, a permanent oxygen enrichment via a closed circle of probes was installed (see Fig. 2). Wells M1 to M3 had no technical oxygen enrichment installations, and at well M4, only a semicircle of aeration probes was present. Mean dissolved oxygen ranged from 0.7 mg/L (M2) to 11.3 mg/L (G) and was associated with the type of oxygen enrichment (see Table 1). The average extracted water volume at M4 (44,000 m^3 per month) was within the range of the remaining investigated wells (6000 to 190,000 m^3 per month).

An analysis of the precipitates from the affected well using electron microscopy and energy-dispersive X-ray spectroscopy (EDX) revealed that the precipitates were composed of 33 % oxygen (O), 17 % carbon (C), 14 % manganese (Mn), 5 % silicon (Si), 10 % iron (Fe) and 3 % calcium (Ca) (mass fractions, see Supplementary Fig. S7). Epifluorescence microscopy analysis revealed filamentous cell structures with compartments (see Supplementary Fig. S8). Light microscopy images of the particles from the filters showed filamentous structures, which were associated with the particles (see Supplementary Fig. S9).

3.2. Significant differences in the microbial community structure

The comparative analysis of the microbial community composition

Table 1

Basic comparison of the investigated alluvial groundwater wells: Alluvial groundwater well characteristics of the affected well M4, its proximal wells in the same well field M (M1, M2, and M3) and the distal wells (A, E, D, and G) with semiquantitative particle formation estimation (+++ strong/excessive, ~ minimal/in-significant, and - no particles detectable), as well as the distance to well M4, the setback distance to the closest surface water body, the well depth, the type of oxygen enrichment, dissolved oxygen concentration and the temperature in the monitoring timeframe.

Alluvial groundwater wells									
type	well	particle formation	distance to affected well M4	Setback distance backwater/river	well depth	average monthly withdrawal	subsurface oxygen enrichment	oxygen mean (min - max)	temperature mean (min - max)
affected well	M4	+++	0 m	90 m	12.2 m	44,000 m^3	semicircle, constant	4.5 mg/L (2.2–11.3 mg/L)	13.1 °C (9.4–15.6 °C)
proximal wells	M1	~	750 m	430 m	12.2 m	6000 m^3	none	0.8 mg/L (0.0–4.5 mg/L)	11.3 °C (9.4–12.8 °C)
	M2	~	590 m	660 m	11.2 m	84,000 m^3	none	0.7 mg/L (0.1–2.6 mg/L)	11.3 °C (9.4–13.4 °C)
	M3	~	340 m	440 m	12.2 m	163,000 m^3	none	1.9 mg/L (0.3–10.0 mg/L)	11.6 °C (9.8–13.1 °C)
distal wells	A	~	28.5 km	340 m	~ 20 m	190,000 m^3	periodic	2.9 mg/L (1.7–4.1 mg/L)	13.0 °C (12.0–14.4 °C)
	E	~	70 km	50 m	~ 20 m	165,000 m^3	periodic	2.4 mg/L (0.8–4.8 mg/L)	14.2 °C (11.8–17.0 °C)
	D	-	20.3 km	1 050 m	20 m	15,000 m^3	closed circle, constant	8.7 mg/L (6.8–10.0 mg/L)	12.5 °C (11.6–13.8 °C)
	G	-	20.5 km	67 m	20 m	105,000 m^3	closed circle, constant	11.3 mg/L (10.0–13.0 mg/L)	12.4 °C (11.8–12.6 °C)

revealed differences between the affected well M4 and the proximal wells (M1, M2 and M3) via both metrics: “within sample/site” alpha diversity and “between sample/site” beta diversity (see Fig. 3). A significant difference in alpha diversity was observed between well M4 and wells M1 and M3 and surface waters m1 and m2 (inverse Simpson diversity index, Kruskal–Wallis and post hoc Dunn test, $n = 20–27$, $p \leq 0.01$; see Fig. 3A). When the community characteristics of wells M1 and M2/M3 were compared, no significant differences in either alpha or beta diversity were observed (as indicated by their clustering via Bray−Curtis dissimilarity index and NMDS display; see Fig. 3B). The surface waters showed significant differences in both metrics for all the groundwater wells. The variability in both alpha and beta diversity was highest for the groundwater samples from well M4, thus indicating notably greater temporal dynamics over the investigation time frame of 2.5 years. Lower variations, and therefore dynamics in the microbial community compositions, were observed for wells M1, M2 and M3, with the lowest variation in the surface water samples (m1 and m2), both of which were sampled for 2 or 2.5 years, respectively.

3.3. Striking seasonal microbial community dynamics in well M4

Temporal display of the microbial community compositions from the affected well M4 and the proximal wells revealed striking differences in their variation over time (see Fig. 4). In well M4, notable high temporal changes in the relative abundance of the phylum *Proteobacteria* with seasonally recurring shifts were observed (highest relative abundance between June and October). On the family level, these shifts were attributed to *Methylomonadaceae* (see Fig. 4, well M4). Conversely, decreasing relative abundance in the summer months and increasing abundance in winter were observed for the phylum *Verrucomicrobiota,* family *Omnitrophaceae,* as well as for the phylum *Patescibacteria*, not further specified at the family level (other *Patescibacteria*). In contrast, the microbial community compositions of the proximal wells M1, M2, and M3 were shown to be temporally stable. Overall, for all four wells in field M, five of the six most abundant phyla were identified as being from kingdom Bacteria (*Verrucomicrobiota, Proteobacteria, Patescibacteria, Myxococcota* and *Acidobacteriota*), and one was from the kingdom Archea (*Nanoarcheota*).

3.4. Seasonally recurring dominance of *Crenothrix* spp.

At the genus level of the microbial community composition of well M4, the strong temporal changes were attributable to the variations in the relative abundance of the genus *Crenothrix,* which dominated the microbial community in the summer months (see Fig. 5). Other taxa (*e.g., Methylophilaceae,* genus MM1 and *Methylotenera)* also showed seasonal variation with similar trends, but less distinct seasonal patterns than from genus *Crenothrix*. Diametrically reverse trends (*i.e.*, lower relative abundance in summer months and higher abundance in winter) were observed for Candidatus *Omnitrophus* and *Nitrospira*. The relative abundance of other genera, such as Candidatus *Methylomirabilis*, showed no seasonality in their relative abundance throughout the study period. The highest yearly relative abundances of the genus *Crenothrix* were 32.8 %, 82.2 % and 68.5 % in June 2017, June 2018, and September 2019, respectively. The lowest relative abundance of this genus in the water from well M4 was reproducibly measured in winter (0.7 % in February 2018 and <0.1 % in January 2019). In the other wells of well field M, the genus *Crenothrix* was observed only at very low relative abundances, with the highest proportions of ≤2 % in well M1, ≤4 % in well M2 and ≤4 % in well M3. Hence, this remarkable seasonal pattern

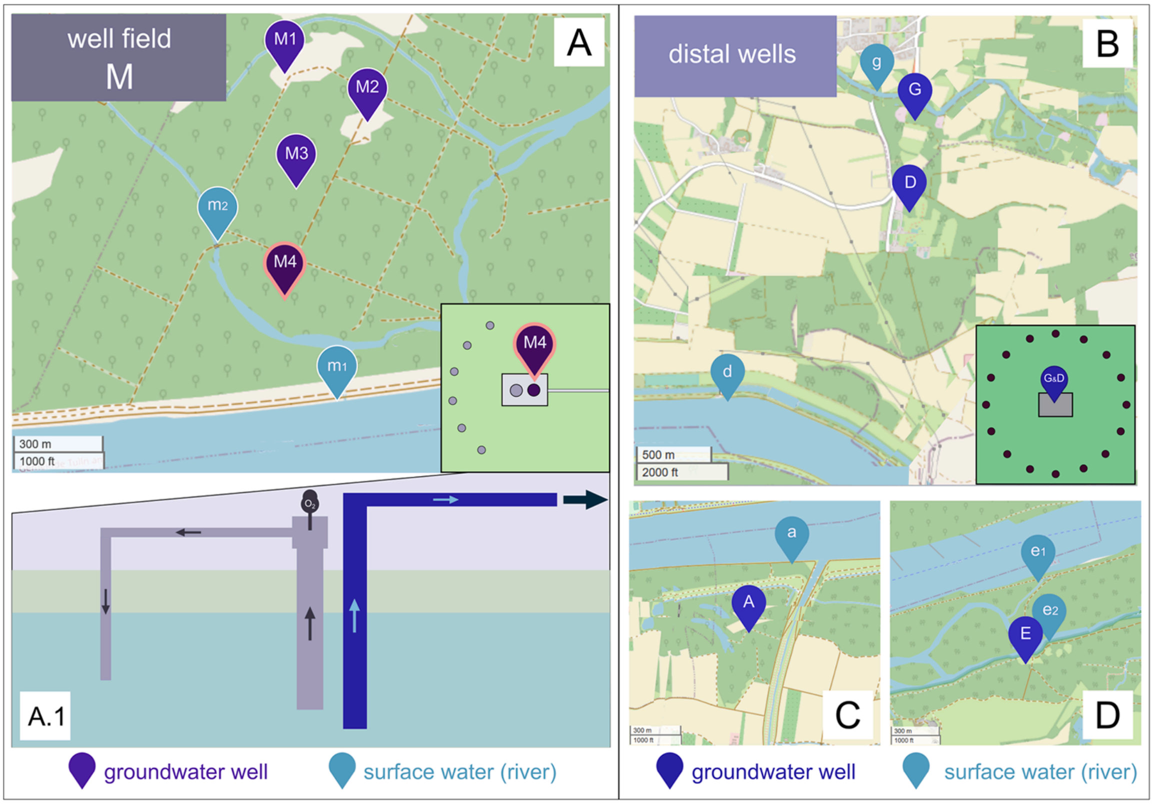

Fig. 2. A: Schematic map of the sampling sites: well field M with wells M1, M2, and M3 and well M4 affected by black-brown particles, visualization of the oxygen enrichment probes in a semicircle around well M4 and surface water sampling sites m1 (Danube River) and m2 (slow flowing backwater). A.1: Schematic overview depicting the oxygen enrichment installations where water is enriched with air at the surface and pumped back into the aquifer. B, C, D: Schematic maps of the transregional reference well locations D, G, A, and E with their adjacent surface waters d, g, a, e1 & e2. Wells D and G have a closed circle of oxygen enrichment probes (see lower right in B). Wells A and E have periodic oxygen enrichment (not shown).

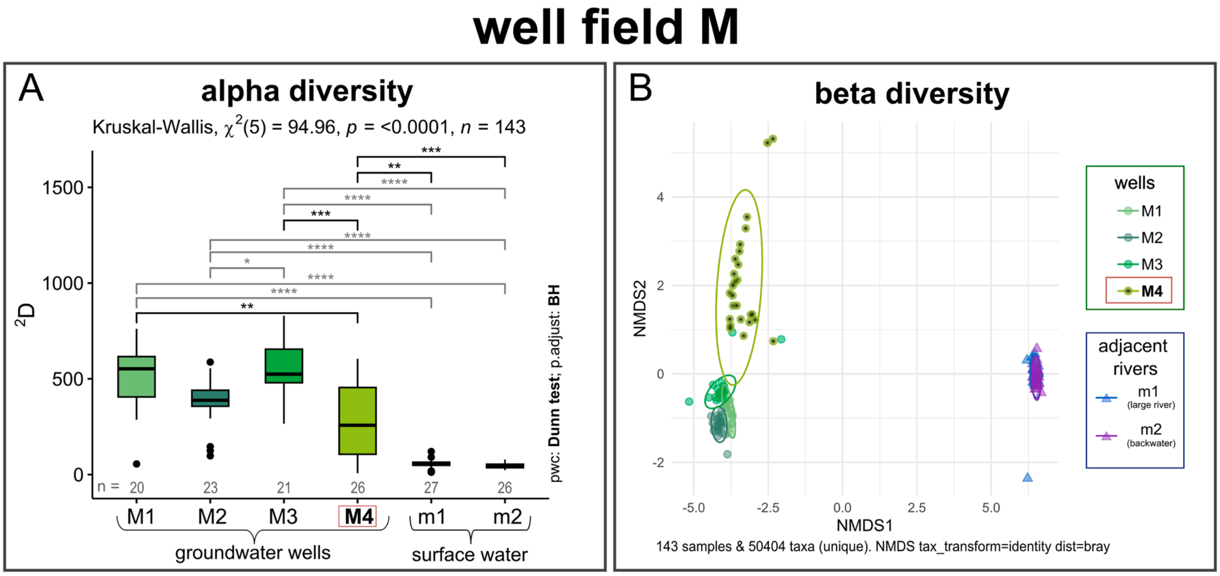

Fig. 3. Within sample/site alpha diversity (panel A) and between sample/site beta diversity (panel B) of the microbial communities at the six sampling sites at well field M, including the affected well M4 and the proximal wells M1, M2, and M3 and the surface waters m1 and m2. A: Box plots of inverse Simpson diversity index values (^{2}D) displaying alpha diversity. Significance levels: * p.adj ≤ 0.05, ** p.adj ≤ 0.01,*** p.adj ≤ 0.001, **** p.adj ≤ 0.0001. B: Non-metric multidimensional scaling (NMDS) using the Bray−Curtis dissimilarity index with ellipsoids (*i.e.*, 95 % confidence intervals) to visualize beta diversity, *i.e.*, community similarities of samples from groundwater wells M1, M2, M3, and M4 as well as from surface waters m1 (large river) and m2 (back water).

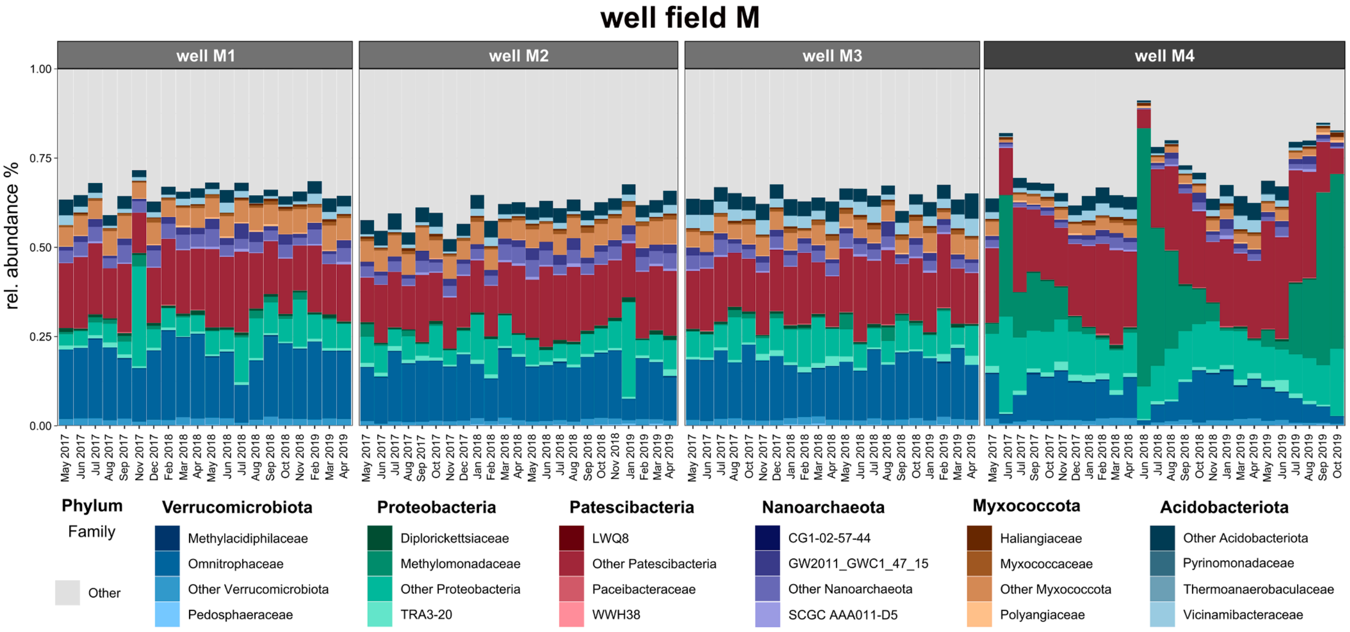

Fig. 4. Nested bar chart displaying the temporal development of the relative abundances of the microbial communities in water from wells M1, M2, M3 and M4 of well field M at the phylum level (top 6) with nested families (top 3).

of dominance in summer and almost absence in winter of the genus *Crenothrix* in the microbial community was observed only in well M4.

Seven unique ASVs assigned to the genus *Crenothrix* had an abundance exceeding 1 % in samples from well M4. To achieve more refined taxonomic identification, certain ASVs were compared to those in the NCBI database using the BLAST function. The ASV with the highest overall proportion (39.2 % of all *Crenothrix* ASVs in well M4) revealed a sequence similarity of 100 % overlap with a sequence previously assigned to a *Crenothrix polyspora* clone reported by Stoecker et al., (2006) (BLAST performed in March 2024). All seven *Crenothrix* spp. ASVs and BLAST results are listed in Supplementary S3.4, Table S4, and Fig. S10.

3.5. *Significant correlations of Crenothrix abundance with total cell count (TCC), water temperature and elemental/ion concentrations*

The analysis of the TCC and temperature in the well M4 water samples revealed seasonal variation, with higher values in summer and lower values in winter (see Fig. 6). The TCC of the water samples from the other proximal wells did not show this trend of apparent seasonal changes, even though comparable temperatures were measured (see Supplementary Fig. S11). This observed TCC pattern resembled the pattern of the relative abundance of *Crenothrix* spp. (compare Figs. 5 and 6). Correlation analysis of the centred log-ratio transformed *Crenothrix* abundance values, TCC, and temperature revealed a significant positive

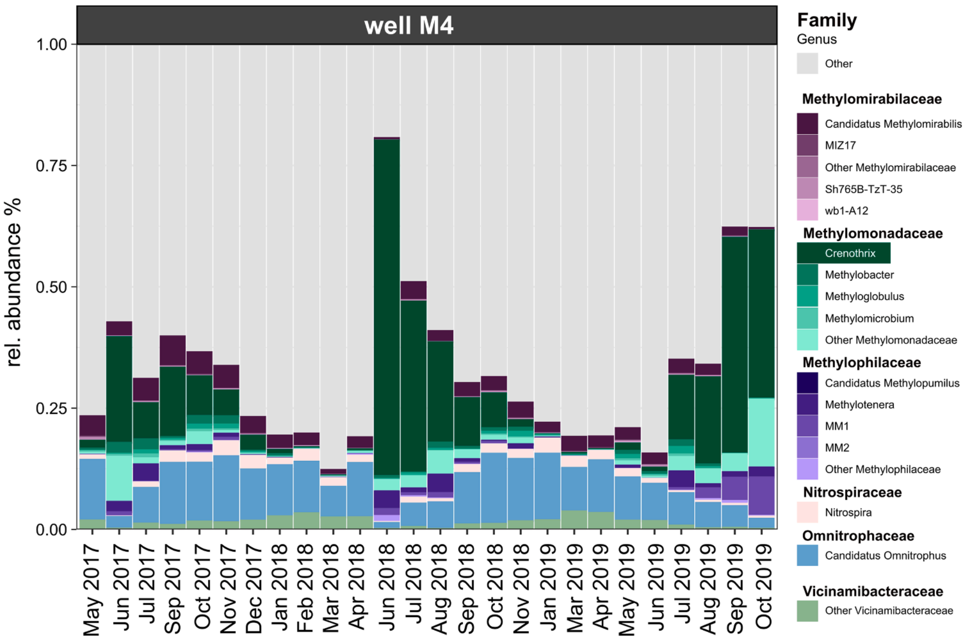

Fig. 5. Nested bar chart displaying the temporal dynamics of the relative abundance of the microbial communities in water from the affected well M4 at the family level (top 6) with nested genera (top 4).

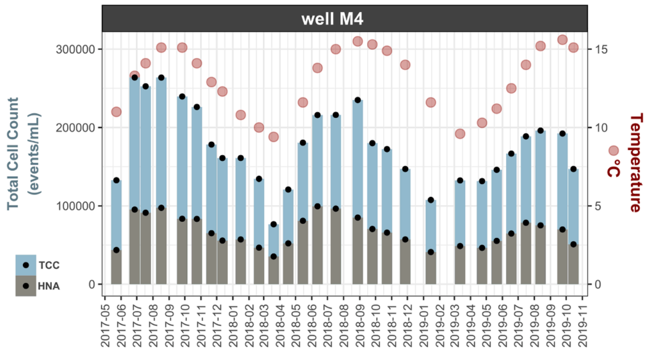

Fig. 6. Bar chart displaying temporal changes in the water temperature (red circles; right y-axis), TCC (events per mL; blue bars with upper black dots), and cells with high nucleic acid content (HNA grey bars with lower black dots; left y-axis).

correlation between *Crenothrix* and the TCC (Spearman rank, $n = 12$, rho $= 0.83$, adjusted p-value (p.adj) $= 0.002$), between *Crenothrix* and temperature (Spearman rank, $n = 12$, rho $= 0.87$, p.adj ≤ 0.001) and between the TCC and temperature (Spearman rank, $n = 12$, rho $= 0.82$, p.adj $= 0.005$) (see Supplementary Fig. S12).

Compared with well M4, all four distal wells showed lower total cell counts without an increase in the summer months, and no correlation with temperature (Supplementary Figs. S12 and S13).

Additional correlations with *Crenothrix* spp. abundance and several elemental/ion concentrations were observed in well M4. A significant positive correlation with arsenic (rho $= 0.62$, p.adj $= 0.038$), manganese (rho $= 0.68$, p.adj $= 0.035$), molybdenum (rho $= 0.73$, p.adj $= 0.035$) and silicon (rho $= 0.76$, p.adj $= 0.001$) and a significant negative correlation with nitrate (rho $=$ - 0.78, p.adj $= 0.005$) and sulfate (rho $= -0.61$, p.adj $= 0.001$) were revealed via Spearman rank correlation ($n = 12$) (see Supplementary Fig. S14). No such high number of correlations were observed for all the other proximal or distal wells (see Supplementary Figs. S14 and S15). Principal component analysis for all chemical/elemental parameters revealed a distinct clustering of these eight wells, highlighting differences in their water chemistry (see Supplementary Fig. S16). It can therefore be stated with high confidence that the water chemistry in well M4 is different from that in the other wells, with the distinct descriptive statistics of several chemical elements/ions and the physicochemical parameters given in Supplementary Table S5.

3.6. Peculiarity of the seasonal *Crenothrix* pattern in well M4

All four distal wells (D, G, A, and E) showed some distinction in their respective alpha diversity (within sample/site), but clearly clustered separately from the wells in well field M (M1, M2, M3 and M4, see Fig. 7) when beta diversity (between samples/sites) was calculated. Significant differences in alpha diversity were observed between well M4 and wells A and E, whereas no significant differences were obtained between well M4 and wells D and G. Beta diversity analysis revealed a clear separation of all the well clusters, with a greater distance between the clusters of wells D and G and well M4 than between those of wells A and E.

An investigation of the temporal dynamics in the microbial community composition of the distal wells and well M4 revealed that all four distal wells showed consistent community compositions over time in contrast to well M4 (see Fig. 8). The genus *Crenothrix* was also observed in well A (between August and October 2019), with a maximum of ≤ 6 %, and in well E, with low relative abundances ($<$ 0.4 % in 3 samples; both wells exhibited minor degrees of particle formation (see Table 1)). Considering the top six phyla in the water samples from the distal wells, the community composition was relatively similar. Only some minor differences, such as a slightly higher abundance of the phylum *Acidobacteriota* (family *Vicinamibacteraceae*) in wells D and G and a slightly higher abundance of the phyla *Methylomirabilota* (family *Methylomirabilaceae*) and *Proteobacteria* in well A were observed (see Fig. 8). Additionally, an analysis of shared ASVs in well water and surface water samples was performed (see Supplementary Fig. S17). This analysis considered only the number of different ASVs and shared ASVs across sample sites and was not including the abundance of single ASVs. In water from well M4, 128, and in the backwater m2, 11 individual ASVs assigned to *Crenothrix,* were detected. Two of these ASVs were shared, hence detected in both groundwater and surface water samples (see Supplementary Fig. S17). In samples from wells A and E and their adjacent surface water *Crenothrix* ASVs, a lower number of individual

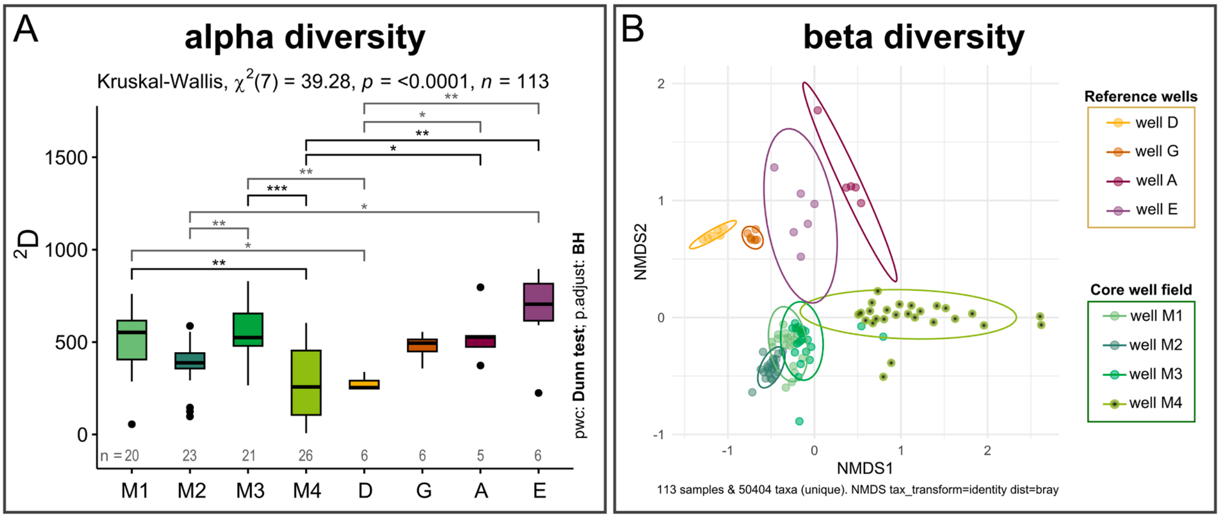

Fig. 7. A: Within sample alpha diversity and between sample beta diversity of the bacterial communities at the wells from well field M (including M4 with precipitation issues) and distal wells D, G, A and E. Panel A: Box plots of inverse Simpson diversity index values (^{2}D) displaying alpha diversity at the proximal wells and distal wells. Significance levels: * p.adj ≤ 0.05, ** p.adj ≤ 0.01, *** p.adj ≤ 0.001, and **** p.adj ≤ 0.0001. B: Non-metric multidimensional scaling (NMDS) using the Bray−Curtis dissimilarity index with ellipsoids (*i.e.*, 95 % confidence intervals) to visualize beta diversity, *i.e.*, community dissimilarities of samples from the alluvial groundwater wells: the affected well M4; the proximal wells M1, M2, and M3; and the distal wells D, G, A, and E.

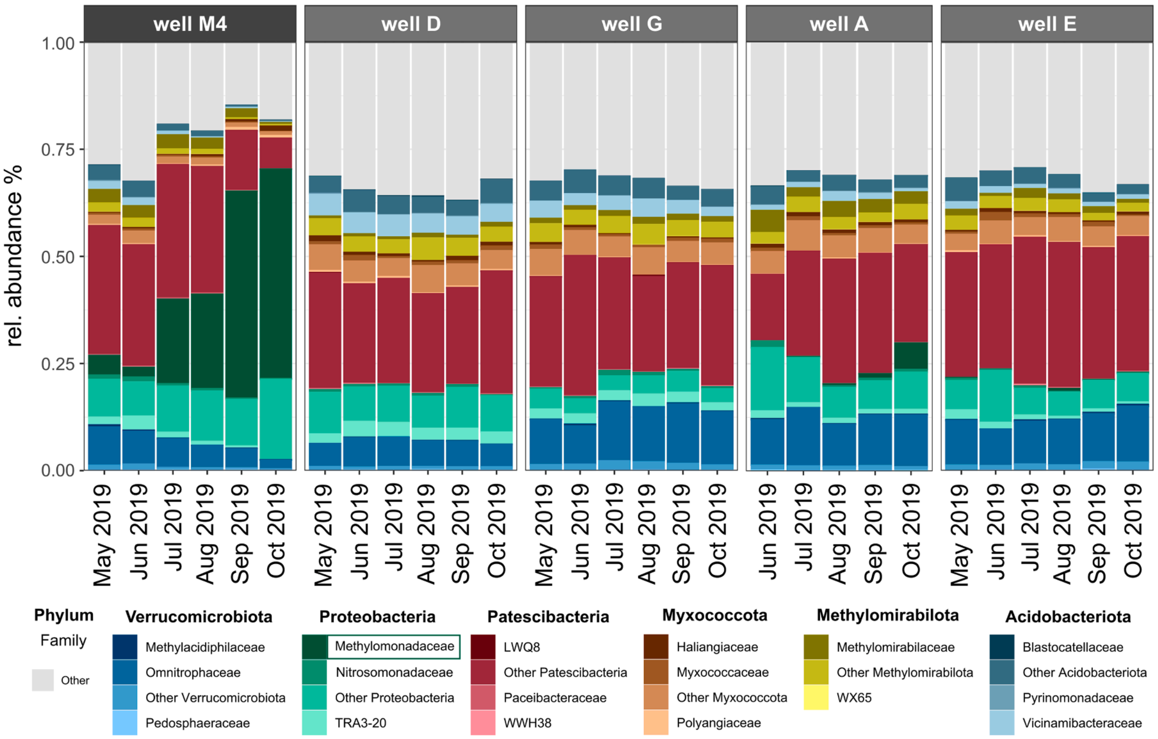

Fig. 8. Nested bar chart displaying the temporal dynamics in the relative abundance of the microbial communities in water obtained from well M4 and distal wells D, G, A and E at the phylum level (top 6) with nested families (top 3).

ASVs (A = 11, a = 1; E = 3, e1&e2 = 4) were detected and none of them were shared (see Supplementary Fig. S17). In samples from wells D and G, as well as in samples from their adjacent surface waters (d and g) no ASVs assigned to *Crenothrix* spp. were detected.

3.7. Groundwater modelling

A numerical groundwater model at the well field M revealed surface water infiltration from the east, south and west of the well M4 (see Supplementary Fig. S18). This highlighted that surface water can flow from the backwater into well M4 from directions where no oxygen enrichment probes were positioned (probes only positioned in the east of the well, see Supplementary Fig. S18). To further investigate potential influences from the surface water on the increase of *Crenothrix* spp., changes in concentrations of the chemical elements/ions in the well water and its adjacent surface/backwater m2 were analysed.

3.8. Hydrogeochemical dynamics

The analysis of the hydrogeochemical dynamics focussed on parameters which showed a correlation with *Crenothrix* spp. (see 3.5 and Supplementary Fig. S14). For nitrate, sulfate and dissolved manganese, data was available for a period of two years, while 12 months of measurements could be used for the other remaining parameters (see Supplementary Fig. S19). Nitrate and sulfate showed similar temporal trends in the backwater and in the water of well M4, thereby, further indicating the link with surface water infiltration (see Fig. 9). Sulfate, on the other hand, was observed in comparably high concentrations, while nitrate was detected at lower concentrations in the well water. Dissolved manganese concentrations in the well were noted as elevated in summer months (not observed in the surface water), thereby further reflecting a potential increase during riverbank filtration (see Fig. 9). Samples obtained from one probe south of the well M4, where no oxygen enrichment probes were positioned ($n = 6$), indicated low amounts of dissolved oxygen, which decreased from 2.5 mg/L in May 2019 down to 0.06 mg/L in September 2019 (see Supplementary Fig. S20).

4. Discussion

4.1. *Crenothrix spp. occurrence associated with deterioration of aesthetic drinking water quality*

The comparative and multiparametric time series investigation of the deterioration of aesthetic drinking water quality revealed unexpected, and to date unreported, seasonally recurring and dominating patterns of the genus *Crenothrix* in the particle affected well M4. Pronounced seasonal changes with peaks in the relative abundance of the genus *Crenothrix* reaching up to 82.2 % were observed in the affected well M4.

It should be mentioned that both black−brown precipitates and the presence of *Crenothrix* spp. had no noticeable direct impact on the hygienic quality of the drinking water, as all the legally mandated chemical and microbiological parameters remained within the limits implemented by Austrian law (*Trinkwasserverordnung, BGBl.II Nr.304/2001,* 2024) and the European Union (EU) drinking water directives (The European Parliament and the Council of the European Union, 2020).

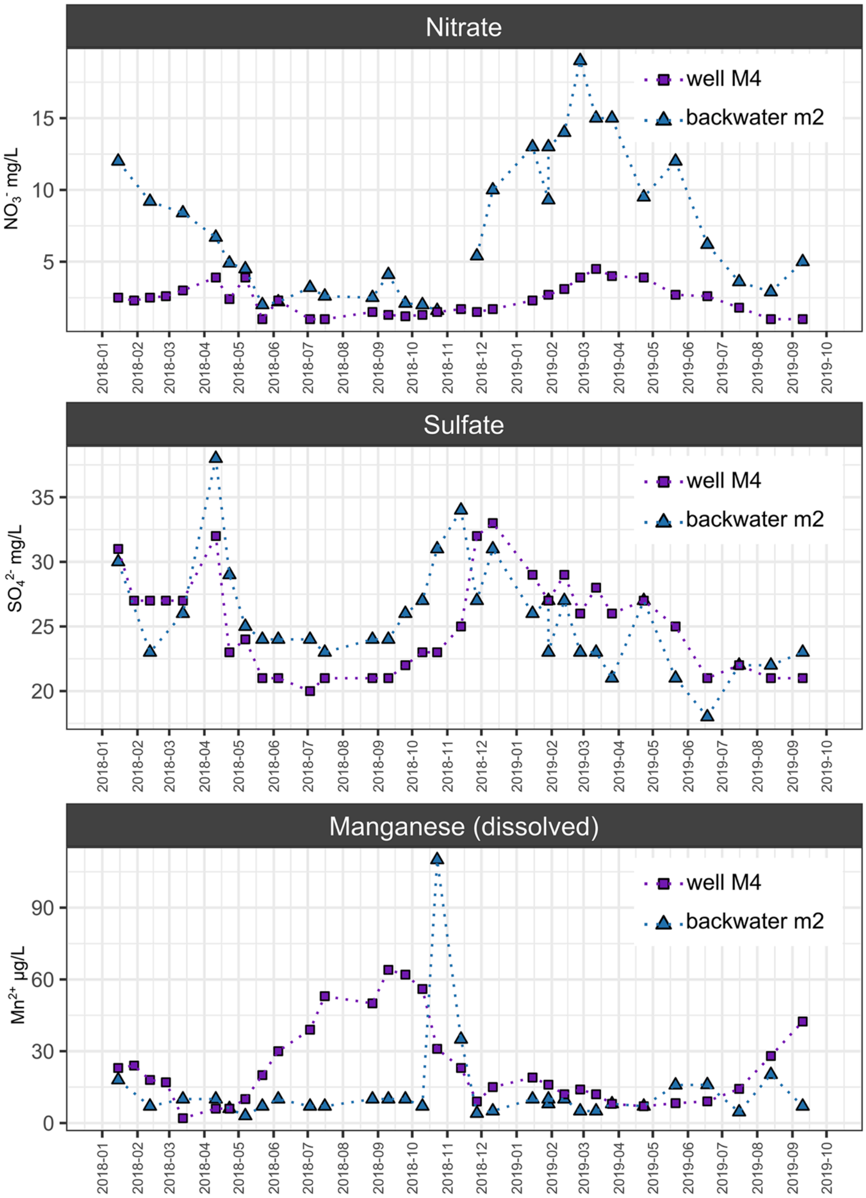

Fig. 9. Temporal trend of the mass concentrations of nitrate, sulfate and dissolved manganese in surface water samples from backwater m2 (blue triangles) and water samples from well M4 (purple squares) over a period of almost two years.

Nevertheless, water containing any particles and high amounts of iron or manganese is not appropriate for distribution and consumption. Firstly, higher turbidity is problematic for adequate dosing and application of disinfection, and secondly, the unaesthetic appearance can result in customer mistrust of the quality of the drinking water provided by the public utilities. Therefore, the manganese content and aesthetic appearance are relevant for potable water and are specifically addressed in the drinking water guidelines of the WHO and EU (The European Parliament and the Council of the European Union, 2020; World Health Organization, 2022). To remove the particles, water from well M4 was passed through filtration devices that needed regular replacement because of their complete blocking (up to 23 times per year during the investigation period, according to the operator, see Supplementary Fig. S1). This resulted in increased costs for drinking water production, whereas the pipes and valves also needed to be cleaned regularly as biofouling occurred (dates given in Supplementary Table S1), which was not the case for the other proximal and distal wells.

In water from well M4, temperature and some elements such as manganese or molybdenum were shown to be significantly correlated with the rise in abundance of *Crenothrix* spp., thereby highlighting the potential influence of these abiotic factors on their seasonal dominance of community composition. Nevertheless, correlations could also have been coincidental, thereby not having an influence on *Crenothrix* spp. proliferation as they might only co-occur due to lower concentrations of, for example, sulfate in the infiltrating surface water during the summer months. Furthermore, temperature or other factors changing during the summer months could have also have had an influence on the mobilisation of some elements during riverbank filtration such as silica, arsenic or manganese. The rise and decline in TCC at well M4 highlighted that microbial activity was more variable here than in the other proximal and distal wells over time. Considering the total absence of *Crenothrix* spp. in the community composition of the distal wells without any particle formation (namely, wells D and G) and the low relative abundances in the distal and proximal wells with minor particle formation (*i.e.,* wells A, E, M1, M2 and M3), a strong association of *Crenothrix* spp. with black-brown particles was indicated. This was further supported by historical reports from circa 1900, where *Crenothrix* spp. were first described, also in association with the presence of black-brown particles in the drinking water in Germany and the Netherlands (Cohn, 1870; de Vries, 1887).

4.2. *Crenothrix* in groundwater; known for a long time, but hardly ever addressed

In 1870, *Crenothrix polyspora* was characterized for the first time in a drinking water well, and referred to as the “Brunnenfaden” (well threads) by Cohn (1870). Initially, it was thought to be a fungus or plant due to its particulate appearance as a filamentous sporulating organism. In the following years, *Crenothrix* spp. were frequently reported in drinking water distribution networks across Europe and North America (Brown and Trax, 1929; de Vries, 1887), where they were linked to water quality deterioration and the occurrence of black-brown particles. Examples include case reports from 1882 in Amsterdam and 1887 in Rotterdam (both in the Netherlands), as well as 1886 in the Berlin water works in Germany (de Vries, 1887). In Rotterdam in particular, the massive increase of *Crenothix polyspora* (also named *Crenothrix kühniana*) in the hot summer months led to the formation of a *“Crenothrix* Commission*”* by local municipalities to investigate the phenomenon. This occurrence also indirectly led to the incorporation of aquatic bacteriology as a scientific discipline (Frankland and Ward, 1892). The “*Crenothrix* Commission” concluded that the organic content of plants and their decay are associated with the growth of *Crenothrix* and that any kind of organic contamination of drinking water must be prohibited (de Vries, 1887). The association of *Crenothrix* spp. with manganese, iron and aluminium precipitates in groundwater wells was also initially reported more than a century ago (de Vries, 1887; Jackson, 1902). Later, more detailed descriptions of its morphology as a filamentous, sheathed prokaryote (*i.e.*, gram-negative bacterium with a distinct arrangement of cells within a tube of extracellular material in which iron or manganese oxides are imbedded) were published (Vigliotta et al., 2007a; Voelker et al., 1977). The rather unusual propagation of the bacterium by the shedding of nonmotile, spherical cells that arise from simple or multiple septations of the filamented tip (also referred to as “macro- or microgonidia”) was described by Cohn (1870) and, later, in more detail (Voelker et al., 1977).

To date, only a limited number of reports on *Crenothrix* spp. in groundwater wells have been published, with most reports based on physiological appearance (Taylor et al., 1997; Tuhela et al., 1997; Vigliotta et al., 2007a; Voelker et al., 1977), and only some using DNA-based methods, such as qPCR or 16S rRNA gene metabarcoding of grab samples (Braun et al., 2016; Wang et al., 2016). Their occurrence in groundwater wells with subsurface oxygen enrichment and proximity to surface waters has thus far been reported only by Braun et al., (2016). Herein, *Crenothrix* spp. were detected via 454 pyrosequencing and qPCR in some of the 22 single timepoint water samples from well fields next to the Amur River in Russia, whereas the presence of traces of methane was also reported by Braun et al., (2016). Case studies performing long-term series investigations and reporting similar recurring seasonal patterns of *Crenothrix* spp., as reported here, have not been found in the literature. The approach chosen in the current study, is thus proposed a novel approach that could be helpful for other researchers and/or well operators to study this presumably widespread but rarely ever investigated phenomenon.

4.3. Reported metabolic properties of *Crenothrix* spp

Most of the literature on *Crenothrix* spp. is from research in microbial ecology focussing on stratified lakes or lake sediments in association with their methane uptake under oxygen-depleted and oxygen-rich conditions (Frindte et al., 2016; Mayr et al., 2020; Oswald et al., 2017; Su et al., 2023). Their metabolic potential for methane oxidation (*i.e.*, type I methanotrophs) has been assessed via genetic analysis, revealing the presence of *pmoA* genes encoding for a particulate methane monooxygenase A (Oswald et al., 2017; Stoecker et al., 2006). Additional investigations with radioactive-labelled carbon sources (methane/methanol) have confirmed the methanotrophic lifestyle of *Crenothrix* spp. or the closely related species, *Clonothrix fusca* (Oswald et al., 2017; Vigliotta et al., 2007a). Oswald *et al.,* (2017) reported not only the presence of *pmoA* genes encoding a methane monooxygenase but also the occurrence of genes associated with the usage of nitrate as electron acceptors (*e.g., narGHI* encoding nitrate reductase) for methane oxidation in the absence of oxygen. Mayr et al., (2020) investigated the presence of methane-oxidizing bacteria along the oxygen-methane counter gradient of stratified lakes, supporting the niche differentiation theory of methane-oxidizing bacteria including *Crenothrix* spp.

Frindte *et al.,* (2016) highlighted the ability of *Crenothrix* spp. to grow under various redox conditions, *i.e.*, aerobic/anaerobic conditions, which, to our knowledge, is one of the only reports of successful quasi-cultivation in mesocosms using sediment cores. They reported an increase in the active microbial community composition after 7 days from 4–5 % to 69 % relative abundance of *Crenothrix* spp. under changing oxic/anoxic conditions. The authors additionally stated that the high manganese concentrations of Lake Stechlin may have promoted the growth and activity of *Crenothrix*-like organisms, which seem to be well adapted to frequent redox changes.

Besides other bacterial populations *Crenothrix* spp. were frequently observed in sand filters used for manganese and iron removal (Cai et al., 2015; Cheng et al., 2017; Madoni et al., 2001; Stoecker et al., 2006). In these studies these bacteria were referred to as manganese oxidizing bacteria (Cheng et al., 2017), whereas little information on their specific mechanism exists (Tuhela et al., 1997). However, they are known to deposit manganese or iron oxides within their sheathed filaments, indicating an association with these minerals and likely a dependence on them for growth (Frindte et al., 2016). Reported cultivation attempts of *Crenothrix* spp. were not successful (Vigliotta et al., 2007a), limiting research on their characteristics, especially on their use of essential elements or nutrients. In contrast, other filamentous sheathed bacteria, such as *Leptothrix* spp. (Suzuki et al., 2012) or *Clonothrix fusca* (Vigliotta et al., 2007b), were successfully cultivated in a pure culture. Also, no genome data on manganese or iron oxidation has been reported for *Crenothrix* spp. In contrast, for other filamentous sheathed bacteria, such as *Leptothrix ochracea*, a mixotrophic lifestyle has been identified, based on the presence of genes related to iron oxidation and carbon fixation (Tothero et al., 2024).

These metabolic characteristics can add important information for the interpretation of our observed results at the affected well M4. The presence of nitrate found in the well water and the infiltrated surface water would make methane consumption under anoxic conditions for *Crenothrix* spp. possible (see Fig. 9). Additionally, the oxygen enrichment probes, positioned only in a semicircle, are probably inducing changing aerobic and anaerobic conditions, which is reported as beneficial for the proliferation of *Crenothrix* spp. (see Supplementary Figs. S17 and S18). Furthermore, manganese concentrations, potentially being essential for growth, significantly correlated with seasonal patterns of genus *Crenothrix* (see Supplementary Figs. S14 and S18).

4.4. Explanation for seasonally recurring patterns of Crenothrix spp

Considering the reported metabolic characteristics of *Crenothrix* spp. and the results presented here, a model was formulated in an attempt to explain their seasonally recurring rise and decline in community composition in association with particle formation for the conditions found in well M4 (summer and winter), as well as for the absence of both in distal wells D or G.

In Fig. 10, three different exemplary scenarios (A, B, and C) for alluvial groundwater wells in proximity to surface waters are presented: A) strong particle formation and excessive *Crenothrix* proliferation, as found in summer months in well M4; B) minor insignificant particle formation and no detectable *Crenothrix* proliferation, but presence in a persistent state, as in winter months in well M4 and other wells; and C) no particles and no *Crenothrix* detectable for an situation in summer months and wells with sufficient subsurface aeration, as observed in wells G and D.

SCENARIO A: Strong particle formation and *Crenothrix* proliferation. In the summer months, the organic content in the backwater and its sediment, in combination with high temperatures, results in high microbial activity in the hyporheic zone and in the aquifer, leading to oxygen consumption. Water at relatively high temperatures already has low amounts of dissolved oxygen; therefore, anaerobic conditions and low redox potentials, *i.e.*, reducing conditions, are reached during riverbank filtration. These changes result in the conversion of manganese compounds to their reduced and soluble state (Mn^{2+}), enabling its mobilization in the aquifer (see *e.g.,* manganese migration model by Yang et al., (2023) or Paufler and Grischek, (2018). Under strong oxygen depletion, redox potentials reach levels where the formation of methane by microbial activity is supported (panel A, Fig. 10). The co-occurrence of manganese and molybdenum has previously been shown to promote the anaerobic conversion of carbon sources, particularly via biogenic

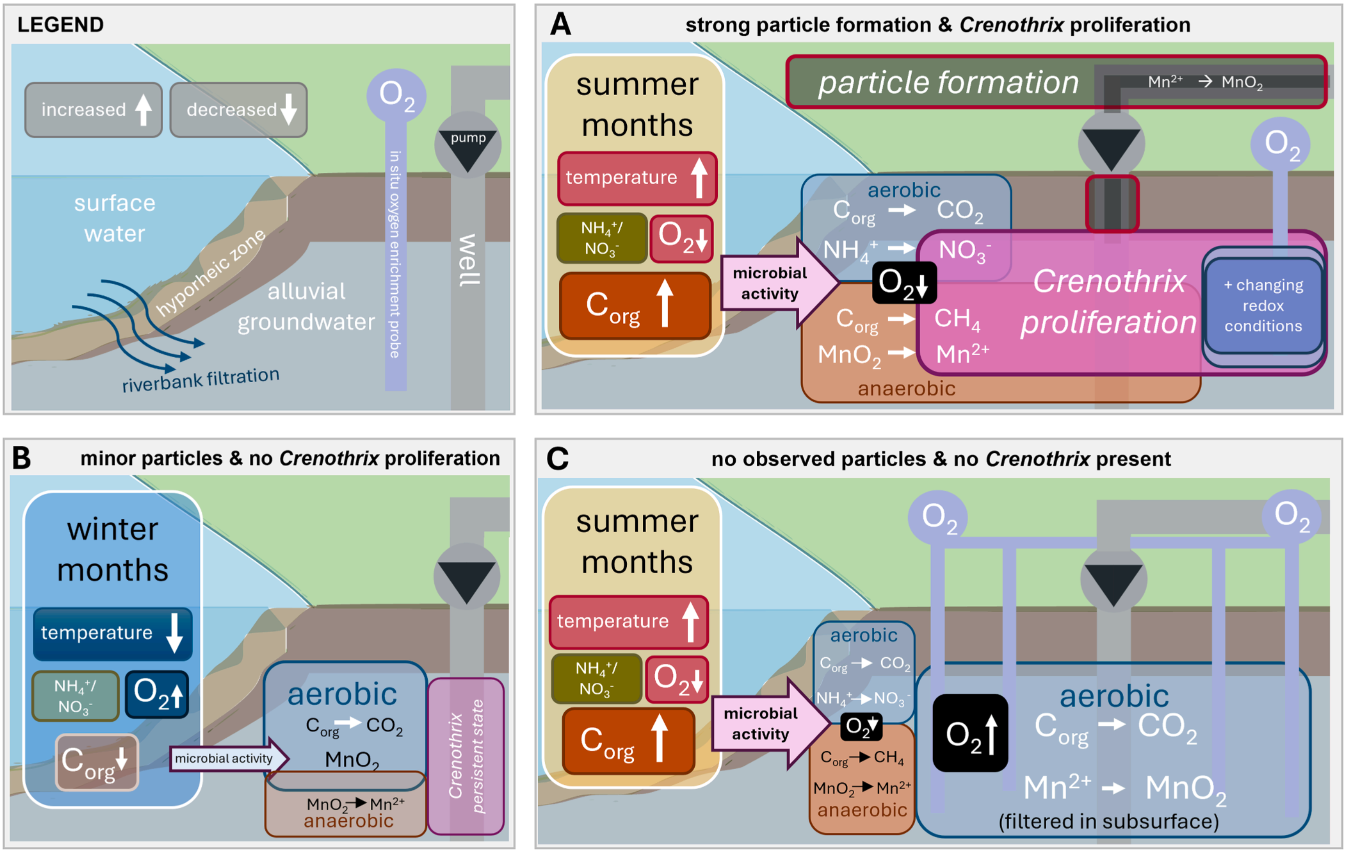

Fig. 10. Graphical visualization of the explanation model for different scenarios, with the legend given in the upper left corner. A) Scenario "strong particle formation and *Crenothrix* proliferation": summer months, high organic content in the surface water, high temperature, and therefore low dissolved oxygen and high microbial activity leading to oxygen depletion in the aquifer and manganese dissolution and methane formation, representing ideal conditions for excessive *Crenothrix* spp. proliferation associated with particle formation. B) Scenario "minor particles & no *Crenothrix* proliferation": Winter months, lower temperature, higher oxygen and lower organic matter concentrations in surface water, and therefore no methane formation and manganese reduction. C) Scenario "no observed particles and no *Crenothrix* present": Summer months with higher temperatures but sufficient oxygen enrichment in the aquifer; therefore, aerobic conditions.

methane formation (Cai et al., 2018). *Crenothrix* spp. oxidize methane either under oxygen-rich or oxygen-depleted conditions using nitrate as an alternative electron acceptor (Oswald et al., 2017). Nitrate can be introduced by riverbank filtration directly or is resulting from the nitrification of ammonia in the subsurface. Insufficient oxygen enrichment via probes can induce changing anaerobic aerobic conditions in the aquifer, further enhancing proliferation of *Crenothrix* spp. (Frindte et al., 2016). The formation of micro- and macrogonidia for the proliferation and spread of *Crenothrix* spp. (Cohn, 1870; Voelker et al., 1977), can further lead to their fast growth and strong increase in relative abundance in the microbial community. *Crenothrix* spp. are associated with manganese or iron by way of the deposition of the minerals in their sheathed filaments (Jackson, 1902; Voelker et al., 1977), while the role of manganese and associated mechanisms are yet to be explored. In the case of insufficient oxidation and removal of manganese in the subsurface, black–brown manganese-containing particles are present in the abstracted well water, or formed further along in the drinking water distribution system, posing major problems there (see panel A, Fig. 10).

This scenario was postulated for the situation at well M4. Even though methane was not directly measured, its formation during the summer months was highly likely. This theory was supported by an observed increase in methanotroph genera, in addition to *Crenothrix*, such as *Methylotenera* and MM1, both belonging to the family *Methylophilaceae*, known for utilizing C1 carbon sources (Kalyuhznaya et al., 2009). Another genus increasing in abundance, Candidatus *Methylomirabilis*, was also reported to use methane under oxygen depleted conditions, using nitrite as an alternative electron acceptor (Mayr et al., 2020; Su et al., 2023). Methane, in combination with changing oxygen rich/oxygen depleted conditions and in the presence of nitrate, was possibly promoting *Crenothrix* spp. growth, outcompeting other bacteria. Measurements taken over a shorter timespan also supported the explanation model. Low oxygen concentrations in samples withdrawn from a probe in the aquifer south of the well highlighted oxygen depletion (see Supplementary Fig. S20). Single timepoint analysis of dissolved organic carbon (DOC) concentrations in the riverbed of the backwater showed concentrations of 14–20 g/L. This supported the hypothesis of induced oxygen depletion during riverbank filtration and changed redox potentials in the summer months, leading to manganese reduction to soluble/mobile Mn^{2+}, and methane production. This theory was further supported by the measurements of dissolved manganese peaking in the summer months, showing the same pattern as *Crenothrix* spp. (see Figs. 5 and 9).

SCENARIO B: Minor insignificant particles and no detectable *Crenothrix* proliferation. In winter, owing to lower temperatures and therefore decreased overall microbial activity and higher oxygen concentrations in the surface water, aerobic conditions in aquifers are maintained. Redox potentials may nevertheless be in a range where manganese is reduced to its soluble form, whereas no methane is formed, as necessary anaerobic/anoxic conditions (highly reducing, low redox potentials) are not reached (see Stumm, 1995). Minor particles may nevertheless be present due to their insufficient removal during soil passage, but conditions for *Crenothrix* spp. proliferation are less favourable due to the absence of methane (see panel B, Fig. 10). Even though a decrease in the relative abundance of *Crenothrix* spp. in the water matrix is visible in the winter months, it can be assumed that the filaments are still present and remain attached to the well pipes or the gravel of the aquifer in a persistent state. Such a scenario could also have been the case in the proximal wells M1, M2, and M3 or the distal wells A and E, where redox potentials were at times within the range, also producing traces of methane, and therefore leading to the growth of *Crenothrix* and the minor formation of particles. Given the periodic oxygen enrichment at the wells A and E, also changing aerobic and anaerobic conditions could be the case. Additionally, the overall dissolved oxygen concentrations in the well water were lower (see Table 1), but low dissolved manganese concentrations were observed (see Supplementary Table S5).

SCENARIO C: No detectable particles and no *Crenothrix* occurrence. In the case of sufficiently stable aerobic conditions, manganese, which could be present in a reduced state, is oxidized and removed by passage through the subsurface. No methane is formed, and therefore, no favourable conditions for *Crenothrix* spp. are present. This could have been the case at distal wells D and G, which had constant oxygen enrichment via the probes in a full circle (see panel C, Fig. 10), due to which aerobic conditions in the aquifer were maintained and therefore no methane or soluble manganese (Mn^{2+}) were formed. The conditions required for both *Crenothrix* spp. proliferation and particle formation were therefore not present and were not observed during the sampling period.

4.5. Proposing Crenothrix spp. as an indicator for groundwater management problems

On the basis of the findings of this study, it can be postulated that the dominant recurring patterns of *Crenothrix* spp. in the wells under investigation were associated with: i) black–brown particles in drinking water, which was already described in the context of *Crenothrix* spp. discovery 150 years ago (de Vries, 1887; Zopf, 1879), ii) organic matter and strong anaerobic conditions in the aquifer, as the formation of methane promotes their growth (de Vries, 1887; Schweisfurth, 1975), iii) manganese and iron oxides (*i.e.*, black–brown particles) by *e.g.,* way of their incorporation in the sheathed filaments of *Crenothrix* spp. (Vigliotta et al., 2007a; Voelker et al., 1977). Additionally, owing to their specific lifestyle, *Crenothrix* spp. have been observed to persist even under unfavourable conditions by attaching their filaments to surfaces (*e.g.*, well walls and water pipes). Once favourable conditions return (due to seasonal changes or the influx of surface water containing the required elements), they can massively proliferate by spreading via micro- and macrogonidia, resulting in their dominance in the microbiome. The conditions found in well M4 highlighted the interplay of factors promoting the excessive growth of *Crenothrix* spp. in an artificially established “environmental reactor”. In fact, the system reported herein demonstrated a very efficient *in situ* cultivation of *Crenothrix* spp., which could provide relevant information for future laboratory cultivation attempts.

In summary, the strong particle formation and prevalence of *Crenothrix* spp. in well M4 was most likely the result of: **i**) the infiltration surface water rich in organic carbon, which was enhanced by high groundwater withdrawal; **ii**) elevated microbial activity at higher temperatures in the summer months; **iii**) leading to oxygen depletion and low redox potentials; **iv**) resulting in manganese mobilisation and methane formation; and **v**) additional apparently changing aerobic and anaerobic conditions, which, on the one hand, **vi**) highly efficiently promoted *Crenothrix* spp. proliferation; and, on the other hand, **vii**) resulted in particle formation due to insufficient oxidation and removal of manganese.

Problems arising from all these potentially interrelated factors could be indicated by a quantitative analysis of only one indicator taxon, namely, *Crenothrix* spp. Therefore, we propose *Crenothrix* spp. as a potential indicator of operational issues associated with manganese and iron contents, organic carbon content and inconsistent redox conditions, hence fluctuating aerobic/anaerobic conditions, in alluvial aquifers used for drinking water production.

The suggested use of *Crenothrix* spp. as a technical treatment indicator is facilitated by the use of molecular methods (*i.e.*, qPCR or sequencing), which are increasingly implemented in practical water quality testing (Demeter et al., 2023). Other easy-to-use assays for analyzing potentially adverse bacteria associated with technical drinking water issues, such as the Laboratory Biological Activity Reaction Test (LAB-BART), have analytical limitations in terms of specificity and quantifiability (Oudega et al., 2023). In contrast, genetic methods offer great specificity and sensitivity, enabling targeted and quantitative analysis. Another advantage of molecular based methods is the possibility to measure several targets in a single sample (toolbox approach). Furthermore, the samples can be stored and analyzed at a later time, enabled by biobanking *i.e.,* storage of frozen DNA extracts (< 1 mL). However, further studies are needed to confirm the suggested treatment indicator capacity of *Crenothrix* spp. globally and the suitability of associated genetic assays.

For drinking water utilities in comparable biogeochemical contexts, the occurrence of *Crenothrix* spp. in alluvial wells can be considered a sensitive indicator for the high possibility of associated particle formation issues. In the case of low detected concentrations (as at wells A, E, M1 – M3), conditions were not (yet) causing particle problems but could do so if, for example, if withdrawal volumes would be increased. The total absence of the proposed indicator, as observed at wells D and G, would indicate that there is a low potential for operational particle-associated issues.

5. Conclusions

- This comprehensive and systematic study of drinking water wells revealed to date unreported, seasonally recurring patterns of *Crenothrix* spp. These patterns were associated with the deterioration of aesthetic drinking water quality from alluvial groundwater wells used for drinking water production. The comparative and long-term investigations presented here involved not only the analysis of water from the affected well, but also from proximal and distal wells. These had similar hydrogeological and technological characteristics, even though they boasted minimal or no particle issues and only minor *Crenothrix* spp. in water from these wells.
- An explanatory model for the high rise and decline of *Crenothrix* spp. and the formation of black−brown particles, based on the observed results and the reported metabolic characteristics of *Crenothrix* spp., was established. In summary, the strong particle formation and high prevalence of *Crenothrix* spp. in well M4 were most likely driven by a complex interplay of factors that led to operational challenges. These factors include: i) infiltration of surface water rich in organic carbon; ii) increased microbial activity during the warmer summer months; iii) oxygen depletion and low redox potentials leading to; iv) manganese mobilisation and - most likely - methane formation; v) apparently transitory aerobic and anaerobic conditions that most probably promoted the proliferation of *Crenothrix* spp.; vii) particle formation due to inadequate manganese oxidation and removal.
- Based on the results of this study *Crenothrix* spp. is proposed as a potential and easy-to-measure genetic indicator/marker (*e.g.,* using qPCR or dPCR) for groundwater resource management and treatment failure, *i.e.*, involving technical issues associated with the deterioration of aesthetic drinking water quality. In contrast to cultivation-based microbial diagnostics, biobanking can be also an option when using nucleic acid extracts (*i.e.*, storage of samples over month to years at appropriate temperatures). However, the selected monitoring strategy has to be adapted to the respective background situation and question (*e.g.,* evaluating the specific monitoring setup, including factors such as sampling volume, temporal frequency and combinations with other parameters/real-time sensors).
- Although *Crenothrix* spp. were first described over a century ago (also in association with drinking water deterioration), similar in-depth studies using novel molecular-based microbiological and chemical analyzes to explore their role in drinking water production, have not been conducted to date.

CRediT authorship contribution statement

Sophia D. Steinbacher: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Katarina Priselac:** Visualization, Formal analysis, Data curation. **Wolfgang Kandler:** Formal analysis, Data curation. **Domenico Savio:** Investigation, Formal analysis, Data curation. **Julia Vierheilig:** Writing – review & editing, Investigation. **René Mayer:** Investigation. **Katalin Demeter:** Writing – review & editing. **Rita B. Linke:** Writing – review & editing, Resources. **Robert L. Mach:** Writing – review & editing. **Regina Sommer:** Writing – review & editing. **Gerhard Lindner:** Investigation. **Karen Zuser:** Resources. **Claudia Kolm:** Writing – review & editing. **Margaret E. Stevenson:** Writing – review & editing, Formal analysis. **Alfred P. Blaschke:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Alexander K.T. Kirschner:** Writing – review & editing, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Mats Leifels:** Writing – review & editing, Supervision, Conceptualization. **Andreas H. Farnleitner:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123406.

Data availability

Data will be made available on request.

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