Large and interacting effects of temperature and nutrient addition on stratified microbial ecosystems — results from an aquatic model micro-ecosystem

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Competing interests

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Abstract

Aquatic ecosystems are often stratified, with cyanobacteria in oxic layers and phototrophic sulfur bacteria in anoxic ones. Changes in stratification caused by global environmental change are an ongoing concern. Understanding how such aerobic and anaerobic microbial communities, and associated abiotic conditions, respond to multifarious environmental changes is an important endeavor in microbial ecology. Insights come from observations of naturally occurring stratified aquatic ecosystems, and from theoretical models of ecological processes. Here we complement such studies with an experimental approach in the laboratory, using a novel aquatic micro-ecosystem, with distinct oxic/anoxic strata. Our two main objectives are to 1) describe the features of this promising micro-ecosystem and 2) report how the microbial

community composition (full-length 16S rRNA-sequencing) and the abiotic conditions responded to two globally relevant environmental changes (temperature and nutrient addition). The composition of the strongly stratified microbial communities was highly affected by temperature and by the interaction of temperature and nutrient addition. Dissolved oxygen and pH responded to the treatments, with differences in effect between the oxic and anoxic zone. Further research with this experimental system could focus on effects of environmental change on spatial distribution and on stability of the communities to press and pulse perturbations.

Introduction

Microorganisms are key players in nearly all ecosystems, ranging from the human gut to marine and freshwater habitats. The functioning of microbial communities is critical for many ecosystem services, since microorganisms are the driving force behind biogeochemical cycles [1–4]. Nevertheless, microbes are highly dependent on environmental conditions; even slight changes can lead to taxonomic and functional community shifts [5, 6]. Consequently, microbial communities and their functions are significantly affected by global change [7], which has recently led to the emergence of the research field termed “Global Change Microbiology” [8].

Stratification of productive lakes is a common phenomenon observed in summer, resulting in warm and oxygen-rich upper water layers, dominated by cyanobacteria and algae, and colder anoxic deeper layers, that harbor heterotrophic biomass-degraders and sulfate-reducing bacteria. In between these layers, the

metalimnion can be found, which is formed by decreasing light-intensities and oxygen concentrations (microaerophilic), and an increasing pool of reduced sulfur compounds, creating ideal niches for phototrophic sulfur bacteria and chemolithoautotrophic microorganisms [9–11].

Beside chemical and physical parameters that influence the presence of oxic and anoxic layers, it was recently postulated that mutual inhibition between cyanobacteria and anaerobic sulfur-dependent bacteria creates and maintains the distinct oxic and anoxic zones of such ecosystems [12]. The mathematical model used in that study predicts abrupt transitions between aerobic and anaerobic microorganisms and the occurrence of oxic-anoxic regime shifts. Moreover, studies have demonstrated that global change consequences could have strong impacts on this sensitive ecosystem, including dominant growth of harmful cyanobacteria due to warming [13], or increasing primary production and creation of anoxic layers due to increased nutrient input [14].

The mentioned studies show that important insights come from analyses of observations of naturally occurring aquatic ecosystems, and from theoretical models of the plausible and relevant ecological and biochemical processes. In addition, in the field of global change biology, targeted experiments that employ a limited number of standardised synthetic model ecosystems have been described as an “entirely different approach” [15] and are considered vital alongside studies of naturally occurring ecosystems [16] and hybrids of both approaches [17]. Key challenges in global change microbiology that are amenable to research with synthetic ecosystems include the role of evolutionary processes, the role of historical contingency [18] and interactions among microorganisms [19], and the integration of data and theory [18].

71 Experiments about the role of environmental, organismal interactions and biochemical
72 feedbacks in stratified aquatic micro-ecosystems appear absent, however.
73 Establishing suitable such standardised-model ecosystem for analyzing
74 potential responses of a broad range of microorganisms remains crucial. Being able
75 to monitor global change responses of diverse functional groups of potentially
76 interacting microbes is a desirable feature of such an experimental system, which point
77 towards systems with strong abiotic gradients in space and or time. In this work, we
78 get inspiration from an “old” but highly valuable approach, the Winogradsky column
79 [20, 21]. We constructed a modified and smaller version that allows a highly replicable
80 development of a broad range of complex and dynamic microbial groups in one
81 experimental unit. In contrast to classical Winogradsky columns, our micro-
82 ecosystems are mostly liquid, with only a small sediment layer (~ 6 % v/v of the
83 column). Thus, we created a highly replicable liquid oxic-anoxic interphase and self-
84 developing model systems.
85 By applying this approach, we were able to analyze microbial responses to
86 multifarious environmental change (temperature and nutrient addition manipulated
87 factorially), including the responses of community composition and abiotic
88 environmental conditions. As well as showing how microbial community composition,
89 dissolved oxygen, pH, and hydrogen sulphide respond to temperature and nutrient
90 addition, we highlight the potential for global change microbiology research of this new
91 approach coupled with state-of-the-art sequencing technologies.
92 We hypothesize that higher temperature will increase the dominance of
93 anaerobic microbes due to an increase in the extent of the anoxic zone, due to the
94 lower solubility of oxygen in warmer water. We hypothesize that nutrient addition
95 (addition of ammonium phosphate) will alter community composition, but we cannot *a*

priori say how, as this would depend on features of the organisms, such as competitive and facilitative interactions among them, which we do not have sufficient information about. We did not have any *a priori* expectation of whether the combined effect of temperature and nutrient addition would be additive (no interaction), more than additive (positive interaction), or less than additive (negative interaction). We anticipated the potential for micro-ecosystems to become entirely oxic or entirely anoxic, and for changes in composition to be non-linear and to potentially include discontinuous responses that are predicted by theory.

Materials and methods

Preparation of micro-ecosystems

The vessels used to house the micro-ecosystems were standard glass test-tubes (diameter 13 mm, height 16 cm). Each was equipped with two oxygen sensors (PreSens Precision Sensing GmbH, Germany), one at a height of 4 cm (bottom sensor) and the other at 14 cm (top sensor), both stuck to the inside surface of the test-tube wall. These sensors allow optical measurements of oxygen to be taken through the wall of test tube.

Each test-tube (which we also refer to as a “column”) had a butyl rubber stopper on top, with two canulas. The longer canula (StericanR, length 120mm, diameter 0.8 mm) reached to the depth of the bottom O_2 sensor and was covered with an oxygen-protecting plug (BD Plug). The shorter canula (length 40 mm, 0.9 mm diameter) was always open and extended only into the headspace of the micro-ecosystem. This

shorter canula therefore allowed for gas exchange between the headspace and the atmosphere (Fig. 1a).

Micro-ecosystems were established from sediment and water samples taken in July from a small pond in Zürich, Switzerland (47°23'51.2"N 8°32'33.3"E, temperature of 24.6 °C, pH of 6.7.) at a depth of 30 cm. In total, 200 g sediment and 2 L water were collected. The sediment sample was supplemented with sterile 0.5 % crystalline cellulose, 0.5 % methyl-cellulose, 1 % $CaSO_4$, 0.2 % $CaCO_3$ and 0.01 % $NH_4H_2PO_4$. Sediment was homogenized by mixing for 30 min, before each test-tube was filled with sediment up to a height of 1.5 cm (in 0.5 cm steps, with constant mixing of the source sediment bottle between steps). Sediment that stuck to inner wall of the test-tube was removed carefully in all cases. Columns were filled with well-homogenized 16 mL pond water (supplemented with 0.01 % $NH_4H_2PO_4$), leaving approximately 1 cm of headspace. In contrast to classical Winogradsky columns [20–22], our set-up is dominated by liquid and contains a small amount of sediment. Columns were incubated at room temperature for two hours without light to allow the sediment to settle fully, after which an initial oxygen concentration of the top and bottom sensor was recorded and the columns were placed in incubators.

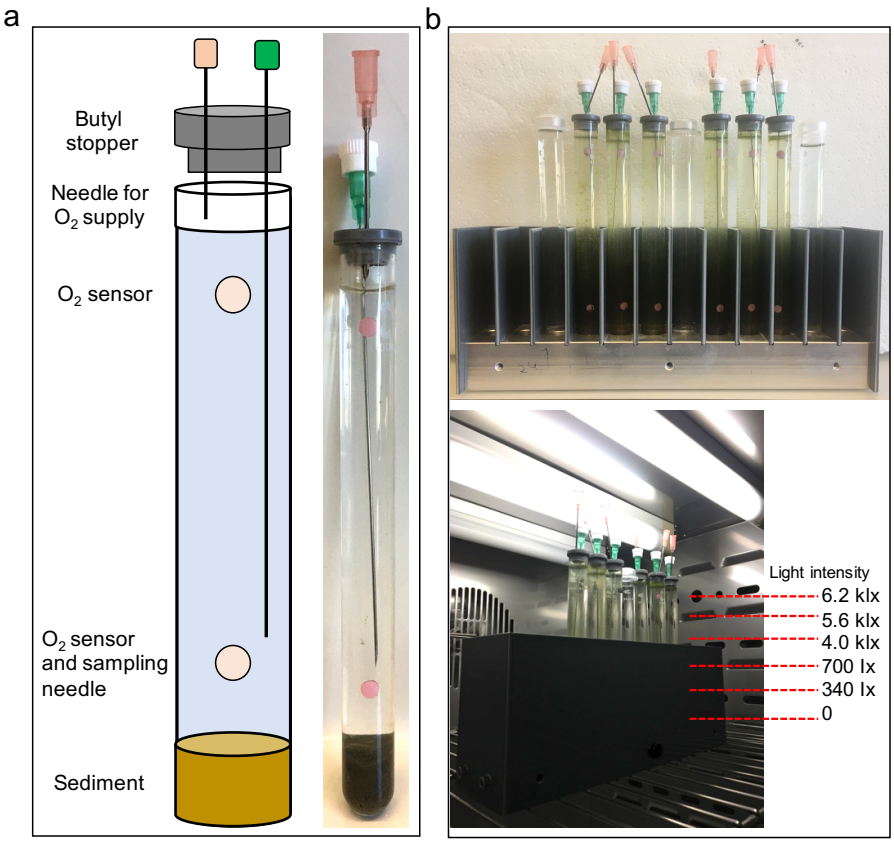

Fig. 1 Preparation and incubation conditions of the micro-ecosystems

(**a**) Columns were filled with 1.5 cm sediment and pond water with supplementary resources (see main text for details). Columns were equipped with two oxygen sensors (top and bottom), and closed with a butyl rubber stopper. One canula allow gas exchange between the headspace and the atmosphere (pink). A second canula is used for taking samples at the bottom sensor (green, anoxic stopper). (**b**) Micro-ecosystems were incubated in light-gradient-producing holders. Sediment was covered inside the metal base (1.5 cm depth) for incubation without light. Columns were thereby incubated with a light gradient ranging from 6.2 kilo lux (klx) to 0 lux (lx).

151 ## Incubation and treatments

152 Each column was incubated at one of seven temperatures: 12, 16, 20, 24, 28, 32 or
153 36 °C. Incubators (Pol Eco Apparatura sp.j.) were equipped with two fluorescent light
154 sources (PHILIPS Master TL8W/840) with a dark-light cycle of 8:16 hours. Columns
155 were placed in a 1.5 cm hole in a metal base, so that the sediment layer was
156 continuously kept in darkness. Up to a height of 10 cm, the columns were surrounded
157 by a plastic holder that causes a gradient of light, ranging from 340 lx at the intersphere
158 of sediment:water at the bottom and up to 700 lx at the top of the light-protector (Fig.
159 1b). The top of the column experienced fluorescent light intensity of 6200 lx. Each
160 holder contained six columns, in two groups of three replicates, separated by a water-
161 filled column. This arrangement was placed in the middle of both light sources in the
162 incubator, allowing the same light-intensity at the back and front of each column.

163 A second treatment was the manipulation of nutrient concentration. For this, 0.1
164 % $NH_4H_2PO_4$ was added to half of the columns by taking 16 µL of a sterile 10 %
165 $NH_4H_2PO_4$ solution, while 16 µL sterile water was used for the controls. This treatment
166 was performed once per week (day 1, 8, 14, 20) and was factorially crossed with the
167 temperature treatment, resulting in 7 (temperature) x 2 (nutrient levels) and 3
168 replicated, giving a total of 42 columns. These were incubated for 22 days.

169

170 ## Sampling and measurements

171 Liquid samples were taken on day 8, 14 and 20 by collecting 200 ul of liquid from the
172 bottom of the columns by using the installed canula.This liquid was used to measure
173 H_2S (Microsensor, AMT Analysenmesstechnik GmbH, Germany) and was replaced

174 with anoxic sterile lake water. Non-invasive oxygen measurements were performed at
175 the top and bottom sensor of the columns (everyday except of day 5, 11, 12 and 17).
176 After incubation, all columns were destructively sampled. For each column, the
177 whole liquid part (16 mL) and the sediment layer were processed separately. One
178 replicate of each treatment combination had the liquid part separated at the height of
179 the bottom oxygen layer, giving a sample of the upper and lower liquid layer as well
180 as a sample for the sediment. Each sample was centrifuged (16,000 rpm, 3 min) and
181 pellets were frozen at -80 °C until DNA extraction. Supernatant was used for pH
182 measurements, as well as total nitrogen and total organic carbon analysis. While
183 sampling, the H_2S concentration at the sediment-water interphase was measured
184 under anoxic conditions.
185

186 **DNA extraction, 16S rRNA amplification**

187 DNA extractions were performed with ZymoBIOMICS DNA Microprep Kit
188 (ZymoResearch), following the manufacturer`s instructions. 16S rRNA gene
189 amplifications were performed with 27F forward primer (5`-
190 AGRGTTYGATYMTGGCTCAG-3`) and 1592R reverse primer (5`-
191 RGYTACCTTGTTACGACTT-3`), resulting in an amplification product of ~1500 bp.
192 Primers were equipped with 5`phosphate and a 5`buffer sequence of GCATC.
193 Barcodes, PCR chemicals and conditions (27 cycles) were performed as suggested
194 by Pacific Biosciences (PacBio, 2020). PCR products were visualized on an 1 % (w/v)
195 agarose gel and were pooled with equal concentrations. Afterwards, PCR products
196 were purified using AMPure®PB beads (PacBio), following manufacturer`s guidelines.
197

198 ## Library Preparation and Sequencing

199 Sequencing was performed at the Functional Genomic Center Zürich, Switzerland.
200 The SMRT bell was produced using the SMRTbell Express Template Prep Kit 2.0
201 (PacBio). The input amplicon pool concentration was measured using a Qubit
202 Fluorometer dsDNA High Sensitivity assay (Life Technologies). A Bioanalyzer 12Kb
203 assay (Agilent) was used to assess the amplicon pool size distribution. 0.75µg of
204 amplicon pool was DNA damage repaired and end-repaired using polishing enzymes.
205 A ligation was performed to create the SMRT bell template, according to the
206 manufacturer's instructions. The SMRT bell library was quality inspected and
207 quantified using a Bioanalyzer 12Kb assay (Agilent) and on a Qubit Fluorimeter (Life
208 technologies) respectively. A ready to sequence SMRT bell-Polymerase Complex was
209 created using the Sequel II Binding Kit 2.1 and Internal Control 1.0 (PacBio) according
210 to the manufacturer instruction. The Pacific Biosciences Sequel II instrument was
211 programmed to sequence the library on 1 Sequel II SMRT Cell 8M, taking 1 movie of
212 15 hours per cell, using the Sequel II Sequencing Kit 2.0 (PacBio). Sequencing data
213 quality were checked, via the PacBio SMRT Link software, using the "run QC module".
214 Sequencing raw data are deposited at NCBI SRA under the BioSample accession
215 numbers SAMN16774060 to SAMN16774152.
216

217 ## Bioinformatics

218 Bioinformatics were performed using R and the package Dada2 [23]. In short, reads
219 were filtered regarding primer sequences, length (1300-1600 bp), quality, error rates
220 and chimeras. The outcome of the filter processes can be observed in Supplementary
221 data for each sample. Finally, the constructed sequence table was aligned with the

SILVA ribosomal RNA database [24], using version 138 (non-redundant dataset 99). Afterwards, a phyloseq formatted data object was constructed, containing the OTU table, taxonomy table and sample metadata, using the package phyloseq [25], which was also used for microbial community analysis together with the vegan package [26]. All sequences that were assigned to “chloroplast” were removed from the phyloseq file, since primers were chosen for bacterial 16S rRNA gene. Microbial community composition was analysed with non-metric multidimensional scaling (NMDS), using the metaMDS function from the vegan package. The metaMDS function was instructed to use Bray-Curtis distance, to transform the data to have 2 dimensions, and to try 200 different starting positions in order to achieve convergence. NDMS was performed on relative abundances of OTUs with a relative abundance of at least 1% in at least one sample. Linear models with appropriate error structures were used to assess the weight of evidence supporting causal effects of treatments and treatment interactions on response variables. We intentionally avoid referring to effects as significant or not significant, and rather use the terms: no evidence $p > 0.1$, weak evidence $0.1 > p > 0.05$, moderate evidence $0.05 > p > 0.01$, strong evidence $0.01 > p > 0.001$, very strong evidence $p < 0.001$.

Results

Macroscopic observations

All columns developed a stratified growth of colorful microorganisms within the incubation time, regardless of the temperature and nutrient addition treatment (Fig. 2a & b). Macro- and microscopical observation of morphology and color indicated that the micro-ecosystems likely consisted of a relatively large upper layer of cyanobacteria and algae and phototrophic sulfur bacteria in a smaller lower layer. Moreover, a white layer occurred in all columns during the first 10 days (Fig. 2c) at different positions depending on the incubation temperature (12 °C and 16 °C below the bottom-sensor, and at higher temperatures above the bottom-sensor). DGGE analysis identified this layer as a consortium of species of the microaerophilic genus *Magnetospirillum*, indicating that this layer is the oxic-anoxic interphase of the micro-ecosystems. In general, all micro-ecosystems developed an oxic and anoxic part (Fig. 5), and triplicates of each treatment combination predominantly showed comparable growth and development. Gas formation was observed in the sediment layers of the columns, indicating an active microbial community (Fig. 2d).

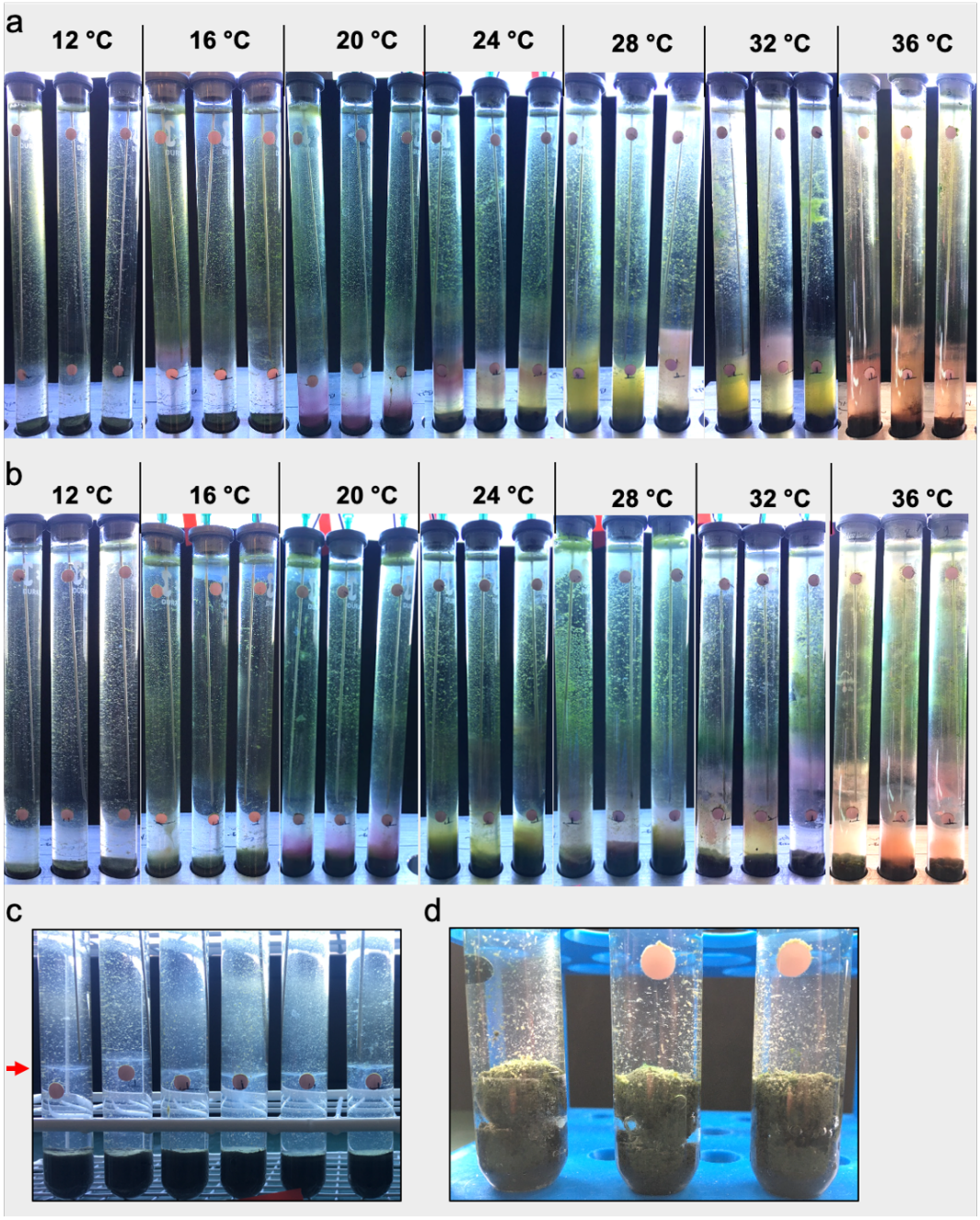

Fig. 2 Macroscopical observation of the micro-ecosystems. **(a)** Control micro-ecosystems (triplicates) after incubation at 12-36°C for 22 days. **(b)** Nutrient addition micro-ecosystems (triplicates) after incubation at 12-36 °C for 22 days. **(c)** Observation of a white bacterial cloud within 10 days of incubation. Exemplary columns incubated at 20 °C of day 10 are shown. Red arrow highlights the position of cloud. **(d)** Gas formation in the sediments, exemplary picture taken at day 12 of columns incubated at 28 °C.

271 **Relative abundances of microbial taxa of the liquid part**

272 In order to study the microbial communities in detail, full-length 16S rRNA sequencing
273 was performed. Removal of sequences that did not occur at more than 1 % in at least
274 one sample resulted in 343 different OTUs (from initial 5633 unique sequences in all
275 samples).

276 All of the micro-ecosystems at 12 °C were dominated by members of the order
277 *Burkholderiales* (54 – 69 %) (Fig. 3a, Fig. S1 for details). Relative abundance of
278 *Burkholderiales* decreased to 15 – 37 % (control) and 29 – 53 % (nutrient addition) at
279 16 °C, and relative abundance of *Rhodospirillales* increased up to 23 %. At 20 °C,
280 members of *Chlorobiales* and *Chromatiales* became more abundant. Members of
281 *Chlorobiales* were dominant in all micro-ecosystems at 24 °C (33 – 64 % controls, 62
282 – 64 % nutrient addition-treatments), 28 °C (42 – 92 % controls, 35 – 70 % nutrient
283 addition-treatments), and 32 °C (78 – 90 % controls, 18 – 50 % nutrient addition). At
284 36 °C, members of *Chlorobiales* disappeared, and in no-nutrient-addition treatments,
285 members of the cyanobacterial order *Limnotrichales* were dominant (up to 25 %; but
286 none occurred in nutrient addition treatment).

287 The full-length 16S rRNA amplicon sequencing resulted in a detailed account
288 of the microbial community to genus level (Fig. 3b, Fig. S2-S8 for details). All micro-
289 ecosystems at 12 °C (Fig. 3b, Fig. S2) were dominated by members of the aerobic
290 genus *Giesbergeria* (controls 22 – 34 %, nutrient addition 57 – 67 %), followed by
291 *Aquaspirillum* in the controls and *Flavobacterium* in the nutrient-addition micro-
292 ecosystems. At 16 °C (Fig. 3b, Fig. S3), control micro-ecosystems were dominated by
293 members of the aerobic genus *Uliginosibacterium* (12 – 27%), which had a very low

294 abundance in the nutrient addition treatment (less than 2 %). In contrast, *Giesbergeria*
295 and *Aquaspirillum* (4 – 35 %) were very abundant in the nutrient addition treatment.
296 Genus composition at 20 °C (Fig. 3b, Fig. S4) included anaerobic phototrophic
297 sulfur bacteria, in particular the green sulfur bacteria *Chlorobium* appeared highly
298 abundant in the nutrient addition treatments (up to 46 %) and the purple sulfur bacteria
299 *Chromatium* in all micro-ecosystems at this temperature (up to 28 %). With increasing
300 temperature, green sulfur bacteria *Chlorobium* and *Chlorobaculum* genera increased
301 in relative abundance. At 24 °C (Fig. 3b, Fig. S5), *Chlorobium* and *Chlorobaculum*
302 were detected in the controls, with 11 – 39 % and 6 – 25 % relative abundance,
303 respectively, and in the nutrient addition treatments with 39 – 65 % and 27 – 60 %.
304 Furthermore, the picocyanobacterial genus *Cyanobium* appeared (up to 7 %) in the
305 control micro-ecosystems.
306 At 28 °C (Fig. 3b, Fig. S6), green sulfur bacteria continued to increase their
307 dominance, especially *Chlorobium* (up to 92 %). Interestingly, the *Control_3* column
308 consisted of a comparable lower amount of green sulfur bacteria, but additionally of
309 the purple sulfur bacteria *Allochromatium* (41 %). This finding can be confirmed by the
310 macroscopical observation of the pink layer (Fig. 2a). Additionally, the *Nutrient*
311 *addition_2* column consisted of *Chlorobaculum* instead of *Chlorobium* in contrast to
312 the *Nutrient addition_1* and *Nutrient addition_3* columns.
313 At 32 °C (Fig. 3b, Fig. S7), *Chlorobium* dominated the control-micro-
314 ecosystems, while this genus also had lower though still high relative abundance in
315 the nutrient addition treatments (up to 52 %).
316 At 36 °C (Fig. 3b, Fig. S8), the microbial community changed again: *Chlorobium*
317 and *Chlorobaculum* disappeared totally. Present instead were the facultatively
318 anaerobic, sulfur-oxidizing bacterium *Sulfuricurvum* (up to 49 %), the cyanobacterial

genera *Nostoc* (up to 36 %) and *Limnothrix* (up to 25 %), and the phototrophic sulfur bacteria *Phaeospirillum* (up to 11 %).

Separate sequencing and analysis of the upper and lower layer of liquid revealed dominance of phototrophic sulfur bacteria in the lower anoxic layer and of cyanobacteria in the upper oxic layer of the columns (Fig. S9).

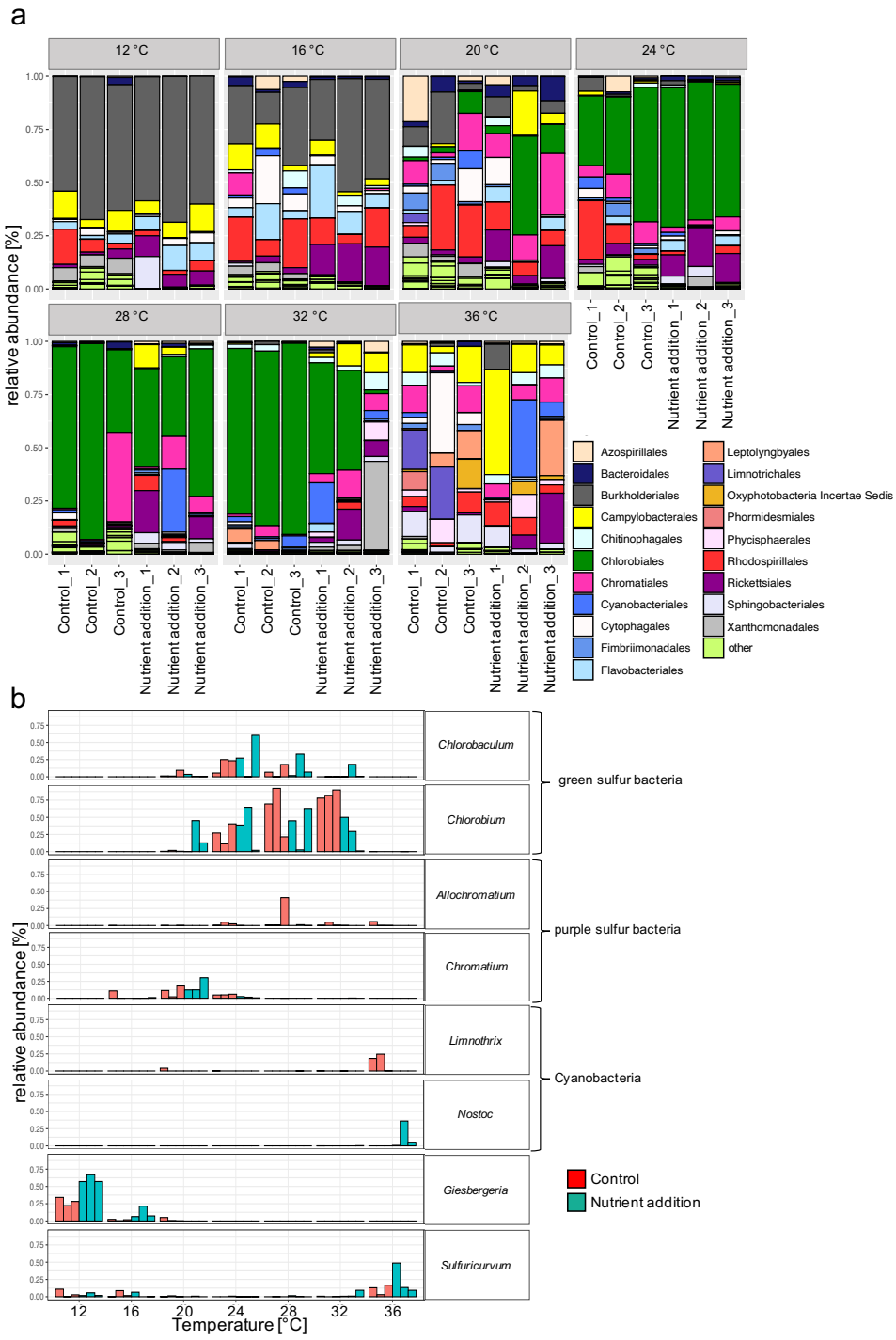

Fig. 3 Microbial community composition of the micro-ecosystems. (a) Relative abundance on order level is shown for each micro-ecosystem. Orders with relative abundances < 7 % in all samples were assigned to “other”. **(b)** Relative abundance of 8 dominant and functional important genera depending on the temperature and treatment conditions. Red bars represent control triplicates, blue bars nutrient addition triplicates.

332 **Community composition (NMDS) of the liquid part**

333 The effects of temperature and nutrient addition on the relative abundance described
334 in the previous sections were very evident in the analyses of community composition
335 via NMDS (Fig. 4a). Compositional variation along the first NMDS axis (hereafter
336 NMDS1) was strongly associated with temperature, but not with the nutrient addition
337 treatment, nor the interaction of the temperature and nutrient addition treatment (Fig.
338 4b) (Table 1). In contrast, compositional variation along NMDS2 was affected by a
339 strong interaction between temperature and the nutrient addition treatment (Fig. 4c)
340 (Table 1). Effects of temperature on NMDS2 scores were non-linear in both the control
341 and the nutrient addition treatments. In the control treatment, the NMDS2 score
342 remained constant from 12 to 24 °C and then decreased up to 36 °C. In contrast, in
343 the nutrient addition treatment NMDS2 values first decrease (12 to 16 °C), then
344 increase (16 to 28/32 °C) and then decrease again (32 to 36 °C).
345

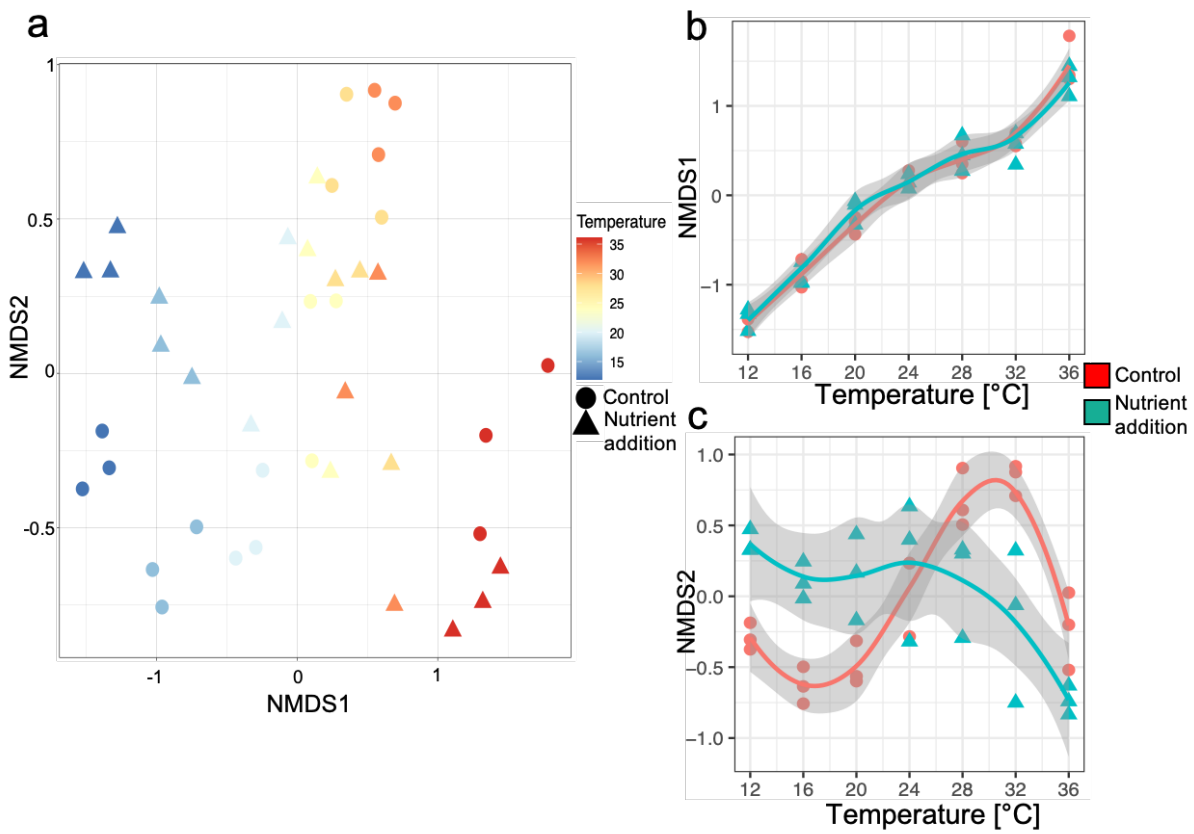

Fig. 4 Variation in microbial community composition. (**a**) NMDS scores based on Bray-Curtis distance showing the variation in microbial community composition of the liquid part of the columns. (**b**) The effect of temperature and nutrient addition on NMDS1. (**c**) The effect of temperature and nutrient addition on NMDS2. (**b** & **c**) Control micro-ecosystems are shown as red circles, nutrient addition-treated micro-ecosystems as blue triangles. Lines are local polynomial regressions; grey ribbons show the 95% confidence intervals.

Microbial communities within the sediments

The community composition of taxonomic orders in the sediment was comparable to that in the liquid part (Fig. S10), though a high relative abundance of *Chlorobiales* and *Chromatiales* was detected between 20 and 32 °C. All ecosystems (except 12 °C) were rich in relative abundance of members of the anaerobic order *Spirochaetales* (up to 48 %). On genus level, however, the community consisted of a majority of sequences that could not be assigned to known taxonomic groups. With focus on sulfate-reducing microorganisms, the sediment harbored members of the order *Desulfutomaculales* up to 2 %. Besides that, genera including *Desulfobulbus*, *Desulfovibrio*, *Desulfovirga, Desulfomicrobium*, *Desulforhabdus,* were detected*,* but all in a low relative abundance of less than 1 %. Furthermore, members of the anaerobic biomass-degrading family *Clostridiaceae* made up to 5 % of the communities, depending on the temperature.

Oxygen dynamics

Oxygen concentration in the top of the columns increased within the first 10 days in all incubations, and then remained oxic until the end of the experiment (Fig. 5a). Moreover, oxygen concentrations in the upper part of the columns were in general higher than 21% (concentration expected if in equilibrium with the atmosphere), and sometimes reached up to 60 %. We assume that oxygen production took place at the upper part of the columns by the algae and cyanobacteria, causing this high level of dissolved oxygen. The controls and the nutrient addition-treated columns showed only slight differences in their oxygen levels.

The long-term effect of temperature and nutrient addition on oxygen concentration in the upper part of the columns was calculated as the mean of the oxygen concentration during the last three days of the experiment (Fig. 5c). There was very strong evidence that temperature decreased the oxygen concentration from about 35 % at 12 °C to about 29 % at 36 °C (Table 1). There was moderately strong evidence that the nutrient addition treatment reduced oxygen concentration by about 4 % irrespective of temperature (i.e. there was no evidence of an interaction; Table 1).

Dynamics of the oxygen concentration of the bottom part of the micro-ecosystems were very different, and were strongly affected by temperature and nutrient addition (Fig. 5b). At 12 °C, a hump-shaped dynamic can be observed, going from anoxic to oxic and back to anoxic during the experiment. The same trend can be observed at 16°C and 20 °C, but took place earlier in the experiment. At 24-36°C there was no hump, and conditions were almost entirely anoxic, though with some rapid anoxic-oxic shifts at 16 °C, 20 °C, 24 °C, 28°C and 36 °C at the end of the experiment. These rapid shifts between oxic and anoxic states correspond with shifts in the position of the white band from above (anoxic) to below (oxic) the bottom oxygen sensor (Fig. 2c).

The long-term effect of temperature and nutrient addition on the oxygen concentration of the bottom part of the columns was assessed by transforming the measured oxygen concentration into a binary variable: oxic (O_2 concentration > 2 %) or anoxic (O_2 concentration < 2%). There was moderate evidence of an interaction between temperature and nutrient addition in determining oxic or anoxic state (Table 1). The anoxic state became more likely as temperature increased in the control treatment, but temperature did not have such an effect in the nutrient addition treatment (Table 1).

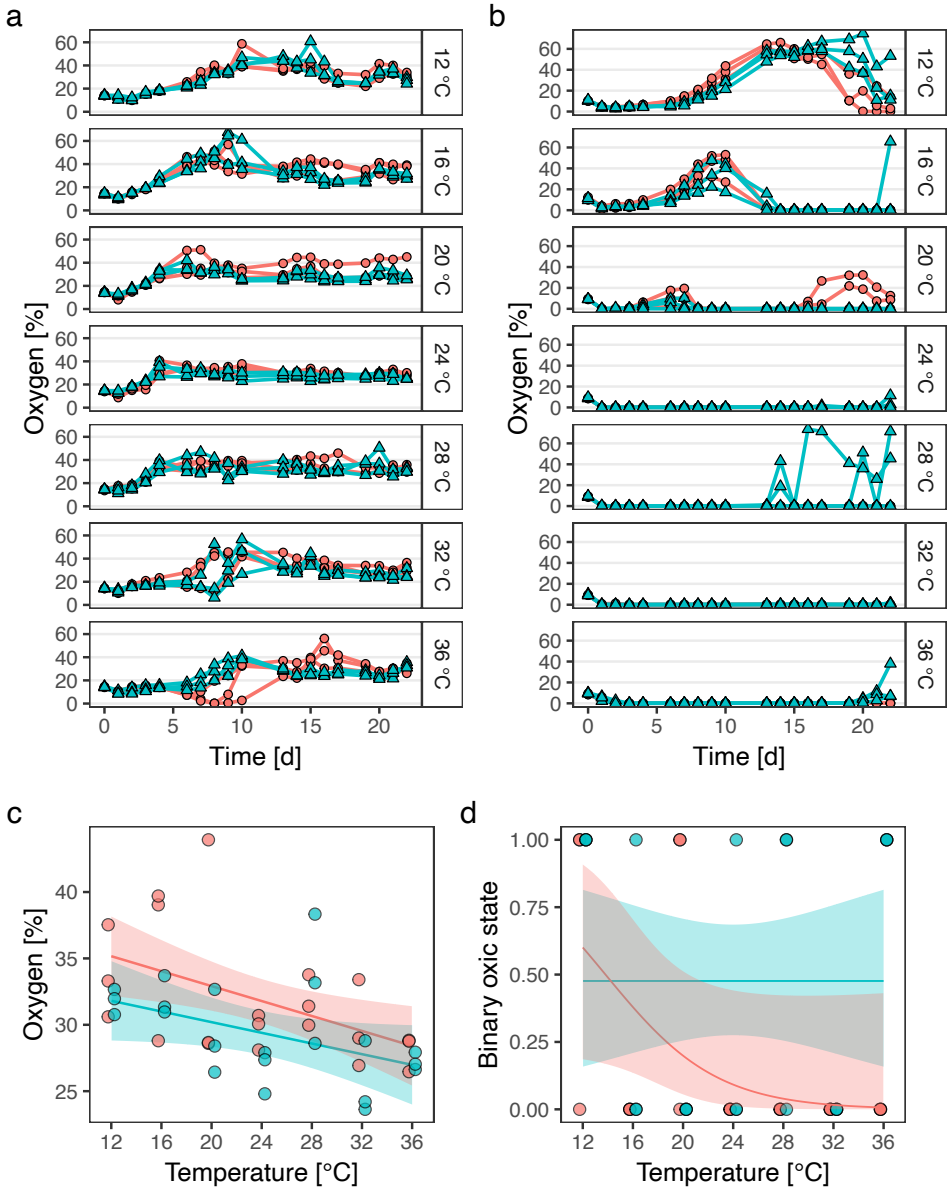

Fig. 5 Oxygen-dynamics and long-term oxygen-states of the micro-ecosystems. In all panels, control incubations are shown in red circles (●), nutrient addition treatments in blue triangles (▲). The oxygen concentration [%] of the micro-ecosystems incubated at different temperatures was measured at the top (**a**) and bottom (**b**) sensor over time. (**c**) The mean of the oxygen concentrations of the top sensor during the last three days of the experiment, lines represent the linear model fit and colored ribbons the 95 % confidence intervals. (**d**) The binary oxygen state of the at the bottom sensor, lines represent the regression line of the used model and colored ribbons the 95 % confidence intervals.

Table 1. Statistical analysis of the various response variables used in this study. Estimates of the temperature treatment are in units of the response variable per degree celcius, and estimates of the nutrient addition treatment are the effect of nutrient addition on the response variable. The interaction estimate is the effect of the nutrient addition on the estimate of the temperature effect. Terms in the main text used for p-values: no evidence $p > 0.1$, weak evidence $0.1 > p > 0.05$, moderate evidence $0.05 > p > 0.01$, strong evidence $0.01 > p > 0.001$, very strong evidence $p < 0.001$. All models were general linear model with F-tests, except for the bottom oxygen analysis, which was a generalised linear model with binary response variable with likelihood ratio tests (LR-test). The total nitrogen response variable was arcsine squareroot transformed prior to analysis.

		Temperature	**Nutrient addition**	**Interaction**
Response variable				
Oxygen top	Estimate	-0.28	-4.32	0.08
	Standard error	0.10	3.66	0.14
	F-test p-value	1.9e-03	0.05	0.58
Oxygen bottom	Estimate	-0.23	-3.2	0.23
	Standard error	0.12	2.64	0.13
	F-test p-value	0.17	0.05	0.05
H_2S at sediment-water interphase	Estimate	-0.17	-12.56	0.43
	Standard error	8.59	0.25	0.35
	F-test p-value	0.43	0.71	0.21
pH top	Estimate	-4e-03	3.01	-6e-04
	Standard error	9.1e-03	0.33	0.01
	F-test p-value	0.49	<2e-16	0.97
pH bottom	Estimate	0.04	-0.78	-0.03
	Standard error	0.01	0.36	0.01
	LR-test p-value	2.6e-03	7.27e-15	0.07
Total nitrogen (TN)	Estimate	-7.8e-04	0.70	-2.7e-03
	Standard error	1.7e-03	0.06	2.3e-03
	F-test p-value	0.08	< 2e-16	0.26
Total organic carbon (TOC)	Estimate	-0.2	12.43	-0.02
	Standard error	0.45	15.63	0.63
	F-test p-value	0.52	0.02	0.98
NMDS1	Estimate	0.11	0.22	-0.00825
	Standard error	0.005749	0.21	0.008130
	F-test p-value	<2e-16	0.99	0.30
NMDS2	Estimate	0.04	1.77	-0.07
	Standard error	0.01	0.41	0.02
	F-test p-value	0.85	0.87	6.49e-05

Chemical parameters

Highest H_2S concentrations at the height of the bottom oxygen sensor were measured at 32 °C on day 8 (max 6 mg/L) and day 14 (max 5 mg/L), before the H_2S concentration decreased strongly by day 20 (Fig. 6a). H_2S on day 22 at the sediment-water interphase was detectable in most micro-ecosystems (Fig. 6b), with frequently high concentrations with more than 15 mg/L. However, there was no evidence for an effect of temperature, nutrient addition, or of their interaction on H_2S at the end of the experiment (Table 1).

There was very strong evidence of that nutrient addition affected the top (Fig. 6c) and bottom pH (Fig. 6d, Table 1), and additionally strong evidence that temperature affected the bottom pH value. Moreover, the pH of top and bottom part of the micro-ecosystems showed clear differences (compare Fig. 6c & d). While the control micro-ecosystems had a pH gradient from ~pH 7.5 at the bottom to ~ pH 9 at the top, no such gradients occurred in the nutrient addition-treatment, which exhibited a pH of ~ 6 in both the top and bottom parts of the columns.

In addition, total nitrogen (TN) and total organic carbon (TOC) were analyzed for the liquid part of the micro-ecosystems (Fig. 6e & f), and data indicated a very strong evidence that nutrient addition increased TN concentration and a strong evidence for increasing the TOC concentration (Table 1).

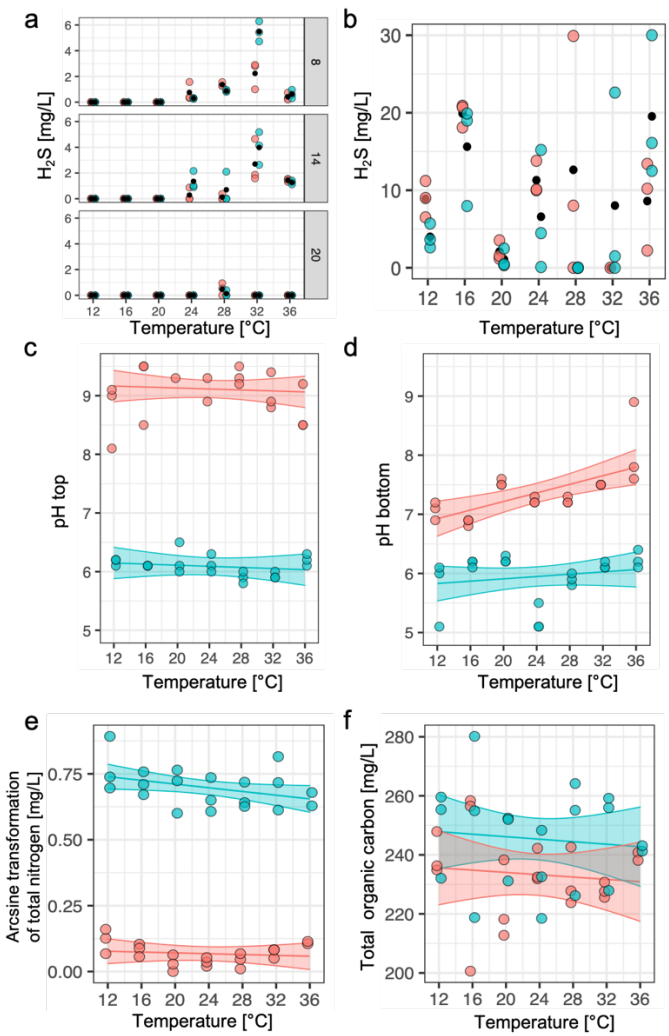

Fig. 6 Chemical parameters of the micro-ecosystems.

(**a**) Measurement of the Net-H_2S concentration [mg/L] at the bottom oxygen sensor per temperature at incubation day 8, 14 and 20. **(b)** Measurement of the Net-H_2S concentration [mg/L] at the soil-water interphase per temperature at final incubation day 22. (**c**) pH of the columns per temperature at the upper part. (**d**) pH of the columns per temperature at the bottom part. (**e**) Total nitrogen [mg/L] concentration [mg/L] of the micro-ecosystems depending on incubation temperature. Data were arcsine transformed for statistical analysis. (**f**) Total organic carbon concentration [mg/L] of the micro-ecosystems depending on incubation temperature. Controls are shown in red dots (●), nutrient addition treatments with blue dots (●). Small black points indicate mean values. Fitted models and confidence intervals (95 %) are shown as respectively lines and shaded areas for plots for which there was evidence of their significance.

469 # Discussion

470 Understanding and predicting the consequences of multifarious environmental change
471 is very important in a broad range of research disciplines, and investigations should
472 be made from global- to micro-scales [27]. In contrast to existing work on higher-
473 developed organisms [28–30] less is known about how bacterial ecosystems react to
474 multifarious global change scenarios [7, 31–33]. In addition, there is a need of
475 experimental studies of complex microbial model ecosystems that can potentially
476 display diverse responses to changing environmental conditions for various microbial
477 groups in one experimental unit. With inspiration from Winogradsky columns [34], we
478 introduce a new experimental micro-ecosystem, that includes the development of
479 aerobic and anerobic microbial communities in one experimental ecosystem in a
480 replicable manner. This system represents an appropriate model system for analyzing
481 responses of complex and stratified microbial communities to global change
482 scenarios.

483 We used this model system to shed light on the consequences of multifarious
484 environmental change, namely simultaneous warming and nutrient deposition. We
485 hypothesized a simultaneous effect on oxygen dynamics and microbial community
486 compositions. While temperature is considered to be a main driver for shaping
487 soil/sediment communities [35–37], we furthermore observed non-additive effects of
488 warming and nutrient addition on the aquatic microbial community (Fig. 4c, Table 1).
489 This highlights the importance of studying potential interactions of multiple
490 environmental change parameters simultaneously [38], given that they can differ in
491 effect compared to the sum of individual disturbances. Environmental change cannot
492 be reduced to the sums of individual factors. It is multifarious, providing the opportunity

493 for “risk multiplication” and for greater challenges when attempting to understand and
494 predict ecosystem responses to environmental change [39].

495 Besides the effect on overall microbial community composition, our results
496 additionally indicated that multiple drivers affect the oxic and anoxic layers differently.
497 The oxic layer of the system was affected by temperature and nutrient addition
498 additively (Fig. 5a & c, Table 1). In contrast, the anoxic layers non-additive effects (Fig.
499 5b & c). These observations might be due to the different biogeochemistry processes
500 in the two strata, such as the interplay of specific microbial communities, and their
501 interplay with available nutrients, trace elements, as well as abiotic factors and the
502 presence of specific oxygen-reducing sulfur compounds [9, 12, 40–43].

503 The microbial community composition and the oxygen dynamics are tightly
504 coupled in a feedback loop. Key players in this feedback loop are oxygen-producing
505 cyanobacteria, sulfate-reducing bacteria and phototrophic sulfur bacteria [12, 44].
506 While cyanobacteria and especially green and purple sulfur bacteria were common in
507 the micro-ecosystems, the abundance of sulfate-reducing microorganisms was
508 comparably low. This observation could be explained by the sampling schedule:
509 detection of high H_2S concentration within the micro-ecosystems indicates a nutrient
510 rich habitat for phototrophic sulfur bacteria [45, 46], which suggests previous high
511 activity of sulfate-reducing microorganisms [47, 48]. Earlier or later sampling may have
512 revealed more oxidized sulfur-compounds and higher abundance of sulfate-reducing
513 microorganisms. Further explanation of this unbalanced ratio between these two
514 functional groups could probably be found in the light-dark- and the sediment-water
515 ratio of our model system.

516 Some results hint at the presence of alternate stable states [49] in the
517 phototrophic sulfur bacterial communities. There was considerable variation among

518 some of the replicates of the same treatment combination, despite them having very
519 similar initial conditions. For example, some contained either the green phototrophs
520 *Chlorobium* or *Chlorobaculum*, or the purple phototroph *Allochromatium* (based on
521 sequencing and macro/microscopical observation of the respective columns). Beside
522 these alternative compositions within the same functional group, also the oxygen
523 dynamics indicated interesting patterns, particularly at 28 °C, where two of three
524 replicates of the nutrient addition-treated micro-ecosystems became oxic at the bottom
525 sensor. Since no significant differences in the microbial community can be observed
526 in these incubations, vertical movements of the oxic-anoxic interphase may have
527 occurred, which was also visible by the different height of the white microbial
528 consortium, consisting of *Magnetospirillum* representatives. The role and relevance of
529 *Magnetospirillaceae* [50] should be investigated further. Additionally, sinking of
530 cyanobacteria into the anoxic layer could also result in temporary changes of oxygen
531 dynamics in our dynamic system.

532 This study reveals that two environmental change factors caused large and
533 non-additive variation in the composition of aquatic microbial communities and in the
534 abiotic conditions of the ecosystem. We believe that these insights are only the tip of
535 the iceberg of what can be learned from such model micro-ecosystems with strong
536 and even stratified spatial environmental gradients. Further research with this new
537 experimental system could take many paths, including elements that we wished to but
538 could not include in the current study due to limited resources. These include:
539 increasing the temporal and spatial resolution of the sampling, in order to understand
540 more about the shift in the anoxic-oxic boundary, and the microbes associated with it;
541 studying the stability of the communities to press and pulse perturbations, and how
542 this stability may depend on aspects of community composition, such as functional

composition and intraspecific diversity; the extent and significance of evolutionary processes such as mutation and selection for mediating effects of environmental change; and observation of community composition via metagenomic methods, in order to capture not just the bacterial component of the micro-ecosystems, but also to research the functional significance of other likely inhabitants, such as viruses. Finally, one could research why these micro-ecosystems did not become entirely oxic or entirely anoxic, and why there was little evidence of the discontinuous responses to environmental change that are predicted for systems with strong positive feedbacks, such as this one.

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Competing interests

The authors declare no competing financial interests.

Author contributions

OP and MS planned the experimental set-up. MS performed all experiments in the lab. MS performed the upstream bioinformatics. OP and MS performed the downstream analyses of microbial community compositions. UD supported statistical analysis and modelling of the oxygen data and general coding in RStudio. YC performed total nitrogen and organic carbon measurements and provided technical support. OP and MS drafted the manuscript. All authors confirmed the final version of the manuscript.

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