Large and interacting effects of temperature and nutrient addition on stratified microbial ecosystems in a small, replicated, and liquid dominated Winogradsky column approach

Marcel Suleiman, Yves Choffat, Uriah Daugaard, Owen L Petchey

Department of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland

Corresponding author: Marcel Suleiman, Department of Evolutionary Biology and Environmental Studies, University of Zurich, Switzerland

eMail: marcel.suleiman@ieu.uzh.ch Tel: +41 44 635 4773

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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. Increasing 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 can come from observational and experimental studies of naturally occurring stratified aquatic ecosystems, from theoretical models of ecological processes, and from experimental studies of replicated microbial communities in the laboratory. Here we demonstrate a laboratory-based approach with small, replicated, and liquid dominated Winogradsky columns, with distinct oxic/anoxic strata in a highly replicable manner. Our objective is to apply simultaneous global change scenarios (temperature, nutrient addition) on this micro-ecosystem to report how the microbial communities (full-length 16SrRNA-seq.) and the abiotic conditions (O_2, H_2S, TOC) of the oxic/anoxic layer responded to these environmental changes. Composition of the strongly stratified microbial communities was greatly affected by temperature and by the interaction of temperature and nutrient addition, demonstrating the need of investigating global change treatments simultaneously. Especially phototrophic sulfur bacteria dominated the water column at higher temperatures, and may indicate the presence of alternative stable states. We show that the establishment of such a micro-ecosystem has potential to test global change scenarios in stratified eutrophic limnic systems.

49 # Introduction

50 Microorganisms are key players in nearly all ecosystems, ranging from the human gut
51 to marine and freshwater habitats. The functioning of microbial communities is critical
52 for many ecosystem services, since microorganisms are the driving force behind
53 biogeochemical cycles (Kertesz 2000; Weiss et al. 2003; Falkowski et al. 2008; Stein
54 and Klotz 2016). Nevertheless, microbes are highly dependent on environmental
55 conditions; even slight changes can lead to taxonomic and functional community shifts
56 (Allison et al. 2013; Evans and Wallenstein 2014). Consequently, microbial
57 communities and their functions are significantly affected by global change (Cavicchioli
58 et al. 2019), which has recently led to the emergence of the research field termed
59 "Global Change Microbiology" (Boetius 2019).

60 Stratification of productive lakes is a common phenomenon observed in
61 summer, resulting in warm and oxygen-rich upper water layers, dominated by
62 cyanobacteria and algae, and colder anoxic deeper layers, that harbor heterotrophic
63 biomass-degraders and sulfate-reducing bacteria. In between these layers, the
64 metalimnion can be found, which is formed by decreasing light-intensities and oxygen
65 concentrations (microaerophilic), and an increasing pool of reduced sulfur
66 compounds, creating ideal niches for phototrophic sulfur bacteria and
67 chemolithoautotrophic microorganisms (Jorgensen et al. 1979; Morrison et al. 2017;
68 Savvichev et al. 2018; Vigneron et al. 2021).

69 Beside chemical and physical parameters that influence the presence of oxic
70 and anoxic layers, it was recently postulated that mutual inhibition between
71 cyanobacteria and anaerobic sulfur-dependent bacteria creates and maintains the
72 distinct oxic and anoxic zones of such ecosystems (Bush et al. 2017). 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 (Posch et al. 2012), or increasing primary production and creation of anoxic layers due to increased nutrient input (Luek et al. 2017).

The mentioned studies show that important insights come from analyses of observations of naturally occurring aquatic ecosystems (Vigneron et al. 2021), 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” (Hutchins et al. 2019) and are considered vital alongside studies of naturally occurring ecosystems (Lahti et al. 2014) and hybrids of both approaches (De Vos et al. 2017). 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 (Widder et al. 2016) and interactions among microorganisms (Overmann and van Gemerden 2000), and the integration of data and theory (Widder et al. 2016). Experiments about the role of environmental, organismal interactions and biochemical feedbacks in stratified aquatic micro-ecosystems appear absent, however.

Establishing suitable such standardised-model ecosystem for analyzing potential responses of a broad range of microorganisms remains crucial. Being able to monitor global change responses of diverse functional groups of potentially interacting microbes is a desirable feature of such an experimental system, which point towards systems with strong abiotic gradients in space and or time. In this work, we

98 get inspiration from an “old” but highly valuable approach, the Winogradsky column
99 (Zavarzin 2006; Dworkin 2012; Pagaling et al. 2017). We constructed a modified and
100 smaller version that allows a highly replicable development of a broad range of
101 complex and dynamic microbial groups in one experimental unit. In contrast to
102 classical Winogradsky columns, our micro-ecosystems are mostly liquid, with only a
103 small sediment layer (~ 6 % v/v of the column). Thus, we created a highly replicable
104 liquid oxic-anoxic interphase and self-developing model systems.

105 By applying this approach, we were able to analyze microbial responses to
106 multifarious environmental change (temperature and nutrient addition manipulated
107 factorially), including the responses of community composition and abiotic
108 environmental conditions. As well as showing how microbial community composition,
109 dissolved oxygen, pH, and hydrogen sulphide respond to temperature and nutrient
110 addition, we highlight the potential for global change microbiology research of this new
111 approach coupled with state-of-the-art sequencing technologies.

112 We hypothesize that higher temperature will increase the dominance of
113 anaerobic microbes due to an increase in the extent of the anoxic zone, due to the
114 lower solubility of oxygen in warmer water. We hypothesize that nutrient addition
115 (addition of ammonium phosphate, highly used fertilizer (Cao et al. 2018)) will alter
116 community composition, but we cannot *a priori* say how, as this would depend on
117 features of the organisms, such as competitive and facilitative interactions among
118 them, which we do not have sufficient information about. We did not have any *a priori*
119 expectation of whether the combined effect of temperature and nutrient addition would
120 be additive (no interaction), more than additive (positive interaction), or less than
121 additive (negative interaction). We anticipated the potential for micro-ecosystems to

122 become entirely oxic or entirely anoxic, and for changes in composition to be non-
123 linear and to potentially include discontinuous responses that are predicted by theory.
124

125 Materials and methods

126 Preparation of micro-ecosystems

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

133 Each test-tube (which we also refer to as a “column”) had a butyl rubber stopper
134 on top, with two canulas. The longer canula (StericanR, length 120mm, diameter 0.8
135 mm) reached to the depth of the bottom O_2 sensor and was covered with an oxygen-
136 protecting plug (BD Plug). The shorter canula (length 40 mm, 0.9 mm diameter) was
137 always open and extended only into the headspace of the micro-ecosystem. This
138 shorter canula therefore allowed for gas exchange between the headspace and the
139 atmosphere (Fig. 1a).

140 Micro-ecosystems were established from sediment and water samples taken in
141 July from a small pond in Zürich, Switzerland (47°23'51.2"N 8°32'33.3"E, temperature
142 of 24.6 °C, pH of 6.7.) at a depth of 30 cm. In total, 200 g sediment and 2 L water were
143 collected. The sediment sample was supplemented with sterile 0.5 % crystalline
144 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 (Zavarzin 2006; Dworkin 2012; Rundell et al. 2014), 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.

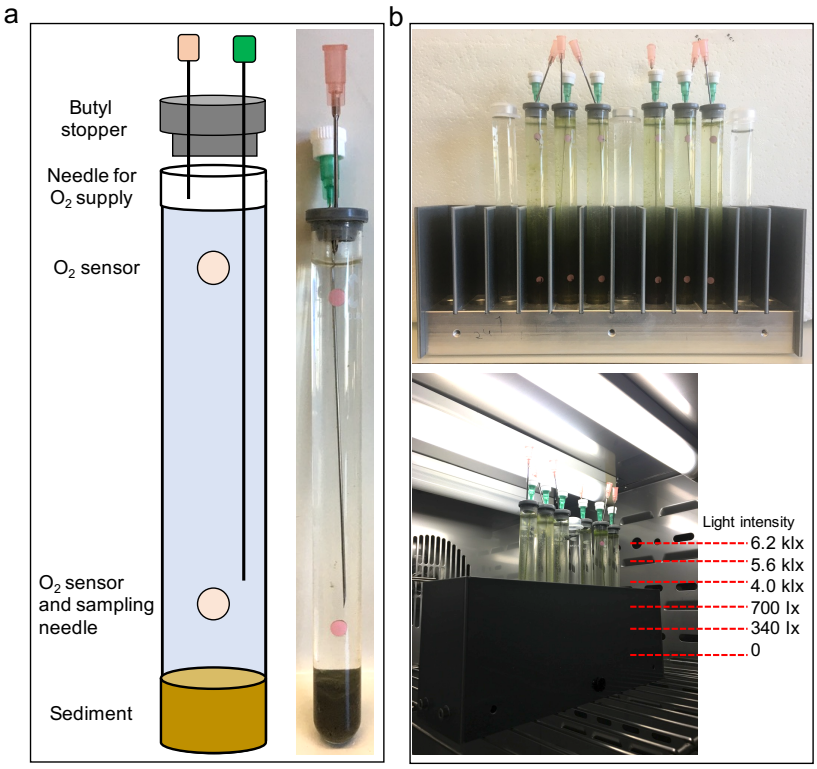

Fig. 1 Preparation and incubation conditions of the micro-ecosystems

(**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).

Incubation and treatments

Each column was incubated at one of seven temperatures: 12, 16, 20, 24, 28, 32 or 36 °C. Incubators (Pol Eco Apparatura sp.j.) were equipped with two fluorescent light

174 sources (PHILIPS Master TL8W/840) with a dark-light cycle of 8:16 hours. Columns
175 were placed in a 1.5 cm hole in a metal base, so that the sediment layer was
176 continuously kept in darkness. Up to a height of 10 cm, the columns were surrounded
177 by a plastic holder that causes a gradient of light, ranging from 340 lx at the intersphere
178 of sediment:water at the bottom and up to 700 lx at the top of the light-protector (Fig.
179 1b). The top of the column experienced fluorescent light intensity of 6200 lx. Each
180 holder contained six columns, in two groups of three replicates, separated by a water-
181 filled column. This arrangement was placed in the middle of both light sources in the
182 incubator, allowing the same light-intensity at the back and front of each column.
183 A second treatment was the manipulation of nutrient concentration. For this, 0.1
184 % $NH_4H_2PO_4$ was added to half of the columns by taking 16 µL of a sterile 10 %
185 $NH_4H_2PO_4$ solution, while 16 µL sterile water was used for the controls. This treatment
186 was performed once per week (day 1, 8, 14, 20) and was factorially crossed with the
187 temperature treatment, resulting in 7 (temperature) x 2 (nutrient levels) and 3
188 replicated, giving a total of 42 columns. These were incubated for 22 days.
189

190 **Sampling and measurements**

191 Liquid samples were taken on day 8, 14 and 20 by collecting 200 ul of liquid from the
192 bottom of the columns by using the installed canula.This liquid was used to measure
193 H_2S (Microsensor, AMT Analysenmesstechnik GmbH, Germany) and was replaced
194 with anoxic sterile lake water. Non-invasive oxygen measurements were performed at
195 the top and bottom sensor of the columns (everyday except of day 5, 11, 12 and 17).
196 Oxygen concentration is presented in % saturation adjusted for the prevailing
197 temperature and pressure (e.g. 21% saturation occurs when water is in equilibrium

198 with the surrounding atmosphere). Thus the known effect of temperature on oxygen
199 solubility does not affect our analyses.
200 After incubation, all columns were destructively sampled. For each column, the
201 whole water column (16 mL) and the sediment layer were processed separately. One
202 replicate of each treatment combination had the water column separated at the height
203 of the bottom oxygen layer, giving a sample of the upper and lower liquid layer as well
204 as a sample for the sediment. Each sample was centrifuged (16,000 rpm, 3 min) and
205 pellets were frozen at -80 °C until DNA extraction. Supernatant was used for pH
206 measurements, as well as total nitrogen and total organic carbon analysis. While
207 sampling, the H_2S concentration at the sediment-water interphase was measured
208 under anoxic conditions.
209

210 DNA extraction, 16S rRNA amplification

211 DNA extractions were performed with ZymoBIOMICS DNA Microprep Kit
212 (ZymoResearch), following the manufacturer`s instructions. 16S rRNA gene
213 amplifications were performed with 27F forward primer (5`-
214 AGRGTTYGATYMTGGCTCAG-3`) and 1592R reverse primer (5`-
215 RGYTACCTTGTTACGACTT-3`), resulting in an amplification product of ~1500 bp.
216 Primers were equipped with 5`phosphate and a 5`buffer sequence of GCATC.
217 Barcodes, PCR chemicals and conditions (27 cycles) were performed as suggested
218 by Pacific Biosciences (PacBio, 2020). PCR products were visualized on an 1 % (w/v)
219 agarose gel and were pooled with equal concentrations. Afterwards, PCR products
220 were purified using AMPure®PB beads (PacBio), following manufacturer`s guidelines.
221 Besides 16S rRNA amplification for PacBio long read sequencing, also DGGE

222 analysis performed as described previously (Antranikian et al. 2017; Suleiman et al.
223 2019) in order to identify the white bacterial population.
224

225 **Library Preparation and Sequencing**

226 Sequencing was performed at the Functional Genomic Center Zürich, Switzerland.
227 The SMRT bell was produced using the SMRTbell Express Template Prep Kit 2.0
228 (PacBio). The input amplicon pool concentration was measured using a Qubit
229 Fluorometer dsDNA High Sensitivity assay (Life Technologies). A Bioanalyzer 12Kb
230 assay (Agilent) was used to assess the amplicon pool size distribution. 0.75µg of
231 amplicon pool was DNA damage repaired and end-repaired using polishing enzymes.
232 A ligation was performed to create the SMRT bell template, according to the
233 manufacturer's instructions. The SMRT bell library was quality inspected and
234 quantified using a Bioanalyzer 12Kb assay (Agilent) and on a Qubit Fluorimeter (Life
235 technologies) respectively. A ready to sequence SMRT bell-Polymerase Complex was
236 created using the Sequel II Binding Kit 2.1 and Internal Control 1.0 (PacBio) according
237 to the manufacturer instruction. The Pacific Biosciences Sequel II instrument was
238 programmed to sequence the library on 1 Sequel II SMRT Cell 8M, taking 1 movie of
239 15 hours per cell, using the Sequel II Sequencing Kit 2.0 (PacBio). Sequencing data
240 quality were checked, via the PacBio SMRT Link software, using the "run QC module".
241 Sequencing raw data are deposited at NCBI SRA under the BioSample accession
242 numbers SAMN16774060 to SAMN16774152.
243

Bioinformatics

Bioinformatics were performed using R and the package Dada2 (Callahan et al. 2016). In short, reads were filtered regarding primer sequences, length (1300-1600 bp), quality, error rates and chimeras. The outcome of the filter processes can be observed in Supplementary data for each sample. Finally, the constructed sequence table was aligned with the SILVA ribosomal RNA database (Quast et al. 2012), using version 138 (non-redundant dataset 99). Afterwards, a phyloseq formatted data object was constructed, containing the amplicon sequence variant (ASV) table, taxonomy table and sample metadata, using the package phyloseq (McMurdie and Holmes 2013), which was also used for microbial community analysis together with the vegan package (Oksanen et al. 2019). All sequences that were assigned to “chloroplast” were removed from the phyloseq file, since primers were chosen for bacterial 16S rRNA gene and bind incorrectly to chloroplast sequences.

Microbial community composition was analysed with non-metric multidimensional scaling (NMDS), using the metaMDS function from the vegan package. NMDS was used because a principal component analysis of the compositional variation exhibited a strong “arch-effect”, which can hinder the use of ordination scores in further analyses that we wanted to make. The metaMDS function was instructed to use Bray-Curtis distance, to transform the data to have 2-dimensions. The metaMDS function was instructed to try 200 different starting positions in order to assure convergence. NDMS was performed on relative abundances of sequences with a relative abundance of at least 1% in at least one sample. The 2-dimensional NDMS solution converged with a stress of 0.15. A 3-D solution converged with stress of 0.11. This suggests the that 2-D solution is a good

representation of the compositional variation among the samples, and that the 3-D solution is not much better. 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. All analyses involving NDMS and variables derived from it were repeated with a different standardization (centred log ratios) and a different distance measure (Aitchison distance) (Gloor et al. 2017). These different methods did not affect the conclusion of strong and relatively linear effect of temperature on composition (NMDS1), and of a nonlinear and interacting effect of temperature and nutrient treatment on composition (NMDS2).

We intentionally avoid referring to effects as significant or not significant, and rather use the terms: no evidence $p > 0.1$, weak evidence $0.1 > p > 0.05$, moderately strong 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,

291 and at higher temperatures above the bottom-sensor). DGGE analysis identified this
292 layer as a community of species of the microaerophilic genus *Magnetospirillum*,
293 indicating that this layer is the oxic-anoxic interphase of the micro-ecosystems. In
294 general, all micro-ecosystems developed an oxic and anoxic part (Fig. 5), and
295 triplicates of each treatment combination predominantly showed comparable growth
296 and development. Gas formation was observed in the sediment layers of the columns,
297 indicating an active microbial community (Fig. 2d).
298

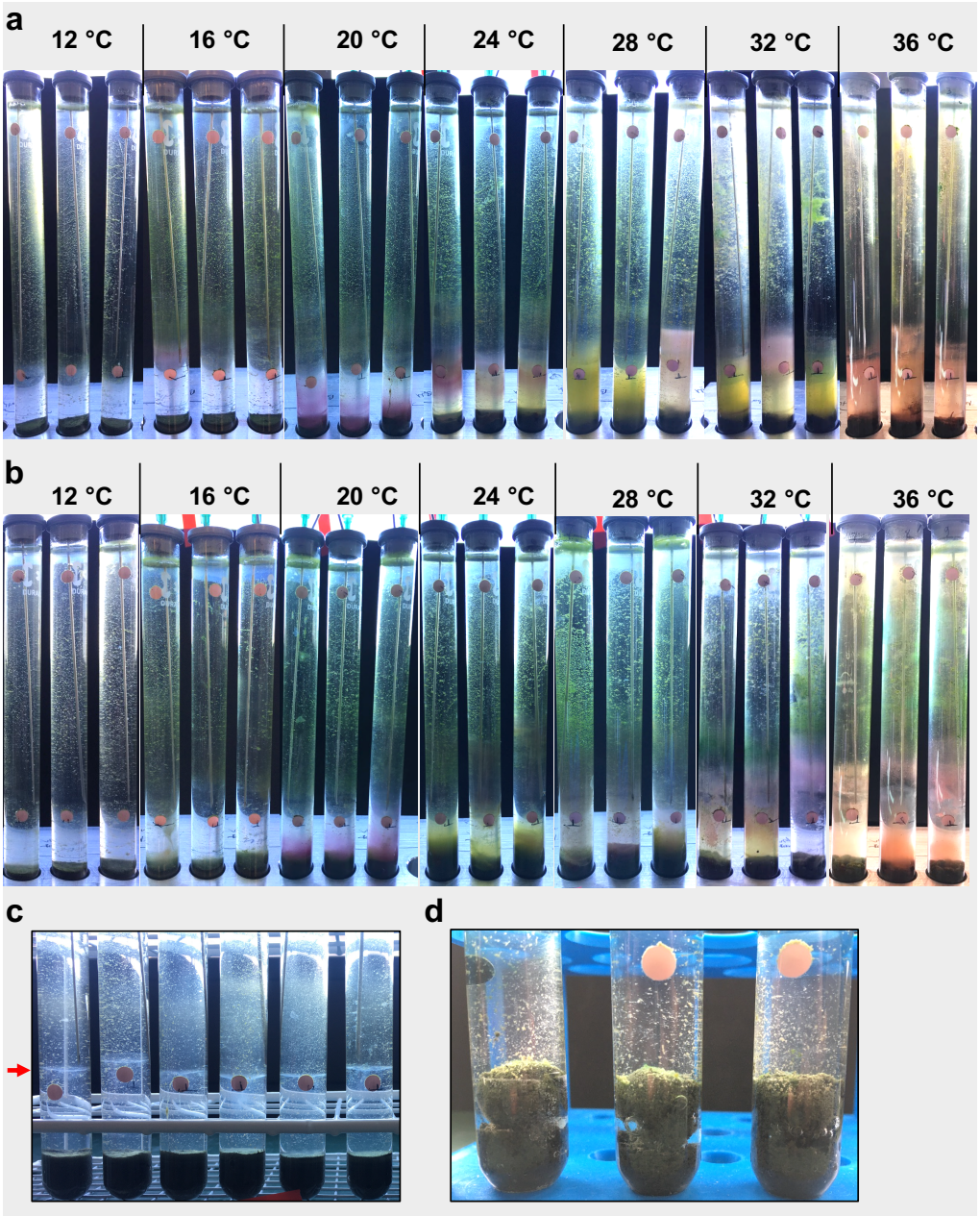

Fig. 2 Macroscopical observation of the micro-ecosystems. **(a)** Control micro-ecosystems (triplicates) after incubation at 12-36°C for 22 days. **(b)** Nutrient addition micro-ecosystems (triplicates) after incubation at 12-36 °C for 22 days. **(c)** Observation of a white bacterial cloud within 10 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.

307 **Relative abundances of microbial taxa of the water column**

308 In order to study the microbial communities in detail, full-length 16S rRNA sequencing
309 was performed. Removal of sequences that did not occur at more than 1 % in at least
310 one sample resulted in 343 different amplicon sequence variants (from initial 5633
311 unique sequences in all samples).

312 All of the micro-ecosystems at 12 °C were dominated by members of the order
313 *Burkholderiales* (54 – 69 %) (Fig. 3a, Fig. S1 for details). Relative abundance of
314 *Burkholderiales* decreased to 15 – 37 % (control) and 29 – 53 % (nutrient addition) at
315 16 °C, and relative abundance of *Rhodospirillales* increased up to 23 %. At 20 °C,
316 members of *Chlorobiales* and *Chromatiales* became more abundant. Members of
317 *Chlorobiales* were dominant in all micro-ecosystems at 24 °C (33 – 64 % controls, 62
318 – 64 % nutrient addition-treatments), 28 °C (42 – 92 % controls, 35 – 70 % nutrient
319 addition-treatments), and 32 °C (78 – 90 % controls, 18 – 50 % nutrient addition). At
320 36 °C, members of *Chlorobiales* disappeared, and in no-nutrient-addition treatments,
321 members of the cyanobacterial order *Limnotrichales* were dominant (up to 25 %; but
322 none occurred in nutrient addition treatment).

323 The full-length 16S rRNA amplicon sequencing resulted in a detailed account
324 of the microbial community to genus level (Fig. 3b, Fig. S2-S8 for details). All micro-
325 ecosystems at 12 °C (Fig. 3b, Fig. S2) were dominated by members of the aerobic
326 genus *Giesbergeria* (controls 22 – 34 %, nutrient addition 57 – 67 %), followed by
327 *Aquaspirillum* in the controls and *Flavobacterium* in the nutrient-addition micro-
328 ecosystems. At 16 °C (Fig. 3b, Fig. S3), control micro-ecosystems were dominated by
329 members of the aerobic genus *Uliginosibacterium* (12 – 27%), which had a very low
330 relative abundance in the nutrient addition treatment (less than 2 %). In contrast,

331 *Giesbergeria* and *Aquaspirillum* (4 – 35 %) were very abundant in the nutrient addition
332 treatment.

333 Genus composition at 20 °C (Fig. 3b, Fig. S4) included anaerobic phototrophic
334 sulfur bacteria, in particular the green sulfur bacteria *Chlorobium* appeared highly
335 abundant in the nutrient addition treatments (up to 46 %) and the purple sulfur bacteria
336 *Chromatium* in all micro-ecosystems at this temperature (up to 28 %). With increasing
337 temperature, green sulfur bacteria *Chlorobium* and *Chlorobaculum* genera increased
338 in relative abundance. At 24 °C (Fig. 3b, Fig. S5), *Chlorobium* and *Chlorobaculum*
339 were detected in the controls, with 11 – 39 % and 6 – 25 % relative abundance,
340 respectively, and in the nutrient addition treatments with 39 – 65 % and 27 – 60 %.
341 Furthermore, the picocyanobacterial genus *Cyanobium* appeared (up to 7 %) in the
342 control micro-ecosystems.

343 At 28 °C (Fig. 3b, Fig. S6), green sulfur bacteria continued to increase their
344 dominance, especially *Chlorobium* (up to 92 %). Interestingly, the *Control_3* column
345 consisted of a comparable lower amount of green sulfur bacteria, but additionally of
346 the purple sulfur bacteria *Allochromatium* (41 %). This finding can be confirmed by the
347 macroscopical observation of the pink layer (Fig. 3a). Additionally, the *Nutrient*
348 *addition_2* column consisted of *Chlorobaculum* instead of *Chlorobium* in contrast to
349 the *Nutrient addition_1* and *Nutrient addition_3* columns.

350 At 32 °C (Fig. 3b, Fig. S7), *Chlorobium* dominated the control-micro-
351 ecosystems, while this genus also had lower though still high relative abundance in
352 the nutrient addition treatments (up to 52 %).

353 At 36 °C (Fig. 3b, Fig. S8), the microbial community changed again: *Chlorobium*
354 and *Chlorobaculum* disappeared totally. Present instead were the facultatively
355 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).

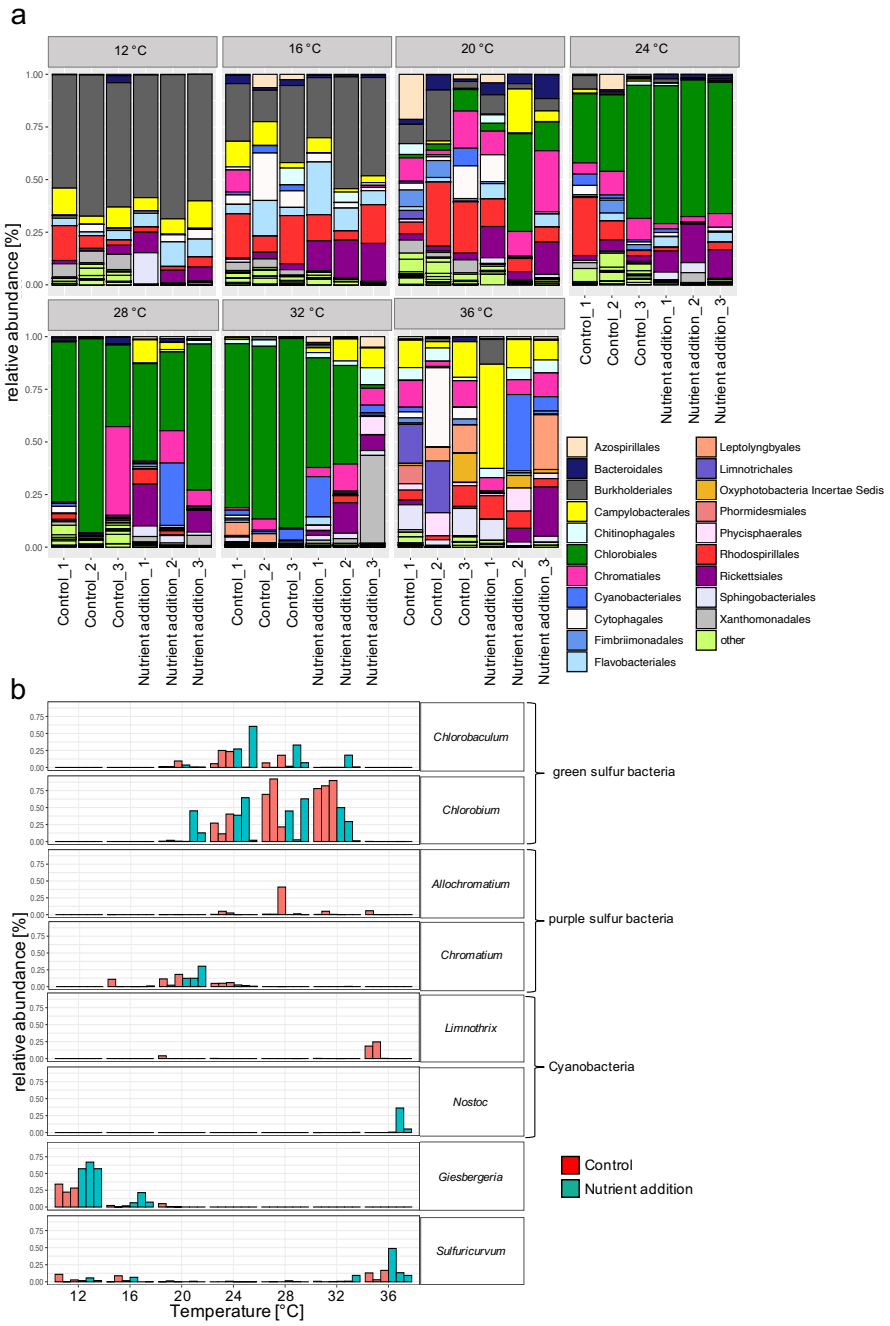

Fig. 3 Microbial community composition of the micro-ecosystems. (a) Relative abundance on order level is shown for each micro-ecosystem. Orders with relative abundances < 7 % in all samples were assigned to “other”. **(b)** Relative abundance of 8 dominant and functional important genera depending on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

382 **Community composition (NMDS) of the water column**

383 The effects of temperature and nutrient addition on the relative abundance described
384 in the previous sections were very evident in the analyses of community composition
385 via NMDS (Fig. 4a). Compositional variation along the first NMDS axis (hereafter
386 NMDS1) was strongly associated with temperature, but not with the nutrient addition
387 treatment, nor the interaction of the temperature and nutrient addition treatment (Fig.
388 4b) (Table 1). In contrast, compositional variation along NMDS2 was affected by a
389 strong interaction between temperature and the nutrient addition treatment (Fig. 4c)
390 (Table 1). Effects of temperature on NMDS2 scores were non-linear in both the control
391 and the nutrient addition treatments. In the control treatment, the NMDS2 score
392 remained constant from 12 to 24 °C and then decreased up to 36 °C. In contrast, in
393 the nutrient addition treatment NMDS2 values first decrease (12 to 16 °C), then
394 increase (16 to 28/32 °C) and then decrease again (32 to 36 °C).

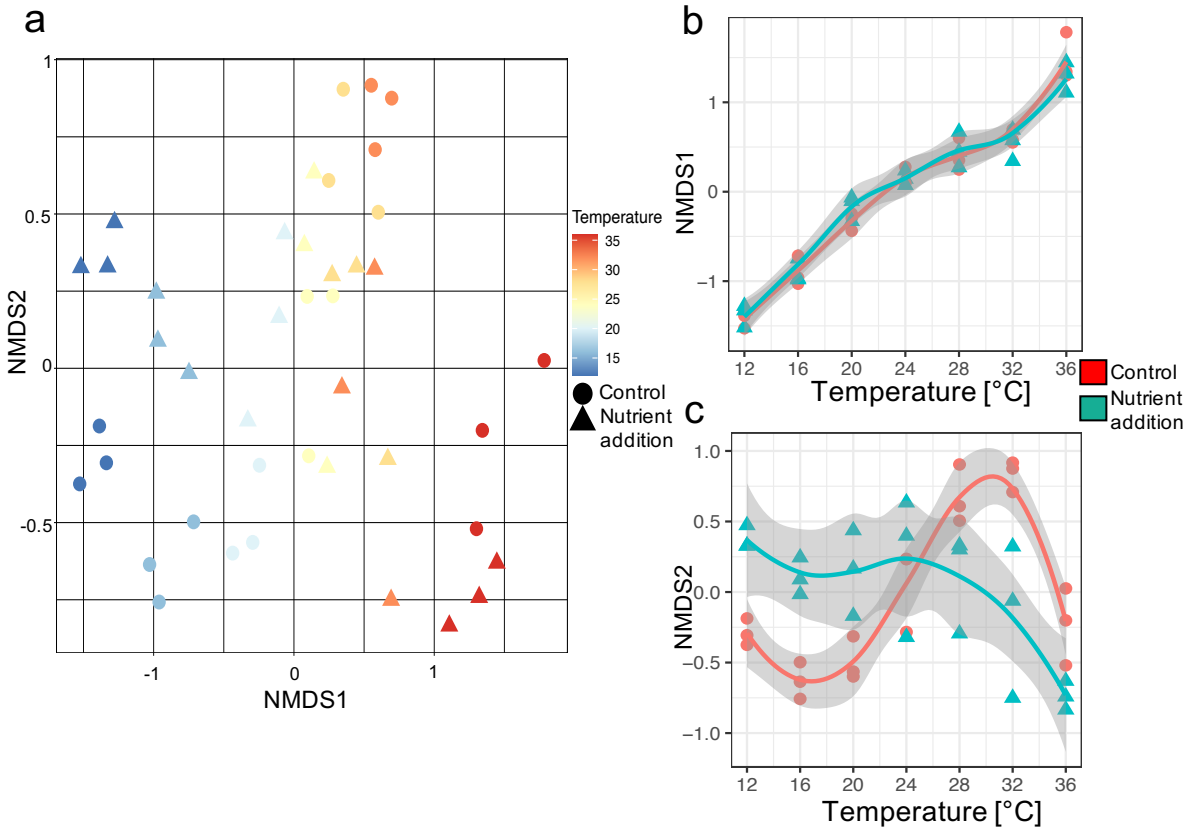

Fig. 4 Variation in microbial community composition. (**a**) NMDS scores based on Bray-Curtis distance showing the variation in microbial community composition of the water column (**b**) The effect of temperature and nutrient addition on NMDS1. (**c**) The effect of temperature and nutrient addition 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 water column (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.

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, moderately strong 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

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

467 of the white microbial population from above (anoxic) to below (oxic) the bottom
468 oxygen sensor (Fig. 2c).
469 The long-term effect of temperature and nutrient addition on the oxygen
470 concentration of the bottom part of the columns was assessed by transforming the
471 measured oxygen concentration into a binary variable: oxic (O_2 concentration > 2 %)
472 or anoxic (O_2 concentration < 2%). There was moderate evidence of an interaction
473 between temperature and nutrient addition in determining oxic or anoxic state (Table
474 1). The anoxic state became more likely as temperature increased in the control
475 treatment, but temperature did not have such an effect in the nutrient addition
476 treatment (Table 1).
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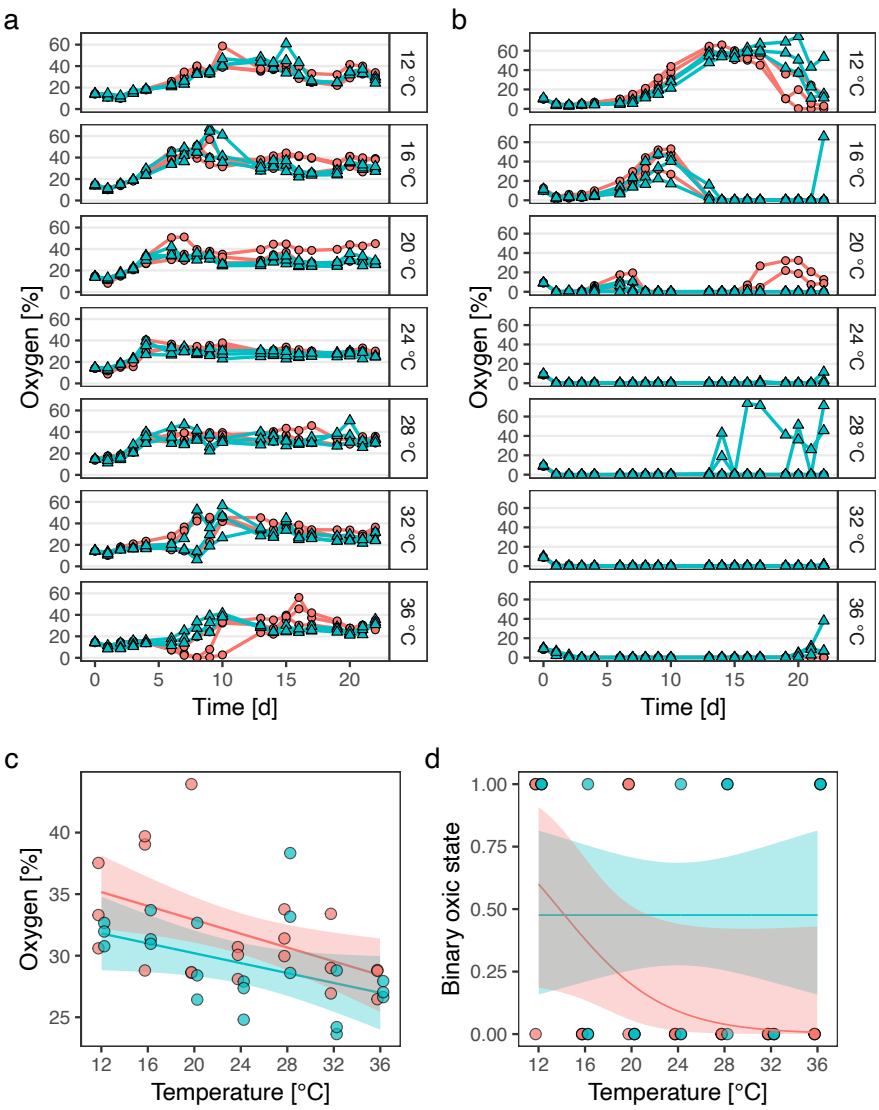

Fig. 5 Oxygen-dynamics and long-term oxygen-states of the micro-ecosystems. In all panels, control incubations are shown in red circles (●), nutrient addition treatments in blue triangles (▲). The oxygen concentration [%] of the micro-ecosystems incubated at different temperatures was measured at the 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 at the bottom sensor; lines represent the regression line of the used model and colored ribbons the 95 % confidence intervals.

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 water column 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).

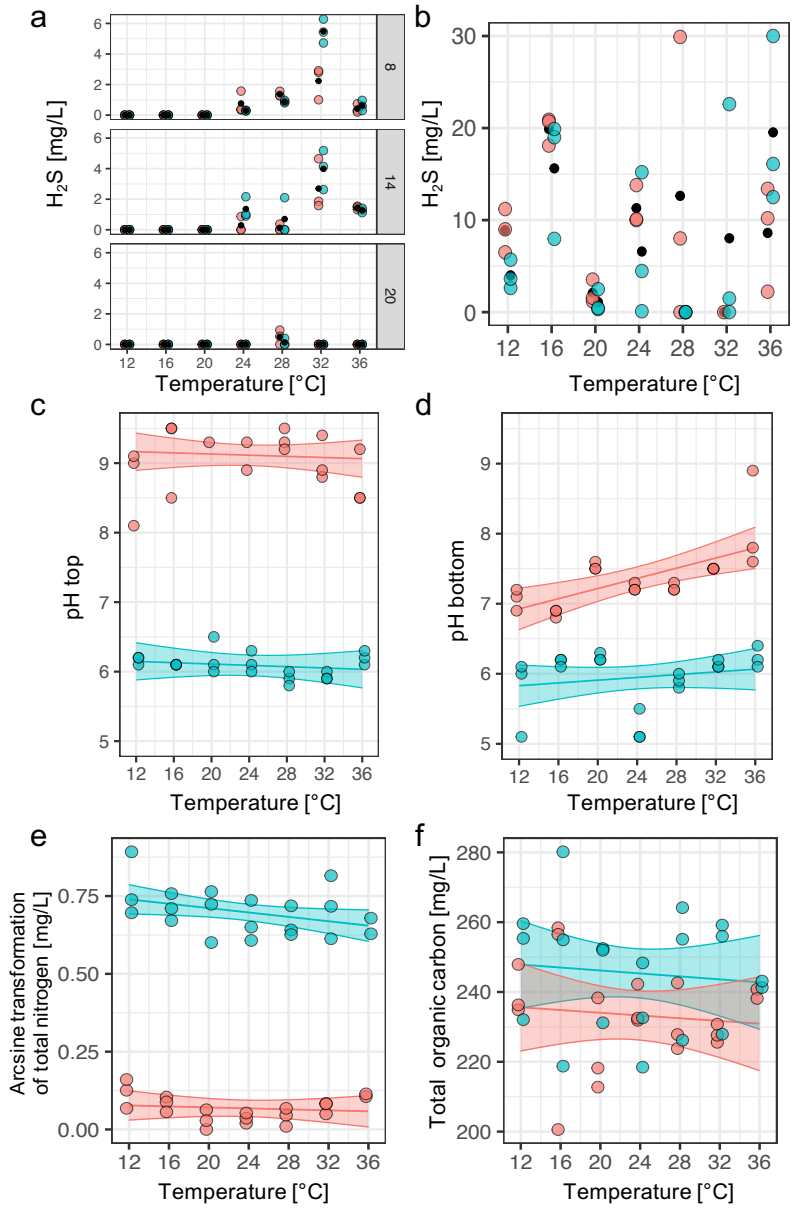

Fig. 6 Chemical parameters of the micro-ecosystems.

(**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.

Discussion

Understanding and predicting the consequences of multifarious environmental change is very important in a broad range of research disciplines, and investigations should be made from global- to micro-scales (Balser et al. 2006). In contrast to existing work on higher-developed organisms (Tylianakis et al. 2008; González-Varo et al. 2013; Pennekamp et al. 2018) less is known about how bacterial ecosystems react to multifarious global change scenarios (Coyle et al. 2017; Hutchins and Fu 2017; Cavicchioli et al. 2019; Rillig et al. 2019). In addition, there is a need of experimental studies (in addition to natural systems and mathematical models) of complex microbial model ecosystems that can potentially display diverse responses to changing environmental conditions for various microbial groups in one experimental unit. With inspiration from Winogradsky columns (Dworkin and Gutnick 2012; Pagaling et al. 2017), we introduce a modified experimental micro-ecosystem, that includes the development of aerobic and anerobic microbial communities in one experimental ecosystem in a replicable manner. In this study we demonstrate that such an approach represents an appropriate model system for analyzing responses of complex and stratified microbial communities to global change scenarios.

544 Some of our results, for example the stratification and temperature effects, may
545 be considered unsurprising and “textbook knowledge”. This is good evidence that
546 these small and liquid dominated microbial ecosystems are appropriate analogues of
547 naturally occurring communities. Furthermore, we used this model system to shed light
548 on the consequences of multifarious environmental change, namely simultaneous
549 warming and nutrient deposition—a relatively novel area of ecology, evolution, and
550 microbiology. We hypothesized a simultaneous effect on oxygen dynamics and
551 microbial community compositions. While temperature is considered to be a main
552 driver for shaping soil/sediment communities (Cole et al. 2013; Garcia-Pichel et al.
553 2013; Deng et al. 2018), we furthermore observed non-additive effects of warming and
554 nutrient addition on the aquatic microbial community (Fig. 4c, Table 1). This highlights
555 the importance of studying potential interactions of multiple environmental change
556 parameters simultaneously (Niiranen et al. 2013), given that they can differ in effect
557 compared to the sum of individual disturbances. Environmental change cannot be
558 reduced to the sums of individual factors. It is multifarious, providing the opportunity
559 for “risk multiplication” and for greater challenges when attempting to understand and
560 predict ecosystem responses to environmental change (Schulte to Bühne et al. 2020).
561 Besides the effect on overall microbial community composition, our results
562 additionally indicated that multiple drivers affect the oxic and anoxic layers differently.
563 The oxic layer of the system was affected by temperature and nutrient addition
564 additively (Fig. 5a & c, Table 1). In contrast, the anoxic layers were affected by non-
565 additive effects of temperature and nutrient addition (Fig. 5b & c). These observations
566 might be due to the different biogeochemistry processes in the two strata, such as the
567 interplay of specific microbial communities, and their interplay with available nutrients,
568 trace elements, as well as abiotic factors and the presence of specific oxygen-reducing

569 sulfur compounds (Jorgensen et al. 1979; Chapra and Canale 1991; Luther et al. 2003;
570 Garcia et al. 2013; Yu et al. 2014; Bush et al. 2017).

571 The microbial community composition and the oxygen dynamics are tightly
572 coupled in a feedback loop. Key players in this feedback loop are oxygen-producing
573 cyanobacteria, sulfate-reducing bacteria and phototrophic sulfur bacteria (Lee et al.
574 2014; Bush et al. 2017). While cyanobacteria and especially green and purple sulfur
575 bacteria had high relative abundance in the micro-ecosystems, the relative abundance
576 of sulfate-reducing microorganisms was comparably low. This observation could be
577 explained by the sampling schedule: detection of high H_2S concentration within the
578 micro-ecosystems indicates a nutrient rich habitat for phototrophic sulfur bacteria
579 (Guerrero et al. 1985; Hamilton et al. 2014), which suggests previous high activity of
580 sulfate-reducing microorganisms (Sass et al. 1997; Pimenov et al. 2014). Earlier or
581 later sampling may have revealed more oxidized sulfur-compounds and higher
582 abundance of sulfate-reducing microorganisms. Further explanation of this
583 unbalanced ratio between these two functional groups could probably be found in the
584 light-dark- and the sediment-water ratio of our model system. With increasing
585 temperature, microorganisms changed from aerobic respresentatives (*Giesbergeria*,
586 *Uliginosibacterium*) to anaerobic ones (*Chlorobaculum*, *Chlorobium*), which is also
587 confirmed by the oxygen measurements. Higher turnover rate of organic substrates of
588 the microbes in the sediment and higher H_2S production supports the dominant growth
589 of phototrophic sulfur bacteria at moderate and higher temperatures (Nedwell et al.
590 1994; Findlay and Kamyshny 2017; Velthuis et al. 2018). The dominant blooms of
591 *Chlorobium* with increasing temperature is an important finding and its ecological
592 consequences should be investigated for future global change scenarios.

593 Some results hint at the presence of alternate stable states (Beisner and
594 Cuddington 2010) in the phototrophic sulfur bacterial communities. There was
595 considerable variation among some of the replicates of the same treatment
596 combination, despite them having very similar initial conditions. For example, some
597 contained either the green phototrophs *Chlorobium* or *Chlorobaculum*, or the purple
598 phototroph *Allochromatium* (based on sequencing and macro/microscopical
599 observation of the respective columns). Beside these alternative compositions within
600 the same functional group, also the oxygen dynamics indicated interesting patterns,
601 particularly at 28 °C, where two of three replicates of the nutrient addition-treated
602 micro-ecosystems became oxic at the bottom sensor. Since no significant differences
603 in the microbial community can be observed in these incubations, vertical movements
604 of the oxic-anoxic interphase may have occurred, which was also visible by the
605 different height of the white microbial community, consisting of *Rhodospirillaceae*
606 representatives. Additionally, sinking of cyanobacteria into the anoxic layer could also
607 result in temporary changes of oxygen dynamics in our dynamic system.

608 An interesting aspect could be observed regarding the pH gradient of the water
609 column, which showed a slightly alkaline pH on the water surface in the controls,
610 probably due to photosynthesis reactions of cyanobacteria/algae and HCO_3^- in the
611 unbuffered system, and a slightly acidic pH at the bottom, probably due to organic
612 matter degradation. This gradient was removed by the addition of $NH_4H_2PO_4$, which
613 decreases the pH due to its acidic character and buffering activity on the surface to
614 pH ~6.

615 This study reveals that two environmental change factors (i.e. temperature and
616 nutrient availability) caused large and non-additive variation in the composition of
617 aquatic microbial communities and in the abiotic conditions of the ecosystem.The

advantages of this system include high parallelization and replication, easy and non-destructive sampling, versatility of testable conditions and manipulations, and are of additional interest besides *in situ* ecosystem studies. We believe that these insights are only the tip of the iceberg of what can be learned from such model micro-ecosystems with strong and even stratified spatial environmental gradients. Even in the described study we could have included many more elements, such as characterization of the conditions at the sampling site, measurements of organic compounds composition in the water column, and a control without cellulose. Further research with this new experimental system could take many paths, including: studying the stability of the communities to press and pulse perturbations, and how 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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The authors declare that there is no conflict of interest.

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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