

# 1 How are oxygen budgets influenced by dissolved iron and 2 growth of oxygenic phototrophs in an iron-rich spring system? 3 Initial results from the Espan Spring in Fürth, Germany

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11 **Abstract.** At present most knowledge on the impact of iron on <sup>18</sup>O/<sup>16</sup>O ratios (i.e.  $\delta^{18}\text{O}$ ) of dissolved oxygen (DO)  
12 under circum-neutral conditions stems from experiments carried out under controlled laboratory conditions. These  
13 showed that iron oxidation leads to an increase in  $\delta^{18}\text{O}_{\text{DO}}$  values. Here we present the first study on effects of  
14 elevated Fe(II) concentrations on the  $\delta^{18}\text{O}_{\text{DO}}$  in a natural, iron-rich circum-neutral watercourse. Our results show  
15 that iron oxidation was the major factor to cause rising oxygen isotopic signatures in the first 85 meters of the  
16 system in the cold season (February) and for the first 15 meters during the warm season (May). Further along the  
17 course of the stream, the  $\delta^{18}\text{O}_{\text{DO}}$  decreased towards values known for atmospheric equilibration at 24.6 ‰ during  
18 both seasons. Possible drivers for this decrease may be reduced iron oxidation, increased atmospheric exchange  
19 and DO production by oxygenic phototrophic algae mats. In the cold season, the  $\delta^{18}\text{O}_{\text{DO}}$  values stabilized around  
20 atmospheric equilibrium, whereas in the warm season stronger influences by oxygenic photosynthesis caused  
21 values down to +21.8 ‰. In the warm season after 145 meters downstream of the spring, the  $\delta^{18}\text{O}_{\text{DO}}$  increased  
22 again until it reached atmospheric equilibrium. This trend can be explained by a respiratory consumption of DO  
23 combined with a relative decrease in photosynthetic activity and increasing atmospheric influences. Our study  
24 shows that dissolved Fe(II) can exert strong effects on the  $\delta^{18}\text{O}_{\text{DO}}$  of a natural circum-neutral spring system even  
25 under constant supply of atmospheric O<sub>2</sub>. However, in the presence of active photosynthesis, with active supply  
26 of O<sub>2</sub> to the system, direct effects of Fe oxidation on the  $\delta^{18}\text{O}_{\text{DO}}$  value becomes masked. Nonetheless, critical Fe(II)  
27 concentrations may indirectly control DO budgets by enhancing photosynthesis, particularly if cyanobacteria are  
28 involved.

## 29 1 Introduction

30 Oxygen is the most abundant (45.2 %) and iron the fourth most abundant (5.8 %) element on earth (Skinner, 1979).  
31 Such huge global reservoirs render these elements critically important in global biogeochemical cycles. In addition,  
32 their reactivity is exceptional: O<sub>2</sub> is a powerful oxidation agent while Fe can cover oxidation states from -4 to +7  
33 in extreme cases, with the most commonly known ones being 0, +2 and +3 (Lu et al., 2016).

34 Iron is also an essential trace element in many biological processes, including photosynthesis, oxygen transport  
35 and DNA biosynthesis (Kappler et al., 2021). This closely links to the formation and dissolution of Fe oxides.  
36 These common forms of metal oxides may enhance or reduce availabilities of both elements in the water column  
37 and pore waters and thus may largely regulate aqueous life.

38 In aqueous environments, dissolved oxygen (DO) is one of the most essential ecosystem parameters and, despite  
39 its moderate solubility (e.g. 9.3 mg/L at 20 °C), it assumes a central role in respiration, primary production and  
40 Fe-oxidation (Pusch, 1996). The concentration of DO coupled to its stable isotope <sup>18</sup>O/<sup>16</sup>O ratios (i.e.  $\delta^{18}\text{O}$ ) can

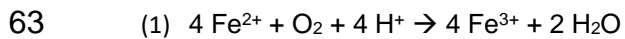
41 yield additional information about sources and sinks, including atmospheric input, photosynthesis, respiration and  
42 mineral oxidation.

43 When equilibrated with the atmosphere,  $\delta^{18}\text{O}_{\text{DO}}$  values typically range around a value of + 24.6 ‰ (Mader et al.,  
44 2017) while photosynthesis and respiration can change these isotope ratios (Guy et al., 1993; Kroopnick, 1975).  
45 The splitting of water molecules during photosynthesis hardly produces an isotope discrimination and the resulting  
46 DO should have the same isotope value as the surrounding water (Guy et. al., 1993; Eisenstadt et al., 2010).  
47 Meteoric water in temperate climates is normally depleted in  $^{18}\text{O}$  and therefore the photosynthetic oxygen in these  
48 areas varies between - 10 to - 5 ‰ (Quay et. al., 1995; Wang and Veizer, 2000). Respiration, on the other hand,  
49 preferentially accumulates  $^{16}\text{O}$  and enriches the remaining DO in  $^{18}\text{O}$ . This process yields  $\delta^{18}\text{O}_{\text{DO}}$  values between  
50 + 24.6 and + 40 ‰ (Guy et. al., 1993).

51 Additionally, oxidation of metals such as Fe also lead to increases in  $\delta^{18}\text{O}_{\text{DO}}$  (Lloyd, 1968; Taylor and Wheeler,  
52 1984; Wassenaar and Hendry, 2007; Oba and Poulsen, 2009 a,b; Pati, 2016). Mostly, the impacts of Fe oxidation  
53 on  $\delta^{18}\text{O}_{\text{DO}}$  values have been investigated experimentally under controlled conditions (Oba and Poulson, 2009b;  
54 Pati et al., 2016). As a new aspect, these dynamics were not studied in open water systems such as springs and  
55 rivers so far. New field investigations might reconcile variations in the fractionation factors obtained in the  
56 abovementioned studies. At current they are thought to result from differences in temperature, pH and initial Fe(II)  
57 concentrations that could be outlined under abiotic conditions.

58 Dissolved Fe(II) in natural systems may have primary and secondary impacts on DO concentration and its  $\delta^{18}\text{O}_{\text{DO}}$   
59 values. The primary influence originates from the  $\text{O}_2$  binding by iron oxidation (equation 1). This leads to decreases  
60 of the DO and causes simultaneous increases of  $\delta^{18}\text{O}_{\text{DO}}$  values (Wassenaar and Hendry, 2007; Smith et al., 2011;  
61 Parker et al., 2012 and Gammons et al., 2014).

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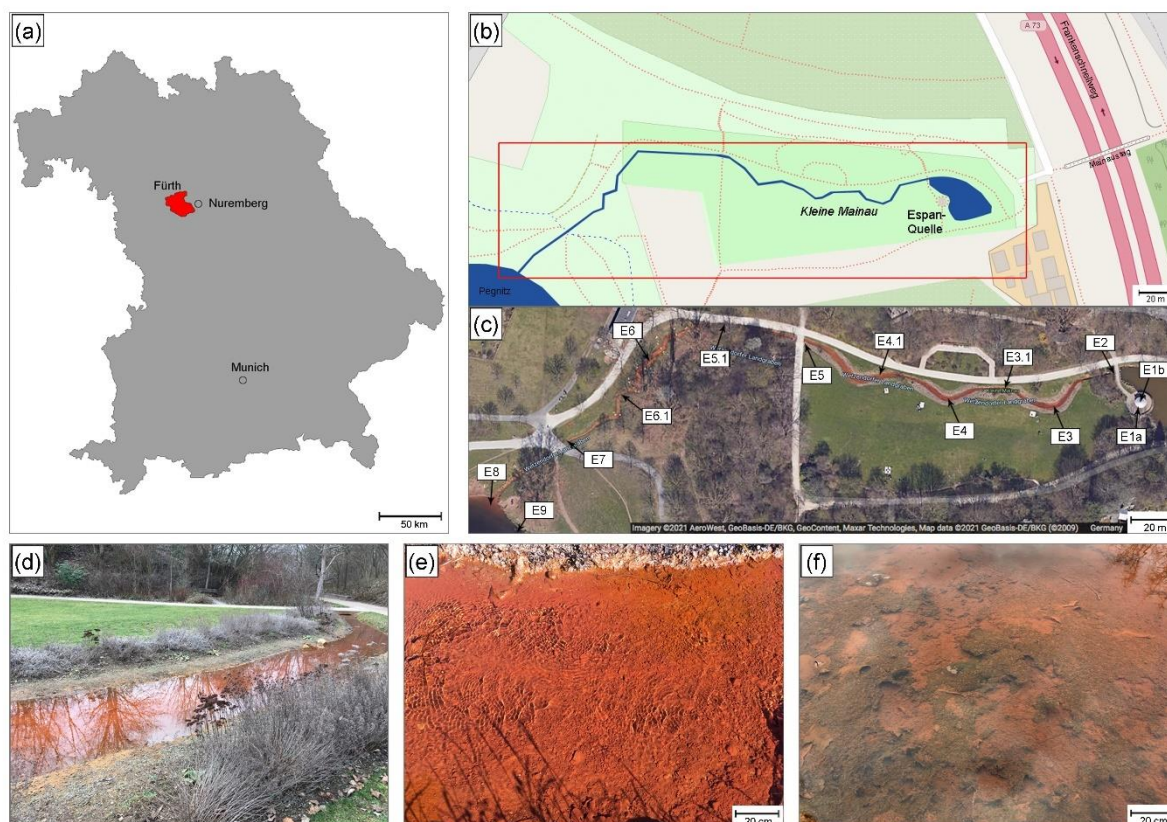


65 Dissolved Fe(II) can also have secondary (i.e. indirect) influences on the DO content and the  $\delta^{18}\text{O}_{\text{DO}}$ . This happens  
66 when it acts as an essential micronutrient to cause growth-stimulating effects on  $\text{O}_2$ -producing and respiring  
67 microorganisms. These influences of Fe(II) on DO and  $\delta^{18}\text{O}_{\text{DO}}$  in circum-neutral aquatic systems have so far  
68 received little attention because of the following reasons:

69 (1) Fe oxidation often masks  $\delta^{18}\text{O}_{\text{DO}}$  values created by respiration, photosynthetic and atmospheric oxygen  
70 and

71 (2) adequate Fe(II)-rich circum-neutral model systems are scarce on modern earth. This is due to the high reactivity  
72 of iron with DO.

73 To the best of our knowledge, no study so far has systematically investigated the influences of elevated Fe(II)  
74 concentrations on  $\delta^{18}\text{O}_{\text{DO}}$  values in a natural and circum-neutral iron-rich system. In order to bridge this gap, we  
75 investigated the aqueous chemistry and  $\delta^{18}\text{O}_{\text{DO}}$  values in the iron-rich Espan Spring in Fürth, Germany (Fig. 1).  
76 This Fe(II)-rich artesian spring offers a complex biogeochemical natural field site to analyse effects of different  
77 Fe(II) contents on the DO and  $\delta^{18}\text{O}_{\text{DO}}$  values.



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 79 **Figure 1** Overview of the Espan Spring in Fürth, Germany. a) and b): Location of the spring in Bavaria and the  
 80 city of Fürth. c) Satellite image of the spring by Google maps showing the distinct red colour. d) to f) Detailed  
 81 photos of the system. d) displays the stream between sampling points E4 and E5, e) shows sampling point E3 (with  
 82 the bank of the water line in the upper part of the picture) and f) displays sampling point E4.1 with algae and  
 83 cyanobacteria mats.

84  
 85 The aims of this study were to establish an inventory of biology together with Fe and oxygen budgets in this natural  
 86 spring and stream system. We further aimed to investigate how increased Fe(II)-levels influence the oxygen budget  
 87 of the system and whether a combination of DO and  $\delta^{18}\text{O}_{\text{DO}}$  measurements can help to assess this effect. This is  
 88 also timely because environmental impacts of Fe(II) become increasingly recognised for their negative effects on  
 89 ecosystems such as with the browning or brownification phenomenon (Kritzberg and Ekström, 2011;  
 90 Weyhenmeyer et al., 2014; Kritzberg et al., 2020). During this process, increased iron levels can consume oxygen,  
 91 cause algae blooms and reduce water quality and thus may affect aqueous ecosystems and their services. Here we  
 92 describe a first complete spatial sampling campaign in the cold and warm season with Fe(II), Fe(III), DO and its  
 93 stable  $^{18}\text{O}/^{16}\text{O}$  isotope ratios together with field parameters (pH, T, DO, pe, electrical conductivity).. This study  
 94 contributes to the knowledge of Fe oxidation in natural systems and delivers implications of hardly explored  
 95 seasonal dynamics in Fe(II) rich systems.

## 96 2 Methods

### 97 2.1 Study site

98 The Espan Spring is located in the city of Fürth, Germany (49°28'15.8"N 11°00'53.0"E, Fig. 1). It is an artesian  
99 spring that originates from a confined aquifer that was tapped by a drilling project in 1935 from a depth of 448.5  
100 m below ground. The water originates from the so called "lower mineral water horizon". This horizon is dominated  
101 by artesian inflow from the lower Buntsandstein Formation. The Buntsandstein in Fürth consists of red sandstone  
102 layers that are composed of light reddish to yellowish-white-grey sandstones of different grain sizes. The  
103 sandstones are intercalated with various rubble, conglomerate, and clay layers as well as thin gypsum and salt  
104 (Birzer, 1936). Three noticeable conglomerate layers are present in the sequence of Buntsandstein layers. Birzer  
105 (1936) distinguished the Upper Buntsandstein from the Upper Main Buntsandstein by the Main Conglomerate  
106 which can be found at a depth of 321 to 324 m. The Middle Boulder Layer at a depth of 370 to 371 m separates  
107 the Upper from the Lower Main Buntsandstein and the so-called Eck'sche Conglomerate at a depth of 433 to 440  
108 m which separates the Lower Main Buntsandstein from the Lower Buntsandstein (Birzer, 1936).

109 At a depth of 370 to 439 m, mineral water flows into the borehole from the Upper and Lower Main Bunter  
110 Sandstone and from the Eck conglomerate. This water, which is caught in the red sandstone and has a temperature  
111 of about +22°C, was called the "Lower Mineral Water Horizon"; in 1936 its yield was about 10 L s<sup>-1</sup> at a water  
112 temperature of +23°C (Kühnau 1938). The water of this lower spring horizon is under artesian pressure and exits  
113 the spring with a head of 13 m above ground level (Birzer, 1936). Nowadays the Espan Spring has a constant yield  
114 of about 5 L s<sup>-1</sup>.

115 After the water exits the basin in a pavilion with a temperature of ~ 20 °C, it discharges into a stream of about 300  
116 m length that is known as the "Wetzendorfer Landgraben (WL)". This small stream drains into the Pegnitz River  
117 without any further tributaries (Fig. 1b, c). The water can be classified as a Na-Ca-Cl-SO<sub>4</sub> mineral water with  
118 initially undersaturated DO values of 2.3 mg/L and Fe(II) contents of up to 6.6 mg/L (Table 1). Figure 1c shows  
119 an aerial image of the spring and stream system that shows a distinct red coloring of the stream bed. The most  
120 plausible explanation for this coloring are iron-oxide-precipitates (Fig. 1 d, e). The WL has a water depth between  
121 8-10 cm and shows little fluctuations.

## 122 2.2 Sampling procedures

123 Two field campaigns were performed in February and May 2020, during which water was collected at 14 locations  
124 along the stream. The onsite parameters pH (±0.05; instrument precision), temperature (±0.1 °C), electrical  
125 conductivity, Eh and DO (all ±2 %) were measured with a HACH HQ 40d multi parameter instrument. Alkalinity  
126 titrations were carried out with a Hach Titrator with a bromocresol-green indicator. Fe(II) and Fe(III) contents were  
127 measured using an iron (II/III) cuvette test set by Hach in combination with a portable Hach spectrophotometer  
128 (model DR 2800).

129 Samples for <sup>18</sup>O/<sup>16</sup>O ratios of DO were collected in 12-mL Exetainers (Labco Ltd. Lampeter, U.K.) that were  
130 prepared with 10 µL of a saturated HgCl<sub>2</sub> solution to prevent secondary biological activity after sampling  
131 (Wassenaar and Koehler, 1999; Parker et al., 2005 and 2010). The Exetainers were filled with syringe-filtered  
132 water via 0.45 µm pore size nylon filters until they were entirely full and free of air bubbles. They were then  
133 carefully closed with screw caps with a butyl septum in order to avoid atmospheric contamination. Test series  
134 showed that the amount of atmospheric contamination during this filling procedure is usually negligible (Mader et  
135 al. 2018).

136 Samples for water isotopes were collected in 15 mL Falcon tubes and treated in the same manner as the ones for  
137 DO isotope measurements, except for preservation with HgCl<sub>2</sub>. All samples were stored in a mobile refrigerator  
138 box at 4 °C directly after collection and carried to the laboratory where they were measured within 24 h.

### 139 **2.3 Identification of possible mineral precipitates**

140 In order to determine possible mineral precipitate data for the pH, pe (the negative decadic activity of available  
141 electrons), temperature, alkalinity (as CaCO<sub>3</sub>), as well as cations and anions, the specific sampling points were  
142 fed into the program PhreeqC (Version 3; Parkhurst and Appelo, 2013) for calculation of saturation indices. The  
143 database used for these calculations was Wateq4.

### 144 **2.5 Laboratory methods**

#### 145 **2.5.1 Identification of cyanobacteria**

146 Samples were collected in a preliminary field assessment at the anoxic piping where the spring flows into the creek  
147 (E2), in the middle of the creek at the first small pond after the water had contact to the atmosphere (E3) and about  
148 5 m downstream of this pond from an algal mat with bubbles on the surface (E4). Samples for cyanobacterial  
149 isolation were collected in sterile 2-mL-Sarsted tubes and sealed. Samples for microscopic analysis were collected  
150 with a 75 % ethanol sterilised spatula and placed in a sterile 6 cm petri dish (Sarsted, Germany). Immediately after  
151 returning from sampling, samples were embedded in 1.5 % Agarose in de-ionized water to preserve the structure  
152 of the bio mats during further handling and shipping.

153 Microscopic analysis was performed on thin sections of the embedded mats using a CLSM-type microscope (LSM  
154 880, Carl Zeiss), using modified acquisition settings from Jung *et al.* (2019) to discriminate between cyanobacterial  
155 (chlorophyll-*a* (chl-*a*) and phycobiliproteins (PBP) and green algal (chl *a*) fluorescence. Laser transmission images  
156 were also generated using the 543 nm laser.

157 A spatula tip of green coloured mat was used to inoculate 5 mL of BG11 medium (Stanier *et al.*, 1971) in a well  
158 of a 6-well plate and incubated for 3 weeks at 24 °C on a 16:8 day:night cycle with illumination at 15 μmols  
159 photons m<sup>2</sup>/s under an OSRAM L30W/840 LUMINLUX Cool White bulb. Individual Cyanobacterial species were  
160 picked from the mat cultures under a Nikon SMZ-U Zoom binocular microscope for further subculturing on 1 %  
161 agar solidified BG11 plates, as well as liquid culture. Isolates were observed under an Olympus BX53 light  
162 microscope and their morphologies recorded using an Olympus DP26 Camera. The number of cells per filament  
163 and cell dimensions were measured using ImageJ 1.47v software. DNA was extracted (Gehring *et al.*, 2010)  
164 from one axenic isolate of a microscopically identified *Persinema* species of cyanobacteria. The 16s rDNA gene  
165 and intergenic spacer sequence was amplified by the SSU-4 fwd and ptLSU-C-D rev primer pair (Marin *et al.*,  
166 2005) using the Taq PCR mastermix (Qiagen, Germany). The PCR product was purified (NucleoSpin PCR clean-  
167 up kit, Macherey-Nagel, Germany) and sequenced (Wilmotte *et al.*, 1993). Sequences were merged (HVDR  
168 Fragment Merger tool, Bell & Kramvis, 2013) and the final 16S-ITS sequence submitted to National Center for  
169 Biotechnology Information, National Institute of Health, USA (NCBI).

#### 170 **2.5.2 Isotope measurements**

171 Stable isotope ratios of DO (expressed as  $\delta^{18}\text{O}_{\text{DO}}$ ) were measured on a Delta V Advantage Isotope Ratio Mass  
172 Spectrometer (IRMS; Thermo Fisher Scientific, Bremen, Germany) coupled to an automated equilibration unit  
173 (Gasbench II). Measurements were carried out in continuous flow mode with a modified method by Barth et al.  
174 (2004). Here the isolation of DO into a headspace relies on a helium extraction technique by Kampbell et al.  
175 (1989) and Wassenaar and Koehler (1999). Different portions of laboratory air were injected into helium-flushed  
176 Exetainers and used to correct obtained data sets for linearity and instrumental drift during each run. Here  
177 laboratory air is defined to represent atmospheric oxygen with a ubiquitous value of 23.9 ‰ versus Vienna  
178 Standard Mean Ocean Water (VSMOW) (Barkan and Luz, 2005). Data were normalized to this value.

179

$$180 \quad \delta = (R_{\text{sample}} / R_{\text{SMOW}} - 1) \quad (\text{Clark and Fritz, 1997})$$

181

182 To obtain ratio changes in per mil (‰), the  $\delta$  values were multiplied by factor of 1000.

183 All samples were measured in triplicates and isotope values standard deviations ( $1\sigma$ ) were less than 0.1 and 0.2 ‰  
184 for  $\delta^{18}\text{O}_{\text{H}_2\text{O}}$  and  $\delta^{18}\text{O}_{\text{DO}}$ , respectively.

## 185 **3 Results and discussion**

### 186 **3.1 On-site parameters**

187 The on-site parameters as displayed in Table 1 show a range of pH values between 6.1 and 8.6 in the cold season

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Sampling point	Distance from spring (m)	pH	O <sub>2</sub> (mg/L)	Temperature (°C)	Conductivity (mS/cm)	Alkalinity (mg/L)	Fe <sup>2+</sup> (mg/L)	Fe <sup>3+</sup> (mg/L)	Na <sup>+</sup> (g/L)	Ca <sup>2+</sup> (g/L)	SO <sub>4</sub> <sup>2-</sup> (g/L)	Cl <sup>-</sup> (g/L)	U <sup>6+</sup> (µg / L)
<b>Cold season</b>													
E1a	0	6.1	2.3	19.5	16.8	820	6.6	0.4	2.5	1.2	2.1	4.4	170
E1b	0	6.5	3.4	19.3	16.4	828	6.6	0.4	2.5	1.2	2.2	4.5	170
E2	15	6.5	4.5	19.3	16.5	796	5.6	0.4	2.5	1.2	2.2	4.5	170
E3	45	6.7	5.8	17.5	16.8	790	5.7	0.4	2.5	1.2	2.2	4.5	170
E3.1	65	6.5	7.4	17.3	16.6	810	4.5	0.5	2.5	1.2	2.2	4.5	170
E4	85	7.1	8.0	16.2	16.9	804	3.9	0.6	2.5	1.2	2.2	4.5	170
E4.1	115	7.5	8.7	16.1	17.0	808	3.4	0.6	2.5	1.2	2.2	4.5	170
E5	145	7.9	8.9	15.2	16.8	804	0.9	0.8	2.4	1.2	2.2	4.5	170
E5.1	175	7.6	9.1	15.3	16.8	816	0.4	0.5	2.5	1.2	2.2	4.5	170
E6	205	7.9	9.5	14.1	16.9	760	0.2	0.1	2.5	1.2	2.2	4.5	170
E6.1	235	7.9	9.7	13.3	16.5	770	0.0	0.1	2.5	1.2	2.2	4.5	170
E7	265	8.0	10.1	12.3	16.6	760	0.0	0.1	2.4	1.1	2.2	4.5	170
E8	295	8.0	10.5	10.8	1.1	195	0.0	0.1	0.1	0.1	0.1	0.2	5.0
E9	300	8.6	11.0	7.4	0.5	160	0.0	0.1	0.0	0.1	0.0	0.0	0.4
<b>Warm season</b>													
E1a	0	6.3	3.6	21.3	16.3	874	6.9	0.0	2.4	1.1	2.2	4.5	190
E1b	0	6.4	3.9	21.2	16.4	850	6.7	0.1	2.5	1.1	2.2	4.4	190
E2	15	6.5	5.9	20.6	16.4	846	5.6	0.0	2.5	1.1	2.1	4.4	160
E3	45	6.6	6.6	21.6	16.4	814	4.0	0.0	2.4	1.1	2.2	4.4	160
E3.1	65	6.9	7.6	22.5	16.4	808	2.9	0.1	2.4	1.1	2.2	4.4	160
E4	85	7.2	8.2	22.7	16.4	826	1.5	0.1	2.5	1.1	2.2	4.4	160
E4.1	115	7.3	8.0	23.0	16.4	812	0.7	0.2	2.5	1.1	2.2	4.5	160
E5	145	7.4	8.1	24.0	16.4	786	0.1	0.1	2.4	1.1	2.2	4.5	160
E5.1	175	7.5	8.0	25.6	16.4	804	0.0	0.0	2.5	1.1	2.2	4.5	160
E6	205	7.5	8.1	25.7	16.4	796	0.0	0.0	2.5	1.1	2.2	4.5	160
E6.1	235	7.5	7.9	25.5	16.4	748	0.0	0.0	2.4	1.1	2.2	4.5	150
E7	265	7.5	8.1	24.9	16.4	742	0.0	0.0	2.5	1.1	2.2	4.5	150
E8	295	7.5	8.3	22.8	16.4	708	0.0	0.0	2.5	1.1	2.2	0.3	180
E9	300	8.0	8.8	16.8	0.8	238	0.0	0.0	0.1	0.1	0.0	0.0	1.0

212 **Table 1** On-site parameters: major ion concentrations and Fe(II) and DO concentrations for the Espan Spring.  
213 Note that values before the forward slash are for cold season and after the slash for warm season.

214 and between 6.3 and 8.0 in the warm season. The observed changes of the pH over the course of the spring are  
215 mostly due to the constant degassing of CO<sub>2</sub> from the spring. Oxygen values range from 2.3 mg/L to 11.0 mg/L in  
216 the cold season and from 3.6 mg/L to 8.8 mg/L in the warm season. Differences between the cold and warm season  
217 are due to the fact that cold water can dissolve more O<sub>2</sub> than warm water. The general increase in the amount of  
218 DO over the course of the spring is due to a continuous dissolution of atmospheric O<sub>2</sub> in the spring water and due  
219 to the impact of photosynthesis. Water temperatures ranged between 19.3 and 7.4°C in the cold season and between  
220 21.3 and 25.7°C in the warm season. The conductivity remained stable over the course of the spring and only  
221 showed minor differences between the cold and warm season. The same applies to the alkalinity. The behavior of  
222 the Fe(II) and Fe(III) is described in section 3.5. Values of major ions (Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, NO<sub>3</sub><sup>-</sup>, Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup> and Mg<sup>2+</sup>)  
223 remained constant over the course of the spring and show no differences between the cold and warm season.

### 224 3.2 Precipitation calculations

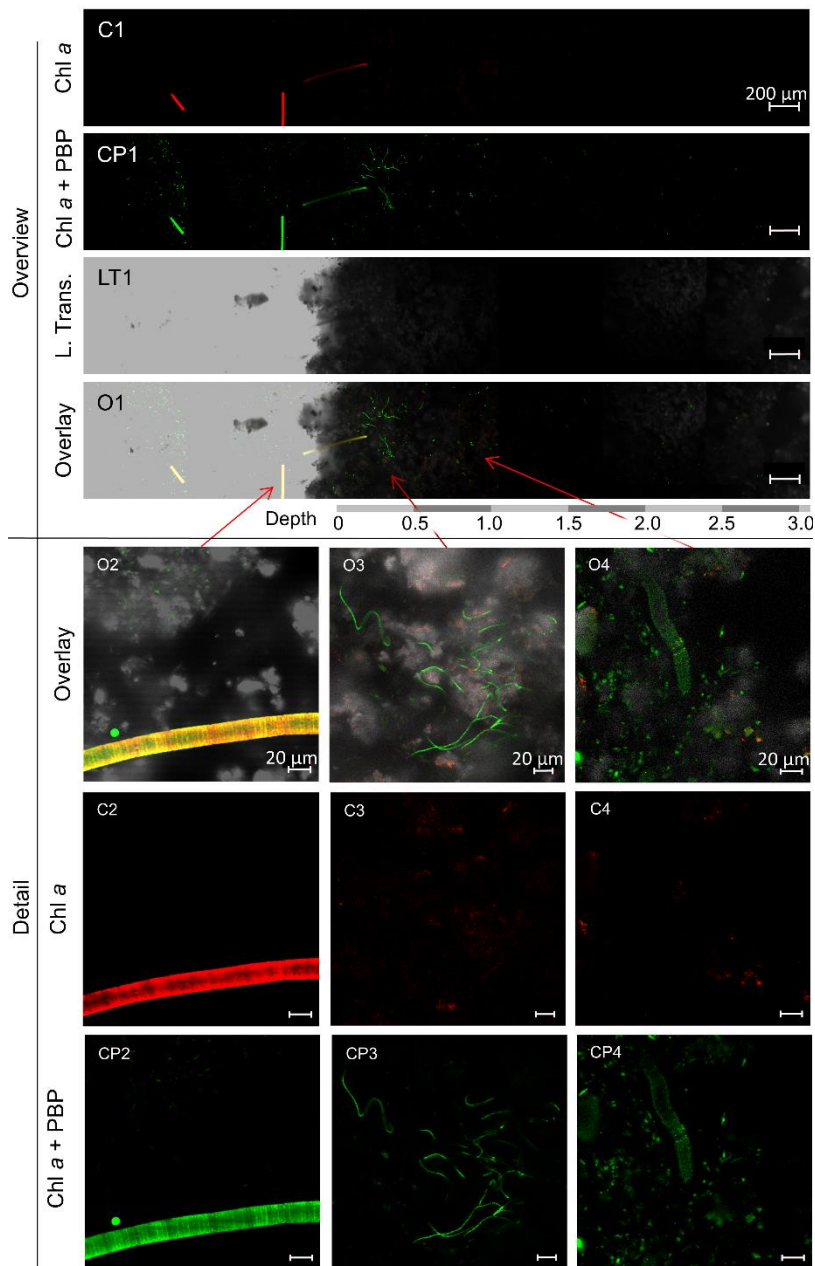
225 Precipitating mineral phases as determined with PhreeqC showed that the dominant phase at all measurement  
226 points was Hematite (Fe<sub>2</sub>O<sub>3</sub>) (Supplementary Information Table S1 and S2). Additionally, Goethite ( $\alpha$ -FeO(OH)),  
227 Ferrihydrite (Fe(OH)<sub>3</sub>), Siderite (FeCO<sub>3</sub>) and K-Jarosite (KFe<sup>3+</sup><sub>3</sub>(OH)<sub>6</sub>(SO<sub>4</sub>)<sub>2</sub>) as well as CaCO<sub>3</sub> and Rhodochrosite  
228 (MnCO<sub>3</sub>) showed elevated SI values and indicated precipitation.

### 229 3.3 Bacterial contents

230 Confocal Laser scanning microscopy (CLSM) showed that only the samples from Site E4.1 have photosynthetic  
231 organisms in significant quantities during the cold period. The photosynthetic community in this biofilm was  
232 dominated by cyanobacteria, with very few eukaryotic algae (Fig. 2). *Lyngby* was observed along the sides of the  
233 fast-flowing stream on the smooth hard canal section at E2, however, the loosely built *Lyngbya* sp. mats were only  
234 observed in the wider, shallower sections from sampling sites E3 to E5, and predominating between sites E3.1 and  
235 E4.1. The *Lyngbya* sp. filaments were not encrusted by oxidized iron as proven by light microscopy. As these are  
236 simple cyanobacterial mats on top of loose iron oxides, with no additional microbial layers beneath them, the  
237 bubbles are presumably oxygen generated during photosynthesis (Supplementary Information Fig. 1)

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240 **Figure 2** CLSM images of mat sample E4.1. Overview: images of the cross-section of the top 3 mm of the biofilm  
 241 with the chl-*a* (C1) and chl-*a* plus PBP (CP1) fluorescence profile, complemented by a laser transmission picture  
 242 (LT1) and the superimposed image (O1). **Detail:** Superimposed images (O2/3/4) of chl-*a* (C2/3/3) and chl-*a* plus  
 243 PBP (CP2/3/4) fluorescence and laser transmission (not shown) of distinct organisms found in the bio mat. **O2:**  
 244 eukaryotic algae. **O3:** Possible *Klisiinema*- or *Persinema*-like sp. and a unicellular cyanobacterium. **O4:** *Lynbya* –  
 245 like sp. and a unicellular cyanobacterium.

246 Most of the cyanobacteria and all eukaryotic algae were located in the topmost 1.2 mm of the biofilms (Fig. 2  
 247 O1). Close-up images show eukaryotic algae (Fig. 2.O2), thin filamentous cyanobacteria, possibly *Persinema* sp.  
 248 or *Klisiinema* sp. (Fig. 2.O3) and *Lynbya* sp. (Fig. 2.O4). All pictures of the top layers of this sample site show an  
 249 abundance of unidentified unicellular cyanobacteria, while images from the other sample sites show very few  
 250 photosynthetic organisms at all (supplementary information Fig. 2).

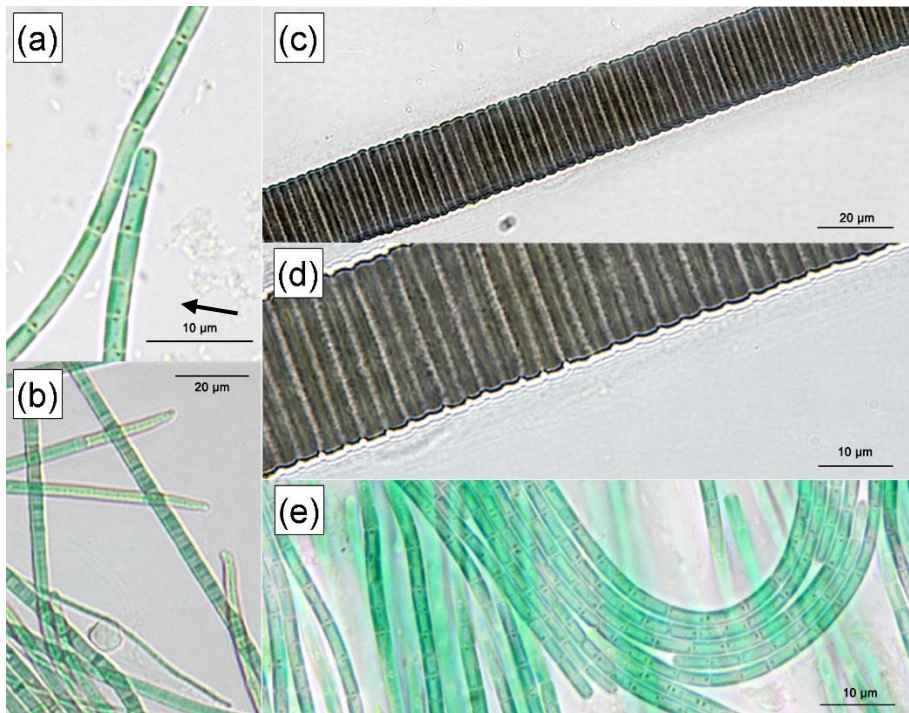
251 In order to determine the identity of the predominant cyanobacterial species isolated from the E4.1 enrichment  
 252 cultures, a determination key was used to compare particular features of an isolate to those already in the literature  
 253 for specific cyanobacterial species (Komárek und Anagnostidis, 2005). Note that enrichment cultures for samples  
 254 E2 and E3 did not yield enough material for cyanobacterial determination after 5 weeks in culture.  
 255 The red-brown filamentous strain (Fig. 3, c, d) exhibits single filaments, without false branching, that are 30.9 to  
 256 38.2  $\mu\text{m}$  wide (Table 2), with a firm, 9.5 to 14  $\mu\text{m}$  thick sheath. The trichomes and single cells are 21.5 to 24.2  $\mu\text{m}$   
 257 wide and 1.5 to 4.1  $\mu\text{m}$  long (Table 2), are red-brown in colour and constricted at the cross-walls. Based on these  
 258 characteristics, the species was attributed to the cyanobacterial genus *Lyngbya*.

259

	Filament length	Filament width ( $\mu\text{m}$ )	Cell width ( $\mu\text{m}$ )	Cell length ( $\mu\text{m}$ )
<i>Lyngbya sp.</i>	Indeterminate	30.9 – 38.2	21.5 – 24.2	1.5 – 4.1
<i>Klisinema sp.</i>	Indeterminate	3.9 – 7.6	12 – 4.5	0.3 – 0.4
<i>Persinema sp.</i>	Indeterminate		0.5 – 1.8	2.7 – 4.7

260 **Table 2** Filament and cell dimensions of the proposed cyanobacterial species.

261 The blue-green filamentous strain (Fig. 3, b) produces single filaments, without false branching, that are 3.9 to 7.6  
 262  $\mu\text{m}$  wide (Table 2) with a firm, 2.7 to 3.1  $\mu\text{m}$  thick sheath. The trichomes and single cells are 1.2 to 4.5  $\mu\text{m}$  wide  
 263 and 0.3 to 0.4  $\mu\text{m}$  long (Table 2), blue-green in colour without constriction at the cross-walls. The terminal cells  
 264 in mature filaments are conical, elongated and bent to one side, corresponding to those of the *Klisinema* genus  
 265 recently described by Heidari et al. (2018). The thin, naked pale green filaments (Figure 3a & e) resembled those  
 266 of *Persinema komarekii* (Heidari et al., 2018) with apical cells flattened at the end. In contrast to the observations  
 267 of Heidari et al. (2018), we observed terminal aerotopes. This species was purified in culture and the 16S-ITS  
 268 (NCBI accession number: MT708471) sequence confirmed its identity to *Persinema komarekii* (MF348313).



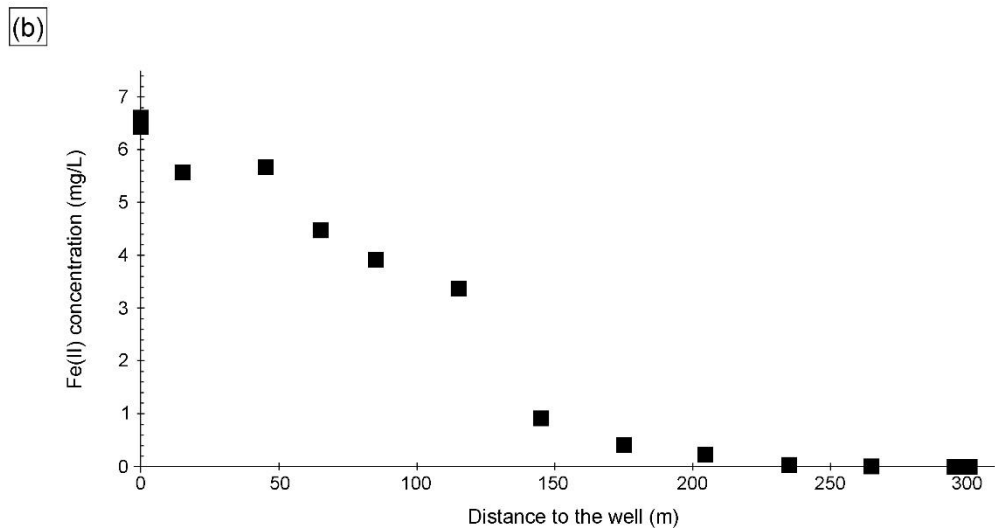
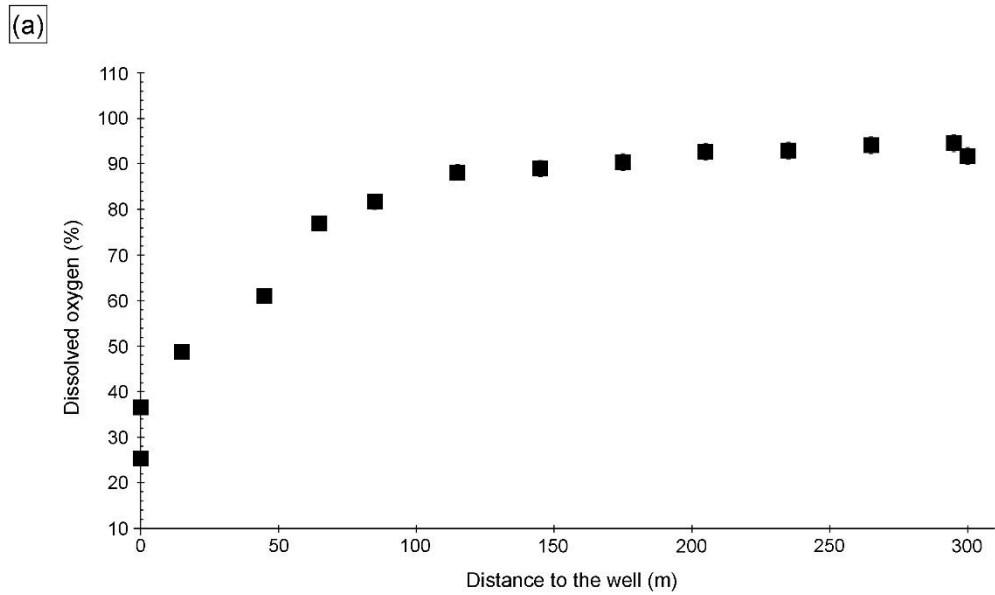
269

270 **Figure 3** Light Micrographs of the predominant isolates from sample E4.1: a) Single filament of *Persinema sp.*,  
 271 arrow indicates aerotopes. b) Biofilm of *Klisinema sp.* interspersed with *Persinema sp.* (arrow) c) *Lyngbya sp.*,  
 272 filament d) *Lyngbya sp.* sheath detail, E: Biofilm of *Persinema sp.*

### 273 3.4 Dissolved oxygen (DO)

274 The DO concentration in the Espan System was lowest at the faucet in the Pavilion (sampling point E1a) with a  
 275 saturation of 25.3 % (2.3 mg/L) (Fig. 4a). Over the following 100 meters DO saturation increased to 88.1 % (8.7  
 276 mg/L) in sampling point E4.1. Afterwards the saturation continually increased to 94.6 % (11.0 mg/L) in point E8.  
 277 From an initial depth of 435 meters with the abundance of reduced species such as Fe(II) and Mn(II), the low DO  
 278 content in sampling point E1a was expected and in the further course, more atmospheric oxygen was able to  
 279 dissolve. In addition, gas bubbles were observed in association with the *Lyngbya* mats. They were most prominent  
 280 at sample site E4.1 and indicate a significant contribution of O<sub>2</sub> from daytime photosynthesis. However, saturation  
 281 with DO was not reached during either of the sampling campaigns.

282



283

284 **Figure 4** a) Dissolved oxygen (%) and b) Fe(II) concentrations over the course of the Espan System in an example  
 285 graph for the cold season In February. The error for DO was 2 % and for Fe(II) it was 0.06 mg/L. Errors are within  
 286 symbol size.

287 **3.5 Fe(II) and Fe(III)**

288 The Fe(II) content was highest at the faucet with 6.6 mg/L while its lowest content was below instrument precision.  
 289 at sampling point E9 at 300 meters from the source (Fig. 4b). Fe(II) concentrations decreased constantly over the  
 290 stream course and were accompanied by increases in DO saturation (Fig. 4a). The decrease in Fe(II) could have  
 291 been caused by three major processes:

- 292 (1) Oxidation of Fe(II) to form ferric iron minerals such as ferrihydrite, hematite and goethite  
 293 (2) Precipitation of Fe(II) minerals such as the iron carbonate siderite ( $\text{FeCO}_3$ ) and/or an amorphous ferrous silicate  
 294 phase or  
 295 (3) Adsorption of Fe(II) on already formed iron minerals.

296  
297 All three possibilities seem plausible when taking into consideration the saturation indices of ferric iron minerals,  
298 goethite, ferrihydrite and hematite precipitate at all sampling points in the system (Köhler et al. 2020). These  
299 calculations furthermore show that siderite can precipitate in almost all sampling points while iron-silicate minerals  
300 are unlikely to precipitate. Therefore, adsorption of Fe (II) onto minerals is also a possible mechanism in the Espan  
301 System. Such adsorption of Fe(II) onto (oxyhydr)oxides was shown to typically occur under neutral conditions  
302 and should increase with rising pH (Zhang et al.,1992; Liger et al., 1999; Appelo et al., 2002; Silvester et al.,  
303 2005). Moreover, of large amounts of sulphate and chloride with average values of 2.2 and 4.5 g/L may have been  
304 responsible for maintaining observed high dissolved Fe(II) contents of the spring system at circum-neutral pH  
305 despite rising DO concentrations. Such elevated Cl<sup>-</sup> and SO<sub>4</sub><sup>2-</sup> contents can delay abiotic Fe(II) oxidation (Millero,  
306 1985).

307  
308 Dissolved Fe(III) was highest (0.8 mg/L) at sampling point E5 after 145 m and lowest (0.05 mg/L) at sampling  
309 point E7 after 265 m flow distance from the spring. The values initially increased from 0.4 mg/L in E1a to a  
310 maximum of 0.8 mg/L in point E5 (+/- 0.03 mg/L) and then decreased to their lowest value in sampling point E7.  
311 The solubility of iron oxides in natural systems at a circum-neutral pH and under aerobic conditions is generally  
312 very low (Cornell and Schwertmann, 2003) with values of the solubility product (K<sub>sp</sub>) between 10<sup>-37</sup> and 10<sup>-44</sup>  
313 (Schwertmann, 1991). However, Fe(III) could still be detected in the water, thus showing that its dissolution was  
314 possible. The dissolution of iron oxides can occur through several pathways including as protonation, reduction  
315 and complexation that create Fe(III) cations, Fe(II) cations as well as Fe(II) and Fe(III) complexes (Schwertmann,  
316 1991; Cornell and Schwertmann, 2003). Both the protonation as well as the reduction would lead to the formation  
317 of dissolved Fe(II). A steep increase in dissolved Fe(III) at 145 m downstream of the spring (from 0.5 mg/L to 0.8  
318 mg/L) also indicated acceleration of this process. One reason for this increase could be available organic matter.  
319 However, further analyses are needed to verify this interpretation.

### 320 3.6 δ<sup>18</sup>O<sub>DO</sub>

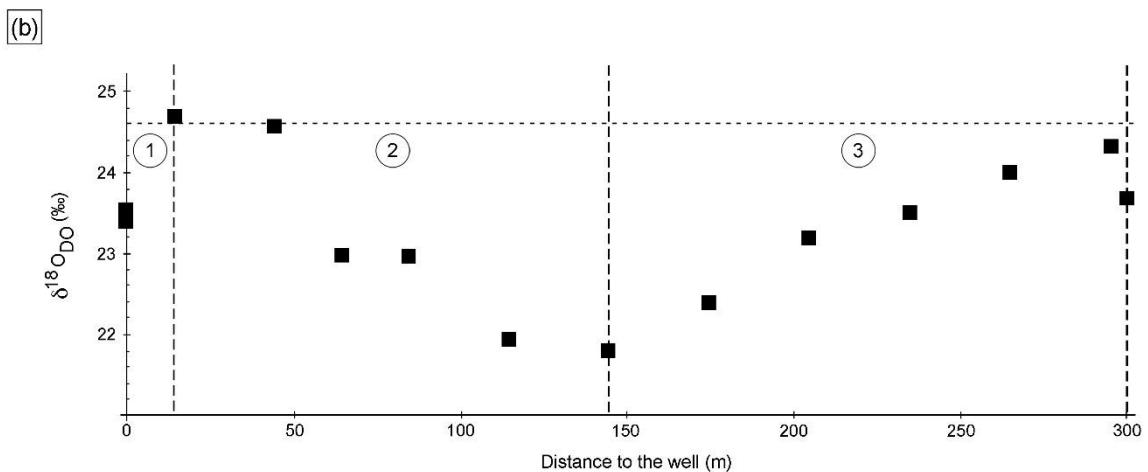
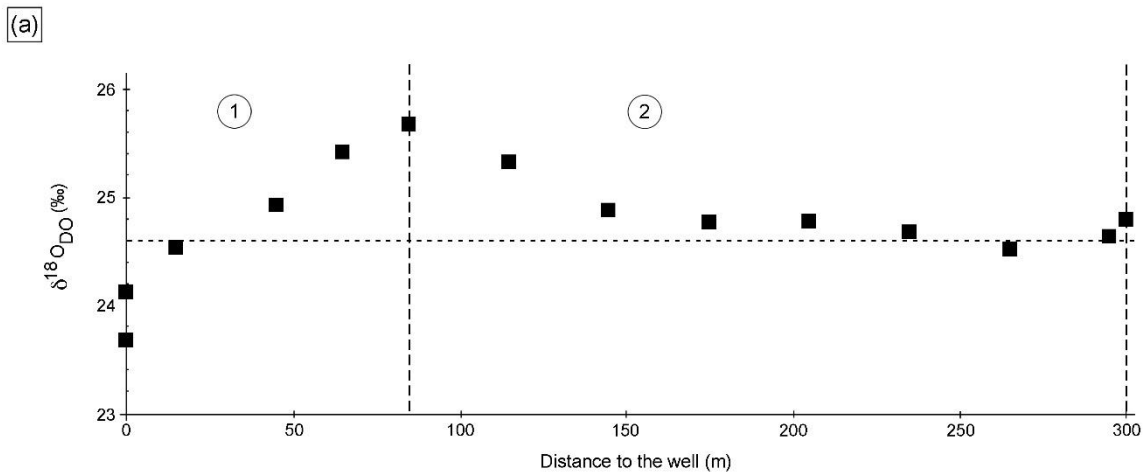
321 Figure 5 a) and b) show δ<sup>18</sup>O<sub>DO</sub> values over the course of the spring for the cold and warm seasons, respectively.  
322 The curves are divided into two zones for the cold season and three zones for the warm season.

#### 323 *Zone 1*

324 In the cold season, zone 1 extended from sampling point E1a to point E4. In these first 85 meters, the δ<sup>18</sup>O<sub>DO</sub>  
325 increased from a value of +23.7 ‰ at the faucet (E1a) to + 25.7 ‰ at E4. In the warm season, zone 1 extended  
326 from E1a to E2 with only 15 m distance from the spring. In this zone the values increased from + 23.4 ‰ at the  
327 faucet to a maximum value of + 24.7 ‰ at E2. In both seasons, δ<sup>18</sup>O<sub>DO</sub> values at E1a were below the value expected  
328 for atmospheric equilibration (+ 24.6 ‰). At first sight such <sup>16</sup>O-enriched δ<sup>18</sup>O<sub>DO</sub> values would suggest  
329 photosynthetic input of DO. However, the water originated from greater depths without any exposure to light and  
330 thus any photosynthetic influence can be ruled out.

331 The occurrence of δ<sup>18</sup>O<sub>DO</sub> values below + 24.6 ‰ in groundwater has been described in the literature (Wassenaar  
332 and Hendry, 2007; Smith et al., 2011; Parker et al., 2014 and Mader et al, 2018) and several explanations for this  
333 phenomenon have been suggested (Wassenaar and Hendry, 2007; Smith et al., 2011; Parker et al., 2014 and Mader  
334 et al, 2018). These include:

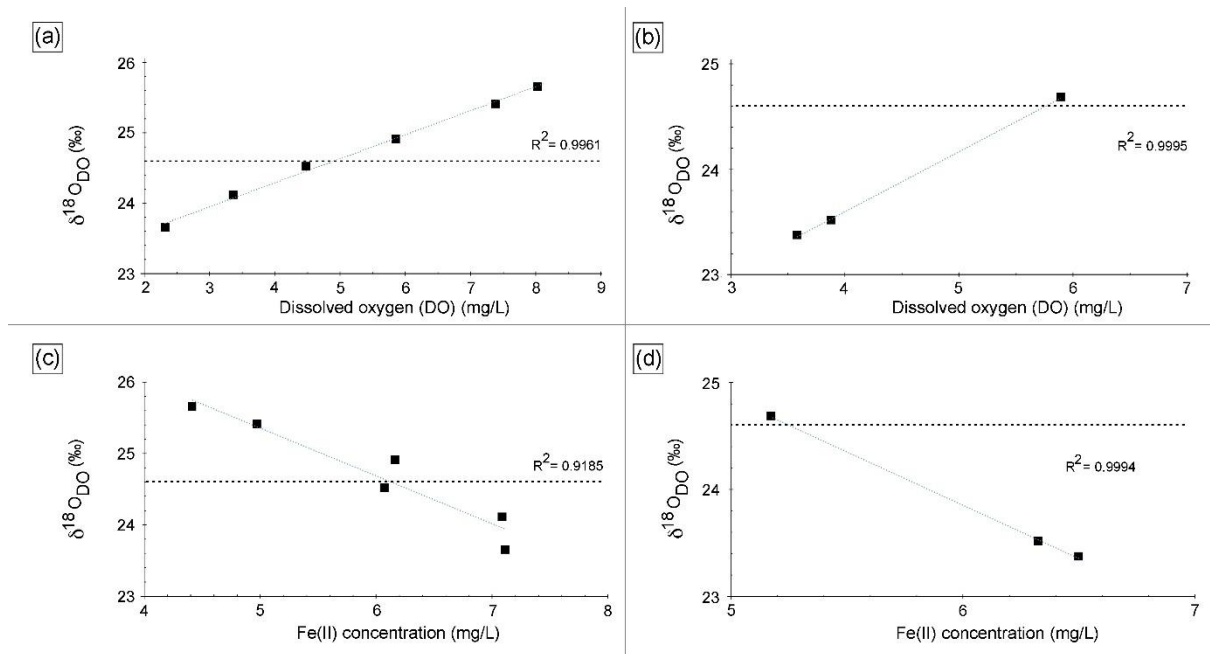
335 (1) possible transfer of photosynthetic or diffusive oxygen into the shallow aquifer (Smith et. al; 2011; Parker et  
 336 al., 2014; Mader et al., 2018),  
 337 (2) radial oxygen loss of plant roots (Teal and Kanwisher, 1966; Michaud and Richardson, 1989; Caetano and  
 338 Vale, 2002; Armstrong and Armstrong, 2005b)  
 339 (3) radiolysis of water (Wassenaar and Hendry, 2007) and  
 340 (4) kinetic gas transfer (Benson and Krause, 1980; Knox et al. ,1992; Mader et al. 2017)  
 341 Explanations (1) and (2) are very unlikely in the Espan Spring, because the water originates from a depth of 435  
 342 meters below ground through pipes that presumably prevent any exchange with surface water or possible impacts  
 343 of plant roots. It should however be noted that water from the Espan Spring contains up to 170 µg/L of uranium  
 344 from easily soluble uranium compounds that are commonly encountered in the Buntsandstein formations (Büttner  
 345 et al. 2006; Meurer and Banning, 2019). The geogenic radiation in the area is rather high because of the high  
 346 uranium content in the Variscian bedrocks of the area (Schwab, 1987; Büttner et al., 2006). Because of this,  
 347 radiolysis could be a possible explanation for the unexpected low  $\delta^{18}\text{O}_{\text{DO}}$  values. Kinetic gas transfer of  
 348 atmospheric oxygen during transport in the pipes or at the faucet might another explanation, since the sample in  
 349 E1a is strongly DO undersaturated. During non-equilibrium gas exchange the kinetically faster  $^{16}\text{O}$  would cause  
 350  $\delta^{18}\text{O}_{\text{DO}}$  below +24.6 ‰ until equilibrium is established (Benson and Krause, 1980; Knox et al. ,1992, Mader et al.  
 351 2017).  
 352



353

354 **Figure 5**  $\delta^{18}\text{O}_{\text{DO}}$  in the cold season a) and the warm season b) over the course of the Espan Spring and stream  
 355 system with the atmospheric equilibrium value of + 24.6 ‰ marked by the horizontal line. Dashed vertical lines  
 356 show borders of the different zones of the fields labelled with 1, 2 and 3. The symbol size is larger than the error  
 357 bars.

358 Increases in  $\delta^{18}\text{O}_{\text{DO}}$  values in zone 1 were accompanied by increases in DO (Fig. 6a). In the cold season, a strong  
 359 positive correlation was evident between points E1a and E4. However, in the warm season, the same correlation  
 360 could only be observed between points E1a and E2 (Fig. 6b). Equilibration with the atmosphere would be  
 361 reasonable explanation for this trend until atmospheric equilibration was reached between point E2 and E3.  
 362 However, the  $\delta^{18}\text{O}_{\text{DO}}$  values, at least in the cold season, increased above this threshold to a value of + 25.7 ‰.  
 363 This shows that another process in addition to atmospheric equilibration must have influenced the  $\delta^{18}\text{O}_{\text{DO}}$  values  
 364 in zone 1. In the warm season, this was less evident, and the isotope atmospheric equilibrium value was only  
 365 marginally exceeded and remained within the range of the analytical uncertainties.



366 **Figure 6** Correlation between  $\delta^{18}\text{O}_{\text{DO}}$  and DO over the course of the spring for zone 1 in the cold season a) and  
 367 the warm season b). Correlation between  $\delta^{18}\text{O}_{\text{DO}}$  and Fe(II) contents over the course of the stream for zone 1 in  
 368 the cold season c) and the warm season d).  
 369

370  
 371 Even though these processes consume DO, both respiration and iron oxidation could be responsible for this trend  
 372 when assuming that they influence the  $\delta^{18}\text{O}_{\text{DO}}$  values, while DO concentrations are constantly replenished by the  
 373 atmosphere. A direct negative correlation between Fe(II) concentrations and  $\delta^{18}\text{O}_{\text{DO}}$  values between point E1a and  
 374 E4 was evident for cold season samples and in point E1a and E2 for warm season samples as shown in Figure 6c  
 375 and d. This correlation between Fe(II) and  $\delta^{18}\text{O}_{\text{DO}}$  in the Espan System corresponds with the experimental  
 376 observations of Oba and Poulson (2009), as well as those of Pati et al. (2016). These studies demonstrate that Fe  
 377 oxidation leads to increases in  $\delta^{18}\text{O}_{\text{DO}}$  values due to preferential consumption of  $^{16}\text{O}$ . The increase in  $\delta^{18}\text{O}_{\text{DO}}$  due  
 378 to iron oxidation in a natural system, which is constantly supplied with fresh oxygen, indicates that Fe(II) oxidation  
 379 must be a dominant control on  $\delta^{18}\text{O}_{\text{DO}}$  in the first 85 meters of the stream in the cold season and in the first 15

380 meters in warm season. It also implies that the direct impact of oxygen addition is subordinate in terms of DO  
381 stable isotope changes. This is shown by iron oxidation being the dominant factor that controls  $\delta^{18}\text{O}_{\text{DO}}$  values,  
382 even though oxygen is constantly supplied from the atmosphere.

383

#### 384 *Zone 2*

385 In the cold season, zone 2 extended from sampling point E4 to point E9 with only minor variations in  $\delta^{18}\text{O}_{\text{DO}}$ . In  
386 this zone, the  $\delta^{18}\text{O}_{\text{DO}}$  decreased from + 25.7 ‰ in sampling point E4 to values around atmospheric equilibrium  
387 with + 24.5 ‰ in E7 and + 24.8 ‰ in the Pegnitz River (Fig. 5a).

388 In the warm season, zone 2 extended from sampling point E2 to point E5 at 145 meters distance from the spring.  
389 In this zone the values decreased from + 24.7 ‰ to a minimum value of + 21.8 ‰ in sampling point E5 (Fig. 5b).  
390 This decrease in  $\delta^{18}\text{O}_{\text{DO}}$  values can be explained by (1) a decrease of the impact of iron oxidation on the  $\delta^{18}\text{O}_{\text{DO}}$   
391 values and (2) a rising impact of atmospheric or photosynthetic oxygen. Even though a decrease in Fe(II) values  
392 was still evident between E4 and E7 in the cold season, as well as between E2 and E5 in the warm season, it is  
393 possible that the decrease was not caused by Fe(II) oxidation and subsequent precipitation as iron oxides.  
394 Alternatively, the decrease could have been caused by adsorption of dissolved Fe(II) onto already existing iron  
395 oxides such as goethite, ferrihydrite and hematite (Zhang, et al., 1992; Liger et al., 1999; Appelo et al., 2002;  
396 Silvester et al. 2005). Because adsorbed Fe(II) is very resistant to oxidation (Park and Dempsey, 2005) the impact  
397 of iron oxidation on the  $\delta^{18}\text{O}_{\text{DO}}$  values would have decrease.

398 No significant changes in the water chemistry were evident and it can be assumed that after sampling point E2  
399 (warm season) or E4 (cold season), a critical value was exceeded with enough Fe(II) having been adsorbed onto  
400 iron oxides. In this case, iron oxidation --- while probably still taking place at small rates--- is no longer an  
401 important factor dominating the  $\delta^{18}\text{O}_{\text{DO}}$  values. Downstream of point E2 and E4 oxygen addition by the atmosphere  
402 or by photosynthesis would become more important.

403 Intensive growth of cyanobacterial and algal mats were observed between point E3.1 and E5 in the cold season  
404 and between E3 and E5 in the warm season (Fig. 1f). Because of this growth it can be postulated that in addition  
405 to the atmospheric  $\text{O}_2$  input, the  $\delta^{18}\text{O}_{\text{DO}}$  values were also influenced by photosynthetically produced oxygen. While  
406 this effect should be less pronounced in the cold and darker season, a stronger influence of photosynthetic oxygen  
407 on the  $\delta^{18}\text{O}_{\text{DO}}$  values would be expected in the warm season with higher light intensity. Such growth of  
408 photosynthetic organisms in the Espan System is not surprising with iron being an important micronutrient  
409 (Andrews et al., 2003).

410 The fact that photosynthesising organisms seem to preferentially grow and impact the  $\delta^{18}\text{O}_{\text{DO}}$  values between  
411 sampling point E3 and E5 may be due to the availability of Fe(II). In addition, the growth could also be controlled  
412 by changes in the pH or other environmental influences, with the site being located in a public park with the  
413 associated perturbations. Cyanobacteria, especially aquatic strains prefer a neutral to alkaline pH (Brock, 1973)  
414 and the shift to higher pH values in this zone could be one of the main factors that drive increased supply of  
415 cyanobacterial  $\text{O}_2$ . For instance, *Lynngbya* spp. are diazotrophic cyanobacteria, capable of fixing nitrogen during  
416 low availability of light, when local oxygen levels are low (Stal, 2012, p. 102). This Oxygen released through  
417 oxygenic photosynthesis would immediately react with Fe(II) and lower the partial pressure of oxygen around the  
418 organisms in a slow flowing stream. This could also favor biological nitrogen fixation and limit carbon loss by  
419 reducing photorespiration. Additionally, the reduced oxygen partial pressure induced by Fe(II) oxidation may



420 minimize the oxygenase activity of ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco), thereby favoring  
421 CO<sub>2</sub>-fixation (Stal, 2012, p. 113).

422

423 A screening of microbial ecology in several iron-rich circum-neutral springs and experiments with the  
424 cyanobacterium *Synechococcus* PCC 7002 (Swanner et al. 2015a) revealed that many cyanobacteria show optimal  
425 growth between 0.4 – 3.1 mg/L Fe(II) and that concentrations above 4.5 mg/L become growth-limiting. The iron  
426 concentrations between point E3.1 and E5 in the cold season and E3 and E5 in the warm season are thus  
427 approximately in the range of optimal cyanobacterial growth. In order to establish a clear correlation between the  
428 iron concentration and the decrease in  $\delta^{18}\text{O}_{\text{DO}}$  values, experiments would need to be carried out with the organisms  
429 found in the Espan System. These have so far have not been assessed for their behaviour under variable iron  
430 concentrations.

431 *Zone 3*

432 In the warm season, zone 3 extended from sampling point E5 to point E8. In this zone the  $\delta^{18}\text{O}$  values rose again  
433 from + 21.8 ‰ to + 24.3 ‰ (Fig. 5B). The renewed increase in values can be explained by the influence of iron  
434 oxidation, respiration and a decrease in photosynthetic activity. Because Fe-contents only decreased marginally,  
435 it can be assumed that decreases in photosynthetic activities are responsible for increase in the  $\delta^{18}\text{O}$  values. This  
436 matches our observations that downstream of point E5, only little or no photosynthetic growth took place. Oxygen  
437 that would dissolve in the water after point E5 would thus most likely stem from the atmosphere. This would also  
438 explain the approach to the equilibrium value of + 24.6 ‰. Reasons for the observed decrease in cyanobacteria  
439 are however not clear and may include changes in temperature, light intensity and shifts in nutrient availability.  
440 The temperature did not change significantly in this part of the watercourse and is therefore unlikely to have caused  
441 a decrease in photosynthetic oxygen production. In contrast, reduced light exposure could have been responsible  
442 as downstream of point E5 trees shade the water course. A decrease in nutrient availability is difficult to determine  
443 because nitrate and phosphate were below the detection limit in the entire spring. Iron starvation could also be a  
444 possible reason for the decrease in activity because only ~0.005 mg/L Fe(II) was left in the system in the lowest  
445 course of the stream.

#### 446 **4 Conclusions**

447 Our study is the first systematic analysis of  $\delta^{18}\text{O}_{\text{DO}}$  values as a function of iron contents and oxygenic  
448 photosynthetic biofilms in a natural iron-rich spring. We were able to confirm from field samples that Fe-oxidation  
449 leads to increases in  $\delta^{18}\text{O}_{\text{DO}}$  values even though oxygen was constantly replenished by atmospheric input. As soon  
450 as photosynthetic oxygen is produced in the system, the effect of iron oxidation on the  $\delta^{18}\text{O}_{\text{DO}}$  values becomes  
451 negligible and can no longer be detected. The fact that photosynthesis has a strong impact on the  $\delta^{18}\text{O}_{\text{DO}}$  values in  
452 specific areas of the system may be controlled by high Fe contents of the system. Similar iron-rich springs show  
453 optimal growth rates of cyanobacteria in the range of 0.4 – 3.1 mg/L Fe(II). The presented  $\delta^{18}\text{O}_{\text{DO}}$  values showed  
454 that photosynthetic activity is also strongest in the Espan System within this range of concentrations.

455 To what extent the changing Fe concentrations (Fe(II)/Fe(III)) influence the growth of cyanobacteria and algae  
456 occurring in the Espan System, requires further investigation. This would ideally include isolating the organisms  
457 from the water course and studying them under varying experimental levels of Fe, pH and temperature while

458 monitoring the  $\delta^{18}\text{O}_{\text{DO}}$  of the system. Further field studies with organic material from the stream bed in  
459 combination with stable carbon isotopes would be promising to narrow down processes for carbon and oxygen  
460 budgets in this environment.

## 461 **5 Author contribution**

462 Inga Köhler, David Piatka and Johannes Barth carried out the sample collection and water analysis for on-site and  
463 isotope data. Raul Martinez carried out the calculation of the saturation index. Michelle Gehringer, Achim  
464 Herrmann and Arianna Gallo performed the analysis and interpretation of cyanobacteria and algae data. Inga  
465 Köhler prepared the manuscript with contributions from all co-authors

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470 declare that they have no conflict of interest.

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