

Moderate oxygen depletion as a factor favouring the filamentous growth of *Sphaerotilus natans*

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1 **Moderate oxygen depletion as a factor favouring the filamentous growth of**

2 ***Sphaerotilus natans***

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20 **Abstract**

21 *Sphaerotilus natans* is a neutrophilic iron-related sheath-forming filamentous microorganism that presents
22 dual morphotype: single cells and ensheathed cells forming filaments. As *S. natans* has been proposed as a
23 sorbent for inorganic pollutants and it is occasionally involved in bulking episodes, elucidating factors
24 affecting its filamentous growth is of crucial interest. The purpose of this work was to evaluate the effect of
25 dissolved oxygen (DO) as a factor affecting *S. natans* filamentation from single cells. A method to quantify *S.*
26 *natans* under its filamentous and single-cell morphotypes, based on a differential filtration procedure coupled
27 with quantitative real-time PCR, was developed here. Scanning Electron Microscopy was used to validate the
28 filtration step. Under actively aerated conditions (DO maintained at $7.6 \pm 0.1 \text{ mg l}^{-1}$), *S. natans* grew mainly
29 as single cells throughout the experiment, while a depletion in DO concentration (to $\sim 3 \text{ mg l}^{-1}$) induced its
30 filamentous growth. Indeed, when oxygen was shortened the proportion of single cells diminished from
31 $83.3 \pm 5.9\%$ to $14.3 \pm 3.4 \%$ while the filaments increased from $16.7 \pm 5.9\%$ to $85.7 \pm 3.4\%$. Our results
32 suggest that oxygen plays a key role in *S. natans* filamentation and contribute to better understand the
33 filamentous proliferation this bacterium. In addition, the proposed method will be helpful to evaluate other
34 factors favouring filamentous growth.

35

36 **Keywords**

37 Filamentous bacteria; dissolved oxygen; *Sphaerotilus natans*; qPCR

38

39

40 **Introduction**

41 Activated sludge process is a widespread biological technology in wastewater treatment plants (WWTP)
42 and it is known that its efficiency depends on the settling ability of the biomass in the clarifier (Silverstein et
43 al. 1990; Patziger et al. 2012). In such process, the invasive proliferation of filamentous bacteria can
44 drastically reduce the settling ability and produce bulking issues (Jenkins 1992; Martins et al. 2004). Despite
45 their negative implications in WWTP, some filamentous bacteria can also potentially be used as biosorbents
46 (e.g. Cd, Zn, Cu, Ag, Cr(III), Cr(VI), Pb, etc.) (Lodi et al., 1998; Pagnanelli et al., 2003). Hence, identifying
47 factors that affect filamentation could help to better understand the filamentous proliferation of these bacteria
48 as well as to improve their application in the field of bioremediation.

49 The identification of filamentous bacteria is often based only on their filamentous morphotype and can
50 lead to ambiguous species assignment (Eikelboom 1975; Martins et al. 2004). Furthermore, some of the
51 filamentous bacteria can also grow as single cells depending on the environmental conditions and cannot be
52 identified by their morphotype as filaments do (Rossetti et al. 1997; Jenkins et al. 2004). In fact, little is
53 known about the factors that govern the induction of filamentous proliferation from such single cells.
54 Moreover, the hypothesis of filament formation by fast chaining of single cells remains to be demonstrated.
55 Elucidating the factors that may favour filamentous over single-cell growth of such bacterial species thus
56 requires to quantify both morphotypes separately and to determine the proportion of single cells versus
57 filaments under varying conditions. However, filamentous bacteria including those involved in bulking
58 episodes and presenting this dual morphotype are not easily grown as axenic cultures (Tomei et al. 1998;
59 Blackall et al. 2000). Hence, the fundamental understanding of the factors inducing filamentous growth from
60 single cells under laboratory conditions is still poorly documented.

61 *Sphaerotilus natans* is chosen here as a model of filamentous sheath-forming bacteria to investigate the
62 triggering factors governing filamentous growth from single cells since it is able to grow either as single cells
63 –measuring around 1 – 2 μm in diameter and 1 – 10 μm in length (Spring 2006, Gridneva et al. 2011)– or as
64 ensheathed cells forming filaments, and it can be easily cultivated under laboratory conditions. [*S. natans*](#)
65 [sheath is a polymer \(termed thiopeptidoglycan\) which consists in a pentasaccharide repeating unit \(Kondo et](#)
66 [al. 2011\) connected to a peptide moiety by a galactosamine residue \(Takeda et al. 2007\). The filamentation](#)

67 [mechanisms can be very different depending on the microorganism \(Claessen et al. 2014\). *S. natans*](#)
68 [filamentous growth consists in the alignment and cell division of single cells inside the sheath \(Takeda et al.](#)
69 [2012\). In contrast, for other well-known microorganisms \(e.g. *Escherichia coli*, Visvalingam et al. 2012 or](#)
70 [Bacillus cereus, den Besten et al. 2009\), the filamentation is the result of cell division inhibition due to stress](#)
71 [conditions \(Bouché and Pichoff, 1998\).](#) The potential of *S. natans* as scavenger for inorganic pollutants has
72 been recently reviewed (Seder-Colomina et al., 2014). In addition, *S. natans* has been occasionally identified
73 in nutrient-rich industrial wastewaters (Van Veen et al. 1978; Pellegrin et al. 1999; Contreras et al. 2000).
74 Therefore, few studies have yet been dedicated to the factors affecting *S. natans* sheath formation and
75 filamentous growth. For instance, it has been demonstrated that at high concentrations of peptone (>0.2%) the
76 filamentation pathway is perturbed (Gaudy and Wolfe 1961). In addition, it has been shown that Ca²⁺
77 concentrations higher than 0.1 mM induce the formation of sheaths and therefore filamentous proliferation
78 (Dias et al. 1968a). The effect of substrate concentration has also been tested and low nutrient-content plates
79 (0.1% NB agar) result in *S. natans* filamentous growth while rich medium (1% NB) leads to single-cell
80 development (Suzuki et al. 2002). Oxygen influence on *S. natans* filamentation has been suggested after
81 observation of bulking in WWTP (Strom and Jenkins 1984; Richard et al. 1985) and in lab-scale activated
82 sludge units (Palm and Parker 1980, Tanaka et al. 1985), as well as in pure and mixed culture experiments
83 (Dias et al. 1968b). However, uncertainties remain on the relative proportions of filaments versus single cells
84 in these and other studies when quantifying filamentous bacteria. The suitability of the procedures used to
85 separate and quantify the two morphotypes in pure or mixed cultures had not been investigated in detail
86 (Gaudy and Wolfe 1961; Dias et al. 1968b; Gino et al. 2010). Furthermore, analysis of microscopy images
87 may be unable to distinguish ensheathed cells inside the filaments from single cells or single cells from
88 different bacterial species (Gaval and Pernelle 2003; Contreras et al. 2004; Jassby et al. 2014). Combining
89 separation techniques with modern strain-specific techniques for bacterial counting may thus help to
90 overcome these difficulties.

91 In the present study we have evaluated the effect of a moderate oxygen depletion as a factor favouring the
92 filamentous or single-cell growth of *S. natans* through an accurate counting of the two morphotypes in axenic
93 cultures, using a differential filtration procedure coupled with quantitative PCR.

94

95 **Materials and Methods**

96 **Bacterial strain and growth conditions**

97 Various strains of *Sphaerotilus* were found to present different responses to oxygen upon preliminary
98 tests, as filamentation from single cells was induced at different oxygen availabilities for each strain tested
99 (Supplementary Fig. S1). The strain presenting the broadest dependence of the morphotype on oxygen
100 concentration was chosen for the present study. The selected strain *Sphaerotilus natans* ATCC 15291 was
101 initially grown in solid CGYA media (0.5% casitone, 1% glycerol, 0.1% yeast extract and 1.2% agar) for ~24
102 hours until it reached the exponential growth phase. Bacteria were then recovered from the plates,
103 resuspended in CGY broth (0.5% casitone, 1% glycerol and 0.1% yeast extract) and filtered through a 3 µm
104 pore-size polycarbonate membrane filter (Isopore™, Millipore®) in order to obtain an inoculum for the batch
105 cultures that mostly contained single cells.

106 Batch experiments were designed to study the role of dissolved oxygen (DO) concentration in influencing
107 *S. natans* filamentation and sheath-formation from single cells. Two types of batch culture conditions were
108 used in triplicate experiments in order to measure the proportion of filaments versus single cells, under
109 oxygen saturated conditions or under depleted oxygen conditions. The first condition, referred to as ‘actively
110 aerated’, corresponds to oxygen-saturated conditions obtained by continuously aerating the growth medium in
111 glass bottles with an active air bubbling system. The second condition referred to as ‘passively aerated’, lead
112 to progressive deoxygenation of the medium upon cell respiration and was obtained by passively aerating the
113 medium by air diffusion through a cotton plug. For both culture conditions, 250 ml CGY broth (0.5%
114 casitone, 1% glycerol and 0.1% yeast extract) were inoculated at an absorbance of 0.03 (at 600 nm) using the
115 previously 3 µm-filtered inoculum of *S. natans*. Bottles were agitated at 100 rpm for 48 hours of incubation.
116 The temperature of 30.0 °C and pH 7.0 were kept constant and were measured using a SevenGo™ SG2
117 (Mettler Toledo). In both cases a YSI Model 57 Dissolved Oxygen Meter (YSI Incorporated) was also used to
118 monitor oxygen as a function of time. Optical density was measured throughout the 48 hours of incubation,
119 but it was found to be inaccurate when quantifying filamentous biomass (Supplementary Fig. S2).

120 **Confocal Laser Scanning Microscopy (CLSM)**

121 In order to initially evaluate the dual morphotype growth of *S. natans* and to distinguish extracellular
122 polymeric substances (EPS) and sheath formation, samples were double-stained with the non-specific bacteria
123 dye Syto[®]61 Red Fluorescent Nucleic Acid Stain (LifeTechnologies[™]) targeting DNA and with two different
124 lectins coupled with green fluorescent FITC. These *Pisum sativum* agglutinin (PSA) and *Wheat germ*
125 agglutinin (WGA) lectins (Sigma-Aldrich[®]) bind sugar residues from the EPS (terminal α -D-mannosyl-like)
126 and sheath (proteins containing β (1 \rightarrow 4)-N-acetyl-D-glucosamine-like residues), respectively. Samples were
127 incubated for 15 minutes with Syto[®]61, PSA and WGA and then washed to remove the unbound Syto[®]61 and
128 lectins. In parallel, Fluorescence *in situ* hybridization (FISH) was used to verify *S. natans* purity in the
129 process of the culture. The specific 16S rRNA-targeting probe SNA –[5'-labeled with Cy3](#)– used in this study
130 (Wagner et al. 1994) was purchased at Eurofins MWG Operon (Ebersberg, Germany). Samples were prepared
131 according to a 3-step protocol (Amann et al. 1990; Manz et al. 1992): 1) cells were dehydrated with ethanol to
132 permeabilize the cell membrane to SNA probe; 2) they were then incubated with the probe for 90 minutes at
133 46°C to hybridize the probe and the targeted 16S rRNA; 3) cells were finally washed to remove the unbound
134 probe. All samples were observed under a CLSM Zeiss Axiovert 200M LSM 510 META.

135 **Filtration procedure coupled to DNA extraction**

136 A specific filtration procedure was developed in the present study in order to quantify separately the total
137 biomass, the filaments and the single cells, in axenic cultures of *S. natans* containing both morphotypes
138 ([Supplementary Fig. S3](#)). This filtration procedure provides three different samples: the total biomass
139 (referred to as ‘global culture’), the fraction of bacteria retained on a 3 μ m pore-size filter (referred to as
140 ‘filaments’) and those who passed through the 3 μ m pore-size filter (referred to as ‘single cells’). To avoid
141 filter clogging in exponential and stationary growth phases, different dilutions of the culture were performed
142 in the experiment prior to the filtration step to a final volume of 5 ml. An aliquot of 1.35 ml of the diluted
143 sample was then transferred to a 2 ml tube containing 135 μ l of SDS 25% and 0.4 g of zirconium beads to
144 disrupt cells to extract DNA (bead-beating tubes). The remaining 3.65 ml were filtered through a 3 μ m pore-
145 size polycarbonate membrane filter (Isopore[™], Millipore[®]). The filtration membrane was then recovered and
146 placed in a bead-beating tube with 1.35 ml fresh CGY medium; 1.35 ml of the 3 μ m-filtered culture were also
147 transferred to a bead-beating tube. Samples were vortexed for 10 minutes in a MO BIO bead-beating adapter

148 for Vortex-Genie2[®] at maximum speed. Phenol-chloroform-isoamyl (PCI) was added to samples (vol:vol 1:1)
149 and centrifuged in Phase-Lock Gel™ tubes (5PRIME), to ensure DNA quality extraction by removing
150 proteins and PCI residues. The DNA was recovered after precipitation in 2-propanol (vol:vol 1:1) at -20°C
151 overnight. Sampling, filtration and DNA extraction were done in triplicate for each sampling time.

152 **Scanning Electron Microscopy (SEM)**

153 SEM was used to validate the efficiency of the above filtration step at various times and dilutions. Two
154 types of samples were observed: 1) samples filtered using 3 µm filters and 2) samples double-filtered, first at
155 3 µm and subsequently at 0.22 µm. Filters were deposited on carbon tape and coated with a thin carbon film.
156 SEM observations were performed with a Field Emission Gun Scanning Electron Microscope (GEMINI
157 ZEISS Ultra55) operating at 2 to 5 kV.

158 **Quantitative real-time PCR (qPCR)**

159 At successive sampling times throughout the batch cultures, DNA was extracted from the three samples
160 separated by our filtration procedure, i.e. global culture, filaments, and single cells. The integrity and quality
161 of extracted DNA were systematically checked on 1% agarose gel electrophoresis (data not shown) prior to
162 the quantification by qPCR analysis. For this purpose we used primers targeting the *sthA* gene of *S. natans*,
163 which is involved in both sheath and EPS synthesis (Suzuki et al. 2002). The choice of *sthA* gene and the
164 design of *sthA* primers are detailed in the Supplementary Fig. S34.

165 Samples were analyzed using Multiplate[®] PCR Plates™ Low-Profile 96-well plates (Bio Rad) in a CFX
166 96™ Real-Time PCR Detection System (Bio Rad). The qPCR reaction mixture (23 µl) was added to global
167 culture, filaments and single cells extracted DNA (2 µl) to give the following final concentrations of
168 components: *sthA* RP and *sthA* FP 300 nM and iQ™ SYBR[®] Green Supermix (Bio Rad) Q.S. to 1U of iTaq
169 DNA polymerase. qPCR was performed with the following cycling program: 3 min at 95°C; 40 cycles
170 consisting of 15 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C followed by a melt curve from 60°C to 95°C
171 (with increasing steps of 0.5°C) at intervals of 10 sec. NTC controls (2 µl of molecular biology grade DNA-
172 free water) were also run. Tests were conducted in triplicate.

173 The standard curves for each assay were generated by the amplification of serial 10-fold dilutions of a
174 linearized plasmid pEX-A containing a sequence of 243 bp encompassing the amplicon. The original plasmid

175 was synthesized by Eurofins MWG Operon (Ebersberg, Germany). It was then linearized with the restriction
176 enzyme HindIII in order to perform an accurate quantitative PCR (Hou et al. 2010). Its concentration was
177 fixed at 33 ng μL^{-1} (1.12×10^{10} copies μL^{-1}). This value was determined using the Biowave II
178 spectrophotometer (Biochrom). After that, the 10-fold dilution series from 10^8 to 10^2 copies *sthA* μL^{-1} was
179 prepared in molecular biology grade DNA-free water. The quantification limit is defined as the lowest
180 concentration of standard in the linear range that differed at least from 1 log unit (equivalent to 3.3 threshold
181 cycles) from the NTC (Smith et al. 2006); in this case, 10^2 copies of *sthA* per qPCR reaction. Each standard
182 was tested in triplicate.

183 Data analysis was carried out with CFX Manager™ V1.0 software (Bio-Rad). The level of contamination
184 –given by negative controls and NTC– and the qPCR efficiency were checked for each assay. The qPCR
185 efficiency (E) was calculated from standard curves using the following formula

$$186 \quad E = (-1 + 10^{-1/\text{slope}}) \times 100 \text{ (eq. 1)}$$

187 where the slope was obtained by the regression analysis between the threshold cycles (Ct) and the
188 logarithm number of *sthA* copies. The qPCR efficiency of all plates run was between 90% and 98% with a
189 regression coefficient value (R^2) systematically above 0.98. Results were validated only when NTC controls
190 were detected after 33 Ct. qPCR results were converted to *sthA* copies mL^{-1} culture depending on the dilution
191 factors applied during the sampling process. Arithmetic means and standard deviation were calculated from
192 qPCR and sample triplicates.

193 **Results**

194 **Evolution of pH and oxygen availability in batch cultures**

195 Under actively aerated conditions, the medium was saturated in oxygen with $[\text{O}_2]_{\text{aq}} = 7.6 \pm 0.1 \text{ mg l}^{-1}$
196 throughout the 48 hours of incubation. In contrast, in the passively aerated culture, the DO concentration
197 decreased from 6.4 mg l^{-1} to values below 4 mg l^{-1} after 4 hours of incubation due to bacterial activity, and
198 remained at $2.9 \pm 0.4 \text{ mg l}^{-1}$ after 8 hours (Fig. 12). Under both batch culture conditions, pH remained
199 constant throughout the experiments, at 7.1 ± 0.1 , indicating that potential changes in filamentation could not
200 be attributed to a pH-related stress but rather to the significant difference in DO.

201 **Qualitative evaluation of the dual morphotype in batch cultures**

202 Depending on the culture conditions, CLSM analyses displayed different contrasted fluorescence for *S.*
203 *natans* morphotypes, sheath and EPS (Fig. 23), which suggested that oxygen availability influenced single-
204 cell vs. filamentous growth, EPS synthesis and sheath formation by *S. natans*. The inoculum (Fig. 23a) was
205 composed mostly of single cells, which did not have any sheath or EPS and therefore did not present lectin
206 staining. After 48 hours of incubation, in the actively aerated culture (Fig. 23b) single cells were the
207 predominant morphotype. No sheath was observed, whereas lectins were slightly bound on the periphery and
208 deeply at the poles of the single cells, suggesting an EPS production concentrated in these parts of the cells.
209 Under passively aerated conditions (Fig. 23c) *S. natans* was present mainly as filaments and the fluorescence
210 of the lectins revealed the sheath formation and EPS synthesis. In all cases, the use of *S. natans*-specific FISH
211 (data not shown) made it possible to successfully discard any contamination problems that could have
212 affected growth patterns.

213 **Selective filtration**

214 SEM images (Fig. 34) show that the inoculum contained almost exclusively single cells that passed
215 through the 3 μm pore-size filter (Fig. 34a), and that were further retained on the 0.22 μm filter (Fig. 34b).
216 However, a few cells forming aggregates were occasionally observed on the surface of the 3 μm filter (Fig.
217 34a, Insert). After 48 hours of incubation, the actively aerated culture was predominantly composed of single
218 cells that passed through the 3 μm filter and that were retained on the 0.22 μm filter (Fig. 34d). Occasionally,
219 a few aggregates of single cells were observed after 48 hours of incubation on the 3 μm filter surface (Fig.
220 34c, Insert). In contrast, after 48 hours of incubation, the passively aerated culture contained mostly filaments
221 retained by the 3 μm filter (Fig. 34e). The remaining single cells and some rare filaments were observed on
222 the 0.22 μm filter (Fig. 34f). No significant clogging of the filter was observed in any case. Altogether, these
223 SEM observations validate the filtration procedure, showing that most filaments were retained on the 3 μm
224 filter, whereas the corresponding filtrate consisted almost exclusively of single cells that could be further
225 retained by the 0.22 μm filtration.

226 **Filamentous vs. single-cell growth**

227 Growth curves of global culture as well as filaments and single cells of *S. natans* obtained from qPCR
228 data after differential filtration are displayed in Figure 45. They demonstrate significant differences in the
229 proportions of the two morphotypes as a function of oxygen availability in the batch cultures.

230 Under actively aerated conditions (Fig. 45a), a lag phase of at least 8 hours was observed. The global
231 culture then yielded a rapid growth phase, parallel to the single cells growth curve, and reached a maximum
232 value of 6.4×10^6 copies of *sthA*. Filaments in this case represented a low proportion of the culture. Indeed,
233 the fractions of single cells and filaments in the culture (Fig. 54a, Insert) remain rather constant throughout
234 the incubation ($83.3 \pm 5.9\%$ and $16.7 \pm 5.9\%$ on average, respectively). The increase in the number of copies
235 of *sthA* corresponding to filaments after 24 hours of incubation could be explained by the presence of small
236 aggregates of single cells on the filter (Fig. 34c, Insert).

237 In contrast, under passively aerated conditions (Fig. 45b), the global culture as well as the filaments
238 presented a classical bacterial growth curve, with a lag phase that lasted for approximately 4 hours. The
239 proportion of *sthA* copies from single cells dramatically decreased with time and became much lower than
240 that of filaments especially after 16 hours of incubation. Indeed, in the stationary phase, i.e. after 24 hours of
241 incubation, the proportion of single cells was $14.3 \pm 3.4\%$ while filaments accounted for up to $85.7 \pm 3.4\%$ of
242 the total biomass (Fig. 45b, Insert). Finally, under passively aerated conditions the maximum total number of
243 *sthA* copies was 1.9×10^8 that is 30 times higher than under actively aerated conditions, suggesting that
244 saturated oxygen conditions are not the most favourable conditions for *S. natans* growth.

245

246 Discussion

247 Differential quantification of single cells and filaments

248 The selective filtration procedure coupled to DNA extraction and amplification, as developed in the
249 present study, is shown to be an effective and accurate method to evaluate factors favouring the filamentous
250 growth of *S. natans*. This differential quantification methodology is faster than imaging methods and
251 overcomes most of their limitations. For instance, manual counting under the microscope and the sludge
252 volume index (SVI) have been used to highlight correlations between both total filament length and total

253 number of filaments per mg TSS (Palm and Parker 1980). In the same way, a filament index (FI) varying
254 from 0 (no filament) to 5 (sludge population extremely dominated by filaments) has been often used for
255 characterizing activated sludge, but it is rather qualitative and influenced by the subjectivity of the
256 experimenter (Eikelboom and Van Buijsen 1981).

257 More recently, image processing methods have been focused on the quantification of filamentous bacteria.
258 FISH imaging was used for filament counting but this method was found to be time consuming since a ten-
259 fold manual counting on a whole microscope slide was necessary to obtain reliable data for each sample
260 (Gaval and Pernelle 2003). Automatic image analyses to quantify filamentous bacteria from activated sludge
261 have significantly improved the method (Contreras et al. 2004; Jassby et al. 2014). In these latter studies,
262 phase contrast microscopy was used and 9 to 15 microscope field images were processed using Global Lab
263 Image 2.10 (Data Translation, Inc.) or ImageJ V1.37 (National Institutes of Health) image analyses software.
264 Filament quantification was related to the number of pixels corresponding to filaments using a correction
265 factor. While Gaval and Pernelle (2003) and Jassby et al. (2014) did not take into account the single-cell
266 microorganisms present in the samples, Contreras et al. (2004) developed a model to discern a single-cell
267 small cocci (strain E932) from *S. natans* ensheathed cells found inside the filaments. Unfortunately, this
268 method by itself could not be applied to quantify separately both morphotypes of *S. natans*, as the single cells
269 have the exact same shape as the ensheathed ones forming the filaments.

270 In the present study, quantitative PCR was successfully used to quantify the two morphotypes after they
271 were separated by a suitable filtration procedure. The optimal size of the generated amplicons recommended
272 by qPCR commercial kits (e.g. SYBR[®] Green or Taqman[®]) is between 50 and 150 bp as too long amplicons
273 may lead to poor amplification efficiency. The amplification products of the *sthA* primers developed here
274 have a length of 143 bp, which is optimal for qPCR results. The qPCR reaction values obtained for the
275 average slope, E, R² and NTC controls also assure the effectiveness of the amplification reaction and therefore
276 the accuracy of the quantification.

277 **Oxygen as a factor influencing filamentation**

278 The present results clearly demonstrate that dissolved oxygen concentration plays a crucial role in *S.*
279 *natans* filamentation from single cells. Indeed, under saturated oxygen conditions ($7.6 \pm 0.1 \text{ mg l}^{-1}$), *S. natans*

280 grew under its single-cell form, while a moderate oxygen depletion ($2.9 \pm 0.4 \text{ mg l}^{-1}$) triggered filamentous
281 growth from the inoculum of single cells. Few studies have similarly reported that low oxygen concentrations
282 could induce the proliferation of *S. natans* filaments. For instance, Palm and Parker (1980) reported *S. natans*
283 bulking due to its filamentous proliferation (measured as SVI), in laboratory activated sludge at DO
284 concentrations $\leq 5.5 \text{ mg l}^{-1}$. In the same way, Gino et al. (2010) found that *S. natans* grew predominantly as
285 filaments forming biofilms in a groundwater well with DO values of $6.4 - 3.6 \text{ mg l}^{-1}$. Dias et al. (1968b)
286 reported filamentous growth of *S. natans* at DO concentrations of $6.9 - 7.8 \text{ mg l}^{-1}$ (~ oxygen saturated
287 conditions), which seems in contradiction with our and previously mentioned studies. However, Dias et al.
288 results were based on the measurements of dry weight of total biomass, which cannot discriminate between
289 single cells and filaments. In addition, oxygen was not supplied during the first 24 hours of batch culture in
290 their experiments. As shown in the present study, after 24 hours of incubation without any active supply of air
291 the dissolved oxygen concentration is depleted due to bacterial activity, and hence the filamentation of *S.*
292 *natans* is induced.

293 In addition, here we showed that oxygen affected not only the growth morphotype but also the quantity of
294 total cells. Indeed, saturated oxygen conditions negatively impacted total bacterial growth (30 times less
295 growth in the actively aerated culture). This fact suggests that elevated DO stresses *S. natans*. Hence, single-
296 cell growth would be a response to stress and filaments would be the predominant morphotype under
297 favourable culture conditions. This hypothesis is supported by a recent publication (Park et al. 2014) in which
298 *S. natans* changed from filaments to single cells in response to the stress produced by full anoxic culture
299 conditions.

300 The method developed here makes it possible to accurately quantify *S. natans* under its two growth
301 morphotypes, which may help in identifying other factors favouring filamentous growth for this and other
302 microorganisms of socio-economical and ecological interest. Furthermore, future investigations of a
303 hypothetical reversibility of the filamentous growth may also help to better understand the mechanisms of its
304 recurrent filamentous proliferation.

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318

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