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1 Towards a consensus-based biokinetic model for green 2 microalgae – the ASM-A

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9

10 **ABSTRACT**

11 Cultivation of microalgae in open ponds and closed photobioreactors (PBRs) using wastewater
12 resources offers an opportunity for biochemical nutrient recovery. Effective reactor system design
13 and process control of PBRs requires process models. Several models with different complexities
14 have been developed to predict microalgal growth. However, none of these models can effectively
15 describe all the relevant processes when microalgal growth is coupled with nutrient removal and
16 recovery from wastewaters. Here, we present a mathematical model developed to simulate green
17 microalgal growth (ASM-A) using the systematic approach of the activated sludge modelling
18 (ASM) framework. The process model – identified based on a literature review and using new
19 experimental data – accounts for factors influencing photoautotrophic and heterotrophic
20 microalgal growth, nutrient uptake and storage (i.e. Droop model) and decay of microalgae. Model
21 parameters were estimated using laboratory-scale batch and sequenced batch experiments using

22 the novel Latin Hypercube Sampling based Simplex (LHSS) method. The model was evaluated
23 using independent data obtained in a 24-L PBR operated in sequenced batch mode. Identifiability
24 of the model was assessed. The model can effectively describe microalgal biomass growth,
25 ammonia and phosphate concentrations as well as the phosphorus storage using a set of average
26 parameter values estimated with the experimental data. A statistical analysis of simulation and
27 measured data suggest that culture history and substrate availability can introduce significant
28 variability on parameter values for predicting the reaction rates for bulk nitrate and the
29 intracellularly stored nitrogen state-variables, thereby requiring scenario specific model
30 calibration. ASM-A was identified using standard cultivation medium, and it can provide a
31 platform for extensions accounting for factors influencing algal growth and nutrient storage using
32 wastewater resources.

33

34 **KEYWORDS**

35 Process modelling; Green microalgal growth; Nutrient storage; Parameter identifiability;
36 Uncertainty and global sensitivity analysis.

37 **1. INTRODUCTION**

38 Cultivation of green microalgae has been proposed as a suitable technology for wastewater
39 remediation due to their capacity to remove nitrogen and phosphorus (Markou et al., 2014).
40 Consequently, several studies have explored the integration of microalgal cultivation in existing
41 wastewater treatment plants (WWTPs), focusing on high pollutant removal from high strength
42 streams, e.g. effluent from anaerobic digester, or as a tertiary treatment step (Wang et al., 2010;
43 Van Den Hende et al., 2014; Boelee et al., 2011). However, due to an increasing global population,
44 climate change and industrialization, in the near future, we will be facing new global challenges,
45 such as severe water scarcity (Bixio et al., 2006; Verstraete et al., 2009) or the depletion of non-
46 renewable phosphorus resources (Verstraete et al., 2009; Desmidt et al., 2015). Consequently,
47 sewage, referred to as used water, should be considered as a source of energy, nutrients and fresh
48 water rather than a waste (Verstraete and Vlaeminck, 2011). Cultivation of microalgae offers the
49 potential to recover water, nitrogen and phosphorus from used water providing an opportunity for
50 residual nutrient recycling (Shilton et al., 2012; Cai et al., 2013; Samorí et al., 2013; Mehta et al.,
51 2015). Moreover, it has been demonstrated that microalgal biomass can be used as a slow-leaching
52 fertilizer (Mulbry et al., 2005). Hence, as an alternative to the conventional algal cultivation for
53 nutrient removal from used water, Valverde-Pérez et al. (2015) propose an enhanced biological
54 phosphorus recovery and removal (EBP2R) process, able to provide optimal cultivation media for
55 green microalgal growth. The EBP2R combined with an algal PBR, referred to as TRENS system
56 (Fang et al., 2016), is then able to produce an algal suspension where nutrients are stored in the
57 algal biomass, which can be used for fertigation. Additionally, algal biomass can be used for biogas
58 or biodiesel production (Mata et al., 2010; Wijffels et al., 2010; Perez-Garcia et al., 2011). Unlike
59 crop-based biofuels, microalgal biomass does not compete with agricultural land used for food

60 production, qualifying it as a third generation biofuel (Clarens et al., 2010). Nevertheless, typical
61 cultivation of microalgae can have a high water and energy demand and a high greenhouse-gas
62 footprint associated with the production of fertilizer used for cultivation (Clarens et al., 2010;
63 Guieysse et al., 2013; Markou et al, 2014). Hence, large-scale microalgal cultivation for biofuel
64 production appears neither energetically nor economically favourable, unless it is coupled with
65 used water resource recovery and treatment (Lundquist et al., 2010; Pittman et al, 2011; Chen et
66 al., 2015).

67 The existing process modelling approaches (Table S1, SI) range in complexity, comprising models
68 that account for either the influence of a single variable on growth, e.g. light exposure (Grima et
69 al., 1994; Huesemann et al., 2013), or the combined influence of multiple variables, such as light,
70 nutrient availability, temperature or pH (Ambrose, 2006; Wolf et al., 2007; Quinn et al., 2011;
71 Broekhuizen et al., 2012; Guest et al., 2013; Decostere et al., 2013; Adesanya et al., 2014; Coppens
72 et al., 2014; Fachet et al., 2014). Although the latter group of models includes more complex
73 approaches, they all show some structural deficiency required to predict the performance of PBRs
74 employed for used water management. The biofilm model PHOBIA (Wolf et al., 2007), for
75 instance, includes the growth of heterotrophs, nitrifiers and microalgae on inorganic carbon, light
76 and nitrogen, but disregards algal growth dependency on phosphate, a key aspect for applications
77 in used water treatment. The model by Broekhuizen et al. (2012) accounts for the effects of pH,
78 inorganic carbon, oxygen, nitrogen, phosphate and light on microalgal growth. However, growth
79 and nutrient uptake are considered directly coupled, and storage of nutrients and growth on the
80 stored nutrients is not considered. To this end, the model by Droop (1973) proposes an approach
81 describing microalgal growth on stored nutrients as well as nutrient uptake and storage. This is an
82 important structural attribute because the calibration of the microbial growth process rate can be

83 done independently from the process rates identified for nutrient uptake and storage.
84 Consequently, the model can describe growth in the absence of external nitrogen or phosphorus –
85 observed in real system – using the internally stored nitrogen and phosphorus, also referred to as
86 quota (Bernard, 2011). Based on the Droop model, when nutrients are persistently limiting, the
87 minimum internal nutrient quota is gradually reached and the growth rate converges to zero. As
88 for the replenishment of the quota, when nutrients in the bulk medium are available in excess, after
89 the nutrient limitation, the maximum internal quota is reached, thereby reaching the maximum
90 growth rate, at which algal growth becomes independent from the nutrient availability (Bernard et
91 al., 2011). There are several models with multiple substrate limitations in accordance to Droop’s
92 approach (Ambrose, 2006; Bernard, 2011; Quinn et al., 2011; Guest et al., 2013; Fachet et al.,
93 2014). Although nitrogen can be stored in the form of amino acids (Romero-García et al., 2012)
94 or nitrate (Coppens et al., 2014), literature is not conclusive about the presence of a possible
95 nitrogen quota for microalgae (Richmond, 2004).

96 Although growth of algae on different organic substrates is well documented (Mata et al., 2010;
97 Brennan and Owende, 2010; Perez-Garcia et al., 2011; Van Wagenen et al., 2015a), none of the
98 above mentioned models combines mixotrophic and heterotrophic growth processes. Moya et al.
99 (1997) propose a simple model for microalgal growth as a function of light (autotrophic growth)
100 and acetate (heterotrophic growth) – the latter expressed using Haldane kinetics. Whilst this
101 approach is useful to predict heterotrophic algal growth in nutrient excess conditions, it does not
102 account for the effects of nitrogen and phosphorus, thereby limiting the model applicability (see
103 e.g. Adesanya et al., 2014).

104 Béchet et al. (2013) propose three different approaches to model the effect of light on algal growth:
105 i) type I: models accounting for an average light intensity and its impact on the algal growth; ii)

106 type II: models accounting for the light gradient in the PBR and the effect on the photosynthetic
107 rate; and iii) type III: models that consider the photosynthetic rate of an individual algal cell as a
108 function of the light history. The effect of light on algal growth can be modelled by taking into
109 account photo-inhibition using the Steele, Peeters-Eilers and Haldane kinetics (Bouterfas et al.,
110 2002; Ambrose, 2006), or omitting the inhibition term using the Monod, Platt-Jassby, Poisson
111 single-hit and Smith models (Bouterfas et al., 2002; Ambrose, 2006; Skjelbred et al., 2012).

112 Design, operation and control of PBRs require process models that are able to predict microalgal
113 growth, as well as the nutrient uptake and storage from used water. Whilst such consensus models
114 already exist for bacterial processes, i.e. the Activated Sludge Models (ASMs) (Henze et al., 2000),
115 for algal systems there is still a lack of a consistent and consensus-based modelling approach. Thus
116 the primary objective of the study is to develop such modelling approach. This is necessary for the
117 development and assessment of operation and control structures for nutrient removal and recovery,
118 which are poorly developed for PBRs, or the generate input data for life cycle assessment studies
119 relevant to PBRs (e.g., Olivieri et al., 2014; Valverde-Pérez et al., 2016; Fang et al., 2015).

120 Proper sets of experiments have to be designed to identify unique sets of model parameters. Whilst
121 optimal experimental design for parameter identification is widely reported for conventional
122 activated sludge models (e.g. Checchi and Marsili-Libelli, 2005; Chandran and Smets, 2005), this
123 aspect has been seldom studied in algal models. Muñoz-Tamayo et al. (2014) reported optimal
124 experimental design to estimate parameters related to algal growth dependence on light and
125 temperature while Decostere et al. (2016) looked into the identifiability of inorganic carbon related
126 parameters using a novel respirometric-titrimetric assay (Decostere et al., 2013). To our
127 knowledge, only one study has dealt with the identifiability analysis of nutrient related parameters
128 (Benavides et al., 2015). However, this study is based on synthetic data generated by simulating

129 an arbitrary chosen model structure. Therefore, a secondary objective of our study was to design
130 experiments that can be used to infer data to analyse the identifiability and the reliability of the
131 parameter estimates. Consequently, to assess the reliability of the parameter estimates,
132 uncertainties imposed by factors known to affect them in activated sludge models, such as culture
133 history or substrate availability (Grady et al., 1996) should be assessed.

134 The main objectives of the present work are (i) to carry out an exhaustive literature review on
135 process models of algal growth, nutrient uptake and storage; (ii) to identify and evaluate a
136 biokinetic process model – based on the state-of-the-art and using novel formulations of process
137 rate equations – for photoautotrophic and heterotrophic microalgal growth in the ASM framework;
138 (iii) to assess the impact of culture history and substrate availability on parameter estimates and
139 their effects on the accuracy of predicting microalgal growth and nutrient storage; (iv) to assess
140 the model identifiability using data obtained from three different laboratory-scale experimental
141 setups, thereby identifying the sources of parameter variability.

142 **2. ASM-A MODEL DEVELOPMENT**

143 **2.1. Modelling in the ASM framework**

144 The systematic model development in this study was carried out as an extension to the well-
145 established Activated Sludge Model, ASM-2d (Henze et al., 2000). By using the ASM framework,
146 we facilitate the integration of the microalgal model into the existing benchmark models (e.g.
147 Nopens et al., 2010). ASM-2d includes all the bacterial groups involved in enhanced biological
148 phosphorus removal systems (EBPR), i.e. ordinary heterotrophs, nitrifiers and polyphosphate
149 accumulating organisms. The expressions included in this study do not consider the above
150 mentioned bacteria, but only the biochemical processes catalysed by green microalgae (Gujer
151 matrix shown in Table 1). Special attention has been paid to the typical challenges faced when

152 extending ASM type models (Snip et al., 2014), including: i) units, in accordance with the ASM
153 framework, are expressed in chemical oxygen demand (g-COD), g-N and g-P per cubic metre; and
154 ii) the continuity of the mass balances in the model is checked (Hauduc et al., 2010). To make the
155 integration of the algal model into the existing model structures straightforward, ASM
156 nomenclature (Table 2) was followed (Corominas et al., 2011).

157 **Uptake and storage of nitrogen (R1 and R2):** ASM-A considers the microalgal uptake and
158 storage of both ammonia (R1) and nitrate (R2) nitrogen (Table 1). The uptake and storage of
159 nitrogen depends on the availability of external nitrogen (S_{NH4} or S_{NO}), as well as on the internal
160 cell quota of nitrogen ($X_{Alg,N}$) – the latter being defined as the total intracellularly stored nitrogen.
161 Nitrogen uptake rate decreases as the stored nitrogen approaches the maximum internal cell quota,
162 $X_{Alg,Nmax}$, in the biomass (X_{Alg}). Typically, ammonia is preferred over nitrate for most algal species
163 (Cai et al., 2013; Markou et al., 2014). Therefore, a competitive inhibition term by ammonia is
164 included in the nitrate uptake process rate (R2, Table 1).

165 **Uptake and storage of phosphorus (R3):** The uptake and storage of phosphorus (R3, Table 1)
166 depends on the availability of external soluble orthophosphate (S_{PO4}), and on the internal cell quota
167 of phosphorus ($X_{Alg,PP}$) – the latter being defined as the total intracellularly stored phosphorus.
168 Accordingly, the phosphorus uptake rate decreases as the stored phosphorus approaches the
169 maximum internal cell quota, $X_{Alg,PPmax}$.

170 **Photoautotrophic growth (R4):** Nutrient limitations are described according to Droop (1973).
171 The specific growth rate decreases as the internal cell quota approaches the minimum internal
172 quota ($X_{Alg,Nmin}$ or $X_{Alg,PPmin}$). The consumption of inorganic carbon (S_{Alk}) is modelled using Monod
173 kinetics. Light limitation is determined by the photo-synthetically available irradiance passing

174 through the PBR. In this study, we assume that the microalgae are exposed to a constant average
175 light intensity (type I light model, Béchet et al., 2013), denoted as I_{Av} . To identify a suitable model
176 structure that describes the light influence on microalgal growth, six different model equations
177 were fitted to the obtained experimental data. Light dependence is modelled using the Steele
178 equation, which was identified through an extensive model discrimination exercise using
179 experimental results (section 4.1.1).

180 The COD mass-balance cannot be closed for the photoautotrophic microalgal growth, which is
181 explained as follows. During the photophosphorilation, algae produce the energy needed for
182 carbon fixation through the Calvin cycle and release oxygen as a by-product. In addition, the
183 energy produced can also be used to build macromolecules (e.g. lipids or starch), to assimilate
184 nitrate, etc. (Wilhelm and Jakob, 2011). The energy not used via the Calvin cycle yields to a higher
185 oxygen production without contributing to biomass production (i.e. COD production), and thus
186 preventing the mass-balance to be closed. Since carbon dioxide, light and water, the substrates in
187 this process do not contribute to the COD balance, they cannot be used either to close the balance.
188 Therefore, the stoichiometry for photoautotrophic growth is set according to literature (Park and
189 Craggs, 2011), and, in this case, the continuity check is only used to close the mass balances for N
190 and P.

191 **Heterotrophic algal growth (R5):** Acetate is used as the organic carbon substrate (S_A), state-
192 variable included in the ASM-2d. The Monod kinetics is used to model the heterotrophic growth
193 as a function of the substrate concentration. Oxygen serves as a terminal electron acceptor for
194 heterotrophic growth (S_{O_2}), and its effect is modelled by Monod kinetics. Inhibition of the
195 heterotrophic growth by light intensity is modelled using the competitive inhibition term. The

196 nutrient consumption associated with algal growth is included analogously to that described in the
197 photoautotrophic growth.

198 **Algal decay (R6):** The algal decay process rate includes the internal resources used for
199 maintenance, biomass loss during dark respiration and death and lysis that reduces the amount of
200 active biomass in the culture. In addition, the term includes reduction in biomass due to predators
201 grazing on the algal biomass. The decay process is modelled following the dead-regeneration
202 principle, which states that a fraction of the products from decay become available for microbial
203 growth (van Loosdrecht and Henze 1999).

204 **2.2.Limitations of the model**

205 ASM-A was identified using experimental data inferred using synthetic growth medium.
206 Conversely, in real systems, factors related to light attenuation (e.g., chromophores) and toxicity
207 (e.g., pharmaceutical residues), occurring in (treated) used water can significantly influence
208 growth conditions that the present model and its calibration do not account for and future model
209 identification studies should quantify them. Furthermore, although the model is implemented as
210 an extension of the ASM-2d and predicts bacterial growth and some interactions between bacteria
211 and algae (e.g. support of heterotrophic bacterial growth via oxygen supply from the algae), direct
212 interactions between algal and bacterial growth, are not considered in this study, and bacterial
213 processes are assumed negligible during the experiments. Further details about how bacterial-algal
214 interactions are accounted for by means of the ASM-A model are described in the Supporting
215 Information (SI, pages S29-S31).

216 High oxygen levels can cause photo-oxidative damage on microalgae (Muñoz and Guieysse,
217 2006). Photo-oxidative damage caused by elevated O₂ levels is reported at significantly higher

218 levels of oxygen in the liquid phase (e.g. 24.5 mg O₂·L⁻¹ reported in Alcántara et al., 2013) than
219 that observed in our study (10 mg O₂·L⁻¹), and mostly it occurs in photobioreactors with poor
220 mixing. This can be avoided with adequate mixing as was the case in our study. The effect of O₂
221 inhibition thus could not be measured, and targeted experiments should be done – in the future –
222 to extend the application of the model to account for photo-oxidative inhibition during autotrophic
223 algal growth. Elevated organic carbon content can potentially inhibit autotrophic microalgal
224 growth (Alcántara et al., 2013). Van Wagenen et al. (2015b) reported no decrease in
225 photoautotrophic growth and nutrient removal in the presence of sufficient light intensity and up
226 to 400 mg·L⁻¹ volatile fatty acids (VFA). This concentration is significantly higher than what is
227 expected in effluents from domestic wastewater treatment systems (Tchobanoglous et al., 2004).
228 Therefore, we contend that autotrophic growth inhibition by VFAs can be ignored.

229 The charge balances have not been tracked through model development. Hauduc et al. (2010)
230 suggest using alkalinity as a sink to close charge balance, leading to stoichiometric coefficients
231 that disregard the biological processes. In the future, the charge balance should be closed using
232 methods for pH estimation (e.g. Flores-Alsina et al., 2015), thereby achieving more accurate
233 estimation of the carbon speciation which might additionally affect microbial growth rates
234 (Decostere et al., 2013). Moreover, the model currently does not consider temperature effects on
235 model parameter values, which is particularly important when considering open pond type
236 systems. This must be addressed in next model generations.

237 <Table 1>

238 <Table 2>

239

240 **3. MATERIALS AND METHODS**

241 **3.1. Microalgae and culture media**

242 The mixed green microalgal consortium used in this study was isolated in a natural pond in contact
243 with used water. The culture mainly consists of *Chlorella sorokiniana* (identification made by the
244 PCR method after isolation of the species as described in the SI, page S24, Fig. S1, SI) and
245 *Scenedesmus sp.* (based on microscopic observations, Fig. S2, SI). The algal culture grows strictly
246 in suspension, without significant biofilm or aggregate formation. The mixed culture was
247 cultivated using the MWC+Se synthetic medium (Guillard and Lorenzen, 1972), unless otherwise
248 specified.

249 **3.2. Experimental design and description of the reactors**

250 **3.2.1. Microbatch experiments**

251 Microbatch experiments were set up in 24-well black microtiter plates (VisiPlate, PerkinElmer
252 Inc., Waltham, MA) in a temperature controlled room at 20 °C. The microbatches – placed on a
253 shaker table operated at 160 rpm – were inoculated with 2 mL samples with 14 mg NO₃⁻-N·L⁻¹
254 and 1.55 mg PO₄-P·L⁻¹. Thereby, nutrients were available in excess in the medium. The light was
255 supplied by cool white LEDs (Werner Co., USA).

256 To assess the effect of light intensity on the microalgal growth, neutral density filters were attached
257 to the bottom of the microbatches to create different light intensities (Van Wageningen et al., 2014).

258 Two sets of experiments were carried out, resulting in twelve different light intensities ranging
259 from 12 to 870 μmol photons m⁻² s⁻¹.

260 In addition, microbatch experiments were set up to assess the heterotrophic growth of microalgae
261 in darkness. The MWC+Se culture medium was modified by adding acetate as organic carbon

262 supplied at different concentrations (10-1000 mg·L⁻¹). The cultures were grown on the same shaker
263 table, and kept in complete darkness (Van Wagenen et al., 2015a). Moreover, two sets of
264 measurements at two different light intensities (120 μmol photons m⁻² s⁻¹ and 450 μmol photons
265 m⁻² s⁻¹) were conducted to assess the effect of light intensity on the acetate uptake rate (200 mg·L⁻¹
266 acetate in each well) and heterotrophic growth. At each measurement point, the algal biomass
267 was measured and the content of three wells were removed and prepared for acetate measurement.
268 This method allowed us to monitor the growth rate together with the acetate removal rate in the
269 microbatch scale.

270 **3.2.2. Sequenced batch experiments in 1-L PBRs**

271 Batch experiments were set up using 1-L wide-neck glass bottles (Duran[®], Germany) with constant
272 stirring at 180 rpm using magnetic stirrers and with a multi-port system, allowing for sample
273 extraction and aeration with CO₂ enriched air (5 % CO₂) at a flow rate of 10 L·h⁻¹. pH was kept
274 between 6.5-8. Light was supplied from the two sides of the batches using 18-W fluorescent lamps
275 (GroLux, Sylvania[®], USA), providing 160 μmol photons m⁻²s⁻¹ continuously. Three parallel batch
276 reactors were run where the nitrogen source in one was ammonium while in the others nitrate. The initial
277 concentration of nutrients was varied by decreasing the nitrogen (either ammonium or nitrate) or
278 phosphorus levels 3 times. Initial nitrogen and phosphorus concentrations ranged between 0.3-14
279 mg·L⁻¹ as N and 0.1-1.55 mg·L⁻¹ as P, respectively. Microalgal biomass was diluted when optical
280 density (OD) reached a value of 0.4 (corresponding to 0.21 g TSS·L⁻¹, Fig. S3). 90% of the volume
281 was replaced with fresh cultivation medium thereby avoiding self-shading in the culture (and thus
282 light limitation). Temperature was kept constant in the room at 20°C. During the batch experiments
283 the limiting and the non-limiting nutrients as well as cell density were monitored.

284 Heterotrophic growth and the acetate uptake were assessed in 1-L batches under dark conditions
285 at 20 °C with concentration of 14 mg NO₃⁻-N·L⁻¹ and 1.55 mg PO₄-P·L⁻¹, ensuring nutrients to be
286 in excess. Initial acetate concentrations were set to 200 and 400 mg·L⁻¹. Constant air was supplied
287 to the cultures to avoid limitation by oxygen and the batches were kept in complete darkness.

288 **3.2.3. Sequential batch experiments in 24-L PBRs**

289 Experimental data were collected for model calibration and validation in a 24 L laboratory-scale
290 airlift PBR. In the first four cycles (Descending cycles), the initial ammonia and nitrate
291 concentration decreased in sequential cycles from 10 to 5 to 2.5 to 0.5 mg-N·L⁻¹. In the following
292 four cycles (Ascending cycles), the initial ammonia and nitrate concentration increased from 0.5
293 to 2.5 to 5 to 10 mg-N·L⁻¹ (Fig. 1). Each cycle was run once in a consecutive manner. The reactor
294 was operated with constant aeration with CO₂ enriched air (5% CO₂) with 600 mL·min⁻¹ flow rate.
295 pH varied between 6.2-7. The temperature varied between 17-21 °C. A custom-built lamp,
296 providing 600 ± 50 μmol photons m⁻² s⁻¹, with two metal-halide light bulbs (OSRAM[®], Germany),
297 was placed on top of the reactor.

298 <Figure 1>

299 **3.3. Analytical methods**

300 *In-vivo* fluorescence (IVF) at 440 nm excitation and 690 nm emission was used to measure and
301 estimate directly the algal growth in microplates due to its high sensitivity at low biomass
302 concentrations (Van Wagenen et al., 2014). Acetate was measured using HPLC (Van Wagenen et
303 al., 2015a). Biomass in the 1-L and 24-L batch reactors was analysed by measuring the OD at 750
304 nm and by total suspended solids (TSS) measurement using glass fibre filter (Advantec[®], USA)
305 with a pore size of 0.6 μm (APHA, 1995). TSS units were converted to COD using a conversion

306 factor of 0.72 gTSS/gCOD (estimated as explained in the SI). Total nitrogen and phosphorus
307 measurements in the suspension were done using commercial test kits (Hach-Lange[®], USA).
308 Following sample filtration (0.2 µm filter), ammonium, nitrate, nitrite and phosphate
309 concentrations were measured using test kits supplied by Merck[®] (USA). The internal cell quota
310 of nitrogen was calculated based on the difference of total nitrogen in the algal suspension
311 (algae+medium) and total soluble nitrogen in the filtrate (soluble organic N, NH₄⁺, NO₂⁻ and NO₃⁻
312). The internal cell quota of phosphorus was obtained by the difference of total phosphorus in the
313 algal suspension and soluble phosphate in the filtrate.

314 **3.4. Model implementation and calibration**

315 **3.4.1. Calibration procedure for 1-L and 24-L batch experiments**

316 A model identifiability analysis was carried out to determine if the information gathered from the
317 1-L and 24-L batches was rich enough, both quantitatively and qualitatively, to estimate
318 parameters. The methodology developed, adapted from literature, is referred to as the Latin
319 Hypercube Sampling based Simplex (LHSS). It comprises 5 modular steps (Fig. 2): Step 1: the
320 parameter space for the parameters to be estimated in each experiment is defined, based on the
321 extensive literature review presented in section 2.1; Step 2: Latin Hypercube Sampling (LHS,
322 Helton and Davis, 2003) is used to select values from the parameter space; Step 3: the parameter
323 sets are used as initial values (*a priori*) for the local optimization algorithm, Simplex (Nelder and
324 Mead, 1965). The objective function to be minimized is the root mean square normalized error
325 (RMSNE) relative to the measured value (y_m):

$$326 \quad RMSNE = \sqrt{\frac{1}{n} \sum_{i=1}^n \left(\frac{y_m - \hat{y}}{y_m} \right)^2} \quad (1)$$

327 where n is the number of measurement points, and \hat{y} is the predicted value.

328 Simplex can identify different optimal parameter sets. Step 4: Thresholds are set by visualization
329 of the distribution of the RMSNE (histogram) for the estimated parameter subsets resulting in
330 different cut-off values in the two scales (1% and 10% of the minimum RMSNE, Fig. S4, SI).
331 Parameter subsets resulting in an error higher than these thresholds are considered as local minima
332 and omitted in further steps; Step 5: The distribution of the optimal parameter set values obtained
333 through Simplex, combined with the average parameter values, standard deviations and correlation
334 matrix are used for identifiability assessment. The distributions of parameter value estimates are
335 plotted as histograms that are interpreted according to their relative wideness, i.e. the narrower the
336 histogram, the more identifiable the parameter is (Van Daele et al., 2015). Parameter
337 identifiability, in this step, is assessed by additionally considering the standard deviations and the
338 correlation matrix, as suggested by Sin et al. (2010). Based on the correlation matrix, if the
339 correlation of parameters is comparably high, then the parameter identifiability should be assessed
340 by analysing the impact of setting one of the parameters to its minimum and maximum boundaries
341 defined by the standard deviation on the simulation output. The Janus coefficient (J) is used to
342 assess the difference in model predictions (Sin et al., 2007). The Janus coefficient describes the
343 accuracy of the model prediction, and for reliable predictions its value is close to 1. Janus
344 coefficients higher or lower than 1 indicate that predictions are worse or better than the original
345 model approximations obtained through parameter estimation, respectively. We find that 500 LHS
346 samples are sufficient to reach convergence on the parameter distributions.

347 <Figure 2>

348 **3.4.2. Autotrophic growth model calibration**

349 Specific growth rates were obtained as a function of light intensity using microplate experimental
350 data. Six different expressions (specified in the model development section), describing the effect

351 of light on algal growth, were tested by approximating the experimental data. Parameter values for
352 the maximum photoautotrophic growth rate ($\mu_{A,max}$) and the saturation light intensity (I_s) were
353 obtained from the fitting.

354 $X_{Alg,Nmax}$, $X_{Alg,Nmin}$, $X_{Alg,PPmax}$ and $X_{Alg,PPmin}$ were approximated as the observed maximum and
355 minimum quota reached overall in the 1-L and 24-L sequenced batch experiments. The
356 stoichiometric parameters, nitrogen and phosphorous content of algal biomass (i_{Nxalg} and i_{Pxalg})
357 were set as the minimum observed quota of nitrogen and phosphorus. Parameter values for $\mu_{A,max}$
358 and half saturation coefficients of ammonium ($K_{NH_4,Alg}$), nitrate ($K_{NO_3,Alg}$) and phosphate ($K_{PO_4,Alg}$)
359 and the maximum specific uptake rates of ammonium ($k_{NH_4,Alg}$), nitrate ($k_{NO_3,Alg}$) and phosphate
360 ($k_{PO_4,Alg}$) were obtained in the 1-L batches with the LHSS parameter estimation method. Microalgal
361 decay rate (b_{Xalg}) was set at $2\% * \mu_{A,max}$ as suggested by Quinn et al. (2011).

362 Parameters $\mu_{A,max}$, $K_{NO_3,Alg}$, $K_{NH_3,Alg}$, $K_{PO_4,Alg}$, $k_{NO_3,Alg}$, $k_{NH_3,Alg}$, $k_{PO_4,Alg}$ and b_{Xalg} were also estimated
363 using the experimental data obtained using the 24-L reactor setup. Average light intensity was set
364 in each cycle, calculated based on Benson et al. (2007), i.e. by integrating the Lambert-Beer
365 equation over the culture depth. The parameter estimates used to calibrate the Lambert-Beer
366 equation are those presented by Wágner et al. (2014).

367 **3.4.3. Heterotrophic growth model calibration**

368 The Monod kinetics was fitted on the results obtained in microbatch experiments. Data obtained
369 in 1-L batch experiments were used to estimate kinetic parameters using the LHSS method.
370 Parameter values for minimum and maximum quotas, half saturation coefficients and maximum
371 specific uptake rates of N and P are taken from the autotrophic growth process rate. Parameter
372 values for the maximum heterotrophic growth rate ($\mu_{H,max}$) and the half saturation coefficient of

373 acetate (K_A) were estimated using data obtained in the microbatch and 1-L batch experiments. The
374 observable yield on acetate (Y_{AC}) was calculated from the 1-L batch experiments as the ratio of g
375 biomass produced as COD and g acetate consumed as COD.

376 In the presence of light and acetate, we observe mixotrophic growth. To assess the effect of light
377 on heterotrophic growth kinetics (described in section 3.2.1), we calculated the heterotrophic
378 biomass production based on the acetate consumption in the microbatch experiments. The
379 observed value of $\mu_{H,max}$ was estimated using the data from the exponential growth phase for both
380 light intensities using the estimated heterotrophic biomass production. The value of the half-
381 saturation coefficient for light inhibition (K_I) was estimated by approximating the observed $\mu_{H,max}$
382 at different light intensities from the microbatch experiments, including $\mu_{H,max}$ estimated in 1-L
383 batch in darkness, using the competitive inhibition term.

384 **3.4.4. Literature values**

385 Remaining model parameters were taken or calculated based on literature (specified in Table 3).
386 In ASM-A, the half-saturation coefficient of inorganic carbon (K_{Alk}) is according to Broekhuizen
387 et al. (2012). The microbial growth yield on inorganic carbon ($Y_{Xalg,SAIk}$) was calculated based on
388 the stoichiometry presented in Park and Craggs (2011). The half-saturation coefficient for oxygen
389 (K_{O_2}) in the heterotrophic growth is based on the minimum operational oxygen level reported in
390 literature, and is given as 20% of the saturation oxygen concentration (Morales-Sánchez et al.,
391 2013). The inert fraction of the biomass (f_{XI}) produced via decay is accounted for according to
392 Henze et al. (2000). The nitrogen and phosphorus released during the decay process in the form of
393 inert and biodegradable matter (iN_{XalgI} , iN_{XalgS} , iP_{XalgI} and iP_{XalgS}) is based on Henze et al. (2000).

394 **3.4.5. Model implementation**

395 The ASM-A model was developed as an extension of the simulation model ASM-2d (Henze et al.,
396 2000), which was implemented in Matlab (The MathWorks, Natick, MA; Flores-Alsina et al.,
397 2012). The Matlab solver ode15s was used (see e.g. Flores-Alsina et al., 2012).

398 **3.5. Model evaluation**

399 The experimental design developed for the 24-L sequenced batch PBR (Fig. 1) was used through
400 a two-step model evaluation. To this end, the hypothesis tests set for the two model evaluation
401 steps comprise the questions (I) Do culture history and/or substrate availability significantly
402 influence parameter estimates?; (II) What are the practical consequences for model calibration?,
403 i.e. can we use a mean parameter set to accurately predict algal cultivation in PBRs?; (III) Can we
404 explain inaccuracies as a result of parameter variability? To answer hypothesis-I, it is noteworthy
405 that the experimental design (Fig. 1) used with different initial substrate to biomass ratio in each
406 cycle allows decoupling the culture history from the substrate availability impact. Through the
407 first evaluation step, parameter sets obtained through each descending cycle (Table 4) were
408 confronted with data obtained in the corresponding (same initial substrate concentrations)
409 ascending cycle (Fig. 1). To assess model accuracy, we used the Janus coefficient (Sin et al., 2007).
410 To answer hypothesis-II and III, in the second evaluation step, Monte Carlo simulations were
411 performed to obtain a confidence interval of model predictions (Sin et al., 2009). The probability
412 range of ASM-A parameters was assigned by calculating the minimum/maximum parameter
413 values as the mean estimated parameter values minus/plus the standard deviation, respectively.
414 The mean and standard deviation values were calculated through the initial descending cycles
415 (Table 3). The uncertainty classes were assigned to each parameter based on previous knowledge,
416 as suggested by Sin et al. (2009), and are reported in Table S2.

417 For those state-variables that failed both evaluation steps global sensitivity analysis (GSA) was
418 carried out. The GSA method applied in this study is linear regression of Monte Carlo simulations
419 (Saltelli et al., 2008) – also referred to as the standard regression coefficient (SRC) method (more
420 details on the method are present in the SI, pages S27-S28). Only the parameters for which $\beta_i \geq$
421 0.1 are considered to be influential (Sin et al., 2011). In this study, 1000 Monte Carlo simulations
422 are found to be sufficient to achieve convergence.

423 **4. RESULTS AND DISCUSSION**

424 **4.1. Model identification**

425 **4.1.1. Autotrophic growth**

426 The Steele expression (included in R4) was found to most accurately ($R^2=0.995$) describe the light
427 dependence of algal growth (Table S3, SI; Fig. 3). We note, however, that the R^2 obtained with all
428 six expressions is comparably high, i.e. $R^2>0.99$. The Steele equation accounts for the photo-
429 inhibition on algal growth, a factor not fully supported by the measured data, and hence, further
430 assessment at higher light intensities is necessary to understand better the inhibition by light. In
431 full-scale systems, however, the prevalence of such high average light intensity ($>900 \mu\text{mol m}^{-2} \text{s}^{-1}$)
432 is assumed to be negligible. The estimated values for $\mu_{A,max}$ and I_s are $3.6 \pm 0.04 \text{ d}^{-1}$ and 758 ± 23
433 $\mu\text{mol m}^{-2} \text{s}^{-1}$, respectively (Table 3). It should be noted that we used observed growth rates to
434 calibrate phototrophic growth, disregarding the effect of the decay rate. Therefore, the maximum
435 growth rate may be underestimated.

436 **<Figure 3>**

437 **4.1.2. Heterotrophic growth**

438 According to the microbatch experimental results, for $S_A = 0-180 \text{ mg COD}\cdot\text{L}^{-1}$, heterotrophic
439 growth can be effectively described using the Monod expression (suggested by Turon et al. (2015))
440 (Fig. S5, SI). The approximation of the experimental data ($R^2=0.7$) results in $\mu_{H,max}=0.7\pm0.06 \text{ d}^{-1}$
441 and $K_A=10.7\pm3.6 \text{ g COD}\cdot\text{m}^{-3}$ (Table 3). We note that our measurements show a plateau (Fig. S5,
442 SI) – with growth rates around 0.38 d^{-1} – above approx. $180 \text{ mg}\cdot\text{L}^{-1}$ acetate concentration. Species
443 that are capable of growing under both heterotrophic and photoautotrophic conditions are reported
444 to have similar heterotrophic and photoautotrophic growth rates (Ogawa and Aiba, 1981; Van
445 Wageningen et al., 2015a) – as opposed to our case. The value of $\mu_{A,max}$ is significantly higher than
446 that obtained for $\mu_{H,max}$ (i.e. $\mu_{A,max}=3.6\pm0.04 \text{ d}^{-1}$ and $\mu_{H,max}=0.7\pm0.06 \text{ d}^{-1}$). However, when
447 microbatches with acetate were exposed to different light intensities, the observed $\mu_{H,max}$ is
448 comparably higher (i.e. $2.8\pm0.8 \text{ d}^{-1}$ and $2.14\pm0.6 \text{ d}^{-1}$). Under mixotrophic growth conditions, the
449 oxygen needed to support heterotrophic growth in microbatch experiments is overcompensated by
450 the oxygen produced during the autotrophic growth. Therefore, it is suggested that heterotrophic
451 microbatch experiments were limited by the oxygen level, thereby decreasing the observed $\mu_{H,max}$
452 (i.e. $0.7 \pm 0.06 \text{ d}^{-1}$). It is also hypothesised that the plateau observed in Fig. S5 (μ around 0.38 d^{-1})
453 is caused by the inefficient oxygen transfer in the microplates.

454 The kinetic parameters obtained from the measurements conducted in 1-L batches (Fig. S6a, SI)
455 are the heterotrophic growth on acetate and the affinity coefficient for acetate, i.e. $\mu_{H,max}=4.5\pm0.05$
456 d^{-1} and the $K_A = 6.3\pm0.52 \text{ gCOD}\cdot\text{m}^{-3}$. The estimated parameters were evaluated using an
457 independent set of experimental data (Fig. S6b, SI), and results obtained show low discrepancy
458 between measured and simulated data (J~1, Table S4, SI). However, the value of $\mu_{H,max}$ obtained
459 at this scale is significantly higher than that obtained in the microbatch experiments
460 ($\mu_{H,max}=0.7\pm0.06 \text{ d}^{-1}$). Since 1-L batch reactors were continuously aerated, oxygen was not

461 limiting heterotrophic growth. Typical values of COD measured in influent domestic used water
462 are in the range of 250-800 mg·L⁻¹ (Tchobanoglous et al., 2004). Thus in used water treatment
463 processes acetate and other volatile fatty acids are not expected to inhibit heterotrophic growth.
464 Y_{AC} was calculated to be 0.42 gCOD·g⁻¹COD (Table 3) from the 1-L batch experiments. K_I was
465 determined using measured data inferred in both microbatch (mixotrophic growth, at two different
466 light intensities) and 1-L batch experiments (heterotrophic growth, no light), estimated to be
467 $331 \pm 160 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Fig. 4). Due to the low experimental data considered in this study, K_I should
468 be interpreted with caution.

469 <Figure 4>

470 4.1.3. Nutrient uptake and storage

471 The kinetic parameters were estimated using the LHSS method (Step 1-3, Fig. 2) using data
472 obtained in the 1-L batch experiments, resulting in $\mu_{A,max} = 3.54 \pm 0.05 \text{ d}^{-1}$, $K_{NH_3,Alg} = 6.7 \pm 1.63 \text{ gN} \cdot \text{m}^{-3}$,
473 $K_{NO,Alg} = 6.87 \pm 2.56 \text{ gN} \cdot \text{m}^{-3}$, $K_{PO_4,Alg} = 4.71 \pm 0.65 \text{ gP} \cdot \text{m}^{-3}$, $k_{NH_3,Alg} = 2.55 \pm 0.61 \text{ gN} \cdot \text{m}^{-3}$, $k_{NO,Alg} =$
474 $2.13 \pm 0.86 \text{ gN} \cdot \text{m}^{-3}$ and $k_{PO_4,Alg} = 4.84 \pm 0.67 \text{ gP} \cdot \text{m}^{-3}$ (Table 3). The specification of the experimental
475 data that are used to calculate the objective function in each of the 1-L batch experiments is
476 included in the supporting information (Table S5). Initial conditions for the 1-L and 24-L batch
477 experiments are reported in the supporting information (Table S6). The upper and lower
478 boundaries of the LHSS simulations are included in the supporting information in Table S7. In
479 accordance with the standard deviations of the parameter values (for $\mu_{A,max}$, the average standard
480 deviation is 1.5%, and for uptake rates and affinity coefficients it is below 40%), the histograms
481 (Table S8, SI) show a relatively narrow parameter distribution, thereby indicating that the
482 parameters are identifiable (Step 5, Fig. 2). The low standard deviation obtained can be attributed

483 to the fact that parameter estimation was carried out by omitting measurement noise. The cut-off
484 value of 1% is set as a general threshold for local minima rejection (Step 4, Fig. 2) in all three
485 experiments (Table S8, Fig. S4a, S6b, SI). This included more than 70% of the parameter sets in
486 the ammonium and phosphorus limiting 1-L batches (Table S8, SI). However, in the nitrate batch,
487 as a result of the high number of local minima identified and rejected (Fig. S4a, SI), even though
488 convergence was reached in the RMSNE distribution, only approx. 15% of the parameter sets were
489 included in the 1% range. Based on the correlation matrix, the parameters can be considered highly
490 correlated, i.e. the matrix elements are close to 1 (Table S8, SI). Therefore, we further assessed the
491 impact of the parameter variability on the model output (Step 5, Fig. 2). To this end, we compared
492 the simulation results using parameter values on the boundaries given by their standard deviation.
493 We show one example (Fig. S7, SI), where we altered one parameter that is highly correlated with
494 another (in this case we alter $k_{NO,Alg}$ that is correlated with $K_{NO,Alg}$). We set $k_{NO,Alg}$ to its maximum
495 and minimum value (i.e. mean \pm standard deviation), and the effect of this manipulation was
496 assessed using the simulation outputs (including algal biomass concentration, soluble nitrate
497 concentration and nitrogen storage). We found comparably low variation in the model outputs
498 when altering $k_{NO,Alg}$ (Janus coefficient ~ 1 , Fig. S7, SI). This approach was also employed to test
499 all experimental data (Table S8, SI) and all highly correlated parameters. The discrepancies
500 obtained between the outputs are comparably low in all cases (Janus coefficients ~ 1 , not shown;
501 Step 5, Fig. 2), thus suggesting parameters are identifiable. The minimum and maximum nitrogen
502 content observed throughout these experiments are $X_{Alg,Nmin}=0.012\pm 0.003$ gN \cdot g $^{-1}$ COD and
503 $X_{Alg,Nmax}=0.09\pm 0.004$ gN \cdot g $^{-1}$ COD, respectively. The minimum and maximum phosphorus content
504 measured throughout these experiments are used as minimum and maximum quotas in the final
505 model calibration set, i.e. $X_{Alg,PPmin}=0.0021\pm 0.0005$ gP \cdot g $^{-1}$ COD and $X_{Alg,PPmax}= 0.019\pm 0.0006$

506 $\text{gP}\cdot\text{g}^{-1}\text{COD}$, respectively (Table 3). Any phosphorus content above this minimum quatum is
507 referred to as “phosphorus storage” for the algae, which can include polyphosphate (Powell et al.,
508 2008).

509 Experiments assessing the effect of nutrient limitation on microalgal growth were conducted in a
510 24-L batch reactor (Fig. 5; Fig. S8-S10, SI). The lowest and highest levels of nitrogen quota found
511 are $X_{Alg,Nmin}=0.00936\pm 0.002 \text{ gN}\cdot\text{g}^{-1}\text{COD}$ and $X_{Alg,Nmax}=0.13\pm 0.016 \text{ gN}\cdot\text{g}^{-1}\text{COD}$, respectively
512 (Table 3), which are included as the minimum and maximum quotas in the model calibration
513 exercise as final parameter values. Any nitrogen content above this minimum quatum is referred
514 to as “nitrogen storage” for the algae, which can be in different forms (Romero-García et al., 2012;
515 Coppens et al., 2014). The minimum and maximum phosphorus content observed throughout the
516 24-L batch experiments are $X_{Alg,PPmin}=0.0028\pm 0.0006 \text{ gP}\cdot\text{g}^{-1}\text{COD}$ and $X_{Alg,PPmax}=0.016\pm 0.0006$
517 $\text{gP}\cdot\text{g}^{-1}\text{COD}$, respectively, which were within the range found for the 1-L batch data. The affinity
518 coefficients, $K_{NH4,Alg}$, $K_{NO,Alg}$ and $K_{PO4,Alg}$ and the uptake rates, $k_{NH4,Alg}$, $k_{NO,Alg}$ and $k_{PO4,Alg}$ were
519 estimated to evaluate and possibly validate the values obtained using the 1-L batch data. We
520 assumed that, in the 24-L batch experiments, the culture was exposed to an average light intensity
521 (estimated for each of the batches – Table S9, SI) in the PBR and that there was no inorganic
522 carbon limitation. Additionally, the RMSNE values obtained through parameter estimation are
523 presented in Table 5. Based on experimental data obtained in the 1-L and 24-L batches (Table 3),
524 a comparative assessment of parameter estimates was carried out, indicating significant
525 discrepancy for only nutrient uptake process rate parameters, i.e. $k_{NH4,Alg}$, $k_{NO,Alg}$ and $k_{PO4,Alg}$ (up to
526 50-times difference). This discrepancy could be explained as a consequence of the different
527 hydrodynamics of the batch reactors. That is, 1-L reactors are well mixed by a magnetic stirrer and
528 bubbling. However, the 24-L reactor mixing only relies on the advective flow induced by the

529 bubbling in the inner side of the reactor. Therefore, there is a higher chance to induce dead zones
530 in the second reactor. Poor mixing has been reported to affect parameter estimates, showing
531 apparent slower dynamics (Arnaldos et al., 2015). Moreover, the discrepancy may be due to the
532 lack of accounting for the impact of light attenuation under dynamic conditions along the
533 experiment in the 24-L PBR. Additionally, the lack of temperature control in the in the 24-L batch
534 reactors resulted in oscillating temperatures below 20 °C during the experiments. The lower
535 temperature may have caused reduced microbial activity (Ras et al., 2013) that could have
536 contributed to the discrepancy between the parameter values.

537 <Figure 5>

538 In an effort to benchmark parameter values obtained herein, literature values (Table S2, SI)
539 selected from studies focusing on *Chlorella sp.* and/or *Scenedesmus sp.* were used. A close
540 agreement is found between parameter values estimated in this study and those in literature - also
541 the case for nutrient uptake rates ($k_{NH_4,Alg}$, $k_{NO,Alg}$ and $k_{PO_4,Alg}$) obtained using the 24-L batch data.
542 Our results suggest that, in the absence of dissolved nitrogen species, microalgal growth can be
543 sustained by accessing intracellularly stored nitrogen. A similar case holds for dissolved
544 phosphate, indicating growth utilising internally stored phosphorus (Fig. 6). These findings agree
545 well with published observations (Powell et al., 2008; Coppens et al., 2014), and highlights the
546 relevance of using the Droop model in ASM-A, which uncouples nutrient uptake and storage from
547 microalgal growth.

548 Subsequently, a default parameter set is selected from the different sets obtained in different scales,
549 and the rationale for the selection approach is elucidated in the following. The I_{Av} of
550 photoautotrophic growth and the K_I for heterotrophic growth parameters are inferred from the set

551 estimated using microbatch experiments. The short light path of the microbatches results in an
552 even light distribution. Hence the entire culture is expected to be evenly exposed to the same light
553 intensity. $X_{Alg,Nmax}$, $X_{Alg,Nmin}$, $X_{Alg,PPmax}$ and $X_{Alg,PPmin}$ were set as the overall minimum and maximum
554 values reached and were inferred from sets estimated using 1-L (P quota) and 24-L (N quota) batch
555 experimental data. The heterotrophic growth kinetic parameters and Y_{Ac} are inferred from sets
556 estimated using 1-L batch data as we found oxygen limitation under microbatch scale. For model
557 evaluation purposes we selected as default, the above mentioned parameters and the literature
558 values (Table 3, bold values).

559 <Table 3>

560 <Table 4>

561 4.2. Model evaluation

562 An independent experimental data set (i.e. data obtained in the ascending cycles in the 24-L batch
563 reactor, Fig. 6) is employed as a means for model evaluation (described in section 3.5). In the first
564 evaluation step, the RMSNE values obtained by approximating the experimental data using the
565 simulation model – calibrated with the specific parameter sets obtained through each respective
566 descending cycle (Table 4) - are relatively low and, for algal biomass concentration, ammonium
567 and phosphate concentrations as well as the nitrogen and phosphorus storage, $J_{\sim 1}$ (Table 5). This
568 outcome indicates that culture history does not significantly affect parameters that the
569 aforementioned outputs are sensitive to (hypothesis-I). In most cases, the RMSNE value for the
570 ammonium concentration state-variable is comparably high (Table 5). This is the consequence of
571 normalizing the error using observed values that gives more weight to low magnitude values
572 (Hauduc et al., 2015). As the ammonium bulk concentration decreases below ~ 0.1 in most cycles

573 (e.g. Fig. 5 and 6), the calculated RMSNE value is high (Eq. 1). Hence, J becomes more sensitive
574 in the case of ammonia, giving relatively high values for the evaluation of cycle 7 (Table 5). To
575 further support this hypothesis, the J for cycle 7 is re-calculated using the mean absolute error
576 (MAE), which gives higher penalty to large errors. As expected, the J , based on MAE, indicates
577 high accuracy in the validation step ($J=1.65$). The experimental values of microalgal biomass
578 concentration, bulk ammonium and phosphate concentration and phosphorus storage are in the
579 proximity of the best fit (lowest RMSNE) of the Monte Carlo simulation results (Fig. 6a, 6b, 6d
580 and 6f).

581 **<Table 5>**

582 This outcome therefore suggests that ASM-A calibrated using the selected mean default parameter
583 set - with the associated uncertainties (Table 4) – can be used to predict algal cultivation in PBRs,
584 in which *Chlorella* and *Scenedesmus* are the dominating species (hypothesis-II). This, however, is
585 not the case for predicting the nitrate concentration and, to a lesser extent, the internal nitrogen
586 storage, indicated by experimental data located outside the confidence interval.

587 **<Figure 6>**

588 Nitrogen storage can be predicted in the ascending cycles using the parameters estimated from the
589 parallel descending cycles, i.e. $J \sim 1$ (Table 5). In the second evaluation step, however, the
590 discrepancy between the predicted and measured nitrogen storage cannot be explained through
591 parameter variability (i.e. most data falls outside the predictive confidence interval, hypothesis-III,
592 Fig 3e). Consequently, substrate availability is assumed to significantly affect the predicted
593 nitrogen storage, thereby indicating the need for case-specific calibration of the nitrogen storage
594 process (hypothesis-II). Finally, the bulk nitrate concentration prediction fails for both evaluation

595 steps ($J \gg 1$ Table 5, experimental data falls outside the predictive confidence interval, Fig. 6c).
596 This outcome suggests that culture history can significantly impact parameter values associated
597 with bulk nitrate prediction (hypothesis-I).

598 According to the GSA results (Fig. 7; Fig. S11, SI), the most sensitive model parameter, affecting
599 the soluble nitrate concentration is the uptake rate of nitrate $k_{NO,Alg}$, which also affects nitrogen
600 storage (Fig. 7a and 7b). Therefore the identifiability of $k_{NO,Alg}$ is subsequently assessed using the
601 LHSS method (Table S10-S13, SI; Step 5, Fig. 2). We find that based on the histograms obtained
602 (Table S10-S13, SI), the distribution of parameter values estimated is relatively narrow, and
603 standard deviations calculated for each cycle are relatively low ($< 40\%$), thus suggesting that $k_{NO,Alg}$
604 is identifiable (Step 5, Fig. 2). Based on the correlation matrix (Table S10-S13, SI), $k_{NO,Alg}$ is highly
605 correlated with the affinity for nitrate. Thus, we assessed the impact of parameter variability on
606 model outputs – analogously to the procedure described in section 4.1.3 (Step 5, Fig. 2). $k_{NO,Alg}$
607 was altered to its maximum and minimum value given by the standard deviation separately using
608 the $k_{NO,Alg}$ estimated in each cycle (1-4) (Table 4). Comparably low variation in the outputs ($J \sim 1$)
609 is obtained (illustrated with an example drawn from cycle 1, Fig. S12, SI), thereby indicating $k_{NO,Alg}$
610 as identifiable (Step 5, Fig. 2). Since $k_{NO,Alg}$ is identifiable, the case specific calibration of $k_{NO,Alg}$
611 is recommended. To this end, $k_{NO,Alg}$ was estimated for each cycle, leaving the rest of the parameter
612 kept at the mean values, and results show hysteresis in the parameter value (Fig. 7c). This outcome
613 can serve as a possible explanation to the observations related to the impacts of culture history and
614 substrate availability on nitrate and nitrogen storage predictions.

615 According to Fig. 8 and Fig. S13- S15, using the case-specific calibration of $k_{NO,Alg}$, the increased
616 model accuracy in terms of bulk nitrate does not necessarily translate into improved prediction of
617 the nitrogen storage, possibly as consequence of the scattered data obtained in the 24-L batches

618 (Fig. 8a and 8c). Therefore, future research should further assess the dynamics of internal nitrogen
619 storage in green microalgal cells that could lead to the re-identification or possible extension of
620 the Droop model.

621 <Figure 7>

622 <Figure 8>

623 5. Conclusions

624 This study presents the identification and evaluation of a biokinetic model for photoautotrophic
625 and heterotrophic microalgal growth, developed in the activated sludge modelling framework
626 (ASM-A), thereby facilitating coupled implementations with the already existing simulation
627 model platforms. We conclude that:

- 628 • Through the specific experimental design and data treatment, the model parameters could be
629 estimated and were identifiable. Furthermore, the experimental design permitted the
630 quantification of model parameter variability caused by culture history and substrate
631 availability.
- 632 • The average parameter estimates can be used to predict microalgal biomass growth, effluent
633 ammonium and phosphate concentrations and phosphorus storage. This is not the case for the
634 nitrogen storage and soluble nitrate concentration, which depends on the culture history and
635 substrate availability.
- 636 • The most sensitive parameter affecting the prediction of the soluble nitrate concentration and
637 nitrogen storage is the maximum uptake rate of nitrate. The case specific re-estimation of
638 $k_{NO,Alg}$ can potentially explain the observations related to the impacts of culture history and
639 substrate availability on nitrate and nitrogen storage predictions.

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