

1 **Elucidating the competition between heterotrophic denitrification and**
2 **DNRA using the resource-ratio theory**

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14 **Abstract**

15 Denitrification and dissimilatory nitrate reduction to ammonium (DNRA) are two microbial
16 processes competing for nitrate and organic carbon (COD). Their competition has great
17 implications for nitrogen loss, conservation, and greenhouse gas emissions. Nevertheless, a
18 comprehensive and mechanistic understanding of the governing factors for this competition is
19 still lacking. We applied the resource-ratio theory and verified it with competition
20 experiments of denitrification and DNRA reported in the literature. Based on this theory, we
21 revealed how COD/N ratio, influent resource concentrations, dilution rate, and stoichiometric
22 and kinetic parameters individually and collectively define the boundaries for different
23 competition outcomes in continuous cultures. The influent COD/N ratio alone did not drive
24 competition outcome as the boundary COD/N ratio for different competition outcomes
25 changed significantly with influent resource concentrations. The stoichiometry of the two
26 processes was determinative for the boundaries, whereas the affinity for the resources (K_s),
27 maximum specific growth rate (μ_{max}) of the two species and the dilution rate had significant
28 impacts as well but mainly at low influent resource concentrations (e.g., <100 μM nitrate).
29 The proposed approach allows for a more comprehensive understanding of the parameters
30 controlling microbial selection and explains apparently conflicting experimental results. The
31 results from this model also provide testable hypotheses and tools for understanding and
32 managing the fate of nitrate in ecosystems and for other species that compete for two
33 resources.

34 **Keywords:** Nitrate reduction; Chemostat; Resource concentration; COD/N ratio; Dilution
35 rate; Mathematic model

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37 **1. Introduction**

38 Denitrification (DEN) and dissimilatory nitrate reduction to ammonium (DNRA, also termed
39 as dissimilatory/respiratory/nitrate ammonification) are two main microbial processes
40 competing for nitrate as an electron acceptor [1]. During denitrification, nitrate is converted to
41 nitrogen gas, thereby leading to nitrogen loss in natural and engineered ecosystems such as
42 wastewater treatment plants (WWTPs). Nitrous oxide, a potent greenhouse gas, can be
43 emitted during this process, posing an increasing concern [2]. In contrast, DNRA retains
44 nitrogen locally by converting nitrate to bioavailable ammonium, which may be beneficial for
45 natural ecosystems but unwanted for WWTPs [3]. Besides, DNRA seems not to contribute to
46 N₂O emissions [1]. Growing evidence suggests that DNRA can be significant in both aquatic
47 and terrestrial ecosystems [4, 5]. Nevertheless, little is known about the importance of DNRA
48 and its relative contribution to global N-cycling [1, 6, 7]. Therefore, there is a pressing need to
49 better comprehend the factors influencing the competition between denitrification and DNRA
50 for nitrate.

51 Energetics and kinetics are the general physiological features of microorganisms that
52 explain and regulate the outcome of competition [8]. Theoretically, the catabolic reaction of
53 the denitrification pathway yields more free energy per unit of organic carbon oxidized (e.g.,
54 802 vs. 505 kJ per mole acetate) whereas for the DNRA pathway slightly more free energy is
55 liberated per unit of nitrate reduced (505 vs. 501 kJ per mole nitrate with acetate as electron
56 donor) [3, 9]. Moreover, the biomass yield per mole nitrate is 0.2-2 times higher from the
57 DNRA process than that of the DEN process [3, 9]. Therefore, from a thermodynamic
58 standpoint, it can be justified that DEN should occur under organic carbon-limiting conditions
59 (i.e., low COD/N), while DNRA is promoted under nitrate-limiting conditions (i.e., high
60 COD/N) [3, 10–12]. In addition, Tiedje [12] proposed a theory that high labile carbon
61 availability would favor organisms that use electron acceptors most efficiently; DNRA

62 transfers eight electrons per mole of nitrate reduced, whereas denitrification only transfers
63 five. According to this theory, DNRA should be more efficient and abundant under nitrate-
64 limiting conditions. Previous studies also suggest that DNRA bacteria generally have a lower
65 maximum specific growth rate but a higher affinity for nitrate compared to denitrifiers [8, 13].
66 The higher affinity for nitrate may also explain the observed dominance of DNRA over
67 denitrification under nitrate-limiting conditions [13]. In opposition to the theoretical
68 explanations that suggest DNRA dominance under nitrate-limiting conditions, results have
69 shown that high COD/N ratios do not necessarily lead to a shift from DEN to DNRA [14, 15].

70 Apart from energetics and kinetics, environmental conditions affect the biological
71 nitrate partitioning as well. There are multiple studies suggesting that the competition depends
72 on the dilution rate [11, 16] and initial resource concentration [8]. In addition, other studies
73 conducted with a pure culture that encompasses a dual pathway showed that COD/N ratio
74 alone was insufficient to explain pathway selection as at low resource concentrations the
75 culture disproportionately utilizes DNRA rather than denitrification [17]. These results
76 delineate that a comprehensive understanding of the factors that drive the partitioning or
77 coexistence of both pathways is lacking, and a mathematical approach to explain competition
78 outcome may be helpful.

79 Over the years, theoretical frameworks have been developed to predict the outcome of
80 interspecies microbial competition for the same resources. One example is the resource-ratio
81 theory, which describes the interactions between resources and growth of two or more
82 competing species and can predict the outcomes of microbial competition for resources, in
83 advance of actual competition experiments [18–20]. This theory takes both physiological
84 properties and environmental conditions into account. It has been successfully demonstrated
85 in predicting the outcome of microbial competition for a single nutrient [21] as well as in an
86 ecological competition between algae for two resources (phosphate and silicate) [22]. The

87 analytical solutions of generalized competition scenarios in continuous systems (e.g.,
88 chemostat) have been investigated at steady state, and results revealed survival of one or
89 coexistence of both species at given circumstance (e.g., [19, 23]).

90 This study investigates the potential of the resource-ratio theory in elucidating the
91 competition between denitrification and DNRA. More specifically, it is studied whether this
92 mathematical approach can match and explain the underlying principle for the seemingly
93 conflicting measurements conducted at different COD/N ratios in different studies. To this
94 end, the resource-ratio theory was applied to predict the experimental competition outcome of
95 heterotrophic denitrifiers and DNRA bacteria in continuous cultures [3, 13]. After verification,
96 the theory was used to test different conditions to understand what may drive pathway
97 partitioning or coexistence. The results highlight the impact of COD/N ratio, resource
98 concentrations, dilution rate, and microbial stoichiometric and kinetic parameters on the
99 competition outcome. Moreover, a generalized spreadsheet was created and supplied to ease
100 the application of this mechanistic theory to similar competition scenarios.

101

102 **2. Materials and methods**

103 After introducing the basics of the resource-ratio theory (section 2.1), this theory was
104 implemented to predict the competition outcome of heterotrophic denitrification and DNRA
105 (section 2.2). Its applicability was subsequently evaluated with experimental data available
106 from literature case studies [3, 13] (section 2.3).

107 **2.1 The resource-ratio theory**

108 The resource-ratio theory describes the interaction between resources and growth of
109 competing species and enables to predict the outcome of microbial competition for shared
110 resources, instead of or prior to actual competition experiments [18, 19]. The growth of the
111 microorganisms on the limiting resources was assumed to follow Monod kinetics (Eq. 1) [24].

$$\mu_i = \mu_{max,i} \frac{S}{K_{S_i} + S} \quad (1)$$

112 Where μ_i is the specific growth rate of species i (h^{-1}); $\mu_{max,i}$ is the maximum specific growth
113 rate of species i (h^{-1}); K_{S_i} is the half-saturation constant (i.e., affinity constant) of species i for
114 S (μM); S is the concentration of resource S (μM).

115 For every limiting resource in a continuous system, there is a subsistence resource
116 concentration at which the growth rate balances the dilution rate (D), which is defined as the
117 parameter J_S (Eq. 2). J_S also represents the concentration of resource S at steady state [25].
118 Below this concentration, the net growth rate would be negative, and thus, the species cannot
119 sustain. If n species are competing for a single limiting resource (S), the species i with the
120 lowest subsistence resource concentration J_{S_i} can utilize the limiting resource to the lowest
121 level at a given dilution rate and influent resource concentration and thus is the only possible
122 winner at steady-state. This has been previously proven mathematically [25] and
123 experimentally [21].

124

$$J_{Si} = K_{Si} \cdot \frac{D}{r_{Si}} = K_{Si} \cdot \frac{D}{\mu_{max,i} - D} \quad (2)$$

125 Where J_{Si} is the subsistence concentration of growth-limiting resource S for species i (μM); D
126 is the dilution rate (h^{-1}); r_{Si} is the intrinsic growth rate and equals to $(\mu_{max,i} - D)$ (h^{-1});

127 The competition of two species (N1 and N2) for two resources (S and R) in a
128 continuous culture is illustrated in Fig. 1, following a graphical-mechanistic approach that
129 was developed to study the competition and predation in macroecology [18]. In this two
130 resources plane (Fig. 1), for every species i, the so-called ‘Zero Net Growth Isoclines’ (ZNGIs)
131 are drawn, which are defined by the subsistence resource concentrations (i.e. J parameter) for
132 the two complementary resources. A species i cannot survive outside the boundary ZNGIs,
133 i.e., for $S < J_{Si}$ and/or $R < J_{Ri}$, even in the absence of a competing species. Stable coexistence
134 only occurs when the ZNGIs of the two species coincide (as in Fig.1), i.e. when each species
135 has lower subsistence concentration (J) for one of the two resources. If the ZNGIs of two
136 competing species do not cross, it means one species must have lower J parameters for both of
137 the two resources, and as a result it would always win the competition [18, 19]. Moreover, it
138 is assumed that the growth is restricted by the most limiting resource (i.e., the one that results
139 in lower growth rate in Eq.1), as described by Eq. 3 [19]. To maintain an equilibrium
140 population, the resource consumption rate must balance the resource supply rate. The
141 consumption vector (defined as C_i , Eq.4 and in Fig. 1) and the ZNGIs define the regions in
142 which either one of the two dominates or two species coexist (Fig. 1). The model based on
143 this theory was further detailed in the Supplementary Information (SI, section S1).

$$\mu_i(S, R) = \min \left(\mu_{max,i} \frac{S}{K_{Si} + S}, \mu_{max,i} \frac{R}{K_{Ri} + R} \right) \quad (3)$$

$$C_i = \frac{Y_{Si}}{Y_{Ri}} \quad (4)$$

144 Where Y_{Si} , Y_{Ri} are the yield of species i per unit of resource S or R consumed (mole biomass
145 per mole S or R); therefore C_i represents the ratio of the consumption of resource R to
146 resource S by species i (mole R per mole S).

147 Overall, with kinetic and stoichiometric parameters of the competing species (e.g.
148 μ_{max} , Ks and Y) and environmental conditions (e.g., influent resource concentration and
149 dilution rate), the resource-ratio theory enables to qualitatively and quantitatively predict the
150 competition outcomes, the status of the competing species and resources (e.g., concentrations)
151 at steady state (i.e., equilibrium points) and the dynamic (i.e. how the steady state is reached).

152 **2.2 Application of the theory for the competition between denitrification and DNRA**

153 In this study, heterotrophic denitrification and DNRA were assumed to be carried out by two
154 distinct specialist species and directly compete for nitrate and organic carbon (COD, e.g.,
155 acetate). Their competition in continuous culture (i.e., chemostat) can be regarded as a
156 specific case of the general resource-ratio theory for two species competing for two resources
157 (Fig. S1). The kinetic and stoichiometric parameters used in this study for the application of
158 this theory are presented in Table S2.

159 These values were used as the default (i) to verify the theory (section 3.1), (ii) to
160 analyze the impact of influent resource concentration and dilution rate on the boundary
161 COD/N ratios and thus the competition outcome (section 3.2 and 3.3), and (iii) to study the
162 dynamic system behaviour (section 3.5). To date, kinetic and stoichiometric parameters (e.g.,
163 Y , μ_{max} , and Ks) of heterotrophic DNRA microorganisms have only been limitedly reported.
164 Therefore, a local sensitivity analysis was performed to investigate the potential impact of
165 these parameters on the competition outcome (section 3.4).

166 **2.3 Experimental case studies for theory verification**

167 To verify the resource-ratio theory, two experimental studies on the competition between
168 heterotrophic denitrification and DNRA processes by van den Berg et al. [3, 13] were used. In
169 these studies, chemostat enrichment cultures were fed with different levels of acetate and
170 nitrate (COD/N=1.8-8.5 g COD g N⁻¹), with an averaged dilution rate of 0.026 h⁻¹. The
171 experimental conditions, observed competition outcomes, and measured biomass
172 concentrations at steady state are summarized in Table S3.

173 **3. Results and discussion**

174 **3.1 Verification of the resource-ratio theory**

175 This study used the results from two previously published chemostat enrichment cultures as
176 case studies for theory verification [3, 13]. These cultures were fed with different levels of
177 acetate and nitrate (COD/N=1.8-8.5 g COD g N⁻¹, Table S3). It was concluded that
178 denitrifiers dominated under carbon-limiting (i.e., high COD/N) conditions, whereas DNRA
179 bacteria dominated under nitrate-limiting (i.e., high COD/N) conditions [3, 13]. Moreover, the
180 coexistence of denitrifiers and DNRA bacteria was found for a wide range of intermediate
181 influent COD/N ratios (Table S3).

182 Fig. 2 compares the measured and predicted competition outcomes at 12 conditions
183 tested in the case studies. The predictions agreed with the measurements under 11 conditions
184 tested (Fig. 2). The only condition (influent 5857 μM nitrate and 6278 μM acetate) where
185 denitrification dominance was observed whereas coexistence was predicted (Fig. 2), was close
186 to the predicted boundary, and microbial community analysis clearly evidenced the strong
187 presence of DNRA bacteria at that point [3], implying that steady state may not have been
188 reached yet experimentally at this point. Quantitatively, the predicted steady-state biomass
189 concentrations and abundance were in good agreement with the measurements under all 7
190 conditions tested in case study 1 (Fig. 3A and 3B, where influent resource concentrations
191 were converted to COD/N ratio for simplicity). Overall, the predictions of this study were
192 both qualitatively and quantitatively in close alignment with the measured data (Fig. 2 and 3).
193 Therefore, the resource-ratio theory was considered valid for predicting the outcome of the
194 competition between heterotrophic denitrifiers and DNRA bacteria in continuous systems.

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197 **3.2 Impact of resource concentration on competition outcome**

198 The boundaries of the different regions in Fig. 2 can be expressed by the COD/N ratio, which
199 is often used in literature for competition and field studies [3, 4, 10, 11]. Fig. 4 illustrates the
200 influent COD/N ratios of the boundaries at different influent nitrate concentrations. These
201 boundaries COD/N ratios define the tipping point at which one process prevails over or
202 coexists next to the other. For instance, the upper boundary of the region for coexistence (i.e.,
203 region 4) represented the minimum influent COD/N ratio for DNRA dominance, whereas its
204 lower boundary represented the maximum influent COD/N ratio for DEN dominance (Fig. 4).
205 Overall, the boundaries influent COD/N ratios changed significantly at low influent nitrate
206 concentrations (e.g., $< 100 \mu\text{M}$) and gradually stabilized at high influent nitrate concentrations
207 (e.g., $> 1000 \mu\text{M}$). With the increase of influent nitrate concentration, the region for
208 coexistence (region 4) gradually widened and its upper and lower boundary influent COD/N
209 ratios asymptotically approached the stoichiometric values of C_{DNRA} (corresponds to COD/N
210 of 6.15) and C_{DEN} (corresponds to COD/N of 4.04), respectively. The stabilized boundaries at
211 high influent nitrate concentration (Fig. 4) were also confirmed in Fig. 3B. Despite the large
212 difference in influent nitrate concentration ($1000 \mu\text{M}$ used for prediction vs. $11790 \mu\text{M}$ in the
213 experiments, Table S3), the predicted DNRA biomass fraction from the model agreed with the
214 measurements (Fig. 3B). The trend also held for different influent COD concentrations (Fig.
215 S4)

216 Overall, the results clearly illustrate that, as a governing factor of the competition
217 between the two nitrate partitioning pathways, the boundary influent COD/N ratios were not
218 constant but could change significantly with influent resource concentrations. At high
219 influent resource concentrations, process stoichiometry (reflected in C_i) of the two competing
220 processes was the determining factor of the boundary influent COD/N ratios, whereas kinetics
221 (i.e., K_S and μ_{max} , reflected in J_S and thus the ZNGIs, Fig. 2 & 4) were important as well but

222 only at low influent resource concentrations. This implies that influent COD/N ratio alone is
223 not sufficient to predict the competition outcome of heterotrophic denitrification and DNRA.
224 Different competition outcomes (resource limitation) could occur at the same influent COD/N
225 ratio but varying influent resource concentrations (e.g., at the same influent COD/N ratio of
226 6.86, all four possible competition outcomes could occur for points a, b, c, and d in Fig. 4,
227 detailed Fig. S5). In this theoretical study, the competition between DEN and DNRA is
228 determined by both stoichiometries and growth kinetics. The stoichiometries were assumed to
229 be constant. The change of the boundary COD/N ratio with influent nitrate level was a result
230 of the change of growth rate of two species and thus the contribution of the two processes at
231 different influent nitrate concentrations.

232 The result also raises the question of how to anticipate the threshold of resource
233 limitation in continuous cultures. Resource limitation is normally anticipated based on the
234 process stoichiometry; for instance, nitrate is expected to be the limiting resource when it is
235 lower than the stoichiometry would require in relation to COD [17]. Our results show that this
236 stoichiometry-based definition is inadequate. For example, nitrate limitation (and thus DNRA
237 dominance) would occur when the influent COD/N ratio was above 6.16 (close to the DNRA
238 stoichiometry) at influent nitrate concentration of 1000 μM , whereas it would only occur with
239 the COD/N ratio above 8.05 at influent nitrate concentration of 10 μM (Fig. 4).

240 The impact of resource concentrations on competition outcomes has significant
241 implications, as different ecosystems have various nitrate availability (i.e., influent nitrate
242 concentration) (Table 1) and therefore possibly different boundary COD/N ratios for nitrate
243 partitioning. High nitrate concentrations have been reported in some ecosystems, for example,
244 in groundwater at a nuclear waste site (up to 233mM [26]), in soil adjacent to bats guano
245 caves [27], and in coastal rockpools affected by gull guano where high level of ammonium
246 that can further result in high nitrate level due to nitrification was observed (e.g., 1600 μM

247 [28]). However, the nitrate concentrations in natural aquatic and terrestrial ecosystems are
248 normally low (e.g., $<100 \mu\text{M}$, Table 3), at which the boundaries of different competition
249 outcomes changed dramatically (Fig. 4). Lab-scale competition studies often supply high
250 concentrations of nitrate ($> 1000 \mu\text{M}$, e.g., in [3, 10, 16]) at which the boundaries were rather
251 stable and mainly defined by the stoichiometries of denitrifiers and DNRA bacteria (Fig. 4).
252 The thresholds obtained from these high-nitrate environments were closely resembled with
253 our model. However, little experimental data is available for environmentally relevant
254 scenarios with low nitrate conditions. It would be interesting to design experiments to check
255 the theory under these conditions.

256 In the context of WWTPs, nitrate concentrations and COD/N ratios could change in a
257 wide range along the treatment line. DNRA bacteria were shown to be enriched from
258 activated sludge in chemostats with high COD/N ratio influent [3] and coexisted with
259 denitrifiers in wastewater treatment wetlands [34, 35]. Besides, the use of biofilm reactors is
260 increasing, where substrate gradients can be formed within the biofilm and may thus create
261 microenvironments with high COD/N for DNRA to proliferate. The role of DNRA in
262 WWTPs needs to be further characterized.

263 Some of the seemingly conflicting results concerning the impact of COD/N ratio may
264 partially attribute to the type of system used for investigation, i.e., continuous (i.e., chemostat)
265 versus batch cultures. In continuous cultures, the competition outcome is determined by the
266 subsistence concentration for the limiting resource (J_s , Eq. 2), as shown in this study. Stable
267 resource limitation can be reached in continuous cultures but not in batch cultures [13, 36]. In
268 batch cultures, the competition outcome of different microorganisms is determined by their
269 maximum growth rate [37]. Using both systems with a dual-pathway pure culture, Yoon et al.
270 [10] suggested that the batch systems cannot resolve the impact of COD/N ratio on pathways
271 selection between denitrification and DNRA. In a batch incubation system, the shift from

272 DEN to DNRA with increasing initial COD/N ratios, as expected in continuous cultures, was
273 not established [14]. Fig. S6 demonstrates a straightforward comparison between these two
274 systems. With the same initial conditions (COD/N ratio of 6.86, same of amount of DNRA
275 and DEN bacteria), DNRA outcompeted DEN in a continuous culture at steady state with
276 nitrate being the limiting substrate, whereas the opposite competition outcome was obtained
277 in a batch culture (Fig. S6). Therefore, caution is required when comparing the results
278 obtained from batch and continuous cultures.

279 **3.3 Impact of dilution rate on competition outcome**

280 In chemostats, the dilution rate (D) dictates the rate of resource supply and biomass washout.
281 A species cannot survive in chemostats above a certain dilution rate (lower than its μ_{max}). The
282 impact of D on single species has been well documented, for instance, in Kuenen and Johnson
283 [38]. The impact of D on the coexistence of two species was therefore investigated closer.
284 According to the resource-ratio theory, stable coexistence is only possible when denitrifiers
285 and DNRA bacteria each have lower subsistence concentration for one of the two resources,
286 i.e., $J_{NO_3}^{DEN} > J_{NO_3}^{DNRA}$ and $J_{COD}^{DEN} < J_{COD}^{DNRA}$. A critical dilution rate for coexistence ($D_C =$
287 0.0435 h^{-1}) was thus calculated as a function of the μ_{max} and K_S for nitrate of the two species
288 (detailed in section S7). Below D_C , all four possible competition outcomes could occur,
289 whereas above D_C , DNRA could not outcompete denitrification (Fig. 5A).

290 The boundaries of the region for coexistence (i.e., region 4 in Fig. 4) were used for
291 illustrating the impact of investigated factors on competition outcomes since it is the
292 conjunction region. Fig. 5B illustrates the impact of D on the boundaries of coexistence when
293 D was lower than D_C . Firstly, with the increase of D, the J_s also increased (Eq. 2) and thus the
294 minimum requirement for resources to sustain the biomass. Secondly, the impact of D was
295 marginal at high influent nitrate concentrations (e.g., $>1000 \mu\text{M}$) but significant at low
296 concentrations (Fig. 5B). At high influent concentrations, the upper and lower boundary

297 COD/N ratios were asymptotically approaching the stoichiometric values of C_{DNRA} and C_{DEN} ,
298 respectively. At low influent concentrations, the impact became increasingly profound as D
299 was approaching the critical dilution rate ($D_C = 0.0435 \text{ h}^{-1}$, Fig. 5B). For instance, the
300 boundary COD/N ratios (g COD g N^{-1}) for coexistence were 4.3-6.4 and 7.8-9.6 for a dilution
301 rate of 0.026 and 0.043 h^{-1} (at influent nitrate concentration of $100 \mu\text{M}$, Fig. 5B), respectively.

302 Overall, the results highlight the importance of dilution rate on competition outcome,
303 especially at low influent resource concentration and/or at high dilution rate. The critical
304 dilution rate for coexistence (D_C) enabled to justify the measured competition outcomes by
305 Rehr and Klemme, (1989). In mixed pure cultures of DNRA bacteria (*Citrobacter freundii*)
306 and denitrifiers (*Pseudomonas stutzeri*) competing for nitrate and lactate, stable coexistence
307 was obtained at low dilution rate (0.05 h^{-1}) whereas DNRA bacterium started to be washed
308 out at a dilution rate (0.1 h^{-1}) much lower than its μ_{max} (0.19 h^{-1}) [16]. The results on the
309 impact of dilution rate were in agreement with the observations in environmental enrichments
310 by Kraft et al. [11] where denitrifiers outcompeted DNRA bacteria at lower generation time
311 (thus higher dilution rate) even under nitrate-limiting conditions. Regarding the COD/N range
312 for coexistence, van den Berg et al. [13] suggested that it should be independent of the
313 dilution rate. Apparently, this only holds at high resource concentrations (as used in their
314 study) but not at low resource concentrations (e.g., $< 1000 \mu\text{M}$, Fig. 5B). In a similar
315 competition scenario, Tilman [39] studied the impact of the ratio of two nutrients on the
316 competition outcomes of two algae species and found an apparent curvature of the boundary
317 between stable coexistence and one species dominance at high flow rate (i.e., dilution rate),
318 confirming the impact shown in Fig. 5B.

319 **3.4 Impact of kinetic and stoichiometric parameters on competition outcome**

320 A sensitivity analysis was conducted to investigate the impact of kinetic and stoichiometric
321 parameters (i.e., K_s , μ_{max} , and Y) on the competition outcome (Fig. 6). The default values of
322 these parameters (Table S2) were used for the reference case. These parameters are species-
323 specific and may change between different denitrifiers and DNRA bacteria. The fate of nitrate
324 is therefore subject to the local communities in an (micro-) ecosystem. The parameters for the
325 same bacteria may also be affected by the environmental conditions (e.g., temperature and
326 pH). For instance, the μ_{max} increases with temperature within a certain temperature range.
327 Consequently, the difference between the μ_{max} of DNRA and DEN may also increase due to
328 global warming and thus affect the fate of nitrate. The sensitivity analysis has the power to
329 unravel the trend in response to the variation of the parameters and can thus give insight into
330 their potential impact on the competition outcome.

331 **3.4.1 Affinity constants for the resources**

332 The ratio of the affinity constants of the two species for nitrate/COD was changed in two
333 magnitudes (Fig. 6A). Stable coexistence (i.e., $0 < \text{fraction of DNRA} < 1$) was only possible
334 when the ratio of the affinity constant for nitrate (i.e., $K_{NO_3}^{DNRA} / K_{NO_3}^{DEN}$) was lower than
335 0.43 (Fig. 6A, section S8), indicating that a sufficiently higher affinity of DNRA for nitrate
336 relative to DEN is required. In contrast, the ratio of the affinity constant for COD (i.e.,
337 $K_{COD}^{DNRA} / K_{COD}^{DEN}$) had to be higher than 0.43 for coexistence (Fig. 6A, section S8). This
338 threshold (i.e., 0.43) was determined by the μ_{max} of the two species and the D of the
339 continuous culture (detailed in section S8). Regarding the absolute values of affinity constants,
340 with the simultaneous increase of $K_{NO_3}^{DNRA}$ and $K_{NO_3}^{DEN}$ (at fixed $K_{NO_3}^{DNRA} / K_{NO_3}^{DEN}$ ratio),
341 the pattern changed from DNRA-favored (reference case) to coexistence-favored and further
342 to DEN-favored pattern (Fig. 6B). This implies that the lower the affinity for nitrate of the

343 two competing species, the lower the threshold (minimum COD/N ratio) for DNRA
344 dominance, especially at low nitrate concentration.

345 Affinity for the competing resources is often used to predict competition outcomes [40,
346 41]. The result demonstrated that the species with higher affinity (i.e., lower K_s) for the
347 limiting resources did not necessarily outcompete other species in continuous cultures (e.g.,
348 when $K_{NO_3}^{DNRA} / K_{NO_3}^{DEN} = 0.2$ and $K_{COD}^{DNRA} / K_{COD}^{DEN} = 0.5$, DNRA bacteria would have a
349 higher affinity for both nitrate and COD. Nevertheless, stable coexistence rather than the
350 displacement of DEN was possible (Fig. 6A)). This illustrates that affinity alone was not
351 sufficient to predict the competition outcome in continuous cultures. The μ_{max} and D need to
352 be taken into account as well, as expressed by J_s parameter (Eq. 2) [19, 21, 42].

353 3.4.2 Maximum specific growth rate

354 The difference between the μ_{max} of the two species (i.e., $\Delta\mu_{max} = \mu_{max}^{DEN} - \mu_{max}^{DNRA}$) was used for
355 sensitivity analysis (Fig. 6C). The increase of $\Delta\mu_{max}$ led to no pattern change but a higher
356 threshold for coexistence, whereas the decrease of $\Delta\mu_{max}$ resulted in a gradual shift towards
357 the coexistence-favored pattern. This implies that the bigger the difference between the μ_{max}
358 of the two competing species, the more likely the dominance of denitrification at low resource
359 concentrations would be (i.e., the higher the maximum COD/N ratio for DEN dominance).
360 The constraint for μ_{max} to allow stable coexistence was detailed in section S8.

361 3.4.3 Yield coefficient

362 Regarding the yield coefficient, the C criterion (i.e., the ratio of Y_{NO_3} to Y_{COD} , Eq. 4) of the two
363 competing species was used for sensitivity analysis (Fig. 6D). Results show that it only
364 affected the upper or lower limits. The higher the difference between C_{DNRA} and C_{DEN} (i.e.,
365 higher ΔC), the broader the region for coexistence (Fig. 6D). This was in line with the
366 observations of the two case studies used for theory verification (Table S3). A lower ΔC was
367 measured in case 2 [3] relative to case 1 [13] and thereby a narrowed region for coexistence in

368 case 2. Noteworthy, if C_{DNRA} were lower than C_{DEN} , stable coexistence would no longer be
369 possible [18, 19], which in turn supported the measured higher biomass yield over nitrate of
370 DNRA bacteria than that of denitrifiers [3, 9].

371 **3.4.4 Overall impact of kinetic and stoichiometric parameters**

372 The sensitivity analysis illustrated that kinetic and stoichiometric parameters (i.e., K_s , μ_{max} ,
373 and Y) affected both the possibility and the boundaries of stable coexistence of denitrifiers and
374 DNRA bacteria. In the region for stable coexistence, K_s and μ_{max} of the two competing
375 species had a significant impact on the boundaries and thus the competition outcome mainly
376 at low resources concentrations (e.g., $<100 \mu\text{M}$ nitrate). The yield coefficients (reflected on C_i)
377 could shift the boundaries across all concentration spectra and had a greater impact at high
378 concentrations than at low influent concentrations.

379 **3.5 Dynamic system behaviour**

380 Fig. 7 demonstrates the trajectories to stable coexistence at steady state, with the evolution of
381 the two competing species and two resources in Fig. 7A and the calculated growth rates (μ ,
382 Eq.1&3) in Fig. 7B. In the dynamic system behaviour, four phases could be distinguished
383 based on the limiting resource for the growth of DNRA(Fig. 7B).

384 In phase I, the growth of DNRA was limited by acetate ($\mu_{\text{DNRA}}=\mu_{\text{DNRA,C}}$, Fig. 7B). The
385 concentration of nitrate and acetate in the chemostat decreased with the growth of denitrifiers
386 and DNRA bacteria (Fig. 7A), which in turn resulted in their decreased growth rates (Fig. 7B).
387 By the end of phase I, nitrate concentration reached $J_{\text{NO}_3}^{\text{DNRA}} (< J_{\text{NO}_3}^{\text{DEN}})$, at which the growth
388 rate of denitrifiers (μ_{DEN}) could not balance the loss rate ($\mu_{\text{DEN}}<D$, Fig. 7B) and the biomass
389 concentration of denitrifiers thus decreased (Fig. 7A). In phase II, the growth of DNRA was
390 limited by nitrate ($\mu_{\text{DNRA}}=\mu_{\text{DNRA,N}}$, Fig. 7B) and the low nitrate concentration favored DNRA
391 bacteria, i.e., $\mu_{\text{DNRA}}>\mu_{\text{DEN}}$ (Fig. 7B). Meanwhile, acetate concentration decreased further and
392 reached a point where the growth of DNRA bacteria shifted to become acetate-limited again

393 ($\mu_{\text{DNRA}} = \mu_{\text{DNRA,C}}$, phase III, Fig. 7B). In phase III, μ_{DNRA} started decreasing with decreasing
394 acetate concentration, resulting in a lower nitrate consumption by DNRA bacteria.
395 Consequently, the nitrate concentration gradually recovered to reach $J_{\text{NO}_3}^{\text{DEN}}$. Simultaneously,
396 the acetate concentration further decreased to reach $J_{\text{COD}}^{\text{DNRA}} (> J_{\text{NO}_3}^{\text{DEN}})$ by the end of phase
397 III. From this point, the growth rate of the two competing species became identical and
398 equaled to dilution rate of the chemostat and thereby reached the steady state (phase IV).

399 Noteworthy, both nitrate and acetate were limiting (i.e., dual limitation) in phase
400 III&IV, with DNRA being acetate-limited ($J_{\text{COD}}^{\text{DNRA}} > J_{\text{COD}}^{\text{DEN}}$) and DEN nitrate-limited
401 ($J_{\text{NO}_3}^{\text{DEN}} > J_{\text{NO}_3}^{\text{DNRA}}$). Therefore, coexistence occurred at steady state because each species
402 was limited by the resource for which its rival has the lower subsistence concentration (J_S)
403 and thus competitive advantage, i.e., DNRA by acetate whereas DEN by nitrate. This is in
404 line with the proposed theoretical condition for coexistence [18, 19, 43] and observed
405 competition behavior (i.e., dual-limitation of acetate and nitrate at stable coexistence [13]).

406 **3.6 Model assumptions and their implications**

407 In this study, denitrification and DNRA were assumed to directly compete for nitrate and be
408 carried out by two distinct specialist species. This section discusses the role of nitrite, the
409 potential difference between specialist and dual-pathway species and the complexity of
410 electron donor (organics), and their implications in predicting the competition outcome.

411 Nitrite is the common intermediate and the branching point of the two pathways, and
412 both nitrate and nitrite can be the terminal electron acceptors in DEN and DNRA [1].
413 However, there is still a lack of consensus about the role of nitrite in their competition. Kraft
414 et al. [11] found a shift from DNRA to DEN when nitrate was replaced by nitrite in chemostat
415 enrichment systems with marine sediments and postulated nitrite as a determining factor in
416 the selection of the two pathways, suggesting that denitrifiers have a comparatively higher

417 affinity for nitrite. Yoon et al. [44] showed the ratio of nitrite to nitrate was determinative in
418 pathway selection in a chemostat study with dual-pathway pure culture, with DNRA
419 dominated at higher nitrite/nitrate ratios. In contrast, van den Berg et al. [45] demonstrated
420 that nitrite does not generally control the competition between denitrification and DNRA in
421 chemostat enrichment cultures. In general, if there is nitrite accumulation, there is no need to
422 consider nitrite in the model. If the competition of denitrification and DNRA only lies in the
423 nitrite reduction, then the resource-ratio theory could be easily implemented in the same way.
424 However, the parameters related to nitrite (e.g., K_s and yield) are still largely missing and
425 need further determination. In case where nitrite accumulation is observed, the applicability of
426 the resource-ratio theory would be limited as it would not be suitable to describe DEN and
427 DNRA as one-step reactions.

428 The possible difference between dual-pathway and specialist microorganisms deserves
429 further clarification. In dual-pathway microorganisms, the first step (i.e., nitrate reduction to
430 nitrite) may be catalyzed by the same enzyme, and the competition of the two pathways
431 would thus lie on nitrite. For example, the dual-pathway *Shewanella loihica* PV-4 utilizes
432 NapA and *I. calvum* utilizes NarG for nitrate reduction [17]. This may explain the observed
433 determining effect of nitrite on pathway selection in *Shewanella loihica* PV-4 [44] but not in
434 the enrichment cultures where different bacteria are responsible for denitrification and DNRA
435 [45]. Moreover, the competition between two species could result in the displacement of the
436 rival, whereas competition of two pathways within the same microorganism may depend on
437 the maximum benefit (e.g., maximum energy production or electron transfer) for the
438 microorganism under certain conditions.

439 The results in this study revealed what determined the boundary COD/N ratios of
440 different competition outcomes between heterotrophic DEN and DNRA, using a non-
441 fermentative acetate as an example for electron donors (i.e., organics). However, the nature of

442 organics can be complex and have been shown to affect the competition outcome [11, 16, 46].
443 The presence of fermentative organic carbon (e.g., lactate) may stimulate fermentative
444 bacteria which can directly compete for both nitrate and organic carbon through fermentative
445 DNRA process [47] and/or alter the organic carbon available for denitrifiers and DNRA
446 bacteria [46]. Consequently, a higher influent COD/N ratio may be needed for DNRA
447 dominance [46]. Previous study suggested that the nitrogen conversions in the oxygen
448 minimum zones (OMZs) of the ocean was likely regulated by organic carbon [29]. The
449 composition and concentration of organic carbon can change both spatially and temporally
450 and different organic compounds may have different influence on various microbial processes
451 [29, 48, 49]. More detailed organic geochemical analyses in different ecosystems and
452 incorporation of fermentative bacteria in the DNRA modeling are of interest for future studies.

453 **3.7 Potential further applications of the resource-ratio theory**

454 One commonly accepted theory for interspecies competition for the same substrate is the K/r
455 strategist hypothesis [50, 51]. With the default kinetics currently available, DNRA resembles
456 a K-strategist (species with high substrate affinity and low μ_{max}) and DEN a r-strategist
457 (species with low substrate affinity and high μ_{max}). According to this theory, DNRA would
458 win the competition against DEN when both organisms are subjected to low-nitrate conditions
459 (i.e., high COD/N), which agrees with the prediction of the resource-ratio theory that was
460 used here (Fig. 4) and that are also confirmed experimentally [3, 16]. Nevertheless, the K/r
461 strategist hypothesis only considers one limiting substrate (nitrate or COD), whereas the
462 resource-ratio theory simultaneously takes both limiting substrates (and dilution rate) into
463 account and is thus more comprehensive.

464 In this study, the resource-ratio theory was applied to elucidate the competition
465 between denitrification and DNRA for nitrate and organic carbon. Nevertheless, the
466 conclusions and their implications can be extended to other similar competition scenarios, for

467 instance, the competition between ammonia-oxidizing bacteria (AOB), archaea (AOA) and
468 comammox microorganisms for ammonia and oxygen, and the competition between sulfide-
469 based autotrophic denitrification and DNRA. As demonstrated in this study and previously
470 [52–54], the resource ratio-theory offers mechanistic insights and quantitative prediction of
471 competition outcomes between microorganisms for common resources. Despite its relatively
472 easy implementation and great value, its application in the microbial competition is still rather
473 limited. To ease the application, a decision tree (Fig. S2) and a spreadsheet model
474 (Supplementary Information_2) were created and provided for the generalized scenario where
475 two species exploitatively compete for two essential resources, as is the case for DEN and
476 DNRA.

477 **4. Conclusions**

478 The resource-ratio theory was applied to elucidate the competition between heterotrophic
479 denitrification and DNRA in continuous cultures and verified with experimental results. The
480 results highlight the impact of resource concentrations, dilution rate and microbial kinetic and
481 stoichiometric parameters on the boundary COD/N ratios and thus the competition outcome.
482 The COD/N ratio dictated the competition between the two nitrate partitioning pathways,
483 however, the boundary values changed significantly with influent resource concentrations. At
484 high influent resource concentrations, the stoichiometries (i.e., consumption of COD per
485 nitrate) of the two competing processes was the determining factor of the boundary COD/N
486 ratios, whereas kinetics (i.e., K_S and μ_{max}) was important as well but only at low influent
487 resource concentrations. The dilution rate became significant at low influent resource
488 concentration and/or high values close to the critical ones. At stable coexistence, the growth
489 of DNRA and DEN was limited by COD and nitrate, respectively. The results also provide
490 testable hypotheses concerning the nitrate partitioning at environmentally relevant low nitrate

491 conditions for further research. The conclusions based on the verified resource-ratio theory
492 potentially have broad implications for similar competition scenarios.

493 **Conflict of Interest**

494 The authors declare no conflicts of interest.

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500

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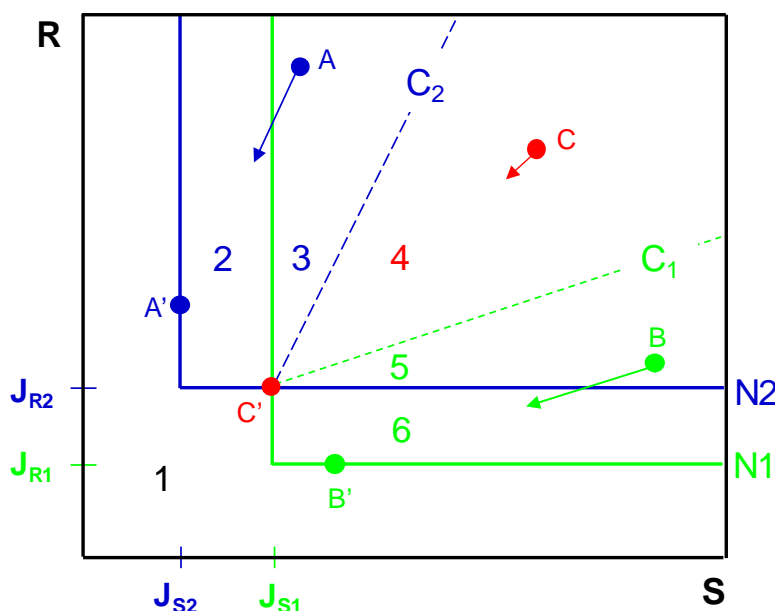
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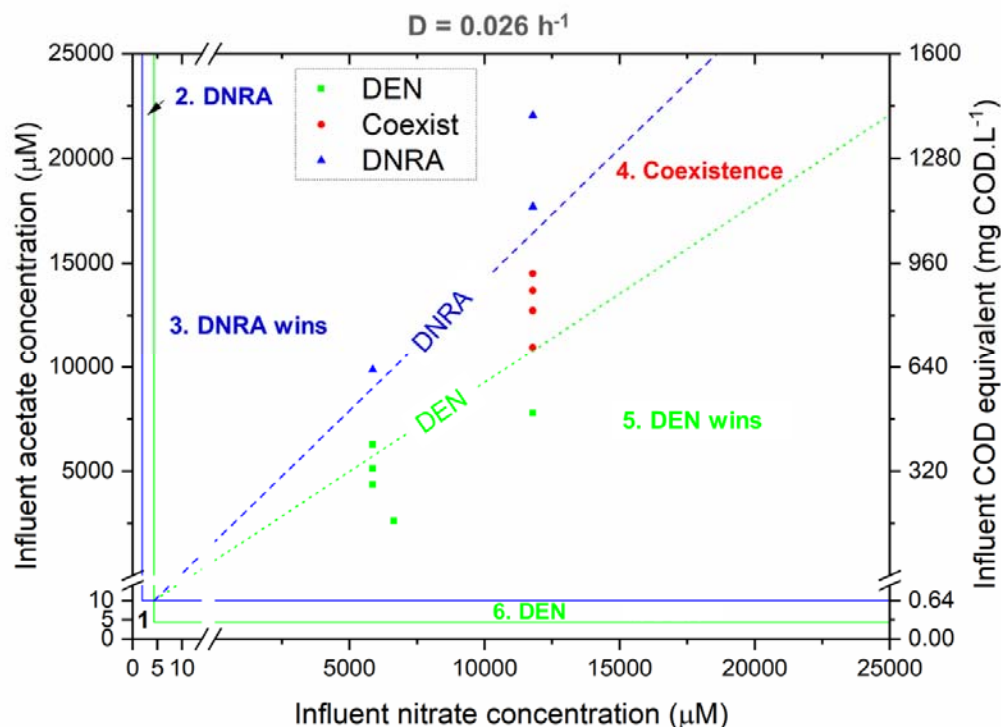


645

646 **Figure 1.** Graphical representation of resource competition of two species (N1 and N2) competing for
 647 two resources (S and R) at a specific dilution rate. The solid lines labeled N1 and N2 are the Zero Net
 648 Growth Isoclines (ZNGIs) for the two species. The dashed lines are the consumption vectors for the
 649 two species, with the slope of C1 and C2, respectively. The competition outcomes can be predicted
 650 from the supply point (defined by the supplied concentration of resource S and R in this S-R plane,
 651 e.g., points A, B and C). Region 1, no species can survive; Region 2, only species N2 can survive;
 652 Region 3, species N2 outcompetes species N1, dynamic behavior (trajectory) governed by slope C₂;
 653 Region 4, the two species stably coexist; Region 5, species N1 outcompetes species N2, dynamic
 654 behavior (trajectory) governed by slope C₁; Region 6, only species N1 can survive. The equilibrium
 655 points always fall on the ZNGIs. Points A', B' and C' represent the corresponding equilibrium points
 656 of supply points A, B and C. Line A-A' has the same slope as C₂, whereas line B-B' has the same
 657 slope of C₁. All supply points in region 4 would reach the same equilibrium point C'.

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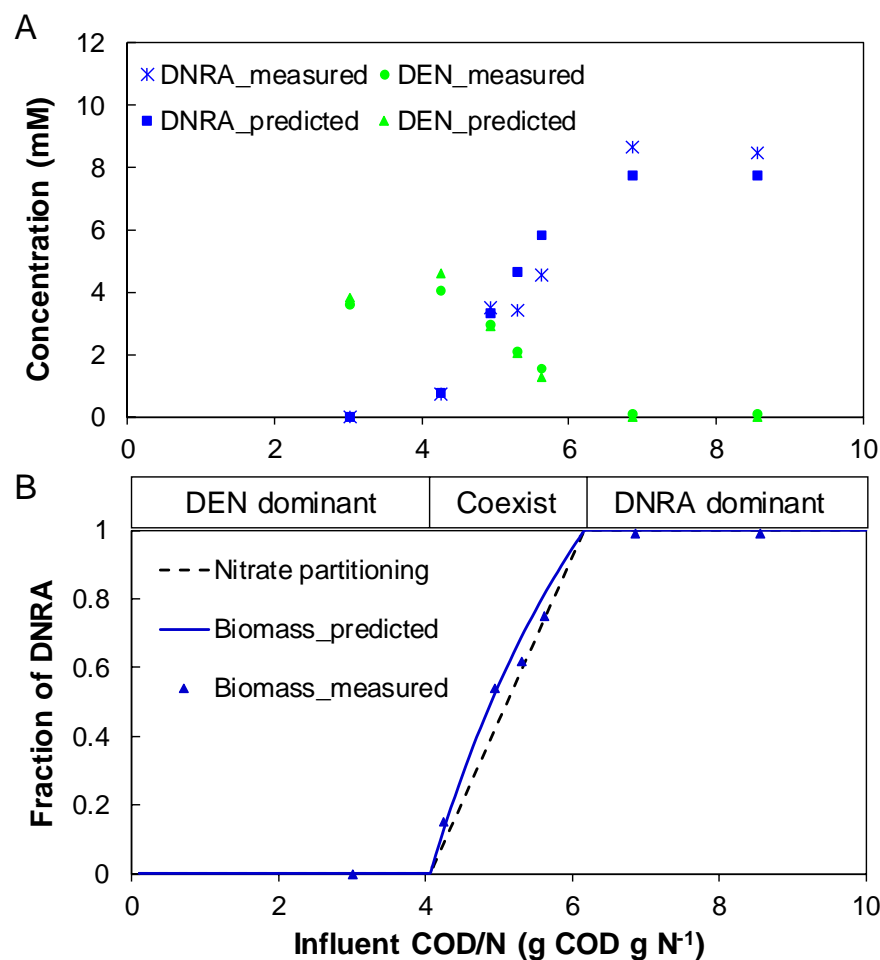


660

661 **Figure 2.** Predicted and observed outcomes of competition for nitrate and organic carbon by
662 heterotrophic denitrifiers and DNRA bacteria in continuous cultures at a dilution rate of 0.026 h^{-1} .
663 Experiments [3, 13] for which DEN was dominant are shown with green squares; those for which
664 DNRA was dominant are shown with blue triangles, and those for which coexistence was observed are
665 shown with red dots. The borders and the meaning of the operating zones distinguished by the
666 resource-ratio theory are detailed in Figure 1. The consumption vectors (broken lines) have a slope of
667 $C_{\text{DEN}} (4.04 \text{ g COD g N}^{-1})$ for denitrifiers and $C_{\text{DNRA}} (6.15 \text{ g COD g N}^{-1})$ for DNRA bacteria.

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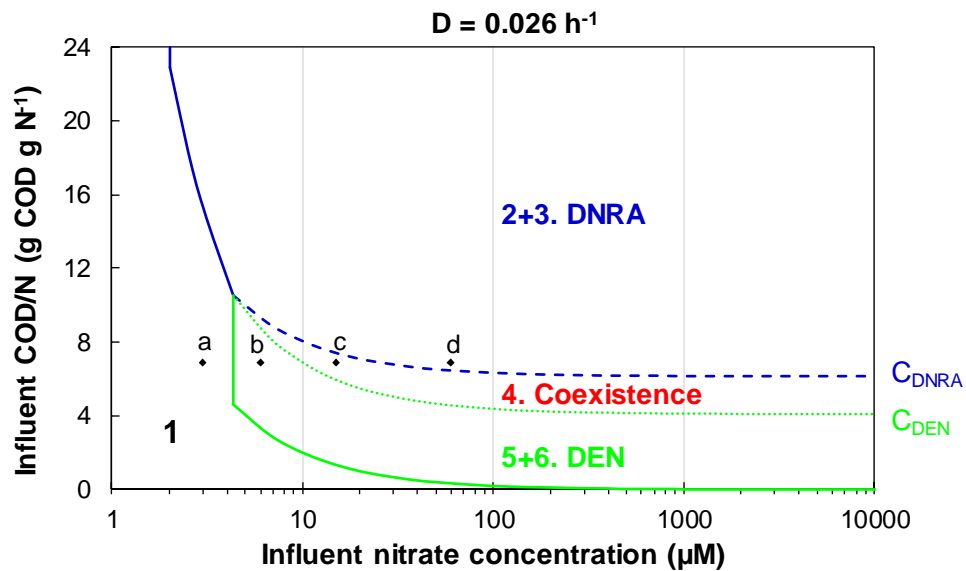


670

671 **Figure 3.** Prediction versus measurement at steady state (case study 1 [13]): (A) concentrations of
672 heterotrophic denitrifiers and DNRA bacteria; (B) relative abundance of DNRA bacteria (to the total
673 of denitrifiers and DNRA bacteria) and contribution of DNRA in nitrate partitioning at different
674 influent COD/N ratios (at influent nitrate concentration of 1000 μM). The black triangles represent the
675 measured DNRA biomass fraction (at influent nitrate concentration of 11790 μM [13])

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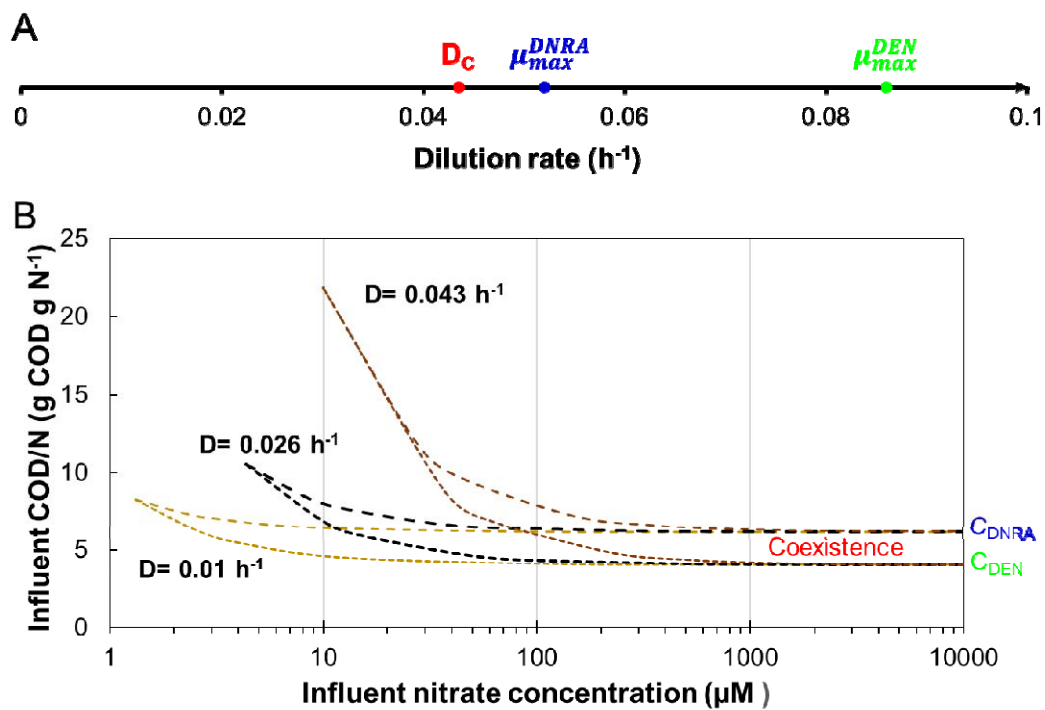


678

679 **Figure 4.** The boundary influent COD/N ratios at different influent nitrate concentrations. The regions
680 correspond to the regions with the same numbers in Fig. 2. Points a, b, c, and d are supply points with
681 the same COD/N ratio but different nitrate concentrations (detailed in Fig. S5).

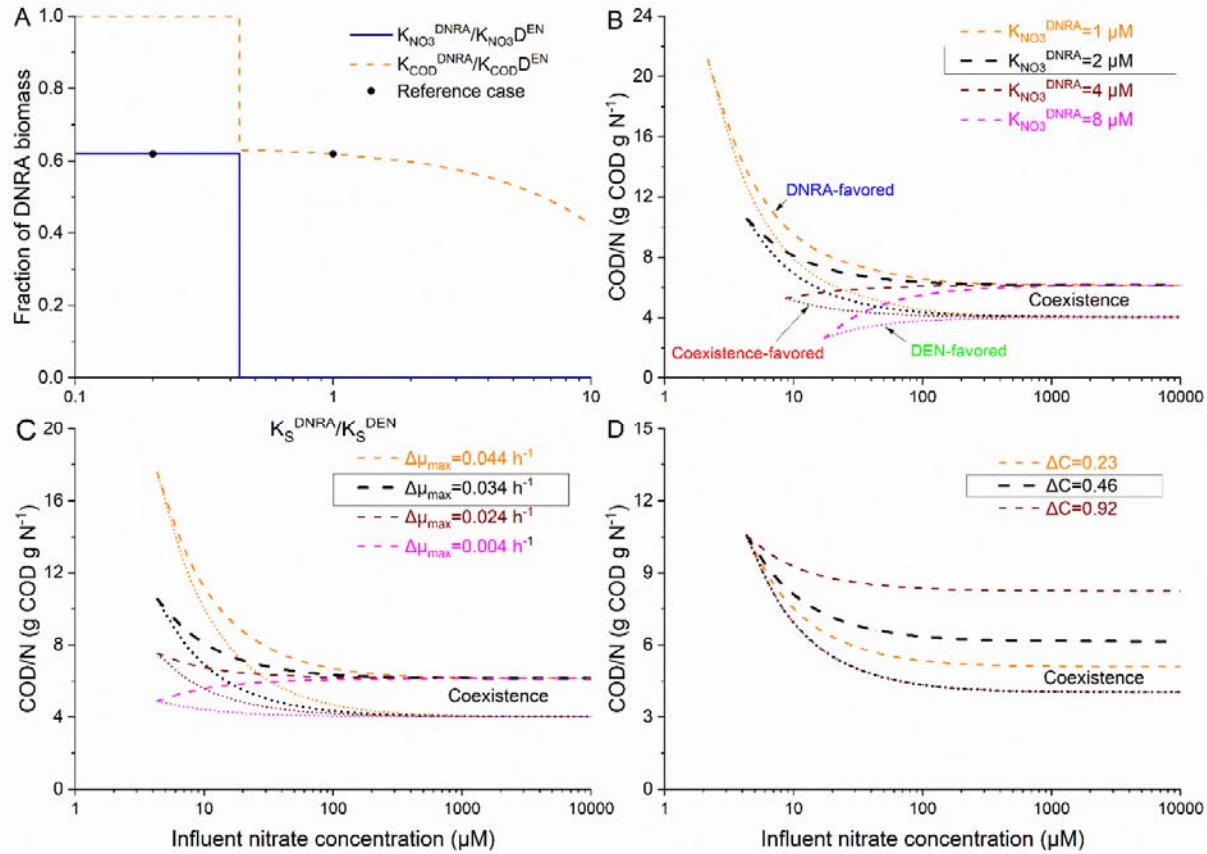
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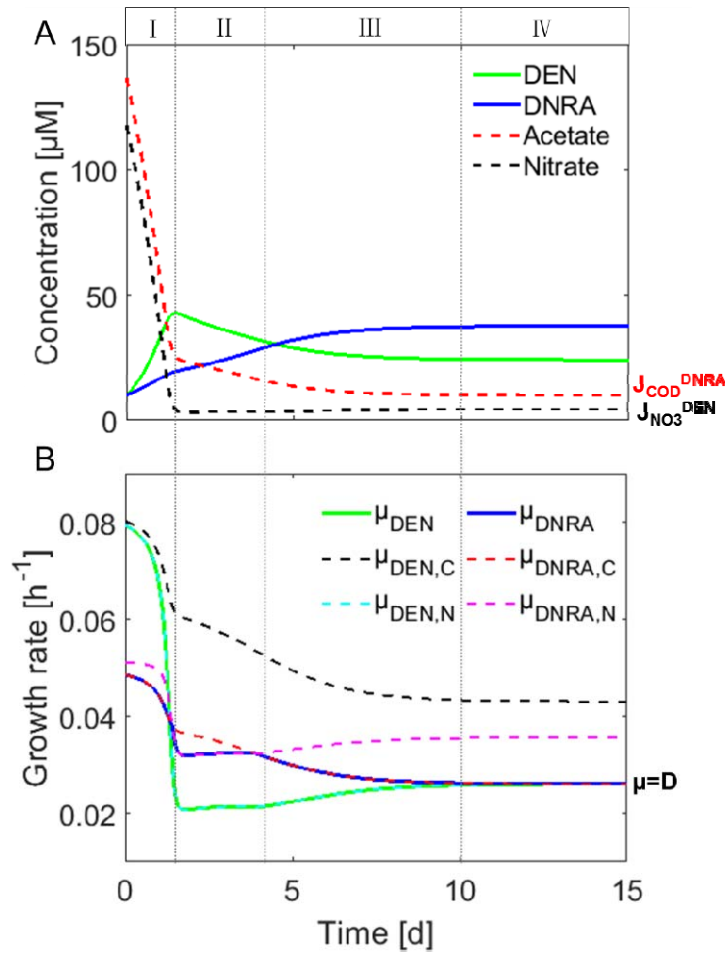
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685 **Figure 5.** Impact of dilution rate on (A) possible competition outcomes; (B) the boundaries of
686 coexistence.



687

688 **Figure 6.** Impact of kinetic and stoichiometric parameters on the boundaries for coexistence: (A) the ratio of the affinity constants of the two species for the
 689 same resource (conditions: influent COD/N=5.3 with 1000 μM nitrate and fixed affinity constants for denitrifiers); (B) affinity for nitrate, expressed as
 690 $K_{NO_3}^{DNRA}$, with fixed $K_{NO_3}^{DNRA}/K_{NO_3}^{DEN}$; (C) maximum growth rate, expressed as $\Delta\mu$ (i.e., $\mu_{max}^{DEN} - \mu_{max}^{DNRA}$) and (D) yield coefficient, expressed as ΔC (i.e.,
 691 $C_{DNRA} - C_{DEN}$). The values in the box were default values at the reference case.



692

693 **Figure 7.** Trajectories of: (A) resources and species concentrations; (B) calculated growth rate in a
694 chemostat fed with acetate and nitrate at a COD/N ratio of 5.3, under which stable coexistence of DEN
695 and DNRA was observed [13] and predicted (this study). The DEN and DNRA species were initially
696 equally presented in a chemostat.

697

698

699 **Table 1.** Typical nitrate concentrations in several ecosystems

Ecosystems	NO₃⁻ (μM)	Source
Seawater	< 30	[29]
Groundwater	< 806	[30]
Surface water	< 161	[31]
Marine sediments	3.7-17.8	[32]
Terrestrial ecosystems ^a	0.01-4.96 ^b	[4]
WWTPs	< 4200 ^c	[33]

a: Forest, grassland, riparian;

b: in μM /g soil

c: assuming all the influent ammonium is converted to nitrate for a medium strength municipal wastewater

700