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Emna Krichen, Alain Rapaport, Emilie Le Floc'H, Eric Fouilland

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E. Krichen[1,2], A. Rapaport[2], E. Le Floc'h[1], E. Fouilland[1*]

[1] UMR MARBEC, Univ. Montpellier, CNRS, IFREMER, IRD, Sète, France
[2] UMR MISTEA, Univ. Montpellier, INRA, SupAgro, Montpellier, France
* Corresponding author: eric.fouilland@cnrs.fr

Abstract

Positive interactions such as facilitation play an important role during the biological colonization and species succession in harsh or changing environments. However, the direct evidence of such ecological interaction in microbial communities remains rare. Using common freshwater microalgae isolated from a High Rate Algal Pond (HRAP) treating wastewaters, we investigated with both experimental and modelling approaches the direct facilitation between two algal strains during the colonization phase. Our results demonstrate that the first colonization by microalgae under a severe chemical condition arose from the rapid growth of pioneer species such as *Chlorella sorokiniana*, which facilitated the subsequent colonization of low growth specialists such as *Scenedesmus pectinatus*. The pioneer species rapidly depleted the total available ammonia nitrogen favouring the specialist species initially inhibited by free ammonia toxicity. This latter species ultimately dominated the algal community in accordance with the competitive exclusion under low nutrient conditions. We show that microbial successions are not only regulated by climatic conditions but also by interactions between species based on the ability to indirectly modify their growth conditions. Furthermore, the theoretical study of algal resilience and succession times as proxies of the facilitation efficiency showed that control of algal production processes might be possible by modifying the initial populations' densities.

Introduction

One of the major challenges in microbial ecology is to understand the dynamics of communities of interacting species. Understanding the biological interactions and the time scales over which they occur is necessary to interpret the results of the directional succession process of communities' development in the natural environment and in artificial ecosystems. In aquatic systems, microalgae are present in natural waters such as oceans, lakes, rivers, and ponds and play a prominent role in the marine and fresh-water ecosystems where they drive major ecosystem processes. Strong similarities exist between marine and freshwater phytoplankton ecology[1] when they face similar change in growth conditions leading to temporal species succession. Abiotic forcing and biotic interactions can both result in successional trends in phytoplankton. The scientific discussion around the phytoplankton growth periodicity and succession has been dominated by the role of the environmental drivers including global climatic change (e.g. light, temperature, wind)[2, 3, 4, 5, 6], local hydrological variations[7, 8] biological disturbances such as species invasion[9], and chemical effects such as toxic pollutants, nutrient enrichment, or change in pH[7, 10, 11, 6, 12]. On the other hand, the conditions governing phytoplankton growth over the seasonal change in plankton communities have mostly been discussed in the context of algae-grazer interactions (e.g. Porter, 1977)[13] or exploitative competition (e.g. Tilman, 1982)[14]. However, few studies have discussed the role of positive interactions (i.e. facilitation), which is the main driving biotic mechanism in plant community succession, particularly

under harsh environmental conditions[15]. The ability to colonize a specific habitat usually explains the dominance and succession of some species under the change in nutrient and/or mixing conditions (see[16, 17, 18]). In marine ecosystems, small-cell diatoms usually grow rapidly in a first stage after a strong nutrient enrichment because of their higher growth rates and are then followed by larger-cell diatoms and dinoflagellates, which are more likely to occur when nutrients are depleted[16, 17]. The seasonal patterns of succession in freshwater ecosystems might be explained by the first occurrence of invasive small-sized stress-tolerant competitor species followed by disturbance-tolerance ruderal species[19].

Because phytoplankton can substantially change its physical and chemical conditions of growth (e.g. by decreasing transparency, increasing pH, or depleting key nutrients), we suggest that this phytoplankton-driven environmental modification can provoke shifts in assemblages of species, thus leading to successions. Hence, we suppose that under highly polluted conditions, similar to strongly anthropized ecosystems, an assemblage of typical pioneer species will first develop because of their potential for rapid dispersal and growth. Species showing the fastest growth rates and the strongest stress tolerance to harsh environments will be able to grow under such conditions, making the ecosystem more favourable for species that are more competitive in stable growth condition through ecological facilitation.

Previous results from a study investigating the biological succession within HRAPs used for wastewater treatment showed a rapid and initial growth of *Chlorella sp*. followed by a low-growth of species *Scenedesmus sp*.[20]. Similar successions have been observed in other studies using HRAPs as well[21, 22]. The successional trends of typical microalgal species growing in HRAPs have generally been interpreted as responses to predation and/or seasonal factors[23, 21]. We hypothesized that during the colonization phase of HRAPs supplemented with wastewaters, *Chlorella* is able to modify its habitat and facilitate growth conditions for *Scenedesmus* by reducing the nutrient stress modulated by free ammonia toxicity[24]. Therefore, we suggest that microbial successions might not be regulated by climatic conditions only, but also through positive interactions between species facing external chemical stress. We conducted a set of laboratory experiments using the species *Chlorella sorokiniana* and *Scenedesmus pectinatus*, isolated from the HRAP[20], to determine the inhibiting factor among ammonium ion, pH, and free ammonia and to determine their respective effects on the growth rates of each species. Then we used a modelling approach to test the magnitude of facilitation/competition on the two microalgae and, further, to explain the observed patterns in HRAPs continuously supplemented with wastewaters. Finally, we studied the resilience and succession times providing informative proxies on the efficiency of the ecological facilitation and the successional trends depending on the initial populations' densities.

Our conclusions supported the theoretical considerations of ecological facilitation between one tolerant and one sensitive organism to a gradient of resource toxicity/bioavailability.

Results and Discussion

Three series of experiments denoted $SE1$, $SE2$, and $SE3$ were performed i) to isolate the inhibitory effects of possible external factors such as high nitrogen concentrations or pH and ii) to demonstrate a facilitation interaction between two species. We then show how to exploit the data using a mathematical modelling approach, providing new insights on the facilitation phenomenon.

No direct toxic effect of high ammonium and pH on microalgae growth rates. Chemical factors such as Total Ammonia Nitrogen (TAN) and pH can affect the rate and efficiency of photosynthesis of microalgae[25, 26, 27]. Negative effects of TAN (referring to nitrogen in two distinct forms: ammonium

ion NH_4^+ and free ammonia NH_3) on algal growth and physiology might occur and vary significantly within classes of microalgae and within species (see[28]). The photosynthesis of different species of marine diatoms was severely inhibited at TAN concentrations in the range of 0.5 to 11 $\mathrm{mgN.L^{-1}}$ [29, 30]. At low pH values (<8), toxicity is likely associated with NH_4^+, while at alkaline pH values (>8), cell growth inhibition is rather due to NH_3.

During the first set of experiments ($SE1$), the potential toxicity of high ammonium ion NH_4^+ was investigated for the two isolated algal species when pH values were adjusted to 7.5 at 25°C. Hence, under such conditions, we ensure that 98% of TAN (ranging from 10 to 110 $\mathrm{mgN.L^{-1}}$) was present as NH_4^+ form. Under this range of concentrations, no significant difference in the growth rates of *Scenedesmus pectinatus* or *Chlorella sorokiniana* was measured (p>0.05, ANOVA from ANOCOVA test results; see Figure 1a). Therefore, the NH_4^+ form at such concentrations, typically found in wastewaters, did not affect the growth rates of both microalgae. Similarly, it was reported that species such as *Chlorella* are very tolerant to high TAN concentrations (max. 140 and 250 $\mathrm{mgN.L^{-1}}$ stated respectively in Collos and Harrison (2014)[28] and Tam and Wong (1996)[31]). However, Przytocka-Jusiak et al. (1977)[32] reported that cell division of $C.vulgaris$ was inhibited at greater TAN concentrations (> 300 $\mathrm{mgN.L^{-1}}$), although such concentrations did not cause any visible lethal effect. The studies performed on *Scenedesmus accuminatus* showed that cell growth was inhibited only when NH_4^+ concentrations were higher than 200 $\mathrm{mg.L^{-1}}$[33]. Interestingly, it has been previously reported that algal photosynthesis of $Scenedesmus\ obliquus$ was inhibited at TAN above 28 $\mathrm{mgN.L^{-1}}$ if the culture pH exceeded 8.0[25].

Because pH can vary during algal growth in ecosystems due to the rapid and large CO_2 consumption of microalgae, this might directly or indirectly affect algal growth rates. The optimal pH of many freshwater algae is about 8[34]. The growth of many algal species is inhibited in waters at pH greater than 8 (reduction of productivity of *Chaetoceros* sp. and *Chlorella* sp. by 22% when pH was raised from 8 to 9), while other species can grow well above pH 8 (e.g. *Amphora* sp. and *Ankistrodesmus* sp. at pH 9 and 10, respectively)[35]. High pH conditions limit the availability of $\mathrm{CO_2}$ while HCO_3^- dominates, and then algae cannot efficiently accumulate carbon and require a high supply of carbonates for maintaining photosynthetic activity[36] or reducing the affinity to free $\mathrm{CO_2}$[37, 38]. During the second set of experiments ($SE2$), the direct effect of pH was tested using pH values ranging from 6 to 9 on algal growth under low initial TAN concentration of about 1 $\mathrm{mgN.L^{-1}}$. As shown in Figure 1b, the growth rates measured for both species, *S. pectinatus* and *C. sorokiniana*, were not significantly different for all the tested pH conditions (p>0.05, ANOVA from ANOCOVA test results). Then, similar to high values of NH_4^+, the results did not support the hypothesis of a negative effect of high pH values on microalgae growth rates. Thus, we assume that our results are consistent with the early study of Azov and Goldman (1982)[37] suggesting that pH did not play a role in the magnitude of inhibition but in the degree of dissociation of nontoxic ammonium to toxic free ammonia NH_3. In other words, the dissociation of TAN as a function of pH is the main determinant of how much NH_3 is available to inhibit photosynthesis. Hence, assuming that NH_3 concentrations in $SE1$ and $SE2$ were likely too low to exhibit algal growth inhibition, the effect of free ammonia at higher concentrations was then tested on both species.

Evidence of species-dependent ammonia effect. NH_3 is considered the TAN's most toxic form for aquatic organisms[39]. The third set of experiments ($SE3$) was then performed on our algal isolates from the HRAP to test algal growth under NH_3 concentrations ranging from 0.56 to 29.42$\mathrm{mgN.L^{-1}}$. The results for the growth rates of both isolates are represented in Figure 1c and showed that the growth rates of *C. sorokiniana* measured under the different unionized ammonia concentrations were similar (p>0.05, ANOVA from ANOCOVA test results). However, the growth rates of *S. pectinatus* were

significantly different (p<0.05, ANOVA from ANOCOVA test results) with an important reduction in growth rates when NH_3 exceeded 8.7 mg.L^{-1}. Similarly, early works reported that free ammonia at concentrations greater than 15 mgN.L^{-1} and at pH values over 8 inhibited the photosynthesis and growth of *Scenedesmus obliquus*[24, 37]. A strong inhibition of *Chlorella sorokiniana* was previously observed at about 77 mgN.L^{-1} of free ammonia[40], although the resistance of *Chlorella sorokiniana* to high NH_3 concentrations (326 mg.L^{-1}) was also previously reported[41], suggesting that species can also adapt their metabolism and becoming more tolerant to high NH_3 environments over time.
In HRAPs initially supplemented with high TAN concentrations, NH_3 toxicity is therefore expected to be associated with elevated pH due to intense photosynthetic activity and could cause the depletion of microalgae culture or promote replacement with other tolerant species to face the prevailing stress. This feature should be magnified considerably during the summer as the conversion between NH_4^+ and NH_3 is also temperature dependent.

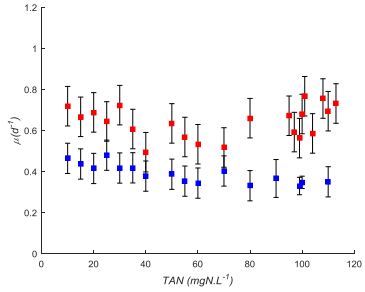
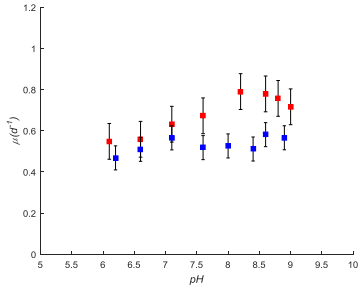
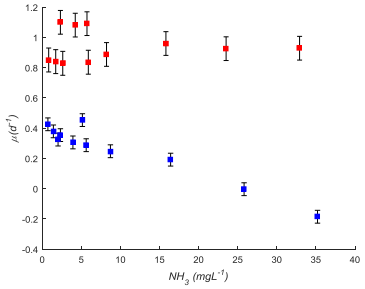
(a) Effect of ammonium concentration	(b) Effect of pH	(C) Effect of free ammonia concentration
		

Figure 1. Growth rates from multiple comparisons Matlab tests on growth rate values estimated based on ANCOVA analyses for $C.\ sorokiniana$ (in red) and $S.\ pectinatus$ (in blue) at different initial conditions of TAN concentrations (a), pH (b), and NH_3concentrations (c).

Evidence of facilitation interactions through a modelling approach. A modelling approach was used to identify the growth characteristics for each species in order to predict their dynamics when they are growing together. We assumed that TAN was the sole limiting substrate driving the algal growth and NH_3 would have a direct inhibitory effect on cell growth as a function of $TAN, pH,$ and temperature T(NH_3-N $= f(TAN, pH, T)$)[42]. Thus, as a first step, species growth rates were related to external TAN concentrations to calibrate one kinetic model, which could represent satisfactorily most of the data points of the previous test experiments obtained in $SE2$ and $SE3$. The proposed model was inspired from Aiba-Edward's model[43] describing the substrate inhibition at high concentrations and consisting of a modified version of the Monod equation, but here it has a slightly different mathematical expression as explained below. While Monod kinetics assumed that only one nutrient limits the growth of cells, the model we propose here includes that a by-product of this limiting nutrient (free ammonia NH_3-N) negatively affects cells growth as given by the following expression:

$$\mu(TAN, pH, T) = \mu_{max} \frac{TAN}{TAN+k} e^{-\frac{f(TAN,pH,T)}{ki}} \quad (*)$$

where μ_{max} is the maximum growth rate (d^{-1}), k is the affinity to substrate (mgL^{-1}), and k_i is the inhibition constant of free ammonia nitrogen (mgL^{-1}). This growth function provided a good fit to experimental data describing the growth kinetics of the two species (see Figure 2). The identified kinetic parameters are given in Table 1. The species *S. pectinatus* showed a strong affinity for nitrogen with a greater $\mu_{max}/_k$ ratio than that obtained for *C. sorokiniana*. In contrast, this latter species has a maximum growth rate (1.04 d^{-1}) much higher than *S. pectinatus* (0.58 d^{-1}). Consequently, *C.*

sorokiniana would grow well at high nitrogen concentrations and would also tolerate high NH_3 concentrations as reported by its highest inhibition constant (k_i =130.25 $mgNH_3\text{-}N.L^{-1}$), while *S. pectinatus* would grow best at low nitrogen concentrations but would show a much faster decline in growth because of its high sensitivity to ammonia toxicity represented by a low k_i (2.31 $mgNH_3\text{-}N.L^{-1}$). Our results are in accordance with older chemostat experiments comparing *Scenedesmus acutus* and *Chlorella minutissima* under P-limited growth[44].

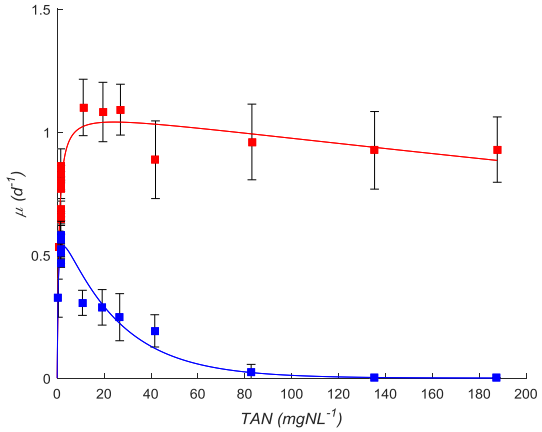


Figure 2. $SE2$ and $SE3$ data sets (full points) compared to the Kinetic model for *C. sorokiniana* (continuous red line) and for *S. pectinatus* (continuous blue line). Data points are the mean of 3 duplicate measurements of growth rate µ.

Table 1: Calibration results on $SE2$ and $SE3$ growth data obtained in batch cultures.

Parameters	***C. sorokiniana***	***S. pectinatus***
μ_{max} (d^{-1})	1.04	0.59
k ($mgN.L^{-1}$)	0.59	0.15
ki ($mgNH_3\text{-}N.L^{-1}$)	130.2	2.313
$\mu_{max}/_k$	1.77	3.93
J (least squares criterion)	0.81	0.29

The ecological succession of species presenting Monod- and Haldane-kind growth functions have already been shown theoretically[45] but not yet experimentally. The Monod and Haldane kinetics were fitted to our data (results not shown). Their graphs closely resemble those given by (*), but with a higher criterion *J*.

Knowing the growth performances of each species in the laboratory (Figure 3a), we proposed a predictive model to explore how the assemblage of the two species might react under a fixed pH (8.6) and temperature (25°C) in continuous culture to check if the hypothesis of ecological facilitation is verified. We used the initial conditions of substrate and biomass and the operational conditions (dilution rate and input substrate concentration) encountered in the previous HRAP study[20]. Our results demonstrated that *S. pectinatus* could not be maintained and is washed out because of the NH_3 toxicity when cultivated alone (Figure 3b). The mathematical simulations predicted that when both microalgae are introduced together, *C. sorokiniana* grows rapidly first while the growth of *S. pectinatus* is inhibited because of high NH_3. The rapid consumption of nitrogen by *C. sorkiniana*

induced low NH_3 and less nitrogen availability, favouring the growth of *Scenedesmus sp.* but not *C. sorokiniana* (Figure 3c).

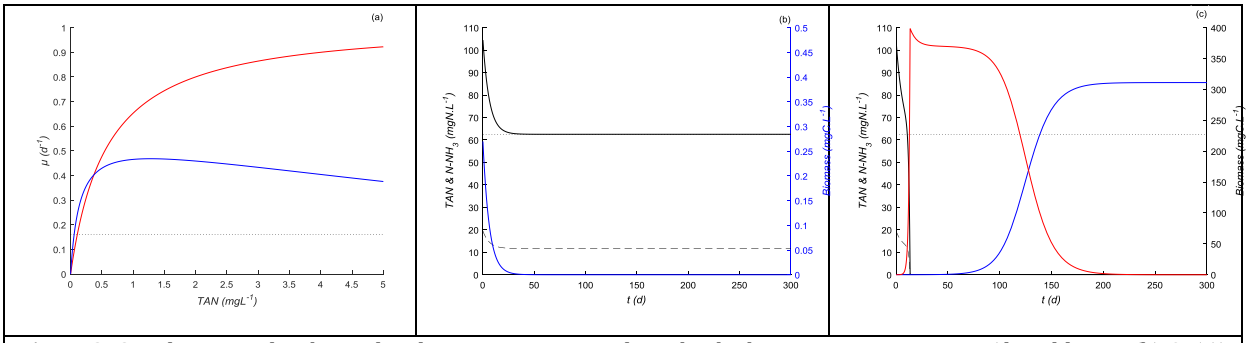


Figure 3. Simulation results obtained under a continuous supply with a high nitrogen concentration (dotted line in (b) & (c)) at a fixed dilution rate (dotted line in (a)). The biomass dynamics of *C. sorokiniana* and *S. pectinatus* are represented in solid red lines and solid blue lines, respectively. The substrate dynamics of TAN is represented with a solid black line while the toxic form (NH_3) is represented with black dashed lines.
(a) growth functions identified in Figure 2,
(b) *S. pectinatus* cultivated alone,
(c) *S. pectinatus* growing with *C. Sorokiniana.*

Validation of facilitation interactions in HRAP. The dynamics of the biomass of *Chlorella sp*. and *Scenedesmus sp*. and the TAN concentrations measured in HRAP operating from 28 April 2015 to 8 September 2015 in Northern France[20] were compared to model simulations (see Figure 4) using the growth functions represented in Figure 2 and Table 1. Some few changes to the previously simulated model were done to take into account the complexity of the biological processes due to the presence of other consumers (e.g. heterotrophic bacteria taking up nitrogen resource) and predators (reducing algal biomass). Indeed, we have added mortality terms to account for the grazing effect on each algal species in an indirect way, keeping the model as simple as possible. Knowing that bacteria were also growing in the pond and consuming nitrogen[20], we supposed that yield coefficients ranged between i) minimal values theoretically calculated during the period of type peak of biomass reached by each species in the HRAP (i.e. 1.3±0.1 gC/gN and 0.5±0.2 gC/gN for *Chlorella sp.* and *Scenedesmus sp.*, respectively) and ii) maximal values identified in our laboratory chemostat experiments at 3 days steady state (i.e. 5.93±0.66 gC/gN and 4.98±0.58 gC/gN for *C. sorokiniana* and *S. pectinatus*, respectively). The estimated parameters of yield and mortality coefficients obtained from the comparison of the model dynamics with data from HRAP are represented in Table 2. The identification results appear to be realistic. Assuming that higher predation pressure corresponds to a higher mortality coefficient, our results suggest that *Chlorella* was likely more sensitive to grazing than *Scenedesmus* known to produce grazer-morphological defense[46]. However, the washout of *Chlorella* at the system steady state is not due to high pressure by a high mortality coefficient but rather to competition with *Scenedesmus* when the environment is depleted of nitrogen resources, as demonstrated through the previous simulation results (see Figure 3c). We noted that the estimated yields coefficients in chemostat experiments were higher than the identified ones in the HRAP which may be explained by the presence of denitrifying bacteria in the HRAP producing N_2 subsequently lost through degassings[20].

The present results confirmed the initial hypothesis that the colonization of hypertrophic ecosystems by pioneer species such as *Chlorella* is a prerequisite for the development of other organisms. *Chlorella* species are usually considered as an invasive phytoplankton or pioneer species because they maintain fast growth rates and assimilate resources with short generation times, and they are able to dominate over slower-growing species[47, 48]. We show here that such pioneer organisms can modify their chemical environment by reducing ammonia toxicity, and then the growth of the sensitive microalgae *Scenedesmus sp.* becomes more efficient than expected in monoculture. At low and nontoxic nutrient levels, *Scenedesmus* is considered an affinity specialist[44], and it is able to dominate in HRAP over *Chlorella* and colonize the HRAP.

There are different advantages to having a *Scenedesmus* dominance in an HRAP supplemented with wastewaters, as this species possesses a high affinity to nitrogen, is strongly resilient to predators, and is readily settleable when the harvested biomass is used for different purposes (e.g. lipid production[49]). Using the mathematical approach, we investigated the influence of the initial density of both species on the time requested for rapid development of *Scenedesmus* in HRAP that might be useful to further explore the optimization strategies under external stress.

Table 2. Calibration results on HRAP data (continuous cultures).

Parameters	***C. sorokiniana*** **(A_1)**	***S. pectinatus*** **(A_2)**
y(gC/gN)	**3.91**	**0.32**
m (d^{-1})	**0.504**	**0.004**
J (least squares criterion)	**102.38**	

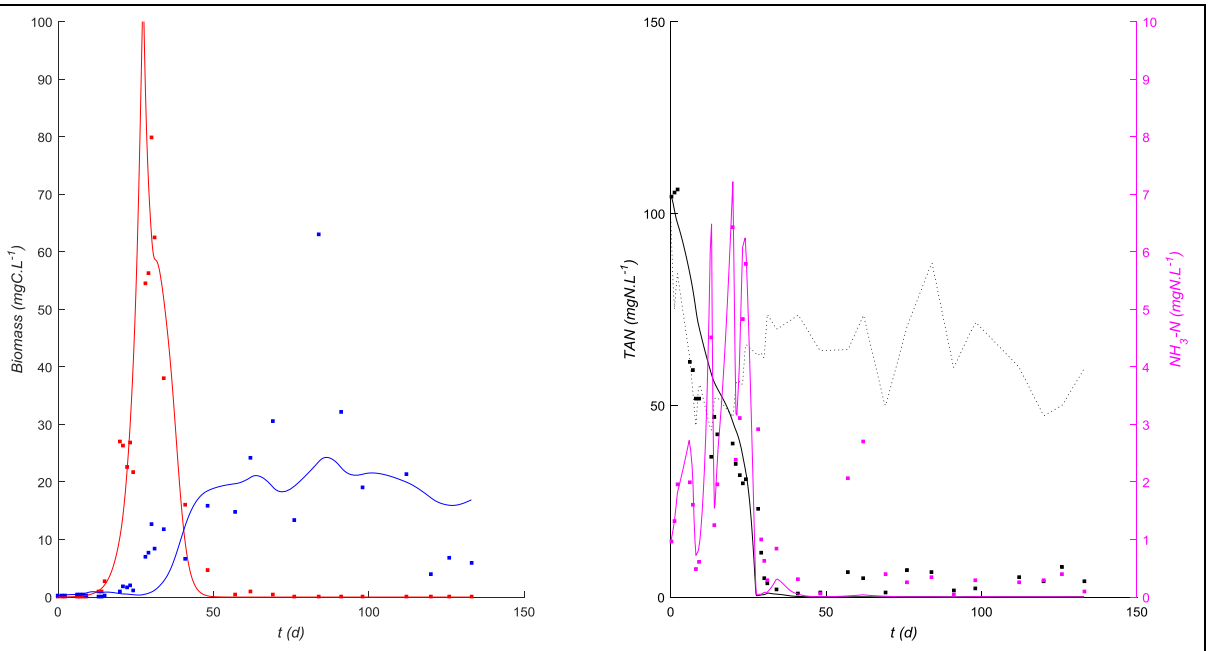


Figure 4. HRAP data points compared to the model prediction (continuous lines). Algal biomasses of *Chlorella sp.* (in red) and *Scenedesmus sp.* (in blue) are grown under a fluctuating continuous supply of nitrogen (dotted black line). The growth of the two microalgae is limited by nitrogen concentration in the pond (in black) and sensitive to free ammonia (in magenta).

Influence of initial biomass values on resilience and succession time. We defined the resilience time as the duration for *Scenedesmus* to reach its initial biomass value under the presence of the toxic NH_3 concentrations. Because the succession of the two microalgae is required for maintaining *Scenedesmus*, we also defined the succession time as the duration for which the species have the same density level for the second time owing to the predominance of *Scenedesmus*. The iso-values diagrams of the resilience time and the succession time are presented in Figure 5 and are obtained for different initial microalgae biomass conditions. The resilience time is more affected by the initial concentrations of *Chlorella* than by the initial biomass concentrations of *Scenedesmus* Figure 5a. This contrasts with the succession time that is more sensitive to initial *Scenedesmus* biomass, especially when the *Chlorella* biomass is initiated at values ≥1 $mgC.L^{-1}$. Such simulation would be of interest in further control of the diversity within a HRAP supplemented with wastewaters, especially for managing the periods of dysfunction (e.g. sudden algal crash, variations in wastewaters inflow). For example, it

could be suggested to increase the initial concentration of *Chlorella* (at a concentration higher than 5 mg L^{-1}) through bioaugmentation to ensure a rapid reduction of NH_3 toxicity and rapid development of *Scenedesmus* in a minimum of 16 days. On the other hand, the time needed for *Scenedesmus* predominance over *Chlorella* will depend on the initial concentration of *Scenedesmus* and the higher the *Scenedesmus* initial concentration would be (>12 mg L^{-1}) the faster the succession would occur (minimum of 60 days). Consequently, we evidence that the algal resilience and succession times within an intensive algal ecosystem are strongly depending on the initial populations' densities that may be used to control algal production processes.

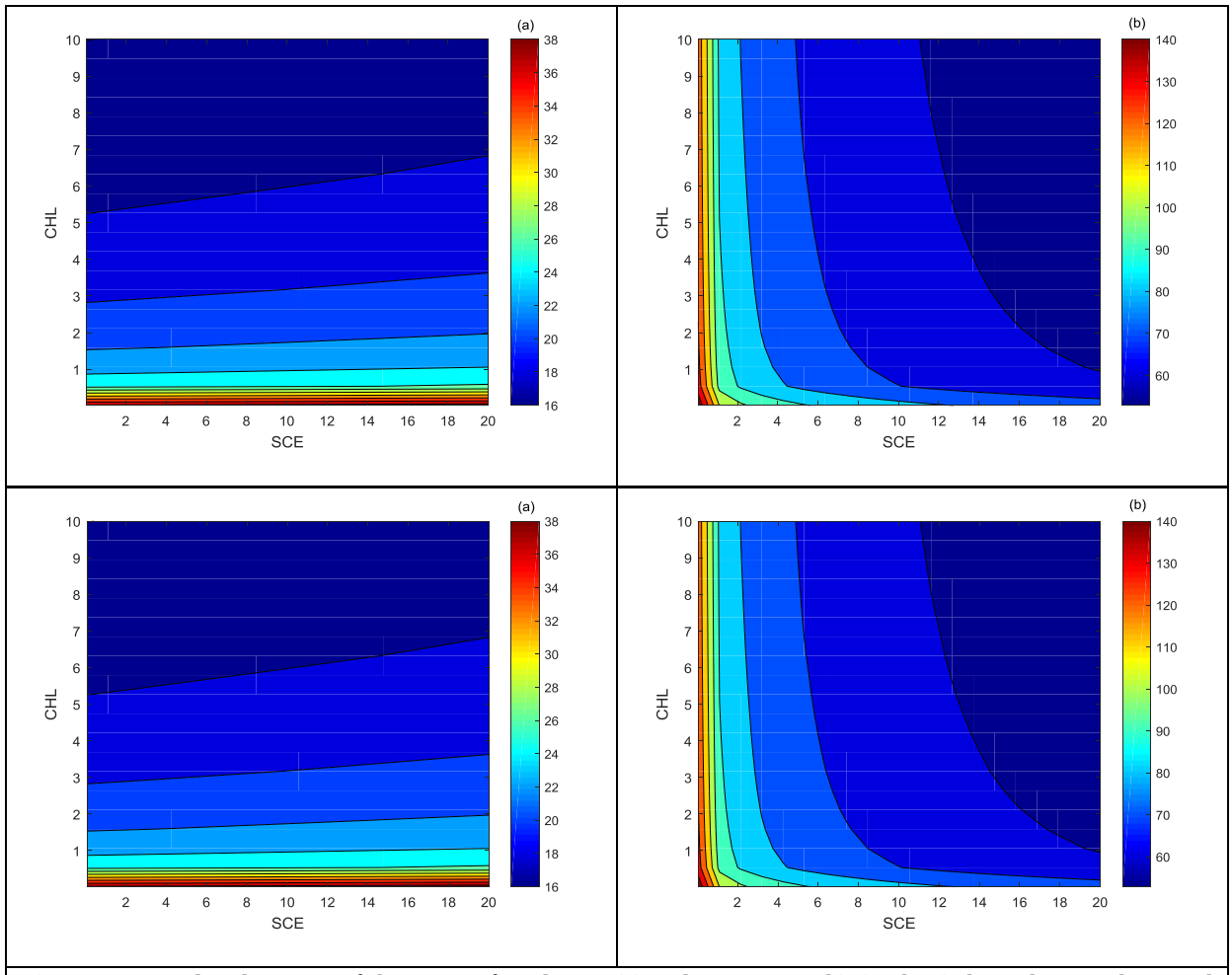


Figure 5. Isovalue diagrams of the times of resilience (a) and succession (b) (in days) depending on the initial biomass densities of *Chlorella* (CHL) and *Scenedesmus* (SCE) (in mgCL^{-1}) under a continuous supply of high nitrogen concentration.

Materials and Methods

HRAP experiment. The pre-existing data used in this study were obtained in HRAP of 1.9 m^3 working volume, continuously fed by pre-treated wastewaters (after screening and removing grit, sand, and grease) with a constant retention time of 6 days (see[20]). Algal blooms occurred naturally in the open pond without any algal inoculation. The period covered by this study was from 28 April 2015 to 8 September 2015. For the present study, we used the data obtained through analytical monitoring that was performed on the influent wastewater and on samples taken from the HRAP. They included water temperature, chemical analyses (TAN and pH), and the algal biomass of the two dominant algal

species (*Chlorella sp* and *Scenedesmus sp*) estimated using cell count from flow cytometry and converted into carbon units.

Microalgae strains, cultivation conditions, and laboratory experiments. Strains of freshwater microalgae *Chlorella sp.* and *Scenedesmus sp.* were isolated from the HRAP samples taken in October 2015. Individual strains were isolated in Z8 media[50]. The Z8 media was modified replacing all nitrogen forms with ammonium salt (NH_4Cl) as the sole source of nitrogen in the growth medium and by adding the HEPES buffer at 20 mM. The two species were maintained and cultivated under continuous light (100 µE m^{-2} s^{-1}) and temperature (20°C).

We performed three series of experiments ($SE1$, $SE2$, and $SE3$) in batch reactors with a working volume of 40 mL. Each series were preceded by a pre-incubation phase in which the two species are preadapted to the fixed cultivation conditions in each set of experiments and providing sufficient fresh volumes for inoculations. The pre-incubations were performed either in continuous mode (in 2L photobioreactors stirred at 300rpm, one-sided illumined at 130 µmol$m^{-2}s^{-1}$, before $SE1$) or in batch mode (in a shaken flask at 150 rpm of 200 mL, before $SE2$ and $SE3$). All batch experiments (in pre-incubation or in the three-test series) were performed in laboratory incubators under a temperature set at 25°C ± 2°C, an orbital agitation at 150 rpm speed and the incident light intensity at 50 µmol$m^{-2}s^{-1}$ in $SE2$ and $SE3$.

The first pre-incubation in continuous photobioreactors were performed to determine the yield constants and provide sufficient fresh volumes for later inoculation in batch culture. Thus, each strain was growing for about 15 days until the biomass stabilization, under a constant temperature of 25°C and a continuous supply of sterilized medium (C:N substrate ratio at about 1:1 and N:P substrate ratio at about 34:1) at a fixed pH value of 7.5 and a fixed dilution rate (0.25 d^{-1}). After that, the growth of the two species was assessed in batch cultures (as described above) under an initial TAN concentration ranging from 10 to 110 mg.L^{-1} keeping constant the concentrations of the other medium components. The pH value was maintained at 7.5 in all batch reactors of $SE1$. In the second series of experiments $SE2$, prior to the experiment, the two species were pre-incubated in batch cultures for about 6 days in a sterilized medium of modified Z8-NH4 with a C:N substrate ratio at about 88:1 and N:P substrate ratio at about 2:1 and pH were set at 7.5. Then, SE2 experiments were performed (as described above) under different pH conditions initially adjusted to 6.0, 6.5, 7.0, 7.5, 8.0, 8.4, 8.7, and 9.0 with NaOH or HCl while using a similar initial concentration of NH_4Cl of 2 mg.L^{-1} that was assumed to be nontoxic for both microalgae strains.

Prior $SE3$, the 6-days pre-incubation of two species was performed in a sterilized medium of modified Z8-NH_4 with C:N ratio substrate at about 88:1 and N:P substrate ratio at about 8:1 at a fixed pH value initially set at 8.6 (corresponding to the pH average measured in HRAP). Finally, in $SE3$, the growth of the two species was assessed in batch cultures under a large range of initial NH_4Cl from 1.2 to 187.7 mg.L^{-1}.

DNA isolation, PCR, and sequencing. Genomic DNA was extracted from a 10-mL sample filtered onto a 0.2 µm membrane (PALL Supor® 200 PES), using the phenol/chloroform method. The 18S and ITS rDNA were amplified in PCR reactions using the *Pfu* polymerase (Promega) with the primers EAF3 and ITS055R[51]. The PCR products were purified and sequenced using specific primers to target the variable V4 region of the 18S rDNA as well as the ITS region. Sanger sequencing was performed at Eurofins Genomics (GATC services).

Sample analyses. The pH in each culture solution was determined daily (pH meter Symphony® SP70P, VWR). For algal biomass estimation, samples were shaken to bring all the cells into suspension and subsamples were daily taken to measure absorbance. In $SE1$, the growth of algae was measured using optical density (OD) of the culture with a microplate reader (FLUOSTAR®, BMG

Labtech) at 650 nm. In SE2 and SE3, cell mass was measured by fluorescence (EX 450 nm, EM 680 nm) and optical density (OD) at 650nm, 730 nm, and 680 nm using a microplate reader (CHAMELEON®, Hidex).

In $SE2$ and $SE3$, subsamples were collected at the beginning and at the end of each experiment, for nutrient and biomass analysis. Samples were then filtered using i) 0.2µm Sartorius filters for measuring nutrients in filtrates and ii) pre-combusted AE filters for measuring carbon biomass onto filters. Ammonia nitrogen was measured with a spectrophotometric test kit (SpectroQuant®, Merck Millipore) and orthophosphate phosphorus according to an optimized molybdenum blue method[52]. After drying the filters (24h, 60°C), the particulate organic carbon representing mainly algal carbon biomass was analysed using an ANCA mass spectrometer (Europa Scientific).

Data Analysis. We performed the covariance analysis using the "aoctool" function of Matlab to compare significant differences in growth rates µ of algae after 48h exposure at each tested condition. The technique required the grouped data of logarithm of the biomass ln(x) measured at time t (during the time period 0 to 48h) for all tested condition. We modelled ln(x) as a linear function of time t to determine whether the slope of the line, which represents an estimate of µ, varies among groups. Based on the model fit of the separate-lines model, the stats output structure from "aoctool" served as input to the multi-compare test "multiconpare" function of Matlab, which allows for testing either slopes or intercepts.

Model description and calibration procedures. The first identifications of the growth function parameters for the two species were performed by fitting the kinetic model (*) to the assessed values of specific growth rate data obtained in $SE2$ and $SE3$ for which cultivation conditions are either identical or different but would not be disruptive of the growth rates except for the initial TAN concentration. The optimal growth parameters were calibrated by the "fmincon" function of Matlab optimization toolbox used in minimizing a mean square criterion $J = \sum_{i=1}^{n}(\mu_{i\_exp} - \mu_{i\_sim})^2$, where μ_{i_exp} and μ_{i_sim} are the normalized experimentally estimated and model generated values of growth rates at the ith experimental condition, and n is the total number of estimated growth rates corresponding to the total number of tested conditions TAN concentrations in $SE2$ and $SE3$.
Secondly, we used the identified growth parameters and the conversion constants y (in gC/gN), theoretically calculated at the steady state of the continuous photobioreactors (performed at pre-incubation for $SE1$) (by $y = \frac{A^*}{N_{in}-N^*}$ where A^* and N^* are the algal biomass concentration (mgC.L^{-1}) and the TAN concentration (mgN.L^{-1}) at steady state and N_{in} is the inlet TAN concentration) to simulate the following model in order to explore the species dynamics under a fixed pH (8.6) and temperature (25 °C) in a homogeneous continuous reactor.

$$\begin{cases} \frac{dA_1}{dt} = (\mu_1(N) - D)\, A_1 \\ \frac{dA_2}{dt} = (\mu_2(N) - D)\, A_2 \\ \frac{dN}{dt} = (N_{in} - N)\, D - \frac{\mu_1(N)}{y_1} A_1 - \frac{\mu_2(N)}{y_2} A_2 \end{cases}$$

This set of equations gives the variations over the time of both algal biomass of *C. sorokiniana* and *S. pectinatus* (in mgC.L^{-1}) and substrate concentrations ($TAN = NH_3 + NH_4^+$) (in mgN.L^{-1}), denoted $A_1(t), A_2(t)$ and $N(t)$, respectively. Growth kinetics $\mu_1(N)$ and $\mu_2(N)$ of the two species depend on TAN, which is the sole source of nitrogen and supplied continuously at fixed dilution rate D=0.16 d^{-1} and a mean concentration value of N_{in}= 62.54 mgN.L^{-1} encountered in the studied HRAP. The model

was solved using ode23t differential equation solver using the following initial conditions of substrate and biomass: N_0= 104.50 mgN.L^{-1}, A_{10}=0.0123 mgC.L^{-1}, A_{20}=0.2698 mgC.L^{-1}.

Third, we validated the hypothesis of ecological facilitation on real dynamics in HRAP. We used the whole dynamics simulated over the time from the given initial condition until the system was about to reach a steady-state and we compared data to the following model equations including terms of mortality m_1 and m_2 on A_1 and A_2:

$$\begin{cases} \frac{dA_1}{dt} = (\mu_1(N,pH,T) - m_1 - D)\,A_1 \\ \frac{dA_2}{dt} = (\mu_2(N,pH,T) - m_2 - D)\,A_2 \\ \frac{dN}{dt} = (N_{in} - N)\,D - \frac{\mu_1(N,pH,T)}{y_1}A_1 - \frac{\mu_2(N,pH,T)}{y_2}A_2 \end{cases}$$

We considered the variations over the time of N_{in}, pH, and T implemented into the model with interpolations performed between the real data points measured over time. We identified the unknown parameters (m_1, m_2, y_1, and y_2) of the dynamic model using "fmincon" function of Matlab optimization toolbox. Optimal parameters assuring the best fit to data were defined in predefined intervals of boundary values after 100 consecutive estimations. The mean squared error was used as the criterion function for the model parameters estimation and was calculated as the square root of the variance of the observations (of A_1, A_2, and N) and divided by the number of measurements.

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Author Contributions

A. R, E. L. F, and E. F participated actively in conceiving the experiments, coordinated the research work, and critically revised the manuscript. E. K designed and conducted laboratory experiments, analyzed data and performed mathematical simulations, and wrote the manuscript.