Farming microalgae; the impact of nitrogen chemical species on nitrogen uptake and assimilation rates by microalgae

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Abstract

Microalgal biofuel technology provides the opportunity to recover nutrients from wastewater. Nitrogen uptake and assimilation rates by microalgae were studied to understand algal growth. Both literature metadata analysis and batch experiments were carried out. *Chlorella vulgaris, Scenedesmus obliquus* and *Micractinium pusillum* were grown in shaken flasks in artificial medium containing nitrate and/or ammonia as the limiting nutrient. Nitrogen availability seems to have regulated algal growth. Exponential growth rates were not significantly different among species. Two distinct *Chlorella vulgaris* strains resuspended in ammonia have shown a significant higher nitrogen uptake rate per cell compared with resuspension in nitrate. The sole use of ammonia led to a decrease in pH that eventually stopped growth for all tested species. *Micractinium pusillum* grown in a mixture of ammonia and nitrate have preferred ammonia over nitrate. Optimization of algal growth should therefore consider the ratio of available nutrient chemical species, and control of pH.

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List of Abbreviations and Symbols

A: algal carbon density
ANOVA: Analysis of variance
As: Arsenic
ATP: Adenosine TriPhosphate
BBM: Bold's basal medium
BOD: Biological Oxygen Demand
C: Carbon
c: fixed algal nutrient quota
CA: Carbonic anhydrase
$CaCl_2$: Calcium chloride
Cd: Cadmium
CO_2: Carbon Dioxide
$CoCl_2 \cdot 6H_2O$: Cobalt(II) chloride hexahydrate
CPCC: Canadian Phycological Culture Centre
Cr: Chromium
Cu: Copper
$CuSO_4 \cdot 5H_2O$: Copper(II) sulfate pentahydrate
C. vulgaris: Chlorella vulgaris
D: Dilution rate
E_0: Incident scalar irradiance
ePBR: environmental PhotoBioReactor
$FeSO_4 \cdot 7H_2O$: Ferrous sulfate heptahydrate
FSC: forward scatter
g: units of gravity
GRH: Growth Rate Hypothesis
GS/GOGAT: Glutamine Synthetase/Glutamine:2-OxoGlutarate AmidoTransferase
H: half-saturation constant for light-dependent algal production
H_{CO2}: Henry constant
H_3BO_3: Boric acid
HCl: Sulfuric acid
HCO^{3-}: Bicarbonate
HRAP: High Rate Algal Ponds
H_2SO_4: Sulfuric acid
I: light intensity
k: specific light attenuation coefficient of algal biomass
K_A: Acidity constants

K_{bg}: background light attenuation coefficient
K_m: Michaelis constant
K_μ: half-saturation constant for growth rate
KH_2PO_4: Monopotassium phosphate
K_2HPO_4: Dipotassium phosphate
KNO_3: Potassium nitrate
L: Liter
M: half-saturation constant for nutrient uptake
$MgSO_4 \cdot 7H_2O$: Magnesium sulfate heptahydrate
$MnCl_2 \cdot 4H_2O$: Manganese(II) chloride tetrahydrate
MRL: Multiple Resource Limitation
mRNA: Messenger RiboNucleic Acid
N: Nitrogen
Nav: Available nitrogen
NaCl: Sodium Chloride
NADPH: Nicotinamide Adenine Dinucleotide Phosphate-oxidase
$NaNO_3$: Sodium nitrate
NH_4Cl: Ammonium chloride
NH_3-N: Ammonia reported with nitrogen basis
NH_4-N: Ammonium reported with nitrogen basis
mg: milligram
mixN: mixture of NO_3 and NH_3
mL: milliliter
mmol: millimole
mV: millivolt
mW: milliwatt
M. pusillum: Micractinium pusillum
$Na_2MoO_4 \cdot 2H_2O$: Sodium molybdate dihydrate
Ni: Nickel
NO_2-N: Nitrite reported with nitrogen basis
NO_3-N: Nitrate reported with nitrogen basis
NRC: National Research Council
NU: nitrogen uptake rate
OD: Optical Density
OECD: Organisation for Economic Cooperation and Development
P: Phosphorus
p: production rate of algae
Pav: Available phosphorus

p_{CO2}: partial pressure of CO_2
P^{C}_{phot}: C- specific rate of photosynthesis
PO_4-P: Phosphate reported with phosphorus basis
Q: cellular quota
R: external nutrient concentration
RNA : RiboNucleic Acid
rpm : rotation per minute
rRNA : Ribosomal RiboNucleic Acid
Rubisco: Ribulose-1,5-bisphosphate carboxylase
RuBP: Ribulose-1,5-BisPhosphate
s: depth below water surface
S1: first stage
S2: second stage
S. obliquus: Scenedesmus obliquus
SSC: side scatter
t_1: initial day
t_2: final day
TN: Total Nitrogen
TP: Total Phosphorus
UK: United Kingdom
US EPA: United States Environmental Protection Agency
v: rate of reaction
wwt: WasteWater Treatment
wwt/a: WasteWater Treatment/Algae
wwt/a/bf: WasteWater Treatment/Algae/BioFuels
x_{CO2}: concentration of CO_2 in liquid
z: depth of mixed water column
Zn: Zinc
$ZnSO_4 \cdot 7H_2O$: Zinc sulfate heptahydrate
α^{Chl}: Chl *a*-specific initial slope of the photosynthesis-light curve
β_N: N-containing compounds other than protein per amount of C
β_P: P-containing compounds other than ribosomes per amount of C
θ^{C}: Chl *a* : phytoplankton carbon ratio
ϕ_{CN}: rate of protein-C synthesis per daily nitrogen assimilation
ϕ_{NP}: rate of protein synthesis by ribosomes
ρ_{Nmax}: nitrate maximum uptake rate
ρ^{Chl}: Chl *a* synthesis regulation term
μ: growth rate

$[H^+]$: hydrogen ions concentration
$[HCO_3^-]$: bicarbonate ions concentration
$[CO_2]$: carbon dioxide concentration in liquid
$[NH_3]$: ammonia concentration
$[NH_4^+]$: ammonium ions concentration
[S]: substrate concentration

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Introduction and overview

The discharge of untreated wastewater causes contamination of water resources and environment. Algal biomass is one option to treat wastewater. Algae will take up and assimilate nitrogen and phosphorus, the two most concerned nutrient removals in wastewater. Algae can then be harvested to produce biofuels and/or other by-products. Wastewater do not contain sufficient nutrients to supply world energy demands but algal biomass is an interesting alternative to actual energy intensive wastewater treatments [1]. This project is related to wastewater treatment with nutrient uptake, but it is also indirectly related to microalgal biofuel technology with biomass production.

First, trends in publications were assessed with different keywords used to describe publications. Number of publications, keywords intensity and richness were analyzed. Published reviews of algae cultivation for wastewater treatment and biofuel production were also summarized (section 1.1). The wide range of sources for wastewater affects greatly the availability of nutrients for algal growth. Data of nitrogen and phosphorus concentrations in wastewater used to cultivate algae have therefore been compiled and analyzed (section 1.2). Both sections formed the basis of two articles published in Algal Research journal.

Literature review of algal growth experiments and modelling has then been carried out to understand wastewater as a source of nutrients (Chapter 2). Chapter 3 defines the methodology of the experimental part of the project. Experimental part was focused on

nitrogen uptake and assimilation rates by microalgae. Chapter 4 and 5 present the results and discussion of the experimental part.

The main objectives of the project were:

- To define and understand wastewater as a source of nutrient for algal cultivation.
- To investigate nitrogen uptake and assimilation rates by different microalgal species.
- To assess algae growth with ammonia, nitrate and a mixture of nitrate and ammonia as the nitrogen source.

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Co-authorship Statement

The literature review (Sections 1.1 and 1.2) was the basis for two published articles:

1. Monfet E, Unc A. 2017. Defining wastewaters used for cultivation of algae. *Algal Research* 24B: 520-526, doi: 10.1016/j.algal.2016.12.008.

Contributions:

EM developed the approach, collected the information, analysed the data and written the manuscript; AU, advised on the development of the approach data collection and analysis and reviewed the writting

2. Unc A, Monfet E, Potter A, Camargo Valero MA, Smith SR. 2017. Microalgae cultivation for wastewater treatment and biofuel production: a bibliographic overview of past and current trends: Note to editor, *Algal Research* 24B: 2-7, doi: 10.1016/j.algal.2017.05.005

Contributions:

EM and AU contributed equally to the project conceptualisation, data collection, data analysis and writing; AP, MACV and SRS advised on the analyses, reviewed the results and reviewed and revised the writing

Chapter 1 Background and Justification

1.1 A review of the history of microalgae cultivation for wastewater treatment and biofuel production [1]

Background

Rigorous interest in the quality of surface waters and the related field of treatment of municipal and industrial wastewaters is not novel. Standards to protect environmental quality were developed by the UK Royal Commission on Sewerage Disposal in 1898 [1]. However related research activities become more obvious in the peer-reviewed publication record after the late 1960's [2,3] reflecting the industrial and urban expansion of the times and the increasing awareness of the impact on surface water. This created the impetus for regulatory authorities to introduce environmental controls on water quality and on urban and industrial emissions. The creation by US EPA of the Clean Water Act of 1972, designed to regulate the restoration and to uphold the quality of the water sources in the United States, is such an example. Related regulations on water discharge stimulated investigations on effective means of nutrient removal, primarily N and P, including the option of microalgae, to mitigate eutrophication of surface waters [2]. Nevertheless, the use of algae to treat wastewaters for reduction of nutrients and biological oxygen demand (BOD) has long been considered as an effective alternative to conventional biological wastewater treatment processes, to achieve environmental quality

[1] A version of this chapter was published as: "Monfet E, Unc A. 2017. Defining wastewaters used for cultivation of algae. *Algal Research* 24B: 520-526, doi: 10.1016/j.algal.2016.12.008."

standards [2,4]. Significant peer reviewed literature targeting the use of [micro]algae as an option for wastewater treatment can be traced to about 1977, and, although mentioned before [5], the first clear statement on the value of wastewater for algal production appeared in 1979 [6]. Subsequently, US national programs aimed at developing algal based biofuels also integrated wastewater research elements, a trend especially evident after 1980 [7,8]. Other bio-products, such as ethanol from residual starches, residual protein for animal feed, nutraceuticals, or even bioplastics may be also obtained from algal residues left behind after the extraction of lipids for biofuel [8]. The significant nutrient demand of large-scale algae biomass production also provided the opportunity to couple the treatment of high nutrient content wastewaters with algal growth [6,7]. An additional benefit of wastewater treatment with algae is the capacity to fix CO_2 [9–12]. Biological nutrient removal from wastewater by a range of algal species is effective in a variety of engineered systems including traditional ponds, high rate algal ponds (HRAP [13]). By combining wastewater treatment with algal biofuel production, biological wastewater treatment processes, which are usually a significant energy sink, can be converted into a positive energy source [8,14].

Therefore, in recent years, research has been devoted to enhancing efficiency of the process of creating biofuels from wastewater derived algal biomass. While other valuable bio-products can and are also obtained from wastewater cultured algae, often from the same harvest [8], the principal driver of our review is the production of biofuels. Literature reviews regularly published on the subject are often written as expert opinions, an approach intrinsically selective. I assessed the current state of the science as published,

by analysing keyword datasets descriptive of peer-reviewed publications as summarised by a publicly available curated database. By not relying on an expert opinion approach, I did not select results based on their perceived quality; articles were retained if they met the respective search criteria, and thus their contribution to the keyword dataset was not-biased by a quality judgment. The apparent historical trends in research on the application of algae in wastewater treatment to possibly identify critical research priority areas were then examined. Methodological details can be found in the Supplementary data - A.

The annualized rate of increase in publication counts can be used to reveal the maturity of a research field. A mature research area, such as “water” or “algal research”, while producing many publications, has a small proportional rate of increase in publication counts from year to year. Interestingly, the broad topic of using algae for wastewater treatment in general, follows the behaviour of a relatively mature field despite the comparatively smaller publication count (Fig. 1.1a). On the other hand, the large annualized increase rates in manuscript counts for algae for biofuel production, with or without wastewater, suggest a new and expanding field. This is confirmed by the similar trend observed in publication rate for wastewater/algae (wwt/a) and wastewater/algae/biofuels (wwt/a/bf), with the latter a 20% subset of wwt/a (Fig. 1.1b). This trend was consistent irrespective of the type of wastewater type considered (municipal, industrial, and farm wastewater streams) for either treatment or biofuel production.

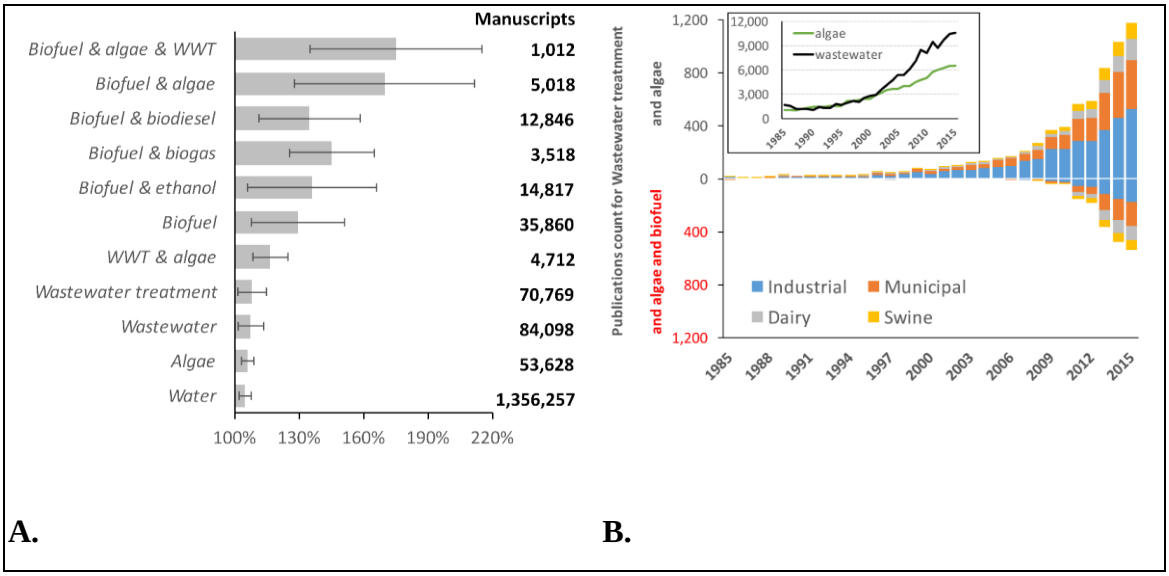


Figure 1-1 Publication for selected research areas (SCOPUS search results obtained on January 18, 2017); error bars are 95% Confidence Intervals); Manuscript counts sum the period from 2007 through 2016. A. Publications; average annual increase rate and total (2007–2016); B. Publications related to algae and biofuels across wastewater types.

The variation in keyword usage intensity conjectures the rationale and context of the associated research area. The analysis confirms that early interest in wastewater treatment was driven primarily by environmental concerns (Fig. 1.2) with less focus on utilization of wastewaters for resource recovery as substrates in bioreactors or like systems. Thus, environmental impact keywords were identified in about 50% of the 1972–1973 related publications (Fig. 1.2). This was followed by a sustained increase in modelling efforts, likely summarizing the extensive modelling of wastewater treatment carried out by the profession of Civil Engineering [3]. It is interesting to also note the sustained and simultaneous increase of environmental impact and modelling research in the 1990–2000 period (Fig. 1.2).

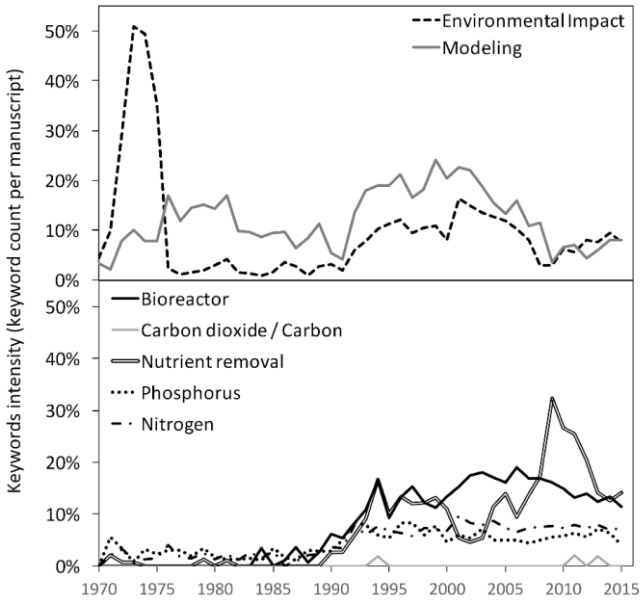


Figure 1-2 Selected keyword utilization rates for the for the "wastewater treatment" query (SCOPUS search results obtained on May 18, 2016).

Wastewater treatment aims to lower BOD and remove nutrients to minimize eutrophication risks [4]. It is noteworthy that the pollution focus of wwt/a publications is also associated with a significantly stronger focus on metal and toxicity terminology (see Supplementary data, Table 6); average abundance for the keywords subsets including As, Cd, Cr, Cu, Ni, Zn, "metals" and "metal ions" was 1.36%±0.69% for wastewater (wwt) and 4.39±1.66% for wwt/a; none were found in the wwt/a/bf publications dataset. This strengthens the notion that addition of algae to the wastewater treatment technologies was initially done with the goal of treatment and not for obtaining algal bio-products.

After 2000 "modelling" dominates the wwt publications (11.5%), "management" and "water pollutants/pollution" are comparably represented in the wwt/a publications (20.1%

and 19.0%, respectively), and “biomass”, at 72.8%, clearly dominates the ww/a/bf publications. Nevertheless, research on modelling of wastewater systems, while relatively constant from 1970 through early 2000's, declined in the last 10 years. This underlying trend, that occurred while publication in the www/a/bf research area accelerated, is a significant concern. It suggests that much of the recent research is exploratory in scope and likely narrative in nature. Therefore, the development of coherent management tools for algal wastewater treatment processes might be justifiably considered as a priority area for future research investment.

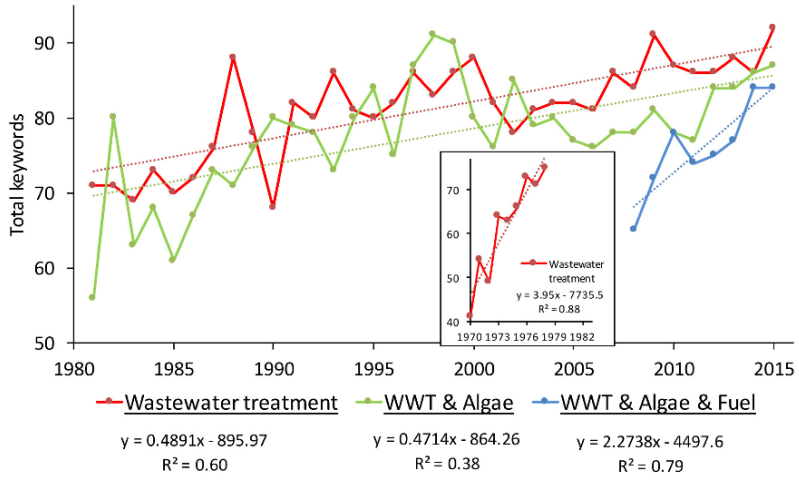
Where wastewaters are primarily employed for algal growth and biomass production the availability of nutrients becomes a critical aspect of the treatment system. More recently “nutrients” and “nutrient removal”, in the context of algal biofuel, have received greater attention by the international research community, and, concomitantly, bioreactor based research has also expanded; the increased intensity of keywords describing bioreactor type (Fig. 1.2a) towards 1996 coincides with the conclusion of the first concerted effort to evaluate the utility of algae for energy production [7]. The intensity of research on nitrogen and phosphorus, in general, follows a similar trend; research on nutrient removal reached its maximum intensity in 2010, coinciding with a significant output of wwt/a/bf research (Fig. 1.2) in the middle of the current surge in wastewater and algae for biofuel research [8]. A closer look at keyword abundance after 2000 shows that while “nutrient removal” dominates (28.1% for wwt/a, and 17.5% for wwt/a/b), “nutrient availability” or “uptake” received very little attention (0.97% and 0.78%, for wwt/a and respectively

wwt/a/bf, and not present in the wwt dataset; Supplementary data, Table 6). This confirms that, whilst nutrient removal, i.e. wastewater treatment, was the key focus of research, the interest in use of wastewater as a nutrient source was only establishing. The increasing use of "nutrient" for the wwt/a/bf literature (18.87%, versus 1.75% for wwt, and 9.38% for wwt/a) also indicated a shift in the approach, but the direction was more difficult to gage without a qualifier term. These trends suggest that despite the increase in research on wastewater usage for algal production the dominant paradigm surrounding wastewater nutrients is still treatment, i.e. the capacity of algae to remove nutrients from wastewaters, and only secondarily the capacity of wastewater to support algal growth, yet not necessarily optimal growth.

The total number of distinct keywords, or keyword richness, increases as the scope of a given research field expands. All three areas of research, wwt, wwt/a and wwt/a/bf, show an increased in keyword richness to reach relatively similar level in 2015 (Fig. 1.3a). For the more established wwt and wwt/a research an average annual increase of approximately 0.5 keywords y^{-1} is recorded. On the other hand, wwt/a/bf showed a rapid increase in distinct keywords at a rate of 2.3 y^{-1}, consistent with a rapidly expanding research field. This pattern is consistent with the early stages of a newly establishing field as shown by the similar rapid increase in keywords of 3.95 y^{-1} in the early period of wwt research (1970–1978). Patterns in keyword richness may also reveal when research areas diversify into new directions; this was evident by the patterns observed for wwt and wwt/a around year 2000. Consequently, the dataset was divided to take this behaviour

into account to allow a more critical analysis of the patterns and trends in research to be scrutinized after 2000.

a. Keyword richness



b. Annualized (year-to-year) dissimilarity. Keyword abundance per manuscript datasets were used in the analysis.

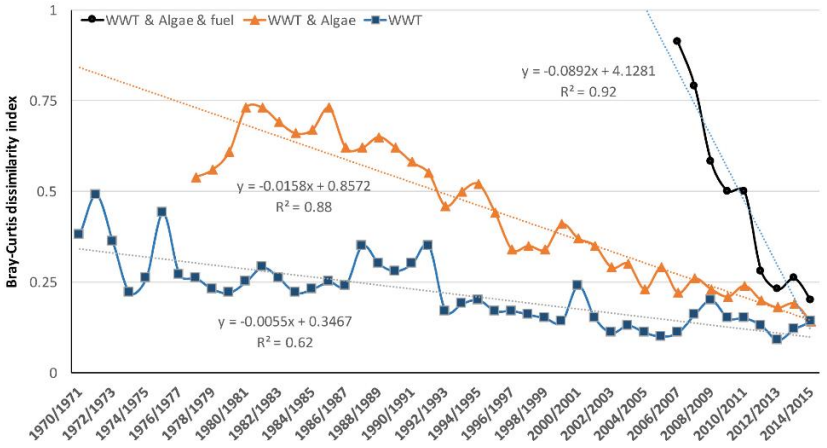


Figure 1-3 Temporal shifts in keyword utilization (SCOPUS search results obtained on May 18, 2016).

Another indicator of a maturing research field is the stabilization of the range of inquiry. As a field of research shifts from discovery to application the diversity of keywords, and thus the range of inquiry, tends to stabilize. A Bray-Curtis dissimilarity index analysis was therefore applied to assess the year-over-year changes in similarity between the annual keyword datasets to assess the running dissimilarity (Fig. 1.3b). The Bray-Curtis index considers both presence and abundance for computation of similarity distances. The index declines with time for all three research areas. The rate of decrease is expectedly greater for faster maturing fields.

Values of dissimilarity above the long-term average trend (i.e. the linear fit line) indicated either that: (1) there was a slower decrease in dissimilarity or, (2) there was an increase in dissimilarity for the pair of years in comparison to the previous period. The second condition applies, for instance, when a set of newly added keywords is significantly different from the keywords found in the previous year. Such patterns in the use of different keywords are indicative of an increase in the scope of research in that area, possibly reflecting a period of innovative development. On the other hand, dissimilarities lower than the multiannual trend indicate a relative stagnation in the scope of research, or more stable, less innovative, research activity. For all three areas of research evaluated here, wwt, wwt/a, and wwt/a/bf, there was a consistent decrease in the year-over-year dissimilarity values, which may reflect the relative decrease in innovation, an indicator of the research scope in these areas reaching a certain steady state. A principal component analysis confirmed that research on biomass production parameters

increased since 2000. *Chlorella* spp. employed for algal research, including cultivation, for over a century [15,16] still dominate as the preferred test organisms (see Supplementary data – A, Table 1.

A survey of published review articles shows that interest in large scale cultivation of algae can be traced to the 1940s; a monograph published in 1953 summarised much of the state of the art research on algal cultivation from laboratory to pilot scale, with a focus on *Chlorella* spp. [15]; the justification of this work was the potential for algae as food source. Research on sewage for cultivation of microalgae followed soon after [17]. In 1978 the Aquatic Species Program [7], a US national funded activity, identified the potential of producing biofuels through algae and microalgae cultivation. In 1979 Beneman et al. [6] also published a conceptual map for the use of wastewater to culture algae on wastewaters for fuel production. Much of the initial focus was on hydrogen production and, subsequently, biodiesel production became more important after 1980. The program was terminated in 1996, but in 2010, a new algae for biofuel program was established [8] that also included integration with water treatment facilities. A query for reviews with the keywords “algae” (including “microalgae” and different spellings) and “wastewater” produced a dataset of 230 reviews. These reviews were examined and only those focusing on growth of algae in wastewater were retained. Reviews dealing with the impacts of wastewaters on the environment and on algal blooms in water bodies, and general wastewater treatment or biosorption reviews were excluded. Just under 80 reviews were identified as relevant to biofuels from algal biomass cultivated in

wastewater (as listed by SCOPUS on March 16, 2016). The bulk of the reviews, many with a (bio)fuel perspective, were published after 2010 and generally focus on the parameters affecting algae production in wastewater from an engineering perspective.

The first review identified considering the growth of algae in wastewater, from a biotechnology perspective, was published in 1997 [18]. This examined the use of microalgae for bio-treatment and by-products with an emphasis on *Chlamydomonas reinhardtii*. The majority of reviews focused on both production and harvesting of algae [9,11,12,19–44] whereas other focus specifically on harvesting issues [45,46]. Several articles after 2010 review biodiesel production [20,25,37] and the effect of light source in bioreactor cultures, although not necessarily for wastewater based systems [47,48].

Many reviews [9,11,18,20–24,26,27,31–35,41–43,46,49–58] consider algae in wastewater treatment systems as a biorefinery strategy considering a range of organic compounds, not only lipids. For example, Markou et al. highlighted the potential production of carbohydrate by algae as an approach to biosynthesising biofuels [59]. Several reviews, after 2014, cover related areas of research on algal biofilms for wastewater systems and biofuel production [60–62], indicating the rapid development of the field and that it is an area attracting interest from different research groups internationally. The application and development of synthetic biology technologies in algal-based bioconversion systems has also received attention [20,23,25–28,32,33,51,56,63,64]. Impacts of large-scale cultures on environmental governance [65],

environment [66,67], resource management (specific to China [68]) and financial viability [67,69] have also been examined.

By contrast, relatively little attention has focussed on the role of algal biodiversity [63] with *Chlorella* spp. confirmed as still the most dominant test species for algal growth as indicated by two reviews from 2013 and 2015 [53,58]. Many studies on algal growth have been performed with artificial media, however a range of wastewaters have also been investigated to reflect conditions more realistic of operational circumstances [68].

More dynamic understanding of algal growth processes and behaviour has been gained through the investigation of biological mechanisms and management systems and their combined impact on process performance, as illustrated by reviews summarizing the interactions among algae [70,71], with microbial consortia [19,38,50,53], and particularly with wastewater associated microbes [72,73]. Consortia of microalgae, compared to single species cultures, are also shown to be advantageous for productivity and biomass stability [71,74]. Several recent reviews advocated mixotrophic cultivation to enhance biomass productivity [56,57,74,75], and two-stage cultivation, with a luxury consumption stage followed by nitrogen limitation, is recommended for lipid production [62,74,75]. Both nutrient removal [76–79] and nutrient uptake [36,80,81] are discussed in the context of lipid production.

Conclusions

This exploratory analysis, shows that growing algae for biofuel on wastewater substrates is a rapidly expanding area of research, with a comprehensive approach extending beyond the conventional scientific disciplines commonly associated with wastewater treatment. However, integrated bioengineering modelling and protocols to effectively manage the incorporation of algae into wastewater treatment for resource recovery and biofuel production have received relatively less attention in the scientific literature. While some modelling efforts are carried out much of the research is still exploratory in scope and narrative in nature. The evidence evaluated here suggests that progress will require translation of the ever-expanding experimental data into the development of management systems based on applied process models. A shift in focus from nutrient removal to optimization of nutrient utilisation may be required. Advancement will also depend on factors outside the strictly scientific activity; however, a focused system approach is required for the successful translation of current understanding into sustainable practice.

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1.2 Literature review: Understanding nutrients in the wastewaters used for cultivation of algae[2]

Wastewaters are the by-product of a wide range of domestic, industrial, commercial or agricultural activities and consequently are of highly variable chemical and biological properties. The content of nitrogen and phosphorus in wastewaters is most concerning from an environmental point of view and extensive research has been directed towards their removal from wastewater [1]. One option is recovery of nutrients by algae or microalgae with the added benefit of producing bio-products and biofuels [2–5]. Consequently, a significant body of scientific literature is dedicated to the capacity of algae to remove nitrogen or phosphorus from wastewaters or to the capacity of wastewaters to sustain algal growth [6]. A query in the SCOPUS database for ["wastewater treatment" AND "algae" AND "biofuels" OR "fuels"] reveals a rapid increase in publications from 5 in 2007 to 87 in 2015, while the ["wastewater" AND "algae"] query shows an increase from 51 in 2000 to 379 in 2015 (Fig. 1.4). A number of peer-reviewed articles describe algal research in artificial wastewaters (e.g. [7–10].), not necessarily always specifying the characteristics of the wastewater or the similarity of the said artificial wastewater to actual wastewaters. The reader is too often left to assume as to what wastewater type is the artificial version alleged to replicate.

[2] A version of this chapter was published as: "Unc A, Monfet E, Potter A, Camargo Valero MA, Smith SR. 2017. Microalgae cultivation for wastewater treatment and biofuel production: a bibliographic overview of past and current trends: Note to editor, *Algal Research* 24B: 2-7, doi: 10.1016/j.algal.2017.05.005"

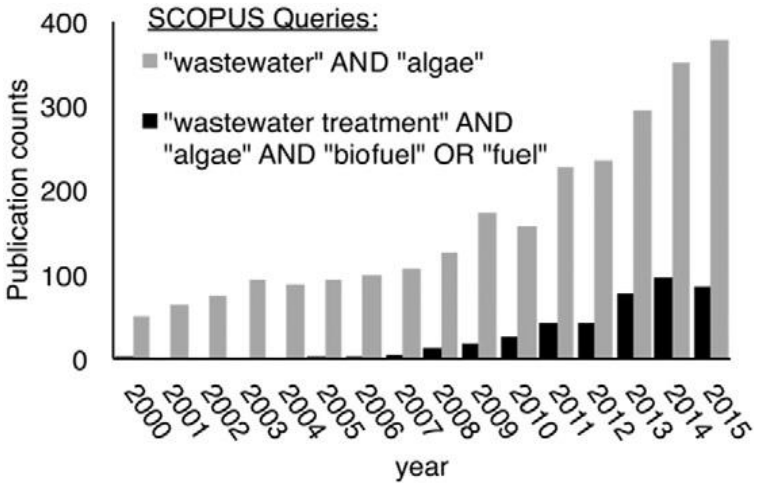


Figure 1-4 Publication counts as identified by SCOPUS.

Removal of nitrogen is described as the balance between the before and after cultivation of either total nitrogen, or the available forms of ammonia or nitrate. Removal of phosphorus is commonly described as the before and after cultivation balance of the total phosphorus. Changes in concentrations in the supernatant are commonly described in terms of absolute mass decline or in terms or proportional mass removal. It was decided to not cite any one peer reviewed article, in support of the statements in the previous two sentences given the very large "wastewater and algae" body of literature [6] and to avoid any perception of undue selectivity.

Given that assimilation of nitrogen and phosphorus is coupled, the N:P ratio of wastewaters is obviously an important parameter to consider. It might be argued that for adequate nutrient removal the N:P ratio in wastewater ought to match the optimal algal

species-specific ratio (Fig. 1.5). The rate of generation of biomass is maximized at optimal N:P ratio [11] but the specific range of concentrations for the unique optimal ratio are not well defined. Published research results might seem to offer divergent information, likely a feature of the inherent variability in the experimental conditions including variability in algal species and strains. As the N:P ratio diverge from the optimal value, algae might accumulate nutrient without biomass production. Biomass productivity might be static at luxury consumption [12]; Wu et al. [13] have shown that while *Scenedesmus* sp. consumed more phosphorus under nutrient replete condition this did not translate into more biomass. A batch study growing *Chlorella kessleri* on artificial wastewater has shown similar cell concentrations independent of the initial nitrate concentrations in the substrate [14]. Nevertheless, in general, augmentation of nutrient quantities is expected to increase biomass productivity, as seen for algae grown long term in continuous culture systems [15]. To further contextualize such nutrient removal-accumulation experimental results it is worth noting that the capacity to store nutrients vary among species and are dependent of environmental conditions [16]. Therefore, for a sound interpretation of results of investigations into biomass productivity and nutrient removal or availability, the distinction between the rate of assimilation into cell constituents, uptake from the substrate, and total accumulation in the algal cell of nutrients in organic and inorganic forms should be considered.

Such inconsistencies complicate directly comparison of results across experiments carried out in wastewaters of variable nutrient ratios, nutrient concentrations and

especially nutrient availability profiles. Synthetic wastewaters are employed as a means to normalize experimental conditions and to simplify nutrient mass balance evaluations. The parameters of these synthetic wastewaters ought to reflect the nutrient availability in a reference wastewater type. Nevertheless, even a casual review of the make-up of synthetic wastewaters can point to inconsistent elements. Firstly, synthetic wastewaters lack an active wastewater microbial population [17]. Secondly, real wastewaters have complex organic matter chemistries that vary widely with source types and extent of treatment [18–20], rather challenging to replicate synthetically.

Given the extensive and rapidly developing field of algal cultivation on wastewaters [6] it is worth pausing to attempt to understand the variability in nutrient profiles in the wastewaters employed for cultivation of microalgae, to eventually support a coherent experimental approach that facilitates comparability and reproducibility of results.

1.2.2 Methodology

A review of the peer-reviewed literature was carried out, with the aim to illustrate the variability in nutrient parameters of a range of wastewaters reportedly employed as a nutrient substrate for cultivation of microalgae. The goal was to identify nutrient parameters for a wide range of wastewaters of various sources as used for algal cultivation for biomass or biofuel production, employing a representative subsample of literature, and not necessarily to comprehensibly summarize the very extensive entire literature available on algae and wastewater treatment research. The units for nutrient

concentrations were re-calculated to molar concentrations, a rather better indicator of algal uptake stoichiometry than the too commonly employed mass per mass or mass per volume units. Ideal molar N:P ratios for a few algal species, as described in selected scientific articles, are also presented here as a means to contextualize the known wastewater nutrient ratios (Fig. 1.5).

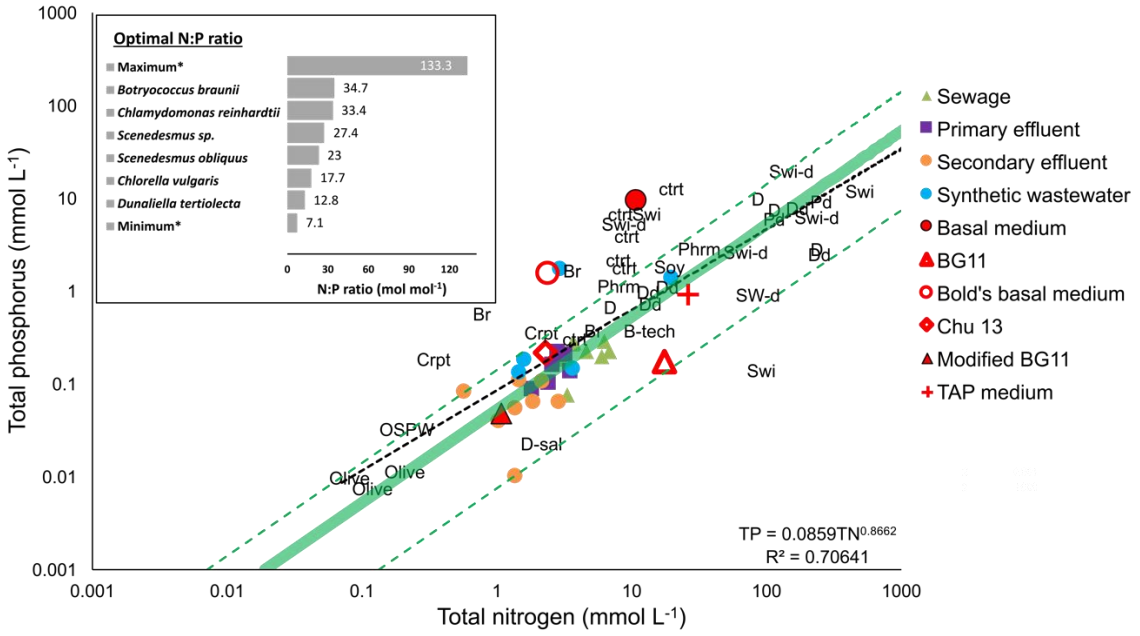


Figure 1-5 Total nitrogen versus total phosphorus (TN:TP) in wastewaters (ww) and optimal TN:TP formicroalgae [25,36–38]. Black dotted line is the best fit line for all wastewaters; artificial media and syntheticwastewaterswere not included in the fit. The green dotted lines encompass the calculated concentration interval between the largest and smallest N:P optimal ratios (i.e. Minimum and Maximum*) for algal growth as described by Klausmeier et al. [25]. The green swath describes the N:P region between the Redfield N:P ratio of 16:1 [21] and the modified 22:1 ratio as described by Martiny et al. [23]. AQ, aquaculture ww; Br, brewery effluent; B-tech, biotechnology effluent; Crpt, carpet manufacture ww; ctrt, centrate; D, dairy manure (liquid); Dd, dairy digestate; D-sal, desalination ww; Olive, olive-oil extraction ww; OSPW, oil sands produced waters; Phrm, pharmaceutical industry effluent; Pd, poultry digestate; Soy, soybean processing effluent; Sw-d, sewage sludge digestate; Swi, swine ww; Swi-d, swine digestate. Basal medium [39], BG11 [13], Bold's basal medium [40], Chu 13 [41], Modified BG11 [42], and TAP medium [43] are artificial algal growth media as used by various researchers.*

1.2.3 Results and discussion

1.2.3.1 Managing nutrients and algal species

The general N:P ratio of 16:1, initially developed for marine phytoplankton and known as the Redfield ratio [21], is a biological constant inherent to the fundamental protein-to-RNA ratio, across living entities on Earth [22]. A more recent, comprehensive revision of ocean organic particulates reported a global median N:P ratio of 22:1 [23]. Differential metabolism under nutrient deficits [22], variable CO_2 availability [24], will affect the measured N:P ratio. Nutrient deficits may be due to variable nutrient concentrations, but also due to variable chemical speciation profiles of nitrogen or phosphorus in diverse wastewaters. A purely physiological control of the N:P ratio might therefore not be necessarily always true [25]. For example the capacity of algae to store unassimilated nutrients, especially nitrogen [26], will skew the N:P ratio in raw biomass. Empirically, phosphorus content in algae has been shown to vary between 0.3 and 3% and nitrogen content between 3 and 12% [27].

Algal growth has been attempted and evaluated in many types of wastewater (see Supplementary Material) but not many studies (e.g. [7,12–14,28–30].) have investigated the effect of the variability of nutrient concentration on algal growth. Moreover, many studies on algal biomass growth and nutrient removal have used synthetic wastewaters (e.g. [7,10,31,32].) but it is often unclear or unspecified if and how nutrient profiles of

such artificial media reflect the nutrient parameters of wastewaters (Fig. 1.5). Often "synthetic wastewater" is assumed to signify municipal wastewaters but this is not always clearly specified. While the N:P ratio and concentrations of some synthetic wastewaters are similar to primary effluents of municipal wastewater treatments this is not always true (Fig. 1.5). Common characteristics of various wastewaters, including examples of synthetic wastewaters used for research into algal biomass growth and nutrient removal are summarized in Table 1.1. Reported concentrations of total nitrogen (TN) and total phosphorus (TP) vary between 0.08 and 491 mmol L^{-1} and 0 to 19.5 mmol L^{-1}, respectively. For most wastewaters more nitrogen than phosphorus is present, which generally corresponds to global cell stoichiometry, albeit not necessarily closely following algal cell stoichiometry. Very generally, there is some consistency in the N:P ratio across wastewaters and concentrations, that can be described as by a direct positive power fit, most likely an indication of the biological origin of wastewater nutrients (Fig. 1.5). The municipal wastewater streams tend to have somewhat similar TN:TP ratios, albeit at concentrations declining along the treatment steps from sewage to primary affluent and then eventually to the secondary effluent. The secondary effluents also tend to have a wider range of the TN:TP ratios, a consequence of the variability in the efficiency of diverse treatment options and their selectivity in removal of N and P. The synthetic wastewaters described here have either a TN:TP ratio and concentrations similar to primary municipal effluent or have greater concentration and lower TN:TP ratios. It is interesting to note that an OECD report [33] recommends synthetic sewage to contain significantly larger TN and TP concentrations (e.g. calculated at an average of

about 3600 mmol L^{-1} TN and 294 TP mmol L^{-1}) than the ones used in algal-wastewater experimentation; thus a calculation using the OECD report recommended substrates suggests that the synthetic sewage would have an average TN:TP ratio of about 12.3, but can vary, depending of the source of the peptone and meat extracts organic substrates, from e.g. 1.8 to 22.6, and an available Nav:Pav ratio (i.e. NH_4-N and PO_4-P) of 6.2 (see Supplementary Materials).

Employing the reported optimal N:P ratios for algal cultivation (Fig. 1.5) for the calculation of a range of nutrient concentrations similar to the ones reported for the wastewaters summarized in Fig. 1.5 allows for the visualisation of a putatively optimal N:P interval. The result of this exercise suggests that some wastewaters, such as dewatering centrate or brewery effluents, might be at or under the minimum preferred ratios. Considering that optimal N:P ratios for various algal species are mostly larger than the 16:1 Redfield ratio (Fig. 1.5) it might be reasonably assume that at least some studies were therefore carried out at sub-optimal N:P ratios. On the other hand, large concentrations of ammonia inhibit photosynthesis and thus are toxic to microalgae. While the threshold of toxicity of free ammonia varies across algal species [34], in general a level above 1.2–2mM for a pH > 8.0 is considered toxic [35]. Thus untreated sewage and most farm and food industry waste (Table 1.1, Fig. 1.5) have ammonia likely at toxic levels, if their pH is not controlled. Such wastewaters would require dilution before being employed for algal cultivation and the N:P ratio becomes a secondary concern. Secondary municipal wastewater effluents, while variable, tend to fall within the mid-

range of optimal N:P ratios and also under the ammonia toxicity threshold. Much of the livestock sourced wastewaters are within the optimal N:P range but require dilution to minimize an eventual ammonia toxicity. Nevertheless, these are general observation and might not be correct for each algal species and strain, under all environmental conditions. Also, it should be noted that while physiologically optimal N:P ratios are a function of available nutrients, much of the data summarized in Fig. 1.5 represents total concentrations.

Table 1-1 Nutrient ranges for various wastewaters used for algal cultivation for biomass or biofuel production (min-max values).

Source	**Nutrient forms (mmol L^{-1})**							**N:P ratios (mol mol^{-1})**	
	NH_4-N	**NO_3-N**	**NO_2-N**	**Organic-N**	**Total-N**	**PO_4-P**	**Total-P**	**Total**	**Available**
Municipal wastewaters									
Sewage	1.51-6.57	0.00036-0.28	0.0036-0.013	0.69	2.90-7.87	0.065-0.46	0.077-0.29	13.1-46	10.2-37.6
Primary effluent	2.19-2.79	0.0057-0.029	0.0014	0.86-0.92	1.82-3.64	0.055-0.13	0.090-0.22	12.6-25	20.4-41.1
Secondary effluent	0.52-1.80	0.0025-1.21	0.00014-0.037		0.57-2.86	0.025-0.13	0.010-0.11	6.8-132.2	12.1-56.4
Centrate	3.94-8.94	0.025			3.79-19.64	1.14-6.94	0.30-12.65	1.4-12.5	1.0-7.8
Agricultural wastewaters									
Aquaculture	0.034-0.30	0.12-2.91	0.0093-0.012		0.49-2.95	0.0069	0.014-0.16	18.4-35.9	24.8
Dairy	3.43-127.29				6.93-236.07	1.56	0.66-9.77	8.8-84.3	1.0-2.2
Piggery	85.5-370.71	0.49	0.12		11.57-491.43	0.14-4.42	0.14-11.84	1.7-659.1	635.5
Industrial wastewaters									
Biotechnology and pharmaceutical effluent					9.71-63.24		0.37-10.52	6.0-36.6	
Brewery					0.77-5.19		0.53-1.75	1.5-7.9	

Source	Nutrient forms (mmol L^{-1})							N:P ratios (mol mol^{-1})	
	NH_4-N	**NO_3-N**	**NO_2-N**	**Organic-N**	**Total-N**	**PO_4-P**	**Total-P**	**Total**	**Available**
Carpet manufacture	0.15-1.55	0.19-1.01			0.34-2.80	0.21-0.29	0.18-0.31	1.9-9.0	1.6-8.8
Desalination	0.075				2.14		0.023	94.9	
Landfill leachate	10.83					0.26			41.1
Olive-oil mill	0.16	2.54			0.081-0.21	0.0021-0.0039	0.0074-0.011	8.3-18.3	42.4-1211.7
Paper mill	11.14								
Soybean processing	3.72				19.08		1.82	10.5	
Steel	4.25	0.43							
Tannery	54.43	0.79				0.16	0.13		337.5
Textile	0.064-15.71	0.24-5.57				0.0016-0.066			31.6-123.4
Anaerobic digestion effluent									
Dairy	6 -159.43	0			13.21-246.86	0.32	0.79-7.74	13.7-93.9	30.1
Piggery	46-235.29	7.93			9.92-236	8.94	2.61-19.48	1.7-37.1	27.2
Poultry	33.21-308.2	0.40			144.39-254.64	2.68	3.10-9.13	24.3-27.9	115.1
Sewage sludge - centrate	18.25-64.71				86.43		0.90-1.25	95.7	

Original data available in the Supplementary Material.

Nutrient concentrations are variable among wastewaters but also variable in time during the growth of algal cultures. Thus nutrient availability is a kinetic parameter dependent not only on algal uptake rates but also on the mineralization rates of any initially unavailable form of nutrients, either organic or mineral. Stability and mineralization rates of organic matter depend on the molecular characteristic of the organic matter, likely dependent on the intensity of wastewater treatment [18– 20]. Variable nutrient availability has a direct impact on algae biochemical composition [30]. The physical and

chemical conditions within the algal culture and the make-up of the microbial community will govern such kinetics. Commonly the temperature and occasionally the pH values are reported. However, the pH, although it is well known to be a kinetic variable, it is not always reported for the entire experimental duration (Supplementary Material). If the main objective is biomass production, addition of nutrients to wastewater is unlikely to be a cost effective solution [45] but modifications of hydraulic retention time in continuous cultivation might be employed to adjust nutrient loads. Up to date, most studies of algal production are batch cultures. Studies with continuous or semi-continuous cycles have nevertheless led to higher biomass productivity compared with batch conditions [46].

Nutrient deficiency is often proposed as a means to increase lipid concentration of algae. When microalgae cells are cultivated under nutrient stress, the fixed carbon seems to be allocated to storage molecules. However, stress conditions on algal cell decrease total biomass production. Nutrient starvation decreases chlorophyll production which in turn reduces biomass productivity and eventually total lipid productivity. Limitation instead of starvation, or the 2 stage-cultivation where sufficient carbon and nitrogen is provided in first stage followed by nitrogen limitation in second stage, has thus been proposed. Phosphorus can also be the limiting nutrient to promote lipid production. Moreover, salinity, light, pH or temperature stresses alone or in combination with nutrient limitation are an alternative to activate lipid production [47]. Many studies show that low nitrogen supply can increase algal lipid content [48]. However, sufficient lipid productivity was attained with *Chlorella sorokiniana* growing in artificial media with either replete or

limited nutrient conditions [12]. Considering the biorefinery strategy, lipids is not the only product that could be generated with algal biomass. Consequently, the operating conditions should maximize biomass productivity and the targeted compound within the algal cells. This means that static values for wastewater nutrients (Figs. 1.5 and 1.6) might not be sufficient to describe the conditions which an algal culture encounters. The fitted line in Fig. 1.5 suggest an N:P ratio of just under 12, significantly lower than the Redfield ratio. Many wastewaters summarized here tend to have an even lower ratio. This suggests that for most wastewaters algae will rapidly reach a state of nitrogen deficiency, ahead of phosphorus deficiency. Of course this is a function of the speciation of these nutrients. Precipitation of phosphorus as insoluble salt is quite likely given the relatively high pH in most wastewaters [49] and actively photosynthesizing algal cultures.

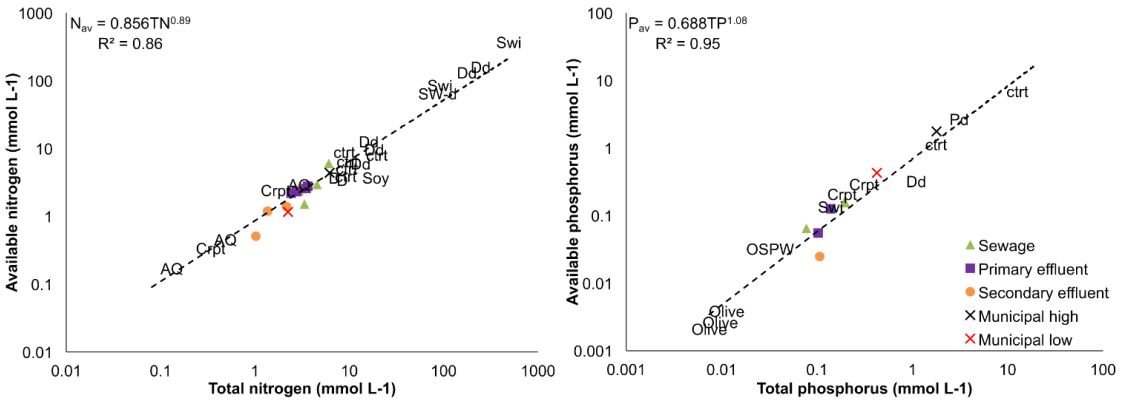


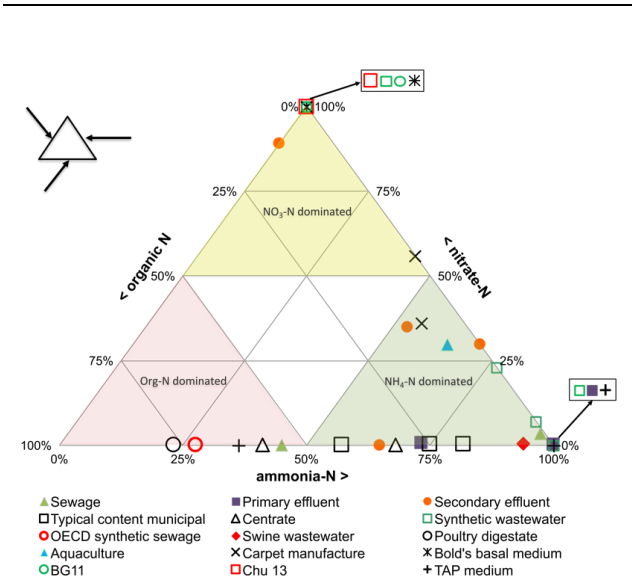
Figure 1-6 Total and available wastewater (ww) nitrogen and phosphorus; the limited data in these graphs reflects the inconsistent data availability: AQ, aquaculture; ww, Crpt, carpet manufacture ww; ctrt, centrate; D, dairy ww; Dd, dairy digestate; Municipal high and low, averages for treated wastewater effluents [44]; Olive, olive-oil processing ww; OSPW, oil sands produced water; Pd, poultry digestate; Soy, soybean processing ww; Swi, swine wastewater; SW-d, sewage digestate.

1.2.3.2 Availability of nutrients

Only inorganic forms of nitrogen and certain inorganic forms of phosphorus, are usually considered to be directly available to algae. The nitrogen compounds that are usually bioavailable are ammonium, nitrate and nitrite. The bioavailable phosphorus is mainly as orthophosphate [27]. When discussing nutrient availability, describing total amounts might be misleading. A short review of the available data suggests that the relationship between the total and available N and P is nearly linear (i.e. power fit at a power close to 1; Fig. 1.6). Thus for nitrogen the data summarized here suggests that about 86% of the TN is available, while for phosphorus about 69% of TP is in available forms. Given that many publications do not explicitly describe all forms of N and P this conclusion might be somewhat speculative.

Many algal experiments do not consider or report all forms of nitrogen. Most artificial media contain either only ammonia or nitrate (Fig. 1.7). Wastewaters, on the other hand, contain both inorganic nitrogen compounds particularly as ammonia, nitrate and nitrite, and also organic nitrogen (Fig. 1.7). Synthetic wastewaters may also contain both ammonium and nitrate or only one of the two. Under acidic conditions ammonia is protonated to ammonium. High pH, common in both wastewaters and algal cultures (Supplementary Material) will favour ammonia volatilization; under such conditions any nitrogen removal calculation must acknowledge and account for such losses. Nitrite is unstable and is rapidly transformed into ammonium or oxidized to nitrate; it is therefore

not separately included in Fig. 1.7. Wastewater organic nitrogen occurs embedded in proteins. Urea can be present in fresh wastewater but it is rapidly ammonified. Most wastewaters tend to be dominated by a combination of ammonia-N and organic-N, with nitrate/ nitrite-N between 0 and 45% of the total (Fig. 1.7). On the other hand certain highly concentrated wastewaters and sewage, including artificial sewage [33] might have N50% of nitrogen in organic forms. For such wastewaters an understanding of the kinetics of organic matter mineralization and the impact on nutrient availability variability during algal growth ought to be considered. It is likely that the consideration of the ecological communities and their interactions might be of greater significance for such organic-N rich substrates. Some organisms may in some conditions have a direct influence on algal biomass [17]. Moreover, the presence of higher trophic level organisms, such as protozoa, arthropods or nematodes, a likely occurrence especially in treatment systems integrating a trickling filter step [50], may act as grazers of both algae and microbes thus affecting microbial functional and diversity balance and also intervening in the nutrient cycle.



Substrate type/ wastewater source		Nitrogen (mmol L^{-1})			
		NH_4-N	NO_3-N	Org.- N	Total-N
Carpet manufacturing [51]		1.55	1.01	0.24	2.80
		0.15	0.19	0.00	0.34
Wastewater centrate (municipal) [52, 53]		6.50	0.03	3.05	9.57
		8.07	0.03	11.55	19.64
Primary effluent [54, 55]		2.54	0.03	0.92	3.49
		2.31	0.01	0.00	2.32
Secondary effluent [56-58]		0.71	0.47	0.16	1.35
		1.40	0.00	0.76	2.16
		0.85	0.36	0.00	1.21
(aeration tank) [59]		0.00	1.21	0.15	1.36
Sewage [57, 60]		5.75	0.21	0.06	6.02
		1.51	0.00	1.85	3.36
Typical municipal wastewater [44]	high	5.36	0.04	1.79	7.18
	medium	3.21	0.01	0.71	3.94
	low	1.43	0.01	1.07	2.51
Aquaculture [61]		0.30	0.14	0.03	0.48
Poultry digestate [62]		33.21	0.40	110.78	144.39
Swine wastewater [63]		85.50	0.49	5.33	91.31
Synthetic wastewaters [64],[65],[9, 10],[8]		0.00	2.94	0.00	2.94
		1.50	0.11	0.00	1.61
		1.46	0.00	0.00	1.46
		2.85	0.85	0.00	3.69
Bold's basal medium [40]		0.00	2.94	0.00	2.94
BG11 [13]		0.00	17.66	0.01	17.66
Chu 13 [41]		0.00	3.96	0.00	3.96
TAP medium [43]		7.01	0.00	0.00	7.01
		9.43	0.00	16.58	26.00

Figure 1-7 Ternary plot of nitrogen in wastewater and artificial media [8–10,13,40,41,43,44,52–66].

Uptake of nitrogen by algae is always in form of ammonia compounds mainly via the GS/GOGAT pathway (glutamine synthetase and glutamine:2-oxoglutarate amidotransferase pathway). Nitrate and nitrite will therefore be converted to ammonium ion before being acquired by algal cells, mostly in the form of glutamine. Thus the optimal TN:TP ratio varies if the source of mineral nitrogen is NH_4-N or NO_3-N, with a larger ratio for the latter [41]. Algae will therefore prefer ammonia over nitrate and nitrite because its assimilation requires less energy. Studies confirm that algae will take up nitrate only after ammonium is depleted [51]. For many algal species, when sufficient ammonium is available nitrate uptake might not occur; however, for nitrogen limited

conditions, algae may assimilate ammonium and nitrate simultaneously. Moreover, for highly carbon-deficient cells, ammonium does not inhibit nitrate uptake.

The mechanisms involved in the inhibition of nitrate uptake when ammonium is present are not completely understood but nitric oxide seems to be part of the inhibitory effect [67]. When the objective of the algae production is to maximize biomass, all forms of nitrogen and the rate of nutrient uptake must be considered. Even if only inorganic forms of nitrogen are considered directly available to algae, some algae can take up organic forms of nitrogen, especially amino acids, urea or purines [27,68]. Care must also be taken to prevent ammonia volatilization as under certain conditions, for example in high rate algal ponds (HRAP), ammonia air stripping might be the primary nitrogen removal mechanism [69]. Soluble phosphorus may precipitate in the presence of a range of cations as aluminium, calcium or iron. Precipitation reactions are governed by pH and thus affected by CO2 concentrations and algal photosynthesis rates. In the presence of magnesium and ammonia, and increased pH associated with accelerated algal photosynthetic activity [70], orthophosphate can precipitate as magnesium ammonium phosphate (struvite) [71], incidentally, a mechanism also employed for recovery of wastewater phosphorus [72]. This impacts phosphorus speciation and may therefore have a major effect on phosphorus recovery rates and production of biomass.

1.2.3.3 Other considerations

Nutrient can be assimilated with the energy provided by photosynthesis which required light and carbon dioxide (CO_2). Light and nutrients are therefore interlinked as photosynthesis can be light-limited thus affecting nutrient fixation and eventually determining nutrient content of biomass in autotrophs [16]. Optimal light intensity is specific for each species [73] and can be affected by the optical parameters of wastewaters, raw or diluted. Cultivation temperature and pH conditions affect algal growth but it is yet unclear how these factors interplay with the nutrient uptake, or how they might influence algal growth and the optimal N: P ratios. Moreover, the complex microbial community including bacteria, yeasts and fungi will compete with algae for nutrients and survival; nutrients and light availability can modify the abundance of all microbes, bacteria and algae and thus affect their direct or indirect interactions and their impact on organic matter degradation rates and nutrient availability kinetics [17].

1.2.4 Conclusion

Nutrient concentrations and availability vary across the wide range of wastewaters available and considered for the cultivation of algae for biomass and bio-products, including biofuels. Simple reporting of nutrient removal, while possibly valuable for very well defined applied scenarios, does not offer sufficient support to advancing the field and hampers comparability across wastewater types and algal species. It is thus propose that any such experimental activity ought to clearly characterize nitrogen and phosphorus

concentrations and offer a detailed description of the speciation of these nutrients in the wastewater substrate employed. Synthetic wastewater as surrogates of real wastewaters ought to explicitly replicate such nutrient speciation, or justify the experimental value of any deviation from a defined wastewater substrate. Clear reporting of experimental conditions is required to insure comparability and replicability and to facilitate an efficient advancement of algal cultivation in wastewaters. Moreover, the research community might benefit from a clearer distinction between the "algae for removal of wastewater nutrients" and "wastewater nutrient for algal production" paradigms.

1.2.5 References

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Chapter 2 : Hypothesis development: Understanding wastewaters as a source of nutrients for autotrophic algal cultivation

2.1 Microalgae growth and nutrients

Microalgae are unicellular photosynthetic organisms that use light energy to fix atmospheric carbon dioxide (CO_2) and accumulate biomass. Algal growth rate describes the change in biomass with time, and biomass accumulation is governed by the availability of resources.

Autotrophic growth of microalgae is governed by the supply of nutrients, essentially nitrogen (N), phosphorus (P), carbon (C), micronutrients, and light. Photosynthesis converts light energy into chemical energy driving fixation of CO_2-carbon in organic forms. Light energy is also necessary to transform inorganic carbon in organic forms. This process, called carbon dioxide fixation, is part of photosynthesis. Light and nutrients are interlinked as photosynthesis can be light-limited thus affecting nutrient fixation and eventually determining nutrient content and proportions of biomass in autotrophs [1].

Algal growth, like for any other plant, is regulated by the law of minimum which states that growth is controlled by the scarcest resource. Droop [2] has assessed the role of the interaction between vitamin B_{12} and P for the growth of *Monochrysis lutheri* and he first demonstrated that algal growth is also regulated by one limiting resource. In 1978, Rhee

[3] evaluated growth of *Scenedesmus* sp. during the transition between the N and P limited states and found results to agree with the law of minimum. The law of minimum has also been confirmed for algae grown in wastewater, in photobioreactors, under varying light or nutrient availability conditions [4].

Note that this must not be interpreted only in terms of nutrient availability but can also be interpreted in terms of lowest uptake rate. For the latter case plant growth shall be limited if uptake rate is slower than the capacity of the plant to assimilate the respective nutrient/factor [5]. The capability of plants to store nutrients might alter the apparent reliance of biomass growth on environmental availability of nutrients [5].

The presence of a large central vacuole in algae generate the potential for storage of organic compounds and inorganic nutrients, which can be used later when the external concentration of nutrients would otherwise limit algal growth. Such capacity to store nutrients varies however among species and is dependent on environmental conditions [1]. Phosphorus content in algae varies between 0.3 and 3%, and nitrogen content between 3 and 12% [6]. Sometimes algae will accumulate nutrients in the form of special storage compounds. Phosphorus can be accumulated as polyphosphate, a salt or ester of polyphosphoric acid. Nitrogen can be stored as nitrate, ammonium or low molecular mass organic compounds [1,7].

The multiple resource limitation hypothesis (MRL) proposed to supplant the law of the minimum states that growth can be limited by scarcity of multiple resources (MRL) [8]. The pattern and response of plants to MRL varies, but is generally described as the

plants' capability to re-allocate resources between tissues and organs to enhance access to metabolically expensive limiting resource [8]. In the case of microalgae such a scenario can be described as a stress response to resource limitation and it usually manifests itself by shifts in the types of organic compounds produced [9]; this phenomenon is relied upon in practice to manage the production of the desired algal compounds.

2.2 Modeling algal growth

To understand, predict and eventually manage algal growth, mathematical models were developed. Ecological models must consider interactions between nutrients and light availability. The theory of ecological stoichiometry, defined as the study of the elements and energy balance, is therefore employed. The elemental content of an organism, as the difference between uptake and losses, is essential for identifying the limiting factor for biomass production.

Mathematical models are mostly based on basic equations of Droop, Monod, Michaelis-Menten and Lambert-Beer's law.

2.3 Growth rate is a function of nutrient concentration

2.3.1 Intracellular control of nutrient

The Droop model [10] is a well-established equation describing the relationship between growth rate and cellular quota for algal cells. Cellular quota defines the intracellular

nutrient concentration of an organism. The equation defines growth rate as a hyperbolic function of nutrient quota:

$$\mu = \mu'_{\max} \quad \frac{Q\text{-}Q_{\min}}{Q} \qquad \text{Eq. 2.1}$$

Q: cellular quota (mol L^{-1})

Q_{min}: minimum cellular quota (mol L^{-1})

μ: specific growth rate (h^{-1})

μ'_{max}: maximum growth rate (h^{-1})

Droop equation can explain the ability of algae to continue to grow few days after nutrient depletion in the medium. As demonstrated by Droop with his experiment with *Monochrysis lutheri* and vitamin B_{12}, a minimum cell quota is required for algae to grow and while, mathematically, μ'_{max} is reached at infinite quota, a maximum practical quota can be assessed for each species. Many empirical studies have confirmed the Droop equation at steady-state [7].

2.3.2 Substrate control on nutrient uptake

Mechanistic modeling of nutrient uptake from substrates assumes that growth rates are limited only by the availability of nutrients, assuming all other factors at ideal steady state.

The Michaelis-Menten enzyme kinetics equation [11] can be employed to describe the initial rate of an enzymatic reaction, when substrate concentration is much greater, and thus not limiting, than the enzyme concentration.

$$v_0 = \frac{v_{\max}\ [S]}{K_m + [S]} \qquad \text{Eq. 2.2}$$

K_m: Michaelis constant (mol L^{-1})

v_0: initial rate of reaction (mol s^{-1})

v_{max}: maximum initial rate of reaction (mol s^{-1})

[S]: substrate concentration (mol L^{-1})

Growth rate under-steady state can be described empirically with Monod's equation [11] as a function of external nutrient concentration:

$$\mu = \mu_{\max}\ \ \frac{R}{K_\mu + R} \qquad \text{Eq. 2.3}$$

K_μ : half-saturation constant for growth rate (mol L^{-1})

R: external nutrient concentration (mol L^{-1})

μ: specific growth rate (h^{-1})

μ_{max}: maximum growth rate (h^{-1})

While Michaelis-Menten mechanistic equation describes the kinetics for a single enzyme, Monod's empirical equation can represent more complex processes with multiple enzymes.

2.3.3 Light as governing factor for nutrient uptake kinetics

2.3.3.1 Light and chlorophyll

Absorption of light by chlorophyll drives photosynthesis. Some models [12,13] consider light absorption to be proportional to the chlorophyll a content of the cells. Chlorophyll a is the primary photosynthetic pigment and can be, most often, considered as the main pigment [11]. Geider et al. [12] used the following equation for the C-specific rate of photosynthesis:

$$P_{phot}^{C} = P_{\max}^{C} \left[1 - \exp\left(-\frac{\alpha^{Chl}\theta^{C}E_0}{P_{\max}^{C}}\right)\right] \quad \text{Eq. 2.4}$$

E_0: Incident scalar irradiance (μmol photons m^{-2})

P^C_{phot}: C- specific rate of photosynthesis (d^{-1})

P^C_{max}: maximum value of P^C_{phot} at temperature T (d^{-1})

α^{Chl}: Chl *a*-specific initial slope of the photosynthesis-light curve (g C m^2 (µmol photons g Chl a^{-1}))

θ^C: Chl *a* : phytoplankton carbon ratio (g Chl a g C^{-1})

Photosynthesis may then be linked to nitrogen (N) assimilation and irradiance:

$$\rho_{Chl} = \theta^N_{\max} \quad \frac{C}{\alpha^{Chl}\theta^C E_0} \qquad \text{Eq. 2.5}$$

C: phytoplankton carbon (g C m^{-3})

E_0: Incident scalar irradiance (µmol photons m^{-2})

α^{Chl}: Chl *a*-specific initial slope of the photosynthesis-light curve (g C m^2 (µmol photons g Chl a^{-1}))

ρ^{Chl}: Chl *a* synthesis regulation term

θ^C: Chl *a* : phytoplankton carbon ratio (g Chl a g C^{-1})

θ^N_{max}: maximum value of Chl *a* : phytoplankton nitrogen ratio (g Chl a g N^{-1})

2.3.3.2 Factors affecting light penetration and attenuation

2.3.3.2.1 Distance/depth

The Lambert-Beer's law is used to describe the relationship between light intensity and material thickness, in our case depth in water. Huesemann et al. [14] used a simple version of the equation to develop a screening model to predict microalgae biomass growth in photobioreactors and raceway ponds. Light intensity decreases exponentially with depth.

$$I(s) = I_{in}\exp(-kAs) \qquad \text{Eq. 2.6}$$

A: algal carbon density (mg C m^{-3})

I: light intensity (μmol photons m^{-2} s^{-1})

I_{in}: light intensity at surface (μmol photons m^{-2} s^{-1})

k: specific light attenuation coefficient of algal biomass (m^2 mg C^{-1})

s: depth below water surface (m)

2.3.3.2.2 Density dependent light attenuation

More complex models [15-18] also consider light attenuation by non-algal components, by employing a background attenuation coefficient (K_{bg}).

$$I(s) = I_{in}\exp\left[-\left(kA + K_{bg}\right)s\right] \qquad \text{Eq. 2.7}$$

Monod's equation can be employed to also link specific production rate as a hyperbolic function of light.

$$p(I) = \frac{p_{\max} \; I}{H+I} \qquad \text{Eq. 2.8}$$

p: specific production rate of algae (d^{-1})

p_{max}: maximum specific production rate of algae (d^{-1})

I: light intensity (μmol photons m^{-2} s^{-1})

H: half-saturation constant for light-dependent algal production (μmol photons m^{-2} s^{-1})

More complex mathematical equations that describe the specific production rate as a function of light have been developed (see Table 2 [19]).

2.3.3.3 Light and nutrient interactions

2.3.3.3.1 Light and intracellular nutrient

Biomass growth model is thus a function of intracellular nutrient (nutrient quota), external nutrient concentration, and light.

Diehl et al. [15] describes the dynamics of phytoplankton, light, and the flexible nutrient quota in a well-mixed water column. A closed system for nutrient was designed and phosphorus was considered as the limiting nutrient.

In the model of Diehl et al. [15], the specific production rate of algae is a function of light and nutrient quota (Q):

$$\int_0^s p[I(s), Q]ds = \frac{p_{\max}}{kA+K_{bg}} ln\left(\frac{H+I_{in}}{H+I_{out}}\right)\left(1-\frac{Q_{\min}}{Q}\right) \quad \text{Eq. 2.9}$$

k: specific light attenuation coefficient of algal biomass (0.0003 m^2 mg C^{-1})

I: light intensity (photon flux) (μmol photons m^{-2} s^{-1})

p: specific production rate of algae (d^{-1})

p_{max}: maximum specific production rate of algae (1.0 d^{-1})

s: depth below water surface (m)

A: algal carbon density (mg C m^{-3})

H: half-saturation constant for light-dependent algal production (120 μmol photons m^{-2} s^{-1})

I_{in}: light intensity at surface (300 μmol photons m^{-2} s^{-1})

I_{out}: light intensity at bottom of mixed layer (μmol photons m^{-2} s^{-1})

K_{bg}: background light attenuation coefficient (0.9 m^{-1})

Q: flexible algal nutrient quota (g P g C^{-1})

Q_{min}: algal nutrient quota at which growth ceases (0.004 g P g C^{-1})

2.3.3.3.2 Light and extracellular nutrients

A second equation is necessary in the Diehl's model to include the external nutrient concentration:

$$\frac{c}{z}\int_0^s p[I(s),R]ds = \frac{c}{z}\frac{p_{\max}}{kA+K_{bg}} ln\left(\frac{H+I_{in}}{H+I_{out}}\right)\frac{R}{M+R} \quad \text{Eq. 2.10}$$

c: fixed algal nutrient quota (0.02 g P g C^{-1})

k: specific light attenuation coefficient of algal biomass (0.0003 m^2 mg C^{-1})

I : light intensity (photon flux) (μmol photons m^{-2} s^{-1})

p: specific production rate of algae (d^{-1})

p_{max}: maximum specific production rate of algae (1.0 d^{-1})

s: depth below water surface (m)

z: depth of mixed water column (m)

A: algal carbon density (mg C m^{-3})

H: half-saturation constant for light-dependent algal production (120 μmol photons m^{-2} s^{-1})

I_{in}: light intensity at surface (300 μmol photons m^{-2} s^{-1})

I_{out}: light intensity at bottom of mixed layer (μmol photons m^{-2} s^{-1})

K_{bg}: background light attenuation coefficient (0.9 m^{-1})

M: half-saturation constant for nutrient uptake (1.5 mg P m^{-3})

R: dissolved mineral nutrient concentration (mg P m^{-3})

The predictions of the model of Diehl et al. [15] has been compared with data of a P-deficient lake. The model correlates the data of field experiment for background turbidity and mixing depth variations.

2.3.4 CO_2 control of growth

2.3.4.1 CO_2 fixation

CO_2 fixation is a critical part of photosynthesis where inorganic carbon is transformed in organic carbon. This process is carried out in the Calvin-Benson cycle dependent on the ribulose-1,5-bisphosphate (RuBP) and driven by the enzyme ribulose-1,5-bisphosphate carboxylase (Rubisco). Rubisco catalyzes CO_2 fixation using ATP as energy and NADPH as reductant. The rate of photosynthesis can be limited by CO_2 concentration fed to Rubisco.

Rubisco, the most abundant protein in all photosynthetic organism, is a slow enzyme with a low specificity for CO_2. When the concentration of CO_2 is low, Rubisco catalyzes RuBP with oxygen (O_2). This process named photorespiration reduces efficiency of photosynthesis. In water, the available carbon for Rubisco is mainly in the form of bicarbonate (HCO^{3-}). Carbonic anhydrase (CA), a buffering enzyme, equilibrates bicarbonate and CO_2 that is supplied to Rubisco. The thylakoidal CA, the most important isoform in algae for providing CO_2 to Rubisco (Hanson et al., 2003) can limit the photosynthetic capacity at high CO_2 concentration. Thus to maintain a stable operation of Rubisco, the CO_2 concentration must be adequate. If the concentration is too low, the enzyme Rubisco will fix O_2 and if it is too high, the pumping capacity of thylakoidal CA will limit the CO_2 fixation [20].

2.3.5 Resource allocation

2.3.5.1 Redfield ratio and luxury consumption

Redfield [21] has empirically developed a stoichiometric ratio of carbon (C), nitrogen (N) and phosphorus (P) for internal phytoplankton composition in deep oceans. The C:N:P ratio (106:16:1) is considered to be constant across the bulk of the ocean. When algae are grown at high growth rates or nutrients are supplied at Redfield ratio, the C:N:P ratio will be close to Redfield proportions. Under nutrient limitation, the actual ratio can diverge strongly from the Redfield ratio [1].

When nutrients are not limiting, phytoplankton will take up and store excess nutrients, a phenomenon is known as luxury consumption. Such storage can lead to the apparent total elemental composition of phytoplankton to diverge from the Redfield ratio [1].

Rhee [3] suggests that there is a species specific optimal cellular N:P ratio. Phytoplankton is however plastic and, under suboptimal conditions, can adapt the nutrient allocation among cellular compartments. The degree of plasticity varies with species. C:N:P stoichiometry depends on physiological response of organism and nutrient supply. Variable resource allocation strategies may be linked to physiological traits (i.e. algal cell size) and life histories. In multiple species algal communities, a particular shift in nutrient supply can lead to species shifts.

2.3.5.2 Resource allocation models

Algal growth modeling must consider unbalanced growth linked to luxury consumption. To explain the variability in C:N:P ratios, Sterner and Elser [1] stated the Growth Rate Hypothesis (GRH) according to which "differences in organismal C:N:P ratios are caused by differential allocations to RNA necessary to meet the protein synthesis demands of rapid rates of biomass growth and development". GRH links the biochemical allocations to growth rate and P content of organisms. The flexibility of allocations can thus explain the variation of C:N:P stoichiometry.

According to the central dogma of biology there are two steps involved in protein synthesis, transcription and translation [22]. In transcription information from DNA is copied to messenger RNA (mRNA) which in turn is translated into proteins synthesized at ribosomes with the help of ribosomal RNA (rRNA). GRH implies that under P depleted conditions RNA production is limited, which in turn limits protein synthesis. Thus the C:N:P ratio of the biomass can be linked to protein synthesis rates.

Many models have been developed with consideration to the concept of resource allocation [1,12,23-28). Resource allocation models describe the optimal strategy for growth and how organisms allocate resources between different cellular functional and structural components. Metabolic energy can be assumed as biomass (carbon) equivalent and partitioned in the equation of growth rate.

For example, Klausmeier et al. [24] developed a model to account for four cellular machineries for phytoplankton growth:

$$R_a + R_N + R_P + R_I = p \qquad \text{Eq. 2.11}$$

p: proportion of cell's dry mass

R_a: assembly machinery (ribosomes) (g g^{-1} dry mass)

R_N: resource-acquisition N-uptake (g g^{-1} dry $mass^{-1}$)

R_P: resource-acquisition P-uptake (g g^{-1} dry mass)

R_I: resource-acquisition light (chloroplasts) (g g^{-1} dry mass)

Chemical composition varies for each machinery. Nutrient quotas vary as a function of nutrient uptake (Droop equations) and photosynthesis (Michaelis-Menten equation). Phytoplankton allocation strategy will determine assembly and uptake rates and therefore growth rate.

2.3.5.3 Relation between N:P ratio and protein-RNA ratio

Loladze and Elser [29] demonstrated the N:P ratio to be related to, and thus describe, the protein-RNA ratio. Their model also demonstrates that under N limitation, constrained protein synthesis leads to an N:P ratio below Redfield ratio. When the RNA synthesis rates are constrained by limited P the model predicts N:P ratio above Redfield ratio. This

confirms that growth requires N (proteins) and protein synthesis requires P (ribosomes RNA).

A model based on biochemical considerations has been developed by Ågren [30]. Ågren assumed protein synthesis, dependent on the amount of ribosomes, to be described through P and the growth rate, a reflection of the rate of C assimilation in proteins, to be described through N. Under stable and balanced growth, N:C ratio increases linearly and P:C ratio increases quadratically with growth rate. This means that N:P ratio increases at low growth rate and decreases at high growth rate. High growth rate requires more RNA, thus more P. Thus, internal quota of N and P can be described as functions of growth rate:

$$Q_N = \frac{\mu}{\varphi_{CN}} + \beta_N \qquad \text{Eq. 2.12}$$

$$Q_P = \frac{\mu^2}{\varphi_{CN}\varphi_{NP}} + \beta_P \qquad \text{Eq. 2.13}$$

Q_N: quota of N (mol N mol^{-1} C)

Q_P: quota of P (mol P mol^{-1} C)

μ: growth rate (d^{-1})

ϕ_{CN}: rate of protein-C synthesis per daily nitrogen assimilation (mol mol^{-1} d^{-1})

ϕ_{NP}: rate of protein synthesis by ribosomes (mol mol^{-1} d^{-1})

β_N: N-containing compounds other than protein per amount of C (mol mol^{-1})

β_P: P-containing compounds other than ribosomes per amount of C (mol mol^{-1})

Growth requires protein, expressed by N concentration, and protein synthesis needs RNA, expressed by P concentration. Protein synthesis is thus proportional to the amount of ribosomes.

$$RNA\ (P) \underset{(ribosomes)}{\Longrightarrow} Proteins(N) \underset{Rubisco}{\Longrightarrow} Biomass(C) \qquad \text{Eq. 2.14}$$

The parameters (ϕ_{CN}, ϕ_{NP}, β_N, β_P) were estimated through regressions (linear and quadratic). The estimated rate of protein-C synthesis per daily nitrogen uptake (ϕ_{CN}) is half of a theoretical protein-C synthesis rate as estimated from observed rates of the catalyzing capacity of Rubisco [31]. The estimated rate of protein synthesis by ribosomes (ϕ_{NP}) was half the rate observed by Sterner and Elser [1]; nevertheless, given the crude estimates, the authors consider the discrepancy between the parameters values not unreasonable, but needs more investigations.

The model of Ågren has also been tested by Bi et al. [32] for three algal species (*Rhodomonas* sp., *P. tricornutum, I. galbana*). Their observed rate of protein-C synthesis per daily nitrogen uptake (ϕ_{CN}) was lower by a factor of 2 to 5 compared with the theoretical rate based of observed rates of the catalyzing capacity of Rubisco [31]. Their observed rate of protein synthesis by ribosomes (ϕ_{NP}) was lower by a factor of 6-14 versus the rate reported by Sterner and Elser [1].

2.3.5.4 Multiple limitation hypothesis

There are a number of papers [33-36] proposing a complex of interactions between nutrients and supporting the multiple limitation hypothesis. Bougaran et al. [35] developed a model with N and P colimitation. They transformed the model of Klausmeier et al. [37] which describes phytoplankton growth under two nutrients according to Liebig's law, and assumes that phytoplankton takes up nutrients at an optimal ratio when no nutrients are limiting.

Bougaran et al. [35] developed a model with the assumption that both N and P will affect nucleic acids and especially RNA associated to growth. The perceived co-limitation is driven by N uptake only. Under P-limited conditions P uptake is controlled by P quota growth rate as described by the Droop model. N uptake is a function of N availability and the P controlled ATP pool. Thus, assuming that N uptake requires energy in the form of ATP, in P starved cells N assimilation is regulated by N availability and P quota.

$$q_N^* = \frac{\rho_{Nmax} q_P^*}{\mu(q_{NL}-q_{N0})(q_{PL}-q_{P0})+\rho_{Nmax} q_P^*} q_{NL} \qquad \text{Eq. 2.15}$$

q^*_N: N saturated quota at steady-state

q^*_P: P saturated quota at steady-state

q_{NL}: hypothetical maximum N quota (mol N mol C^{-1})

q_{N0}: N subsistence quota (mol N mol C^{-1})

q_{PL}: hypothetical maximum P quota (mol P mol C^{-1})

q_{P0}: P subsistence quota (mol P mol C^{-1})

ρ_{Nmax}: nitrate maximum uptake rate (mol N mol C^{-1} d^{-1})

μ: hypothetical growth rate when quota is infinite (d^{-1})

Saturated quota is defined for non-limiting nutrient conditions. In the previous equation, N saturated quota is a function of down-regulating terms (q_{NL} - q_{N0} and q_{PL} - q_{P0}). The down-regulating terms allow for a decreased uptake rate as N and P quotas shift from optimal to minimum; this allows for correction of the common overestimates obtained with the Droop and Monod equations.

The P saturated quota which controls the N saturated quota:

$$q_P^* = \frac{\rho_{Pmax} q_{PL}}{\rho_{Pmax} + D(q_{PL} - q_{P0})} \qquad \text{Eq. 2.16}$$

D: dilution rate (d^{-1})

One can thus assume nutrient saturation under non-limiting nutrient conditions, and limiting nutrient at their minimum quota [35]. Under very high N:P input, the effect of P quota on N uptake has to be included to fit data. Under an species-specific N:P ratio, Droop equation should be used for P; as long as P saturated quota is not reached, N uptake is regulated by P resource.

Isochrysis affinis galbana was grown in a photobioreactor under high and low N:P ratio to validate Bougaran's model. Results have shown that luxury consumption was higher for P compared with N. The model also agrees with results obtained with *Isochrysis*

affinis galbana and *Selenastrum minuturn* [38] for saturated and limiting quota at steady-state.

2.3.6 Experimental evidence on nutrient uptake kinetics

One might hypothesize that for an efficient uptake of nutrient, the concentration ratios of N and P in wastewaters should match the intracellular N:P in algae. Most studies show that algae adjust their intracellular contents of nitrogen and phosphorus to the nitrogen and phosphorus contents in wastewater [39]. Klausmeier et al. [37] determined that phytoplankton adjust their stoichiometry at low growth rates but their stoichiometry remains more stable at high growth rates.

Both nitrogen and phosphorus are important in algae metabolism; as shown above, assimilation of nitrogen and phosphorus are coupled.

2.3.6.1 Nutrient uptake

Efficient removal of nitrogen requires phosphorus. Wastewater from a steel plant containing no phosphate showed a very slow ammonia removal rate [40]. In an experiment with *Scenedesmus* sp. grown in autoclaved medium, phosphorus limitation led to limited nitrogen removal [41]. A similar result was obtained with *Scenedesmus obliquus* grown in nutrient-supplemented autoclaved wastewater, where nitrogen removal was dependent of initial phosphorus [42]. Ammonia removal rate from an industrial

wastewater by *Chlorella vulgaris*, was dependent on phosphate concentration until the phosphate reached a concentration of 15.3 g m^{-3}, ostensibly the saturation quota for P in the said system [40]. Moreover, ammonium uptake is a very variable mechanism strongly influenced by environmental conditions [7].

There are fewer studies on nitrite uptake since nitrite is easily reverted to ammonium and rarely accumulates. The amount of nitrite reductase is higher than nitrate reductase; transformation of nitrate to nitrite seems thus to be the controlling step in the reduction reaction [7].

Batch culture observations suggest a faster uptake of ammonium and nitrate for nitrogen-starved cells compared with replete conditions. When nitrogen is deficient or limiting, the assimilation of nitrogen is limited by the rate of protein synthesis [7].

Phosphorus uptake is also dependent on nitrogen availability. In a study growing two different algae separately, *Chlorella* and *Scenedesmus*, in artificial wastewater, algae have adjusted their intracellular phosphorus concentration in function of their intracellular nitrogen concentration. When nitrogen concentration in the biomass was high, algae could accumulate more phosphorus. However, a low nitrogen concentration in the biomass decreases the phosphorus uptake [43].

In most studies, the rate of phosphorus removal is proportional to the initial phosphorus concentrations [39]. One study with *Scenedesmus obliquus*, cultured in a mineral

medium, showed that phosphorus uptake rate increases with the initial concentration until it reaches a certain constant value [44].

2.3.6.2 Optimal N:P ratio

There are differences in nutrient removal among species. Different metabolic pathways induces a species-specific N:P optimal ratio [39]. Optimal N:P is however not a fixed parameter according to Sterner and Elser [1], it declines as growth rate increases. Algae are more limited by phosphorus at fast growth rates and more easily limited by nitrogen at slow growth rates. This mechanism is linked to the kinetics of the production of phosphorus-rich ribosomal RNA [1]. For *Scenedesmus dimorphus* in an artificial medium the optimal N:P ratio for growth decreased as the dilution rate (1- 4 d^{-1}) increased and the growth rate improved [45].

2.3.6.3 Light and nutrients interactions

Light intensity influences algal nutrient content [1]. Light is generally capable of stimulating inorganic P uptake directly [7]. At lower light intensity (e.g. 25-60 µmol m^{-2} s^{-1}) however, an increase in light intensity has a negative effect on phosphorus luxury uptake. Studies have shown that at lower light levels, microalgae contain more phosphorus. When light increases, a rapid accumulation of phosphorus is observed but a rapid consumption in the metabolism for growth is performed [46,47]. Studies also propose that light can enhance nitrogen uptake [39].

2.3.6.3.1 Light:dark cycle vs continuous illumination

In the absence of light, mixotrophic algae can continue to grow by fixing organic carbon. They therefore use the same metabolic pathways as heterotrophic algae, which require an external organic carbon source. Mixotrophic culture conditions can offer some advantages [48]. For example, *Chlorella kessleri* grown in artificial wastewater have shown greater removal efficiency of organic carbon with light:dark cycle compared to continuous lighting. Nitrate removal was however higher with continuous illumination [49].

2.3.6.3.2 Nutrient and pigments

Nutrient limitation can decrease chlorophyll content of algae and thus photosynthesis rate [7]. Chlorophyll is a nitrogenous pigment and is affected by nitrogen limitation. E.g. nitrogen limitation affected chlorophyll a content of *Chlorella sorokiniana* grown in artificial media but the chlorophyll a content was not disturbed by phosphorus limitation [50].

2.3.7 Managing nutrients

2.3.7.1 Biochemical composition

Carbon allocation to different biomass components depends on growth conditions [51] and species [52]. Photosynthesis fixes CO_2 into sugars which can be synthesized with nitrogen into proteins. Alternatively, carbon can also be channeled into lipid or

carbohydrate molecules according to gross composition [11]. C:N ratio is therefore of major importance to maximize a targeted compound within the algal cells. Considering that algae cell ratio will match the ratio in medium or wastewater, high C:N ratio in medium or wastewater will lead to less proteins in algal cells. Fernandes et al. [53] have confirmed this trend for 3 species, *Nannochloropsis gaditana*, *Rhodomonas marina* and *Isochrysis* sp., with constant aeration and decrease of nutrient concentrations. This nutrient variation did not translate into more lipids for all species. Some species might thus have different responses with different nutrients as all nutrients were reduced in artificial medium [53].

Nutrient availability is therefore another factor that has a direct impact on algae biochemical composition. For replete nutrient conditions, the growth rates stay relatively constant even if the nutrient uptake varies [50], but the biochemical compositions vary strongly [43]. Metanalysis of data from many studies of *Chlorella* has also shown that a higher ammonium concentration can lead to a higher lipid production and lipid productivity. These results probably correlate lipid production and lipid productivity with biomass production and biomass productivity [54]. On the other hand, Li et al. [55] showed that optimization of low nitrogen stress and high photosynthetic capacity adjusted with the initial nitrogen supply led to higher lipid yield for a culture of *Chlorella vulgaris* grown in artificial medium. This technique allows to minimize nutrient requirement and limit stress on algal cells.

2.3.8 Wastewaters as a source of nutrients

2.3.8.1 Managing wastewaters as nutrient sources

2.3.8.1.1 Dilution rate

Most studies of algal production are batch cultures. Studies with continuous or semi-continuous cycles have nevertheless led to higher biomass productivity compared with batch conditions [56].

In continuous mode, the adequate dilution rate must be determined. Dilution rate will have an impact on biomass concentration, biomass productivity, biochemical composition and thus nutrient uptake. The main impact is nevertheless on the biochemical profile of algal cells [57,58].

High dilution rates enhanced nutrient uptake and biomass productivity in many studies. Ammonium uptake of *Desmodesmus communis* grown in primary municipal effluent decreased with the reduction of dilution rate [58]. Nitrogen content of *Scenedesmus dimorphus* grown in artificial medium was increased as dilution rate increases; this was true for a range of N:P ratios. The study shows an increase of phosphorus content with the increase of N:P ratio at high dilution rate (4 d^{-1}) but the inverse trend was seen at low dilution rate (1 d^{-1}) [45]. Also, Samorì et al. [58] and Kunikane et al. [45] have observed that as the dilution rate increases, the nitrogen and phosphorus uptake rates increases.

When dilution rate was increased, from 0.1 d^{-1} to 0.3 d^{-1} (corresponding to 10-30% of volume renewal per day), total biomass of *Chlorella vulgaris* grown in concentrated

desalination brine was lower but the biomass productivity, which is defined as the rate of generation of biomass expressed per volume (mg L^{-1} d^{-1}), increased. The increase of dilution rate also led to a lower protein and a higher lipid content of algal cells [59]. Sobczuk and Chisti [60] obtained similar results for biomass concentration (expressed as mg L^{-1}) and biomass productivity (expressed as mg L^{-1} d^{-1}) for the microalga *Choricystis minor* grown in artificial medium under replete nutrient conditions. Samorì et al. [58] had also obtained a lower protein content when varying dilution rate from 0.14 and 0.67 d^{-1} for *Desmodesmus communis* in primary municipal effluent. However, the biomass productivity remained stable over different dilution rates.

2.3.9 Wastewater and Biomass production

Biomass yields do not necessarily vary with variation of nutrient in wastewater. The biomass productivity stays similar because of the luxury consumption [50].

The review of Chiu et al. [54], summarized the impact of ammonium and total phosphorus on biomass production and productivity across multiple studies on *Chlorella* grown in wastewater. The influence of ammonium and total phosphorus for biomass production and biomass productivity was comparable. However, those studies had different growing conditions and used different species of *Chlorella*.

The biomass expressed as unit cell weight (unit cell weight = dry weight/cell density) can be employed as an indicator of algal biomass yield. Biomass compounds expressed per

unit cell weight can also indicate luxury consumption in cells. Even if results with unit cell weight can bring important information, there are only few studies reporting results with this parameter [55].

2.3.10 Wastewater, other considerations

2.3.10.1 Light interference

At high light intensity, the photosynthetic system of algae can be negatively affected and lead to photoinhibition. On the other hand, too low light levels will limit photosynthesis. Algae cells have however the capacity to adapt their photosynthetic response to light variability. They will adjust their light absorption, i.e. photoacclimation, to limit photosynthetic damages. Optimal light intensity is specific for each species [61].

When algae are grown in diluted cultures, there is no significant light gradient. However, high density culture will lead to light changes in layers. In batch cultures algal density increases over time; the light is attenuated by absorption by the algal pigments and through scattering which will impact negatively biomass production. An incremental light intensity strategy can therefore avoid photoinhibition at the early stage of the cultivation and provide sufficient light at the following stages of the algal cultivation [62]. Light availability is declining exponentially with depth for reactors or ponds illuminated from the top. Therefore, depth of the culture and mixing must be taken into consideration to maximize algae growth. Mixotrophic/heterotrophic cultivation mode and

vertical mixing have been proved to overcome light limitation and improve biomass productivity [63].

High turbidity of agricultural wastewater caused by high amount of dissolved organic compounds can also limit photosynthesis. Agricultural wastewater is therefore diluted, filtered or centrifuged and settled to enhance light penetration and algal growth [64].

2.3.10.2 Toxicitiy

Many toxic compounds present in certain wastewaters can compromise algal growth. For example, heavy metals can inhibit photosynthesis, and viruses can stop algal growth [65]. High ammonia concentration will also cause toxicity and inhibit algal growth especially when algae are grown in undiluted anaerobic digestion effluents. This toxicity intensifies with pH and temperature.

2.3.10.3 pH stability

pH is an important parameter for algae cultures and the optimal pH varies among species. When it is not controlled, algal photosynthetic activities induce an increase of pH. Omitting to maintain a stable pH during algal cultivation can affect algal growth because changes in pH affect carbon dioxide availability and thus decrease photosynthetic rates. Moreover, high pH can lead to volatilization of ammonia and precipitation of phosphate. However, high pH has proven helping to prevent contamination and increase lipid accumulation in algal cells grown in outdoor cultures with anaerobic digested effluent

[66] and olive-mill wastewater [67]. A pH control strategy can thus be necessary for some types of cultivation.

Some forms of nitrogen can nonetheless produce hydrogen ions and acidify the culture during algal photosynthesis. If one includes splitting of water and reduction of electron carriers as proposed by Scherholz and Curtis [68], photosynthesis equation will include production of hydrogen ions.

$$\alpha_i CO_2 + \lambda_i N_i + \delta_i H_2O \xrightarrow{h\nu} CH_xN_yO_z + \lambda_i O_2 + \varphi_i H^+ \qquad \text{Eq. 2.17}$$

Algae consuming nitrate show an increase of pH that might indicate that the produced hydrogen ions are used to reduce nitrate to ammonium for assimilation [68]. The inverse trend of pH for algae growing on ammonia indicates however a generation of free hydrogen ions.

Most of pH declines observed during algae growing on ammonia are with artificial medium [e.g. 69,70]. Many types of wastewaters containing ammonia alone or with other nitrogen forms resulted in an increase of pH and the latter had to be controlled with CO_2 (Supplementary data- B). The presence of a microbial community in wastewaters might affect uptake of nitrogen and avoid or compensate the release of hydrogen ions. Some centrate containing ammonia had however experienced pH decrease [71,72]. Wang et al. [72] had explained the decrease of pH observed during light period with a highest proton concentration released by nitrification/nitritation compared with hydroxide ions

concentration released by algal photosynthesis. A minor pH increase during the dark period would have be caused by denitritation which produce hydroxide ions.

2.3.10.4 Competition for nutrients

A complex microbial community including bacteria, yeasts and fungi is present in wastewater. This population will compete with algae for nutrients and survival. Bacteria and algae communities can lead to complex relationships of commensalism, mutualism, parasitism or antagonism [73]. Therefore, algae can help to promote bacteria growth by providing oxygen and organic compounds and bacteria can provide carbon dioxide to algae. Cultivation conditions and nutrient availability can promote competition for nutrients but some co-culture have also been reported to enhance removal nutrients and algal growth [74]. Moreover, a controlled zooplankton community in high rate algal ponds (HRAPs) can help to maintain an ecological balance [75].

Consequently, to avoid microbial contamination of algal cultures, proper operating conditions should be maintained. Selecting microalgal strains isolated from the local environment or mixed cultivation is also recommended to improve cultivation stability of the system [63].

2.4 Summary

Given the variability of nutrient concentrations, forms, and availability in wastewater streams the reporting of nutrient removal by cultivation of algae may be a) inaccurate as, most often, organic forms of nutrients and changes in their concentrations are not considered, and b) of limited value for the development of an efficient biomass production management system, as most are rather descriptive in nature [76,77].

While algae adapt to sub-optimal concentrations and nutrient ratios, this is generally associated with slow growth. Best algal growth for enhanced biomass productivity and removal of nutrients will likely occur within a range near the optimal conditions for the respective algal species.

Thus understanding, and accordingly, correcting for nutrient deficiencies can maximize algal biomass and enhance the overall quality of wastewater treatment.

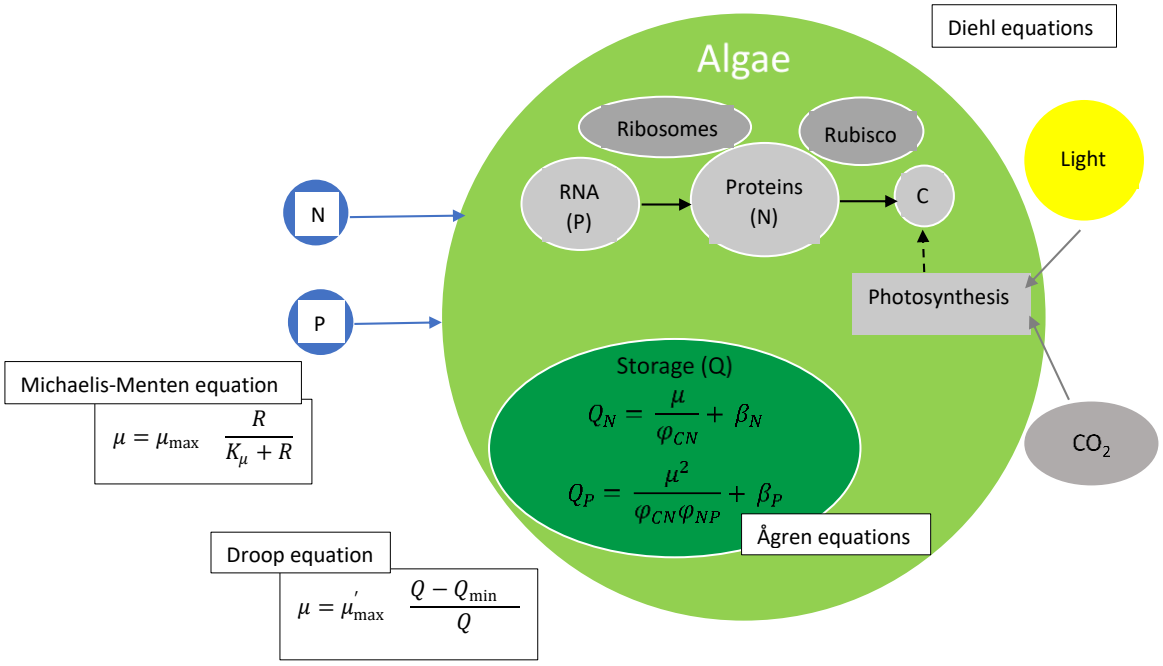
2.5 Hypothesis:

- As most wastewaters do contain both nitrate and ammonium it is expected that in the presence of algae there will be a preferential depletion of one of these nitrogen species (more commonly ammonium) before the other nitrogen species is significantly removed.

- Thus it is hypothesized that algal growth and nitrogen uptake kinetics in substrates that contain a mix of nitrate and ammonium is governed by the availability of the preferred chemical species
    - Nitrate uptake is accelerated in the absence of ammonia
    - A shift from an NH_3-N rich substrate to a 100% NO_3-N substrate (e.g. after selective depletion of NH_3-N) will induce a permanent or reversible stress, species dependent, evident in the algal growth and algal stoichiometric balance.

Notes:

By measuring carbon, nitrogen and phosphorus in the growth medium and within algal cell, one can differentiate between assimilated and accumulated nutrient in algal cells. Nutrient concentrations and cell biochemistry are therefore linked to nutrient uptake, nutrient assimilation and photosynthesis in algal cells (Figure 2.3).



c: fixed algal nutrient quota
k: specific light attenuation coefficient of algal biomass (0.0003 m^2 mg C^{-1})
p: specific production rate of algae (d^{-1})
p_{max}: maximum specific production rate of algae (1.0 d^{-1})
s: depth below water surface (m)
z: depth of mixed water column (m)
A: algal carbon density (mg C m^{-3})
H: half-saturation constant for light-dependent algal production (120 µmol photons m^{-2} s^{-1})
I : light intensity (photon flux) (µmol photons m^{-2} s^{-1})
I_{in}: light intensity at surface (300 µmol photons m^{-2} s^{-1})
I_{out}: light intensity at bottom of mixed layer (µmol photons m^{-2} s^{-1})
K_{bg}: background light attenuation coefficient (0.9 m^{-1})
K_μ : half-saturation constant for growth rate (mol L^{-1})
M : half-saturation constant for nutrient uptake(1.5 mg P m^{-3})
Q: cellular quota (mol L^{-1}) (Diehl: g P g C^{-1})
Q_{min}: minimum cellular quota (mol L^{-1}) (Diehl: 0.004 g P g C^{-1})
Q_N: quota of N (mol N mol^{-1} C)
Q_P: quota of P (mol P mol^{-1} C)µ: growth rate (d^{-1})
R: external nutrient concentration (mol L^{-1}) (Diehl: mg P m^{-3})
β_N: N-containing compounds other than protein per amount of C (mol mol^{-1})
β_P: P-containing compounds other than ribosomes per amount of C (mol mol^{-1})
µ: specific growth rate (h^{-1})
μ_{max}: maximum growth rate (h^{-1})
μ'_{max}: theoretical maximum growth rate (h^{-1})
ϕ_{CN}: rate of protein-C synthesis per daily nitrogen assimilation (mol mol^{-1} d^{-1})
ϕ_{NP}: rate of protein synthesis by ribosomes (mol mol^{-1} d^{-1})

Figure 2-1 Nutrients in algal cell

2.6 References

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Chapter 3 Methodology

3.1 Algal species and experiment

Chlorella vulgaris CPCC90 and *Scenedesmus obliquus* CPCC5 have been provided by Canadian Phycological Culture Centre at the University of Waterloo. *C. vulgaris* and *S. obliquus* have been extensively studied and have proved to be adequate species for wastewater treatment [1]. They also seem to be a good option for wastewater with variable concentration due to their flexible internal nitrogen:phosphorus composition [2]. Another 10 isolates were offered by the Institute for Marine Biosciences - National Research Council (NRC, Halifax, NS) from their own collection. Of these three isolates, *Micractinium pusillum* MCWW-S27, *Chlorella vulgaris* SMC-2M and *Scenedesmus obliquus* SMC-6M, have been chosen to perform the experiments. Growth of algae was first evaluated in flasks. *Chlorella vulgaris* CPCC90 growth have also been evaluated in environmental photobioreactors (ePBR101, Phenometrics). Experiments were thereafter performed in ePBRs with different nitrate/ammonia (NO_3/NH_3) ratios and in flasks with nitrate or ammonium. Treatments are summarized in Table 3.1.

Table 3-1 Tests performed

Species	Growth evaluation in flasks	Growth evaluation in ePBRs	Two-stage nitrogen treatment in flasks (Grown in – Resuspended in)	Tests in ePBRs
Chlorella vulgaris CPCC90	100% NO_3	100% NO_3 100% NH_3	NO_3 – NO_3 NO_3 – NH_3 NH_3 – NO_3 NH_3 – NH_3 NO_3 – 0N	100% NO_3 100% NH_3 66% NO_3-N and 34% NH_3-N 34% NO_3-N and 66% NH_3-N
Scenedesmus obliquus CPCC5	100% NO_3			
MCWW-S3: *Pseudotetracystis* sp.	100% NO_3			
MCWW-S10: *Chlorella* sp.	100% NO_3			
MCWW-S11: *Dictyophaerium* sp.	100% NO_3			
MCWW-S12: *Tetracystis vinatzeri*	100% NO_3			
MCWW-S27: *Micractinium pusillum*	100% NO_3		NO_3 – NO_3[1] NO_3 – NH_3[1] NO_3 – mixN[2]	
MCWW-S30: *Tetracystis vinatzeri*	100% NO_3			
SMC-2M: *Chlorella vulgaris*	100% NO_3		NO_3 – NO_3[1] NO_3 – NH_3[1] NO_3 – mixN[2]	
SMC-6M: *Scenedesmus obliquus*	100% NO_3		NO_3 – NO_3[1] NO_3 – NH_3[1]	

[1] 2 batches were carried out

[2] mixN is a a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L).

3.2 Growth conditions

3.2.1 Flasks

Prior to inoculation, the algae were grown on autoclaved modified Bold's basal medium (BBM) composed of 1.29 mmol/L KH_2PO_4, 0.17 mmol/L $CaCl_2 \cdot 2H_2O$, 0.30 mmol/L $MgSO_4 \cdot 7H_2O$, 2.0 mmol/L KNO_3, 0.43 mmol/L K_2HPO_4, 0.43 mmol/L NaCl, 0.018 mmol/L $FeSO_4 \cdot 7H_2O$ with 0.001 mL/L concentrated H_2SO_4, 1 mL/L trace metal solution, 0.13 mmol/L H_3BO_3, 1 mL/L f/2 vitamin solution. Growth evaluation of *C. vulgaris* CPCC90 in flasks was however performed with 2.94 mmol/L $NaNO_3$ instead of 2.0 mmol/L KNO_3. The composition of the trace metal solution was 46.13 mmol/L H_3BO_3, 9.14 mmol/L $MnCl_2 \cdot 4H_2O$, 0.774 mmol/L $ZnSO_4 \cdot 7H_2O$, 1.612 mmol/L $Na_2MoO_4 \cdot 2H_2O$, 0.317 mmol/L $CuSO_4 \cdot 5H_2O$, 0.170 mmol/L $CoCl_2 \cdot 6H_2O$ and the composition of the f/2 vitamin solution was 0.0007 mmol/L vitamin B12, 0.004 mmol/L biotin, 0.6 mmol/mL thiamine HCl. The pH of medium was adjusted to 6.8 ± 0.1. Algae were cultured to log phase under continuous agitation (100 rpm) at room temperature in 250 mL Erlenmeyer flasks. Light was provided by a Morsen 600 W Double Chips 10 W LED Grow Light Full Spectrum with an intensity of 45 ± 3 µmol m^{-2} s^{-1}. Light was measured with an APOGEE MQ-500 Full spectrum quantum sensor. Carbon dioxide available in the air was used as carbon source for photosynthesis.

3.2.2 ePBRs

Algae was also grown in 6 ePBRs (Figure 3.1) equipped with conical vessel cultures (height of 270 mm) and illuminated by a white high power LED through the vessel cap. Light intensity was set to 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ the first two days and was then increased to 100 $\mu E \cdot m^{-2} \cdot s^{-1}$. Temperature control jacket equipped with thermoelectric elements (heaters and coolers) allowed to maintain the temperature at 25 °C. pH was also continuously monitored with pH probes and was adjusted to 6.8±0.1 with addition of carbon dioxide through the top of the reactor. The culture was continuously mixed with a magnetic stir bar (300 rpm).

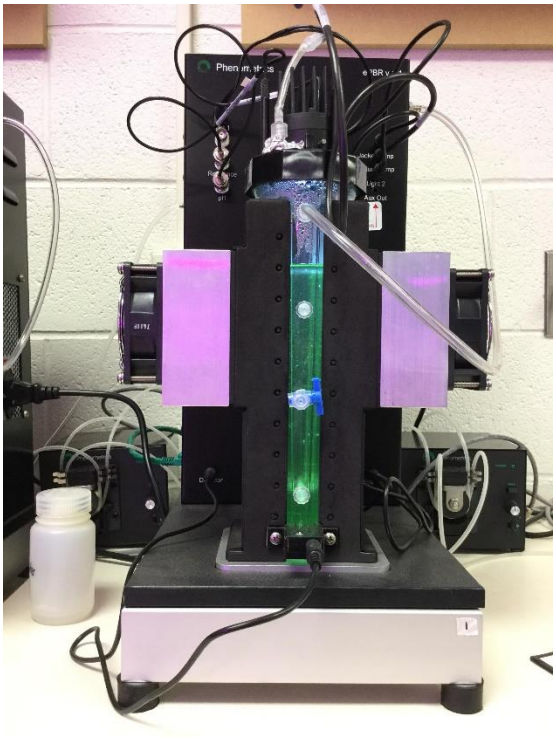


Figure 3-1 Environnemental photobioreactors ePBR101, Phenometrics

3.3 Operating conditions – tests

Laboratory scale experiments were performed in 250 Erlenmeyer flasks under continuous agitation (100 rpm), at room temperature, in 100 mL of medium.

The nitrogen chemical species was the variable parameter in this study. All other nutrients were set to ensure copiotrophic conditions. KNO_3 or NH_4Cl were added to the medium, according to the treatments presented in Table 3.1.

The concentration of ammonia and nitrate was defined according to the average municipal secondary effluent concentration for ammonia or nitrate; 2 mmol N/L (Chapter 1).

The medium of the first batch of the NO_3-NO_3 treatment (i.e. initially grown in NO_3 only substrate and then resuspended in NO_3 only substrate) for MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus*, had however a lower concentration of 1 mmol NO_3-N/L.

3.3.1 Algal transfer for the two-stage experiment

A volume of 50 mL of algal culture collected around mid-exponential phase from the first stage was centrifuged (5000 *g*, 10 minutes) and used to inoculate second-stage flasks. Each treatment was carried out in batch mode and had three replicates. For ePBRs tests, a volume of 1 mL (Run 1), 20 mL (Run 2) and 50 mL (Run 3) of inoculum was added to medium to a final volume of 500 mL in the reactors.

3.4 Laboratory analyses

3.4.1 Algal growth

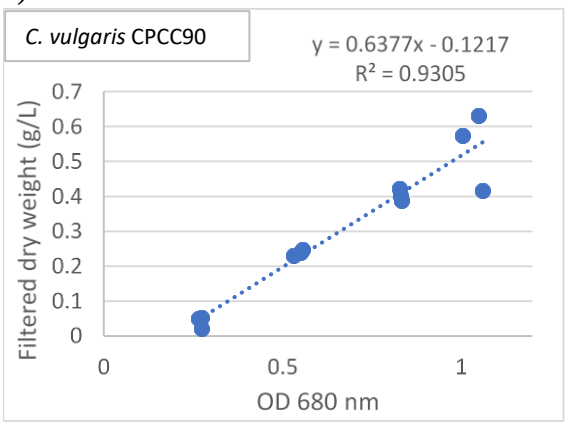
As growth is not only manifested with an increase in the number of cells, but also with an increase in cell volume, multiple measurements were carried out to assess growth kinetics: OD680, OD750, cell count, and dry weight biomass were therefore all considered to improve the understanding of algal growth.

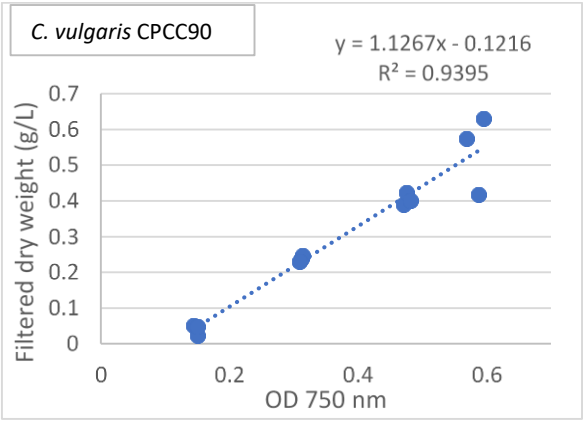
3.4.1.1 Dry weight biomass

Daily dry weight biomass was indirectly evaluated through optical density proxy measurements to overcome the challenge to weigh very small algal biomass (less than 1 mg).

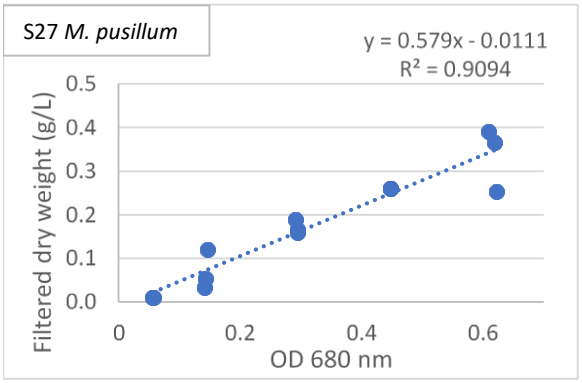
For this a calibration of optical densities (Figure 3.2) as correlated to true dry weight was obtained. For the calibration dry weight was measured by vacuum filtration using a 0.45 µm nylon membrane filter (Whatman 47 mm), with 3 replicates of 9.8 mL. The filters were pre-weighted and then oven dried for 2 hours at 104 °C. Biomass concentration was calculated as the difference in mass divided by volume. Ash content of dry microalgae, another common approach to biomass measurements, as it might lead to a biased assessment of absolute dry weights, usually within an error range of by 8 to 10% [3].

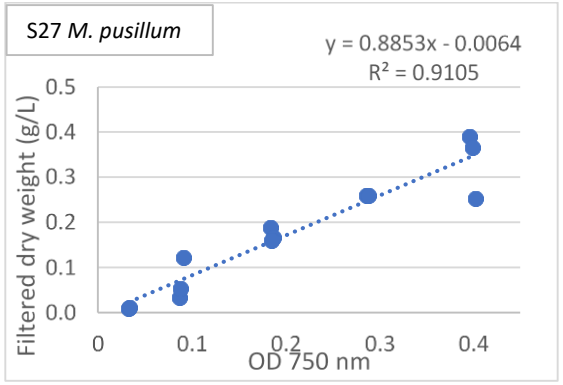
a)


C. vulgaris CPCC90
y = 0.6377x - 0.1217
R² = 0.9305
Filtered dry weight (g/L)
OD 680 nm

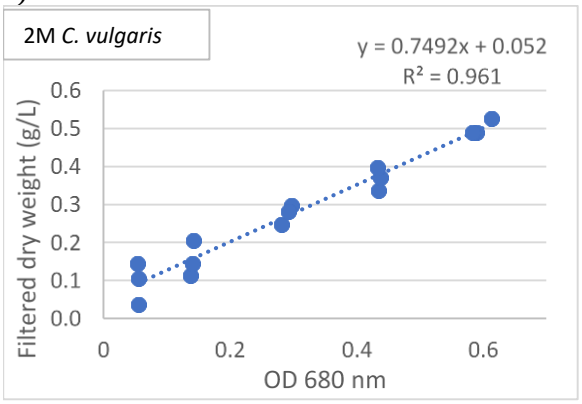

C. vulgaris CPCC90
y = 1.1267x - 0.1216
R² = 0.9395
Filtered dry weight (g/L)
OD 750 nm

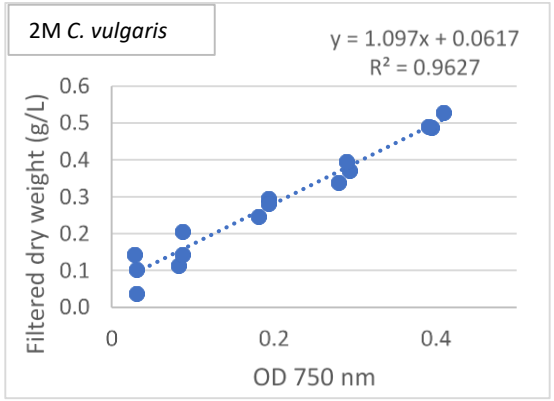
b)


S27 M. pusillum
y = 0.579x - 0.0111
R² = 0.9094
Filtered dry weight (g/L)
OD 680 nm


S27 M. pusillum
y = 0.8853x - 0.0064
R² = 0.9105
Filtered dry weight (g/L)
OD 750 nm

c)


2M C. vulgaris
y = 0.7492x + 0.052
R² = 0.961
Filtered dry weight (g/L)
OD 680 nm


2M C. vulgaris
y = 1.097x + 0.0617
R² = 0.9627
Filtered dry weight (g/L)
OD 750 nm

d)

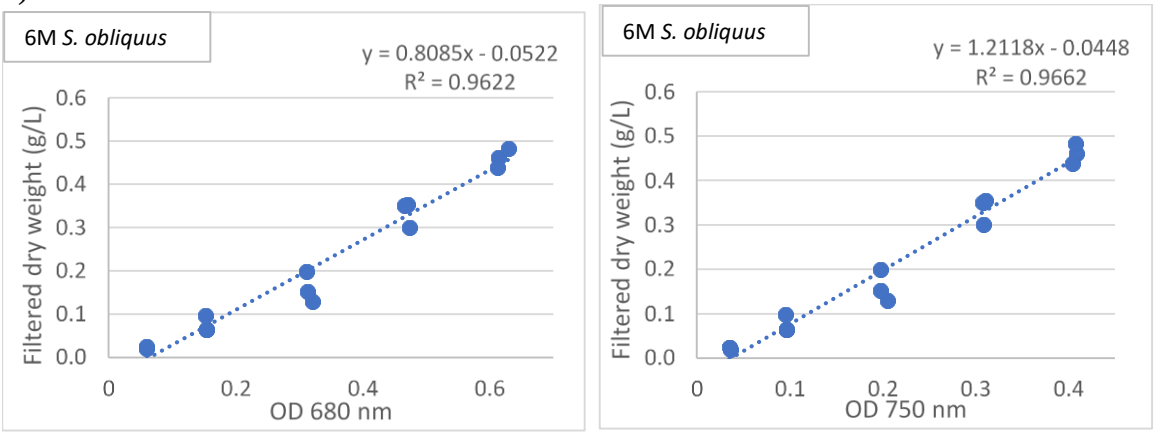


Figure 3-2 Filtered dry weight as a function of OD680 nm and OD750 nm; a) C. vulgaris CPCC90 b) MCWW-S27 M. pusillum c) SMC-2M C. vulgaris d) SMC-6M S. obliquus.

3.4.1.2 Cell counts

Cells counts were also measured, as an indicator of growth [4], on an Attune Acoustic Focusing Cytometer (Applied BioSystems, Life Technologies). Cell count were carried out with a 488 nm (blue) 20 mW laser; the autofluorescence signals were measured through photomultiplier voltage gain parameters on forward scatter (FSC) at excitation of 2750 mV, side scatter (SSC) at excitation of 4450 mV. The BL3 channel using the 640 nm longpass filter (>640 nm) at excitation of 1300 mV. Lower excitation thresholds of 250 mV and 20 mV for SSC and BL3, respectively, were set to remove noise and debris. Calibration was daily performed with Attune performance tracking beads.

3.4.1.3 Optical density measurements

Since chlorophyll fluorescence is absorbed at a wavelength of 680 nm, optical density at 680 nm (OD680) was used as a proxy to measure chlorophyll. Optical density was also determined at

750 nm (OD750) which represents total density of the culture [5]. Optical densities were measured with a Synergy HT microplate reader.

pH

pH was monitored with a Metler Toledo FiveEasy F20 pH-meter.

3.4.1.4 Management of culture contamination

To perform experiments in an environment as sterile as possible, flasks and media were autoclaved before each experiment. Sampling was also done with aseptic techniques and autoclaved equipment. A higher-power compound microscope Nikon was used to visualize algae cells and ensure that no bacteria, or a very low proportion of bacteria were present. Results from the flow-cytometer were also used as an indicator for possible contamination.

3.4.2 Growth substrate nutrient monitoring

During experiments, samples were taken to measure nutrients in medium and algae. For the latter, samples were thereafter centrifuged (10 000 *g*, 5 minutes). Oven dried (60 °C, 1 h) pellets and supernatants were kept frozen for further analyses of nitrate, ammonium, total nitrogen and carbon. Nutrients in supernatants were analyzed on a Lachat Quickchem 8500 Series 2 autoanalyzer.

Evaluation of nitrogen uptake rate by algae (i.e. nitrogen use efficiency) was calculated according to the number of cells (Eq. 3.1).

$$NU = \frac{N\ concentration(t_1)\text{-}N\ concentration(t_2)}{cells\ count_{(t_1)}(t_2\text{-}t_1)} \qquad \text{Eq. 3.1}$$

NU: nitrogen uptake rate (mmol N $cells^{-1}$ d^{-1})

N concentration(t_1): initial nitrogen concentration (mmol N/L)

N concentration(t_2): final nitrogen concentration (mmol N/L)

Cells count(t_1): initial number of cells (cells/L)

t_1: initial day (d)

t_2: final day (d)

3.4.2.1 Cell chemistry survey

Total carbon and nitrogen in cells were analyzed with an elemental analyzer PerkinElmer 2400 Series II CHN. Given the very low mass of algae (less than 1 mg), acid washed sand (12-15 mg) was added to pellets. Carbon:Nitrogen ratio (C:N) have been calculated and changes of C:N ratio (C:N slopes) have been determined with linear correlations representing C:N over time.

To analyze nitrate in algal cells, cells were lysed with freeze/thaw cycles (-80 °C / 38 °C), resuspended in deionized water and centrifuged (10 000 *g*, 5 minutes). The supernatant was then analyzed on a Lachat Quickchem 8500 Serie 2 autoanalyzer.

3.5 Growth rate calculations

Growth rate was evaluated over time with cells count (Eq. 3.2).

$$\mu = \frac{\ln[cells\ counting(t_2)/cells\ counting(t_1)]}{t_2\text{-}t_1} \quad \text{Eq. 3.2}$$

μ: growth rate (d^{-1})

cells counting(t_1): initial number of cells per volume (cells/mL)

cells counting(t_2): final number of cells per volume (cells/mL)

t_1: initial day (d)

t_2: final day (d)

Growth rate during exponential phase was determined as the slope of the linear segment of the natural logarithm of OD 750 nm over time. The linear segment represents the exponential phase growth and it is assumed to be constant over the considered time period.

3.6 Calculation of minimum pH caused by CO_2 and ammonium chloride dissolution

Consumption of ammonium by algae acidifies cultures by the release of hydrogen ions. Moreover, dissolution of carbonic acid can also release hydrogen ions. Considering that algal culture and CO_2 form a gas-liquid system at equilibrium, the concentration of CO_2 that is

dissolved in water can be determined with Henry's law (Eq. 3.3). Equation 3.4 represents the simplified equilibrium for acidic conditions. Thus, to calculate hydrogen ions concentration released with CO_2 dissolution, the concentration of CO_2 in water is calculated (Eq. 3.3), and then concentration of hydrogen ions (H^+) can be calculated (Eq. 3.5).

$$p_{CO2} = x_{CO2} H_{CO2} \quad \text{Eq. 3.3}$$

p_{CO2}: partial pressure of CO_2 (atm); 0.03% of CO_2 in air

x_{CO2}: concentration of CO_2 in liquid

H_{CO2}: Henry constant; CO_2 25 °C, 1 atm: 3.3E-2 mol L^{-1} atm^{-1} [6]

$$CO_2 + H_2O \Leftrightarrow H^+ + HCO_3^- \quad \text{Eq. 3.4}$$

$$K_A = \frac{[H^+][HCO_3^-]}{[CO_2]} \quad \text{Eq. 3.5}$$

K_A: Acidity constants, CO_2 25 °C: 4.45E-7 [7]

$[H^+]$: hydrogen ions concentration (mol/L)

$[HCO_3^-]$: bicarbonate ions concentration (mol/L)

$[CO_2]$: carbon dioxide concentration in liquid (mol/L)

Production of hydrogen ions caused by ammonium chloride dissolution is calculated with equation 3.8 considering the equilibria of equations 3.6 and 3.7. Thereafter, the sum of hydrogen ions can be used to calculate the minimum pH reached with ammonium and CO_2 in water (Eq. 3.9).

$$NH_4Cl \Leftrightarrow NH_4^+ + Cl^- \quad \text{Eq. 3.6}$$

$$NH_4^+ + H_2O \Leftrightarrow NH_3 + H^+ \quad \text{Eq. 3.7}$$

$$K_A = \frac{[NH_3][H^+]}{[NH_4^+]} \quad \text{Eq. 3.8}$$

$$pH = \log[H^+] \quad \text{Eq. 3.9}$$

K_A: Acidity constants, NH_4^+ 25 °C: 5.6E-10 [6]

$[H^+]$: hydrogen ions concentration (mol/L)

$[NH_3]$: ammonia concentration (mol/L)

$[NH_4^+]$: ammonium ions concentration (mol/L)

3.7 Statistical analysis

Statistics were carried out with Minitab 17. Analyses, including ANOVA, were evaluated for 95% confidence intervals. LAB Fit [8] was also used to perform analyses on growth rates and plot 3D graphs.

3.8 Influence of the location of flask on the shaker

A grid was added on the shaker (Figure 3.3) to evaluate if the position on the shaker had an impact on growth. The shaker had an orbital movement and algae growing at the edge of the shaker seemed to produce more clumps. Edges of shaker were therefore avoided for growth experiments. ANOVA have however revealed that the position on the shaker did not have a significant impact on exponential growth rate ($p > 0.05$).

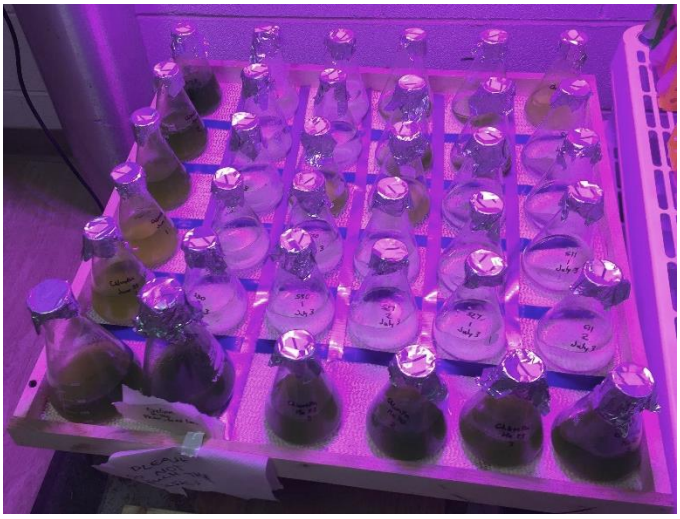


Figure 3-3 Shaker

3.9 Evaluation of environmental photobioreactors (ePBRs)

Three runs growing *Chlorella vulgaris* CPCC90 were performed in ePBRs with different ammonia/nitrate ratios. Optical densities and cell count in ePBRs (Run 1 to 3) were very low; less than 0.3 for OD 680 nm. By contrast on the shaker, OD680 of *Chlorella vulgaris* CPCC90 grown in nitrate can reach about 1 before cells count decreases (Supplementary data - D). To overcome the problem of a long latent period, more inoculum has been added (from 1 mL to 50 mL). The increase of inoculum volume has helped to reduce the latent period but the growth was still low compared with the growth on the shaker (Supplementary data - D).

Mixing in ePBRs were done by a magnetic stirring bar and even if the speed of the bar was increased, the mixing was visually not optimal. Small air pumps were added (Run 3) to enhance mixing in the ePBRs. However, inadequate air filtration had resulted in contamination of algal culture by bacteria (Figure 1 Supplementary data - C).

3.10 References

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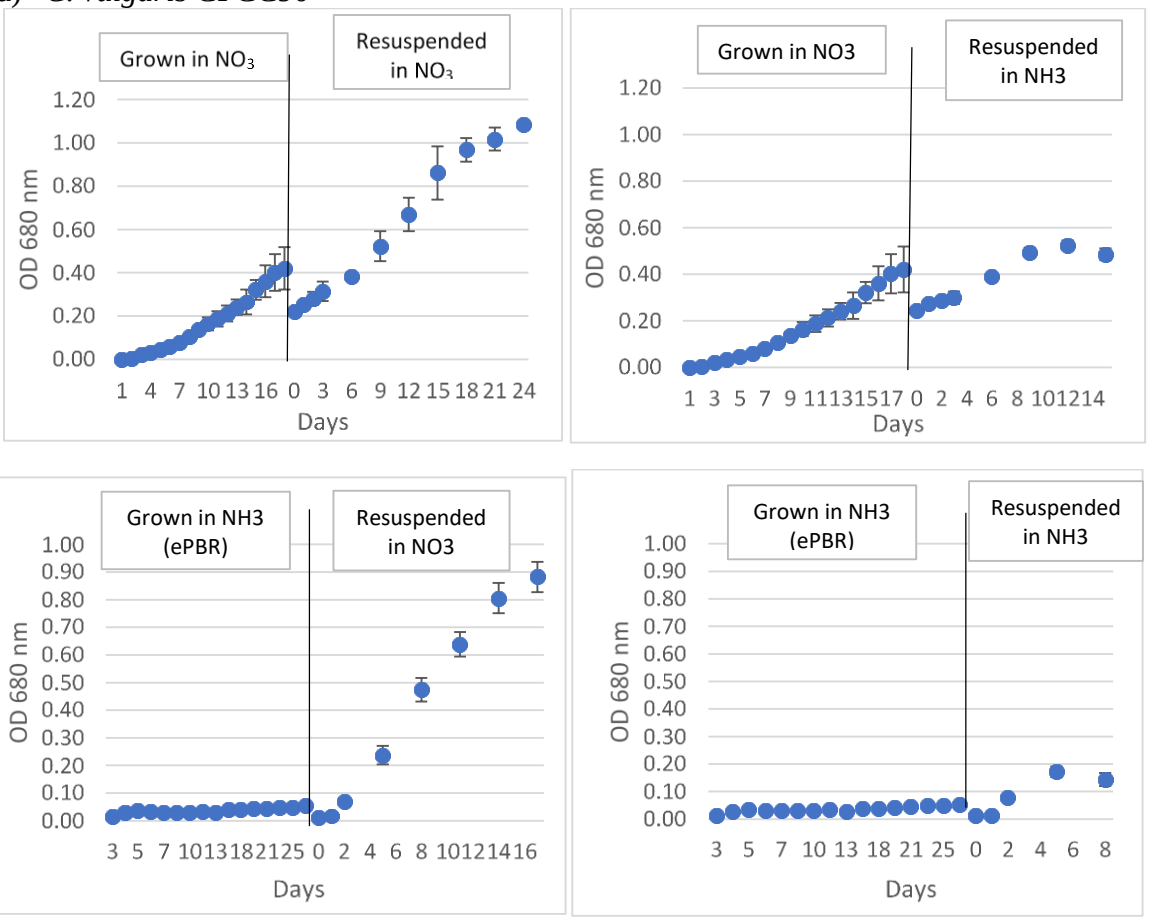
[8] W.P. Silva and C.M.D.P.S. Silva, C.M.D, LAB Fit Curve Fitting Software (Nonlinear Regression and Treatment of Data Program) V 7.2.49 (1999-2016), online, available from world wide web: <www.labfit.net>, date of access: 2017-11-02.

Chapter 4 Results

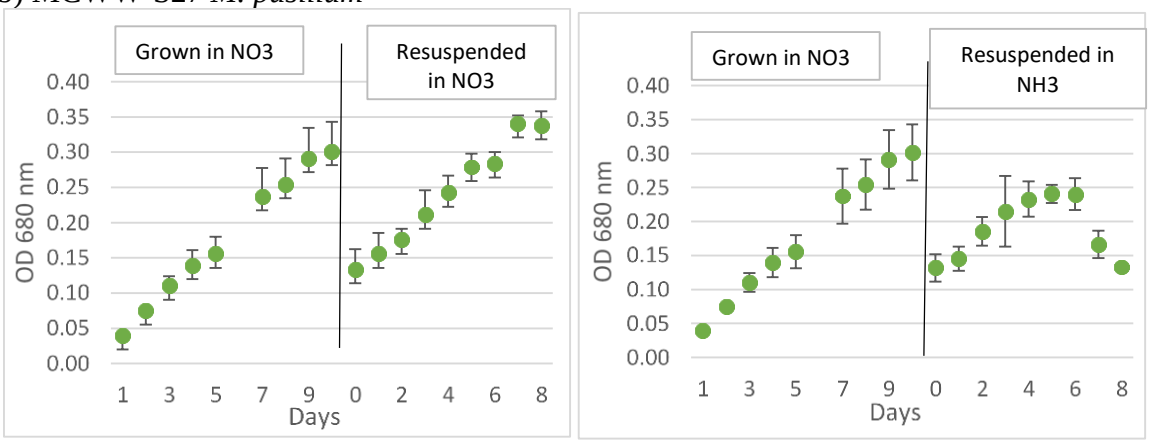
4.1 Growth evaluation

Growth of algae in flasks before (first stage; S1) and after resuspension (second stage; S2) is shown in Figure 4.1. For resuspension, algae from a 50 mL aliquot from the first stage were separated by centrifugation and inoculated in 100 mL of medium.

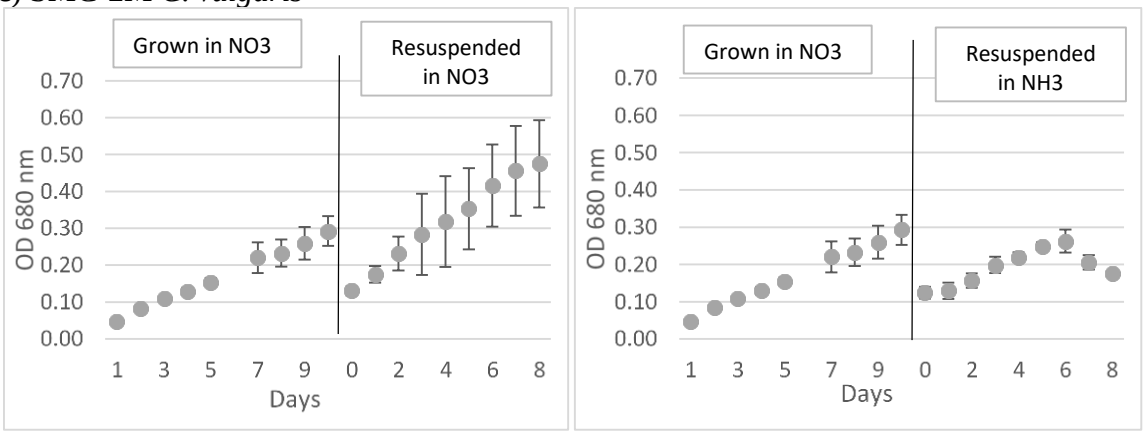
a) *C. vulgaris* CPCC90



b) MCWW-S27 *M. pusillum*



c) SMC-2M *C. vulgaris*



d) SMC-6M *S. obliquus*

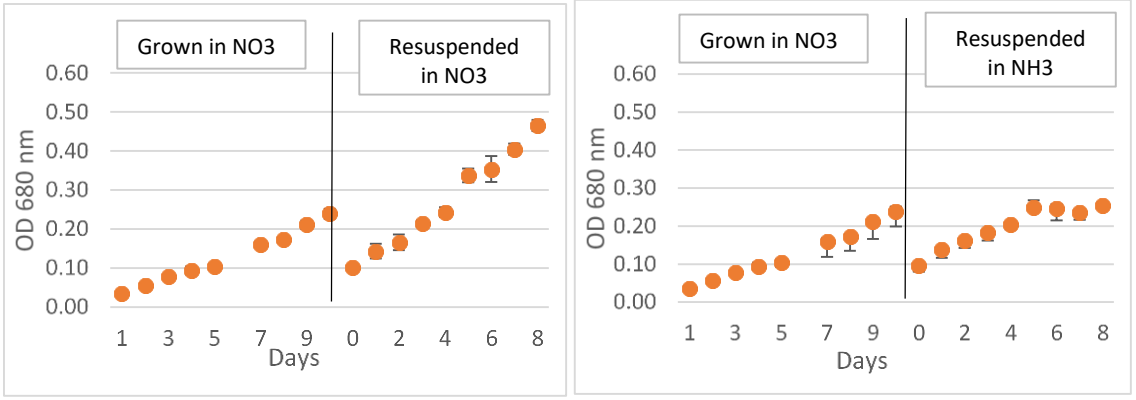


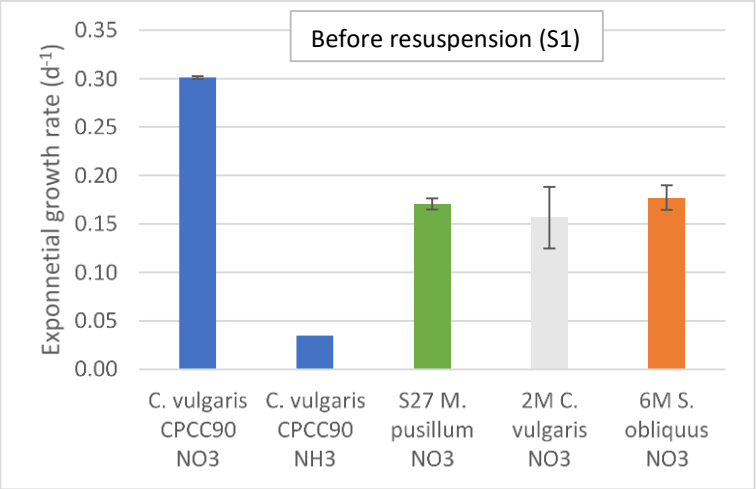
Figure 4-1 Growth of a) C. vulgaris CPCC90, b) MCWW-S27 M. pusillum c) SMC-2M C. vulgaris, d) SMC-6M S. obliquus; inoculum for resuspension was 50 mL of centrifuged algae resuspended in 100 mL of medium (2 mmol N/L except for a) S1 in NO_3 with a concentration of 2.94 mmol NO_3-N/L; b), c) and d): data of batch 1, S2 in NO_3 with a concentration of 1 mmol NO_3-N/L); all growth in flasks except for a) S1 in NH_3 in ePBR); mean of 3 replicates with 95% confidence interval.

OD680 at the beginning (day 0) of S2 should have been half of the value measured at the end of S1. However, most of OD680 measured at day 0 of S2 are less than half of OD680 at the end of S1 which is likely an indicator of the stress caused by the resuspension. Stress of resuspension was also translated into decrease of cell count of *C. vulgaris* CPPC90 the first day in S2 (Supplementary data – D3). Moreover, exponential growth in S2 has resumed after a period of adaptation of 1 to 6 days (Figure 4.1).

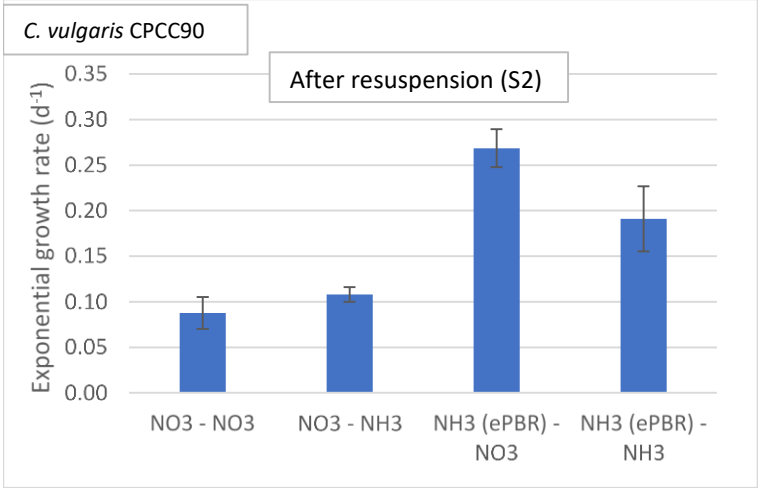
Two-way ANOVA was performed to analyse the impact of treatment factors, i.e. algal species and nutrient condition, on the algal exponential growth rates before (S1) and after resuspension (S2). Exponential growth rate was not significantly different among species ($p > 0.05$) but the treatment had a significant impact ($p < 0.05$). However, when a one-way ANOVA was calculated for each algal species it was found that the treatment had a significant impact on the exponential growth rate only for *C. vulgaris* CPCC90 ($p < 0.05$) (Figure 4.2).

C. vulgaris CPCC90 grown in nitrate in S1 reached the highest exponential growth rate among all treatments. However, this growth occurred with a higher nitrogen concentration (2.94 mmol N/L) compared with other treatments (2 mmol N/L). The lowest exponential growth rate of *C. vulgaris* CPCC90 has been measured in ammonia in S1; however this was performed in an ePBR. As explained earlier (chapter 3), all experiments in ePBRs led to a lower growth rate than the growth rates in flasks. Moreover, significant higher exponential growth rates in S2 were reached when in S1 *C. vulgaris* CPCC90 was grown in ammonia (ePBR) versus when the S1 occurred on a NO_3-N only medium (Figure 4.2b).

a)


Before resuspension (S1)
Exponnetial growth rate (d-1)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
C. vulgaris CPCC90 NO3
C. vulgaris CPCC90 NH3
S27 M. pusillum NO3
2M C. vulgaris NO3
6M S. obliquus NO3

b)


C. vulgaris CPCC90
After resuspension (S2)
Exponential growth rate (d-1)
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
NO3 - NO3
NO3 - NH3
NH3 (ePBR) - NO3
NH3 (ePBR) - NH3

c)

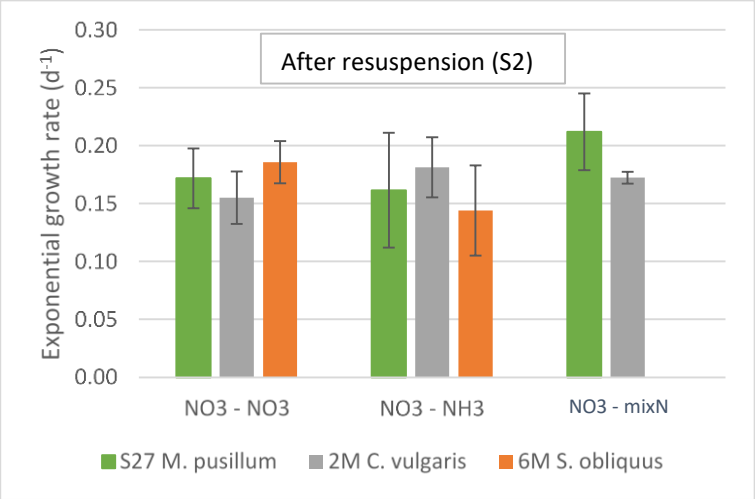
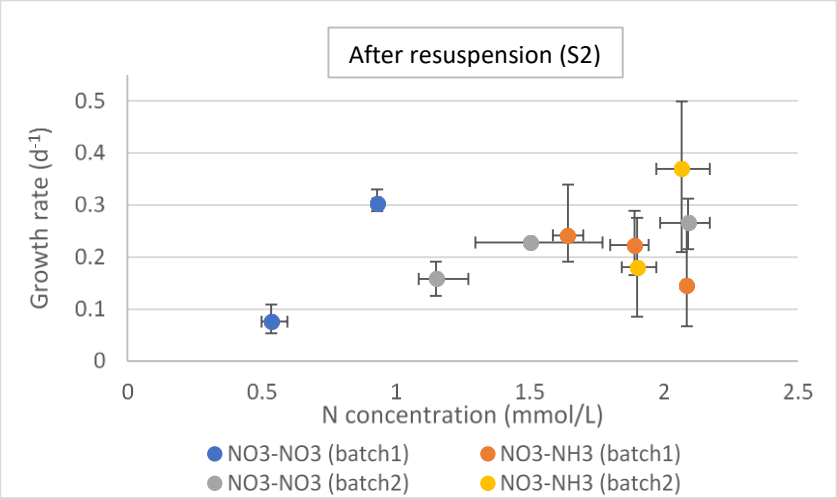


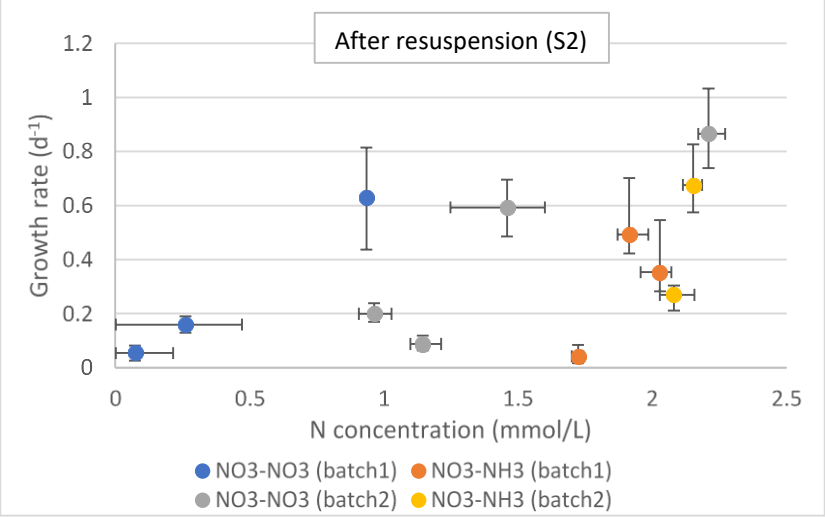
Figure 4-2 Impact of species and treatments on exponential growth rate, mean with 95% confidence interval a) medium was 2 mmol N/L except for CPCC90 S1 in NO_3: 2.94 mmol NO_3-N/L; C. vulgaris CPCC90 S1 in NH_3 was in ePBR *b) C. vulgaris CPCC90 S1 in NH_3 ePBR; c) S2, half of replicate of S27, 2M and 6M NO_3-NO_3 was in 1 mmol NO_3-N/L; mixN is a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L).*

All S2 experiments of algae grown in nitrate have reached higher optical densities (OD680 and OD750) and cells counts compared with S2 in ammonia (Figure 4.1; supplementary data - D). A higher growth rate with a smaller nitrogen concentration in medium was therefore achieved when S2 was in nitrate compared with ammonia. Different inflection points depending on treatment can thus be observed on the growth curves (Figure 4.3).

a) MCWW-S27 *M. pusillum*



b) SMC-2M *C. vulgaris*



c) SMC-6M *S. obliquus*

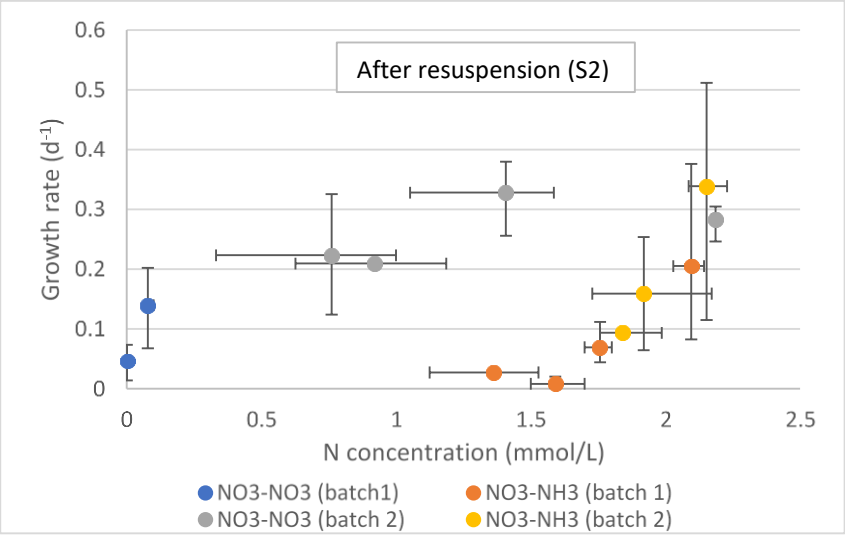
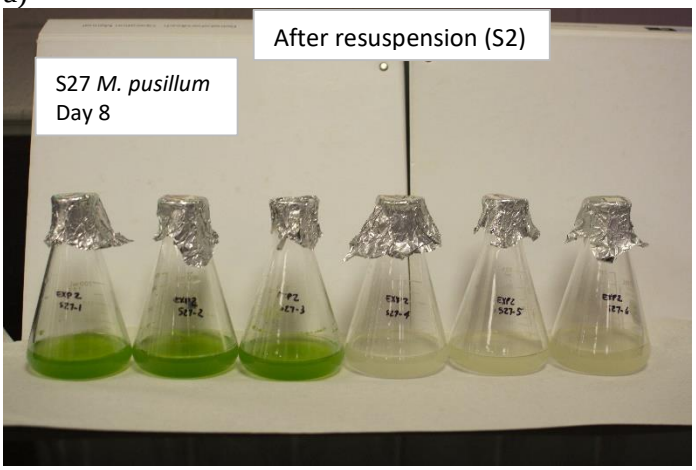


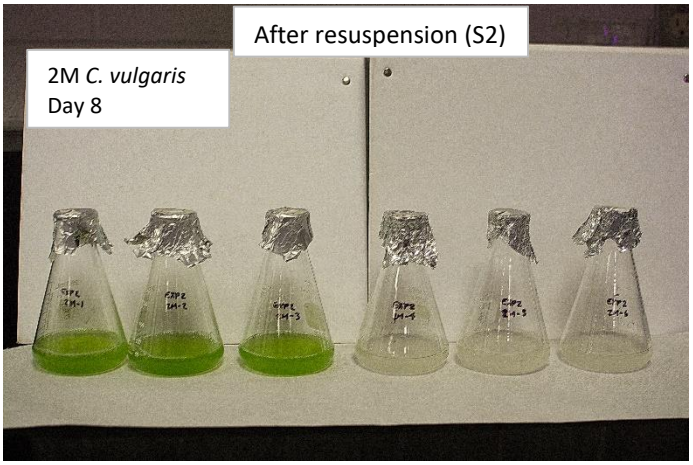
Figure 4-3 Growth rate as a function of nitrogen concentration in medium (S2) a) MCWW-S27 M. pusillum, b) SMC-2M C. vulgaris, c) SMC-6M S. obliquus; mean of 3 replicates with minimum and maximum values.

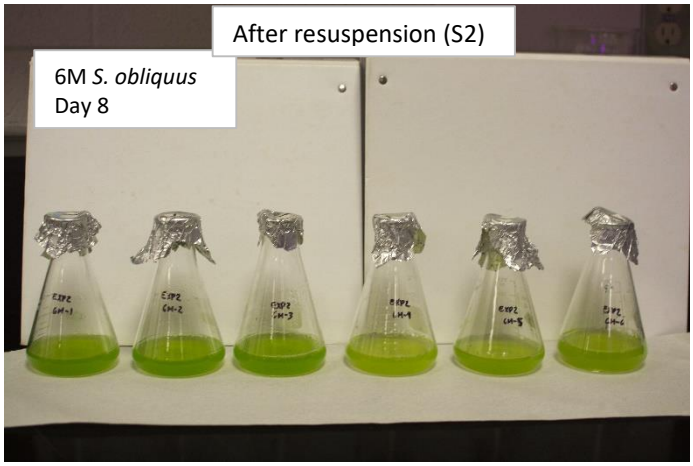
Loss of colour, i.e. culture bleaching, was also observed towards the end of the experiment in S2 (day 8) for MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* resuspended in NH_3-N medium (Figure 4.4). The decrease of pH observed with growth in NH_3-N medium (Figure 4.5) has probably hampered algal growth.

a)



b)





c)

Figure 4-4 Images of replicates 1 to 6 (left to right) at day 8 (batch1); 1 to 3 is NO_3-NO_3; 4 to 6 is NO_3-NH_3; a) MCWW-S27 M. pusillum, b) SMC-2M C. vulgaris and c) SMC-6M S. obliquus.

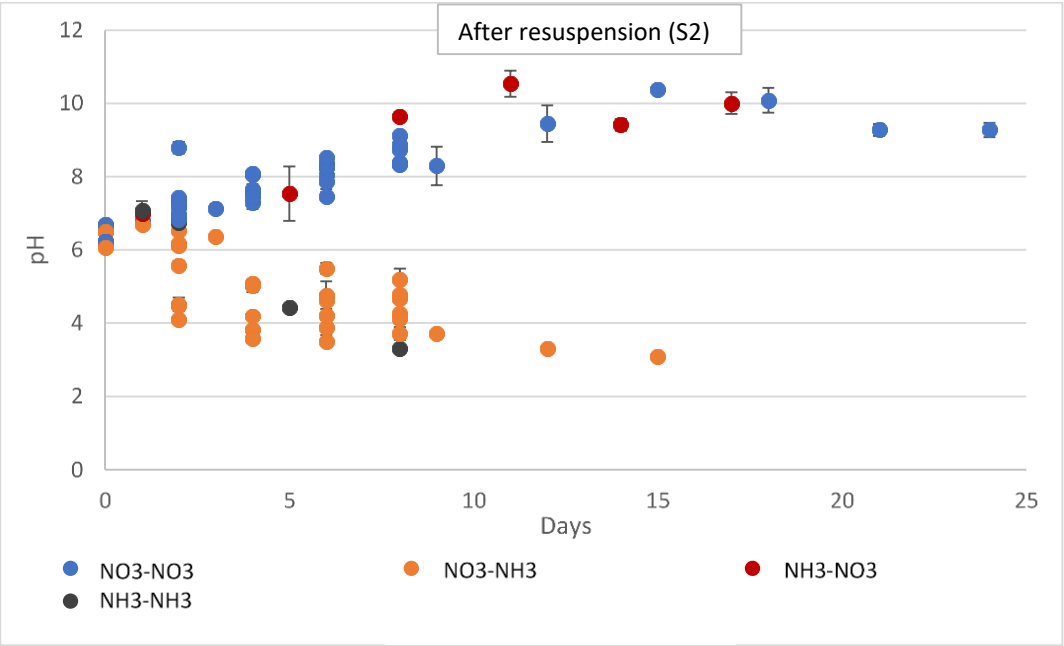


Figure 4-5 pH during growth of C. vulgaris CPCC90, MCWW- S27 M. pusillum, SMC-2M C. vulgaris and SMC-6M S. obliquus; means with 95% confidence interval.

Low pH in solution can partly be explained by dissolution of CO_2 and ammonium releasing hydrogen ions. Theoretically, if considering only dissolution of CO_2 from air and ammonium from ammonium chloride the substrate would be expected to reach a minimum pH of 4. However, assimilation of ammonium by algal cells could also release hydrogen ions.

To overcome the pH decrease, algae have been grown with a mixture of nitrate (1.8 mmol NO_3-N/L) and ammonia (0.2 mmol NH_3-N/L) in S2 (Figure 4.6). In consequence, the pH decrease was avoided but the mixture of nitrate and ammonia did not significantly improve exponential growth rate compared with other treatments (ANOVA, $p > 0.05$; Figure 4.7).

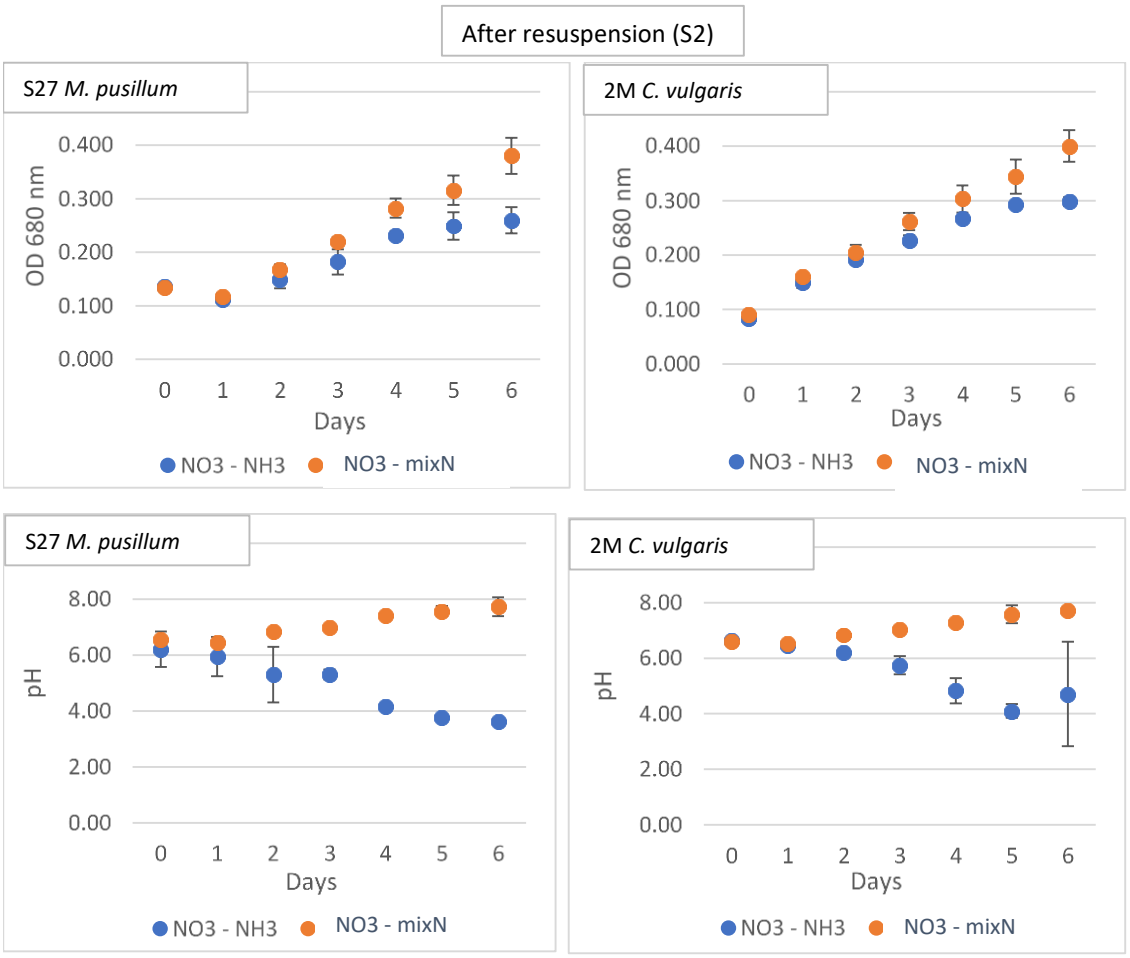


Figure 4-6 OD680 and variation of pH of MCWW-S27 M. pusillum and SMC-2M C. vulgaris; mean of 3 replicates with 95% confidence interval (mixN is a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L)).

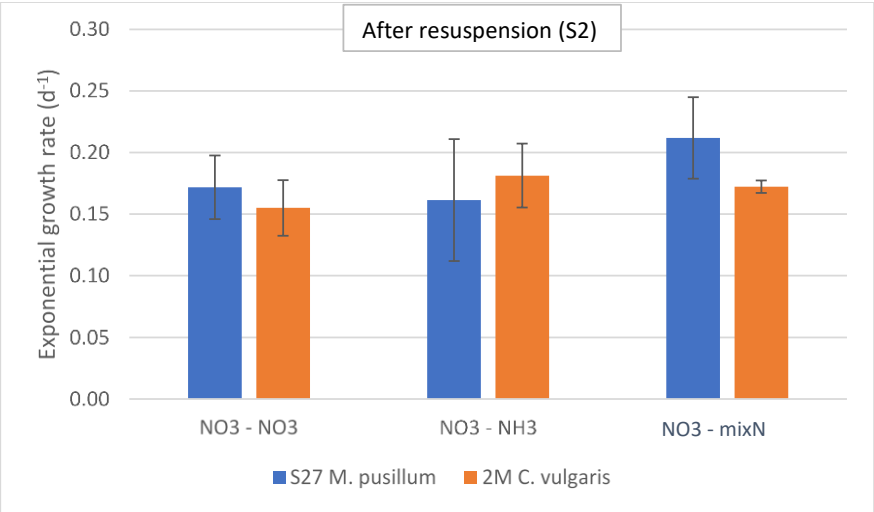


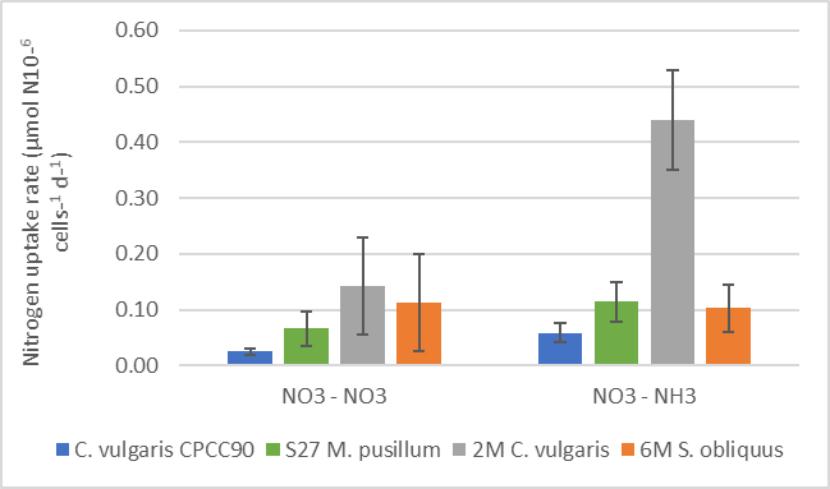
Figure 4-7 Influence of treatment on exponential growth rate for MCWW-S27 M. pusillum and SMC-2M C. vulgaris; means with 95% confidence interval (mixN is in a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L)).

4.2 Removal of nitrogen in supernatant

Nitrogen removal rates have been evaluated as a function of the number of cells during exponential phase (Figure 4.8). This can offer information on a per cell nutrient uptake rate and thus an insight on the nutrient use efficiency. *C. vulgaris* CPCC90 grown in nitrate in S1 and S2 have achieved lower per cell nitrogen uptake rates compared with other species (MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus*) (Figure 4.8a). When in S1 *C. vulgaris* CPCC90 was grown in NH_3-N medium, it has shown higher S2 nitrogen uptake rates per cell than when S1 occurred on NO_3-N medium (Figure 4.8b). SMC-2M *C. vulgaris* grown in NO_3-N medium for S1 reached significantly higher per cell nitrogen uptake rates for the S2 NH_3-N

medium than MCWW-S27 *M. pusillum* and SMC-6M *S. obliquus* (Figure 4.8a) (ANOVA, $p < 0.05$).

a)



b)

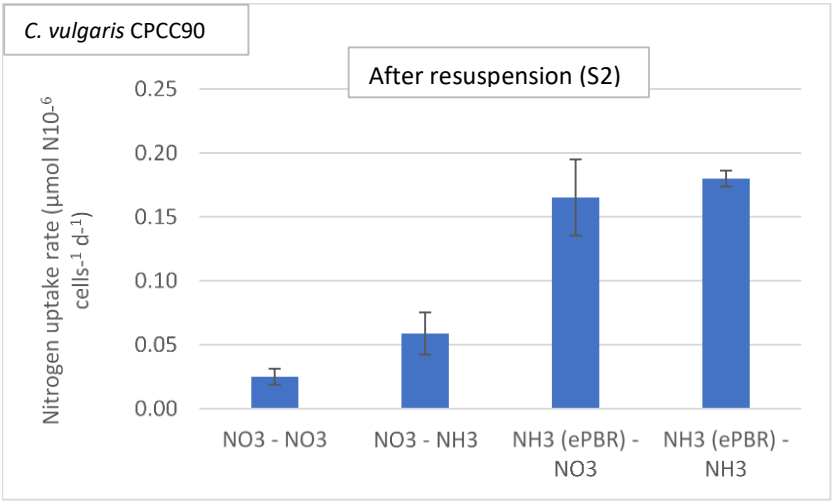


Figure 4-8 Nitrogen uptake rate of nitrogen as a function of the number of cells during exponential growth rate for C. vulgaris CPCC90, MCWW-S27 M. pusillum, SMC-2M C. vulgaris and SMC-6M S. obliquus; means with 95% confidence interval.

4.2.1 Removal of nitrogen in supernatant for algae grown in a mixture of nitrate and ammonia

MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* grown in a mixture of nitrate (1.8 mmol NO_3-N/L) and ammonia (0.2 mmol NH_3-N/L) in S2 have rapidly removed ammonia (from 0.17 ± 0.002 mmol NH_3-N/L to 0.04 ± 0.003 mmol NH_3-N/L for MCWW-S27 *M. pusillum* and from 0.16 ± 0.004 mmol NH_3-N/L to 0.05 ± 0.007 mmol NH_3-N/L for SMC-2M *C. vulgaris*) and some of the nitrate (MCWW-S27 *M. pusillum* have removed 0.06 ± 0.02 mmol NO_3-N/L and SMC-2M *C. vulgaris* 0.06 ± 0.05 mmol NO_3-N/L) the first day. After the first day in S2, when NH_3-N reached a steady state low concentration, removal of nitrate from the medium accelerated (Figure 4.9). MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* seem therefore to have preferred ammonia over nitrate. However by comparing ammonia uptake rate and nitrate uptake rate for the first day in S2, only MCWW-S27 *M. pusillum* had a significantly higher ammonia uptake rate (ANOVA $p < 0.05$; Figure 4.10).

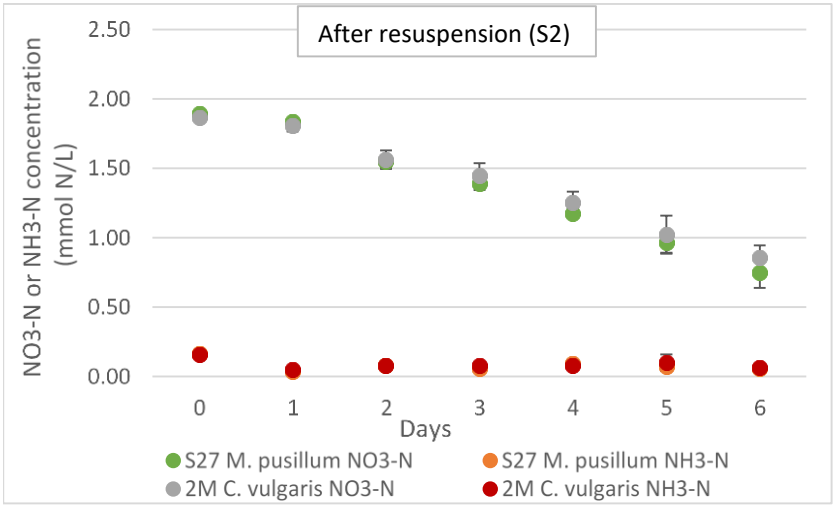


Figure 4-9 Nitrogen concentration over time for MCWW-S27 M. pusillum and SMC-2M C. vulgaris grown in a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L); mean of 3 replicates with 95% confidence interval.

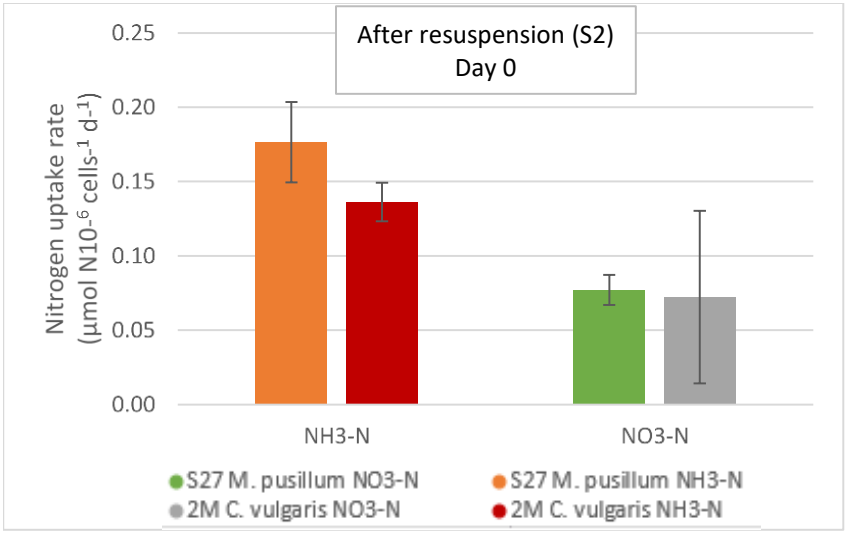


Figure 4-10 Ammonia and nitrate uptake rate per cell at day 0 of MCWW-S27 M. pusillum and SMC-2M C. vulgaris; mean of 3 replicates with 95% confidence interval.

A slight increase of ammonia has been measured in the medium after the first day (Figure 4.9). A low level of nitrite (0.09 – 0.18 mmol N/L) have also been detected at the end of the experiment

(day 6) in the medium (Supplementary data -D13). The autoanalyzer (Lachat Quickchem) can detect as low as 0.01 mmol NH_3-N/L and 0.01 mmol NO_2-N/L under standard parameters. However, the system parameters were adjusted to analyze samples with low volume (1 mL). Considering standard deviations and 99% confidence interval, a detection limit of 0.04 mmol N/L might be considered for ammonia. However, the small volume (1 mL) might have affected the accuracy of the measurements and amino acids might have interfered (interferences were previously reported for ammonia in soil extracts analysis [1]) with ammonia concentration. Even if no bacteria could be seen with the microscope, the algal culture might have been contaminated with low counts of bacteria which might have increased ammonia concentration by decomposing organic matter containing nitrogen or some nitrate might have been reduced to nitrite.

4.3 Intracellular composition

4.3.1 Carbon:Nitrogen ratio

As algae take up and assimilate nutrient, they adjust their internal Carbon:Nitrogen (C:N) ratio to environmental conditions. Changes in C:N ratio over time (C:N slopes) are therefore presented in Figure 4.11 for S2. C:N slopes represent different periods in growth as not all samples were available for this analysis. C:N slopes of *C. vulgaris* CPCC90 include data between days 15 and 21 for the NO_3-NO_3 treatment (growth in NO_3–resuspension in NO_3), between days 9 and 15 for NO_3-NH_3 treatment, between days 2 and 14 for NH_3-NO_3 treatment, between days 5 and 8 for NH_3-NH_3 treatment and between days 11 and 32 for NO_3-0N treatment (0N does not contain nitrogen). C:N slopes of MCWW-S27 *M. pusillum,* SMC-2M *C. vulgaris* and SMC-6M *S.*

obliquus include data between days 1 and 7 for NO_3-NO_3 and NO_3-NH_3 treatments. C:N slopes of NO_3-mixN treatment (mixN is a mixture of NO_3 (1.8 NO_3-N mmol/L) and NH_3 (0.2 mmol NH_3-N/L)) include data between days 0 and 5.

C:N slopes for S2 were not significantly different between species and nitrogen forms, as described by their means and 95% confidence intervals. However, some trends can be nevertheless observed (Figure 4.11). S2 C:N ratios have increased (positive slopes) for *C. vulgaris* CPCC90 for the NO_3-NO_3 treatments (Figure 4.11a). However for the same treatment the S2 C:N ratios of MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus* have been less affected and C:N slopes were near 0 (Figure 4.11c). The S2 C:N slope of *C. vulgaris* CPCC90 for the NO_3-NH_3 treatment was also stable, near 0 (Figure 4.11c). However, for the NO_3-NH_3 treatment of MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus* the S2 C:N ratios increased (Figure 4.11c). S2 C:N ratio for *C. vulgaris* CPCC90 for the NH_3-NO_3 treatment was near 0. By contrast, *C. vulgaris* CPCC90 have shown a negative S2 C:N slope for the NH_3-NH_3 treatment (Figure 4.11b). As it was expected, growth of *C. vulgaris* CPCC90 for NO_3-0N treatment has led to an increase of the S2 C:N ratios as nitrogen proportion have decreased, a reflection of a stress response (Figure 4.11a). When grown in nitrate (S1) and resuspended (S2) in a mixture of nitrate (1.8 mmol NO_3-N/L) and ammonia (0.2 mmol NH_3-N/L) (NO_3-mixN), MCWW-S27 *M. pusillum* have shown a slight negative S2 C:N slope and SMC-2M *C. vulgaris* had an S2 C:N slope near 0 (Figure 4.11c). C:N slopes directions are summarized in Table 4.1.

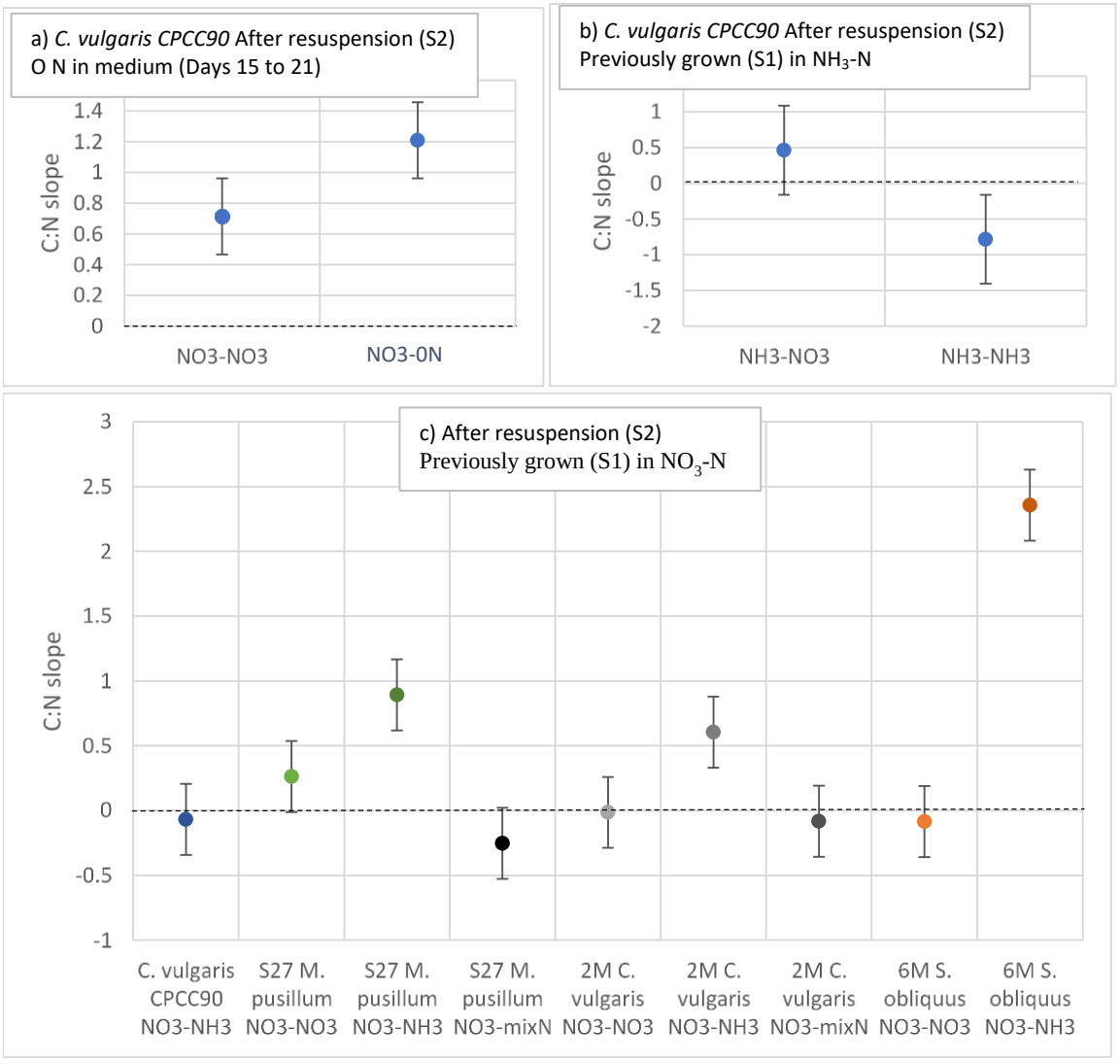


Figure 4-11 C:N slopes; means with standard errors (C. vulgaris CPCC90 NO_3-NO_3: between days 15 and 21, C. vulgaris CPCC90 NO_3-NH_3: between days 9 and 15, C. vulgaris CPCC90 NH_3-NO_3 between days 2 and 14, C. vulgaris CPCC90 NH_3-NH_3 between days 5 and 8, C. vulgaris CPCC90 NO_3-0N between days 11 and 32, MCWW-S27 M. pusillum, SMC-2M C. vulgaris, SMC-6M S. obliquus NO_3-NO_3 and NO_3-NH_3 between days 1 and 7, MCWW-S27 M. pusillum and SMC-2M C. vulgaris NO_3-mixN (NO_3 :1.8 mmol NO_3-N/L and NH_3: 0.2 mmol NH_3-N/L) between days 0 and 5).

Table 4-1 C:N ratios changes over time

	C:N changes (positive (+), negative (-) slopes or 0)					
	NO_3-NO_3	NO_3-NH_3	NH_3-NO_3	NH_3-NH_3	NO_3–0N	NO_3-mixN[1]
C. vulgaris CPCC90	+	0	0	-	+	
MCWW-S27 *M. pusillum*	0	+				-
SMC-2M *C. vulgaris*	0	+				0
SMC-6M *S. obliquus*	0	+				

[1] mixN is a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L)

It should be mentioned that for NO_3-NO_3 the S2 C:N ratios of SMC-2M *C. vulgaris* have not shown a linear behaviour as there was a decrease between days 1 and 3 followed by a small increase between days 3 and 7 (Figure 4.12).

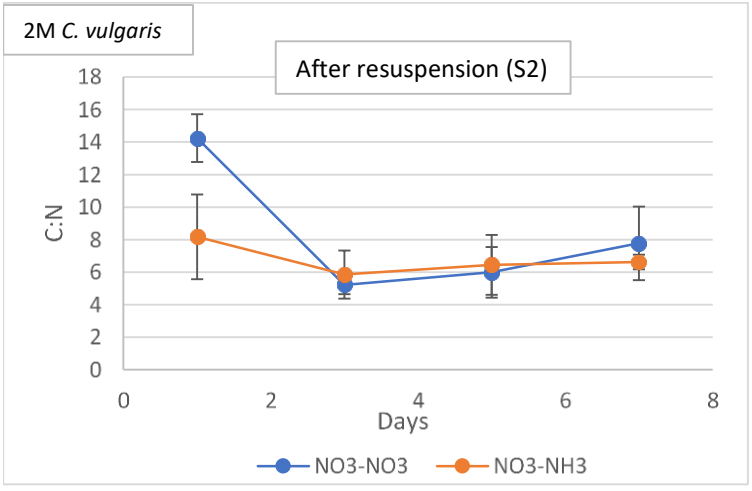


Figure 4-12 C:N ratios of SMC-2M C. vulgaris (data of batch 1); mean of 3 replicates with 95% confidence interval.

4.3.2 Growth rate as related to C:N ratios and nitrogen uptake rate per cell

Growth rates, C:N ratios and nitrogen uptake rates per cell have been plotted in 3D surface plots with a view to study the response of growth of MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris*, SMC-6M *S. obliquus* for S2 between days 2 and 8 (Figure 4.13). Given the use of alternate days for estimation of nitrogen and C:N ratios, for NO_3-NO_3 and NO_3-NH_3 treatments, the C:N ratios were inferred for the missing alternate days, along linear correlations.

Best 3-D fits, as obtained in LAB Fit [2], varied among the datasets. However a best common fit was obtained with a geometric fit (Eq. 4.1):

$$\mu = A \cdot \mathrm{Nuptake}^{B/(C:N)} \qquad \text{Eq. 4.1}$$

μ: growth rate (d^{-1})

Nuptake: nitrogen uptake rate per cell (μmol N 10^{-6} $cell^{-1}$ d^{-1})

C:N: Carbon:Nitrogen ratio

A : constant

B : constant

Constants A and B of equation 4.1, chi-square values and its associated p value are shown in Table 4.2. Chi-square and p values of the equations of growth rates as a function of nitrogen uptake rates per cell and C:N ratios have shown a poor fit of the data ($p > 0.05$, Table 4.2), likely a consequence of the sparse datasets. Consequently, only general visual trends will be considered. Most surface response graphs (Figure 4.13) show some consistencies among species and treatments. Growth rates are directly linked to nitrogen uptake with accelerated growth for

algae with higher C:N ratios. Growth rates of SMC-6M *S. obliquus* have shown a different pattern. For S2 NO_3-NO_3 treatment, growth rates were linked to nitrogen uptake but more accelerated, i.e. a steeper slope for growth rates, at lower nitrogen uptake (Figure 4.13e). Growth seems therefore to have stopped more suddenly for this species, following an initial short-term accelerated growth, compared with other species. Growth rates of SMC-6M *S. obliquus* for S2 NO_3-NH_3 treatment seem independent of ammonia uptake rates and C:N ratios (Figure 4.13f); nevertheless the validity of this conclusion is hampered by the sparse dataset.

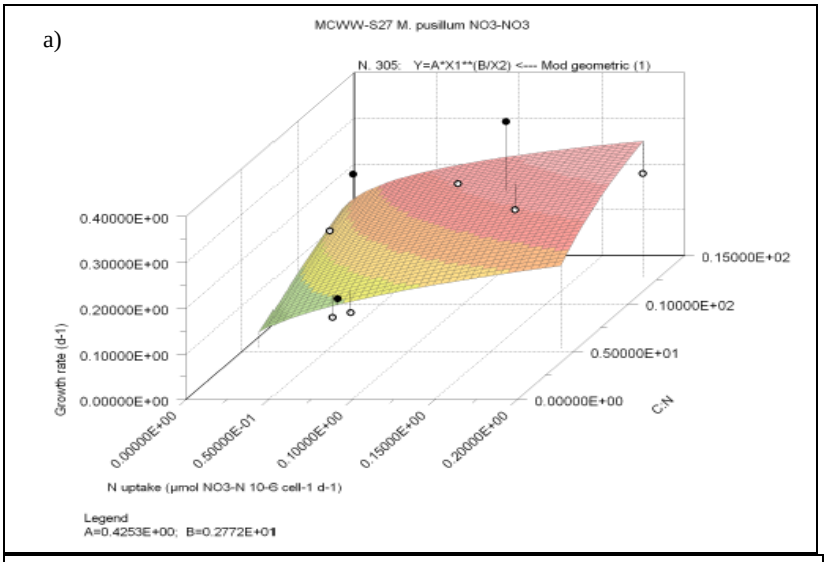
Thus, while the relationship between growth and the type of N source varies with species (Table 4.2) when a single N-source is available, the use of mix N source media seems to favour both *M. pusillum* and *C. vulgaris* (see fitted parameter A in Table 4.2). Consequently the negative role of a large C:N ratio on growth is also mitigated by mix N-source media (see fitted parameter B in Table 4.2).

Attempts were performed to fit the data to functions with 3 or 4 parameters but those equations did not improve the p values which might confirm that growth rates are more likely linked only to nitrogen uptake. The small volume and mass (1 mL and less than 1 mg) of samples might have induced errors which might explain the poor fit of the data ($p > 0.4$).

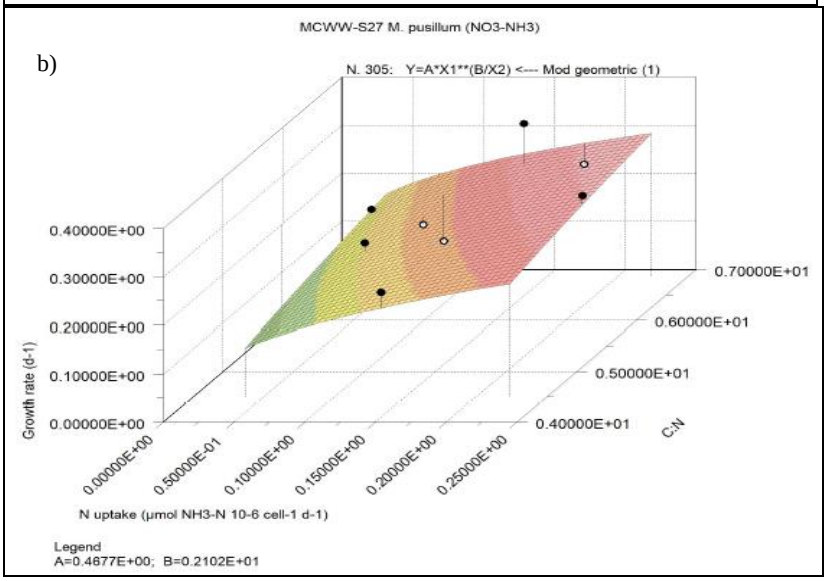
Table 4-2 Constants and evaluation of curve fitting

Species	Treatment	A	B	Chi square	p value
MCWW-S27 *M. pusillum*	NO_3-NO_3	0.4253	2.772	7	0.429
	NO_3-NH_3	0.4677	2.102	6	0.423
	NO_3-mixN	0.5885	4.126	7	0.429
SMC-2M *C. vulgaris*	NO_3-NO_3	0.7321	4.659	8	0.433
	NO_3-NH_3	0.5692	5.123	13	0.448
	NO_3-mixN	1.876	6.295	7	0.429
SMC-6M *S. obliquus*	NO_3-NO_3	0.3421	1.397	9	0.437
	NO_3-NH_3	0.03423	-0.3198	4	0.406
All species	All treatment	0.4341	2.684	75	0.478

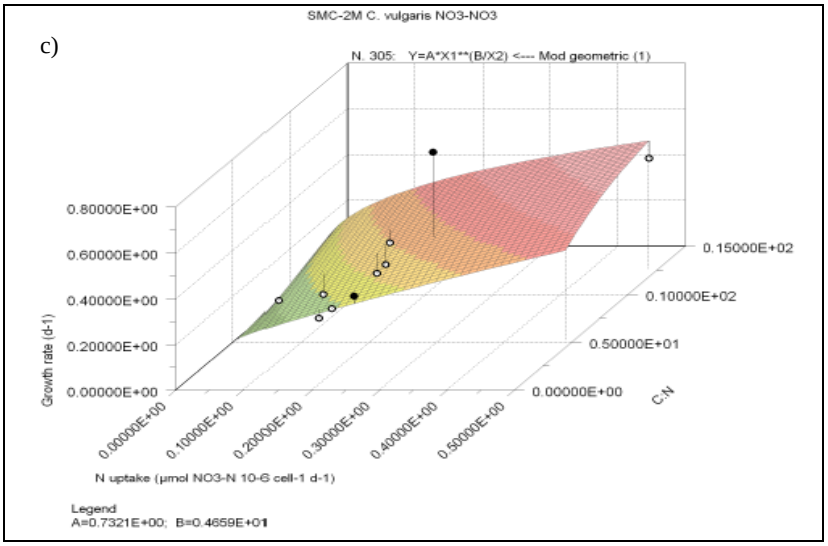
a)


MCWW-S27 M. pusillum NO3-NO3
N. 305: Y=A*X1**(B/X2) <---- Mod geometric (1)
0.40000E+00
0.30000E+00
0.20000E+00
0.10000E+00
0.00000E+00
Growth rate (d-1)
0.00000E+00
0.50000E-01
0.10000E+00
0.15000E+00
0.20000E+00
N uptake (µmol NO3-N 10-6 cell-1 d-1)
0.15000E+02
0.10000E+02
0.50000E+01
0.00000E+00
C:N
Legend
A=0.4253E+00; B=0.2772E+01

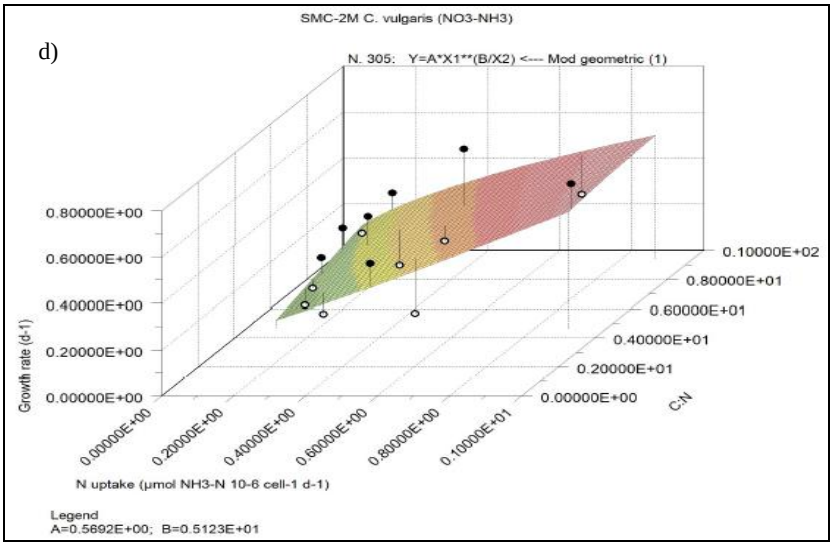
b)


MCWW-S27 M. pusillum (NO3-NH3)
N. 305: Y=A*X1**(B/X2) <---- Mod geometric (1)
0.40000E+00
0.30000E+00
0.20000E+00
0.10000E+00
0.00000E+00
Growth rate (d-1)
0.00000E+00
0.50000E-01
0.10000E+00
0.15000E+00
0.20000E+00
0.25000E+00
N uptake (µmol NH3-N 10-6 cell-1 d-1)
0.70000E+01
0.60000E+01
0.50000E+01
0.40000E+01
C:N
Legend
A=0.4877E+00; B=0.2102E+01

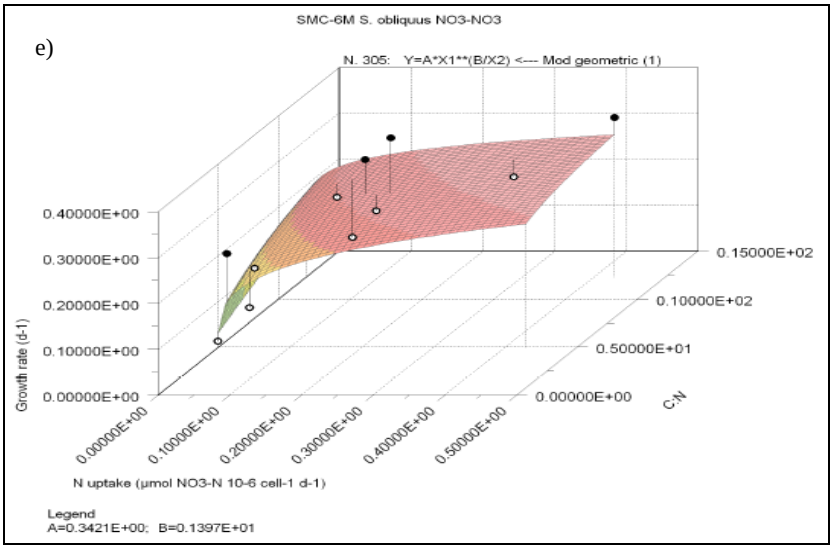
c)


SMC-2M C. vulgaris NO3-NO3
N. 305: Y=A*X1**(B/X2) <--- Mod geometric (1)
0.80000E+00
0.60000E+00
0.40000E+00
0.20000E+00
0.00000E+00
Growth rate (d-1)
0.15000E+02
0.10000E+02
0.50000E+01
0.00000E+00
C:N
0.00000E+00
0.10000E+00
0.20000E+00
0.30000E+00
0.40000E+00
0.50000E+00
N uptake (µmol NO3-N 10-6 cell-1 d-1)
Legend
A=0.7321E+00; B=0.4659E+01

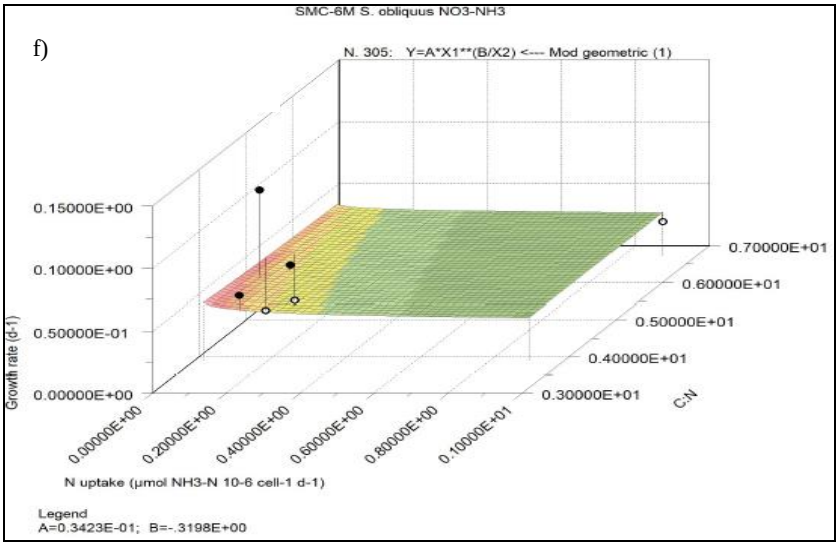
d)


SMC-2M C. vulgaris (NO3-NH3)
N. 305: Y=A*X1**(B/X2) <--- Mod geometric (1)
0.80000E+00
0.60000E+00
0.40000E+00
0.20000E+00
0.00000E+00
Growth rate (d-1)
0.10000E+02
0.80000E+01
0.60000E+01
0.40000E+01
0.20000E+01
0.00000E+00
C:N
0.00000E+00
0.20000E+00
0.40000E+00
0.60000E+00
0.80000E+00
0.10000E+01
N uptake (µmol NH3-N 10-6 cell-1 d-1)
Legend
A=0.5692E+00; B=0.5123E+01

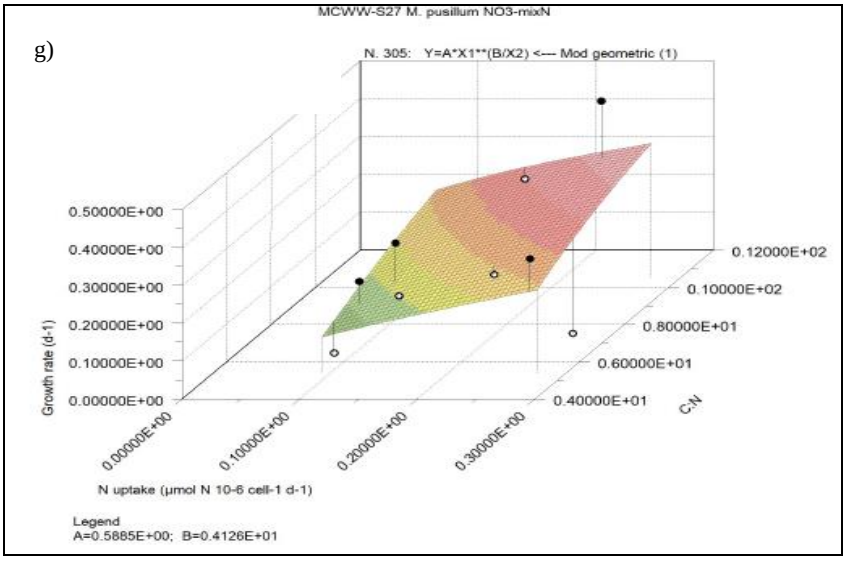
e)


SMC-6M S. obliquus NO3-NO3
N. 305: Y=A*X1**(B/X2) <--- Mod geometric (1)
0.40000E+00
0.30000E+00
0.20000E+00
0.10000E+00
0.00000E+00
Growth rate (d-1)
0.00000E+00
0.10000E+00
0.20000E+00
0.30000E+00
0.40000E+00
0.50000E+00
N uptake (µmol NO3-N 10-6 cell-1 d-1)
0.15000E+02
0.10000E+02
0.50000E+01
0.00000E+00
C:N
Legend
A=0.3421E+00; B=0.1397E+01

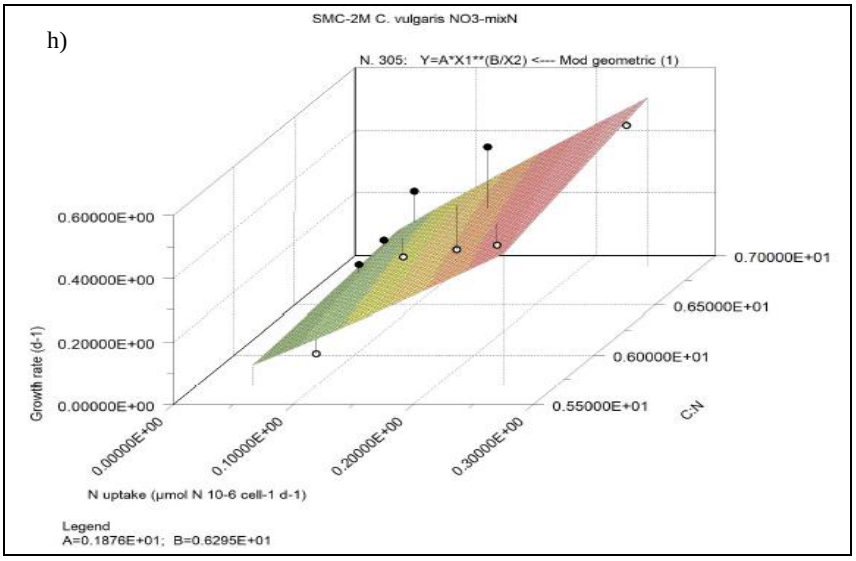
f)


SMC-6M S. obliquus NO3-NH3
N. 305: Y=A*X1**(B/X2) <--- Mod geometric (1)
0.15000E+00
0.10000E+00
0.50000E-01
0.00000E+00
Growth rate (d-1)
0.00000E+00
0.20000E+00
0.40000E+00
0.60000E+00
0.80000E+00
0.10000E+01
N uptake (µmol NH3-N 10-6 cell-1 d-1)
0.70000E+01
0.60000E+01
0.50000E+01
0.40000E+01
0.30000E+01
C:N
Legend
A=0.3423E-01; B=-.3198E+00

g)


MCWW-S27 M. pusillum NO3-mixN
N. 305: Y=A*X1**(B/X2) <--- Mod geometric (1)
0.50000E+00
0.40000E+00
0.30000E+00
0.20000E+00
0.10000E+00
0.00000E+00
Growth rate (d-1)
0.12000E+02
0.10000E+02
0.80000E+01
0.60000E+01
0.40000E+01
C:N
0.00000E+00
0.10000E+00
0.20000E+00
0.30000E+00
N uptake (µmol N 10-6 cell-1 d-1)
Legend
A=0.5885E+00; B=0.4126E+01

h)


SMC-2M C. vulgaris NO3-mixN
N. 305: Y=A*X1**(B/X2) <--- Mod geometric (1)
0.60000E+00
0.40000E+00
0.20000E+00
0.00000E+00
Growth rate (d-1)
0.70000E+01
0.65000E+01
0.60000E+01
0.55000E+01
C:N
0.00000E+00
0.10000E+00
0.20000E+00
0.30000E+00
N uptake (µmol N 10-6 cell-1 d-1)
Legend
A=0.1876E+01; B=0.6295E+01

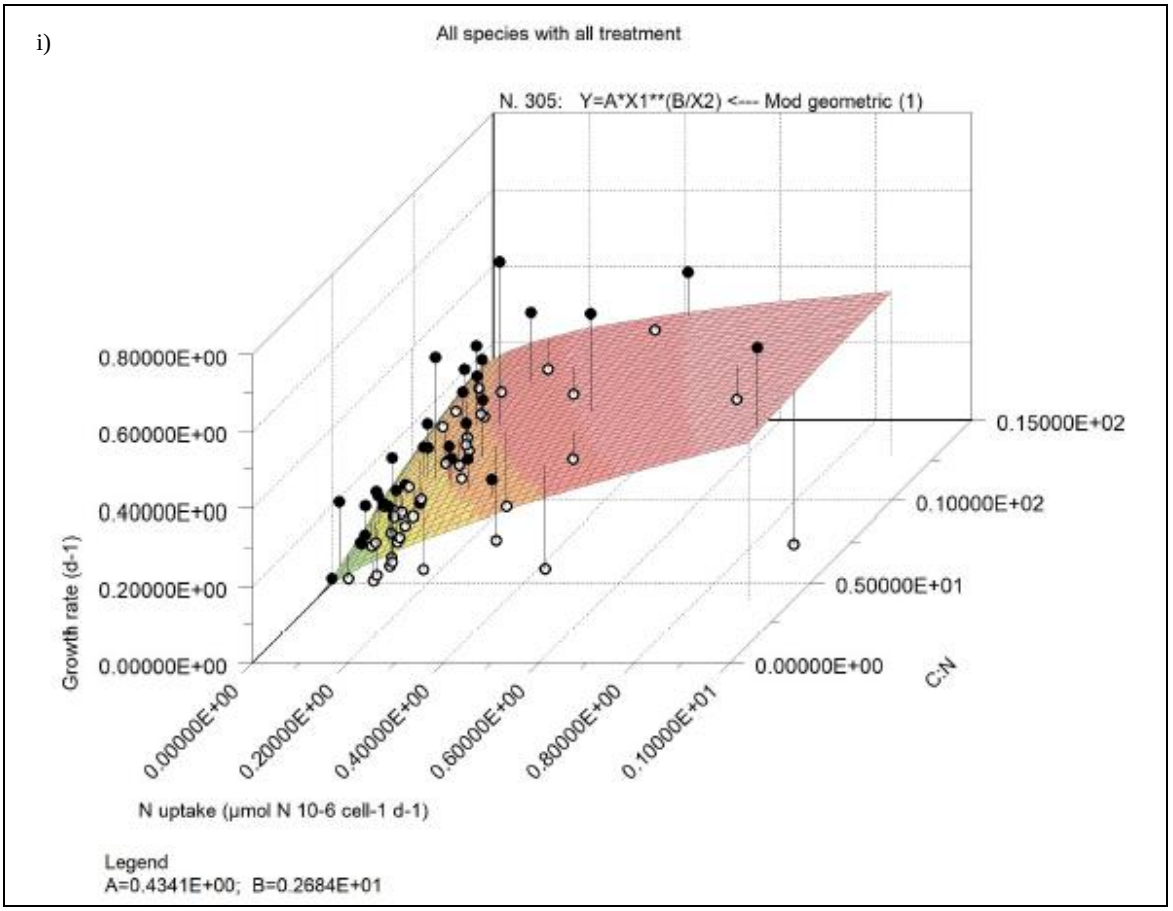
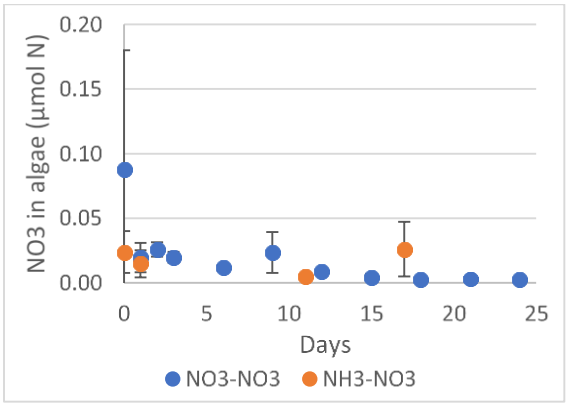


Figure 4-13 3D plot of growth rate (d^{-1}) as a function of N uptake (µmol N 10^{-6} $cell^{-1}$ d^{-1}) and C:N between days 2 and 8; a) MCWW-S27 M. pusillum NO_3-NO_3, b) MCWW-S27 M. pusillum NO_3-NH_3, c) SMC-2M C. vulgaris NO_3-NO_3, d) SMC-2M C. vulgaris NO_3-NH_3, e) SMC-6M S. obliquus NO_3-NO_3, f) SMC-6M S. obliquus NO_3-NH_3, g) MCWW-S27 M. pusillum NO_3-mixN, h) SMC-2M C. vulgaris NO_3-mixN; mixN is a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L), i) All species with all treatment.

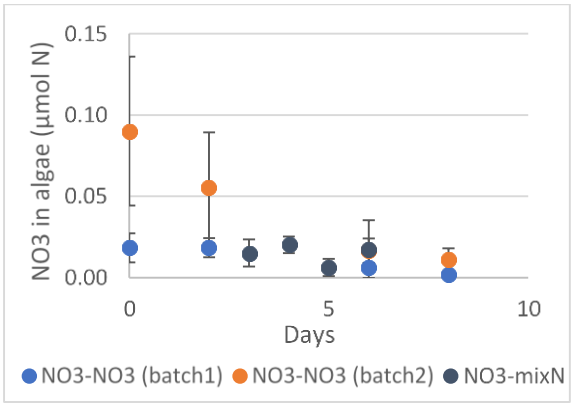
4.3.3 Nitrate in algal cells

Figure 4.14 presents the results of nitrate measured in algae cells. All treatments have shown a decrease of nitrate in cells over time.

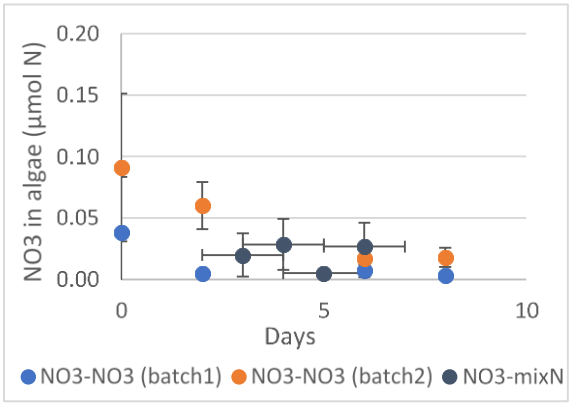
a) *C. vulgaris* CPCC90



b) MCWW-S27 *M. pusillum*



c) SMC-2M *C. vulgaris*



d) SMC-6M *S. obliquus*

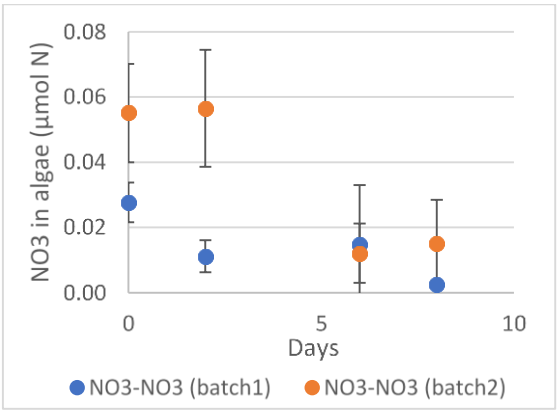


Figure 4-14 Nitrate in algae (sample of 1-2 mL centrifuged, cells broken with freeze/thaw (-80/38 °C) cycles and resuspended in 2 mL on deionized water; supernatant was then analyzed), a) C. vulgaris CPCC90, b) MCWW-S27 M. pusillum, c) SMC-2M C. vulgaris, d) SMC-6M S. obliquus; mean of 3 replicates with 95% confidence interval; mixN is a mixture of NO_3 (1.8 mmol NO_3-N/L) and NH_3 (0.2 mmol NH_3-N/L).

4.4 Fitting the Monod model

The Monod equation correlates growth rates as a function of nitrogen in medium [3] (Figure 4.4, Eq. 4.2).

$$\mu = \mu_{\max} \quad \frac{R}{K_\mu + R} \qquad \text{Eq. 4.2}$$

K_μ : half-saturation constant for growth rate (mol L^{-1})

R: external nutrient concentration (mol L^{-1})

μ: specific growth rate (d^{-1})

μ_{max}: maximum growth rate (d^{-1})

To calculate Monod half-saturation constants, the highest growth rate measured for each algal species was considered as the theoretical maximum growth rate. Results of Monod half-saturation constant (k) are presented in Table 4.3 according to the treatments to reflect the variation among treatments.

Table 4-3 Maximum growth rate and k values of Monod equation

Species	Treatment	μ_{max} (d^{-1})	k (mean) (mmol L^{-1})	k CI95
CPCC90	NO_3-NO_3 (2 mmol N/L)	1707	0.826	0.226
	NO_3-NH_3 (2 mmol N/L)		1.209	0.141
	NH_3-NO_3 (2 mmol N/L)		0.918	0.356
	NH_3-NH_3 (2 mmol N/L)		1.546	0.087
S27	NO_3-NO_3 (1 mmol N/L)	0.499	-0.367	0.088
	NO_3-NO_3 (2 mmol N/L)		-0.778	0.151
	NO_3-NH_3 (2 mmol N/L)		-0.961	0.048
	NO_3-mixN (2 mmol N/L)		-0.828	0.112
2M	NO_3-NO_3 (1 mmol N/L)	1.033	0.018	0.009
	NO_3-NO_3 (2 mmol N/L)		0.047	0.009
	NO_3-NH_3 (2 mmol N/L)		0.064	0.003
	NO_3-mixN (2 mmol N/L)		0.054	0.007
6M	NO_3-NO_3 (2 mmol/L)	0.512	-0.650	0.189
	NO_3-NH_3 (2 mmol N/L)		-0.922	0.060

4.5 Inadequate mixing in ePBRs

Growth evaluation of *Chlorella vulgaris* CPCC90 has shown that it was more difficult to replicate the growth in the ePBRs compared with the growth in the flasks on the shaker. Moreover, biomass obtained with ePBRs was much lower compared with biomass produced in flasks (Supplementary data – C). Mixing in the ePBRs was not optimal. Mixing was initially supposed to be done by injection of CO_2 through the bottom of the reactor. However, the solenoid valve installed to inject CO_2 was not adequate and was causing back pressure in the gas line. To avoid liquid in the valve, the injection of CO_2 was performed through the top of the reactor. Inadequate mixing has probably hampered algal growth as mixing ensure that light is provided to all culture volume. Moreover, adequate mixing improves intracellular activities [4].

4.6 References

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Chapter 5 Discussion

5.1 Algal growth

Centrifugation and resuspension of algae have caused a certain stress on algae. During resuspension (S2), 50 mL of centrifuged algae were inoculated in 100 mL of medium. At the beginning of the resuspension (S2), OD 680 nm, which represents chlorophyll density and is an indicator of algae health, should therefore have been half of the value before resuspension (end of S1). As many algae cultures had an OD 680 nm lower that the theoretical value of 50% and/or there were a few days of slow growth before exponential growth (Figure 4.1), it may be assumed that resuspension had an impact on algae. A decrease in cells count has also been measured for *C. vulgaris* CPPC90 the first day after resuspension (Supplementary data – D3).

Exponential growth rates for S2 of *C. vulgaris* CPCC90 were significantly lower than for other species (MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus*) for both NO_3-NH_3 and NO_3-NO_3 treatments. Growth of *C. vulgaris* CPCC90 in ammonia before resuspension (S1) has however significantly improved exponential growth rates after resuspension (S2) in either nitrate or ammonia (NH_3-NO_3 and NH_3-NH_3; Figure 4.2b). S1 cultivation of *C. vulgaris* CPCC90 in ePBR's led to very low growth rates (Supplementary data – D2). S2 of *C. vulgaris* CPCC90 were performed after 27 days of growth in ePBR (S1) and cells were therefore probably starved. While nutrient concentration in the medium at that time is unknown, extrapolation of previous experiments (Supplementary data -C2) supports this assumption. Starved-cells will

usually take up ammonia and nitrate at an accelerated rate compared to nutrient replete cells [1]. Faster nutrient uptake rate could have translated into a quicker nitrogen assimilation and growth. *C. vulgaris* CPCC90 grown in ammonia (ePBR) during S1 and resuspended in nitrate or ammonia for S2 have indeed taken up nitrogen (nitrate or ammonia) more rapidly during exponential growth. However, for the NO_3-NH_3 treatment *C. vulgaris* CPCC90 has shown a significantly faster S2 nitrogen uptake rate compared with NO_3-NO_3 (Figure 4.8b). Interestingly, SMC-2M *C. vulgaris* have also shown a significantly faster nitrogen uptake rate during the S2 exponential growth for the NO_3-NH_3 treatment (Figure 4.8a). However, results cannot confirm that enhanced growth (as evaluated by cell counts and OD750) of *C. vulgaris* (CPCC90 or SMC-2M) is reached in NH_3-N substrate since growth was probably inhibited with the associated decrease in pH (Figure 4.5). Experiments with pH control would then be necessary to fully evaluate growth in NH_3-N substrates.

Poorer growth of algae grown in NH_3-N media compared with algae grown in NO_3-N media was confirmed by lower optical densities (OD680 and OD750), lower cells counts (Figure 4.1, Supplementary data – D) and lower growth rates corresponding to higher nitrogen concentrations in medium (Figure 4.3). Algae grown in NO_3-N media have taken up more nitrogen compared with algae grown in ammonia (Supplementary data – D) since their growth has not stopped, but, nevertheless, they did not take up nitrogen at a faster rate (Figure 4.8). As mentioned earlier, *C. vulgaris* CPCC90 and SMC-2M had rather a higher nitrogen uptake rate when they were grown in NH_3-N media.

To overcome the decrease of pH experienced with culture grown in NH_3-N media, a mixture of 10% ammonia and 90% nitrate (0.2 mmol NH_3-N/L NH3 and 1.8 mmol NO_3-

N/L) was added in the medium (Figure 4.6) to grow MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris*. The mixture has avoided the pH decrease but exponential growth rates were not significantly increased compared with other treatments (Figure 4.7). While exponential growth rates were not statistically improved, analysis of growth rate as a function of nitrogen uptake rate and C:N ratios (Figure 4.13g and 4.13h) suggest higher growth on a mixture of nitrogen sources. Parameters A and B of the fitted equation (Eq. 4.1, Table 4.2) ($\mu = ANuptake^{B/(C:N)}$) also support higher growth on mixture, i.e. with higher S2 growth values for NO_3-mixN treatment compared with other treatments. Further experiments could validate if a higher proportion of ammonia could enhance growth without reaching a critical minimum pH threshold. Another study [2] has grown *C. vulgaris* in shaken flasks with different proportions of ammonia and nitrate. They have found that approximately 36% of ammonia favor high biomass without an excess of proton excretion. Their experiments were conducted with a total nitrogen concentration of 21.4 mmol N/L and addition of 5% (v/v) CO_2.

5.2 Variation of pH

As expected, algae grown in nitrate have shown an increase of pH culture caused by photosynthetic activity. pH culture of algae grown in ammonia has however fallen (Figure 4.5). A minimum pH value of 4 was theoretically calculated considering CO_2 and ammonia dissolution in the medium. Cultures grown in NH_3-N media have however reached pH as low as 3 (Figure 4.5). The difference between theoretical and experimental values can be explained with the release of hydrogen ions during ammonium assimilation

by algae. Further work to understand nitrogen metabolism and assimilation of CO_2 at cellular level would however be required to understand and predict hydrogen ions excretion [2].

Culture pH has an impact on nutrient uptake, nutrient assimilation and photosynthesis since the transport of nutrients in cells including inorganic carbon might be altered with pH variation [3]. Algal cultures grown in ammonia with low pH have taken up less nitrogen compared with algae grown in nitrate (Supplementary data – D). Transport of nitrogen into the cells and nitrogen incorporation into biomass have therefore been affected. MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* grown in ammonia have also experienced bleaching (Figure 4.4) which means that the chlorophyll was degraded. Low pH has therefore prevented attaining high algal biomass and reinforced the importance of pH control in algal culture.

5.3 Preference of ammonia over nitrate

Many studies have shown that algae prefer ammonia over nitrate and will take up all available ammonia before nitrate [4]. This preference was seen with MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* grown in a mixture of ammonia and nitrate (0.2 mmol NH_3-N/L and 1.8 mmol NO_3-N/L; Figure 4.9). MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* have first depleted ammonia and started to take up nitrate after the first day of the experiment. Nitrogen uptake rates of the first day (Figure 4.10) have shown that ammonia uptake rate was significantly higher than nitrate uptake rate for MCWW-S27 *M. pusillum*. SMC-2M *C. vulgaris* have not shown a significant difference between ammonia

uptake rate and nitrate uptake rate the first day but this is probably because the uptake of ammonia was too fast. Resuspension of SMC-2M *C. vulgaris* in NH_3-N medium led to a nitrogen uptake rate near 4 times higher than the nitrogen uptake by MCWW-S27 *M. pusillum* (Figure 4.8a). Moreover, comparison of nitrate uptake rates between the first day and the following days have revealed that MCWW-S27 *M. pusillum* doubled nitrate uptake rate after the first day but SMC-2M *C. vulgaris* have kept a constant nitrate uptake rate over time (Supplementary data – E). An experiment with a higher sampling frequency would be necessary to confirm the behaviour of SMC-2M *C. vulgaris*.

5.4 Intracellular C:N ratio as related to growth

Intracellular Carbon:Nitrogen (C:N) ratios could be an indicator of how cells respond to their environment as carbon is related to biomass production and nitrogen to uptake and assimilation of nitrogen. There was no statistical significant difference between S2 C:N slopes (changes in C:N over time) at a 95% confidence interval. Nevertheless consistent trends were observed for the C:N ratios changes over time (Figure 4.11).

Increasing C:N ratios might be an indicator of stress since it means a decrease of nitrogen and a proportional increase of carbon. Increase of S2 C:N ratios of *C. vulgaris* CPCC90 for the NO_3-NO_3 treatments was predictable because the ratios have been measured between days 15 and 21 and all substrate nitrate was depleted by this time (Supplementary data -D11). As expected, a positive S2 C:N slope has also been measured for *C. vulgaris* CPCC90 in the NO_3-0N treatment (Figure 4.11a).

Apparently, MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus* have experienced less stress for the NO_3-NO_3 treatments since no change in S2 C:N ratios were measured (Figure 4.11c). However, the S2 C:N ratios of SMC-2M *C. vulgaris* have decreased between days 1 and 3 and slightly increased the remaining days (days 3 to 7) (Figure 4.12). *C. vulgaris* CPCC90 in the NO_3-NH_3 treatments have also shown no change in S2 C:N ratios over time (Figure 4.11c), but again data was collected between days 9 and 15 which was at the end of the experiment. There was still some residual ammonia in the medium at that time and algae were probably adapted to their environment. S2 C:N ratios of MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus* have however increased with NO_3-NH_3 treatment (Figure 4.11c). Change from NO_3-N medium in S1 to NH_3-N medium in S2 has thus induced more stress for those species compared with the same medium (NO_3-N medium) in S1 and S2.

Two experiments (*C. vulgaris* CPCC90 NH_3-NH_3 and MCWW-S27 *M. pusillum* NO_3-mixN) seem to have helped to accelerate nitrogen uptake (negative C:N changes). For the NH_3-NH_3 treatment *C. vulgaris* CPCC90 had a negative S2 C:N slope (Figure 4.11b) and might thus confirm the affinity of this species for the uptake and assimilation of ammonia, especially with starved-cells. However S2 C:N ratios (between days 5 and 8) did not include the first days of the experiment where a stress might have occurred. MCWW-S27 *M. pusillum* for the NO_3-mixN treatment (0.2 mmol NH_3-N/L and 1.8 mmol NO_3-N/L) had also decreased S2 C:N ratios and accelerated nitrogen uptake. The mixture might therefore have slightly helped to improve nitrogen uptake rate. On the other hand for the SMC-2M *C. vulgaris* grown in the same NO3-mixN treatment, the S2 C:N ratios were stable. As discussed above, for this species a higher frequency of

sampling might be necessary to allow to pinpoint variations given that ammonia uptake rate is faster than for other algal species (Figure 4.8a).

Results of nitrate in cells measurements have shown a decrease of nitrate in cells over time (Figure 4.14) which probably means a quick assimilation of nitrate into biomass. Therefore, there was no apparent storage of nitrate compounds in cells. However, the methodology of this analysis might be improved since the small volume of the samples did hinder very accurate measurements; the sample weight was very low (less than 1 mg) and thus a higher biomass would probably be more representative.

Generally, higher growth rates were noticed when nitrogen uptake rates per cell and C:N ratios were high (Figure 4.13) which occurred at the beginning of the growth period. High growth rates could plausibly be linked to nitrogen availability in medium as nitrogen was most available the first days and did not seem to accumulate in cells.

A geometric modelling fit of growth rates as a function of nitrogen uptake rates per cells and C:N ratios has not shown a good curve fitting ($p > 0.05$, Table 4.2). However, general comments can be made for the parameters A and B of the fitted equations (Eq. 4.1) ($\mu = ANuptake^{B/(C:N)}$). The parameter A is directly related to nitrogen uptake rates and the parameter B mitigates the role of high C:N ratios on the nitrogen uptake rates, thus also a reflection of the overall relationship between nitrogen uptake and growth. The fitted parameter A was higher for NO_3-mixN treatments compared with other treatments of MCWW-S27 *M. pusillum* and SCM-2M *C. vulgaris* (Table 4.2) which might confirm the accelerated nitrogen uptake rate when those species were grown in the mixture (0.2 mmol NH_3-N/L NH3 and 1.8 mmol NO_3-N/L).

SMC-6M *S. obliquus* have shown similar ammonia uptake for the NO_3-NH_3 treatment compared with MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* (Supplementary data D12). However, ammonia assimilation for SMC-6M *S. obliquus* might have been lower compared with other species since S2 growth rates of NO_3-NH_3 treatment did not increase with ammonia uptake rates (Figure 4.13f). This behavior is also translated with a lower parameter A of the fitted equation (Eq. 4.1) compared with other species (Table 4.2) and a negative value of the B parameter compared with positive values for other species and treatments (Table 4.2).

5.5 Growth rate as a function on nitrogen concentration in medium

Half-saturation constants of Monod equation have been calculated to determine the relationship between growth rate and nitrogen concentration in medium. As no accumulation seems to have happened in cells, Monod model could probably describe growth. Maximum growth rate in Monod equation is a theoretical value that cannot be reached experimentally. Given the lack of literature data for our strains a maximum growth rate was however hypothesized as the maximum growth rate measured for each species. Maximum growth rates were obviously underestimated for MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* and negative values of half-saturation constants were obtained (Table 4.3). As the value of half-saturation constant decrease, higher growth rates can be obtained with lower nitrogen concentrations in medium. If one omits negative half-saturation constants, higher growth rate with lower nitrogen concentrations

were therefore obtained with resuspension (S2) in NO_3-N media which correlates with the measured growth.

5.6 Bacterial contamination

Presence of bacteria in algal culture leads to a more complex system. Many precautions were taken to limit the bacterial contamination of algal culture. Experiments with the mixture (0.2 mmol NH_3-N/L and 1.8 mmol NO_3-N/L) seem nonetheless to have been contaminated with bacteria. It is difficult to conclude how bacteria might have affect algal growth and nitrogen uptake. Bacteria can favour or inhibit algal growth [5]. Moreover, algae and bacteria could compete for nutrients [5]. Identification of bacteria might help to understand algal-bacterial interactions and their influence on the algal cultures.

5.7 References

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Chapter 6 Summary

Current state of algae cultivation as an option for wastewater treatment and production of biofuels was assessed through an analysis of the utilisation of keywords in the relevant scientific literature. Wastewaters used for cultivation of algae, as listed in the literature, were also characterized.

- Algae cultivation was first developed for environmental purposes. Thereafter, algal cultivation has expanded to algae bio-products production but research has concentrated more on nutrients removal than nutrients uptake or availability.

- Algal research is associated with biomass production but wastewaters are often not seen as a source of nutrients for algal production. Algal cultivations are consequently not operated at optimal conditions specific to algal species.

- Kinetics properties of algae growing in wastewaters are important as nutrient concentrations and algae requirements vary among wastewaters but also in time during algal growth.

Experimental research was focused on batch cultures and the influence of nitrogen chemical species on growth, nutrient uptake and assimilation.

- Algae have experienced growth stress with resuspension (S2). Resuspension from nitrate to ammonia (NO_3-NH_3 treatment) has caused more stress compared with resuspension from nitrate to nitrate (NO_3-NO_3 treatment) in MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus* cultures. Stress was translated into decrease of OD680 (proxy of chlorophyll) and increase of C:N ratios.

- Most experiments have shown a simultaneous decrease in growth rate and nitrogen uptake rate per cell. Since all nutrients except nitrogen were considered in excess, nitrogen availability appears to have regulated growth rate.

- Exponential growth rates did not vary significantly with species studied in this work (*C. vulgaris* CPCC90, MCWW-S27 *M. pusillum*, SMC-2M *C. vulgaris* and SMC-6M *S. obliquus*). *C. vulgaris* CPCC90 was the only species to have shown significant differences of exponential growth rates among different nitrogen treatments. The difference could probably be explained with starved-cells grown in ePBRs that have uptake more nitrogen than nutrient replete cells grown in flasks.

- Algal growth in NH_3-N media has stopped due to low pH in culture. Experiments with pH control should be performed to assess the difference of growth among nitrogen treatments.

- In this study, a medium containing 10% ammonia (0.2 mmol NH_3-N/L) and 90% nitrate (1.8 mmol NO_3-N/L) have prevented decrease of pH and death phase of

culture and increased growth rates, but not significantly from a statistical point of view. Experiments with higher proportion of ammonia should be performed to evaluate the feasibility to increase growth rate with ammonia, in mixed N-source media.

- Both *C. vulgaris* (CPCC90 and SMC-2M) have taken up ammonia at a higher rate compared with nitrate when nitrogen uptake rate per cell. This species had more affinity to uptake and assimilate ammonia efficiently compared with other species (MCWW-S27 *M. pusillum* and SMC-6M *S. obliquus*) of this study.

- MCWW-S27 *M. pusillum* have clearly preferred ammonia over nitrate when grown in medium composed of both nitrogen chemical species with ammonia depleted before nitrate; nitrate uptake rate per cell increased after depletion of ammonia. SMC-2M *C. vulgaris* also seem to have preferred ammonia over nitrate but experiments with higher sampling frequency should be performed to confirm this trend since this species had an ammonia uptake rate per cell more rapid than the measurements steps presented here.

- Stress caused by the change of nitrogen metabolism from ammonia to nitrate was not possible to assess since the handling during resuspension (S2) caused a simultaneous stress (first day of S2). A direct transition to ammonia during the experiment, without resuspension, should be performed to evaluate the impact of nitrogen metabolism shift from ammonia to nitrate.

Supplementary data

A. Supplementary data Chapter 1 (Bibliographic overview)

Supplementary Table 1: Keywords inducing dissimilarities between publication datasets for years 2000 to 2015 (SIMPER analysis[1] carried out on keyword intensity dataset).

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
1	Modelling	wwt	0.74	1.20	11.50	10.40	0.42
2	Activated sludge	wwt	0.50	0.81	9.56	4.54	4.79
3	Oxidation	wwt	0.45	0.74	6.68	3.57	2.27
4	Membrane bioreactor	wwt	0.36	0.59	4.91	0.11	1.75
5	Sludge	wwt	0.31	0.50	5.44	1.66	0.42
6	Water supply	wwt	0.29	0.48	5.15	2.85	1.94
7	Filtration	wwt	0.29	0.47	4.90	2.93	0.83
8	Phenols	wwt	0.27	0.44	4.23	0.00	0.00
9	Biofilm	wwt	0.27	0.43	3.71	2.11	2.72
10	Water management	wwt	0.26	0.43	4.28	3.69	1.90
11	Denitrification	wwt	0.25	0.40	4.22	1.01	0.00
12	Nitrification	wwt	0.22	0.36	3.77	0.96	0.29
13	Reaction Kinetics	wwt	0.22	0.35	3.30	3.24	0.00
14	Optimization	wwt	0.19	0.31	2.78	0.76	1.93
15	Oxidation-Reduction	wwt	0.17	0.28	2.69	0.48	0.00
16	Coagulation	wwt	0.16	0.25	2.07	0.70	1.11
17	Groundwater	wwt	0.15	0.25	2.42	0.20	0.00
18	Iron	wwt	0.15	0.25	2.36	1.36	0.00
19	Catalysis	wwt	0.13	0.22	1.74	0.00	0.83
20	Isolation and purification	wwt	0.13	0.21	1.04	0.91	1.01
21	Microbial activity	wwt	0.11	0.18	1.07	0.92	0.47

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
22	Mass spectrometry	wwt	0.10	0.17	1.61	0.14	0.00
23	Microbial community	wwt	0.10	0.16	1.06	0.48	0.64
24	Water sampling	wwt	0.10	0.16	1.18	0.74	0.00
25	Irrigation	wwt	0.10	0.16	1.46	0.20	0.00
26	Contamination	wwt	0.09	0.14	1.09	0.57	0.00
27	Wastewater disposal	wwt	0.07	0.12	1.07	0.19	0.00
28	Soil	wwt	0.07	0.12	1.07	0.20	0.00
29	Aeration	wwt	0.07	0.12	0.95	0.00	0.38
30	Hydrogen peroxide	wwt	0.07	0.12	1.14	0.00	0.00
31	Ozonation	wwt	0.07	0.11	1.09	0.00	0.00
32	Ultraviolet radiation	wwt	0.07	0.11	0.98	0.19	0.00
33	*Gadus morhua*	wwt	0.07	0.11	0.97	0.19	0.00
34	Waste disposal	wwt	0.06	0.10	0.74	0.37	0.00
35	Photocatalysis	wwt	0.06	0.10	0.92	0.00	0.00
36	Ultrafiltration	wwt	0.06	0.09	0.86	0.00	0.00
37	*Escherichia coli*	wwt	0.05	0.08	0.57	0.30	0.00
38	Engineering	wwt	0.05	0.08	0.67	0.22	0.00
39	X ray diffraction	wwt	0.05	0.08	0.71	0.00	0.00
40	Titanium dioxide	wwt	0.05	0.08	0.71	0.00	0.00
41	Wastewater, textile mills	wwt	0.04	0.07	0.59	0.18	0.00
42	Reactors	wwt	0.04	0.07	0.69	0.00	0.00
43	Acids	wwt	0.04	0.07	0.48	0.29	0.00
44	Drinking water	wwt	0.04	0.07	0.38	0.36	0.00
45	Nanoparticles	wwt	0.04	0.06	0.30	0.16	0.21
46	Chlorine/chloride	wwt	0.03	0.05	0.51	0.00	0.00
47	Fouling	wwt	0.03	0.05	0.31	0.16	0.00
48	Wastewater, papermill	wwt	0.03	0.04	0.40	0.00	0.00

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
49	Anaerobic metabolism	wwt	0.02	0.04	0.26	0.16	0.00
50	Sequencing Batch reactors	wwt	0.02	0.03	0.30	0.00	0.00
51	Liquid chromatography	wwt	0.02	0.03	0.23	0.00	0.00
52	Photodegradation	wwt	0.01	0.02	0.23	0.00	0.00
53	Chromatography	wwt	0.01	0.02	0.23	0.00	0.00
54	Aromatic compounds	wwt	0.01	0.02	0.21	0.00	0.00
55	Transmission electron microscopy	wwt	0.01	0.02	0.19	0.00	0.00
56	Sorption	wwt/a	1.43	2.32	1.10	24.60	0.00
57	(Waste) Nutrient removal	wwt/a	1.26	2.04	15.30	28.10	17.53
58	Water Pollutants/pollution	wwt/a	1.25	2.03	19.00	28.90	5.89
59	Adsorption	wwt/a	1.22	1.97	9.70	23.40	0.00
60	Management	wwt/a	0.98	1.59	20.10	26.30	20.70
61	pH	wwt/a	0.87	1.41	11.50	20.80	5.36
62	Heavy metals	wwt/a	0.85	1.38	5.97	16.40	0.21
63	Environmental Impact	wwt/a	0.62	1.00	8.66	10.60	0.35
64	Industrial waste	wwt/a	0.59	0.95	7.88	10.00	7.28
65	Water Purification	wwt/a	0.53	0.85	8.02	11.70	7.02
66	Isotherms	wwt/a	0.49	0.79	0.24	8.17	0.00
67	Biodegradation	wwt/a	0.44	0.72	10.50	12.50	6.90
68	Water quality	wwt/a	0.43	0.69	7.74	11.30	3.44
69	Oxygen demand	wwt/a	0.41	0.66	5.53	5.95	4.82
70	Pollutants/pollution	wwt/a	0.40	0.65	5.43	6.23	5.41
71	Chromium	wwt/a	0.39	0.63	2.32	6.90	0.00
72	Eutrophication	wwt/a	0.38	0.61	0.00	6.27	1.66
73	Kinetics	wwt/a	0.38	0.61	4.51	8.22	0.78
74	Bioremediation	wwt/a	0.37	0.60	2.66	6.84	6.23
75	Copper	wwt/a	0.36	0.59	2.82	7.09	0.00

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
76	Toxicity	wwt/a	0.36	0.59	3.13	7.36	0.98
77	Cadmium	wwt/a	0.36	0.59	1.38	6.61	0.00
78	Pond	wwt/a	0.35	0.57	0.00	4.60	3.60
79	Dyes	wwt/a	0.35	0.56	4.47	4.81	0.83
80	Microbiology	wwt/a	0.31	0.50	4.42	4.46	3.14
81	Drug	wwt/a	0.28	0.45	3.69	3.73	0.96
82	Ammonia(um)	wwt/a	0.27	0.44	5.32	6.39	4.75
83	Stabilization Pond	wwt/a	0.27	0.43	0.09	4.56	0.00
84	Temperature	wwt/a	0.26	0.42	4.00	6.32	3.10
85	Rivers	wwt/a	0.25	0.40	2.38	3.47	0.70
86	Zinc	wwt/a	0.24	0.39	2.18	4.61	0.00
87	Wetlands	wwt/a	0.23	0.37	1.05	1.64	0.00
88	Metals	wwt/a	0.23	0.37	0.94	3.83	0.00
89	Ecosystems	wwt/a	0.22	0.35	0.44	3.62	0.00
90	Toxicity testing	wwt/a	0.20	0.33	0.00	3.55	0.00
91	Thermodynamics	wwt/a	0.19	0.31	0.12	3.08	0.00
92	Metals ion	wwt/a	0.18	0.30	0.86	2.85	0.00
93	Nickel	wwt/a	0.18	0.29	0.39	2.98	0.00
94	Activated Carbon	wwt/a	0.17	0.28	2.36	2.63	0.00
95	Wastewater, industrial	wwt/a	0.17	0.27	1.77	2.18	0.00
96	Organic matter	wwt/a	0.17	0.27	2.25	2.40	0.00
97	Bioaccumulation	wwt/a	0.17	0.27	0.00	2.61	0.38
98	Scanning electron microscopy	wwt/a	0.17	0.27	1.51	1.99	0.00
99	Water contamination	wwt/a	0.16	0.25	1.63	2.09	0.00
100	Dissolved Oxygen demand	wwt/a	0.12	0.19	0.67	1.58	0.29
101	Bioassay	wwt/a	0.11	0.18	0.00	2.00	0.00
102	Risk assessment	wwt/a	0.11	0.18	0.31	1.69	0.00

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
103	Surface waters	wwt/a	0.11	0.18	0.42	1.62	0.00
104	Disinfection	wwt/a	0.10	0.16	0.46	0.85	0.83
105	Infrared spectroscopy	wwt/a	0.10	0.15	0.11	1.52	0.00
106	Fresh Water	wwt/a	0.09	0.14	0.00	0.99	0.93
107	Performance assessment	wwt/a	0.08	0.14	0.49	1.10	0.00
108	*Lemna*	wwt/a	0.08	0.13	0.00	1.36	0.00
109	*Daphnia*	wwt/a	0.07	0.11	0.00	1.17	0.00
110	Ecotoxicology	wwt/a	0.07	0.11	0.00	1.11	0.00
111	Absorption	wwt/a	0.06	0.09	0.00	0.97	0.00
112	Animal	wwt/a	0.05	0.09	0.10	0.60	0.35
113	Fisheries	wwt/a	0.05	0.08	0.00	0.89	0.00
114	Fourier transform infrared spectroscopy	wwt/a	0.05	0.08	0.24	0.59	0.00
115	Immobilization	wwt/a	0.05	0.07	0.00	0.77	0.00
116	Toxic materials	wwt/a	0.04	0.07	0.00	0.74	0.00
117	Precipitation	wwt/a	0.04	0.07	0.22	0.48	0.00
118	Dewatering	wwt/a	0.04	0.06	0.13	0.39	0.29
119	Lagoons	wwt/a	0.03	0.05	0.00	0.58	0.00
120	Calcium	wwt/a	0.03	0.05	0.14	0.44	0.00
121	Marine environment	wwt/a	0.03	0.05	0.00	0.55	0.00
122	Liquid-solid separation	wwt/a	0.03	0.04	0.12	0.35	0.00
123	Macrophyte	wwt/a	0.03	0.04	0.00	0.47	0.00
124	Turbidity	wwt/a	0.03	0.04	0.00	0.47	0.00
125	Seawater weed	wwt/a	0.02	0.04	0.00	0.46	0.00
126	Acidity	wwt/a	0.02	0.04	0.00	0.41	0.00
127	Zooplankton	wwt/a	0.02	0.04	0.00	0.41	0.00
128	Antibiotics	wwt/a	0.02	0.03	0.13	0.16	0.00
129	Arsenic	wwt/a	0.02	0.03	0.00	0.28	0.00

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
130	Biomass	wwt/a/bf	3.11	5.04	5.33	21.30	72.80
131	CO2/carbon	wwt/a/bf	1.25	2.02	0.23	2.02	34.90
132	Bacteria	wwt/a/bf	1.13	1.83	16.40	24.80	30.40
133	Microorganisms	wwt/a/bf	1.09	1.76	2.57	7.79	23.80
134	*Chlorella*	wwt/a/bf	1.05	1.70	0.00	3.96	23.20
135	Nutrient	wwt/a/bf	0.99	1.60	1.75	9.38	18.30
136	Phosphorus	wwt/a/bf	0.83	1.34	5.35	13.60	17.00
137	Cultivation	wwt/a/bf	0.81	1.32	0.00	2.34	17.10
138	Lipid	wwt/a/bf	0.80	1.29	0.00	2.62	16.60
139	Anaerobic digester	wwt/a/bf	0.79	1.28	4.19	2.24	25.50
140	Energy	wwt/a/bf	0.76	1.24	0.00	0.16	18.30
141	Fatty acid	wwt/a/bf	0.73	1.18	0.00	0.56	23.80
142	Nitrogen	wwt/a/bf	0.71	1.16	7.55	12.80	16.50
143	Renewable resources	wwt/a/bf	0.65	1.05	0.13	0.65	19.20
144	Bioreactor	wwt/a/bf	0.63	1.02	14.90	10.30	16.80
145	Wastewater reclamation	wwt/a/bf	0.63	1.02	7.12	4.73	9.56
146	Electricity	wwt/a/bf	0.62	1.00	0.00	0.00	15.20
147	Photobioreactor	wwt/a/bf	0.56	0.90	0.00	1.30	11.90
148	Energy production	wwt/a/bf	0.54	0.88	0.00	0.00	18.90
149	*Cyanobacteria*	wwt/a/bf	0.52	0.84	0.00	6.37	6.82
150	Growth rate	wwt/a/bf	0.52	0.83	0.00	3.38	9.57
151	Photosynthesis	wwt/a/bf	0.51	0.82	0.00	3.32	12.70
152	Biotechnology	wwt/a/bf	0.50	0.81	0.68	3.71	10.20
153	Chlorophyll	wwt/a/bf	0.49	0.80	0.00	6.09	7.28
154	Metabolism	wwt/a/bf	0.48	0.79	3.11	4.70	12.30
155	Ethanol	wwt/a/bf	0.48	0.77	0.00	0.00	15.30
156	Methane	wwt/a/bf	0.43	0.70	1.55	0.48	14.00

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
157	Oil content	wwt/a/bf	0.41	0.67	0.00	0.00	7.37
158	Wastewater, Municipal	wwt/a/bf	0.41	0.66	0.86	0.61	9.03
159	BOD	wwt/a/bf	0.41	0.66	4.87	5.28	8.85
160	Fermentation	wwt/a/bf	0.40	0.65	0.00	0.00	16.80
161	Ecology	wwt/a/bf	0.40	0.65	0.09	2.62	7.79
162	*Scenedesmus*	wwt/a/bf	0.36	0.58	0.00	1.88	6.53
163	Greenhouse gases	wwt/a/bf	0.36	0.58	0.00	0.00	13.50
164	Nitrogen removal	wwt/a/bf	0.33	0.53	3.13	2.75	6.18
165	Agriculture	wwt/a/bf	0.32	0.52	2.14	2.32	8.74
166	Water recycling	wwt/a/bf	0.30	0.49	2.46	1.57	3.20
167	Carbon	wwt/a/bf	0.28	0.46	4.16	3.78	6.75
168	Anaerobiosis	wwt/a/bf	0.28	0.45	1.42	0.00	7.92
169	Phytoplankton	wwt/a/bf	0.27	0.44	0.00	2.36	4.23
170	Bioprocess	wwt/a/bf	0.27	0.43	1.35	2.67	2.68
171	Electron transport	wwt/a/bf	0.25	0.41	0.00	0.00	11.00
172	Lake	wwt/a/bf	0.25	0.41	0.00	2.58	3.64
173	Growth	wwt/a/bf	0.25	0.41	0.11	1.25	4.59
174	Carbohydrate	wwt/a/bf	0.24	0.39	0.00	0.00	8.18
175	Light	wwt/a/bf	0.23	0.37	0.00	0.15	5.43
176	Hydraulic retention time	wwt/a/bf	0.22	0.36	0.54	1.36	5.39
177	Nitrates	wwt/a/bf	0.22	0.35	2.75	1.75	2.87
178	Electrochemistry	wwt/a/bf	0.21	0.33	0.50	0.24	4.70
179	Organic Carbon	wwt/a/bf	0.20	0.33	1.03	0.34	5.74
180	Calorimetry	wwt/a/bf	0.19	0.31	0.00	0.00	9.30
181	Sugars	wwt/a/bf	0.19	0.31	0.00	0.16	9.09
182	Cell Cultivation	wwt/a/bf	0.19	0.30	0.00	0.84	3.90
183	Bioelectric	wwt/a/bf	0.19	0.30	0.00	0.00	5.17

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
184	Flocculation	wwt/a/bf	0.18	0.30	2.08	1.90	3.49
185	Wastewaters, dairy	wwt/a/bf	0.18	0.29	0.00	0.00	4.98
186	Life Cycle Assessment (LCA)	wwt/a/bf	0.15	0.25	0.00	0.00	3.75
187	Extraction	wwt/a/bf	0.15	0.25	0.86	0.00	4.76
188	Nutrition	wwt/a/bf	0.13	0.21	0.00	0.47	4.85
189	Lipid production	wwt/a/bf	0.13	0.21	0.00	0.00	2.88
190	Microalgae cultivation	wwt/a/bf	0.12	0.20	0.00	0.11	2.68
191	Water resources	wwt/a/bf	0.12	0.20	0.97	0.21	1.63
192	Anaerobic growth	wwt/a/bf	0.12	0.19	0.00	0.00	5.20
193	Energy crops	wwt/a/bf	0.12	0.19	0.00	0.00	5.24
194	Lipid content	wwt/a/bf	0.12	0.19	0.00	0.00	2.97
195	Batch reactors	wwt/a/bf	0.11	0.18	0.63	0.37	1.70
196	Fungi	wwt/a/bf	0.11	0.18	0.00	1.00	1.65
197	Seawater	wwt/a/bf	0.10	0.16	0.00	0.78	1.55
198	Costs	wwt/a/bf	0.09	0.15	0.85	0.17	1.14
199	Biochemistry	wwt/a/bf	0.09	0.14	0.20	0.80	0.93
200	High Rate Pond	wwt/a/bf	0.09	0.14	0.00	0.22	1.81
201	Dry weight	wwt/a/bf	0.09	0.14	0.00	0.00	2.37
202	Glucose	wwt/a/bf	0.08	0.14	0.00	0.00	2.05
203	Manure	wwt/a/bf	0.08	0.13	0.00	0.00	1.95
204	Mixotrophy	wwt/a/bf	0.08	0.13	0.00	0.00	1.89
205	Eukaryota	wwt/a/bf	0.08	0.12	0.00	0.42	1.53
206	Lipid metabolism	wwt/a/bf	0.08	0.12	0.00	0.00	1.79
207	Growth medium	wwt/a/bf	0.07	0.11	0.00	0.00	1.90
208	Wastewater Swine	wwt/a/bf	0.07	0.11	0.00	0.00	1.49
209	Bioconversion	wwt/a/bf	0.06	0.10	0.11	0.00	1.53
210	Flue gases	wwt/a/bf	0.06	0.10	0.00	0.00	1.67

	Keyword (2000 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
211	Proteins	wwt/a/bf	0.06	0.10	0.00	0.00	1.42
212	Microbial Biomass	wwt/a/bf	0.05	0.09	0.00	0.22	0.96
213	Sodium	wwt/a/bf	0.05	0.09	0.26	0.22	0.70
214	Physiology	wwt/a/bf	0.05	0.08	0.16	0.14	0.82
215	Lipid composition	wwt/a/bf	0.05	0.08	0.00	0.00	1.24
216	Nutrient availability	wwt/a/bf	0.04	0.07	0.00	0.00	0.97
217	*Acutodesmus obliquus*	wwt/a/bf	0.04	0.07	0.00	0.00	1.02
218	Lipid storage	wwt/a/bf	0.04	0.07	0.00	0.00	0.95
219	Spirulina	wwt/a/bf	0.04	0.07	0.00	0.00	1.01
220	Animal feed	wwt/a/bf	0.04	0.06	0.00	0.00	1.00
221	*Chlorella pyrenoidosa*	wwt/a/bf	0.04	0.06	0.00	0.00	0.85
222	Environment	wwt/a/bf	0.04	0.06	0.00	0.18	0.70
223	Biodiversity	wwt/a/bf	0.04	0.06	0.00	0.16	0.70
224	Sludge digestion	wwt/a/bf	0.04	0.06	0.14	0.00	0.70
225	Nutrient uptake	wwt/a/bf	0.03	0.05	0.00	0.00	0.78
226	Bioethanol	wwt/a/bf	0.03	0.05	0.00	0.00	0.77
227	Phycoremediation	wwt/a/bf	0.03	0.04	0.00	0.00	0.54
228	Bioactivity	wwt/a/bf	0.02	0.04	0.20	0.00	0.35
229	Genetics	wwt/a/bf	0.02	0.04	0.13	0.00	0.37
230	Acetic acid	wwt/a/bf	0.02	0.04	0.09	0.00	0.38

Supplementary Table 2: Keywords inducing dissimilarities between publication datasets for years 1970 to 2015 (SIMPER analysis[1] was carried out on keyword intensity datasets).

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
1	Environmental impact	wwt	0.89	1.25	9.32	7.40	0.35
2	Industrial waste	wwt	0.77	1.09	9.37	7.33	7.28
3	Activated sludge	wwt	0.66	0.92	9.11	4.65	4.79
4	Filtration	wwt	0.53	0.75	5.15	4.95	0.83
5	Oxidation	wwt	0.42	0.59	3.52	2.69	2.27
6	Wastewater, paper mill	wwt	0.40	0.57	2.74	2.35	0.00
7	Sludge	wwt	0.34	0.48	3.83	2.11	0.42
8	Water supply	wwt	0.33	0.46	3.63	1.87	1.94
9	Nitrification	wwt	0.29	0.41	3.13	1.96	0.29
10	Denitrification	wwt	0.28	0.40	2.89	1.78	0.00
11	Membrane bioreactor	wwt	0.26	0.37	2.63	0.05	1.75
12	Water management	wwt	0.25	0.35	1.91	1.77	1.90
13	Reaction kinetics	wwt	0.24	0.33	2.49	1.78	0.00
14	Phenols	wwt	0.23	0.32	2.50	0.54	0.00

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
15	Irrigation	wwt	0.22	0.31	1.78	1.33	0.00
16	Aeration	wwt	0.22	0.31	1.62	1.23	0.38
17	Costs	wwt	0.21	0.30	1.80	1.03	1.14
18	Activated carbon	wwt	0.20	0.28	2.13	1.17	0.00
19	Sanitation	wwt	0.19	0.27	1.23	0.94	0.00
20	Waste disposal	wwt	0.18	0.25	1.70	0.37	0.00
21	Groundwater	wwt	0.17	0.23	1.48	0.81	0.00
22	Ozonation	wwt	0.16	0.22	1.07	0.67	0.00
23	Soil	wwt	0.14	0.20	1.22	0.55	0.00
24	Sludge disposal	wwt	0.14	0.19	0.93	0.70	0.00
25	Iron	wwt	0.12	0.16	0.98	0.88	0.00
26	Law and regulations	wwt	0.12	0.16	0.73	0.62	0.00
27	Oxidation-reduction	wwt	0.11	0.15	1.15	0.21	0.00
28	Wastewater disposal	wwt	0.09	0.13	0.92	0.29	0.00
29	Contamination	wwt	0.07	0.10	0.57	0.39	0.00
30	Hydrogen peroxide	wwt	0.07	0.10	0.64	0.17	0.00
31	*Gadus morhua*	wwt	0.07	0.09	0.66	0.17	0.00
32	Ultraviolet radiation	wwt	0.06	0.09	0.49	0.27	0.00
33	Reactors	wwt	0.06	0.08	0.64	0.00	0.00
34	Ultrafiltration	wwt	0.06	0.08	0.67	0.00	0.00
35	Mass spectrometry	wwt	0.06	0.08	0.58	0.14	0.00
36	Reverse osmosis	wwt	0.06	0.08	0.54	0.00	0.00
37	Diseases	wwt	0.04	0.06	0.25	0.13	0.00
38	Photocatalysis	wwt	0.03	0.04	0.33	0.00	0.00
39	Acids	wwt	0.02	0.03	0.19	0.12	0.00
40	X ray diffraction	wwt	0.02	0.03	0.26	0.00	0.00

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
41	Titanium dioxide	wwt	0.02	0.03	0.26	0.00	0.00
42	Methanogenesis	wwt	0.02	0.03	0.13	0.08	0.00
43	Volatile Pollutants/pollution	wwt	0.02	0.02	0.20	0.00	0.00
44	Sequencing batch reactors	wwt	0.02	0.02	0.11	0.07	0.00
45	Desalination	wwt	0.02	0.02	0.14	0.00	0.00
46	Water reclamation	wwt	0.01	0.02	0.15	0.00	0.00
47	Fouling	wwt	0.01	0.02	0.11	0.07	0.00
48	Detergents	wwt	0.01	0.02	0.08	0.07	0.00
49	Sludge dewatering	wwt	0.01	0.02	0.13	0.00	0.00
50	Drainage	wwt	0.01	0.01	0.10	0.00	0.00
51	Liquid chromatography	wwt	0.01	0.01	0.08	0.00	0.00
52	Photodegradation	wwt	0.01	0.01	0.08	0.00	0.00
53	Aromatic compounds	wwt	0.01	0.01	0.07	0.00	0.00
54	Transmission electron microscopy	wwt	0.01	0.01	0.07	0.00	0.00
55	Water reuse	wwt	0.01	0.01	0.06	0.00	0.00
56	Sulfur	wwt	0.00	0.01	0.05	0.00	0.00
57	Polymers	wwt	0.00	0.01	0.05	0.00	0.00
58	Biological filtration beds	wwt	0.00	0.01	0.04	0.00	0.00
59	Aerobic treatment	wwt	0.00	0.01	0.04	0.00	0.00
60	Nutrient removal	wwt/a	1.64	2.30	7.57	19.90	16.70
61	Management	wwt/a	1.59	2.23	18.80	26.90	20.70
62	Water Pollutants/pollution	wwt/a	1.28	1.80	16.40	20.40	5.89
63	Sorption	wwt/a	1.08	1.52	0.50	13.50	0.00
64	Modelling	wwt/a	1.06	1.49	11.90	14.20	0.42
65	pH	wwt/a	1.04	1.46	5.76	12.90	5.36
66	Adsorption	wwt/a	1.02	1.43	5.07	12.40	0.00

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
67	Heavy metals	wwt/a	0.74	1.04	3.70	9.83	0.21
68	Pond	wwt/a	0.70	0.99	0.17	8.01	3.60
69	Water quality	wwt/a	0.68	0.96	5.50	10.50	3.44
70	Eutrophication	wwt/a	0.63	0.88	0.12	6.96	1.66
71	Biodegradation	wwt/a	0.62	0.87	5.65	7.23	6.90
72	Toxicity	wwt/a	0.57	0.81	2.03	7.26	0.98
73	Pollutants/pollution	wwt/a	0.53	0.75	4.53	6.60	5.41
74	Chemical Oxygen Demand	wwt/a	0.53	0.75	6.17	6.57	6.39
75	Ammonia(um)	wwt/a	0.51	0.72	3.27	7.00	4.75
76	Lake	wwt/a	0.48	0.67	0.09	4.30	3.64
77	Microbiology	wwt/a	0.46	0.65	4.03	4.06	3.14
78	Stabilization pond	wwt/a	0.44	0.62	0.18	5.45	0.00
79	Rivers	wwt/a	0.42	0.60	1.28	4.77	0.70
80	Copper	wwt/a	0.40	0.56	1.53	5.16	0.00
81	Bioprocess	wwt/a	0.39	0.54	1.53	3.55	2.68
82	Cadmium	wwt/a	0.38	0.54	0.86	4.78	0.00
83	Kinetics	wwt/a	0.37	0.52	2.29	4.11	0.78
84	Temperature	wwt/a	0.35	0.49	2.04	4.05	3.10
85	Fisheries	wwt/a	0.33	0.47	0.28	3.59	0.00
86	Chromium	wwt/a	0.31	0.43	1.09	3.60	0.00
87	Lagoons	wwt/a	0.30	0.43	0.08	3.10	0.00
88	Isotherms	wwt/a	0.30	0.42	0.09	3.65	0.00
89	Wastewater, industrial	wwt/a	0.29	0.40	2.58	2.74	0.00
90	Chlorine/chloride	wwt/a	0.29	0.40	0.70	2.53	0.00
91	Dyes	wwt/a	0.28	0.40	1.84	2.24	0.83
92	Ecosystems	wwt/a	0.28	0.40	0.25	3.42	0.00

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			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
93	Drug	wwt/a	0.25	0.36	1.40	2.11	0.96
94	Organic matter	wwt/a	0.25	0.35	1.29	2.78	0.00
95	Surface waters	wwt/a	0.25	0.35	0.60	2.44	0.00
96	Coliforms	wwt/a	0.24	0.34	0.39	2.53	0.00
97	Zinc	wwt/a	0.23	0.33	1.17	2.65	0.00
98	Food industry	wwt/a	0.23	0.32	0.52	1.79	0.83
99	Aquaculture	wwt/a	0.22	0.31	0.06	2.41	0.00
100	*Daphnia*	wwt/a	0.20	0.29	0.00	2.38	0.00
101	Disinfection	wwt/a	0.19	0.27	0.92	1.32	0.83
102	Toxicity testing	wwt/a	0.19	0.26	0.02	2.32	0.00
103	Metals	wwt/a	0.18	0.26	0.38	2.18	0.00
104	Dissolved Oxygen demand	wwt/a	0.18	0.25	0.43	1.82	0.29
105	Oxidation pond	wwt/a	0.17	0.24	0.00	1.84	0.00
106	Bioaccumulation	wwt/a	0.17	0.24	0.00	1.99	0.38
107	Bioassay	wwt/a	0.17	0.24	0.02	2.00	0.00
108	Wetland	wwt/a	0.16	0.22	0.47	0.70	0.00
109	Viruses	wwt/a	0.15	0.21	0.17	1.36	0.00
110	Nickel	wwt/a	0.15	0.20	0.21	1.74	0.00
111	Thermodynamics	wwt/a	0.14	0.20	0.18	1.64	0.00
112	Drinking water	wwt/a	0.14	0.19	0.60	1.12	0.00
113	Metals ion	wwt/a	0.13	0.19	0.31	1.41	0.00
114	*Lemna*	wwt/a	0.13	0.18	0.00	1.53	0.00
115	Engineering	wwt/a	0.13	0.18	0.74	0.81	0.00
116	Arthropod	wwt/a	0.12	0.17	0.02	1.28	0.00
117	Water contamination	wwt/a	0.12	0.17	0.74	1.09	0.00
118	Land application	wwt/a	0.11	0.16	0.54	0.64	0.00

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
119	Marine biology	wwt/a	0.11	0.15	0.01	1.11	0.00
120	Fertilizers	wwt/a	0.11	0.15	0.18	0.93	0.00
121	Scanning electron microscopy	wwt/a	0.11	0.15	0.55	0.84	0.00
122	Performance assessment	wwt/a	0.10	0.15	0.65	0.85	0.00
123	Invertebrate	wwt/a	0.10	0.14	0.04	0.97	0.00
124	Risk assessment	wwt/a	0.10	0.13	0.21	1.03	0.00
125	Sludge stabilization	wwt/a	0.09	0.13	0.03	0.98	0.00
126	Wastewater, textile mills	wwt/a	0.09	0.12	0.42	0.53	0.00
127	*Escherichia coli*	wwt/a	0.08	0.11	0.35	0.58	0.00
128	Ecotoxicology	wwt/a	0.08	0.11	0.00	0.91	0.00
129	Chemical industry	wwt/a	0.07	0.11	0.22	0.64	0.00
130	Coastal	wwt/a	0.07	0.10	0.05	0.87	0.00
131	Water sampling	wwt/a	0.07	0.10	0.43	0.58	0.00
132	Calcium	wwt/a	0.07	0.10	0.05	0.77	0.00
133	Immobilization	wwt/a	0.07	0.10	0.00	0.84	0.00
134	Precipitation	wwt/a	0.07	0.09	0.38	0.48	0.00
135	Zooplankton	wwt/a	0.06	0.09	0.00	0.81	0.00
136	Enzyme	wwt/a	0.06	0.09	0.05	0.61	0.00
137	Seawater weed	wwt/a	0.06	0.09	0.00	0.74	0.00
138	Odor	wwt/a	0.06	0.08	0.14	0.55	0.00
139	Animal	wwt/a	0.06	0.08	0.18	0.39	0.35
140	Infrared spectroscopy	wwt/a	0.06	0.08	0.04	0.65	0.00
141	Food	wwt/a	0.06	0.08	0.00	0.59	0.00
142	Macrophyte	wwt/a	0.06	0.08	0.00	0.68	0.00
143	Wastewater reuse	wwt/a	0.05	0.07	0.18	0.40	0.00
144	Septic tank	wwt/a	0.05	0.07	0.10	0.44	0.00

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145	*Selenastrum*	wwt/a	0.05	0.07	0.00	0.51	0.00
146	*Chlamydomonas*	wwt/a	0.05	0.07	0.00	0.44	0.38
147	Wastewater, poultry	wwt/a	0.05	0.07	0.03	0.45	0.00
148	Farm waste treatment	wwt/a	0.04	0.06	0.01	0.45	0.00
149	Nitrite	wwt/a	0.04	0.06	0.07	0.46	0.00
150	Wastewater standard	wwt/a	0.04	0.06	0.13	0.42	0.00
151	Chromatography	wwt/a	0.04	0.06	0.18	0.31	0.00
152	Operational regime	wwt/a	0.04	0.06	0.00	0.50	0.00
153	Wastewater, mine	wwt/a	0.04	0.06	0.05	0.35	0.00
154	Hazardous materials	wwt/a	0.04	0.06	0.24	0.29	0.00
155	Mercury	wwt/a	0.04	0.06	0.04	0.35	0.00
156	Absorption	wwt/a	0.04	0.05	0.01	0.50	0.00
157	Phosphoric acid	wwt/a	0.04	0.05	0.04	0.30	0.00
158	*Ceriodaphnia*	wwt/a	0.04	0.05	0.00	0.38	0
159	Isotopes	wwt/a	0.04	0.05	0.05	0.34	0.00
160	Alkalinity	wwt/a	0.03	0.05	0.04	0.37	0.00
161	Crustacea	wwt/a	0.03	0.05	0.01	0.34	0.00
162	Radioactive	wwt/a	0.03	0.05	0.05	0.29	0.00
163	Periphyton	wwt/a	0.03	0.05	0.01	0.37	0.00
164	Turbidity	wwt/a	0.03	0.05	0.00	0.42	0.00
165	Polychlorinated bisphenols	wwt/a	0.03	0.05	0.03	0.29	0.00
166	Diatom	wwt/a	0.03	0.04	0.00	0.33	0.00
167	Carageenan	wwt/a	0.03	0.04	0.00	0.32	0.00
168	Toxic materials	wwt/a	0.03	0.04	0.00	0.39	0.00
169	Sargassum	wwt/a	0.03	0.04	0.00	0.32	0.00
170	Fourier transform infrared spectroscopy	wwt/a	0.03	0.04	0.09	0.25	0.00

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			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
171	Mollusks	wwt/a	0.03	0.04	0.00	0.30	0.00
172	15N tracer	wwt/a	0.03	0.04	0.00	0.30	0.00
173	Organization and management	wwt/a	0.03	0.04	0.12	0.13	0.00
174	Nitrogen fixation	wwt/a	0.03	0.04	0.00	0.31	0.00
175	Slurry	wwt/a	0.03	0.04	0.00	0.29	0.00
176	Trace element	wwt/a	0.03	0.04	0.00	0.29	0.00
177	Magnesium	wwt/a	0.03	0.04	0.00	0.28	0.00
178	Sludge settling tanks	wwt/a	0.02	0.04	0.08	0.18	0
179	Sea	wwt/a	0.02	0.03	0.04	0.21	0.00
180	*Eichhornia crassipes*	wwt/a	0.02	0.03	0.00	0.24	0.00
181	*Bacillariophyta*	wwt/a	0.02	0.03	0.00	0.26	0.00
182	Calcium (bi)Carbonate	wwt/a	0.02	0.03	0.00	0.23	0.00
183	Alginate	wwt/a	0.02	0.03	0.00	0.29	0.00
184	Clarifiers	wwt/a	0.02	0.03	0.02	0.20	0.00
185	Slaughterhouse	wwt/a	0.02	0.03	0.00	0.21	0.00
186	Microcystis	wwt/a	0.02	0.03	0.00	0.21	0.00
187	Protozoa	wwt/a	0.02	0.03	0.00	0.21	0.00
188	Mining	wwt/a	0.02	0.03	0.03	0.20	0.00
189	Gas chromatography	wwt/a	0.02	0.03	0.05	0.17	0.00
190	Biomonitoring	wwt/a	0.02	0.03	0.00	0.21	0.00
191	Anaerobic metabolism	wwt/a	0.02	0.03	0.09	0.16	0.00
192	Wastewater, canning	wwt/a	0.02	0.03	0.00	0.19	0.00
193	Spectroscopy	wwt/a	0.02	0.03	0.02	0.18	0.00
194	Bloom	wwt/a	0.02	0.03	0.00	0.18	0.00
195	Limnology	wwt/a	0.02	0.03	0.01	0.17	0.00
196	Fixed-bed Reactors	wwt/a	0.02	0.03	0.02	0.16	0.00

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			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
197	*Giardia*	wwt/a	0.02	0.02	0.00	0.18	0.00
198	Marine environment	wwt/a	0.02	0.02	0.00	0.23	0.00
199	Fly ash	wwt/a	0.02	0.02	0.02	0.13	0.00
200	Poultry	wwt/a	0.02	0.02	0.00	0.16	0.00
201	Marine Pollutants/pollution	wwt/a	0.02	0.02	0.00	0.21	0.00
202	*Tracheophyta*	wwt/a	0.02	0.02	0.00	0.15	0.00
203	Potassium	wwt/a	0.02	0.02	0.00	0.18	0.00
204	*Ulva*	wwt/a	0.01	0.02	0.00	0.16	0.00
205	Liquid-solid separation	wwt/a	0.01	0.02	0.04	0.15	0.00
206	*Cladocera*	wwt/a	0.01	0.02	0.00	0.16	0.00
207	Enzyme kinetics	wwt/a	0.01	0.02	0.00	0.13	0.00
208	Acidity	wwt/a	0.01	0.02	0.00	0.18	0.00
209	Wastewaters, cyanide	wwt/a	0.01	0.02	0.04	0.09	0.00
210	Pesticide	wwt/a	0.01	0.02	0.03	0.10	0.00
211	Cation	wwt/a	0.01	0.02	0.00	0.16	0.00
212	Sulfide	wwt/a	0.01	0.02	0.02	0.13	0.00
213	Antibiotics	wwt/a	0.01	0.02	0.05	0.07	0.00
214	Wastewater, process	wwt/a	0.01	0.02	0.03	0.09	0.00
215	Cattle	wwt/a	0.01	0.02	0.00	0.13	0.00
216	Hazardous waste	wwt/a	0.01	0.02	0.04	0.09	0.00
217	Arsenic	wwt/a	0.01	0.01	0.00	0.12	0.00
218	Sludge bulking	wwt/a	0.01	0.01	0.02	0.09	0.00
219	Calcium oxide	wwt/a	0.01	0.01	0.01	0.09	0.00
220	Surfactant	wwt/a	0.01	0.01	0.02	0.08	0.00
221	Aerobic metabolism	wwt/a	0.01	0.01	0.04	0.05	0.00
222	Biomass	wwt/a/bf	2.84	4.00	3.39	17.80	72.80

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223	Bacteria	wwt/a/bf	1.63	2.30	8.72	20.40	30.40
224	Phosphorus	wwt/a/bf	1.13	1.59	4.31	14.30	17.00
225	Nutrient	wwt/a/bf	1.04	1.46	1.15	8.59	18.30
226	Nitrogen	wwt/a/bf	1.02	1.44	4.81	12.70	16.50
227	Microorganisms	wwt/a/bf	0.93	1.31	2.77	5.63	23.80
228	*Chlorella*	wwt/a/bf	0.91	1.28	0.00	4.70	23.20
229	Bioreactor	wwt/a/bf	0.87	1.22	7.94	6.68	16.80
230	CO2/carbon	wwt/a/bf	0.87	1.22	0.12	1.59	34.90
231	Anaerobic digester	wwt/a/bf	0.70	0.98	3.60	1.84	25.50
232	Cultivation	wwt/a/bf	0.68	0.95	0.06	2.58	17.10
233	Chlorophyll	wwt/a/bf	0.61	0.86	0.00	6.02	7.28
234	Cyanobacteria/ bluegreen algae	wwt/a/bf	0.60	0.85	0.00	5.78	0.0682
235	Water purification	wwt/a/bf	0.59	0.83	2.90	4.96	7.02
236	Ecology	wwt/a/bf	0.57	0.80	0.84	4.73	7.79
237	Photosynthesis	wwt/a/bf	0.55	0.77	0.01	3.95	12.70
238	Lipid	wwt/a/bf	0.54	0.76	0.00	1.12	16.60
239	Energy	wwt/a/bf	0.54	0.76	0.22	0.55	18.30
240	Wastewater reclamation	wwt/a/bf	0.54	0.75	3.36	2.40	9.56
241	Bod	wwt/a/bf	0.53	0.75	4.09	7.04	8.85
242	Fatty acid	wwt/a/bf	0.50	0.70	0.00	0.56	23.80
243	Oxygen demand	wwt/a/bf	0.44	0.62	3.07	3.49	4.82
244	Electricity	wwt/a/bf	0.44	0.61	0.07	0.28	15.20
245	Growth rate	wwt/a/bf	0.43	0.61	0.00	2.47	9.57
246	Biotechnology	wwt/a/bf	0.43	0.61	0.58	2.57	10.20
247	Metabolism	wwt/a/bf	0.43	0.61	1.16	2.13	12.30
248	Renewable resources	wwt/a/bf	0.41	0.58	0.05	0.28	19.20

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
249	Bioremediation	wwt/a/bf	0.40	0.56	0.96	3.42	6.23
250	Photobioreactor	wwt/a/bf	0.38	0.54	0.00	0.66	11.90
251	Wastewater, municipal	wwt/a/bf	0.37	0.52	0.56	1.43	9.03
252	Nitrogen removal	wwt/a/bf	0.36	0.50	1.56	2.36	6.18
253	Carbon	wwt/a/bf	0.34	0.48	2.17	2.41	6.75
254	Energy production	wwt/a/bf	0.34	0.48	0.00	0.07	18.90
255	*Scenedesmus*	wwt/a/bf	0.34	0.47	0.00	2.03	6.53
256	Methane	wwt/a/bf	0.32	0.46	1.16	0.36	14.00
257	Agriculture	wwt/a/bf	0.32	0.45	1.21	2.27	8.74
258	Phytoplankton	wwt/a/bf	0.31	0.44	0.03	2.82	4.23
259	Ethanol	wwt/a/bf	0.31	0.43	0.00	0.00	15.30
260	Oil content	wwt/a/bf	0.30	0.42	0.12	0.07	7.37
261	Fermentation	wwt/a/bf	0.28	0.40	0.18	0.35	16.80
262	Flocculation	wwt/a/bf	0.28	0.40	1.31	2.69	3.49
263	Growth	wwt/a/bf	0.28	0.40	0.09	1.92	4.59
264	Nitrates	wwt/a/bf	0.28	0.39	1.80	2.18	2.87
265	Biofilm	wwt/a/bf	0.28	0.39	2.57	1.15	2.72
266	Cell cultivation	wwt/a/bf	0.25	0.35	0.00	2.02	3.90
267	Economics	wwt/a/bf	0.24	0.34	1.26	1.16	1.65
268	Agricultural wastes	wwt/a/bf	0.23	0.32	0.17	1.13	9.09
269	Water recycling	wwt/a/bf	0.22	0.31	1.33	0.87	3.20
270	Greenhouse gases	wwt/a/bf	0.22	0.31	0.00	0.00	13.50
271	Water resources	wwt/a/bf	0.21	0.30	1.49	0.71	1.63
272	Light	wwt/a/bf	0.19	0.27	0.00	0.69	5.43
273	Anaerobiosis	wwt/a/bf	0.17	0.24	0.54	0.00	7.92
274	Carbohydrate	wwt/a/bf	0.17	0.24	0.00	0.20	8.18

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
275	Coagulation	wwt/a/bf	0.16	0.22	0.90	1.05	1.11
276	Electron transport	wwt/a/bf	0.15	0.22	0.00	0.00	11.00
277	Optimization	wwt/a/bf	0.15	0.22	1.22	0.51	1.93
278	Hydraulic retention time	wwt/a/bf	0.15	0.22	0.20	0.75	5.39
279	Proteins	wwt/a/bf	0.15	0.22	0.00	1.23	1.42
280	High rate pond	wwt/a/bf	0.14	0.20	0.02	1.08	1.81
281	Fungi	wwt/a/bf	0.14	0.19	0.09	1.16	1.65
282	Organic carbon	wwt/a/bf	0.13	0.18	0.39	0.15	5.74
283	Wastewaters, dairy	wwt/a/bf	0.13	0.18	0.00	0.20	4.98
284	Seawater	wwt/a/bf	0.13	0.18	0.09	1.08	1.55
285	Electrochemistry	wwt/a/bf	0.13	0.18	0.22	0.10	4.70
286	Manure	wwt/a/bf	0.12	0.17	0.03	0.88	1.95
287	Sugars	wwt/a/bf	0.12	0.17	0.01	0.14	9.09
288	Calorimetry	wwt/a/bf	0.12	0.16	0.00	0.00	9.30
289	Biochemistry	wwt/a/bf	0.12	0.16	0.30	0.82	0.93
290	Bioelectric	wwt/a/bf	0.11	0.16	0.00	0.00	5.17
291	Life cycle assessment (lca)	wwt/a/bf	0.10	0.14	0.00	0.00	3.75
292	Solar radiation	wwt/a/bf	0.10	0.14	0.00	0.80	1.65
293	Swine	wwt/a/bf	0.10	0.13	0.00	0.62	1.49
294	Extraction	wwt/a/bf	0.10	0.13	0.36	0.07	4.76
295	Petrochemical industry	wwt/a/bf	0.09	0.13	0.35	0.46	0.56
296	Isolation and purification	wwt/a/bf	0.09	0.12	0.44	0.39	1.01
297	Lipid production	wwt/a/bf	0.08	0.12	0.00	0.00	2.88
298	Nutrition	wwt/a/bf	0.08	0.12	0.00	0.26	4.85
299	Fresh water	wwt/a/bf	0.08	0.12	0.00	0.73	0.93
300	Coal gasification	wwt/a/bf	0.08	0.11	0.18	0.50	0.70

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
301	Animal feed	wwt/a/bf	0.08	0.11	0.00	0.56	1.00
302	Carbonate	wwt/a/bf	0.08	0.11	0.00	0.11	3.31
303	Lipid content	wwt/a/bf	0.08	0.11	0.00	0.00	2.97
304	Anaerobic growth	wwt/a/bf	0.07	0.10	0.02	0.00	5.20
305	Batch reactors	wwt/a/bf	0.07	0.10	0.23	0.16	1.70
306	Glucose	wwt/a/bf	0.07	0.10	0.03	0.20	2.05
307	Energy crops	wwt/a/bf	0.07	0.10	0.00	0.00	5.24
308	Catalysis	wwt/a/bf	0.07	0.10	0.67	0.00	0.83
309	Microbial activity	wwt/a/bf	0.07	0.10	0.39	0.39	0.47
310	Dry weight	wwt/a/bf	0.07	0.10	0.00	0.19	2.37
311	Sludge digestion	wwt/a/bf	0.06	0.09	0.55	0.00	0.70
312	Microbial community	wwt/a/bf	0.06	0.09	0.38	0.20	0.64
313	Sodium	wwt/a/bf	0.06	0.09	0.09	0.41	0.70
314	Acetic acid	wwt/a/bf	0.06	0.08	0.34	0.28	0.38
315	Eukaryota	wwt/a/bf	0.05	0.07	0.00	0.25	1.53
316	Mixotrophy	wwt/a/bf	0.05	0.07	0.00	0.00	1.89
317	Lipid metabolism	wwt/a/bf	0.05	0.07	0.00	0.00	1.79
318	Environment	wwt/a/bf	0.05	0.07	0.02	0.37	0.70
319	Bioconversion	wwt/a/bf	0.05	0.07	0.04	0.10	1.53
320	Waste Nutrient removal	wwt/a/bf	0.05	0.07	0.34	0.00	0.83
321	Fluidized bed Reactors	wwt/a/bf	0.05	0.07	0.24	0.12	0.83
322	Wastewater, distillery	wwt/a/bf	0.05	0.06	0.00	0.29	0.83
323	*Chlorella pyrenoidosa*	wwt/a/bf	0.05	0.06	0.00	0.26	0.85
324	Air Pollutants/pollution	wwt/a/bf	0.04	0.06	0.26	0.09	0.47
325	Growth medium	wwt/a/bf	0.04	0.06	0.00	0.00	1.90
326	Physiology	wwt/a/bf	0.04	0.06	0.06	0.17	0.82

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
327	Flue gases	wwt/a/bf	0.04	0.05	0.00	0.00	1.67
328	Lipid composition	wwt/a/bf	0.04	0.05	0.00	0.05	1.24
329	Microbial biomass	wwt/a/bf	0.04	0.05	0.00	0.09	0.96
330	Aerobiosis	wwt/a/bf	0.04	0.05	0.02	0.00	1.65
331	Dewatering	wwt/a/bf	0.03	0.05	0.18	0.17	0.29
332	Nutrient availability	wwt/a/bf	0.03	0.04	0.00	0.00	0.97
333	Genetics	wwt/a/bf	0.03	0.04	0.05	0.17	0.37
334	*Acutodesmus obliquus*	wwt/a/bf	0.03	0.04	0.00	0.00	1.02
335	Nutrient uptake	wwt/a/bf	0.03	0.04	0.00	0.08	0.78
336	Pilot scale/plant	wwt/a/bf	0.03	0.04	0.04	0.13	0.37
337	Lipid storage	wwt/a/bf	0.03	0.04	0.00	0.00	0.95
338	Carotenoid	wwt/a/bf	0.03	0.04	0.00	0.11	0.70
339	*Spirulina*	wwt/a/bf	0.03	0.04	0.00	0.00	1.01
340	Biosynthesis	wwt/a/bf	0.02	0.03	0.00	0.07	0.64
341	Hydrolysis	wwt/a/bf	0.02	0.03	0.05	0.11	0.35
342	Brewery wastewater	wwt/a/bf	0.02	0.03	0.00	0.15	0.21
343	Nanoparticles	wwt/a/bf	0.02	0.03	0.11	0.07	0.21
344	Biodiversity	wwt/a/bf	0.02	0.03	0.00	0.07	0.70
345	Brackish water	wwt/a/bf	0.02	0.03	0.00	0.06	0.70
346	Heterotrophy	wwt/a/bf	0.02	0.03	0.00	0.09	0.46
347	Bioethanol	wwt/a/bf	0.02	0.03	0.00	0.00	0.77
348	Land use	wwt/a/bf	0.02	0.03	0.00	0.09	0.47
349	Design	wwt/a/bf	0.02	0.03	0.01	0.00	0.83
350	Bicarbonate	wwt/a/bf	0.02	0.02	0.00	0.09	0.35
351	Phycoremediation	wwt/a/bf	0.02	0.02	0.00	0.00	0.54
352	Glycerol	wwt/a/bf	0.02	0.02	0.00	0.07	0.28

	Keyword (1970 to 2015)	Dataset where keyword is dominant	Dissimilarity		Mean abundance (%)		
			Average	Contribution (%)	wwt (2000-2015)	wwt/a (2000-2015)	wwt/a/bf (2006-2015)
353	Forestry	wwt/a/bf	0.01	0.02	0.03	0.00	0.47
354	Bioactivity	wwt/a/bf	0.01	0.02	0.07	0.00	0.35

Reference:

[1] Ø. Hammer, D.A.T. Harper, P.D. Ryan, PAST: Paleontological Statistics software package for education, 2001.

B. Supplementary data Chapter 1 (Defining wastewaters used for cultivation of algae)

Selected literature summary of reported nutritional parameters; N:P ratios calculated

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Municipal wastewaters													

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Sewage[1]					95.9		7	30.3[g]		NC 7.12	Tolerance to salt tested	Antibiotic sensitivity test	With and without filtration, with and without dilution
Sewage[2]	80.5± 6.62[a]	2.94 ±0.60[a]	0.18±0.23[a]		88.47± 3.18 84.42± 2.65[a]	4.93± 0.06[a]	8.91±0.38 6.07±0.26[a]	22[g]	36.2[ag]	NC 8.0			
Sewage[2]	39.55± 4.21[a]	< 0.5[a]	0.02 ±0.01[a]		52.08± 9.48 41.96± 5.47[a]	4.89±0.12[a]	8.81±0.15 5.93±0.18[a]	13.1[g]	17.9[ag]	NC 8.1			
Sewage[3]	41.3± 12.79			9.7±4.9	51.0± 14.2		8.5	13.3[g]		NC			
Sewage[4]	61.7-63.5	2.3 -2.8				6.5-21.9			9.8[g]	C (CO_2) 7.8 – 8.0		Bacteria, competing micro - organisms	Filtered
Sewage[5]	92.0	3.9			110.2		5.3[o]	46[g]		NC 7.5			Filtered, mixed with seawater
Sewage[6]	41.11[b]				64[c]		6.92	20.5[g]		C (NaOH + CO_2) 7.5			Trace elements added
Sewage[7]	21.14[b]	0.05[d]	0.005[e]		47.04[c]	2.0[f]	2.4[fo]	43.4[g]	23.4[g]	NC 6.78	486 mg L^{-1}		
Sewage[8]	33.4±0.6	nd	nd		40.65± 0.07		5.66±0.08	15.9[g]		NC			Filtered Metal ions removal
Primary effluent[2]	30.6±0.1[a]	< 0.5[a]	< 0.02[a]		35.6±1.0 33.9± 0.83[a]	1.7±0.1[a]	5.08±0.2 3.20±0.1[a]	15.5[g]	39.9[ag]	NC 7.2			
Primary effluent[9]	35.5	0.40		12.9	48.4[c]	3.89	4.29	25[g]	20.2[g]	NC 7.10			
Primary effluent[10]					25.5±0.2		2.8±0.2	20.2[g]		NC 9.3±0.0			Filtered, sterilized, with and without dilution
Primary effluent[11]	32.39± 1.05	0.08± 0.03	0.02± 0.01			2.39±0.67			30[g]	C (CO_2) < 8.3			
Primary effluent[12]					45±12[c]		6.5±1.6	15.3[g]		NC			

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Primary effluent[8]	32.2±0.4	nd	nd		38.95±1.91		6.86±0.05	12.6[g]		NC			Filtered Metal ions removal
Primary effluent[13]	39	< 0.01	< 0.01	12	51	2.1			41.1[g]	C (CO_2) 7.0-8.0			Filtered
Secondary effluent[14]					24.92 – 26.16		1.77-2.23	28.3[g]		NC			
Secondary effluent[15]	10.0±7.1	6.6±4.0			18.9±4.1		1.7±0.3	24.6[g]		NC			Filtered and UV
Secondary effluent[16]	24.1±0.7					2.4±0.14			22.2[g]	C (CO_2) 7			Autoclaved and filtered
Secondary effluent[17]	7.43 – 16.23	1.56 – 8.52	0.18 – 0.85			0.99 – 2.14			16.7[g]	C 7.2-8.5			
Secondary effluent[6]	7.23[b]				14.30[c]		1.25	25.3[g]		C (NaOH + CO_2) 7.5			Trace elements were added
Secondary effluent[18]					8		2.6	6.8[g]		NC 7.40			Autoclaved and diluted 1:10
Secondary effluent[7]	19.58[b]	0.035[d]	0.002[e]		30.24[c]	0.77[f]	3.3[fo]	20.3[g]	56.3[f]	NC 7.74			
Secondary effluent[19]	21.3[k]	< 0.2[d]	< 0.3[e]			3.9[f]			12.1[g]	NC 9.3	565 mg L^{-1}		Filtered and autoclaved
Secondary effluent[20]	21.6-228.85					2.22 – 3.51			18.1-24.3	C (CO_2) 6.2, 6.6 and 7			After UV disinfection and ultrafiltration Metals removal
Secondary effluent[21]	7.73	2				1.73			9.9[g]	NC			Filtered
Secondary effluent[22]					20.4±4.6		3.5±0.9	12.9[g]		NC			Fish-amended reactors
Secondary effluent (in the aeration tank)[8]	nd	16.95± 0.07	0.074±0.003		19.1±0.1		0.32±0.04	132.2[g]		NC			Filtered Metal ions removal
Secondary effluent[23]					40		2.0	44.3[g]		NC			Autoclaved, addition of Fe and P
Centrate[2]	125.1± 2.1[a]	< 0.5[a]	< 0.02[a]		130.1±1.4 123.9± 1.5[a]	35.3±1.5[a]	60.49±1.7 55.01±1.0[a]	4.8[g]	7.8[ag]	NC 7.1			
Centrate[6]	55.18[b]				128.60[c]		120.60	2.4[g]		C (NaOH + CO_2) 7.5			Trace elements were added

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Centrate[18]					53		9.4	12.5[g]		NC 9.47			Autoclaved and diluted 1:10
Centrate[24]	113±18	0.35± 0.36	< 0.03		275±151[c]	215±135	392±82[n]	1.6[g]	1.2[g]	C (CO_2) 7.0-7.5		Seems that CO_2 injection repress bacteria pollution Metallic inhibitors	Settling pre-treatment
Centrate[8]	71.8±1.1	nd	nd		131.5±2.1		201.5±10.6	1.4[g]		NC			Filtered Metal ions removal
Centrate[25]	91±4.4	0.35±0.36	< 0.03		134±7.1[c]	211±3.8			0.95[g]	NC			Autoclaved and filtered
Agricultural wastewaters													
Aquaculture[26]	4.24± 0.38	2.00± 0.23	0.13± 0.07		6.81±0.68		0.42±0.05	35.9[g]		C (CO_2) 6.8-7.2	2.8% (2 freshwater species adapt well)		Settled
Aquaculture[27]	0.529	1.697	0.164			0.213			5.5[g]	NC			
Aquaculture[28]	0.48	40.7	0.146		41.3[i]		4.96[j]	18.4		C (CO_2) 8.40			Ultrafiltration Orthophosphate supplemented
Dairy[29]	48±1.5				118.0±2.8	48.6±0.9[f]			2.2[g]	NC 8.3±0.2 (CO_2 or acetic acid addition to decrease pH)			Centrifuged, autoclaved
Dairy[30]	51.9				97.0[c]		20.6[fo]	10.4[g]		NC			Sedimentation, filtration
Dairy[31]					1600		230	15.4[g]		C (CO_2) 7.0-7.5			Dilution
Dairy[32]	1782				3305[c]		86.8[fo]	84.3[g]					
Dairy[33]	306±49	< 1			1210±194		303±55	8.8[g]		C (HCl) 7.0-7.5			
Piggery[34]	5190± 9.21				6880± 6.14		367±1.46	41.5[g]		NC 7.45±0.31			
Piggery[35]	1197±6[a]	6.8±1.0[a]	1.6±0.2[a]		1280±15[a]	4.2±0.3[a]	4.3±0.5[a]	659[g]	631[g]	NC 8.1		NH_4-N (1197 mg/L) reduce algal growth	Treated and filtered Dilution with synthetic medium : 0, 20, 40, 60, 80, 100%

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Piggery[36]					341	137[f]				NC 6.0		COD inhibition	Settled and diluted with distilled water (250, 400, 520, 650, 800, 1100 COD mg L^{-1})
Piggery[37]					162.0 ±8.0		209 ±5.5[o]	1.7[g]		NC 6.2			Autoclaved and filtered Dilution with distilled water: 2500, 1900, 1300, 800 and 400 mg L^{-1} COD
Biotechnology facility effluent[38]					190		11-12	36.6[g]		C (CO_2) 7±0.3	3.34±0.6%		Wastewater has high salinity: dilution for salinity adjustment Effluent of internal circulation reactor
Brewery[39]					72.6±0.1		54.4±0.2	3[g]		NC 8.6±0.1 C (HCl, NaOH) 8, 10 and 11			Centrifuged, filtered, autoclaved
Brewery[40]					50-75		15-20	7.9[g]		NC 6.5 – 7.5			Anaerobically digested Filtered, centrifuged and sterilized
Brewery[41]					7.16-14.5		14.28-18.49	1.5[g]		NC 6.3-6.4			Settled and filtered
Carpet dyeing[42]	17.58-25.85	0.21-28.13			32.6-45.9[c]	6.63-11.45[f]	5.47-13.83	9[g]	5.3[g]	NC 6.54-7.18			untreated

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Carpet dyeing[42]	0.57-3.61	1.39-3.91			3.97-5.53[c]	5.74-7.16[f]	3.47-7.89	1.9[g]	0.7[g]	NC 6.88-8.04	Marine algae seem to have osmotic adjustment and regulation mechanisms for salinity change		Treated Filtered and sterilized
Desalination[43]	1.05[k]				30.0		0.70[o]	94.9[g]		NC 8.11			Treated and filtered Na: 987.5 mg/L Cl: 1691.3 mg/L Dilution: 25%
Herbal pharmaceutical effluent[44]					444		88[fo]	11.2[g]		NC 3.9-4.0			Raw
Herbal pharmaceutical effluent[44]					136		36[fo]	8.4[g]		NC 6.4			Physico-chemically treated
Herbal pharmaceutical effluent[44]							21[fo]			NC 6.9			Biologically treated
Industrial[45]	63.3	6.8				43.6			3.2[g]	NC			Untreated wastewater
Landfill leachate[46]	151.66± 39.52					8.18±1.06			41.1[g]	NC 6.81±0.12		Toxicity evaluation (IC_{50})[h]	Treated, different loadings
Olive-oil[47]	2.3±0.67	99.13±5.13[l]			2.90± 0.46[c]	0.12±0.01[fm]	0.35±0.02	18.3[g]	42.4[g]	NC 5.37 (initial pH adjusted to 8.0)			Raw
Olive-oil centrifuged[47]	nd				1.13±0.1[c]	0.082±0.007[fm]	0.30 ±0.02	8.3[g]		NC			
Olive-oil, settled 10 days[47]	nd	35.57± 4.04[l]			1.67± 0.08[c]	0.065±0.007[fm]	0.23±0.03	16.1[g]		NC		Decrease of phenol concentration and turbidity with NaOCl	
Paper mill[48]	156[k]	< 0.5	< 0.01							NC 9.2 (CO_2 addition)		Al, Mn	Diluted with medium Heavy metals: Fe, Mn

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Pharmaceutical (Riboflavin production)[49]					885.3± 36.2		326±18.3	6[g]		NC 4.7±0.8			microfiltration
Pulp and paper mill[50]										NC			Diluted Color: 4018 PtCo AOX
Steel[51]	45.0-74.1[b]	4.0-8.0[d]					nd			NC 7.0-8.5 (but a buffer (HEPES) was added)			P addition
Soybean processing[52]	52.1				267.1		56.3	10.5[g]		NC		Comparaison of growth for toxicity (between 3 dyes)	Centrifuged, autoclaved
Tannery[53]		11[d]					3.90			NC 5.6			Diluted with distilled water Heavy metals: Cr, Cu, Pb, Zn
Tannery[54]	762[b]					5[f]			337[g]	NC 7.40	Impact of salinity		Diluted with distilled water Heavy metals: Fe, Cr
Textile[55]	0.90	< 0.30				0.05[f]			39.9[g]	NC 8.4 (with and without buffering solution)	0		Color: 500 PtCo
Textile[56]	0.47-50.83	1.23-5.60				0.07-4.01			27.8[g]	NC 3.85-11.40			Treated
Textile[57]	220									NC 8.9	Impact of different salt species and concentrations studied	Phytotoxicity tests	Filtered, autoclaved, diluted with medium Apparent color: 169.67 – 1937.33 PtCo True color: 76.00 – 1777.73 PtCo

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Textile[58]		78±2[d]				1.4±0.03[f]				6.7			Filtered and autoclaved Chloride: 847±30 mg/L
Oil and gas produced waters													
Oil sands process water[59]		3				1				NC			Addition of $NaNO_3$ and KH_2PO_4
Anaerobic digestate													
Dairy[60]	136±8	0			257±16[c]	10±1[m]	34±2	16.7[g]	30.1[g]	NC 7.89	EC: 2510±10 µS/cm		
Dairy[32]	2232				3456[c]		81.5[fo]	93.9[g]		NC			Filtered, 4 dilution
Dairy[33]	1620± 341	< 1			2370±123		240	21.9[g]		C (HCl) 7.0-7.5			No water is used for flushing = higher concentration
Dairy[33]	178±13	< 1			225±15		24.7±3	20.2[g]		C (HCl) 7.0-7.5			
Poultry[61]	4315± 834					83±3	96±5		115[g]	NC 7.85		Ammonia, fed-batch (daily addition)	K: 2590±74 mg/L
Poultry[62]	1143-1787	0.55-10.7			1570 - 2473		154-214	24.3[g]		NC			Centrifuged K: 1632 - 2100
Poultry[63]	3275				3565		283	27.9[g]		NC			Centrifuged, autoclaved K: 1876 mg/L
Sewage sludge[64]	906				1210		28	95.7[g]		C (CO_2) < 8			Centrifuged and addition of polymer Diluted with wastewater effluent
Sewage sludge[65]	238.6 - 272.5						35.2 -42.6			C (NaOH/ HCl) 5 levels: 5.7 to 6.5, 6.8 to 7.3, 7.6 to 8.1, 8.3 to 8.8, 9.1 to 9.6		Bacteria/grazers pH, free ammonia	1.5x diluted in secondary treated effluent Settled and filtered
Swine[66]	3294	111[l]				277			26.3[g]	NC 7.2		Cu had probably been toxic	Diluted (manure concentration up to 6%)

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Swine[34]	1576± 6.00				2140± 4.21		604±2.38[o]	7.8[g]		NC 8.31±0.29			Centrifuged, supernatant used, diluted with deionized water
Swine[67]	644±11				981±1		81±4	26.8[g]		NC		Chloride: 52524 mg/L Color: 6175±26 PtCo/L	Filtered
Swine[68]		< 0.6[d]			3304± 195[c]		192±20	38.1[g]		NC 7.6			Autoclaved Different feeding frequency
Swine[69]					138.83± 17.03		185.37± 7.85	1.7[g]		NC 6.31±0.12			Autoclaved and filtered Dilution with distilled water: 2200, 1600, 1200, 800, and 400 mg L^{-1} COD
Swine co-digested with microalgae biomass[16]	38.8±1.6					5.66±0.81			15	C (CO_2) 7			Secondary autoclaved and filtered municipal wastewater + autoclaved digestate (1.6x concentration of ammonia)
Swine co-digested with microalgae biomass[16]	58.8±1.8					9.55± 0.59			13.6[g]	C (CO_2) 7		fouling	Secondary autoclaved and filtered municipal wastewater + autoclaved digestate (2.4x concentration of ammonia)
Synthetic wastewater													

Wastewater type	NH_4-N	NO_3-N	NO_2-N	Total org. N	TN	PO_4-P	TP	TN:TP	Available N: Available P	pH Controlled: (C, set point; NC, initial pH, Not controlled	Salinity	Toxicity	Other comments
	(mg L^{-1})							(mol mol^{-1})					
Synthetic wastewater[70]	21	1.6			22.6	5.6	5.6	8.9	8.9	NC			
Synthetic wastewater[71]	20.4				20.4	4.1	4.1	11	11	C			
Synthetic wastewater[72]	39.83	11.83			51.66	4.46	4.46	25.6	25.6	NC			
Synthetic wastewater[6]		41.2			41.2	53.3	53.3	1.7	1.7	C (NaOH)			
Synthetic wastewater[37]		102				7.69		29.3	29.3				

nd: Not detected/reported
[a] Filtered
[b] Calculated (raw data as NH_3)
[c] Total Kjeldahl nitrogen (TKN)
[d] Calculated (raw data as NO_3)
[e] Calculated (raw data as NO_2)
[f] Calculated (raw data as PO_4)
[g] Calculated (average of multiple values)
[h] Inhibition concentration
[i] Dissolved inorganic N
[j] Dissolved inorganic P
[k] Calculated (raw data NH_4)
[l] NO_3-N +NO_2-N
[m] Reactive phosphate
[n] Soluble
[o] Expressed as total phosphate

Synthetic sewage example

OECD recommendations for synthetic sewage[73]:
For 1L: peptone, 160 mg; meat extract, 110 mg; urea, 30 mg; anhydrous dipotassium hydrogen phosphate (K_2HPO_4), 28 mg; sodium chloride (NaCl), 7 mg; calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), 4 mg; magnesium sulphate heptahydrate ($Mg_2SO_4 \cdot 7H_20$), 2 mg.

The variability in the commercial peptones and meat extracts can lead to variable nutritional profiles. The table below summarizes the total nitrogen and phosphorus in a series of animal-origin peptones and beef extracts manufactured by BD-Biosciences[74].

BD-Biosciences meat extracts and animal-origin peptones;	Product content[74]		Calculated nutrient content according to OECD recipe			
	N%	P%	N (mg L^{-1})	P (mg L^{-1})	N (mM)	P (mM)
Meat/Beef extracts						
BD BBL™ Beef Extract Powder	12.40%	3.22%	19.84	5.152	1416.46	166.33
BD Bacto™ Beef Extract, Desiccated	13.90%	0.43%	22.24	0.688	1587.81	22.21
Animal origin peptones						

BD BBL™ Gelysate™ Peptone	17.00%	0.18%	27.2	0.288	1941.93	9.30
BD Bacto™ Neopeptone	13.60%	2.59%	21.76	4.144	1553.54	133.79
BD Bacto™ Peptone	15.40%	0.40%	24.64	0.64	1759.16	20.66
BD BBL™ Polypeptone™ Peptone	13.10%	3.40%	20.96	5.44	1496.43	175.63
BD Bacto™ Proteose Peptone	14.30%	0.64%	22.88	1.024	1633.50	33.06
BD BiTek™ Proteose Peptone	13.10%	0.94%	20.96	1.504	1496.43	48.56
BD Bacto™ Proteose Peptone No. 2	12.90%	1.88%	20.64	3.008	1473.58	97.11
BD Bacto™ Proteose Peptone No. 3	13.40%	0.51%	21.44	0.816	1530.70	26.34
BD BiTek™ Proteose Peptone No. 3	12.80%	1.22%	20.48	1.952	1462.16	63.02
BD Bacto™ Proteose Peptone No. 4	14.30%	0.72%	22.88	1.152	1633.50	37.19
D Bacto™ Tryptose	13.30%	2.05%	21.28	3.28	1519.27	105.90

Available N (urea-N) and P (anhydrous dipotassium hydrogen phosphate - P) as per OECD recipe:

N (mM)	999.08	
P (mM)	160.76	N_{av}:P_{av} = 6.21

Organic and total N and P

		Organic N and P (mM)		Total N and P (organic and mineral) (mM)		
Peptone	Beef Extract	N	P	TN	TP	TN:TP
BD BBL™ Gelysate™ Peptone	BD BBL™ Beef Extract Powder	3358.39	175.63	4357.47	336.39	12.95
BD Bacto™ Neopeptone	BD BBL™ Beef Extract Powder	2970.01	300.12	3969.09	460.88	8.61
BD Bacto™ Peptone	BD BBL™ Beef Extract Powder	3175.62	187.00	4174.70	347.75	12.00
BD BBL™ Polypeptone™ Peptone	BD BBL™ Beef Extract Powder	2912.89	341.97	3911.97	502.72	7.78
BD Bacto™ Proteose Peptone	BD BBL™ Beef Extract Powder	3049.97	199.39	4049.05	360.15	11.24

BD BiTek™ Proteose Peptone	BD BBL™ Beef Extract Powder	2912.89	214.89	3911.97	375.65	10.41
BD Bacto™ Proteose Peptone No. 2	BD BBL™ Beef Extract Powder	2890.05	263.45	3889.12	424.21	9.17
BD Bacto™ Proteose Peptone No. 3	BD BBL™ Beef Extract Powder	2947.16	192.68	3946.24	353.44	11.17
BD BiTek™ Proteose Peptone No. 3	BD BBL™ Beef Extract Powder	2878.62	229.36	3877.70	390.11	9.94
BD Bacto™ Proteose Peptone No. 4	BD BBL™ Beef Extract Powder	3049.97	203.53	4049.05	364.28	11.12
D Bacto™ Tryptose	BD BBL™ Beef Extract Powder	2935.74	272.23	3934.82	432.99	9.09
BD BBL™ Gelysate™ Peptone	BD Bacto™ Beef Extract, Desiccated	3529.74	31.51	4528.82	192.27	23.55
BD Bacto™ Neopeptone	BD Bacto™ Beef Extract, Desiccated	3141.35	156.00	4140.43	316.76	13.07
BD Bacto™ Peptone	BD Bacto™ Beef Extract, Desiccated	3346.97	42.87	4346.05	203.63	21.34
BD BBL™ Polypeptone™ Peptone	BD Bacto™ Beef Extract, Desiccated	3084.24	197.84	4083.32	358.60	11.39
BD Bacto™ Proteose Peptone	BD Bacto™ Beef Extract, Desiccated	3221.32	55.27	4220.39	216.03	19.54
BD BiTek™ Proteose Peptone	BD Bacto™ Beef Extract, Desiccated	3084.24	70.77	4083.32	231.53	17.64
BD Bacto™ Proteose Peptone No. 2	BD Bacto™ Beef Extract, Desiccated	3061.39	119.33	4060.47	280.08	14.50
BD Bacto™ Proteose Peptone No. 3	BD Bacto™ Beef Extract, Desiccated	3118.51	48.56	4117.59	209.31	19.67
BD BiTek™ Proteose Peptone No. 3	BD Bacto™ Beef Extract, Desiccated	3049.97	85.23	4049.05	245.99	16.46
BD Bacto™ Proteose Peptone No. 4	BD Bacto™ Beef Extract, Desiccated	3221.32	59.41	4220.39	220.16	19.17
D Bacto™ Tryptose	BD Bacto™ Beef Extract,	3107.08	128.11	4106.16	288.87	14.21

Desiccated					
Maximum	**3529.74**	**341.97**	**4528.82**	**502.72**	**23.55**
Minimum	**2878.62**	**31.51**	**3877.70**	**192.27**	**7.78**

Thus the TN:TP ratio varies widely with the selection of the organic substrates products, from 7.8 to 23.55. Furthermore, the organic compounds from different sources might mineralize at different rates under different environmental parameters. If organic N and P forms are ignored then an N:P ratio of 6.21 can be calculated. This simple exercise highlights the necessity for clarity in the description of the experimental setup for any experiment whose results depend on the N and P availability kinetics. A simple reporting of the general recipe without a reporting of the actual product employed might render results non-replicable, and non-comparable.

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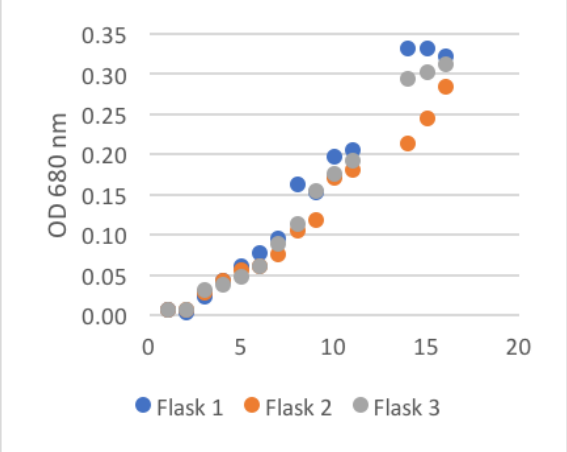
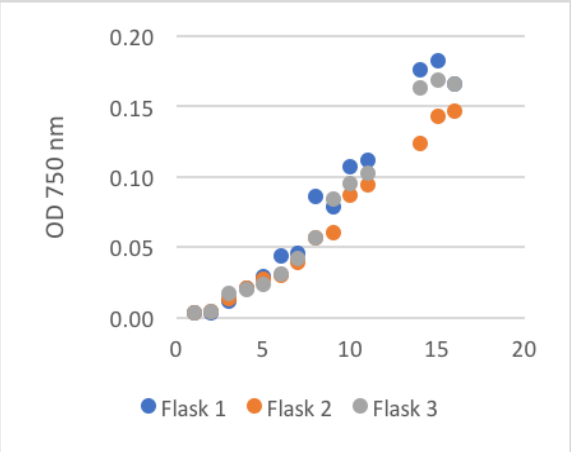
C. Method development

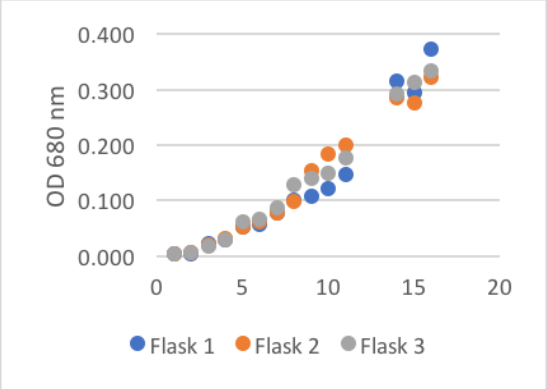
1. Growth evaluation of *Chlorella vulgaris* CPCC90 and *Scenedesmus obliquus* CPCC5 in flasks and ePBRs (NO_3-N medium)

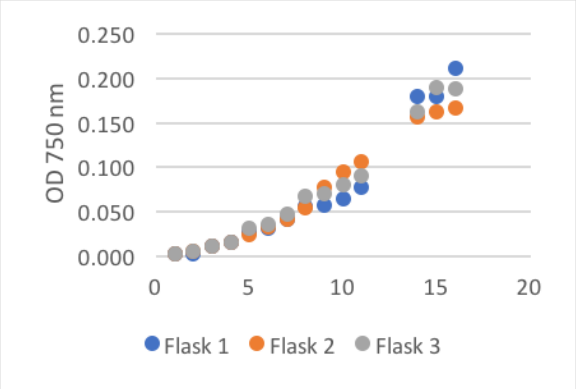
Chlorella vulgaris CPCC90 in flasks – first evaluation



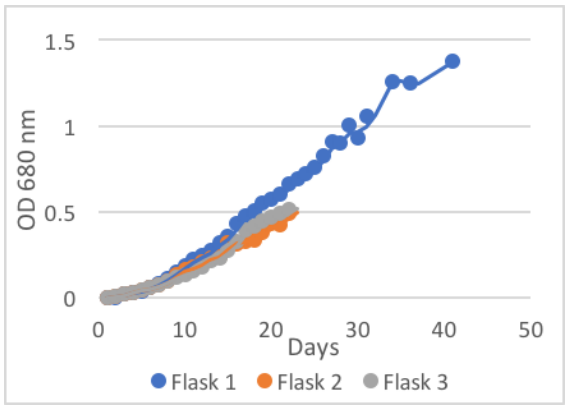


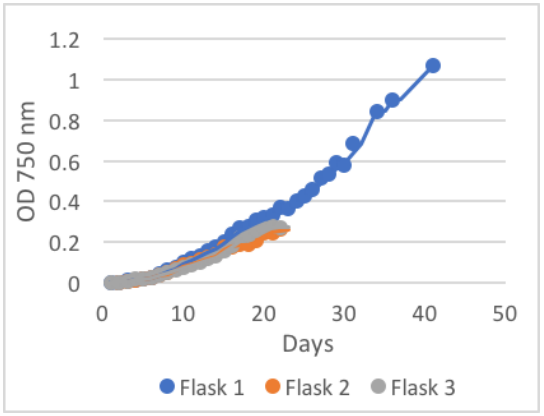
Scenedesmus obliquus CPCC5 in flasks – first evaluation

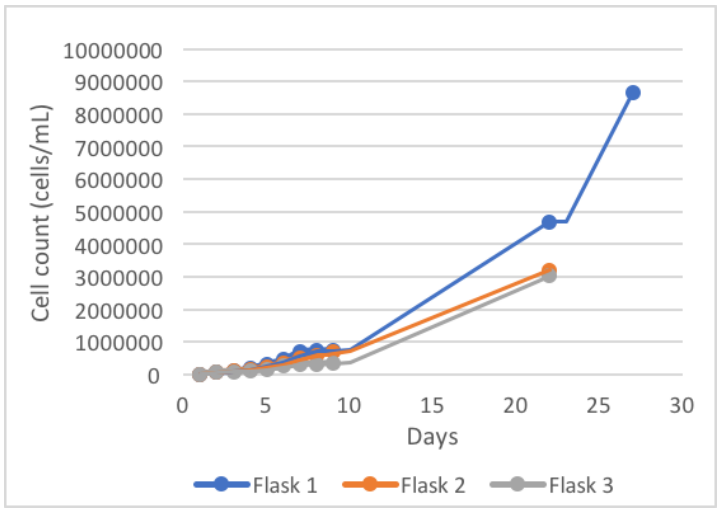




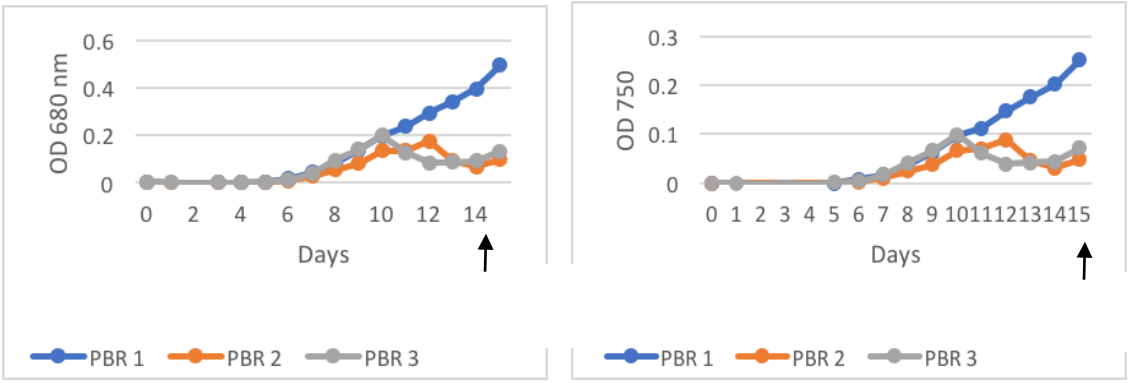
Chlorella vulgaris CPCC90 in flasks – second evaluation







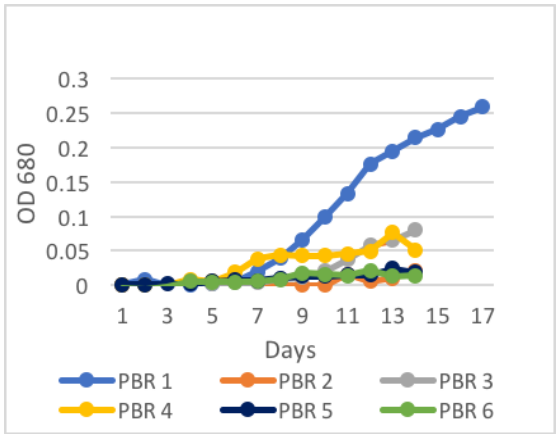
Chlorella vulgaris CPCC90 in ePBRs – growth evaluation

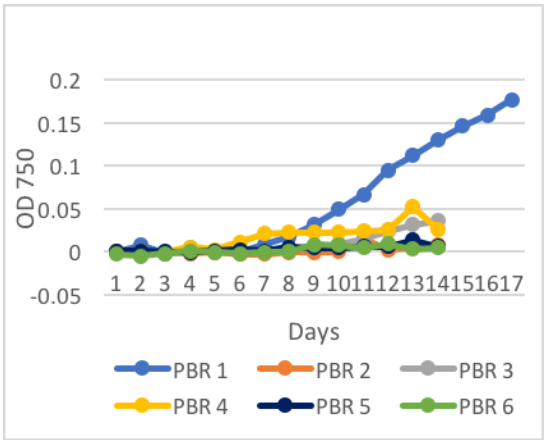


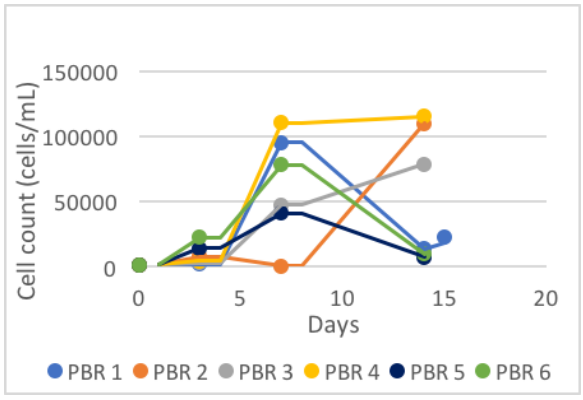
2. Runs performed in ePBRs with *Chlorella vulgaris* CPCC90 to evaluate the influence of nitrogen

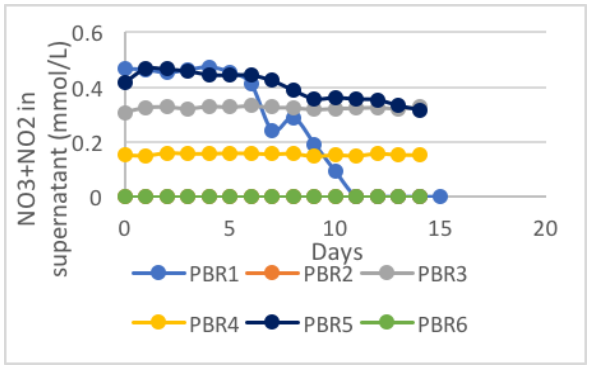
Run1

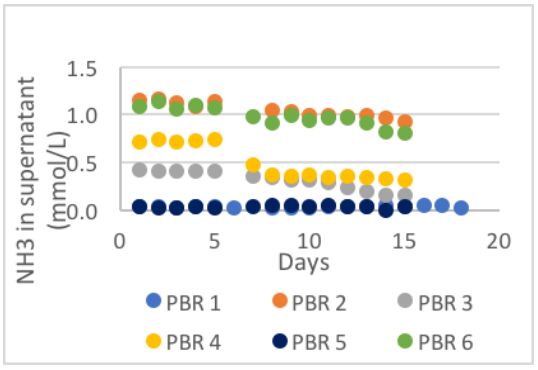
PBR1: 100% NO_3-N
PBR2: 100% NH_3-N
PBR3: 66% NO_3-N and 34% NH_3-N
PBR4: 34% NO_3-N and 66% NH_3-N
PBR5: 100% NO_3-N
PBR6: 100% NH_3-N





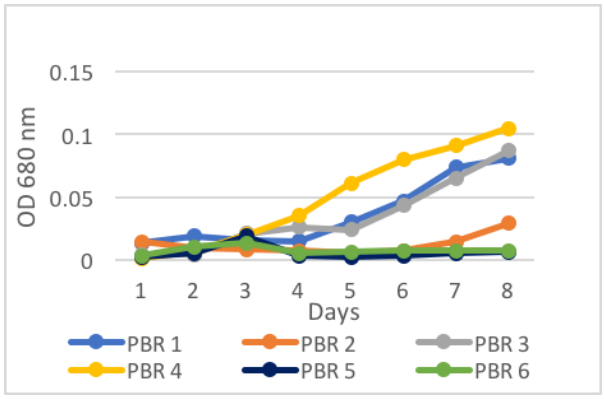


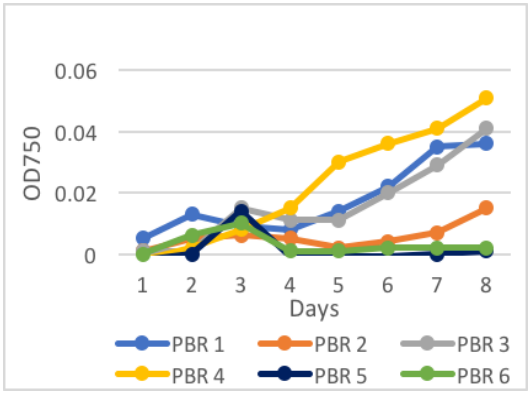


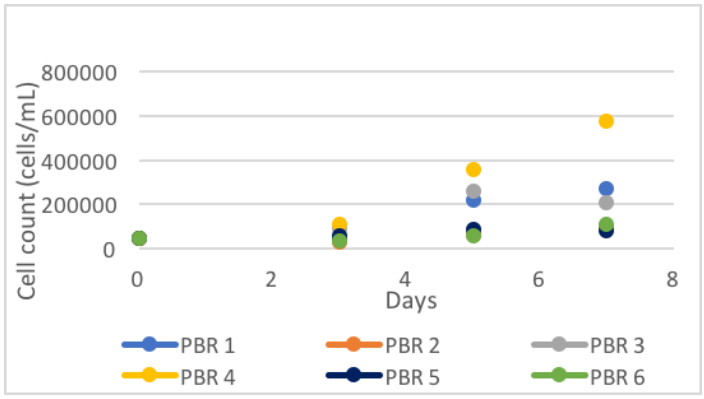


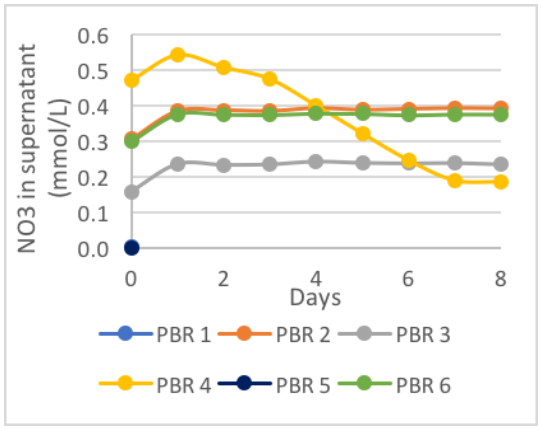
Run2

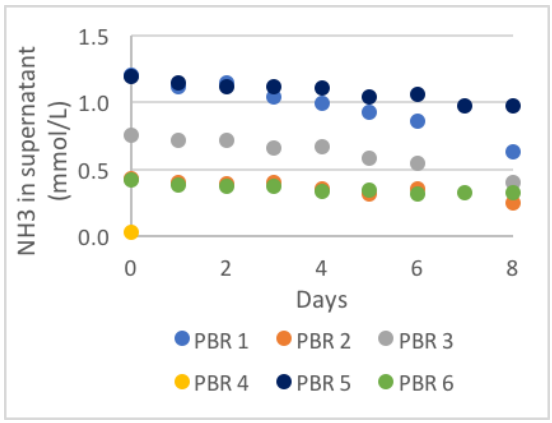
PBR1: 100% NH_3-N
PBR2: 66% NO_3-N and 34% NH_3-N
PBR3: 34% NO_3-N and 66% NH_3-N
PBR4: 100% NO_3-N
PBR5: 100% NH_3-N
PBR6: 100% 66% NO_3-N and 34% NH_3-N





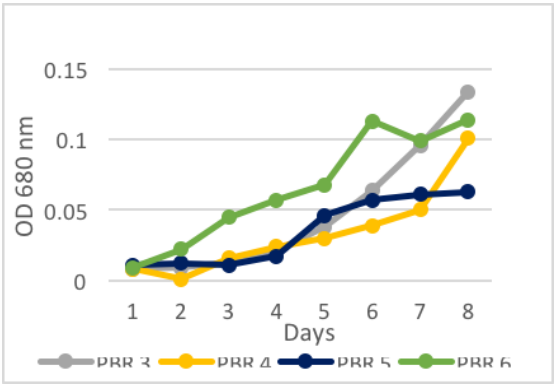


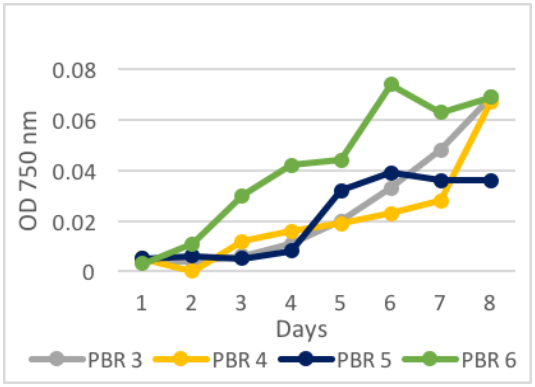


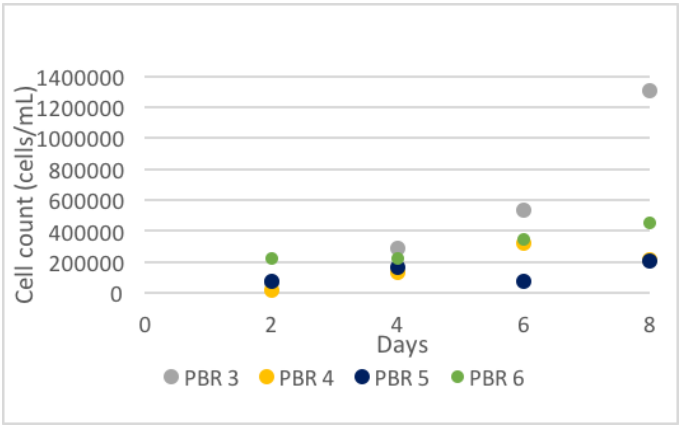


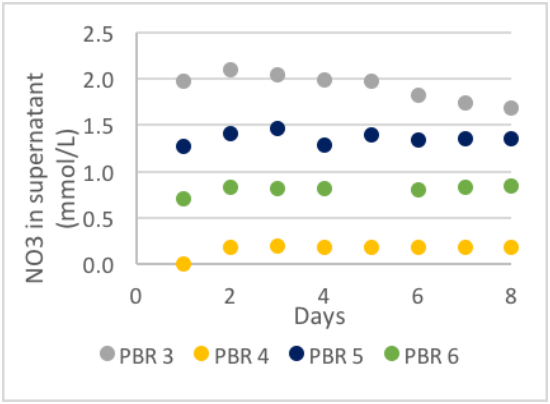
Run 3

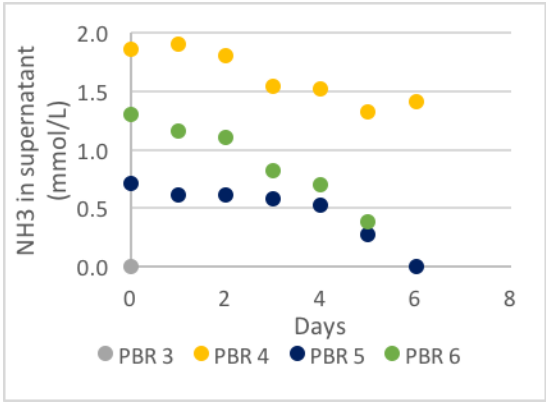
PBR3: 100% NO_3-N
PBR4: 100% NH_3-N
PBR5: 66% NO_3-N and 34% NH_3-N
PBR6: 34% NO_3-N and 66% NH_3-N











a) b)

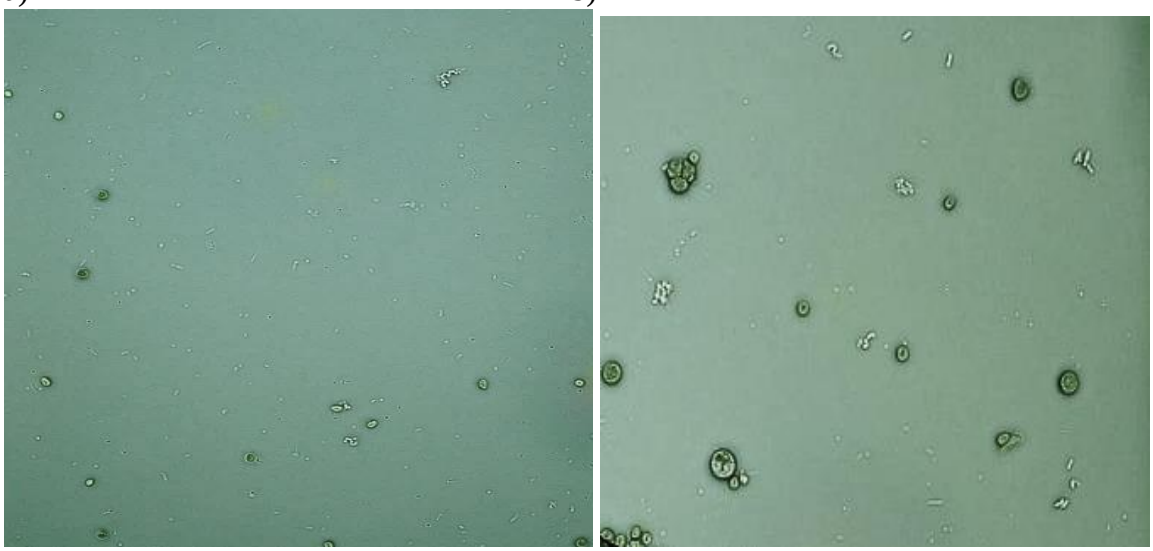
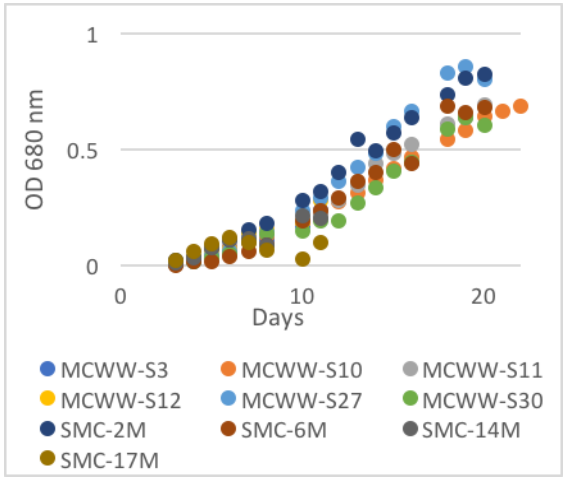


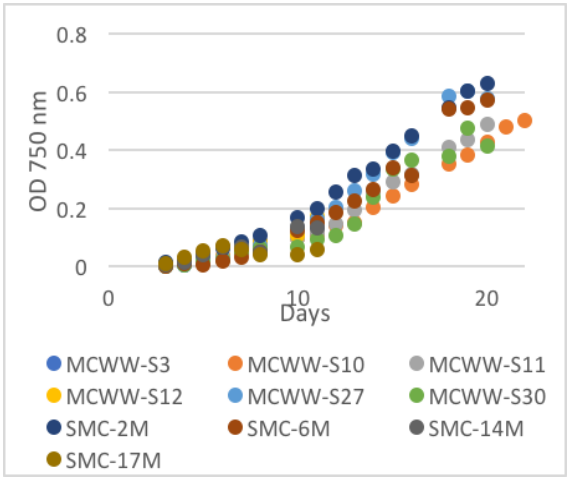
Figure 1 Images from environmental photobioreactors 40x Run3 day 8; a) 66% NO_3-N and 34% NH_3-N, b) 34% NO_3-N and 66% NH_3-N.

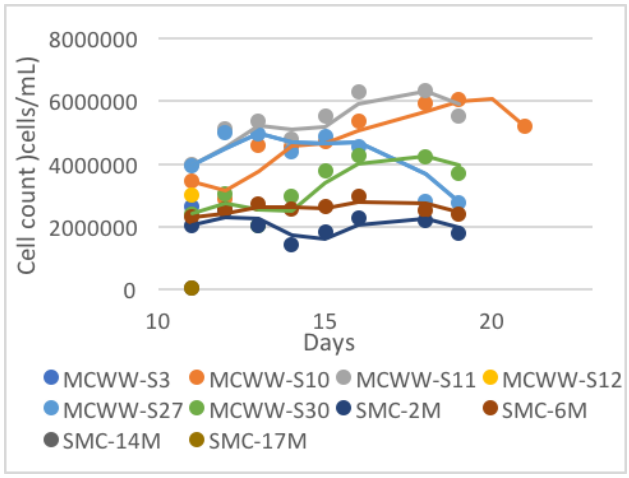
Growth evaluation of the species from NRC (NO_3-N medium)

First evaluation

MCWW-S3: *Pseudotetracystis* sp.
MCWW-S10: *Chlorella* sp.
MCWW-S11: *Dictyophaerium* sp.
MCWW-S12: *Tetracystis vinatzeri*
MCWW-S27: *Micractinium pusillum*
MCWW-S30 : *Tetracystis vinatzeri*
SMC-2M: *Chlorella vulgaris*
SMC-6M: *Scenedesmus obliquus*

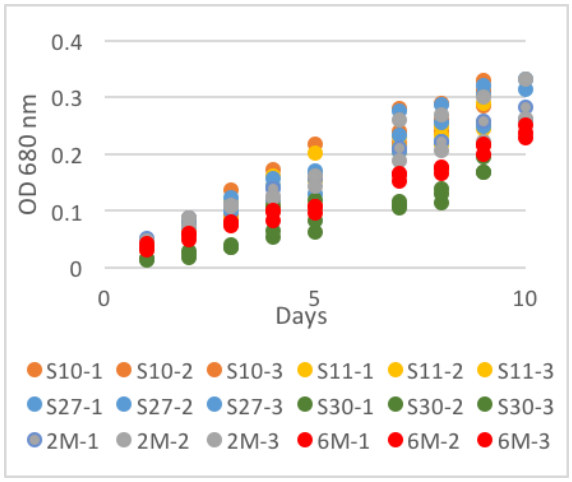


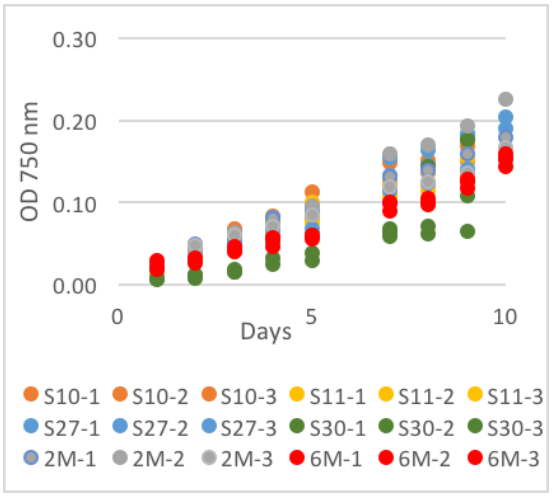


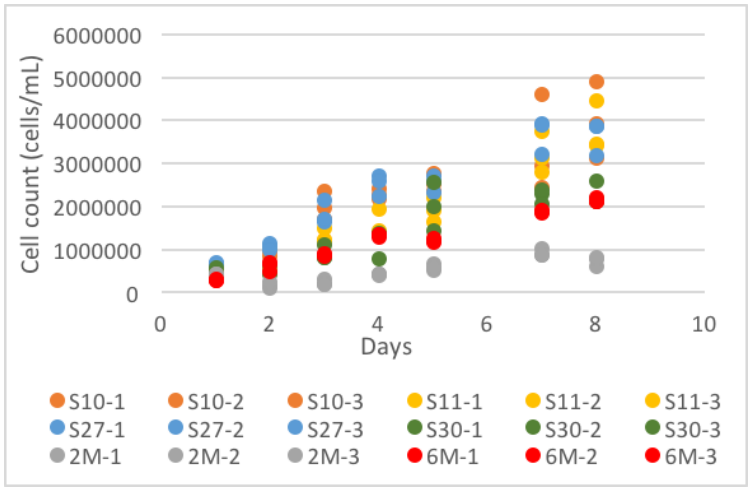


Four species (MCWW-S3, MCWW-S11, SMC-14M and SMC-17M) have produced clumps and were discarded for the next growth evaluation (see below Second evaluation).

Second evaluation







D. Algal growth data

1. *C. vulgaris* CPCC90 grown in NO_3 (before resuspension)

	OD 680 nm			OD 750 nm			Cell count (cells/mL)		
Days / Replicate	1	2	3	1	2	3	1	2	3
1	0.001	0	0.001	0	-0.001	0	6960	6540	7260
2	0.004	0.006	0.006	0.001	0.002	0.001	61120	79460	79360
3	0.022	0.022	0.021	0.011	0.009	0.009	102400	103520	85600
4	0.034	0.032	0.035	0.014	0.014	0.016	184100	159640	107000
5	0.041	0.047	0.045	0.016	0.02	0.02	323780	241560	162200
6	0.06	0.059	0.059	0.026	0.027	0.028	453820	366780	256800
7	0.085	0.077	0.078	0.042	0.039	0.04	706440	526320	325820
8	0.117	0.102	0.101	0.06	0.048	0.052	727240	585000	327260
9	0.149	0.137	0.123	0.078	0.072	0.065	756220	722120	356140
10	0.19	0.165	0.137	0.1	0.087	0.076			
11	0.222	0.18	0.161	0.121	0.097	0.089			
12	0.247	0.208	0.181	0.134	0.113	0.103			
13	0.276	0.234	0.214	0.156	0.125	0.121			
14	0.323	0.24	0.232	0.178	0.138	0.13			
15	0.36	0.323	0.279	0.203	0.179	0.16			
16	0.435	0.317	0.33	0.239	0.174	0.185			
17	0.479	0.33	0.396	0.268	0.191	0.222			
18	0.509	0.335	0.418	0.276	0.186	0.234			

2. *C. vulgaris* CPCC90 grown in NH_3 in ePBR (before resuspension)

Days	OD680	OD750	Cell count (cells/mL)
3	0.015	0.009	
4	0.028	0.016	
5	0.035	0.02	
6	0.032	0.017	
7	0.031	0.017	
8	0.03	0.016	
10	0.031	0.017	
11	0.033	0.018	297560
13	0.029	0.013	287260
15	0.039	0.022	382360
18	0.04	0.021	499600
20	0.043	0.023	
21	0.046	0.024	336280
23	0.048	0.025	391560
25	0.049	0.026	592980
26	0.054	0.029	

3. *C. vulgaris* CPCC90 grown in NO_3 and resuspended in NO_3 (replicates A) or NH_3 (replicates B)

	OD 680 nm						OD 750 nm					
Days/ Replicates	1A	2A	3A	1B	2B	3B	1A	2A	3A	1B	2B	3B
0	0.227	0.224	0.21	0.238	0.247	0.242	0.12	0.118	0.108	0.134	0.135	0.129
1	0.267	0.243	0.246	0.263	0.286	0.268	0.145	0.129	0.134	0.141	0.155	0.148
2	0.301	0.294	0.251	0.273	0.294	0.291	0.164	0.156	0.137	0.139	0.158	0.163
3	0.35	0.323	0.272	0.288	0.326	0.283	0.199	0.169	0.142	0.157	0.18	0.159
6	0.401	0.376	0.374	0.385	0.403	0.383	0.227	0.212	0.216	0.22	0.232	0.222
9	0.59	0.51	0.47	0.48	0.514	0.487	0.315	0.291	0.273	0.311	0.328	0.308
12	0.684	0.729	0.595	0.507	0.544	0.522	0.388	0.413	0.343	0.343	0.364	0.357
15	0.904	0.942	0.738	0.462	0.506	0.488	0.499	0.533	0.416	0.356	0.379	0.38
18	0.965	1.018	0.922				0.56	0.599	0.546			
21	0.965	1.055	1.034				0.606	0.655	0.651			
24	1.07	1.101	1.081				0.702	0.734	0.719			

	Cell count (cells/mL)						pH					
Days/ Replicates	1A	2A	3A	1B	2B	3B	1A	2A	3A	1B	2B	3B
0	2665200	2536140	2656140	2431280	2189820	2741540						
1	2279920	2211020	2251460	1267080	1373580	1827520	6.87	6.74	6.82	6.67	6.70	6.69
2	2761000	2838820	2518100	1675360	1982760	1913340	6.90	6.96	6.97	6.53	6.56	6.53
3	3227780	3125440	2464140	2259140	1870840	2647840	7.25	7.09	7.05	6.31	6.39	6.40
6	2906100	2852960	2836480	1795480	1971200	2315560	7.58	7.39	7.42	4.49	4.66	5.14
9	4741220	3764120	3582560	1875860	2367280	2861860	8.83	8.01	8.04	3.72	3.72	3.72
12	5443160	5328780	4447120	1765160	2206600	2425940	9.90	9.42	9.02	3.31	3.35	3.27
15	5100220	5881240	4827540	1576840	1768540	1891120	10.42	10.44	10.26	3.15	3.08	3.04
18	6119820	6061360	5982200				10.27	10.24	9.74			
21	5377120	5502360	5153780				9.41	9.28	9.13			
24	4726460	4258080	4856100				9.44	9.10	9.28			

4. *C. vulgaris* CPCC90 grown in NH_3 and resuspended in NO_3 (replicates C) or NH_3 (replicates D)

	OD 680 nm						OD 750 nm					
Position on shaker	C1	B1	B2	B3	B4	B5						
Days / Replicates	1C	2C	3C	1D	2D	3D	1C	2C	3C	1D	2D	3D
0	0.01	0.014	0.013	0.018	0.013	0.013	0.004	0.006	0.006	0.009	0.006	0.006
1	0.012	0.02	0.018	0.013	0.014	0.015	0.004	0.009	0.007	0.005	0.006	0.006
2	0.06	0.079	0.074	0.083	0.081	0.076	0.028	0.037	0.036	0.042	0.042	0.036
5	0.265	0.24	0.206	0.164	0.16	0.191	0.14	0.12	0.103	0.09	0.086	0.105
8	0.516	0.462	0.444	0.129	0.134	0.168	0.287	0.253	0.246	0.12	0.117	0.141
11	0.668	0.654	0.594				0.399	0.389	0.348			
14	0.811	0.852	0.755				0.512	0.554	0.451			
17	0.889	0.927	0.831				0.628	0.646	0.557			

	Cell count (cells/mL)						pH					
Days / Replicates	1C	2C	3C	1D	2D	3D	1C	2C	3C	1D	2D	3D
0	176020	193620	177960	207240	141000	170800						
1	195680	298180	275040	170580	205300	188500	6.63	7.13	7.19	7.06	7.09	7.08
2	754780	989480	781500	889640	831060	1038740	7.35	7.40	7.18	6.74	6.75	6.75
5	990000	682900	627080	1081740	874080	1097100	6.78	7.97	7.85	4.47	4.39	4.46
8	1740900	1768140	1378200	83140	92760	212480	9.68	9.53	9.72	3.29	3.33	3.29
11	3204900	3090620	2944400				10.78	10.65	10.18			
14	2869140	3680460	3138860				9.29	9.57	9.38			
17	2151920	2772680	3094720				9.86	9.85	10.31			

5. *C. vulgaris* CPCC90 grown in NO_3 and resuspended in a medium with no nitrogen

	OD 680 nm			OD 750 nm			Cells count			pH		
Days / Replicates	1E	2E	3E	1E	2E	3E	1E	2E	3E	1E	2E	3E
0	0.221	0.225	0.22	0.118	0.122	0.118	2324680	2631560	2296420			
1	0.236	0.242	0.241	0.133	0.142	0.141	1888760	2231900	1947440	6.92	6.93	6.90
2	0.275	0.237	0.275	0.18	0.143	0.166	1834380	1722560	2188800	6.93	6.90	6.90
5	0.319	0.305	0.313	0.225	0.211	0.22	1170840	1134480	1046720	6.94	6.93	6.95
8	0.354	0.334	0.363	0.268	0.247	0.279	827380	730920	620640	6.94	6.93	6.91
11	0.386	0.35	0.394	0.313	0.274	0.323	535640	466940	432220	6.82	6.89	6.89
14	0.418	0.389	0.431	0.355	0.319	0.367	364840	399960	270540	6.73	6.83	6.76
17	0.448	0.423	0.452	0.39	0.362	0.394	345800	253940	217800	6.74	6.74	6.68
20	0.492	0.452	0.479	0.439	0.4	0.432	169160	116320	105360	6.65	6.56	6.54
23	0.535	0.487	0.507	0.492	0.442	0.467	114380	76280	74440	6.61	6.44	6.43
26	0.536	0.523	0.526	0.5	0.485	0.492	41880	34700	28040	6.37	6.32	6.33
29	0.564	0.528	0.544	0.536	0.495	0.518	17600	13200	9920	6.13	6.16	6.22
32	0.588	0.556	0.563	0.558	0.527	0.535	14840	9820	8480	5.05	5.97	6.07

6. MCWW-S27 *M. pusillum* grown in NO_3 and resuspended in NO_3 (replicates 1 to 3, 7 to 9) or NH_3 (replicates 4 to 6, 10 to 12)

	OD680						OD750					
Days / Replicate	1	2	3	4	5	6	1	2	3	4	5	6
0	0.105	0.153	0.143	0.147	0.136	0.112	0.057	0.086	0.083	0.086	0.077	0.061
1	0.128	0.180	0.159	0.163	0.133	0.140	0.069	0.105	0.089	0.090	0.075	0.085

2	0.164	0.191	0.171	0.203	0.188	0.166	0.087	0.110	0.097	0.117	0.111	0.098
3	0.189	0.246	0.198	0.232	0.250	0.163	0.101	0.148	0.114	0.137	0.158	0.093
4	0.230	0.267	0.230	0.256	0.233	0.210	0.130	0.163	0.142	0.163	0.148	0.129
5	0.272	0.298	0.266	0.254	0.236	0.232	0.172	0.180	0.171	0.176	0.152	0.141
6	0.271	0.282	0.299	0.221	0.262	0.238	0.165	0.179	0.193	0.174	0.180	0.158
7	0.334	0.352	0.337	0.146	0.174	0.179	0.204	0.227	0.216	0.141	0.152	0.146
8	0.358	0.325	0.331	0.133	0.137	0.129	0.230	0.217	0.218	0.125	0.130	0.123
Position on shaker	F1	E1	D1	F2	E2	D2						

	Cell count (cells/mL)						pH					
Days / Replicates	1	2	3	4	5	6	1	2	3	4	5	6
0	1307120	1433820	1060960	1153420	836800	1032600	6.70	6.70	6.63	6.55	6.49	6.47
2	2327140	2565320	2053620	1940060	957540	1288900	6.52	7.04	6.92	6.10	6.12	6.19
4	3417700	3425780	3250580	2700380	1705560	1981940	7.36	7.36	7.20	4.69	5.26	5.23
6	4247340	3815100	3707800	3960020	3361480	2926880	7.97	8.06	8.10	4.40	4.70	4.74
8	3637440	2910920	2770420	3480	1120	700	8.35	8.41	8.36	4.40	4.86	4.78

	OD680						OD750					
Days / Replicate	7	8	9	10	11	12	7	8	9	10	11	12
0	0.086	0.163	0.170	0.160	0.175	0.152	0.039	0.116	0.111	0.108	0.120	0.110
1	0.141	0.172	0.149	0.213	0.172	0.160	0.096	0.121	0.109	0.146	0.125	0.111
2	0.179	0.216	0.172	0.179	0.185	0.149	0.113	0.134	0.115	0.129	0.136	0.105
3	0.210	0.265	0.205	0.195	0.213	0.154	0.135	0.175	0.144	0.145	0.158	0.111
4	0.273	0.296	0.206	0.197	0.188	0.161	0.182	0.200	0.145	0.154	0.144	0.122

5	0.296	0.302	0.246	0.185	0.194	0.162	0.204	0.202	0.168	0.144	0.146	0.126
6	0.419	0.290	0.237	0.143	0.150	0.145	0.276	0.192	0.153	0.123	0.123	0.116
7	0.445	0.335	0.253	0.105	0.119	0.127	0.290	0.222	0.165	0.102	0.109	0.103
8	0.549	0.384	0.353	0.103	0.110	0.105	0.382	0.256	0.236	0.108	0.106	0.098
Position on shaker	F4	E4	D4	F3	E3	D3						

	Cell count (cells/mL)						pH					
Days / Replicate	7	8	9	10	11	12	7	8	9	10	11	12
0	912980	995220	880640	459460	710740	528160	6.21	6.27	6.24	6.19	6.14	6.16
2	1705360	1708120	1353620	1247320	1578380	803520	7.39	7.09	6.97	4.08	3.86	4.38
4	2707340	2680820	2133700	1479680	1184660	1394580	7.82	7.55	7.30	3.93	3.73	3.85
6	3966660	3444940	1498300	348760	553560	812640	8.84	8.10	7.87	3.94	3.77	3.93
8	3358300	2975840	3288980	1940	200	300	9.60	9.30	8.50	4.86	4.00	3.95

7. SMC-2M *C. vulgaris* grown in NO_3 and resuspended in NO_3 (replicates 1 to 3, 7 to 9) or NH_3 (replicates 4 to 6, 10 to 12)

	OD680						OD750					
Days / Replicate	1	2	3	4	5	6	1	2	3	4	5	6
0	0.119	0.140	0.131	0.120	0.113	0.140	0.071	0.088	0.077	0.069	0.066	0.085
1	0.154	0.178	0.194	0.126	0.112	0.150	0.087	0.100	0.111	0.061	0.053	0.067
2	0.201	0.216	0.278	0.149	0.145	0.176	0.127	0.123	0.160	0.076	0.075	0.091
3	0.206	0.252	0.393	0.187	0.188	0.221	0.117	0.147	0.237	0.104	0.107	0.127
4	0.232	0.282	0.441	0.213	0.212	0.233	0.139	0.170	0.287	0.128	0.129	0.145
5	0.270	0.328	0.460	0.249	0.240	0.258	0.155	0.201	0.310	0.161	0.157	0.175
6	0.313	0.426	0.509	0.294	0.252	0.242	0.189	0.265	0.334	0.190	0.174	0.184

7	0.339	0.478	0.551	0.223	0.204	0.189	0.212	0.318	0.394	0.200	0.182	0.175
8	0.364	0.489	0.572	0.181	0.174	0.171	0.231	0.338	0.415	0.173	0.167	0.163
Position on shaker	F3	E3	D3	F4	E4	D4						

	Cell count (cells/mL)						pH					
Days / Replicate	1	2	3	4	5	6	1	2	3	4	5	6
0	176120	106380	217600	176740	250560	151440	6.64	6.66	6.68	6.53	6.53	6.45
2	422180	543020	772400	383480	351120	414820	7.26	7.51	7.53	6.30	6.22	5.99
4	929880	1212000	945660	1225520	1206460	725900	7.38	7.59	7.97	5.10	5.19	4.99
6	1204420	1769980	708400	1450900	1253360	758060	8.03	8.32	8.39	4.80	4.72	4.65
8	1419760	867120	746180	1420	2240	200	8.35	8.32	8.36	4.77	4.93	4.63

	OD680						OD750					
Days / Replicate	7	8	9	10	11	12	7	8	9	10	11	12
0	0.144	0.136	0.143	0.130	0.100	0.100	0.097	0.086	0.093	0.084	0.061	0.062
1	0.171	0.156	0.157	0.141	0.127	0.154	0.103	0.094	0.097	0.083	0.071	0.093
2	0.179	0.173	0.215	0.176	0.170	0.194	0.109	0.110	0.131	0.107	0.103	0.114
3	0.242	0.226	0.232	0.194	0.198	0.212	0.149	0.139	0.142	0.125	0.129	0.144
4	0.283	0.256	0.263	0.203	0.208	0.233	0.176	0.158	0.161	0.136	0.142	0.165
5	0.321	0.305	0.320	0.188	0.187	0.193	0.203	0.191	0.201	0.155	0.159	0.170
6	0.349	0.368	0.343	0.154	0.163	0.160	0.215	0.222	0.207	0.149	0.153	0.152
7	0.418	0.419	0.382	0.157	0.143	0.166	0.265	0.269	0.241	0.151	0.135	0.158
8	0.487	0.449	0.415	0.145	0.102	0.140	0.308	0.283	0.266	0.136	0.098	0.135
Position	C2	C3	C4	F5	E5	D5						

on shaker							

	Cell count (cells/mL)						pH					
Days / Replicate	7	8	9	10	11	12	7	8	9	10	11	12
0	66760	54260	48020	113660	107200	50160	6.21	6.25	6.25	6.22	6.20	6.18
2	347340	237660	378660	358700	375800	261820	6.98	6.97	6.98	4.41	4.69	4.44
4	1147080	956300	999200	547720	676640	480240	7.47	7.39	7.49	3.69	3.55	3.50
6	1349920	1087280	1265960	22520	14020	15240	7.85	7.92	7.86	3.62	3.42	3.44
8	1893020	1589140	2041840	1020	300	0	8.99	8.69	8.51	3.98	3.54	3.63

8. SMC-6M *S. obliquus* grown in NO_3 and resuspended in NO_3 (replicates 1 to 3, 7 to 9) or NH_3 (replicates 4 to 6, 10 to 12)

	OD680						OD750					
Days / Replicate	1	2	3	4	5	6	1	2	3	4	5	6
0	0.097	0.103	0.107	0.101	0.094	0.092	0.056	0.049	0.057	0.057	0.052	0.047
1	0.163	0.134	0.134	0.146	0.141	0.128	0.084	0.072	0.071	0.081	0.078	0.072
2	0.177	0.176	0.146	0.162	0.163	0.159	0.094	0.095	0.079	0.090	0.093	0.085
3	0.210	0.222	0.210	0.187	0.180	0.184	0.112	0.115	0.116	0.112	0.102	0.102
4	0.248	0.251	0.231	0.208	0.196	0.211	0.136	0.143	0.120	0.125	0.117	0.122
5	0.329	0.355	0.327	0.249	0.230	0.270	0.185	0.194	0.180	0.140	0.131	0.163
6	0.322	0.380	0.358	0.256	0.236	0.246	0.191	0.221	0.210	0.168	0.149	0.158
7	0.393	0.418	0.403	0.220	0.234	0.254	0.249	0.265	0.260	0.159	0.154	0.168
8	0.479	0.461	0.455	0.228	0.258	0.275	0.315	0.295	0.297	0.163	0.177	0.177
Position on shaker	F5	E5	D5	C2	C3	C4						

	Cell count (cells/mL)						pH					
Days / Replicate	1	2	3	4	5	6	1	2	3	4	5	6
0	1176980	1098540	897520	1109280	1247220	895680	6.69	6.70	6.70	6.50	6.48	6.51
2	1864280	2097140	1997600	1516540	1470360	1899820	8.69	8.73	8.95	5.49	5.60	5.67
4	2119160	2437520	2104720	1683040	1836740	2073280	8.06	8.09	8.05	4.92	5.19	4.94
6	2856240	2789880	3151460	1687040	1846360	2157560	8.34	8.34	8.36	5.35	5.56	5.59
8	3164260	3228660	3240020	1780620	1444760	1907600	8.33	8.38	8.36	5.03	5.50	5.03

	OD680						OD750					
Days / Replicate	7	8	9	10	11	12	7	8	9	10	11	12
0	0.121	0.085	0.127	0.094	0.108	0.156	0.077	0.045	0.092	0.052	0.071	0.113
1	0.105	0.158	0.106	0.126	0.160	0.096	0.059	0.061	0.058	0.093	0.119	0.052
2	0.162	0.280	0.145	0.092	0.174	0.119	0.094	0.171	0.081	0.065	0.125	0.067
3	0.191	0.339	0.171	0.127	0.180	0.131	0.107	0.208	0.099	0.093	0.133	0.075
4	0.237	0.407	0.218	0.136	0.189	0.145	0.131	0.250	0.129	0.109	0.145	0.086
5	0.276	0.456	0.247	0.148	0.215	0.174	0.156	0.281	0.142	0.111	0.154	0.101
6	0.324	0.527	0.275	0.139	0.198	0.169	0.188	0.333	0.162	0.110	0.155	0.102
7	0.363	0.310	0.317	0.143	0.205	0.175	0.211	0.183	0.190	0.118	0.162	0.111
8	0.419	0.642	0.390	0.146	0.210	0.186	0.248	0.408	0.243	0.112	0.165	0.120
Position on shaker	F2	E2	D2	F1	E1	D1						

	Cell count (cells/mL)						pH					
Days / Replicate	7	8	9	10	11	12	7	8	9	10	11	12
0	305040	485360	394440	347940	551100	274840	6.24	6.14	6.27	6.00	5.91	6.27
2	560840	794000	715660	757860	693440	764620	7.17	7.68	6.99	4.02	4.02	5.37
4	1198080	1324120	1435440	675320	788360	1269860	8.09	8.63	7.55	4.13	4.03	4.43
6	1812260	2020860	1196320	582140	570980	1529940	7.89	9.61	8.10	4.43	4.15	4.09
8	2815580	2587640	2292720	0	0	100	8.51	9.66	8.45	4.46	4.06	3.89

9. MCWW-S27 *M. pusillum* grown in NO_3 and resuspended in NH_3 (replicates A to C) or mixture of NH_3 (10%) + NO_3 (90%) (replicates D to F)

	OD680						OD750					
Days / Replicate	A	B	C	D	E	F	A	B	C	D	E	F
0	0.138	0.139	0.131	0.131	0.132	0.139	0.081	0.086	0.075	0.071	0.074	0.082
1	0.119	0.108	0.110	0.123	0.111	0.115	0.060	0.052	0.056	0.069	0.064	0.065
2	0.163	0.133	0.154	0.170	0.175	0.159	0.091	0.070	0.088	0.099	0.100	0.090
3	0.193	0.158	0.195	0.218	0.228	0.217	0.107	0.087	0.110	0.127	0.131	0.130
4	0.239	0.225	0.229	0.274	0.301	0.273	0.146	0.136	0.140	0.163	0.180	0.161
5	0.275	0.236	0.236	0.288	0.331	0.329	0.185	0.155	0.156	0.176	0.196	0.197
6	0.280	0.237	0.262	0.353	0.376	0.412	0.205	0.165	0.188	0.215	0.226	0.248
Position on shaker	E2	D2	C2	E1	D1	C1						

	Cell count (cells/mL)						pH					
Days / Replicate	A	B	C	D	E	F	A	B	C	D	E	F
0	814880	944740	924040	689860	699680	854320	5.56	6.51	6.55	6.56	6.58	6.56
1							5.23	6.27	6.34	6.47	6.46	6.48
2	1443220	949340	988360	726320	1281220	1276100	4.29	5.82	5.80	6.86	6.90	6.79
3							5.35	5.44	5.13	6.97	7.02	7.02
4	1832340	1711820	1573680	1895620	2163300	2199640	4.06	4.25	4.18	7.40	7.48	7.41
5							3.68	3.82	3.80	7.39	7.67	7.67
6	1366520	1455200	1508760	2799820	2285260	3015060	3.72	3.56	3.58	7.47	7.67	8.05

10. SMC-2M *C. vulgaris* grown in NO_3 and resuspended in NH_3 (replicates A to C) or mixture of NH_3 (10%) + NO_3 (90%) (replicates D to F)

	OD680	OD750

Days / Replicate	A	B	C	D	E	F	A	B	C	D	E	F
0	0.081	0.088	0.078	0.090	0.089	0.095	0.047	0.050	0.046	0.048	0.046	0.053
1	0.141	0.150	0.160	0.162	0.159	0.161	0.075	0.082	0.087	0.094	0.093	0.092
2	0.184	0.191	0.198	0.210	0.214	0.191	0.103	0.107	0.110	0.120	0.124	0.109
3	0.221	0.226	0.236	0.249	0.277	0.259	0.125	0.128	0.135	0.145	0.163	0.159
4	0.264	0.272	0.267	0.303	0.325	0.281	0.156	0.154	0.166	0.184	0.195	0.171
5	0.292	0.297	0.288	0.336	0.375	0.321	0.183	0.188	0.185	0.202	0.220	0.196
6	0.306	0.293	0.295	0.386	0.430	0.385	0.198	0.190	0.193	0.235	0.258	0.233
Position on shaker	E3	D3	C3	E4	D4	C4						

	Cell count (cells/mL)						pH					
Days / Replicate	A	B	C	D	E	F	A	B	C	D	E	F
0	910420	860660	872960	799020	834440	902740	6.65	6.63	6.62	6.60	6.61	6.64
1							6.52	6.45	6.39	6.50	6.52	6.53
2	1477000	1528920	1627940	2269380	2292720	1654360	6.32	6.22	6.10	6.85	6.90	6.78
3							5.94	5.89	5.42	6.92	7.14	7.00
4	2073600	2383040	2144140	2881420	2803800	2677240	5.27	4.75	4.48	7.27	7.36	7.22
5							4.35	4.01	3.94	7.32	7.89	7.55
6	2290880	2395220	2004360	3822280	3412060	3266660	3.85	3.66	6.63	7.59	7.83	7.72

11. Nitrate and nitrite in supernatant

C. vulgaris CPCC90 grown in NO_3 and resuspended in NO_3

	$NO_3 + NO_2$ (mmol N/L)		
Days / Replicate	A1	A2	A3
0	1.99	2.03	2.01
1	1.86	1.81	1.81
2	1.80	1.84	1.84
3	1.69	1.73	1.76
6	1.44	1.56	1.51
9	0.86	1.10	1.08
12	0.44	0.54	0.67
15	0.001	0.001	0.23
18	0.001	0.001	0.001
21	0.003	0.003	0.002
24	0.002	0.001	0.002

No NO_2 was detected at day 8.

C. vulgaris CPCC90 grown in NH_3 and resuspended in NO_3

	$NO_3 + NO_2$ (mmol N/L)		
Days / Replicate	C1	C2	C3
0	2.03	2.04	2.07
1	1.96	1.99	2.01
2	1.79	1.73	1.86
5	1.31	1.28	1.40
8	0.41	0.49	0.54
11	0.08	0.17	0.00
14	0.00	0.00	0.00
17	0.00	0.00	0.00

MCWW-S27 *M. pusillum* grown in NO_3 and resuspended in NO_3

	$NO_3 + NO_2$ (mmol N/L)					
Days / Replicate	S27-1	S27-2	S27-3	S27-7	S27-8	S27-9
0	0.936	0.914	0.943	2.0	2.1	2.2
1				1.9	1.9	2.0
2				1.3	1.4	1.8
3	0.594	0.582	0.711	1.2	1.4	1.5
4	0.512	0.500	0.596	1.1	1.1	1.3
5	0.324	0.338	0.405	0.7	1.1	1.2
6	0.200	0.156	0.184	0.5	0.9	1.0

7	0.080	0.045	0.019	0.3	0.7	0.8
8	0.007	0.000	0.002	0.1	0.4	0.7

No NO_2 was detected at day 8.

SMC-2M *C. vulgaris* grown in NO_3 and resuspended in NO_3

	$NO_3 + NO_2$ (mmol N/L)					
Days / Replicate	2M-1	2M-2	2M-3	2M-7	2M-8	2M-9
0	0.950	0.929	0.929	2.2	2.3	2.2
1				1.7	1.8	1.6
2				1.2	1.6	1.5
3	0.538	0.446	0.005	1.5	1.5	1.3
4	0.473	0.311	0.002	1.1	1.1	1.2
5	0.345	0.101	0.002	1.0		
6	0.216	0.002	0.003	1.0	0.9	1.0
7	0.076	0.000	0.000	0.7	0.7	0.8
8	0.000	0.002	0.002	0.4	0.5	0.6

No NO_2 was detected at day 8.

SMC-6M *S. obliquus* grown in NO_3 and resuspended in NO_3

	$NO_3 + NO_2$ (mmol N/L)					
Days / Replicate	6M-1	6M-2	6M-3	6M-7	6M-8	6M-9
0				2.2	2.2	2.2
1				2.0	1.6	1.8
2				1.6	1.1	1.6
3	0.065	0.701	0.103	1.5	0.8	1.3
4	0.067	0.068	0.099	1.0	0.6	1.2
5	0.000	0.000	0.079	1.0	0.5	1.3
6	0.000	0.000	0.001	1.0	0.3	1.0
7	0.002	0.000	0.001	0.7	0.1	0.8
8	0.002	0.000	0.002	0.6	0.0	0.7

No NO_2 was detected at day 8.

12. Ammonia in supernatant

C. vulgaris CPCC90 grown in NH_3 and resuspended in NO_3

	NH_3 (mmol NH_3-N/L)		
Days / Replicate	B1	B2	B3
0	2.01	2.06	2.10
1	1.94	2.14	1.93
2	1.80	2.07	1.89
3	1.67	1.69	1.67
6	1.30	1.33	1.38

9	1.04	1.02	1.02
12	0.35	0.68	0.58
15	0.20		

C. vulgaris CPCC90 grown in NH_3 and resuspended in NH_3

	NH_3 (mmol NH_3-N/L)		
Days / Replicate	D1	D2	D3
0	2.33	2.41	2.40
1	2.21	2.30	2.16
2	2.03	1.93	2.06
5	1.30	1.46	1.41
8	1.02	0.98	0.94

MCWW-S27 *M. pusillum* grown in NO_3 and resuspended in NH_3

	NH_3 (mmol NH_3-N/L)								
Days / Replicate	S27-4	S27-5	S27-6	S27-10	S27-11	S27-12	S27-A	S27-B	S27-C
0	2.07	2.10	2.09	2.06	1.97	2.17	1.31	1.21	1.26
1	1.86	2.04	2.03	1.99	1.91	1.94	1.00	1.05	0.96
2	1.80	1.94	1.93	1.84	1.89	1.97	0.79	0.84	0.83
3	1.73	1.73	1.76	2.07	1.66	1.97	0.65	0.70	0.70
4	1.59	1.70	1.64	1.73	1.67	1.87	0.45	0.58	0.55
5	1.32	1.46	1.50	1.69	1.66	1.67	0.12	0.35	0.28
6	1.33	1.39	1.37				0.11	0.14	0.21
7	1.08		0.85						
8				2.06	1.61	1.69			

SMC-2M *C. vulgaris* grown in NO_3 and resuspended in NH_3

	NH_3 (mmol NH_3-N/L)								
Days / Replicate	2M-4	2M-5	2M-6	2M-10	2M-11	2M-12	2M-A	2M-B	2M-C
0	2.06	2.07	1.96	2.11	2.19	2.16	1.44	1.23	1.18
1	1.91	2.03	1.77	2.54	2.26	1.94	1.04	1.09	1.01
2	1.89	1.99	1.87	2.06	2.16	2.03	0.88	0.89	0.84
3	1.71	1.67	1.73	0.00	2.16	1.84	0.73	0.74	0.67
4	1.73	1.70	1.74	1.66	1.70	1.63	0.63	0.61	0.56
5	1.39	1.49	1.43	1.49	1.41	1.39	0.45	0.41	0.37
6	1.37	1.35	1.31	1.39	1.24	1.21	0.22	0.18	0.19
7	0.92	1.01		1.23	1.32	1.26			
8		0.88		1.41	1.47	1.30			

SMC-6M *S. obliquus* grown in NO_3 and resuspended in NH_3

	NH_3 (mmol NH_3-N/L)					
Days / Replicate	6M-4	6M-5	6M-6	6M-10	6M-11	6M-12
0	2.03	2.14	2.11	2.09	2.14	2.23
1	1.81	1.70	1.94	1.80	1.67	2.09
2	1.77	1.70	1.80	1.86	1.73	2.17
3	1.59	1.60	1.57	1.90	1.74	2.14
4	1.57	1.70	1.50	1.77	1.76	1.99
5	1.53	1.56	1.50	1.77	1.61	1.87
6	1.53	1.12	1.43	1.60	1.49	1.69
7		1.09	1.03			
8				1.61	1.57	1.73

13. Nitrate, nitrite and ammonia in supernatant for MCWW-S27 *M. pusillum* and SMC-2M *C. vulgaris* growing in a mixture of nitrate and ammonia

	NH_3 (mmol NH_3-N/L)			NO_3 + NO_2 (mmol N/L)		
Days / Replicate	S27-D	S27-E	S27-F	S27-D	S27-E	S27-F
0	0.17	0.17	0.17	1.87	1.90	1.90
1	0.04	0.04	0.04	1.81	1.84	1.84
2	0.10	0.09	0.06	1.54	1.51	1.59
3	0.05	0.05	0.08	1.42	1.34	1.42
4	0.12	0.10	0.08	1.17	1.15	1.21
5	0.06	0.09	0.07	1.03	0.95	0.91
6	0.03	0.08	0.06	0.85	0.74	0.66

Concentration of NO_2 at day 17: 0.09 – 0.18 mmol NO_2-N/L.

	NH_3 (mmol NH_3-N/L)			NO_3 + NO_2 (mmol N/L)		
Days / Replicate	2M-D	2M-E	2M-F	2M-D	2M-E	2M-F
0	0.16	0.17	0.16	1.86	1.87	1.87
1	0.04	0.05	0.05	1.84	1.77	1.80
2	0.09	0.08	0.07	1.51	1.54	1.63
3	0.05	0.10	0.08	1.44	1.37	1.53
4	0.10	0.06	0.07	1.24	1.19	1.33
5	0.05	0.16	0.09	1.11	0.89	1.06
6	0.08	0.06	0.06	0.86	0.77	0.93

Concentration of NO_2 at day 17: 0.09 – 0.18 mmol NO_2-N/L.

E. Nitrogen uptake rate for mixture treatment

	NH_3 in Mix				
Avg N uptake rate per cell (mmol NH_3-N $cells^{-1}$ d^{-1})	S27	2M		CI95 S27	CI95 2M
Day0	0.18	0.14		0.0270	0.0130

	NO_3 in Mix				
Avg N uptake rate per cell (mmol NO_3-N $cells^{-1}$ d^{-1})	S27	2M		CI95 S27	CI95 2M
Day0	0.08	0.07		0.0101	0.0581
Day2	0.15	0.06		0.0292	0.0244
Day4	0.10	0.08		0.0356	0.0410

F. C:N ratios

Species	Treatment	Replicate	Day	C:N ratio
2M	NO_3-NO_3	2M-1	1	15.0
2M	NO_3-NO_3	2M-1	3	5.8
2M	NO_3-NO_3	2M-1	5	4.8
2M	NO_3-NO_3	2M-1	7	6.0
2M	NO_3-NO_3	2M-2	1	13.5
2M	NO_3-NO_3	2M-2	3	4.8
2M	NO_3-NO_3	2M-2	5	5.7
2M	NO_3-NO_3	2M-2	7	7.4
2M	NO_3-NO_3	2M-3	3	5.2
2M	NO_3-NO_3	2M-3	5	7.5
2M	NO_3-NO_3	2M-3	7	10.0
2M	NO_3-NH_3	2M-4	3	7.3
2M	NO_3-NH_3	2M-4	5	8.3
2M	NO_3-NH_3	2M-4	7	7.1
2M	NO_3-NH_3	2M-5	1	9.5
2M	NO_3-NH_3	2M-5	3	5.3
2M	NO_3-NH_3	2M-5	5	5.2
2M	NO_3-NH_3	2M-5	7	6.4
2M	NO_3-NH_3	2M-6	1	6.8
2M	NO_3-NH_3	2M-6	3	4.9
2M	NO_3-NH_3	2M-6	5	5.9
2M	NO_3-NH_3	2M-6	7	6.3
6M	NO_3-NO_3	6M-1	1	3.7

Species	Treatment	Replicate	Day	C:N ratio
6M	NO_3-NO_3	6M-1	3	5.9
6M	NO_3-NO_3	6M-1	5	6.2
6M	NO_3-NO_3	6M-1	7	7.2
6M	NO_3-NO_3	6M-2	1	4.7
6M	NO_3-NO_3	6M-2	3	5.6
6M	NO_3-NO_3	6M-2	5	5.9
6M	NO_3-NO_3	6M-2	7	7.8
6M	NO_3-NO_3	6M-3	1	5.4
6M	NO_3-NO_3	6M-3	3	5.2
6M	NO_3-NH_3	6M-4	1	3.9
6M	NO_3-NH_3	6M-4	3	4.5
6M	NO_3-NH_3	6M-4	5	6.0
6M	NO_3-NH_3	6M-4	7	7.2
6M	NO_3-NH_3	6M-5	1	19.0
6M	NO_3-NH_3	6M-5	3	5.2
6M	NO_3-NH_3	6M-5	5	5.5
6M	NO_3-NH_3	6M-5	7	6.6
6M	NO_3-NH_3	6M-6	1	7.8
6M	NO_3-NH_3	6M-6	3	5.3
6M	NO_3-NH_3	6M-6	5	6.5
6M	NO_3-NH_3	6M-6	7	7.1
CPCC90	NO_3-NO_3	A1	15	7.4
CPCC90	NO_3-NO_3	A1	21	10.9
CPCC90	NO_3-NO_3	A2	15	8.0
CPCC90	NO_3-NO_3	A2	21	14.0
CPCC90	NO_3-NO_3	A3	15	8.1
CPCC90	NO_3-NO_3	A3	21	11.4
CPCC90	NO_3-NH_3	B1	9	9.3
CPCC90	NO_3-NH_3	B2	9	8.6
CPCC90	NO_3-NH_3	B2	15	8.5
CPCC90	NO_3-NH_3	B3	9	8.3
CPCC90	NO_3-NH_3	B3	15	7.6
CPCC90	NH_3-NO_3	C1	2	3.0
CPCC90	NH_3-NO_3	C1	14	10.1
CPCC90	NH_3-NO_3	C2	2	6.5
CPCC90	NH_3-NO_3	C2	14	10.6
CPCC90	NH_3-NO_3	C3	14	8.1
CPCC90	NH_3-NH_3	D1	5	8.1
CPCC90	NH_3-NH_3	D1	8	9.5
CPCC90	NH_3-NH_3	D2	5	13.0

Species	Treatment	Replicate	Day	C:N ratio
CPCC90	NH_3-NH_3	D2	8	7.5
CPCC90	NH_3-NH_3	D3	5	10.4
CPCC90	NH_3-NH_3	D3	8	7.5
CPCC90	NO_3-0N	E1	11	20.5
CPCC90	NO_3-0N	E1	20	29.0
CPCC90	NO_3-0N	E1	26	38.5
CPCC90	NO_3-0N	E1	32	30.5
CPCC90	NO_3-0N	E2	11	20.0
CPCC90	NO_3-0N	E2	20	18.6
CPCC90	NO_3-0N	E2	26	26.1
CPCC90	NO_3-0N	E2	32	64.5
CPCC90	NO_3-0N	E3	11	22.7
CPCC90	NO_3-0N	E3	20	17.8
CPCC90	NO_3-0N	E3	26	34.4
CPCC90	NO_3-0N	E3	32	44.4
S27	NO_3-NO_3	S27-1	1	6.0
S27	NO_3-NO_3	S27-1	3	5.2
S27	NO_3-NO_3	S27-1	5	5.4
S27	NO_3-NO_3	S27-1	7	6.2
S27	NO_3-NO_3	S27-2	1	5.3
S27	NO_3-NO_3	S27-2	3	5.8
S27	NO_3-NO_3	S27-2	5	6.2
S27	NO_3-NO_3	S27-2	7	7.0
S27	NO_3-NO_3	S27-3	3	5.9
S27	NO_3-NO_3	S27-3	5	5.9
S27	NO_3-NO_3	S27-3	7	6.5
S27	NO_3-NH_3	S27-4	1	4.8
S27	NO_3-NH_3	S27-4	5	5.8
S27	NO_3-NH_3	S27-4	7	5.2
S27	NO_3-NH_3	S27-5	1	4.4
S27	NO_3-NH_3	S27-5	3	6.6
S27	NO_3-NH_3	S27-5	5	6.3
S27	NO_3-NH_3	S27-5	7	8.5
S27	NO_3-NH_3	S27-6	1	3.4
S27	NO_3-NH_3	S27-6	3	5.1
S27	NO_3-NH_3	S27-6	7	7.4
2M	NO_3-NH_3	2M-10	1	5.5
2M	NO_3-NH_3	2M-10	5	8.5
2M	NO_3-NH_3	2M-10	7	32.0
2M	NO_3-NH_3	2M-11	3	9.9

Species	Treatment	Replicate	Day	C:N ratio
2M	NO_3-NH_3	2M-11	5	8.4
2M	NO_3-NH_3	2M-11	7	10.8
2M	NO_3-NH_3	2M-12	3	9.8
2M	NO_3-NH_3	2M-12	5	7.9
2M	NO_3-NH_3	2M-12	7	10.0
2M	NO_3-NO_3	2M-7	3	7.9
2M	NO_3-NO_3	2M-7	5	8.4
2M	NO_3-NO_3	2M-7	7	8.1
2M	NO_3-NO_3	2M-8	1	5.8
2M	NO_3-NO_3	2M-8	3	8.9
2M	NO_3-NO_3	2M-8	5	7.3
2M	NO_3-NO_3	2M-8	7	9.3
2M	NO_3-NO_3	2M-9	1	5.1
2M	NO_3-NO_3	2M-9	3	11.5
2M	NO_3-NO_3	2M-9	5	11.2
2M	NO_3-NO_3	2M-9	7	8.6
6M	NO_3-NH_3	6M-10	1	11.8
6M	NO_3-NH_3	6M-10	7	53.0
6M	NO_3-NH_3	6M-11	3	28.0
6M	NO_3-NH_3	6M-11	5	40.5
6M	NO_3-NH_3	6M-12	5	15.2
6M	NO_3-NO_3	6M-7	1	12.3
6M	NO_3-NO_3	6M-7	3	9.2
6M	NO_3-NO_3	6M-7	5	7.6
6M	NO_3-NO_3	6M-7	7	9.2
6M	NO_3-NO_3	6M-8	1	7.4
6M	NO_3-NO_3	6M-8	3	10.0
6M	NO_3-NO_3	6M-8	5	8.4
6M	NO_3-NO_3	6M-8	7	7.5
6M	NO_3-NO_3	6M-9	3	11.9
6M	NO_3-NO_3	6M-9	5	9.0
6M	NO_3-NO_3	6M-9	7	8.6
S27	NO_3-NH_3	S27-10	1	6.4
S27	NO_3-NH_3	S27-10	3	8.6
S27	NO_3-NH_3	S27-10	5	8.9
S27	NO_3-NH_3	S27-10	7	17.0
S27	NO_3-NH_3	S27-11	1	7.0
S27	NO_3-NH_3	S27-11	3	11.3
S27	NO_3-NH_3	S27-11	5	11.0
S27	NO_3-NH_3	S27-12	1	6.4

Species	Treatment	Replicate	Day	C:N ratio
S27	NO_3-NH_3	S27-12	3	9.5
S27	NO_3-NH_3	S27-12	5	10.4
S27	NO_3-NH_3	S27-12	7	17.5
S27	NO_3-NO_3	S27-7	1	7.7
S27	NO_3-NO_3	S27-7	3	10.6
S27	NO_3-NO_3	S27-7	5	11.1
S27	NO_3-NO_3	S27-7	7	9.0
S27	NO_3-NO_3	S27-8	1	6.7
S27	NO_3-NO_3	S27-8	3	12.4
S27	NO_3-NO_3	S27-8	5	10.6
S27	NO_3-NO_3	S27-8	7	10.1
S27	NO_3-NO_3	S27-9	1	6.0
S27	NO_3-NO_3	S27-9	3	12.4
S27	NO_3-NO_3	S27-9	5	10.3
S27	NO_3-NO_3	S27-9	7	9.9
2M	NO_3-NH_3	2M-A	0	6.8
2M	NO_3-NH_3	2M-A	1	17.0
2M	NO_3-NH_3	2M-A	2	6.8
2M	NO_3-NH_3	2M-A	3	7.0
2M	NO_3-NH_3	2M-A	4	6.6
2M	NO_3-NH_3	2M-A	5	6.3
2M	NO_3-NH_3	2M-B	0	9.4
2M	NO_3-NH_3	2M-B	1	7.4
2M	NO_3-NH_3	2M-B	2	8.0
2M	NO_3-NH_3	2M-B	3	6.7
2M	NO_3-NH_3	2M-B	5	5.8
2M	NO_3-NH_3	2M-C	0	7.4
2M	NO_3-NH_3	2M-C	1	7.5
2M	NO_3-NH_3	2M-C	2	9.0
2M	NO_3-NH_3	2M-C	3	6.1
2M	NO_3-NH_3	2M-C	4	7.7
2M	NO_3-NH_3	2M-C	5	6.1
2M	NO_3-mixN	2M-D	0	6.4
2M	NO_3-mixN	2M-D	2	6.8
2M	NO_3-mixN	2M-D	3	6.6
2M	NO_3-mixN	2M-D	4	6.5
2M	NO_3-mixN	2M-E	0	6.7
2M	NO_3-mixN	2M-E	1	7.8
2M	NO_3-mixN	2M-E	2	6.7
2M	NO_3-mixN	2M-E	3	6.1

Species	Treatment	Replicate	Day	C:N ratio
2M	NO_3-mixN	2M-E	4	6.8
2M	NO_3-mixN	2M-F	0	6.1
2M	NO_3-mixN	2M-F	2	6.9
2M	NO_3-mixN	2M-F	3	6.4
2M	NO_3-mixN	2M-F	4	5.7
S27	NO_3-NH_3	S27-A	0	41.0
S27	NO_3-NH_3	S27-A	1	7.0
S27	NO_3-NH_3	S27-A	2	6.4
S27	NO_3-NH_3	S27-A	3	6.0
S27	NO_3-NH_3	S27-A	4	5.9
S27	NO_3-NH_3	S27-A	5	5.8
S27	NO_3-NH_3	S27-B	1	13.5
S27	NO_3-NH_3	S27-B	3	6.3
S27	NO_3-NH_3	S27-B	4	7.0
S27	NO_3-NH_3	S27-B	5	6.4
S27	NO_3-NH_3	S27-C	2	6.8
S27	NO_3-NH_3	S27-C	3	7.0
S27	NO_3-NH_3	S27-C	4	6.9
S27	NO_3-NH_3	S27-C	5	5.8
S27	NO_3-mixN	S27-D	0	7.0
S27	NO_3-mixN	S27-D	1	7.0
S27	NO_3-mixN	S27-D	2	10.3
S27	NO_3-mixN	S27-D	3	5.8
S27	NO_3-mixN	S27-D	4	6.3
S27	NO_3-mixN	S27-D	5	6.9
S27	NO_3-mixN	S27-E	0	5.4
S27	NO_3-mixN	S27-E	1	8.8
S27	NO_3-mixN	S27-E	2	10.5
S27	NO_3-mixN	S27-E	3	6.2
S27	NO_3-mixN	S27-E	4	5.9
S27	NO_3-mixN	S27-E	5	5.9
S27	NO_3-mixN	S27-F	0	6.6
S27	NO_3-mixN	S27-F	1	10.0
S27	NO_3-mixN	S27-F	2	6.8
S27	NO_3-mixN	S27-F	3	8.0
S27	NO_3-mixN	S27-F	4	6.3
S27	NO_3-mixN	S27-F	5	6.8

G. Nitrate in cells

Species	Replicate	Treatment	Days	NO3+NO2 in algae (mg N/L)
CPCC90	1	NO_3-NO_3	0	0.536
CPCC90	1	NO_3-NO_3	1	0.103
CPCC90	1	NO_3-NO_3	2	0.145
CPCC90	1	NO_3-NO_3	3	0.143
CPCC90	1	NO_3-NO_3	6	0.0631
CPCC90	1	NO_3-NO_3	9	0.106
CPCC90	1	NO_3-NO_3	12	0.0562
CPCC90	1	NO_3-NO_3	15	0.0231
CPCC90	1	NO_3-NO_3	18	0.0194
CPCC90	1	NO_3-NO_3	21	0.0204
CPCC90	1	NO_3-NO_3	24	0.0200
CPCC90	2	NO_3-NO_3	0	0.0846
CPCC90	2	NO_3-NO_3	1	0.218
CPCC90	2	NO_3-NO_3	2	0.213
CPCC90	2	NO_3-NO_3	3	0.162
CPCC90	2	NO_3-NO_3	6	0.0912
CPCC90	2	NO_3-NO_3	9	0.277
CPCC90	2	NO_3-NO_3	12	0.0562
CPCC90	2	NO_3-NO_3	15	0.0190
CPCC90	2	NO_3-NO_3	18	0.0190
CPCC90	2	NO_3-NO_3	21	0.0189
CPCC90	2	NO_3-NO_3	24	0.0169
CPCC90	3	NO_3-NO_3	0	1.22
CPCC90	3	NO_3-NO_3	1	0.0889
CPCC90	3	NO_3-NO_3	2	0.182
CPCC90	3	NO_3-NO_3	3	0.110
CPCC90	3	NO_3-NO_3	6	0.0861
CPCC90	3	NO_3-NO_3	9	0.110
CPCC90	3	NO_3-NO_3	12	0.0649
CPCC90	3	NO_3-NO_3	15	0.0477
CPCC90	3	NO_3-NO_3	18	0.0191
CPCC90	3	NO_3-NO_3	21	0.0205
CPCC90	3	NO_3-NO_3	24	0.0193
CPCC90	1	NH_3-NO_3	0	0.0864
CPCC90	1	NH_3-NO_3	1	0.16
CPCC90	1	NH_3-NO_3	11	0.0264
CPCC90	1	NH_3-NO_3	17	0.28
CPCC90	2	NH_3-NO_3	0	0.279
CPCC90	2	NH_3-NO_3	1	0.116
CPCC90	2	NH_3-NO_3	11	0.0347
CPCC90	2	NH_3-NO_3	17	0.0331

CPCC90	3	NH_3-NO_3	0	0.134
CPCC90	3	NH_3-NO_3	1	0.032
CPCC90	3	NH_3-NO_3	11	0.0442
CPCC90	3	NH_3-NO_3	17	0.232
CPCC90	1	NH_3-NH_3	0	0.0237
CPCC90	2	NH_3-NH_3	0	0.0235
CPCC90	3	NH_3-NH_3	0	0.0238
CPCC90	1	NO_3-NH_3	0	0
CPCC90	1	NO_3-NH_3	1	0
CPCC90	1	NO_3-NH_3	2	0
CPCC90	1	NO_3-NH_3	3	0
CPCC90	1	NO_3-NH_3	6	0
CPCC90	2	NO_3-NH_3	0	0
CPCC90	2	NO_3-NH_3	1	0
CPCC90	2	NO_3-NH_3	2	0
CPCC90	2	NO_3-NH_3	3	0
CPCC90	2	NO_3-NH_3	6	0
CPCC90	3	NO_3-NH_3	0	0
CPCC90	3	NO_3-NH_3	1	0
CPCC90	3	NO_3-NH_3	2	0
CPCC90	3	NO_3-NH_3	3	0
CPCC90	3	NO_3-NH_3	6	0
CPCC90	1	NO_3-0N	0	0
CPCC90	1	NO_3-0N	1	0
CPCC90	1	NO_3-0N	2	0
CPCC90	1	NO_3-0N	5	0
CPCC90	2	NO_3-0N	0	0
CPCC90	2	NO_3-0N	1	0
CPCC90	2	NO_3-0N	2	0
CPCC90	2	NO_3-0N	5	0
CPCC90	3	NO_3-0N	0	0
CPCC90	3	NO_3-0N	1	0
CPCC90	3	NO_3-0N	2	0
CPCC90	3	NO_3-0N	5	0
S27	1	NO_3-NO_3	0	0.0731
S27	2	NO_3-NO_3	0	0.183
S27	3	NO_3-NO_3	0	0.131
2M	1	NO_3-NO_3	0	0.064
2M	2	NO_3-NO_3	0	0.148
2M	3	NO_3-NO_3	0	0.586
6M	1	NO_3-NO_3	0	0.174
6M	2	NO_3-NO_3	0	0.237
6M	3	NO_3-NO_3	0	0.17
S27	1	NO_3-NO_3	2	0.168
S27	2	NO_3-NO_3	2	0.125

S27	3	NO_3-NO_3	2	0.0948
2M	1	NO_3-NO_3	2	0.0456
2M	2	NO_3-NO_3	2	0.0334
2M	3	NO_3-NO_3	2	0.0293
6M	1	NO_3-NO_3	2	0.0523
6M	2	NO_3-NO_3	2	0.0708
6M	3	NO_3-NO_3	2	0.112
S27	1	NO_3-NO_3	6	0.0485
S27	2	NO_3-NO_3	6	0.0512
S27	3	NO_3-NO_3	6	0.037
2M	1	NO_3-NO_3	6	0.0835
2M	2	NO_3-NO_3	6	0.0403
2M	3	NO_3-NO_3	6	0.0217
6M	1	NO_3-NO_3	6	0.065
6M	2	NO_3-NO_3	6	0.23
6M	3	NO_3-NO_3	6	0.0135
S27	1	NO_3-NO_3	8	3.73E-03
S27	2	NO_3-NO_3	8	0.0181
S27	3	NO_3-NO_3	8	0.0178
2M	1	NO_3-NO_3	8	0.0163
2M	2	NO_3-NO_3	8	8.56E-03
2M	3	NO_3-NO_3	8	0.0388
6M	1	NO_3-NO_3	8	0.0257
6M	2	NO_3-NO_3	8	0.0151
6M	3	NO_3-NO_3	8	0.0143
S27	7	NO_3-NO_3	0	0.767
S27	8	NO_3-NO_3	0	0.306
S27	9	NO_3-NO_3	0	0.819
2M	7	NO_3-NO_3	0	0.498
2M	8	NO_3-NO_3	0	1.06
2M	9	NO_3-NO_3	0	0.356
6M	7	NO_3-NO_3	0	0.309
6M	8	NO_3-NO_3	0	0.493
6M	9	NO_3-NO_3	0	0.483
S27	7	NO_3-NO_3	2	0.67
S27	8	NO_3-NO_3	2	0.203
S27	9	NO_3-NO_3	2	0.418
2M	7	NO_3-NO_3	2	0.354
2M	8	NO_3-NO_3	2	0.612
2M	9	NO_3-NO_3	2	0.438
6M	7	NO_3-NO_3	2	0.565
6M	8	NO_3-NO_3	2	0.318
6M	9	NO_3-NO_3	2	0.436
S27	7	NO_3-NO_3	6	0.129
S27	8	NO_3-NO_3	6	0.0779

S27	9	NO_3-NO_3	6	0.181
2M	7	NO_3-NO_3	6	0.0695
2M	8	NO_3-NO_3	6	0.164
2M	9	NO_3-NO_3	6	0.163
6M	7	NO_3-NO_3	6	0.0762
6M	8	NO_3-NO_3	6	0.0427
6M	9	NO_3-NO_3	6	0.164
S27	7	NO_3-NO_3	8	0.031
S27	8	NO_3-NO_3	8	0.127
S27	9	NO_3-NO_3	8	0.0973
2M	7	NO_3-NO_3	8	0.167
2M	8	NO_3-NO_3	8	0.176
2M	9	NO_3-NO_3	8	0.0786
6M	7	NO_3-NO_3	8	0.209
6M	8	NO_3-NO_3	8	0.0231
6M	9	NO_3-NO_3	8	0.117
S27	D	NO_3-mixN	3	0.0775
S27	E	NO_3-mixN	3	0.0941
S27	F	NO_3-mixN	3	0.184
2M	D	NO_3-mixN	3	0.0874
2M	E	NO_3-mixN	3	0.083
2M	F	NO_3-mixN	3	0.294
S27	D	NO_3-mixN	4	0.143
S27	E	NO_3-mixN	4	0.198
S27	F	NO_3-mixN	4	0.133
2M	D	NO_3-mixN	4	0.29
2M	E	NO_3-mixN	4	0.317
2M	F	NO_3-mixN	4	0.057
S27	D	NO_3-mixN	5	0.028
S27	E	NO_3-mixN	5	0.029
S27	F	NO_3-mixN	5	0.091
2M	D	NO_3-mixN	5	0.0403
2M	E	NO_3-mixN	5	0.0403
2M	F	NO_3-mixN	5	
S27	D	NO_3-mixN	6	0.275
S27	E	NO_3-mixN	6	0.0449
S27	F	NO_3-mixN	6	0.0894
2M	D	NO_3-mixN	6	0.145
2M	E	NO_3-mixN	6	0.118
2M	F	NO_3-mixN	6	0.361
S27	4	NO_3-NH_3	0	0.023
S27	5	NO_3-NH_3	0	0.0187
S27	6	NO_3-NH_3	0	0.0176
2M	4	NO_3-NH_3	0	0.0278
2M	5	NO_3-NH_3	0	0.0189

2M	6	NO_3-NH_3	0	0.0219
6M	4	NO_3-NH_3	0	0.0242
6M	5	NO_3-NH_3	0	0.0268
6M	6	NO_3-NH_3	0	0.0226
S27	4	NO_3-NH_3	2	0.0213
S27	5	NO_3-NH_3	2	0.0187
S27	6	NO_3-NH_3	2	0.0154
2M	4	NO_3-NH_3	2	0.0241
2M	5	NO_3-NH_3	2	0.0372
2M	6	NO_3-NH_3	2	0.0199
6M	4	NO_3-NH_3	2	0.0219
6M	5	NO_3-NH_3	2	0.0169
6M	6	NO_3-NH_3	2	0.0224
S27	10	NO_3-NH_3	0	0.024
S27	11	NO_3-NH_3	0	0.0237
S27	12	NO_3-NH_3	0	0.0251
2M	10	NO_3-NH_3	0	0.0183
2M	11	NO_3-NH_3	0	0.0309
2M	12	NO_3-NH_3	0	0.0179
6M	10	NO_3-NH_3	0	0.0201
6M	11	NO_3-NH_3	0	0.0212
6M	12	NO_3-NH_3	0	0.022
S27	10	NO_3-NH_3	2	0.0196
S27	11	NO_3-NH_3	2	0.0189
S27	12	NO_3-NH_3	2	5.75E-03
2M	10	NO_3-NH_3	2	0.0188
2M	11	NO_3-NH_3	2	0.0204
2M	12	NO_3-NH_3	2	3.55E-03
6M	10	NO_3-NH_3	2	0.0162
6M	11	NO_3-NH_3	2	0.0172
6M	12	NO_3-NH_3	2	0.0175
S27	A	NO_3-NH_3	0	0.0178
S27	B	NO_3-NH_3	0	0.0212
S27	C	NO_3-NH_3	0	0.0257
2M	A	NO_3-NH_3	0	0.0251
2M	B	NO_3-NH_3	0	7.33E-03
2M	C	NO_3-NH_3	0	0.0466
S27	A	NO_3-NH_3	2	0.0204
S27	B	NO_3-NH_3	2	0.0197
S27	C	NO_3-NH_3	2	0.0125
2M	A	NO_3-NH_3	2	0.0203
2M	B	NO_3-NH_3	2	0.0218
2M	C	NO_3-NH_3	2	0.0204

H. Shaker

Location of flasks on the shaker was identified as in the table below.

A1	B1	C1	D1	E1	F1
A2	B2	C2	D2	E2	F2
A3	B3	C3	D3	E3	F3
A4	B4	C4	D4	E4	F4
A5	B5	C5	D5	E5	F5
A6	B6	C6	D6	E6	F6