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Microalgae growth on the aqueous phase from Hydrothermal Liquefaction of the same microalgae

Laura Garcia Alba [a,*], Cristian Torri [b], Daniele Fabbri [b], Sascha R.A. Kersten [a], Derk W.F. (Wim) Brilman [a]

[a] Sustainable Process Technology Group, Faculty of Science and Technology, University of Twente, P.O. Box 217, 7500AE Enschede, The Netherlands
[b] Centro Interdipartimentale di Ricerca Industriale, Università di Bologna, Via Sant' Alberto 163, 48123 Ravenna, Italy

HIGHLIGHTS

- Detailed evaluation of the participation of AP-N compounds during growth.
- Micronutrients (e.g. Mg) must be supplied upon AP-recycling to avoid growth reduction.
- Insufficient aqueous phase dilution does not seem to be the cause of growth reduction.
- 50% N recycling was proven to be possible and it can be further improved.
- Algae showed a preference for NH_4-N with no evidence of organic-N consumption.

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ABSTRACT

Cultivation of *Desmodesmus* sp. microalgae in the recycled aqueous phase (AP) recovered after Hydrothermal Liquefaction (HTL) of the same microalgae was studied to evaluate the potential of nutrients recycling. AP dilution ratio was systematically varied, using either water or water enriched with standard medium, while keeping the same N concentration (either as total-N or as sum of ammonia and nitrate) as that in the standard medium. More than 90% of the organic compounds in the AP were indentified and quantified and potential growth inhibiting substances (e.g. phenols) were found. However, the combination of growth and analytical results showed that the lack of (macro-/micro-)nutrients, other than N and P, in the AP is the main cause of growth reduction rather than toxicity due to insufficient AP dilution, as pointed in previous investigations. Therefore, these (macro-/micro-)nutrients such as Mg should be supplied upon AP recycling. For this specific cultivation case, algae production costs related to nutrients consumption can be significantly reduced considering that a 50% N-replacement was achieved, while showing nearly identical growth as that in standard culture medium.

1. Introduction

Algae are the fastest growing photosynthetic organisms on earth and therefore an interesting resource for energy, as they are capable to store sunlight in the form of energy-rich organic compounds and do not directly compete for arable land. The productivity of algae could be 50 times higher than that of switchgrass, one of the fastest growing terrestrial plants [1]. However, the current estimated costs for algal cultivation are significantly higher; 5–10 $ kg^{-1} [2] vs. 0.025–0.1 $ kg^{-1} [3] for switchgrass. Moreover, the energy requirements associated with the production of nitrogen nutrients (e.g. nitrate) represent a significant part of the total energy inputs, comparable to the electrical requirements [4]. Also phosphorus, another essential nutrient, is a non-renewable resource and current global reserves may be depleted in 50–100 years [5]. In our research we aim to alleviate these issues for algae biorefinery concepts by recycling of these nutrients, from downstream processing back to the algae cultivation section.

In an algae biorefinery scheme, several extraction and conversion processes could be combined to co-produce high value-added products, feed/food ingredients and energy carrier products from microalgae. A promising conversion method hereto is Hydrothermal Liquefaction (HTL) to produce an algal bio-crude oil. This process circumvents the costs of energy intensive drying for complete dewatering. Several studies into the hydrothermal conversion of lignocellulosic biomass and algae have been performed in the past [6–8].

The aqueous phase (AP) obtained from HTL of microalgae contains N and P constituents that, ideally, can be recycled, aiming to reduce cultivation costs and energy production costs induced by the continuous use of fresh make-up nutrients. Furthermore, the AP contains several oxygenated hydrocarbons that may be

* Corresponding author. Tel.: +31 53 489 2891; fax: +31 53 489 4738.
E-mail addresses: lgarcialba@gmail.com (L. Garcia Alba), D.W.F.Brilman@utwente.nl (D.W.F. (Wim) Brilman).

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assimilated when a heterotrophic/mixotrophic algae strain is used. In previous work we showed that the molecular and elemental composition of the AP can significantly vary, in particular with HTL operating temperature [9]. The largest N sources in the aqueous HTL product were always organic N-containing compounds and ammonia. Moreover, as shown in the work by Biller et al. [10], AP composition can also vary depending on the algae strain used.

The desirable situation within a biorefinery framework would be performing HTL of algae (–debris) under the adequate process conditions in which all the N in the algae is transferred to the AP in the form of NH_3/NH_4^+, while still producing enough crude bio-oil. In developing such a concept, the algae strain selected for growth might be of importance, as it must, e.g. be able to persist within a medium containing organic constituents from recycled HTL process water and preferably assimilate all the nutrients offered via this HTL aqueous phase recycling.

This idea of nutrients recycling was first proposed and tested in a pioneering investigation of Minowa and Sawayama [11]. In their study, *Chlorella vulgaris* was successfully cultivated (comparable to that in standard medium) in the recycled AP from catalytic *gasification* of the same algae when this one was diluted in nitrogen less standard medium. Recently, Jena et al. [12] and Biller et al. [10] tested similarly the ability to grow algae in diluted aqueous phase from HTL. Both stressed the importance of AP dilution in view of inhibitory effects of potential toxic compounds at high concentrations. Biller et al. [10] stated that heavy dilution of AP is required to avoid growth inhibition. Both observed that the algae in water diluted AP could not grow as fast as that in standard culture medium, even when sufficient amounts of N and P were present in the media. In this work, we also investigate the replacement/recycling of N and P containing nutrients in the growth medium. However, this was done by following a different approach. In our study we used mixtures of standard culture medium, AP and demineralized water with the same nitrogen concentration (either as total N or the sum of ammonia and nitrate) as that in standard growth medium. Consequently, the main differences between the tested media in this work were the concentrations of (macro-/micro-)nutrients and potentially toxic organic compounds.

2. Experimental

2.1. Algae feedstock

The freshwater microalgae used, *Desmodesmus* sp., was provided by Ingrepro B.V. (The Netherlands), where industrial cultivation is performed in raceway ponds and at high pH to maintain monoculture conditions. The batch obtained was centrifuged and maintained in the dark, cold and in COMBO (*CB*) growth medium [13] from which KCl, $Na_2SiO_3{\cdot}9H_2O$, animal trace elements and later $NaHCO_3$ (since CO_2 gas was used as carbon source) were excluded. The constructed growth setup was inoculated with that maintained culture. The proximate and ultimate analyses and biochemical composition of this type of microalgae can be found in our previous work [9].

2.2. Hydrothermal Liquefaction (HTL) and aqueous phase (AP) recovery

From a batch of concentrated *Desmodesmus* sp. solution (7.66 wt% of algae dry ash free), several batch HTL tests (using 20 g of feedstock each time) were performed using a 45 ml stainless steel autoclave, heated by immersion in a hot fluidized sand bed. These experiments were carried out with an inert atmosphere at 300 °C for 5 min reaction time (excluding 6–7 min of heating time). After these 5 min of reaction time, fast quenching (~1–2 min) was performed by inserting the autoclave in a water bath. After gas analysis, a subsequent product collection and separation procedure was carried out to obtain the other three products: oil (crude from dichloromethane soluble fraction), water soluble organics (aqueous phase AP), and solid residue. A detailed explanation of the setup, products separation procedures and typical mass balance closure can be found elsewhere [9]. In the present study, however, additional detailed analyses on the obtained *aqueous phase* fractions were performed.

Nutrients concentration, *Ni and COD (in mg L^{-1})*: These compounds were quantified with standardised tests (HACH LANGE) and measured with a spectrophotometer (DR 5000, HACH Corporation). The compounds quantified were: total nitrogen (TN, LCK 238), nitrate (NO_3-N, LCK 339), ammonia (NH_4-N, LCK 305), phosphate (PO_4-P, LCK 349), Ni (LCK 337), and COD (chemical oxygen demand, LCK 414). Organic nitrogen (org-N) was calculated as: TN–(NO_3-N)–(NH_4-N)–(NO_2-N). Nitrite was below the detection limit for all the aqueous phases analysed. In the labelled "NH_4-N" test, the total nitrogen coming from both NH_3 and NH_4^+ is determined. Both, ammonia and ammonium coexist in equilibrium in water depending on pH.

Total Organic Carbon, *TOC (in mg L^{-1})*, was measured with a Shimadzu TOC/TIC analyser.

Na, K, Ca, S and Mg nutrients were determined by means of Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Varian Vista MPX) and *Cl* by Ion Chromatography (IC, Dionex ICS 2100). Analyses were performed in duplicate.

AP organic compounds: Analyses of volatile compounds (acetic acid, ethanol and acetone) in AP were performed by acidifying the medium to pH = 2 with potassium bisulphate prior to 10 min SPME (Solid Phase Microextraction, polyacrilate coating) immersion sampling and direct GC–MS (equipped with MDN-5S) and GC–FID (equipped with NUKOL™ capillary column) for acetic acid analysis. Quantitative analyses were done by calibration with solutions of known concentrations of pure standards. The non-volatile fraction of water soluble organics was quantified by evaporation of water, derivatization and GC–MS analysis. Derivatization and analysis were performed following a published procedure [14] which was slightly modified in order to obtain complete silylation of cyclic dipeptides and polyhydroxylated compounds.

2.3. Cultivation system

A batch growth setup was designed, constructed and calibrated (Fig. 1). The total reactor volume was distributed over 10 glass reactors (1–10 in Fig. 1a), each having a volume of 2 L. Fluorescent artificial lighting (Philips MASTER TL-D Graphica 58W/9651SL) was used as light source in continuous mode. Light intensities and wavelength spectra inside the reactor compartment and individual reactors were determined using a USB4000 spectrophotometer (Ocean Optics). As a significant drop in received light was measured for reactors 1 and 10, located at both ends of the reactor compartment (see Fig. 1a), these two reactors were not used.

The temperature inside the reactor compartment was kept constant with max. ±1 °C difference throughout the reactor compartment by air circulation (blue[1] arrow in Fig. 1a). For mixing, oxygen stripping and carbon dioxide supply inside the reactors, aeration enriched with pure CO_2 was used. This gas mixture was saturated with water through two saturators (in Fig. 1a, vessels A and B containing demineralized water) to minimize evaporation losses. A gas pressure equalizer ensured equal inlet pressure for all connected

[1] For interpretation of colour in Fig. 1, the reader is referred to the web version of this article.

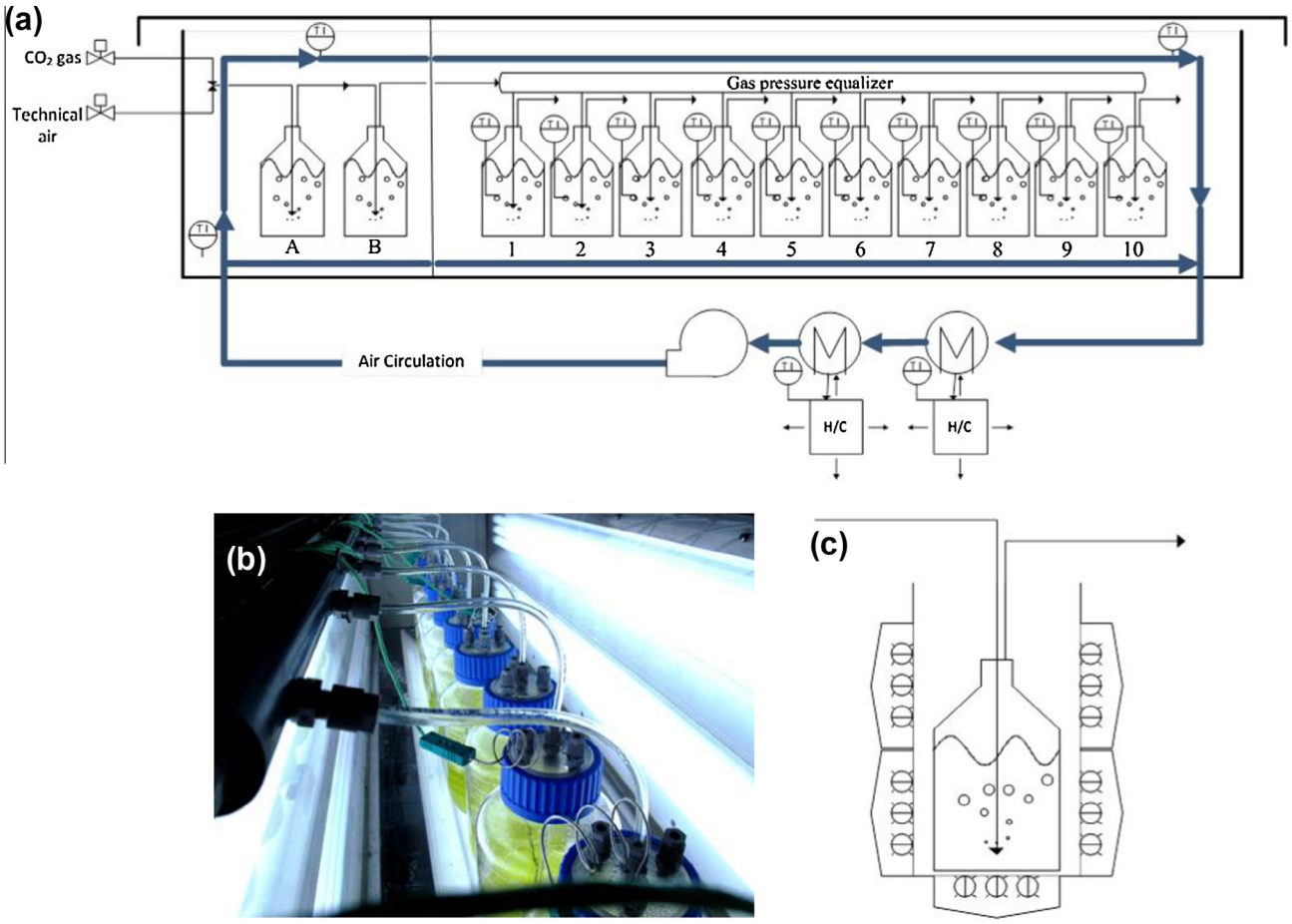

Fig. 1. (a) Growth setup scheme, (b) inoculated individual reactors and (c) lights configuration in the reactor compartment.

reactors. After passing the reactors, the gas was emitted to the general compartment.

2.4. Growth measurements and medium analyses

Algae growth was followed by measuring optical density at 750 nm (OD750) with a spectrophotometer (DR 5000, HACH Corporation). The measured optical density was found to properly correlate with the algae dry weight content of the corresponding solution (after filtrating and drying at 105 °C for 24 h). Up to OD750 = 0.7, a linear relationship was obtained with the following equation:

$$\text{Algae concentration } (\text{g L}^{-1}) = 1.58 \cdot \text{OD750} \quad (1)$$

The constant parameter in Eq. (1) (1.58) has a 95% confidence interval of 1.56–1.61.

Specific growth rate (h^{-1}) of a microalgal population is determined from the exponential growth phase and automatic inline measurements of optical density can lead to a clear identification of this exponential phase. However, this becomes more difficult when taking OD750 measurements between intervals of 6–10 h, as done in this work. Therefore, for our case, we chose *total algae produced* (in g L^{-1}, on algae dry weight) in tests of 68–96 h and *maximum daily productivity* as best indicators for comparing the growth between different culturing experiments. Maximum daily productivity found over the course of a growth test was defined as 'P_{max}' and calculated by a three point approximation of the maximum gradient.

Nutrients consumption was measured by determining the concentration of nutrients in the medium during growth using the same HACH LANGE tests used for aqueous phase analyses. For that, the samples were centrifuged (8228*g*, 5 min) to separate the supernatant from the algal pellet. Nutrients analyses were performed on the recovered supernatant immediately after that separation.

A light intensity of 247 μmol m^{-2} s^{-1} (measured at the reactor wall) was used for all growth experiments leading to an onset of light limitation at a concentration of about 0.3 g L^{-1}. The initial algae concentration for all experiments was always lower to avoid light limitations.

2.5. Algae adaptation

The maintained fresh algae need a period of physiological adjustment due to changes in e.g. nutrients or culture conditions [15]. A sample of maintained culture was centrifuged at 8228*g* for 5 min after which the cell pellet was collected, re-suspended and transferred to the individual growth reactors, each containing 1 L COMBO medium and aerated at 1.8 vvm with an air–CO_2 mixture containing 2 vol% of CO_2 and showing an initial pH of 7.4. The culture was kept under this controlled environment inside the reactor compartment, where the temperature and light intensity were gradually increased up to a final value of 27.0 (±1 °C throughout the reactor compartment) and a light intensity of 247 μmol m^{-2} s^{-1}. Every day during this adaptation time, half of the culture volume was replaced by fresh medium to avoid light limited conditions due to high concentrations of algae. After a period of approximately 15 days, the algae seemed to have adapted to

the new growth environment since a repeatable growth behaviour was observed without fluctuations. By visual inspection using a microscope, the algal cells were the same as the initial feedstock without abnormal morphological characteristics. This algae batch was used as inoculum for the first growth test using aqueous phase (Section 2.6). Every time a new growth test was started, the algae were pre-adapted again (for at least 5 days diluting the culture every day), to the growth conditions as previously mentioned. Algal growth was not performed under axenic conditions, as in an envisaged algae biorefinery, outdoor cultivation under non-axenic conditions is preferable in order to reduce costs.

2.6. Algae growth experiments

The same inoculation method was used for all the growth experiments. In this method, the algae to be used as inoculum is first maintained and adapted to COMBO medium and the growth conditions mentioned in Section 2.5. After that, the culture (growing at exponential phase) is harvested and centrifuged at 8228*g* during 5 min after which the supernatant is removed. The cells pellet is used to inoculate the corresponding reactors until an initial algae concentration of 0.03–0.04 g L^{-1} (algae dry weight).

Three series of growth experiments were performed, with the only difference being the characteristics of the culture medium. Those differences can be seen in Table 1, where all the details concerning media preparation are shown.

The first growth experiment (expt. 1 in Table 1), used as control test, was performed in standard culture medium COMBO (*CB*). Two reactors containing 1 L of the same medium were inoculated. The results of this test were used to compare with the growth experiments adding AP.

In the second growth experiment (expt. 2 in Table 1), performed as proof of concept, two reactors were inoculated containing a medium with a high concentration of AP. The AP was diluted in demineralized water for one reactor and in COMBO-medium for the other (*W + AP_20x* and *CB + AP_20x* respectively). All the phosphorus in the aqueous phase was in the form of phosphate, at a concentration of 159 mg L^{-1}. Therefore, since there was sufficient P supply via the aqueous phase, no extra P was added (see Table 1 phosphorous stock solution).

Finally, in the third growth experiment (expt. 3 in Table 1), the medium of all the reactors had the same initial nitrogen concentration (either as total-N including organic N or as NO_3-N + NH_4-N) and equal to that in the standard COMBO medium (approx. 13 mg L^{-1} of N). To reach that, different amounts of AP were added. For each growth configuration of experiment series no. 3 two reactors were used (as duplo). Hence, each data point in the graphs presented represents the mean value between the results obtained from both reactors, along with their corresponding error bars. Four reactors contained only water-diluted aqueous phase (*W + AP-1* and *W + AP-2*) and the other four supplemented with COMBO (*CB + W + AP-1* and *CB + W + AP-2*). Furthermore, in all media P was supplied to the same final concentration as in COMBO medium (by adding specific amounts of P stock solution, see Table 1), as the purpose of this test was to look at the influence of only the N-substances.

3. Results and discussion

The AP fractions were produced by performing HTL experiments all under the same reaction conditions, 300 °C and 5 min reaction time (excluding heating time). Similar results as those reported in our previous work [9] were obtained, including a mass balance closure of almost 100% and oil yield of 40 wt% with a HHV for the oil of 35 MJ kg^{-1}. Further analyses performed on the oil, gas and residual solids fractions are not presented here, as they are very similar to earlier work [9] and since the focus of this work was on the usage of the aqueous phase for algal production.

3.1. Proof of concept: algae growth using HTL-AP

First, as a proof of concept, the non-optimised (i.e. regardless of the final nutrients concentration in the medium) growth of *Desmodesmus* sp. in a medium containing a significant fraction of AP obtained from HTL of the same microalgae was tested. The main objective was to test if the presence of a large amount of AP would lead to significant adverse effects on growth.

The initial concentrations of NO_3-N and NH_4-N for each medium as well as the amount of organic nitrogen can be seen in Table 2. The difference in NO_3-N between the *W + AP_20x* and *CB + AP_20x* media is the amount of NO_3-N coming from COMBO addition (12.4 mg from *CB* and 5.7 mg from AP). The same occurring for the phosphorous supply, having a higher initial concentration of P in the *CB + AP_20x* medium due to the double contribution from the AP and from COMBO.

Also in Table 2, the total algae produced after 96 h of growth and the maximum daily productivity for the media with AP are shown and compared to those in standard COMBO medium (control test) after 68 h of growth. The growth with AP in COMBO was significantly faster than that with only AP in water. After

Table 1
Media preparation of the various growth experiments.

Expt.	Reference name	wt% of total medium[a]				AP DF[f]	Final N concentration in medium created from…
		CB[b]	W[c]	AP[d]	P[e]		
1	*CB*	100	0.00	0.00	0.00	–	(from NO_3-N) in *CB*
2	*W + AP_20x*	0.00	95.0	5.00	0.00	20	(from NO_3-N + NH_4-N + Org-N[g]) in only AP
	CB + AP_20x	95.0	0.00	5.00	0.00	20	8% N (from NO_3-N) in *CB* + 92% N (from NO_3-N + NH_4-N + Org-N) in AP
3[i]	*W + AP-1*	0.00	99.28	0.62	0.10	160	(from NO_3-N + NH_4-N) in only AP
	W + AP-2	0.00	99.58	0.32	0.10	311	(from NO_3-N + NH_4-N + Org-N) in only AP
	CB + W + AP-1	50.0[h]	49.64	0.31	0.05	320	50% N (from NO_3-N) in *CB* + 50% N (from NO_3-N + NH_4-N) in AP
	CB + W + AP-2	50.0	49.79	0.16	0.05	623	50% N (from NO_3-N) in *CB* + 50% N (from NO_3-N + NH_4-N + Org-N) in AP

[a] Total amount of medium = 1 L.
[b] COMBO standard growth medium.
[c] Demineralised water.
[d] Aqueous phase recovered from HTL experiment.
[e] Phosphorous stock solution.
[f] DF: Dilution Factor.
[g] Organic nitrogen.
[h] As an example: 1 L medium with 500.0 ml *CB*, 496.4 ml W, 3.1 ml AP and 0.5 ml P.
[i] Final target of nitrogen concentration being 13 mg L^{-1} for all the growth media of expt. 3.

Table 2
Initial media composition, total algae produced and maximum daily productivity in standard COMBO (*CB*) and in media with aqueous phase (AP) co-feeding 20× diluted.

Experiment reference[a]	Initial composition in medium (mg L^{-1} of N and P)				Algae[b] (g L^{-1})	P_{max}[c] (g L^{-1} d^{-1})
	NO_3-N	NH_4-N	Org-N	PO_4-P		
CB	13.0	0.00	0.00	1.55	0.81[d]	0.70
W + AP_20x	5.70	47.8	91.7	8.00	0.21[e]	0.14
CB + AP_20x	18.1	47.8	91.7	9.40	0.74[e]	0.62

[a] For abbreviations refer to Table 1.
[b] Total algae produced (on dry weight). Estimated error below 10%.
[c] Maximum daily productivity (on dry weight). Estimated error below 20%.
[d] After a total of 68 h of growth.
[e] After a total of 96 h of growth.

4 days of growth, a total of 0.74 g L^{-1} of algae (dry weight) were produced, almost a factor four higher than that produced in *W + AP_20x*. At the same time, the growth results from *CB + AP_20x* and *CB* appeared comparable. In *CB + AP_20x*, a slightly longer lag phase was observed, thereby taking longer time to reach an algae concentration comparable to that in *CB* after 68 h (see Table 2). However, their maximum daily productivities were in the same range. Therefore, the presence of a large amount of AP in COMBO is apparently not causing a major reduction in growth. The small difference could be related to the fact that the algae was, for the first time, in a medium with AP and at high concentration needing a longer time for adaptation. In *CB + AP_20x* medium, the inoculum was transferred from one set of growth conditions to another (by adding AP) while the cells in COMBO were totally acclimated.

Growth could develop in the *W + AP_20x* medium. However, it did not result in optimal algal growth showing a significantly lower productivity than that in COMBO. The few studies available [10–12], although having different strains; different growth reactor configurations; and different growth conditions, also showed a drop in growth rate when using only water-diluted AP. Biller et al. [10] reported even *no* growth for *Spirulina platensis* in 50× diluted AP, while having a higher N concentration than that in standard medium. Jena et al. [12] reported the same for *Chlorella minutissima* in a 10× water diluted AP with sufficient amounts of both N and P.

In the section below, the possible explanations to the previous observations are discussed.

Ammonia is formed during HTL of microalgae. In the AP, free ammonia (NH_3) exists in equilibrium with ammonium (NH_4^+), depending on temperature and, especially, pH. When the pH increases, the equilibrium shifts towards ammonia, which is the main toxic form for algae [16]. In literature, it was demonstrated that the concentration at which NH_3 inhibits the growth of a specific algae strain is fixed, and pH only affects how much NH_3 is available via the degree of dissociation [16]. The growth of *Scenedesmus obliquus*, an algae strain very similar to the one used in this work, was completely inhibited by NH_3 concentrations above 2 mM [16]. If, for the experiments shown in this section, all N coming from the equilibrium NH_3/NH_4^+ would be in the form of free NH_3, its concentration would be 3.4 mM which could be in the range of toxicity. However, both *W + AP_20x* and *CB + AP_20x* media had the same amount of initial NH_4-N (total N in the form of NH_3/NH_4^+) as they were equally diluted, and the initial pH of only *CB* was similar to that of water. Therefore, although pH was not controlled along the growth period, a pH change leading to ammonia toxicity occurring only in the *W + AP_20x* medium is unlikely to have caused the lowered growth rate.

Following the same reasoning, both media had the same concentrations of potential growth inhibitory compounds such as phenols, metals (e.g. Ni, from reactor wall corrosion) and fatty acids. In contrast with other studies reporting the importance of AP dilution [10,12] to avoid toxicity, this cultivation tests show that, at least for *Desmodesmus* algae strain, dilution is not likely to be the main limiting factor for growth.

Another explanation, alternative to the growth inhibition by poisoning, could be related to the nutrients ratio, rather than their absolute concentration. However, the *W + AP_20x* and *CB + AP_20x* media had comparable N:P ratio (e.g. 6.7 and 7.0 respectively when only counting for inorganic nitrogen NO_3-N and NH_4-N), but were exhibiting large differences in growth.

Finally, although the *W + AP_20x* medium had much larger amount of total N (NO_3-N + NH_4-N + org-N) and P nutrients than that in the standard medium (11 times more N and 5 times more P), the maximum daily productivity was much lower. In preliminary experiments (results not shown here) we observed that cultivating with higher amounts of nitrate and phosphate than that in COMBO did not lead to higher growth rates, thus indicating that the concentrations in standard medium seem quite optimal for the N and P demand. Consequently, the form in which N is available might have played an important role instead. The lower amount of nitrate in the *W + AP_20x* medium (compare to that in *CB*) could have had an effect. The supply of NH_4-N might have not been sufficient to offset for the decline in NO_3-N, which could be due to the possible preference of the algae to this latter nutrient. To investigate this further, a new growth experiment (presented in the following section) was performed focusing on the participation of the various AP nitrogen sources during growth and to find out why AP enriched with COMBO exhibits a better growth performance.

3.2. Analysing the participation of N-compounds in HTL-AP during growth

New HTL AP was produced for these experiments and analysed in detail. Its concentration of N containing compounds (70 mg L^{-1} NO_3-N, 2012 mg L^{-1} NH_4-N and 1964 mg L^{-1} organic N) as well as the chemical oxygen demand (34,897 mg L^{-1} of O_2) were slightly different compared to the AP composition in the earlier growth run, and in the same range of other studies [10]. Nickel was also detected, mostly likely from corrosion or leaching from the reactor walls (INCOLOY 825) during HTL. Biller et al. [10] also reported the presence of Ni in HTL aqueous phase (0–4 ppm). This type of toxic metal can affect almost every aspect of algal metabolism, growth and differentiation [15]. However, in our AP, Ni concentration was still low (1.9 mg L^{-1}) and ended up even much lower in the growth media due to the dilution factors of more than 100 times. Therefore, no inhibitory effects were expected.

The purpose of this experiment was to learn about the participation of the different nitrogen-containing species during the growth of algae, upon recycling HTL aqueous phase. Knowing the HTL-AP composition, it was possible to control the initial amount of the various N constituents in the medium. Instead of adding a large amount of AP (as in the previous tests), we now added a specific amount of it, in order to reach the exact same N concentration (either as total-N or in a given form) as that in the standard COMBO medium. That procedure simultaneously resulted in media with different AP dilution factors. Again, we tested some reactors containing only water-diluted aqueous phase and others supplemented with COMBO.

All the details on media preparation can be found in Table 1 and the exact final concentrations of the various N containing compounds in the initial media are shown in Table 3. The concentrations shown in Table 3 reflect the strategy of changing the form of N available for growth by varying the composition for each of the growth media tested (see highlighted values in Table 3). In the media *W + AP-1* and *CB + W + AP-1*, we targeted a final

Table 3
Initial distribution of N substances, initial phosphorus concentration, total algae produced and maximum daily productivity in standard COMBO (*CB*) and in different media configurations containing HTL aqueous phase (AP).

Experiment reference[a]	Initial N-composition in medium (mg L^{-1} of N)					Initial PO_4-P (mg L^{-1})	Algae[b,c] (g L^{-1})	P_{max}[d] (g L^{-1} d^{-1})
	NO_3-N	NH_4-N	Org-N	NO_3-N + NH_4-N	Total N			
CB	13.0	0.00	0.00	**13.0**	13.0	1.55	0.81	0.70
W + AP-1	0.38	11.9	9.13	**12.3**	21.4	1.55	0.17	0.19
W + AP-2	0.22	6.09	5.79	6.31	**12.1**	1.55	0.18	0.19
CB + W + AP-1	6.82	5.83	5.39	**12.7**	18.0	1.55	0.84	0.61
CB + W + AP-2	6.59	2.97	3.04	9.56	**12.6**	1.55	0.73	0.66

[a] For abbreviations refer to Table 1.
[b] Total algae produced (on dry weight). Estimated error below 10%.
[c] After a total of 68 h of growth.
[d] Maximum daily productivity (on dry weight). Estimated error below 20%.

concentration of around 13 mg L^{-1} of N (as in COMBO), however here just in terms of inorganic nitrogen species (NO_3-N + NH_4-N). Hence, in *CB + AP-1* medium, 50% of the N (in the form of nitrate) in COMBO was replaced by N (in the form of NO_3-N and NH_4-N) from the AP. For *W + AP-2* and *CB + W + AP-2,* the final N concentration of around 13 mg L^{-1} was targeted, but now also taken into account the organic nitrogen present in the AP. That was done to evaluate its possible consumption and thereby resulting in lower concentrations of NO_3-N + NH_4-N.

As can be seen, the design of this experiment allows for the evaluation of several aspects such as: dilution factor effect, water or water + COMBO diluted AP; organic nitrogen presence; and NO_3-N vs. NH_4-N.

This time, the AP was much more diluted leading to lower initial concentrations of NH_4-N than that in the medium of the previous growth experiment (Section 3.1). The highest amount of NH_4-N was therefore found in the *W + AP-1* medium, but a concentration below the estimated toxicity limit for *Scenedesmus* and *Desmodesmus* strains.

The growth curves for the various cultivation tests are shown in Fig. 2, together with the reference run using COMBO, each for a period of nearly 3 days of growth. Very clear trends were obtained with significant differences. The algae could grow much faster in the medium containing a mixture of *CB + W + AP-1* than only in *W + AP-1*, despite having almost identical N concentration from the sum of NO_3-N and NH_4-N (12.3 and 12.7 mg L^{-1} in Table 3). At the same time, the growth curve for *CB + W + AP-1* was very similar to that for COMBO alone. This is also reflected in Table 3, where comparable maximum daily productivities are shown producing nearly the same amount of algae after 68 h of growth. This already excludes the hypothesis that, in previous experiments, the lower amount of nitrate in the *W + AP_20x* medium could have limited the growth, since now the amount of NO_3-N in *CB + AP-1* (6.82 mg L^{-1}) of the new experiment was almost half of that in *CB,* while showing comparable algae growth performance. Moreover, it seems that the standard medium can be further optimised to lower N amounts as the growth rate in the medium *CB + W + AP-2* (with lower inorganic nitrogen) was very similar to that in *CB* alone.

Overall, algae in *CB*, *CB + W-AP-1* and *CB + W + AP-2* exhibited comparable growth with a total algae production (on dry weight) of about 0.80 g L^{-1} (see Table 3). The growth curves for *W + AP-1* and *W + AP-2* were even close to each other (see Fig. 2), showing equal maximum daily productivity (0.19 g L^{-1} day^{-1}) while reaching a total of 0.18 g L^{-1} of algae (dry weight). This indicates that the presence of organic nitrogen did not seem to have any (positive nor

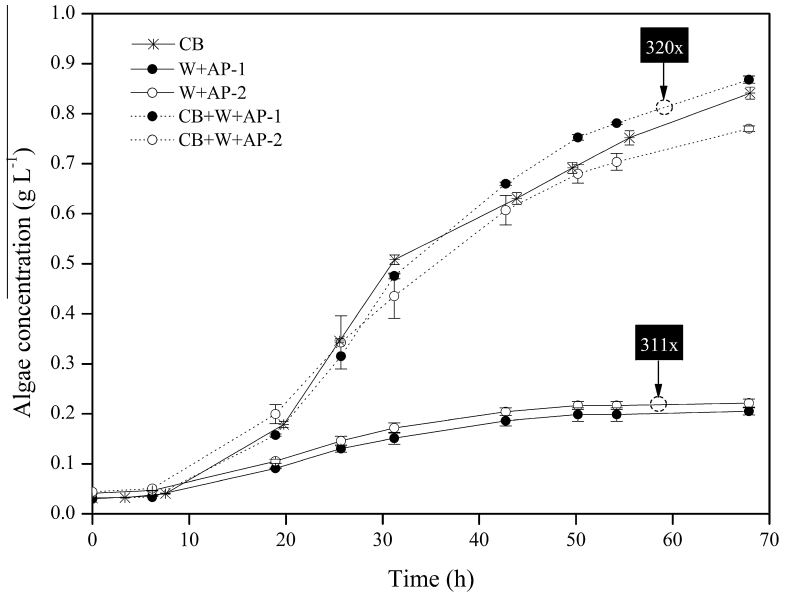

Fig. 2. Algae growth (with algae concentration on algae dry weight basis) in standard COMBO (*CB*) and in different medium configurations containing HTL aqueous phase (AP).

negative) effect on growth rate, suggesting that, most likely, this type of compounds are not toxic, but also not readily metabolised by the algae.

To investigate further which form of N is preferred during growth, several samples were taken during cultivation and analysed for the NO_3-N and NH_4-N content in the medium. As ammonia stripping by the continuous flow of CO_2 enriched air could also lead to a decrease of NH_4-N during growth, first an experiment was performed where 9.4 ml of AP was added to a 1 L demineralised water in a reactor vessel, contained into the same growth system. The same CO_2-areation flow was used and after 68 h of continuous bubbling, still the same amount of NH_4-N as initially was found in the aqueous solution, indicating that no significant ammonia stripping had occurred.

Fig. 3 shows the NO_3-N and NH_4-N consumption results during growth in the media *W + AP-1* and *CB + W + AP-1*. Algal growth was accompanied by decrease in nitrogen content in the medium, indicating that nitrogen removal was due to algal uptake and assimilation. For all media tested, complete NO_3-N and NH_4-N removal was accomplished after 30 h of growth, except for the *W + AP-1* medium where the algae did not consume any nitrate (Fig. 3a). There, the initial amount of NH_4-N was much higher than NO_3-N and after 68 h there was still NH_4-N available and thus no need for the algae to consume nitrate. Possibly, after complete depletion of NH_4-N, the cells would have started consuming NO_3-N, as is e.g. observed in the uptake of N within the *CB + W + AP-1* medium where NH_4-N was consumed faster than NO_3-N and when all the NH_4-N was finished (after 20 h) the remaining NO_3-N was consumed. Consequently, in all the culture media, the microalgae cells preferentially utilize NH_4-N, most likely because it is easier to convert into amino-acids than NO_3-N [17].

Nitrogen depletion (and also P which was also depleted after 30 h) seems to coincide with the decrease of growth rate. For all the reactors, from 20 to 30 h the biomass concentration was increasing at the highest growth rates and after that, a significant decrease of the growth rate occurred (see Fig. 2, change of slope of the curve after 30 h, coinciding with the full depletion of N and P).

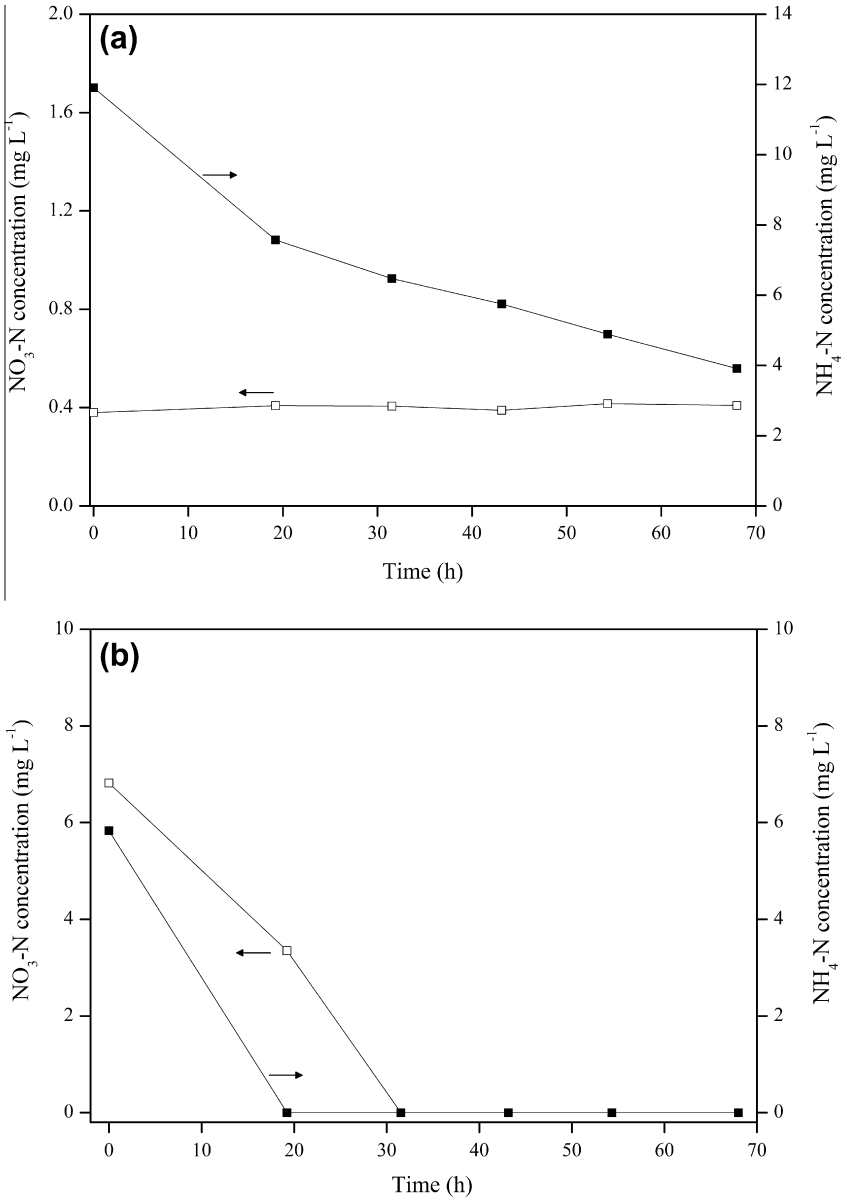

Fig. 3. NO_3-N and NH_4-N consumption during growth in media (a) *W + AP-1* and (b) *CB + W + AP-1*.

Additionally, the total nitrogen content was also monitored along the growth to study the possible consumption of organic nitrogen. For a period of almost 3 days of growth, the amount of org-N remained nearly constant in all the growth media. Hence, no evidence of consumption of organic nitrogen was found, at least over the time used for cultivation. Total organic carbon content was also monitored along the growth in all the cultures, but again no clear evidence of its consumption was found for the cultivation period of 68 h. Therefore, no clear signs of mixotrophic growth were observed. Biller et al. [10] reported consumption of organic carbon by *Scenedesmus dimorphous* (similar strain to the one used in this work) in AP, but the cells were not able to use it to the same extent as the other algae species tested. Mixotrophic growth could lead to higher carbon efficiencies for the combined HTL-cultivation loop. On the other hand, if an algae species with desirable properties is used but cannot assimilate any kind of organic source, still the carbon efficiency can be improved by extracting the energy-containing compounds from the AP by other methods as e.g. supercritical water gasification or anaerobic digestion of the AP prior to recycling to cultivation. These treatments would convert the organic matter into a high heating value gas while, most likely, producing more ammonia dissolved into the AP for further reuse.

The effect of the AP dilution factor by itself can be evaluated as a results of the adjustment of the various media for different nitrogen compositions. Again, and in contrast with earlier literature [10,12], the lowered growth does not seem to be caused by insufficient dilution related to toxicity of certain organic compounds. This is proven by comparing the media *CB + W + AP-1* and *W + AP-2* with comparable AP dilution (320× and 311× respectively) and NH_4-N concentration (the preferable N source) but with algae exhibiting completely different growth performance. Almost five times more algae were produced in the medium enriched with COMBO.

Although the results showed that the toxicity of organics can be excluded, still the AP was analysed in detail to get a general picture of water soluble products of HTL. The dissolved organics fraction was mainly composed of small water soluble compounds, which became GC-detectable by adopting a derivatisation procedure optimised for the determination of cyclic dipeptides [14]. The quantitative analysis of the identified substances provided an exhaustive picture of the water soluble organic matter, with a mass closure of more than 90%. Table 4 shows the concentration (in mg L^{-1}) of organic constituents, grouped into various categories for pure AP and initially in *W + AP-1* medium. This medium had the lowest dilution factor (160×) and hence the highest concentration of potential inhibitory compounds.

Residual solvent, used for the oil recovery in the HTL experiment, was a prominent contaminant detected at a concentration of about 1% in the solution. Among the HTL generated substances, acetic acid was found to be the most abundant compound, followed by pyroglutamic acid, earlier detected in remarkable amount in HTL oil [18]. Expressed as mass yield, the water dissolved fraction of this compound corresponds to about 1.6% of the original microalgae biomass. Other water soluble HTL products with less important contribution (100–600 mg L^{-1}) were found to be sugar-like compounds, hydroxyacids, cyclic dipeptides, small fatty acids, amines and amino acids. Although detectable, only minor amount of phenols (58 mg L^{-1}) ended up in the water phase, even though larger amounts of these compounds were detected in the HTL oils [18]. Their concentration in the AP was almost identical to that in the HTL aqueous phase composition as reported by Jena et al. [12]. In our case, the *W + AP-1* medium contained the highest phenol concentration, 0.3 mg L^{-1}, but still too low to expect any kind of inhibitory effect. Hirooka et al. [19] reported that nitrophenols and chlorophenols are known to be toxic to aquatic organisms within the range from 5 to 25 mg L^{-1}. The EC_{50} for *Scenedesmus quadricauda* (similar to our algae) was 184 mg L^{-1} after 24 h exposure [20], a concentration 600 times higher than that in the *W + AP-1* medium.

Table 4
Concentration of various organic compounds in the aqueous phase after HTL and in the medium *W + AP-1* (160× diluted).

Compounds[a]	In pure AP (mg L^{-1})	Initially in *W + AP-1* medium (mg L^{-1})
Residual solvent (CH_2Cl_2)	13000 ± 120	81.3
Acetic acid	3946 ± 550	24.7
Ethanol	359 ± 50	2.24
Acetone	208 ± 25	1.30
Polyols	558 ± 179	3.49
Amines	138 ± 57	0.86
>C_2 fatty acids	236 ± 92	1.48
Aminoacids	152 ± 35	0.95
Cyclic dipeptides	252 ± 83	1.58
Nitrogen aromatic	223 ± 121	1.39
Hydroxyacids	582 ± 191	3.64
Sugars	136 ± 66	0.85
Pyrrolidones[b]	1394 ± 255	8.71
Phenols	58 ± 14	0.36
Dipeptides	3 ± 3	0.02
Not identified	762 ± 352	4.76
Sum[c]	9006	56.3

[a] For abbreviations refer to Table 1.
[b] Pyroglutamic acid as most abundant compound.
[c] Excluding residual solvent.

The water soluble fraction, formed by a large number of constituents, was characterized by different degrees of toxicity or methabolic activity. Some (sugars, carboxylic acid, aminoacids and hydroxyacids) can definitely be considered non-toxic metabolites and potentially be used as carbon and nitrogen sources. Others, in particular nitrogen containing compounds like pyroglutamic acid, cyclic dipeptides and amines, can still be considered degradable or utilizable (upon hydrolysis they are converted to aminoacids), but they are characterized by well known high biological activity. Finally, a considerable amount of "xenobiotics" (e.g. nitrogen containing aromatics) ended up in the water phase, with concentrations that ranged from 0.09% to 0.12%.

Pyroglutamic acid [21] has antifouling activity against algal spore attachment and is one of the most abundant water soluble compounds produced. Cyclic dipeptides are often characterized by biological activity and some are produced by marine microorganisms [22] as protection toward grazing, which can be beneficial in big scale cultivation. On the opposite, cyclo(Pro-Tyr) showed an herbicidal activity and, therefore, its toxicity could be expected at high concentration. Finally, various aromatic heterocycles (e.g. pyridine derivatives) and phenols detected also showed toxicity toward microalgae at relatively low concentration [23,24].

Relatively high concentrations (close to growth inhibition values) of toxic compounds were detected in the HTL-AP. Yet, due to the 160 times dilution factor, toxicity levels were not reached. Possible inhibition phenomena would occur only after many recycles (e.g. about 100 recycles are needed for achieving the IC_{50} for pyroglutamic acid) and in absence of significant bio-degradation of these pollutants. Testing a series of several cycles of cultivation with liquefaction and AP recycling is however recommendable and activities in this direction are already initiated in our laboratories. Furthermore, the high concentration of the residual dichloromethane solvent (widely used in HTL laboratory studies) in the aqueous phase emphasizes the need of replacement for a greener solvent with low water solubility.

Finally, at this points, we excluded several aspects which, in our view, did not limit algal growth: NH_4-N toxicity, differences in N:P ratio and organic substances toxicity (by insufficient AP dilution).

Table 5
Elements present in standard COMBO (*CB*) and in different media configurations containing HTL aqueous phase (AP).

Elements	In pure AP (mg L^{-1})	Initial concentration (mg L^{-1}) in medium[a]				
		CB	*W + AP-1*	*W + AP-2*	*CB + W + AP-1*	*CB + W + AP-2*
Na	334.5 ± 6.5	28.4	2.09	1.08	15.3	14.7
K	1290 ± 120	3.91	8.05	4.15	5.98	4.03
Ca	17.0 ± 0.4	10.0	0.11	0.05	5.06	5.04
Cl	62.0 ± 0.0	86.4	0.39	0.20	43.4	43.3
Mg	29.3 ± 0.1	3.65	0.18	0.09	1.91	1.87
S	83.0 ± 0.0	7.39	0.52	0.27	3.95	3.83

[a] For abbreviations refer to Table 1.

Instead, there are strong indications suggesting that something specific is missing in the AP, which is required to maintain an optimal growth speed. Besides N, P and C, other nutrients that are essential for algae growth are S, Ca, Mg, Na, K and Cl. These nutrients are required in a relatively large amount. Micronutrients include Fe, B, Mn, Cu, Mo, V, Co, Ni, Si and Se [25]. To evaluate that, Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and Ion Chromatography (IC) analyses were performed on the HTL aqueous phase. Table 5 shows the main micronutrients in pure AP, in the various cultures after addition of AP and in standard COMBO.

The lack of almost all these elements (except potassium) in the media with only water and AP became evident. Many of the trace elements are important in enzyme reactions and for the biosynthesis of many compounds, e.g. Co is essential for vitamin B_{12} production [15], which on their turn can be essential cofactors for carboxylase enzymes involved in fatty acid synthesis [26]. Richmond [15] indicated that autoclaving cultivation media (for sterilization) results in a certain degree of vitamins decomposition, which could also happen when performing HTL where more severe conditions are applied. Growth of *Dunaliella tertiolecta* in standard culture medium was compared to that in the same medium without the presence of Fe^{3+}, Co^{2}+, Mn^{2+} or Mo^{2+} by Chen et al. [27]. Algal growth was substantially reduced when iron was eliminated followed closely by elimination of cobalt, magnesium and molybdenum. Magnesium is essential for the synthesis of chlorophyll and photosynthesis. In conclusion, a lack of other essential nutrients (e.g. Mg), other than N and P, seems to be the primary cause of the growth reduction observed.

Considering that *CB* contained 13 mg L^{-1} of NO_3-N and that *CB + W + AP-1* had 6.59 mg L^{-1} of NO_3-N from *CB* and 0.22 mg L^{-1} of NO_3-N plus 5.83 mg L^{-1} of NH_4-N from AP, 50% of the N in *CB* can be replaced by N in AP while having no detrimental effects on growth rate, thus reducing N nutrient costs by half. However, micronutrients should be supplied upon AP recycling and the dilution factor must be that one that supplies just enough N and P for the algae to grow at rates comparable to that in *CB*.

4. Conclusions

Cultivation of *Desmodesmus* sp. was performed to evaluate nutrients recycling via the AP from HTL of the same microalgae. This was done by systematically varying the AP dilution, using only water or a mixture of water and standard medium (*CB*), while keeping the same nitrogen concentration (either as total N or the sum of ammonia and nitrate) as that in standard growth medium. A substantial reduction in growth was observed when using water diluted AP. In contrast, comparable growth as that in standard culture medium was achieved when the mixture W + AP was enriched with COMBO. Based on our results, differences in N:P ratio and toxicity by too high concentrations of NH_4-N and/or organic compounds can be excluded as the cause of growth reduction. Algae in media with nearly equal AP dilution factor (thus same N:P ratio and same concentrations of organics and NH_4-N) exhibited completely different growth rates. Therefore, we have strong indications that the lack of essential (macro-/micro-)nutrients (other than N and P) in AP, enhanced when diluting with water, is the major cause. These nutrients must be supplied upon AP recycling to avoid growth reduction.

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