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Gonzalez-Camejo, J.; Aparicio, S.; Paches Giner, MAV.; Borrás, L.; Seco, A. (2022). Comprehensive assessment of the microalgae-nitrifying bacteria competition in microalgae-based wastewater treatment systems: Relevant factors, evaluation methods and control strategies. Algal Research. 61:1-15. https://doi.org/10.1016/j.algal.2021.102563

The final publication is available at

https://doi.org/10.1016/j.algal.2021.102563

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Additional Information

Comprehensive assessment of the microalgae-nitrifying bacteria competition in microalgae-based wastewater treatment systems: relevant factors, evaluation methods and control strategies

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Abstract

Due to their capacity to assimilate carbon dioxide and nutrients, microalgae-based cultivation systems have emerged as a green solution for intensive wastewater treatment. However, when large concentrations of ammonium are present the competition between microalgae and ammonium-oxidising bacteria (AOB) plays a significant role. Microalgae (MA) use ammonium to synthesise proteins, photosynthetic pigments and nucleic acids, while AOB use it as a source of electrons and oxidise it to nitrite.

Several authors have studied the isolated factors that influence microalgae-nitrifying bacteria competition, although a comprehensive analysis of this interesting topic is still lacking. This review makes an overall assessment of the competition between microalgae and AOB for ammonium uptake, focusing on: I) factors that influence the competition; II) methods of measuring the activity and concentrations of microalgae and

nitrifying bacteria; III) useful strategies to control nitrification to improve the performance of microalgae-based wastewater treatment systems.

Keywords: ammonium; competition; microalgae; nitrification; wastewater

1. Introduction

Microalgae (MA) cultivation is attracting increasing interest as intensive green tertiary wastewater treatment from the scientific community all over the world [1–10]. Phototrophic microalgae can remove large amounts of nutrients from the medium while fixing carbon dioxide to produce microalgae biomass, which can then be used to obtain by-products such as biofuels and fertilisers [11–14]. Recent studies have focused on the design of different microalgae cultivation technologies, the different wastewater streams to be treated by microalgae and the parameters affecting microalgae performance [4,8,15–20]. However, when microalgae are cultivated outdoors, non-aseptic conditions trigger the proliferation of other microorganisms such as heterotrophic bacteria, protozoa, intrusive microalgae, fungi, etc., competing with microalgae for nutrient assimilation [1,21–26]. With wastewater from anaerobic processes such as digestates, centrates and effluents from anaerobic membrane bioreactor (AnMBR) systems or aerobic secondary effluents, heterotrophic bacteria activity is usually neglected as their organic matter content is mostly recalcitrant to biodegradation [11]. As ammonium (NH_4) is usually present in these wastewater streams [27,28], the competition between microalgae and autotrophic nitrifying bacteria (specifically ammonium oxidising bacteria (AOB)) for ammonium uptake plays a significant role [29–31]. There are several ways of dealing with microalgae-nitrifying bacteria cultivation systems to treat high ammonium-loaded wastewater streams, depending on the final goal of the process:

i) Maximising microalgae activity to improve nitrogen and phosphorus removal, thus reducing the nutrient concentration of the effluent common in wastewaters with middle or low ammonium concentrations, i.e. 20-80 mg $N \cdot L^{-1}$ [32].

ii) Maximising microalgae activity to increase by-products produced by microalgae biomass [33].

iii) Favouring nitrification by the oxygen production of microalgae to reduce the ammonium load, which in turn reduces system aeration requirements [28,34,35].

One or other organism will be more active, according to the goal of the mixed microalgae-nitrifying bacteria system.

Although many studies analyse the factors which affect microalgae (such as temperature, light, nutrient concentration, dilution rate, etc.) [7,8,36,37], their influence on the competition between microalgae and AOB for ammonium uptake is often overlooked, given little attention, or is evaluated by only considering one of the isolated factors. For instance, Choi et al. [38] reported that microalgae growth was favoured over AOB under appropriate lighting and nutrient-replete conditions, while González-Camejo et al. [39] suggested that high temperature peaks could make AOB growth surpass microalgae activity. Other authors have studied the metabolic routes of microalgae [36,40] and nitrifying bacteria [41] separately. However, a comprehensive analysis of this competition is still lacking.

It should be noted that nitrification can be the main ammonium removal pathway in microalgae cultivation systems, even if there is much less AOB biomass than microalgae. Methods of measuring the activity and the abundance of both microalgae and nitrifying bacteria are thus essential to monitor culture dynamics during continuous operation.

This review makes an overall assessment of the competition between microalgae and AOB for ammonium uptake in suspended cultures, focusing on: I) factors that influence MA-AOB competition; II) methods of measuring the concentration and activity of both microalgae and nitrifying bacteria within the mixed culture; III) nitrification control strategies. This is expected to be useful to wastewater treatment systems based on MA-AOB cultures as it could help to improve the process performance by maximising/minimising microalgae activity and/or nitrification.

2. MA-AOB competition for ammonium uptake

Microalgae use ammonium for the synthesis of proteins, photosynthetic pigments and nucleic acids [13,42]. On the other hand, nitrifying bacteria mainly use NH_4 as a source of electrons in the nitrification process, producing nitrite (NO_2) and nitrate (NO_3) (Figure 1). Hence, in high NH_4-loaded wastewater treatment systems based on microalgae cultivation, an equilibrium often exists between microalgae and AOB [39,45]. However, if ammonium is not present in excess, microalgae and AOB reduce the NH_4 concentration of the culture, limiting their activities [46].

[FIGURE 1 NEAR HERE]

As can be seen in Table 1, cultures with higher nitrification usually reach lower microalgae performance in terms of nitrogen removal rate or nitrogen removal efficiency. For instance, González-Camejo et al. [47], who treated AnMBR effluent in an outdoor membrane photobioreactor (MPBR) system, obtained a nitrogen removal rate (NRR) and nitrogen removal efficiency (NRE) of 19.7 mg $N{\cdot}L^{-1}{\cdot}d^{-1}$ and 59.8%, respectively, when nitrification only accounted for 34.6% of the influent ammonium, while they decreased to 14.5 mg $N{\cdot}L^{-1}{\cdot}d^{-1}$ and 32.6%, respectively, when nitrification rose to 57.2% of the influent NH_4 concentration. This suggests partial microalgae

limitation due to an increasing nitrification rate. Under lab-conditions, Rada-Ariza et al. [45] reported an NRR of 5.4 mg $N\cdot L^{-1}\cdot d^{-1}$ when nitrification achieved 40% of total NH_4 removed, while it dropped to 1.6 mg $N\cdot L^{-1}\cdot d^{-1}$ when nitrification accounted for 66% of the total NH_4 removed. The factors that influence microalgae activity and nitrification thus need further study.

[TABLE 1 NEAR HERE]

Microalgae can also have a negative impact on nitrifiers [35,38,50]. Although some authors found no significant influence of nitrification on microalgae activity [34], others obtained symbiotic benefits between microalgae and nitrifying organisms [44,51]. MA-AOB competition must thus be analysed in depth to fully understand the behaviour of microalgae-based wastewater treatment system.

3. Factors influencing microalgae-nitrifying bacteria cultivation

Different medium characteristics and environmental and operational conditions can greatly affect microalgae or nitrifying bacteria, and shift their equilibrium [31,52]. The most important factors related to both microalgae and nitrifying bacteria activity are depicted in Figure 2 and discussed below. In this study, the word "microalgae" is considered to refer to eukaryotic microalgae. The most common indigenous wastewater microalgae are *Chlorella, Scenedesmus, Monoraphium, Chlamydomonas,* etc, [52,53].

[FIGURE 2 NEAR HERE]

3.1. Temperature

Temperature is a key factor in the rate of enzymatic processes [54,55]. Microalgae growth increases with temperature until they reach the optimum, which is strain-specific [56]. *Scenedesmus* and *Chlorella* usually have growth rates in the range of 0.3-0.9 d^{-1} [32,57–59]. However, when temperature is higher than the optimum value even by only

2-4ºC, microalgae growth can fall drastically [60]. In the case of a mixed microalgae culture dominated by *Chlorella*, González-Camejo et al. [39] reported that microalgae viability fell significantly when temperature was over 30 ºC.

The AOB growth rate sharply increases at higher temperatures. In this respect, AOB growth has been reported to be 0.9 d^{-1} at 20 ºC but reaches 2.9 d^{-1} at 30 ºC [61]. Temperature peaks can thus have a noteworthy influence on MA-AOB competition. If high temperatures are maintained, nitrification will increase steadily, and nitrifying bacteria will outcompete the microalgae, while if temperature drops, nitrification will fall and microalgae activity could be able to recover [39,62].

3.2. Light

The importance of light irradiance on the MA-AOB competition is mainly due to microalgae as it is probably the main factor involved in phototrophic growth [63]. In fact, when a culture is light-irradiated under proper conditions, microalgae are usually the dominant organism in MA-AOB competition [64]. However, in an open or closed photobioreactor (PBR), light intensity is exponentially reduced with PBR depth due to the scattering of the culture biomass, the inorganic matter and the microalgae pigments [65,66]. This light attenuation is indirectly measured by the extinction coefficient (K_a) [67], together with other factors that affect light availability, like microalgae adaptation to light changes or the microalgae cells´ age [33]. The Lambert-Beer Law (Eq. S1) is normally used to calculate the average light irradiance ($I_{av,}$), which represents the average irradiance to which the microalgae culture is exposed inside the PBR [67,68]. I_{av} (better than total irradiance) is thus the factor which determines microalgae activity.

Although nitrifying bacteria do not need light for their metabolism, excessive light irradiance can affect them negatively [41,50]. For instance, Akizuki et al. [29] reported nitrification inhibition at incident light intensities over 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$, while Merbt et

al. [69] observed photoinhibition of AOB *Nitrosomonas europaea* and *Nitrosospira multiformis* at light irradiance of 500 μmol·m^{-2}·s^{-1}. Meng et al. [70] found inhibition of *Nitrospiraceae* (nitrite oxidising bacteria (NOB)) under incident light irradiances over 180 μmol·m^{-2}·s^{-1}. In fact, NOB are usually more sensitive to light than AOB [71,72], implying that photoinhibition can lead to partial nitrification, i.e. accumulation of nitrite due to the inhibition of the second step of nitrification [70].

3.3. Nitrogen concentration

Of all the nitrogen sources that microalgae can assimilate, NH_4, NO_2 and NO_3 are the only ones involved in the nitrification process. The distribution of these nitrogen species in the wastewater is highly relevant in the MA-AOB interaction [24].

3.3.1. Ammonium/free ammonia nitrogen (NH_4/FAN)

Ammonium is the preferred nitrogen species for microalgae, since its uptake needs less energy than other sources [55,73]. In this respect, González-Camejo et al. [39] reported ammonium uptake rates of microalgae up to 15-fold higher than nitrite and nitrate uptake rates in lab-scale PBRs.

The influence of NH_4 is analogous in both microalgae and AOB and their growth rate is limited with low ammonium availability. In the case of microalgae, ammonium concentrations under 10 mg N·L^{-1} have been found to significantly affect their growth [53,74]. However, AOB can support NH_4 scarcity more efficiently than microalgae. According to Reichert et al. [75], the semi-saturation constant of AOB growth with respect to ammonium is only 0.5 mg N·L^{-1}. This suggests that under low ammonium concentrations, AOB can induce microalgae growth limitation [8].

It should also be noted that ammonium is in equilibrium with free ammonia nitrogen (FAN), which is toxic to both microalgae and AOB [12,30,76]. This equilibrium favours FAN at high nitrogen concentrations, temperature and pH (over 9) (see Eq. S2).

For this, ammonium-rich wastewater streams such as centrates, which can contain up to 1000 mg $N\text{-}NH_4 \cdot L^{-1}$, have often to be diluted prior to being added to microalgae-nitrifying bacteria cultures [1,58], This way, not only ammonia but also other toxic substances are diluted [51]. FAN has been reported to inhibit NOB more than AOB [14]. In fact, the toxic FAN concentration for NOB has been found to be in the range of 0.1-3 mg $N \cdot L^{-1}$, while AOB can support ammonia concentrations over 10 mg $N \cdot L^{-1}$ [77].

3.3.2. Nitrite/free nitrous acid (NO_2/FNA)

Since nitrite is the product of the first step of nitrification and a substrate for NOB, if NOB are more affected by any biotic or abiotic factor other than AOB, NO_2 can be expected to accumulate [76]. Nitrite can act as a nitrogen source for microalgae [72], although it can also have negative effects on photosynthesis. For instance, González-Camejo et al. [47] reported a decay in the performance of a mixed microalgae culture dominated by *Chlorella* when nitrite concentration was 5-20 mg $N \cdot L^{-1}$, and Yang et al. [78] found reduced growth of green microalgae *Botryococcus braunii* at 70 mg $N \cdot L^{-1}$. It is assumed that this effect is in fact due to an accumulation of free nitrous acid (FNA), which is in acid-base equilibrium with nitrite. FNA has also been found to inhibit nitrifying bacteria at concentrations as low as 200 µg $N \cdot L^{-1}$ and 30 µg $N \cdot L^{-1}$ for AOB and NOB, respectively [79,80].

3.3.3. Nitrate (NO_3)

Nitrate, the final product of nitrification, is usually more innocuous to aquatic life than ammonium and nitrite [30,76]. If residual nitrogen is emitted from a MA-AOB cultivation system, it will be less problematical if the nitrogen is in the form of nitrate. However, microalgae assimilate NO_3 less efficiently than ammonium, as they have to reduce it internally by the action of the nitrate and nitrite reductase enzymes [8,14,73].

3.4. pH

pH affects the MA-AOB culture in two aspects: I) it regulates their metabolic pathways; microalgae present optimum pH at around 7-8 [81,82], while the optimum pH range for AOB and NOB has been reported to be 7.4-7.8 [83] and 7.5-9.95 [84], respectively; II) pH is related to the acid-base equilibrium of the medium [11], so that it is related to the production of FAN and FNA (Section 3.3). It should be remembered that pH not only affects the biological process, but also microalgae autotrophic activity entails pH rises [81] while nitrification reduces pH due to a drop in culture alkalinity [85].

3.5. SRT/HRT/dilution rate

Solids retention time (SRT) and hydraulic retention time (HRT) are essential operating parameters in continuous and semi-continuous operations as they can be used to control factors related MA-AOB systems [34,86]. In cultivation systems with no biomass retention, HRT (or dilution rate as the inverse of HRT) controls the biomass concentration and nutrient loads to the culture. On the other hand, when HRT and SRT are decoupled (for instance, in MPBR systems [32] or in raceway reactors coupled to membrane filtration [87]), SRT appears as the key parameter in the control of biomass concentration to improve light availability and avoid washout [88–90] while HRT is responsible for the nutrient load to the system, thus being a key factor in the nutrient removal efficiency of the system [46,91].

It must be noted that shorter SRTs boost the growth of the fastest microorganisms [92], which can favour bacteria growth with respect to microalgae [47].

3.6. Oxygen concentration

In a microalgae-nitrifying bacteria culture, oxygen is mainly produced from microalgae photosynthesis. Indeed, oxygen concentration is often used as an indirect measurement of microalgae performance [93]. Oxygen is needed to carry out nitrification [24].

Depending on the microalgae activity within the culture, there will be four different situations [29]: I) low microalgae activity, which produces insufficient oxygen to carry out nitrification [93]; II) enough oxygen to develop the first step of nitrification (via nitrite); III) sufficient oxygen to carry out full nitrification (via nitrate); i.e. over 2 $mg \cdot L^{-1}$; IV) production of oversaturated dissolved oxygen, i.e. over 250% (around 20-25 $mg \cdot L^{-1}$), which can reduce microalgae performance [94].

3.7. Soluble microbial products (SMP)/Extracellular polymeric substances (EPS)

Soluble microbial products (SMP) and extracellular polymeric substances (EPS) include proteins, polysaccharides, nucleic acids and are a result of microalgae and bacteria activity [95,96]. It has been reported that stressing factors such as extreme temperatures and nutrient limitation can increase the release of SMP/EPS to the culture [97]. However, microalgae do not usually take advantage of this organic matter since most microalgae species tend to grow autotrophically under lighting [2,15]. On the other hand, excessive SMP/EPS in microalgae-bacteria cultures tends to favour the growth of competing organisms such as heterotrophic bacteria and grazers, reducing microalgae performance [98]. It can also reduce nitrifying bacteria activity due to oxygen depletion [24].

4. Measuring culture activity

The microbial state of the culture will be defined by the activity of both microalgae and nitrifying bacteria. The most active microorganism will dominate the competition, while they will be in equilibrium if they show similar activity. Several parameters are often needed to properly assess the overall activity since some of these parameters are based on analysing microalgae while others focus on nitrifying bacteria. Figure 3 gives a

summary of the methods of measuring microalgae and nitrifying bacteria activity/concentration.

[FIGURE 3 NEAR HERE]

4.1. Nutrient removal

The main goal of microalgae-based wastewater treatment systems is to reduce the nitrogen and phosphorus loads of the influent. This nutrient removal mainly occurs due to: I) microalgae growth; II) nitrifying bacteria growth; III) nitrogen losses due to ammonia stripping; and iv) phosphorus precipitation. During nitrification, nitrogen is not removed from the system, only changes its oxidation state. Other mechanisms of removing nutrients like phosphorus adsorption in the culture biomass or volatilisation of N_2/N_2O are often negligible [99].

There are two different approaches to evaluate nutrient removal: nutrient removal efficiency and nutrient removal rate. The former considers the percentage of nutrient concentration removed from the influent (Eq. S3), while the latter measures the rate at which nutrients are removed from the system (Eq. S4).

4.2. Biomass productivity

Biomass productivity is the culture biomass produced and taken out of the PBRs [100] and can be calculated by Eq. S5. It is usually related to nutrient removal [101] so that it is a useful parameter to assess wastewater treatment processes. It also appears an essential factor in microalgae cultures designed to obtain by-products [13].

4.3. Photosynthetic efficiency

As photosynthetic efficiency measures the capacity of microalgae to use the light applied to the PBRs (Eq. S6), it is a useful parameter to evaluate photolimitation or photoinhibition processes [102]. It is also a relevant parameter in evaluating microalgae production and designing PBRs [103]. Although it gives no direct information on the

activity of nitrifying bacteria, a decay in photosynthetic efficiency can be due to bacteria proliferation.

4.4. Respirometry

Respirometry is a simple and cheap method based on dissolved oxygen (DO) profiles [85,96]. In the case of microalgae-nitrifying bacteria cultures, it consists of batch tests in which light and dark (L/D) phases alternate to obtain the oxygen production rate (OPR) or the oxygen uptake rate (OUR). The L/D phases can be repeated several times during the test to obtain replicates of the OPR and OUR values. Nutrients can be added as substrate to obtain maximum activity while substances like allylthiourea (ATU) can be injected to inhibit AOB [104]. The oxygen profile is the result of several processes: I) OPR by microalgae; II) OUR by nitrification; III) OUR by microalgae respiration; IV) and OUR by heterotrophic bacteria respiration [105,106]. The gross OPR/OUR value differ according to the weight of each component and gives valuable information on the activity of the different organisms in the culture [107].

Although many respirometric protocols have been reported in the literature (Table 2), the results are usually hard to compare [85]. To overcome this issue, Rossi et al. [104] stated that the following data should be reported to make these protocols comparable:

i. Microalgae cultivation system: environmental and operational conditions, composition of the microbial community and culture characteristics.
ii. Respirometric procedure: initial culture concentration, nutrient sources and their concentrations, setpoints for temperature, pH and dissolved oxygen, light intensity, test procedure (protocol and duration of light and dark phases) and processed OPR and OUR data.

The main advantages of respirometry are its: I) low-cost; II) possibility to obtain both OPR/OUR in the same test; III) it can measure specific kinetic parameters; and IV)

good test reproducibility. However it also has some drawbacks: I) off-site test that cannot be carried out in-situ nor continuously monitored; II) it usually includes an acclimatation phase that takes time; III) it can be affected by oxygen mass transfer from the atmosphere; and IV) it sometimes has to be supplied with external aeration or pH control [85,104].

[TABLE 2 NEAR HERE]

4.5. Chlorophyll fluorescence parameters

Chlorophyll fluorescence parameters, i.e. parameters that evaluate variations in the photosystem II (PS II) photochemistry and linear photon flux [112], indirectly measure the adaptability of microalgae to certain environmental and operating conditions [104]. Maximum PS II quantum efficiency (F_v/F_m) is the most frequently used fluorescence parameter and represents the maximum efficiency at which light absorbed by PS II is used for the reduction of the primary quinone electron acceptor (Q_A). In this respect, Sánchez-Zurano et al. [113] suggested that F_v/F_m values below 0.6 indicated photoinhibition. However, Baker [114] found issues related to the accuracy of this value and its relation to the rates of linear CO_2 assimilation. These parameters give no direct information on nitrification. In fact, González-Camejo et al. [32] did not find any statistically significant relationship between F_v/F_m and microalgae-bacteria performance in the continuous operation of a mixed MA-AOB culture.

4.6. pH dynamics

pH data can monitor online the activity of mixed microalgae-bacteria cultures due to the pH variations caused by their metabolic activity. Robles et al. [115] used pH sensors to describe the start-up phase of an outdoor raceway pond treating urban wastewater, while González-Camejo et al. [116] reported a good correlation between pH dynamics and performance of an outdoor MPBR plant treating AnMBR effluent.

4.7. Nitrite and nitrate concentrations

Some authors have used nitrite and nitrate concentrations as an indirect measure of nitrifying bacteria activity [29,48]. However, these parameters cannot be used to directly evaluate nitrification if NO_2/NO_3 are present in the influent in relevant concentrations, since some of the nitrite and nitrate present in the culture would not be due to nitrification but to the influent in which case nitrification rate (Section 4.8) would be a better indicator of nitrifying bacteria activity.

4.8. Nitrification rate

Nitrification rate measures the production of nitrite and nitrate during nitrification (Eq. S7). Microalgae can assimilate nitrite and nitrate simultaneously with ammonium [39] although the amount assimilated by microalgae cannot be assessed by the nitrification rate. When microalgae absorb relevant amounts of nitrite and nitrate, the evaluation of nitrifying bacteria activity should thus be complemented by other methods such as respirometry (Section 4.4).

5. Measuring culture concentration

The culture biomass concentration also gives information on MA-AOB competition, as microalgae and bacteria concentrations are closely related to biomass productivity and nutrient removal rates [52,86]. In microalgae-bacteria cultures, microalgae are usually the dominant organism mainly due to their larger cell size. In this respect, Luo et al. [90] reported only 0.2-3.5% of bacteria in a lab-scale microalgae cultivation system. For this reason, some methods of assessing culture biomass concentration consider the whole biomass as if it were only composed of microalgae, so that several methods should be used to measure the distribution of microalgae and bacteria and evaluate the competition between these microorganisms [22].

5.1. Biomass dry weight

The common method of measuring biomass concentration is by quantifying the total (TSS) or volatile suspended solids (VSS) concentration. This is a low-cost method, although time-consuming: at least 2 hours for TSS and around 24 hours for VSS [22].

This measure will include not only the mass of microalgae and bacteria but also any other compound present in the culture such as cell debris and SMP/EPS as it cannot distinguish between them [52,106].

5.2. Chlorophyll concentration

Chlorophyll concentration has traditionally been used as an indirect measure of microalgae concentration [64]. Although it gives no information on bacteria [104], the relation between chlorophyll and total biomass (mg Chla·mg VSS^{-1}) can serve as a proxy for the microalgae-bacteria ratio [22].

The trichromatic method can be used to measure chlorophyll and consists of extracting the chlorophyll from the culture biomass by solvents such as acetone. The solution composed of the extracted chlorophyll and the solvent is then measured by spectrophotometer analysis [117]. Chlorophyll a concentration can then be calculated by applying Jeffrey and Humphrey's Equation (Eq.S8), being a simple and inexpensive method [22]. However, chlorophyll concentration as a proxy for microalgae biomass content is somewhat controversial since microalgae can vary their intracellular chlorophyll content with the lighting conditions, showing higher chlorophyll a content when microalgae are light-limited to take full advantage of light irradiance [118]. Other limitations of chlorophyll analysis lie in the incomplete efficiency of chlorophyll extraction by the solvent and the presence of molecules that can interfere with the spectrophotometric analysis. Similar limitations are present in measuring the concentration of other pigments such as carotenoids and phycocyanin [22].

5.3. Optical density

Optical density (OD) is based on the linear relationship between light absorbance and particle concentration [22]. It is often used as it is fast and cheap, only requires a small sample and can be monitored online [119]. Selecting the wavelength to be measured is a critical point and should minimise absorbance from the medium, thus reducing background noise. Wavelengths in the range of 600-700 nm are usually applied in bacterial cultures [120]. In those dominated by green microalgae, the most frequently used wavelength is 680 nm, as it coincides with the chlorophyll absorption peak [121,122]. However, some authors have employed other wavelengths to measure biomass concentration in microalgae-based systems (Table 3).

The calibration curve which relates OD and biomass concentration varies throughout the experimental period and is a drawback of using optical density, due to changes in the microalgae pigment content, the dominant microalgae species and the proportion of microalgae and bacteria. This can cause serious errors when measuring biomass concentration [22].

[TABLE 3 NEAR HERE]

5.4. Cell counting

Cell counting under a microscope is another common method of measuring microalgae biomass. It can quantify not only the total microalgae concentration but also distinguish between genera or species on a morphological basis (Figure 4), although it gives no information on bacteria identity [104]. A minimum of 100 microalgae cells of the most abundant genera and at least 300 total microalgae cells need to be counted to achieve less than a 20% error [127]. After counting, microalgae concentration can be correlated with dry weight as long as microalgae are the dominant organism. However, this linear

relationship will not always occur as the method does not consider microalgae cell size, which can vary significantly [22].

With large microalgae colonies or flocs in the sample, serious errors can occur in counting due to the difficulty of counting inside a floc and the lack of random cell distribution in the area counted [22].

[FIGURE 4 NEAR HERE]

5.5. Autofluorescent pigments, fluorescent probes and dyes

Information on some of the most frequently used fluorophores for bacterial cells is given in the Supplementary Data. Fluorescent dyes are not needed for microalgae cells due to the autofluorescence provided by their photosynthetic pigments. Microalgae emit red fluorescence (λ_{em} = 670 nm) when the cells are illuminated by a blue-light excitation filter (λ_{ex}: 460–490 nm), as seen in Figure 5. Although the fluorescent dyes included mainly focus on the detection of total bacterial cells, SYTOX Green and fluorescein diacetate (FDA) have also been used to detect non-viable cells. The simultaneous detection of live and dead microalgae or bacterial cells is therefore possible by applying double fluorescent staining.

Propidium iode (PI) stain is also widely used for cell viability staining [128,129]. However, the maximum emission wavelength is in the same range as microalgae photosynthetic pigments, so that the two signals overlap. On the other hand, SYTOX green only penetrates non-viable cells and does not overlap with photosynthetic pigments [130], so that red autofluorescence and SYTOX green fluorescence can be used as markers for viable and non-viable microalgal cells, respectively (Figure 5B).

Despite the variety of dyes, it is not possible to classify taxonomically bacteria by the generic fluorescent dyes described in the Supplementary Data. The fluorescence in-situ hybridization (FISH) microscopic technique is widely used to identify different

taxonomic groups of bacteria using probes with different non-overlapping fluorochromes [22]. The main FISH probes used to detect the bacterial domain (specifically AOB) are also compiled in the Supplementary Data. This method was used by González-Camejo et al. [131] and Mantovani et al. [28] to detect AOB diversity in mixed microalgae cultures. However, FISH is not normally used to identify photosynthetic organisms due to signal interference with autofluorescence given by the microalgae photosynthetic pigments. Hosoi-Tanabe and Sako [132,133] designed ribosomal RNA-based probes for the detection of phytoplanktonic organisms, specifically dinoflagellates.

[FIGURE 5 NEAR HERE]

Target organisms in stained samples are quantified by epifluorescence microscopy with an image acquisition system and image processing, although this is a time-consuming technique [134].

5.6. Flow Cytometry

5.6.1. Conventional Flow Cytometry

Flow cytometry (FCM) is a powerful tool for single-cell analysis that can provide a rapid, direct and precise quantification of cells in samples containing heterogeneous microbial populations [22]. It can count more than 1000 cells (or events)$\cdot s^{-1}$ directly in the sample by using an automated fluid-optical system [135]. In conventional cytometers, cells suspended in a liquid sample are aspirated and swiped in front of an illumination source with the subsequent detection of light scatter (forward scatter (FSC); side scatter (SSC)) and the fluorescence responses of cells in the sample. Cell or event classification is performed according to their size (FSC), internal complexity (SSC) and through fluorescence emissions (given by cell staining or autofluorescence). The data obtained for analysing the cell or event are plotted from empirical correlations

among FSC, SSC and fluorescence and two dimensional cytograms. Due to the sample complexity, these correlations should be previously determined by a calibration procedure using standard samples as well as negative and positive controls. By way of example, Foladori et al. [136], Luo et al. [137] and Petrini et al. [85] combined FCM with fluorescent molecular probes for rapid quantification of microalgal and bacterial cells and to assess their physiological state for wastewater treatment purposes.

The main limitation of FCM is given by cell aggregation and multicellular organisms. Bacteria cells can live attached to microalgal [138] and some microalgae genera such as *Scenedesmus* or *Coelastrum* can develop a coenobia structure, thus underestimating the analysed populations. Non-ionic surfactants [139] and sonication [140] are also feasible options to pre-treat the samples and disperse cell aggregates.

Conventional flow cytometer also features Fluorescence Activated Cell Sorting (FACS). This is a powerful tool for the rapid identification, separation and recovery of targeted cells from a heterogeneous cell suspension, based on the signal detected [141]. It is widely used for microalgae strain selection and further sequencing of microalgae [142] and combined with FISH, AOB cells can be identified, stored and isolated [141].

5.6.2. Imaging Flow Cytometry (IFC)

Imaging Flow Cytometry (IFC) combines single-cell identification and high FCM throughput with cellular image acquisition of fluorescence microscopy [143]. The images captured can be visualised and analysed by an image processor. IFC is recommended for analysing samples containing cellular aggregates and multicellular organisms, since the true nature of every event read can be verified from the 2D images obtained. IFC has been used to determine the relative abundance of microalgae species; to analyse microalgae cell size; to determine their viability and metabolic activity; and to define different life stages of microalgae [144,145]. Despite all its advantages, the

taxonomic resolution provided by image acquisition is relatively low. In addition, to assess different microalgae cell sizes it is necessary to use a combination of magnification objectives and working modes [144,146]. IFC is not used to quantify free bacteria such as AOB in wastewater as it is complicated and provides no significant information other than that obtained by FCM [146].

5.7. Sequencing-based techniques

5.7.1. Polymerase Chain Reaction (PCR)

Molecular analyses are the most sensitive and specific method for microorganism identification. They are based on targeted sequencing of amplified gene regions or "amplicon sequencing", i.e. they consist of Polymerase Chain Reaction (PCR) amplification of a target gene, followed by short-read sequencing of the obtained amplicon. The PCR technique has been widely used to identify bacteria and archaea [147,148].

5.7.2. Quantitative real time PCR (qPCR)

The microbial community can be classified and detected by PCR, but not quantified. Quantitative real time PCR (qPCR) is a DNA amplification and quantification technique using specific fluorescent dyes to combine PCR amplification and detection stages, allowing the quantification of the concentration of genetic material in samples [148]. qPCR measures the DNA produced through the increase in the fluorescent signal after fluorescent staining.

5.7.3. Next Generation Sequencing (NGS)

PCR and qPCR require previous knowledge of the organisms of interest and provide little information on the general dynamics of the microbial community, while the emergence of NGS helps to understand complex microbial communities. NGS involves

a variety of high-throughput nucleic acid sequencing technique which can retrieve millions of DNA or RNA sequences from environmental samples [149].

5.7.4. Application of sequencing-based techniques

Most studies of AOB populations target the 16S rRNA gene as amplicon sequencing, as it is a universal gene for bacteria and archaea and can determine the taxonomy or phylogeny of microbial population members. The 16S rRNA gene is about 1500-bases long and has relatively conserved regions and nine variable regions (from V1 to V9) which range from 50 to 100 bp in length [150]. Analyses of AOB populations have amplified segments of the following variable regions: V2 [151,152], V3-V4 [153,154], V6 [155] and V7-V8 [156] with the primers listed in the Supplementary Data. Although the 16S rRNA gene has been widely used to study AOB at the phylogenetic level, the amoA gene has also been applied as a molecular marker for AOB organisms through the primers described by Rotthauwe et al. [157] and compiled in the Supplementary Data. The amoA gene encoding the alpha-subunit of ammonia monooxygenase, which is the metalloenzyme responsible for catalysing ammonia oxidation to hydroxylamine [158]. This gene is thus involved in the first step of nitrification and has been found only in organisms capable of oxidising ammonia, such as AOB.

Unlike AOB, no universal standard DNA sequence has been found for microalgae identification and quantification. Intensive research on microalgae identification is currently focused on the search for molecular markers that can be used to successfully identify and/or quantify a wide spectrum of microalgae. However, a universal sequence to establish a single sequence DNA barcode system is apparently not possible. However, in the case of specific taxonomic groups with different phylogenetic positions, the identification of microalgal populations would be performed with different sets of target sequences [159].

Most molecular research on microalgae has used a rather limited set of markers, such as 18S rDNA, rbcL (large subunit of plasma Rubisco), internal transcribed spacers (ITS) or plastid elongation factor (tufA) [160,161].

Fawley and Fawley (2020) recommended starting with sequencing of the 18S rDNA gene for identifying the higher taxonomic groups such as order or class because the available sequences set for this gene is a very large database. The primers selected should include the most variable region of the target gene such as the V4 and V9 region of the 18S rRNA [163].

In case of microalgae genus (or family), more specific primers based on specific protein-coding genes can be selected for species-level identification [164–166].

As the gene region between the large (rbcL spacer) and small (rbcS spacer) subunits of RuBisCO is not variable enough to identify all strains [167]. Chase et al. [168] proposed that the rbcL marker be combined with others. ITS1 and ITS2 markers are sufficiently variable to differentiate algal strains within species [164,167,169]. However, few ITS sequences are available in the databases. The tufA marker appears to be a promising specific marker for class-level classification [160].

At present there is no general classification technique that identifies all microalgal species, so that when a single area of the genetic material is not enough to address questions related to a specific group, it is recommended to combine two or more regions. For this it is necessary to choose the appropriate marker set, based not only on the genetic divergence of the group and the existence of reference sequences in databases, but also on the previous experience of other researchers to facilitate the selection of the appropriate markers for each group. The most commonly used markers (and their combinations) to identify indigenous microalgae from WWTPs are listed in the Supplementary Data.

5.8. Simultaneous measurement of microalgae and AOB concentration

The most promising methods of accurately determining the proportion of microalgae and AOB in a sample are: I) the combination of FISH and autofluorescence of microalgae; II) the combination of FISH and flow cytometry; III) sequencing-based techniques and; IV) respirometries (Section 4.4).

FISH and autofluorescence of microalgae: by combining FISH probe fluorescence for AOB and microalgae autofluorescence, the proportion of AOB (%AOB) and microalgae (%MA) in a sample can be estimated. An image processing system can be used to determine the area occupied by photosynthetic organisms, AOB and the rest of the bacteria (determined by *Eubacteria* FISH probes [170]). This method measures the proportion of AOB and MA in terms of area, which is supposed to be equivalent to the volume and biomass of the populations.

FISH technique and flow cytometry: by combining fluorescence signals and FSC and SSC measurements, the proportion of microalgae and AOB in a biological sample can be quantified. The technique can discriminate not only by colour but also by size and complexity [114], although there are some technical limitations. The main drawback is that to differentiate between both populations the flow cytometer must be calibrated with standard samples (usually pure cultures of the target organism). In samples with high biodiversity, calibration of the flow cytometer is complex, time-consuming and depends on the sample to be analysed in each case. Accurate quantification by cytometry requires the complete disaggregation of the samples (as the cytometer counts events) and there may be a high degree of bias if the aggregates are estimated as single cells instead of a group of cells. In biological samples inorganic compounds (e.g. debris) can interfere with the signal of the instrument. The results of this technique are

given in an event (hopefully cells) basis instead of cell basis, which could be controversial when applying this data directly to mathematical models.

Sequencing-based techniques: The results obtained from metagenomic techniques should be interpreted with caution, since the measured genetic material is not usually correlated to the real sample concentration. The main factors that influence the quantitative results are: the efficiency of the sample extraction process; the kind of primers used for DNA amplification; the different ratios between initial DNA concentration and final concentration after amplification, the sequences available in databases; and the algorithms used for data processing [22]. Depending on the target population, it is recommended to use a different extraction kit, introducing more uncertainty in the biological taxa comparison. The sequencing of microalgae and AOB is carried out separately, i.e., one sample is sequenced to obtain the information related to the Bacterial domain and another sample for the Eukaryotic domain. The results obtained are the relative abundance of AOB compared to bacteria abundance and the relative abundance of microalgae and compared to eukaryotic organisms abundance [171]. The results obtained are thus not comparable and at present it is not possible to determine the ratio of AOB to microalgae in a sample. The main drawback is that these techniques quantify gene copies instead of organisms. As the number of gene copies varies between genes and organisms it is hard to correlate the obtained results with the real number of organisms present in the sample.

Respirometries: The main advantage of this technique is that it is the only one that measures activity instead of the number of microorganisms, which avoids the bias due to the presence of non-active AOB or microalgae in the sample. The results can be applied directly in mathematical models.

6. Nitrification control strategies

Microalgae normally outcompete AOB due to their greater capacity to uptake nitrogen [172]. However, nitrifying bacteria can proliferate faster than microalgae depending on operational and environmental conditions [107]. In fact, nitrifiers can surpass microalgae as the dominant microorganism in the competence. In some cases microalgae can even collapse [39], so that nitrification has to be controlled. Other options can minimise nitrifying bacteria growth, hence minimising the decay in microalgae performance.

6.1. Temperature control

As commented in Section 3.1, temperature is essential in regulating nitrification. González-Camejo et al. [39] reported that microalgae-nitrifying bacteria remained in equilibrium in flat-panel PBRs when temperature was around 20 ºC, but nitrification was favoured when peak temperatures went over 30 ºC. When cultivating microalgae-bacteria cultures in open ponds the temperatures are usually regulated by evaporation. However, closed PBRs can reach temperatures 10 ºC higher than their surroundings [173]. Including cooling systems [39,60] can reduce temperature in closed PBRs although it involves a drastic increase in the process costs.

6.2. SRT/HRT control

As explained in Section 3.6, SRT (or HRT) plays a significant role in MA-AOB competition. In terms of process efficiency, SRT (or HRT) tends to be operated for as short as possible to reduce costs. However, a too short SRT (around 2 days) can favour NO_2 accumulation due to higher AOB activity than microalgae and NOB, which can reduce microalgae activity significantly [48]. On the other hand, longer SRTs tend to increase NOB activity [174], favouring nitrification at the expense of lower microalgae growth. Microalgae have been found to be favoured over nitrifiers at mid-range SRT

values. González-Camejo et al. [47] reported this range to be 2-4.5 d for an outdoor flat-panel MPBR system treating AnMBR effluent.

The most appropriate values of SRT/HRT depend on the culture characteristics (mainly biomass concentration and pigment content) as they affect the average light irradiance of the culture (Section 3.2). These characteristics will in turn be related to other factors such as ambient conditions and nutrient availability [46]. Mathematical models can predict the most appropriate SRT/HRT for variable conditions [68,175].

6.3. Temporary increase of dilution rate

Once a consistent nitrifying bacteria population has been established within the culture, microalgae usually reduce their performance. To change this trend, the dilution rate can be increased to wash out the excess bacteria [176]. Raising the dilution rate will also reduce microalgae biomass and pigment concentrations, which can favour microalgae growth by reducing light attenuation in the reactor (Section 3.2). González-Camejo et al. [47] found a significant reduction in the nitrification rate of a microalgae-nitrifying bacteria system when the dilution rate was temporarily raised from 0.3 d^{-1} to 0.5 d^{-1}. However, the higher dilution rate only benefits the microalgae for a short time. If the operating and ambient conditions favour nitrifying bacteria growth, the nitrification rate will be dominant in the long-term.

6.4. Control of nutrient loads

Nutrient loading is related to the SRT/HRT/dilution rate. However, the competition between microalgae and nitrifying bacteria does not only depend on the influent flow rates but also on the distribution of nutrients, as previously explained in Section 3.3. For instance, if the ammonium loading rate is negligible, AOB growth will be inhibited. However, microalgae will still be able to grow using nitrite or nitrate (although at a lower growth rate). This behaviour was reported by González-Camejo et al. [32], who

observed an equilibrium between microalgae and bacteria when the ammonium loading rate was under 30-35 mg $N \cdot L^{-1} \cdot d^{-1}$, but a significant proliferation of nitrifying bacteria when it went over 40 mg $N \cdot L^{-1} \cdot d^{-1}$. Other authors have also reported nitrification control by adjusting the N:P ratio of the influent [31]. If phosphorus is scarce in the medium, nitrifying bacteria will not be able to grow. However, microalgae can use their intracellular phosphorus when P is lacking [178].

6.5. Addition of nitrification inhibitors

Nitrification can be easily inhibited by adding selective inhibitors. ATU has been reported as a transient inhibitor of AOB activity when the concentration in the medium is around 5-10 $mg \cdot L^{-1}$ [39,41,107], while chlorate compounds inhibit the second step of nitrification [104]. However, chlorate not only inhibits NOB but can also affect microalgae activity and is thus not recommended in MA-AOB systems. On the other hand, ATU does not affect microalgae significantly. As AOB growth is inhibited when ATU is added, nitrite will not be produced and NOB will thus be limited indirectly due to nutrient scarcity, favouring microalgae growth.

Conclusions

When operating mixed microalgae-bacteria systems using feeding media where ammonium is the main nitrogen source, the competition between microalgae and AOB for ammonium uptake is expected. Depending on the final goal of the wastewater treatment process, the growth of one organism or another will be pursued. When nutrient removal and biomass productivity are to be maximised, microalgae activity will be favoured. On the other hand, significant nitrification will be desired when ammonium influent concentration is high (as in centrates) in order to reduce ammonium toxicity and the overall aeration needs of the treatment plant.

The activity of both microalgae and nitrifying bacteria can vary significantly with different medium characteristics and ambient and operational conditions, affecting equilibrium. Some of the important factors that influence (either directly or indirectly) microalgae-AOB competition are: temperature, light, nitrogen concentration (and the distribution of nitrogen species), pH, and dilution rate. Modifying these factors can thus help to improve process performance by maximising/minimising microalgae activity and/or nitrification. In this respect, some system control options are the following: I) control of culture temperature; II) control of SRT/HRT; III) temporary increase of dilution rate; IV) control of nutrient loading rates; and V) adding nitrification inhibitors to the culture. However, some of these operations can increase operating costs and affect the microalgae cultivation system so that they should be carefully evaluated.

To assess microalgae-nitrifying bacteria competition, most of the commonly used methods focus on measuring either microalgae or nitrifying bacteria activity (or concentration). For instance, photosynthetic efficiency and chlorophyll fluorescence parameters are specific indicators of microalgae activity, while NO_2/NO_3 concentrations and nitrification rate are indicators of nitrifying bacteria activity. With respect to nutrient removal, biomass productivity, optical density and pH dynamics, despite being a result of both microalgae and bacteria activities, they are strongly influenced by microalgae in most microalgae-based systems, while respirometries can clearly distinguish between the activity of both microorganisms. Several of these methods should be combined to improve the monitoring of the wastewater treatment process by MA-AOB cultivation. Similarly, most methods of analysing the culture biomass concentration are based on either microalgae (chlorophyll concentration, cell counting and optical density) or bacteria (FISH, flow cytometry and DNA analysis).

Acknowledgements

This research work was supported by the Spanish Ministry of Economy and Competitiveness (MINECO, Projects CTM2014-54980-C2-1-R and CTM2014-54980-C2-2-R) jointly with the European Regional Development Fund (ERDF), both of which are gratefully acknowledged. It was also supported by the Spanish Ministry of Education, Culture and Sport via a pre-doctoral FPU fellowship to authors J. González-Camejo (FPU14/05082) and S. Aparicio (FPU/15/02595).

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Figures

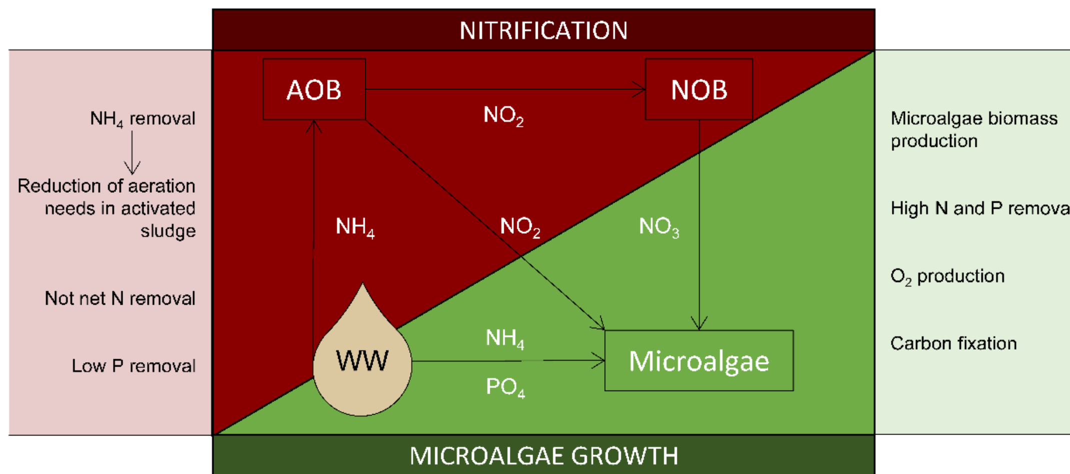

Figure 1. Schematic representation of the nitrogen cycle within the microalgae-ammonium oxidising bacteria competition.

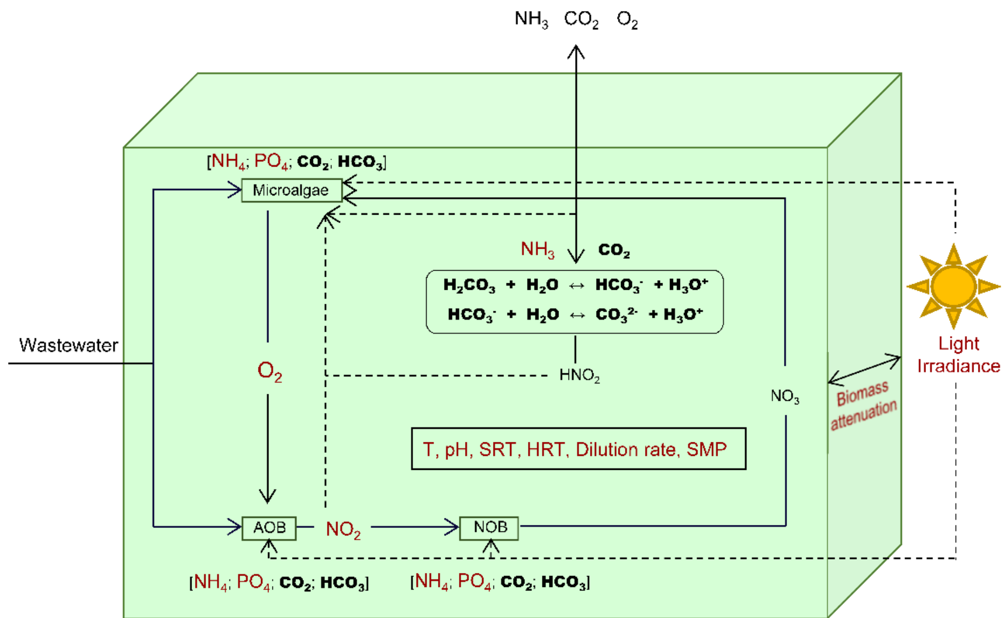

Figure 2. Representation of the main parameters and interactions influencing MA-AOB competition. In red the main environmental and operational parameters that affect metabolism of microalgae and bacteria are highlighted. The carbon species involved in the carbonate/bicarbonate equilibrium that regulates the pH of the culture medium are represented in bold. Legend: HRT: hydraulic retention time; SMP: soluble microbial products; SRT: solids retention time; T: temperature.

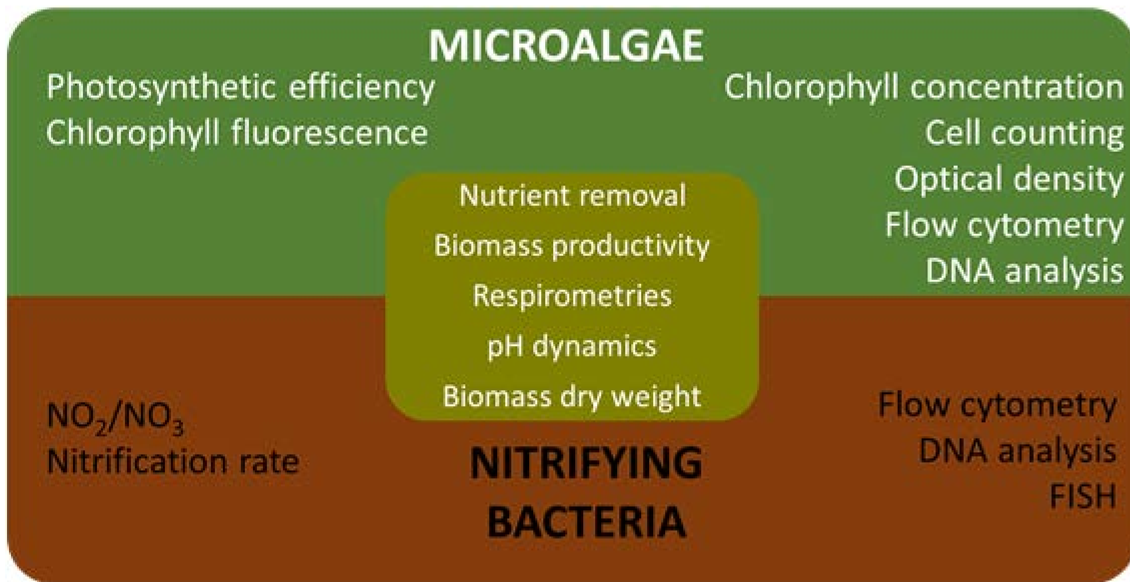

Figure 3. Methodologies to measure the activity and concentration of microalgae and nitrifying bacteria.

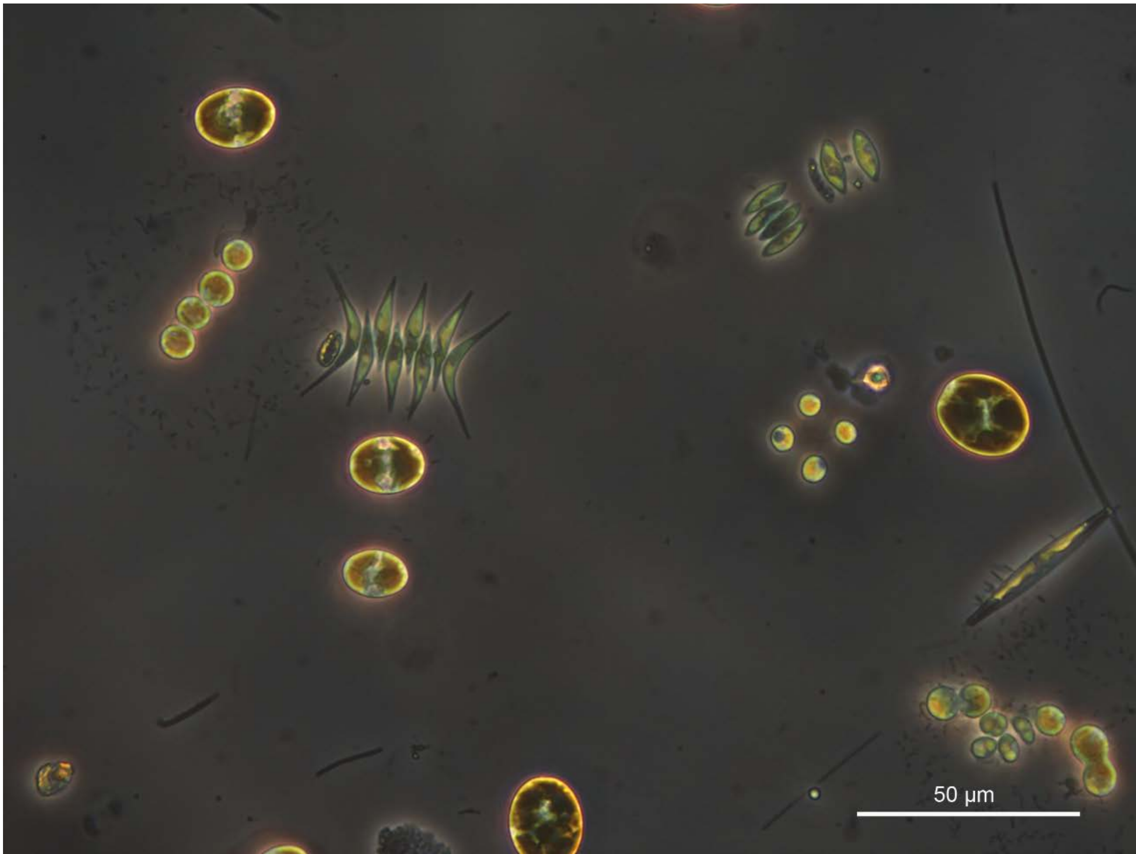

Figure 4. Visualisation of different microalgae genera (Chlorella, Scenedesmus and diatoms*) (400x) from a Leica DM2500 microscope with N2.1 and I3 filters.*

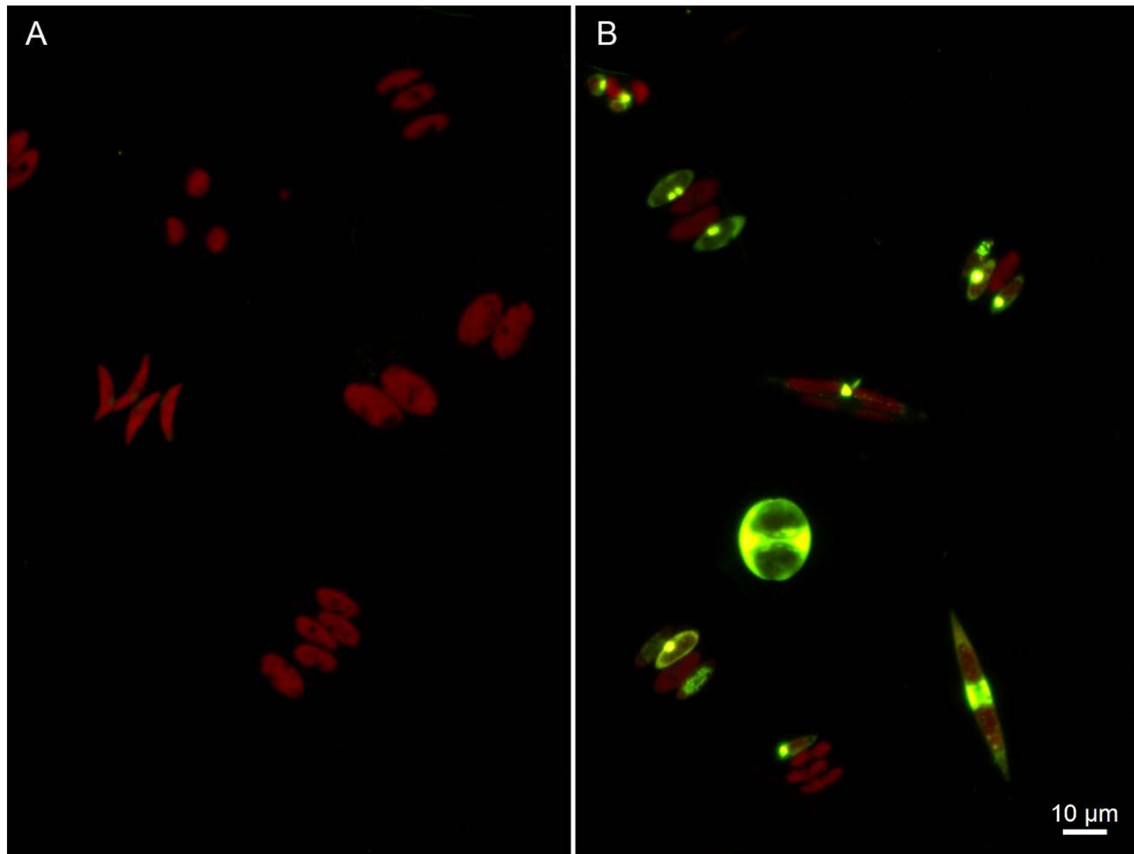

Figure 5. (A) Microalgae autofluorescence; (B) Microalgae sample stains with SYTOX Green, cells with damaged membrane emit a green fluorescent signal, while cells that are considered viable emit a red fluorescent signal associated with microalgal autofluorescence. The microalgae sample was visualized (400x) through a Leica DM2500 microscope when excited with a blue-light excitation filter (λ_{ex} 450–490 nm).

Table 1. Results obtained in previous studies related to MA-AOB competition for ammonium uptake.

Wastewater	Reactor (Volume)	[$NH_{4,i}$] (mg N·L^{-1})	Conditions	MA uptake (%$NH_{4,i}$)	Nitrification (%$NH_{4,i}$)	Stripping (%N)	NRR (mg N·L^{-1}·d^{-1})	NRE (%)	Reference
Artificial	CSTR (1.5 L)	1400	HRT = 10 d	40	60	-	-	98[a]	[44]
Sewage	HRAP (1900 L)	54-63	HRT = 6 d	<50%	-	-	-	>80%[a]	[21]
AnMBR effluent	PBR (550 L)	44.7	T = 18.5ºC	57.4	17.8	0.3	4.3	63.5	[39]
AnMBR effluent	PBR (550 L)	44.7	T = 26.7ºC	44.4	16.3	0.4	3.3	47.0	[39]
Centrate	Bubble column	147	T = 20ºC; pH = 8.6-8.7	35	34	20	20	54.1	[48]
Artificial	Cylindrical reactor (1 L)	23	SRT = 26 d	35	40	-	5.4	89.5[b]	[45]
Artificial	Cylindrical reactor (1 L)	23	SRT = 17 d	10	66	-	1.6	87.0[b]	[45]
AnMBR effluent	MPBR (470 L)	40.0	SRT = 2.5 d; HRT = 1.25 d.	53.7	34.6	0.1	19.7	59.8	[49]
AnMBR effluent	MPBR (470 L)	51.5	SRT = 4.5 d; HRT = 1.25 d.	39.7	57.2	0.1	14.5	32.6	[49]
Centrate	HRAP (1200 L)	244	HRT = 10 d; pH = 8.2	10	45	32	-	86[a]	[28]
Centrate	PBR (35 L)	451	HRT = 10 d; pH = 7.9;	6.0	37.1	0.01	4.7	6.0	[29]

	T = 25ºC	

[a] NH_4 removal efficiency (includes the nitrified NH_4).
[b] Includes denitrification.

AnMBR: anaerobic membrane bioreactor; AOB: ammonium oxidising bacteria; CSTR: continuous stirred tank reactor; HRT: hydraulic retention time; HRAP: high rate algal pond; MA: microalgae; MPBR: membrane photobioreactor; $NH_{4,i}$: ammonium influent concentration; NRE: nitrogen removal efficiency; NRR: nitrogen removal rate; PBR: photobioreactor; SRT: solids retention time.

Table 2. Description of respirometric protocols.

Cultivation system					Respirometric protocol									
Wastewater	**Reactor (L)**	**Conditions**	**HRT/SRT (d)**	**Culture**	**Nutrients ($mg \cdot L^{-1}$)**	**Initial biomass ($mg \cdot L^{-1}$)**	**Light ($\mu mol \cdot m^{-2} \cdot s^{-1}$)**	**T (ºC)**	**pH**	**DO ($mg \cdot L^{-1}$)**	**Duration L/D phases**	**OPR/OUR ($mg \cdot L^{-1} \cdot h^{-1}$)**	**Ref.**
AnMBR effluent	Vertical PBR (7)	Laboratory	-	Mixed microalgae culture	-	833	Only dark	20	7-8.4	1-9	0/> 48 h	0/0.1-0.8	[108]
Artificial	Flasks (0.25)	Laboratory	Batch	*Chlorella vulgaris*	$NaHCO_3$ (0.75 $g \cdot L^{-1}$); NH_4 (10 $mgN \cdot L^{-1}$); NO_3 (10 $mgN \cdot L^{-1}$)	1.06-2.05[a]	135	24	7-8	Non-controlled	24 h (L)/ 0 (D)	0.61 /-	[109]
Centrate	Raceway pond (1,200)	Outdoor	20	Microalgae-bacteria	NH_4Cl (3.2 $gN \cdot L^{-1}$); $NaNO_2$ (8.2 $gN \cdot L^{-1}$)	0.4-0.6[b]	-	-	-	-	15/10 min	10-25/0-4	[110]
Artificial	Flasks (0.25)	Laboratory	Batch	*Mixed Chlorella protothecoides*	$NaCO_3$ (1 $g \cdot L^{-1}$); N (247 $mgN \cdot L^{-1}$); P (5.4 $mgP \cdot L^{-1}$)	200	20-1500	24	7.5	0-11	5/5 min	-	[111]
Primary effluent	SB-PBR (2)	Laboratory	5.6/65	Consortium microalga	Acetate (5-10 $gCOD \cdot L^{-1}$);	900-1000	90	-	-	7.5-8.5	i) based on nutrients	8.3/0.5	[85]

				e-bacteria	NH_4 (1 gN·L^{-1})						ii) based on DO		
Centrate	Raceway pond (1,200)	Outdoor	10	Microalgae-bacteria	$NaHCO_3$ (150 mgC·L^{-1}); NH_4Cl (30 mgN·L^{-1}); K_2HPO_4 (10 mgP·L^{-1})	0.2[b]	110	20	8.5	10	10/20 min	-	[11]
Primary effluent	Raceway (4,400)	Outdoor	5	Consortium microalgae-bacteria	$C_2H_3O_2^-$ (0.3 g·L^{-1}); NH_4Cl (30 mg·L^{-1})	-	200	25	8.0	90-130%sat	4/4 min	16.7/1.9	[107]

[a] Final biomass concentration.

[b] Measured as optical density at 680 nm (OD680)

AnMBR: anaerobic membrane bioreactor; DO: dissolved oxygen; HRT: hydraulic retention time; L/D: light-dark; SB-PBR; sequencing batch photobioreactor; SRT: solids retention time.

Table 3. Optical density used to measure biomass concentration in microalgae-based systems.

OD	Wastewater	Reactor	Microorganism	R^2	Reference
750 nm	Artificial	Airlift reactors (3.2 L)	Green microalgae	-	[120]
750 nm	Artificial	Flask (0.4)	Green microalgae	0.992-0.998	[123]
940 nm	Artificial	ePBR	*C. sorokiniana*	0.938	[124]
683 nm	Artificial	Flask (0.25 L)	*C. vulgaris*	0.991	[109]
680 nm	Artificial	Cylindrical PBR (1 L)	*Chlorella*	0.996-0.999	[125]
550/665/750 nm	Artificial	Tubes (0.1 L)	*Rhodomonas salina*	0.952-0.979	[122]
680 nm	AnMBR effluent	MPBR (470 L)	Microalgae-nitrifying bacteria	0.905	[86]
680 nm	Centrate	Raceway (1200 L)	Microalgae-bacteria	0.87	[28]
680 nm	Synthetic secondary effluent	MPBR (2.5 L)	*C. vulgaris*	0.999	[126]
680 nm	AnMBR	Flask (2 L)	Green	0.891-	[53]

effluent	microalgae	0.992

AnMBR: anaerobic membrane bioreactor; ePBR: environmental photobioreactor; MPBR: membrane photobioreactor; OD: optical density; R2: coefficient of determination.