Universität Hamburg

# Municipal Wastewater Treatment Using Microalgae-Bacteria (MaB) Flocs in the Reduced Hydraulic Retention Time (HRT)

**Cumulative Dissertation**

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# Abbreviations

| | |
|---|---|
| AOB | ammonia oxidizing bacteria |
| BBL | wastewater from lautering process of beer brewing |
| CAS | conventional activated sludge |
| CS | control series |
| CSB | Chemischer Sauerstoffbedarf |
| COD | chemical oxygen demand |
| DO | dissolved oxygen |
| DW | dry weight |
| EPS | extracellular polymeric substance |
| EU | European Union |
| EUS91 | European Council Directive standard 91/271/EEC |
| FA | free ammonia |
| GHG | greenhouse gas |
| HRT | hydraulic retention time |
| IC | inorganic carbon |
| LS | lawn silage (extract) |
| MaB | microalgae-bacteria |
| NOB | nitrite oxidizing bacteria |
| NOx | nitrogen oxides ($NO_2$+$NO_3$) |
| OC | organic carbon |
| p.e. | population equivalent |
| PS | photosystem |
| SBR | sequencing batch reactor |
| SD | standard deviation |
| SDG | Sustainable Development Goals |
| TAN | total ammoniacal nitrogen |
| TIC | total inorganic carbon |
| TN | total (dissolved) nitrogen |
| TSS | total suspended solids |
| UN | United Nations |
| WWTP | wastewater treatment plant |

# Zusammenfassung

Die Zunahme der Erdbevölkerung und die drastische Verstädterungsrate sind mit der Produktion enormer Mengen an kommunalem Abwasser verbunden. Das sechste Ziel der UN für nachhaltige Entwicklung (SDG) ist die „Verfügbarkeit und nachhaltige Bewirtschaftung von Wasser und sanitären Einrichtungen für alle sicherzustellen" als eine der globalen Herausforderungen für unsere Gesellschaften und unsere Umwelt (Qadir et al., 2020). Kläranlagen spielen eine Schlüsselrolle bei der großflächigen Dekontamination kommunaler Abwässer und erfüllen das sechste SDG. Die konventionellen Behandlungssysteme sind auf das Belebtschlammverfahren angewiesen. Dieser Prozess ist stark aerob und erfordert daher eine intensive mechanische Belüftung. Daher ist für den Betrieb herkömmlicher Aufbereitungsanlagen eine hohe und konstante Energiezufuhr unerlässlich. Es gibt zudem einige andere Bedenken hinsichtlich der Umweltauswirkungen der herkömmlichen Behandlungssysteme.

Die fotosynthetische Oxygenierung durch die Mikroalgen kann als nachhaltiger Ersatz für die mechanische Belüftung des Belebtschlamms eingeführt werden. Nicht nur Mikroalgen können die Nährstoffe effektiv entfernen, sondern die Algen und Bakterien des Belebtschlamms können Aggregate (Flocken) bilden, um ihren produzierten und benötigten Sauerstoff (im Austausch gegen $CO_2$) zu recyceln. Da die Anwendung der Algenbiotechnologie eine relativ große Oberfläche erfordert, ist die Optimierung der Abwasserbehandlungsleistung entscheidend, um den Landverbrauch zu minimieren. Die experimentelle Forschung in dieser Arbeit ist ein Versuch, die Herausforderungen bei der Reduzierung der hydraulischen Retentionszeit (HRT) der MaB-Behandlung anzugehen. Eines der Hauptziele dieser Studie ist es, einen empirischen Mechanismus anzubieten, um die HRT auf 2 Tage und kürzer zu reduzieren. Diese Studie führte zur Veröffentlichung von 2 Artikeln in wissenschaftlichen Zeitschriften.

Der erste Artikel (in dieser Dissertation als Artikel 1 bezeichnet) trägt den Titel „Einfluss der hydraulischen Verweilzeit auf die kommunale Abwasserbehandlung unter Verwendung von Mikroalgen-Bakterien-Flocken in sequenzierenden Batch-Reaktoren". Artikel 1 wurde im Journal of Bioresource Technology Reports (Ausgabe 17) veröffentlicht. In diesem Artikel wurde eine MaB-Kultur, bestehend aus einem Konsortium einheimischer Algen und Belebtschlamm, zur Behandlung von echtem kommunalem Abwasser verwendet. Es wurde beobachtet, dass nach Erschöpfung des organischen Kohlenstoffs (OC) durch die heterotrophen Bakterien (hauptsächlich während der ersten Belichtungsperiode) der überschüssige Sauerstoff am zweiten Tag zur Nitrifikation des übrig gebliebenen Ammoniums verwendet wurde. Die höchste biologische Stickstoff- und Phosphorentfernung fand am ersten Tag der Behandlung statt, bevor die Nitrifikation beschleunigt wurde. Die Entfernung der produzierten Nitrate (NOx) auf die EU-Grenzwerte war die größte Herausforderung, um die europäischen Standards innerhalb von 2 Tagen nach der HRT zu erreichen.

Eine Verlängerung der Behandlung auf 3 Tage verbesserte die Nährstoffentfernung nicht, da die Erhöhung des pH-Werts (aufgrund von Photosynthese und Alkalitätsmangel) eine Reihe von Hemmungen der MaB-Mikroorganismen auslöste. Es wird angenommen, dass der hohe pH-Wert und die anschließende Erhöhung der Konzentration an freiem Ammoniak (FA) eine Nitratakkumulation, eine Hemmung der Nitrifikation und eine dauerhafte Unterdrückung der heterotrophen und photoautotrophen Aktivitäten der Algen verursacht hat. Der Zerfall von MaB-Flocken und das Kollabieren der Kultur wurde in den sequenzierenden Batch-Reaktoren mit 3 Tagen HRT beobachtet.

Der zweite Artikel (Artikel 2) mit dem Titel „Einfluss von Kohlenstoff Zugabe auf die Reduzierung der hydraulischen Verweilzeit bei der Behandlung von kommunalem Abwasser durch Mikroalgenbakterien (MaB)" ist im Journal of Water Process Engineering (Ausgabe 51) erschienen. Dieser Artikel stellt das Ergebnis der umfangreichen Experimente vor, die entwickelt wurden, um die übermäßig alkalischen Kulturbedingungen zu verhindern und darüber hinaus die Bildung von Nitraten zu verhindern. Um den Anstieg des pH-Werts (< 8,0) zu verhindern und die Alkalinität aufrechtzuerhalten, wurde die Zugabe von exogenem ($CO_2$) praktiziert. Ohne hohe pH- und freie Ammoniak (FA)-Werte nahm die Aktivität der nitrifizierenden Bakterien zu und am zweiten Tag wurde mehr Nitrate produziert. Aufgrund der Verfügbarkeit von Ammonium konnten Algen das Nitrat nicht innerhalb von 2 Tagen nach der HRT entfernen. Zwei Sequenzierungschargen von 3 Tagen HRT könnten jedoch die EU-Standards erfüllen, indem den Algen genügend Zeit gegeben wird, das verbleibende Nitrat und Phosphat am dritten Tag zu assimilieren.

Mit Artikel-2-Experimente untersuchte ich weiterhin die Wirkung einer Zugabe von OC (Glucose), um die MaB-Behandlung zu verstärken. Das stöchiometrisch ausgeglichene CSB:N-Verhältnis (w:w) von ungefähr 16 wurde eingestellt, um den internen Kreislauf von $C+O_2 \overset{\text{MaB}}{\Longleftrightarrow} CO_2$ zu stützen. Die Ergebnisse zeigten, dass die exogene Zugabe mit OC ein signifikant zuverlässiger Ansatz ist, um die Assimilation sowohl von N als auch von P innerhalb von etwa 24 Stunden zu verbessern, während keine externe Hilfsbelüftung bereitgestellt wurde. Auch die Absetzqualität und Quantität der geernteten MaB-Biomasse war bei diesem Verfahren höher.

Da die Verwendung von Glucose in großem Maßstab zur Ergänzung der kommunalen Abwasserbehandlung wirtschaftlich und ökologisch nicht nachhaltig ist, wurden potenziellen Alternativen aus Abfallquellen getestet (unveröffentlichte Ergebnisse). Glycerin, Rasensilageextrakt und das Abwasser aus dem Läuterprozess einer gewerblichen Bierbrauerei waren die Abwässer mit hohen CSB-Werten, die als externe Quellen für zusätzliches OC für die MaB-Behandlung untersucht wurden. Von diesen Kandidaten könnte letzteres (Abwasser aus der Bierbrauerei) eine mit der Glukose vergleichbare Leistung zeigen und als nachhaltige Alternative eingeführt werden, um die HRT der MaB-Behandlung von kommunalem Abwasser auf weniger als 28 Stunden zu reduzieren.

## Summary


Increase of the population of the Earth and the drastic rate of urbanization is associated with production of enormous amount of municipal wastewater. The sixth goal of the UN Sustainable Development Goals (SDG) is to "ensure availability and sustainable management of water and sanitation for all". Sustainable sanitation is one of the global challenges for our societies and environment which is out to be achieved by 2030 (Qadir et al., 2020). Wastewater treatment plants (WWTP) have a key role in decontamination of the municipal wastewaters in large scales and fulfil the sixth SDG. The conventional treatment systems are dependent on the activated sludge process. This process is heavily aerobic, thus requires intense mechanical aeration. Therefore, to operate the conventional treatment systems, high and constant supply of energy is vital. There are also some other concerns about the environmental impacts of the conventional treatment systems.

The microalgal photosynthetic oxygenation can be introduced as a sustainable substitute for the mechanical aeration of the activated sludge. Not only microalgae can remove the nutrients effectively, but the algae and bacteria of the activated sludge can form aggregates (flocs) to recycle their produced and required oxygen (in exchange for $CO_2$). Since the application of algae biotechnology demands a relatively large surface area, optimizing the wastewater treatment performance is crucial to minimize the land use. This experimental research is an attempt to address the challenges in reducing the hydraulic retention time (HRT) of the MaB treatment in a single stage. Offering an empirical mechanism to reduce the HRT to 2 days and shorter is one of the main objectives of this study. This study led to publication of 2 articles in the scientific journals.

The first article (addressed as Article 1 in this dissertation) is titled 'Influence of hydraulic retention time on municipal wastewater treatment using microalgae-bacteria flocs in sequencing batch reactors'. Article 1 is published in the journal of Bioresource Technology Reports (issue 17). In this article, a MaB culture consisting of a consortium of indigenous algae and activated sludge was used to treat real municipal wastewater. It was observed that after exhaustion of the organic carbon (OC) by the heterotrophic bacteria (mainly during the first illumination period), the surplus oxygen was employed to nitrify the leftover ammonium on the second day. The highest biological nitrogen and phosphorus removal took place on the first day of the treatment before the nitrification accelerates. Removal of the produced nitrates (NOx) to the EU limits was the main challenge to reach the European standards within 2 days of HRT. Extension of the treatment to 3 days did not improve the nutrients uptake as the elevation of the pH (due to photosynthesis and shortage of alkalinity) triggered a series of inhibitions to the MaB microorganisms. It is assumed that the high pH and subsequently raise of the free ammonia (FA) concentration could cause the nitrate accumulation, inhibition of nitrification, and a lasting suppression of the heterotrophic and photoautotrophic activities. Disintegration of MaB flocs and culture collapse was observed in the sequencing batch reactors (SBRs) with 3 days of HRT.

The second article (Article 2) with title 'Influence of supplementary carbon on reducing the hydraulic retention time in microalgae-bacteria (MaB) treatment of municipal wastewater' is published in the Journal of Water Process Engineering (issue 51). This article presents the result of the extensive experiments which were designed to prevent the exceedingly alkaline culture conditions, and furthermore, impede the formation of nitrates. To negate raise of the pH (<8.0) and maintain the alkalinity, regulated addition of exogenous ($CO_2$) was practiced. In absence of high pH and FA levels, the activity of the nitrifying bacteria increased, and more nitrates could be produced on the second day. Due to availability of ammonium, algae could not remove the nitrate within 2 days of HRT. Two sequencing batches of 3 days HRT could however allow the EU standards to be satisfied by giving the algae enough time to assimilate the remaining nitrate and phosphorus on the third day.

Article 2 experiments continued with examination of the effect of supplementation of the municipal wastewater with OC (glucose) to enhance the MaB treatment. The stoichiometrically balanced COD:N ratio (w:w) of roughly 16 is calculated to boost the internal cycle of $C+O_2 \overset{\text{MaB}}{\Longleftrightarrow} CO_2$. The results showed that the exogenous supplementation with OC is a significantly reliable approach to improve the assimilation of both N and P within about 24 hours while no external auxiliary aeration. The settling quality and quantity of the harvested MaB biomass was also higher in this method.

Because large scale utilization of glucose to supplement the municipal wastewater treatment is not economically and environmentally sustainable, the potential waste-sourced alternatives were tested (unpublished results). Glycerol, lawn silage (LS) extract and the effluent from the lautering process of a commercial beer brewer (BBL) were the wastewaters with high COD:N ratios. From among these substrates, the latter (beer brewing wastewater) could show a comparable performance with the glucose and can be introduced as a sustainable alternative for exogenous supplementation of the organic carbon to reduce the HRT of the MaB treatment of municipal wastewater to less than 28 hours.

# 1. Introduction

## 1.1. Sustainable Development Goals (SDGs) and Sanitation

The population of the Earth has recently passed the new milestone of 8 billion persons which signifies the enormity of the resources that are crucial for the welfare and sanitation of this multitude of people. The United Nation's Sustainable Development Goals (SDG) are developed to bring the prosperous and sustainable development and conservation of our planet by 2030 (UN Department of Economic and Social Affairs, 2016). These goals are indeed the blueprint to achieve a better and more sustainable future for all the humans and the natural resources. The SDGs target the global challenges that we as the whole planet face, including poverty, inequality, climate change, environmental degradation, peace, and justice. From among 17 objectives of the SDG, the sixth goal seeks to ensure that people have access to clean water and adequate sanitation services worldwide. Proper sanitation is essential to keep our societies protected from the diseases and environmental disasters. The significance of wastewater treatment is more clear when we observe that 80 percent of the global sewage is released into the oceans and natural water bodies untreated (National Geographic Society, 2022). Inadequate wastewater management leads to the excessive emissions of $CO_2$ and methane ($CH_4$) which are presumably the main contributors to the greenhouse effect and climate change (Salmiati et al., 2015). Environmentally friendly, uncomplicated, inexpensive, and widely accessible wastewater treatment technologies are vital to realize the sixth goal and other related SDG goals by 2030.

## 1.2. Conventional Wastewater Treatment

There is a vast range of mechanical, chemical, and biological technologies developed to treat municipal wastewater. The main goals of such processes are to reduce the health and environmental hazards of wastewater disposal and recovery of the nutrients and energy from the removed sludge (Dos Anjos, 1998; US-EPA, 2013). Municipal wastewater is a cocktail of dissolved and suspended matter and organisms which can be harmful to both the environment and the human health. The variety of the organic and inorganic substances together with pathogens in the municipal wastewater makes a perilous recipe for eutrophication and contamination of water resources (Volterra et al., 2002). Application of sustainable treatment approaches not only hinders the adverse effects of contamination of natural water resources but also enables us to recover and recycle the water and nutrients for promoting the agricultural, energy, and environmental benefits (Goswami et al., 2020; Rashidi et al., 2015).

The conventional wastewater treatment is an effective way to remove contaminants from the municipal sewer. The primary, secondary and tertiary treatment stages are developed to remove the pollutants through physical, biological, and chemical processes. At a wastewater treatment plant (WWTP), the solids and grit removal comprise the primary treatment. The biological treatment after primary treatment is an essential phase to eliminate the dissolved and highly suspensible compounds. The biological treatment at the municipal WWTPs relies on the application of conventional activated sludge (CAS). In this process, high concentration of microorganisms in form settleable flocs, remove the organic and inorganic compounds by oxidizing carbonaceous and nitrogenous matter. The products of CAS treatment are $CO_2$, gaseous nitrogen ($N_2$), highly settleable bacterial biomass, and less complex substances (Jin et al., 2003; Scholz, 2016). Activated sludge treatment is an efficient method to strip nitrogen from the wastewater and break down the biodegradable organic substances. Typically, the CAS process consists of two steps of nitrification and denitrification. The former is aerobic, and the latter is an anaerobic process; hence external aeration is usually necessary to maintain high dissolved oxygen (DO) for nitrification and breakdown of the biodegradable carbon chains. Nitrification is generally handled through autotrophic metabolism while the heterotrophic bacteria activities reduce the nitrogen oxides (NOx) and organic compounds. Ammonium ($NH_4^+$) comprise about 90% of the nitrogen content of the municipal wastewater (Henze, 2008). In the activated sludge process, ammonium is oxidized to nitrite ($NO_2^-$) and nitrate ($NO_3^-$) through nitrification, a highly oxygen demanding activity. The nitrite production takes place by the ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) are responsible for the conversion of nitrite into nitrate. These oxidation reactions are shown as

Nitritation: $2NH_4^+ + 3O_2 \xrightarrow{AOB} 2NO_2^- + 4H^+ + 2H_2O$

Nitratation: $2NO_2^- + O_2 \xrightarrow{NOB} 2NO_3^-$

The sociochemical equation of nitrification can be demonstrated as (Ebeling et al., 2006):

$$NH_4^+ + 1.83\ O_2 + 0.094\ CO_2 + 0.024\ HCO_3^- \rightarrow 0.024\ C_5H_7O_2N + 0.977\ NO_3^- + 0.953\ H_2O + 1.95\ H^+ \quad \text{(Eq. 1)}$$

The $C_5H_7O_2N$ in the equation above represents the bacterial biomass. Both AOB and NOB are susceptible to high pH and free ammonia (FA) concentrations. The tolerance of NOB against these inhibitory factors is considerably less than the AOB which can lead to nitrite accumulation in moderate and even low levels of pH or FA concentrations (Anthonisen et al., 1976; Cho et al., 2014)

Since the nitrogen oxides (NOx) can cause eutrophication, denitrification is normally practiced following to nitrification. Despite nitrification, denitrification is an anoxic process. It is handled by heterotrophic bacteria which utilize nitrate to oxidize the exogenously supplied organic carbon (e.g. methanol). Thus, the nitrate is reduced to gaseous nitrogen ($N_2\uparrow$) which is volatile and released into the atmosphere.

### 1.2.1. CAS Process is Energy Intensive

The nitrogen and carbon in the municipal wastewater range from 20-85 mg N $L^{-1}$ (averagely 43.7 mg N $L^{-1}$ according to Qadir et al., 2020) and 80-290 mg total organic carbon (TOC) $L^{-1}$, respectively (Adams et al., 1999). According to (Eq. 1, nearly 3.2 g of oxygen ($O_2$) is needed to nitrify 1 g of ammonium. Heterotrophic bacteria consume 0.78 g of oxygen to metabolize 1 g of organic carbon (Ebeling et al., 2006). This implies that the CAS oxidation of ammonium and organic compounds is an oxygen intensive process. This oxygen is supplied through mechanical aeration of the activated sludge ponds. This stage of the CAS treatment requires a large amount of energy, claiming 40% to 75% of the electricity demand of a treatment plant (Mamais et al., 2015; Rosso et al., 2008). Municipal wastewater treatment is accountable for up to 5% of total national energy consumption (Longo et al., 2016). Any development that can evade this energy intensive process is a magnificent step towards sustainable materializing of the SDGs in the less developed countries.

### 1.2.2. Environmental Impacts

It is shown that the carbon footprint of conventional wastewater treatment plants is significant (Daelman et al., 2013; Parravicini et al., 2016). The emission of greenhouse gases (GHGs) from the conventional wastewater treatment plants set them as the contributors to climate change and global warming (Maktabifard et al., 2018; Xu et al., 2017). For example, to extract and release every mole of $N_2$, about 1.6 moles of $CO_2$ is released when methanol as the external source of carbon is supplemented the denitrification (Foglar and Briški, 2003). The nitrous oxide ($N_2O$) emission from the wastewater treatment plant can overshadow the carbon footprint of the treatment plant (Daelman et al., 2013)

Because the biological treatment is not adequately efficient to remove the phosphorus, the complementary phosphorus removal (tertiary treatment) would be necessary when the P concentrations in the effluent of secondary treatment are above the standards. This usually implies the application of phosphorus coagulants (e.g. salts containing $Fe^{3+}$ and $Al^{3+}$) to precipitate the dissolved phosphorus (Sedlak, 1991). The surplus coagulants can cause secondary contamination of the treated wastewater.

Although conventional activated sludge (CAS) systems are proven to be efficient in terms of nutrient removal and magnificent liquid-solid separation quality, their relatively energy intensive process and some undesired environmental impacts have intrigued researchers to discover more sustainable and environmentally friendly alternatives. Realizing the United Nations' sustainable development goals (SDG) requires more environmentally friendly and energy efficient technologies for wastewater treatment (Rosemarin et al., 2020; X. Wang et al., 2019).

## 1.3. Microalgae, The Natural Oxygen Supplier

Photosynthetic microorganisms can be considered as invaluable natural substitutes for mechanical oxygenation of the activated sludge. Among which, microalgae are superior in term of affinity to aquatic settings, remarkable capabilities to symbiosis and aggregate with the activated sludge bacteria, and efficient metabolic capacities of the nitrogen, phosphorus and carbon through photosynthesis and heterotrophic uptake. Both phosphorus and nitrogen in wastewater are essential for biosynthesis of vital organic compounds such as amino and nucleic acids (Richmond, 2003). Microalgae can remove a considerable amount of nitrogen, phosphorus, and carbon through assimilation into the biomass which requires no secondary processing of nitrogen (e.g. denitrification). In fact, carbon sequestration and storage of solar energy in the microalgal biomass offers a great potential to produce the third generation of renewable energy (Brennan and Owende, 2010; Chisti, 2007; Markou and Georgakakis, 2011; Pires et al., 2012; Zhang et al., 2014). In principle, algae cells in the flocs use solar energy and the inorganic nutrients for photosynthesis, sequestrating the inorganic carbon which can be the $CO_2$ respired by the bacteria. Microalgae can utilize the solar irradiation and use it to oxidize the water and provide the energy to sequestrate the ambient inorganic carbon into carbohydrates (Richmond and Hu, 2013). This photoautotrophic metabolism (photosynthesis) consists of two distinct phases of Light and Dark Reactions.

During the light reactions, the photosystems (PS) in the photosynthetic membranes (thylakoid) of the chloroplast absorb the light to provide the energy to oxidize water and release $H^+$. The proton which is required to restore the $NADP^+$ molecules into nicotinamide adenine dinucleotide phosphate dihydrogen ($NADPH_2$) and regenerate the high energy molecules of adenosine triphosphate (ATP) from ADP. Both $NADPH_2$ and ATP are essential to convert the $CO_2$ to simple carbohydrates in the dark reaction phase. The active photosynthesis applies the wavelengths of 400 – 700 nm of electromagnetic waves which corresponds to about 45% of direct sunlight irradiance. The reaction centers in the chlorophyll use this light to extract electron from water for generation of $NADPH_2$ and ATP molecules. The light (dependent) reactions can be simplified in equation 1 (Richmond, 2003):

$$2\ NADP + 2\ H_2O + 2\ ADP + 2\ P_i \xrightarrow{light} 2\ NADPH_2 + 2\ ATP + O_2 \qquad \text{(Eq.2)}$$

The surplus oxygen in in this equation is excreted in the medium and increases the concentration of dissolved oxygen (DO).

## 1.4. Light-independent Reactions and the pH

The light–dependent reactions provide the energy and electrons for the light–independent (also known as the dark) reactions. In presence of enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), light–independent reactions assimilate and covert the ambient $CO_2$ into carbohydrates. The general equation for the photosynthesis can be depicted as

$$2H_2O + CO_2 \rightarrow (CH_2O)_n + O_2.$$

The $CO_2$ required for dark reactions can also be supplied from the bicarbonate and even carbonate. This leads to release of hydroxide which would reduce the concentration of hydrogen ion ($H^+$) and alkalinize the medium (Richmond, 2003):

$$HCO_3^- \leftrightarrow CO_2 + OH^- \text{ and } CO_3^{2-} + H_2O \leftrightarrow CO_2 + 2OH^-$$

Aqueous carbon dioxide in forms dissolved carbonic acid ($H_2CO_3$) which is in equilibrium with bicarbonate ($HCO_3^-$) and carbonate ($CO_3^{2-}$). This equilibrium forms the alkalinity buffer:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2\,H^+ + CO_3^{2-} \qquad \text{(Eq. 3)}$$

lower pH $\longrightarrow$ higher pH

When one of these compounds is consumed, other compounds will make up the shortage by absorbing or releasing the hydrogen ion ($H^+$). Interchangeably, varying the pH adjusts the concentration of the substrates in the equilibrium (Markou and Georgakakis, 2011). Carbon dioxide is the dominant form of inorganic carbon at pH <6.4 while bicarbonate takes over the equilibrium in pH range of about 6.4–10.3. For example, at pH 9.6, about 70% of total inorganic carbon is in form of $HCO_3^-$. Microalgae can utilize both dissolved $CO_2$ and $HCO_3^-$ for photosynthesis (Binaghi et al., 2003; Brennan and Owende, 2010). Algae preferably uptake bicarbonate through their cell membranes while photosynthesis depends on sequestration of $CO_2$ in chloroplast to react with RuBisCO to synthesis of glucose (Abinandan and Shanthakumar, 2015; Guo et al., 2015; Kumar et al., 2011). If the rate of $CO_2$ production by aerobic bacteria in MaB culture becomes less than the inorganic carbon uptake by the algae, then microalgae acquire its $CO_2$ by extraction the required $H^+$ from water and react with the carbonates (Eq. 3) to be used in light-independent reactions (Calvin cycle). Subsequently, shortage of the hydrogen ions increases the pH of the culture, even to highly basic (pH>10) levels (Richmond, 2003). Drastic increase of pH in microalgae cultures and in MaB cultures with high ratio of inorganic to organic carbon has been observed (Van Den Hende et al., 2011).

### 1.5. Microalgae – Bacteria (MaB) biocenosis

Although application of solely microalgae for treatment of municipal wastewater requires no intensive aeration, it is not challenge–free. Microalgae are not as capable as the CAS bacteria in removing the organic compounds. Moreover, separation of the microalgal biomass from the treated wastewater in an urban scale is presumed to be exceedingly difficult. Poor settleability of microalgal biomass due to the microscopic size of the cells with a density close to water makes the harvest challenging and sometimes economically or practically unviable (Gerardo et al., 2015; Park et al., 2013).

When microalgae and the activated sludge are mixed together in the wastewater, the produced oxygen in (Eq.2 can be directly employed by the nitrifying and heterotrophic bacteria. In return, biodegradable organic carbon components of the wastewater (e.g. saccharose, fructose, glucose, etc.) are oxidized by the bacteria and carbon dioxide is released in the medium which pushes this symbiotic cycle forward (Fig. 1). However, depending on the strains of microalgae and bacteria (MaB) in the flocs and environmental factors (e.g. light intensity and retention time, etc.), different metabolic pathways take place in a MaB culture concurrently (C. Y. Chen et al., 2011; Evans et al., 2017).

The exemplary symbiosis of microalgae and heterotrophic bacteria does not necessitate the presence of the nitrifying bacteria, however in the normal conditions, autotrophic oxidation of ammonium in the MaB treatment of municipal wastewaters is inevitable. Autotrophic and heterotrophic bacteria serve each other mutually through production of needed nitrogenous organic compounds (Baskaran et al., 2020). When we discuss the heterotrophic activities in the MaB treatment, we should bear in mind that both heterotrophic bacteria and mixotrophic microalgae contribute to removal of organic carbon and ammonium collectively. It is reported that the microalgae is the dominant consumer of the dissolved oxygen under heterotrophic and dark conditions. Flores-Salgado et al. (2021) showed that up to 86% of the total oxygen for the heterotrophic respiration was consumed by the microalgae; therefore, microalgae can outcompete the bacteria in organic carbon removal. Nonetheless, it is reported that not all heterotrophic activities of the microalgae would involve utilization of $O_2$ (Fan et al., 2020).

The microalgae and bacteria can form aggregates (flocs) where they can easily exchange their required oxygen, $CO_2$, and other substances. The extracellular polymeric substance (EPS) which are produced by the microalgae and bacteria, provides the bounding force to hold the flocs structure (Arcila and Buitrón, 2017). Formation of MaB aggregates improves the biomass settling characteristics and reduces the risk of biomass washout (Christenson and Sims, 2011; Gutzeit et al., 2005; Quijano et al., 2017).

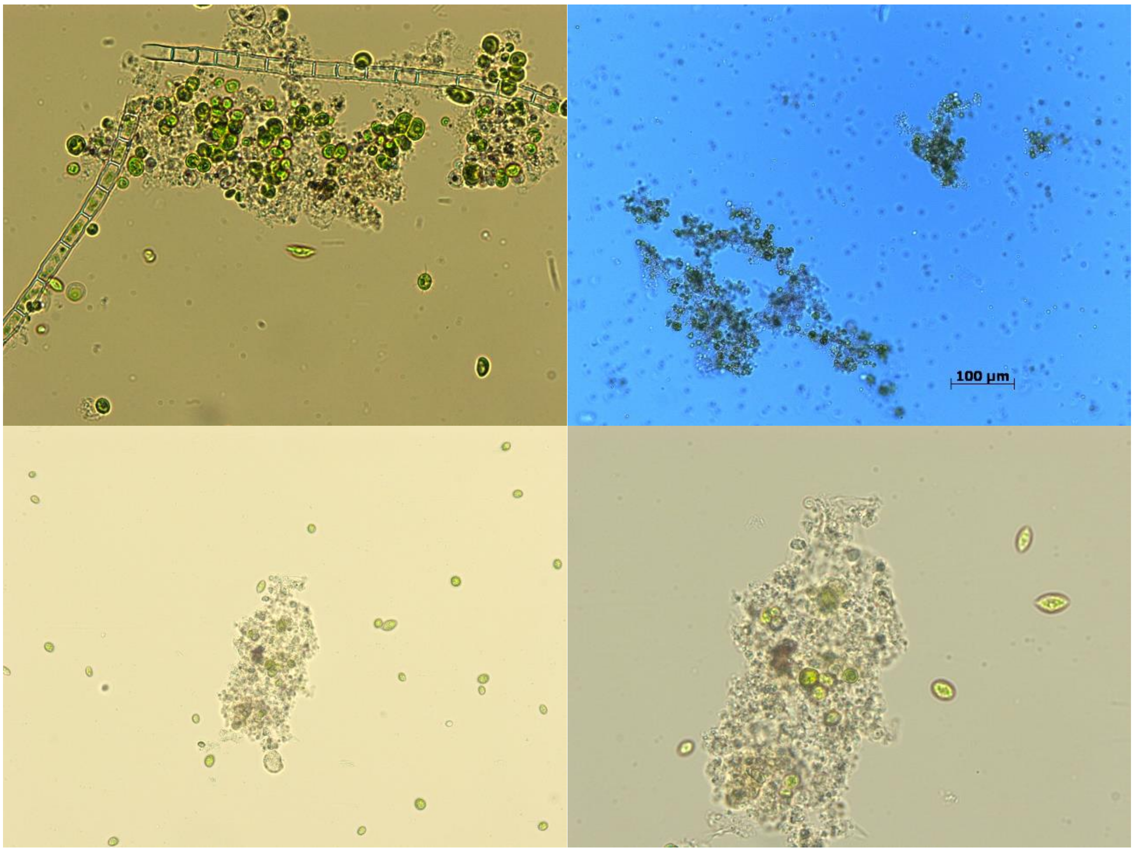


**Fig. 1** The biocenosis in the microalgae-bacteria (MaB) flocs is the basis for the wastewater treatment through nutrients uptake in the MaB biomass.

### 1.6. MaB Treatment of the Municipal Wastewater

Wastewater treatment using MaB saves energy due to independency from external aeration compared to CAS and improves the nutrient removal rate and harvestability of the biomass significantly (Praveen and Loh, 2015; Van Den Hende et al., 2014b, 2010; Zhang et al., 2020).

Replacement of electricity for aeration with sunlight energy makes the MaB system a sustainable substitute for the conventional activated sludge treatment systems. The microalgae-bacteria treatment require a very larger surface to volume ratio to improve the light penetration and decrease the self-shading effect. Saving land is a major challenge for large-scale implementation of this technology in dense urban areas (Muñoz and Guieysse, 2006; Van Den Hende et al., 2014a). Consequently, it is crucial to investigate the successful high performance MaB treatment scenarios with short hydraulic retention time (HRT) of the wastewater to minimize the land use (Foladori et al., 2018; López-Serna et al., 2019; Zeng et al., 2013). For example, increasing the nutrients uptake rate can reduce the hydraulic retention time (HRT) and reduce the land construction of the shallow algal ponds.

### 1.7. The European Standard (EUS91)

The benchmark for the water quality in this study is the current European standard (EUS91) which is introduced as the European Council Directive standard of 91/271/EEC concerning urban wastewater treatment (The Council of the European Union, 1991). European standard of 91/271/EEC limits the concentration of pollutants in the effluent of European WWTPs, based on the population equivalent (p.e.), to the following levels (The Council of the European Union, 1991):

| Parameter | Concentration in effluent | Minimum percentage of reduction |
|---|---|---|
| **COD** | 125 mg $L^{-1}$ $O_2$ | 75 |
| **Total phosphorus** | 2* mg P $L^{-1}$<br>1** mg P $^{L-1}$ | 80 |
| **Total Nitrogen** | 15* mg N $L^{-1}$<br>10** mg N $^{L-1}$ | 70-80 |

**Table 1** European standards (EUS91) for discharges from urban wastewater treatment plants
* For 10 000 < population equivalent (p.e.) ≤ 100 000.
** For p.e. > 100 000

According to our knowledge at this date, there are no major studies with a focus on reducing the municipal wastewater treatment period by means of improving the nutrient removal efficiency of MaB system which would aim to meet this standard.

By setting EUS91 limits as the target for MaB treatment, we investigated the pollutants removal from real wastewater in different scenarios. Among the macronutrients in wastewater to be removed, nitrogen has rather complex metabolic pathways in microalgae and bacteria cultures. Since ammonium is the major nitrogenic compound of municipal wastewater, eliminating the nitrogen in the ammonium can let us reach the EUS91 of 10 mg N $L^{-1}$ in the effluent; given that the concentration of nitrogen in the municipal wastewater is normally far less than 100 mg N $L^{-1}$ (Hammer and Hammer, 2012).

There are not many studies which aim to reduce the contaminants to meet the EUS91 with HRT shorter than 3 days; however, the MaB treatment in less than 3 days has been proven challenging when some efforts were made to achieve this goal (Olguín, 2012; Van Den Hende et al., 2014a). The diverse metabolic activities and their interrelations in the MaB technology, translate into relatively complex alterations of biochemical properties of the culture, such as pH, alkalinity, bioavailability of nutrients, oxygen, and carbon (Ramanan et al., 2016). In this study, MaB treatment of real wastewater is investigated. The real wastewater which is collected from the municipal sewer network contains a very diverse communities of bacteria, protozoa and even microalgae. All these add to the complexity of the biological and biochemical interactions between the MaB elements. The dynamic of the coexistence of photoautotrophic, heterotrophic, autotrophic, and mixotrophic microorganisms in MaB systems in wastewater is not yet completely understood (Flores-Salgado et al., 2021; Unnithan et al., 2014). Besides competition over the nutrients, certain concentrations of one microorganism induce inhibition on some other organism. For example, it is reported that microalgae can inhibit nitrate oxidizing bacteria in MaB culture and that can cause nitrite accumulation and inhibition of microalgae eventually, thus changes the chemistry and biodiversity of microbial community (González-Camejo et al., 2020; Huang et al., 2015; Rada-Ariza et al., 2017). Therefore, empirical investigation of the MaB treatment performance in the real wastewater is meritorious.

This study is an attempt to investigate the single-step MaB wastewater treatment to satisfy the EUS91 in the shortest time possible. Unfortunately, the available literature has primarily examined the MaB performance in synthetic wastewater which leads to unrealistic results due to lack of actual biotic and abiotic properties of real wastewater. The primary goal is to substitute conventional mechanical aeration with MaB photosynthetic oxygenation and to replace nutrient removal from municipal wastewater through nitrification/denitrification with nutrient assimilation into MaB biomass feasibly in a relatively short retention time of 2 days or less. The course of this study led us to experiment the role of pH and bioavailability of carbon in nitrogen removal. This study presents the results of three assays regarding the performance of MaB cultures in wastewater in different hydraulic retention times, pH, and carbon supplementation.

## 1.8. Publications

In this doctoral research the conditions and challenges to reduce the HRT to shorter than 2 days are investigated experimentally. This study led to publication of two journal articles which present and discuss the culture parameters and treatment performance as HRT shortens. The first paper with the title 'Influence of hydraulic retention time on municipal wastewater treatment using microalgae-bacteria flocs in sequencing batch reactors' is published the journal of Bioresource Technology Reports 17 (February 2022). This article is addressed as *Article 1* in this cumulative dissertation (Soroosh et al., 2022a). Article 1 is available on the Elsevier database with the DOI address: https://doi.org/10.1016/j.biteb.2021.100884. Article 1 is also enclosed to this dissertation as 4.1.

The second paper is the continuation of Article 1 and compares the results of the experiments when pH was artificially regulated to prevent inhibitions that can lead to culture failure as what we observed during the experiments of Article 1. This article, which will be called *Article 2* hereafter, is titled 'Influence of supplementary carbon on reducing the hydraulic retention time in microalgae-bacteria (MaB) treatment of municipal wastewater' (Soroosh et al., 2022b). It is published in the Journal of Water Process Engineering 51 (February 2023). Article 2 is enclosed to this dissertation as 4.2 and also available on the Elsevier database (Sciencedirect.com) with the DOI address: https://doi.org/10.1016/j.jwpe.2022.103447.

## 2. Discussion

In this section, the overall results of the experiments of Article 1 and Article 2 will be discussed. More detailed discussion and the measured values are presented in the articles.

### 2.1. pH Inhibition Chain

Removal of nitrate was not however the main challenge that we faced during the Article 1 experiences. The side-effect of photosynthesis affected the wastewater treatment to the degree that nitrogen uptake was suppressed.

Because the heterotrophs could not supply the required $CO_2$ for the algae beyond the first day of the treatment, the pH increased due to dominance of consumption of alkalinity over oxidation of the limited organic carbon ($CO_2$ production). This was in the extent that pH raised extensively which caused a domino of inhibitions. Initially, the sensitive NOB were inhibited which caused that the produced nitrite could not convert to nitrate and therefore the nitrite accumulation influenced the photosynthesis adversely (González-Camejo et al., 2020). The elevated pH also caused the equilibrium of $NH_4^+ \leftrightarrow NH_3$ shift toward higher concentration of free ammonia (FA) which was associated with further inhibition of autotrophic and photoautotrophic activities. This reduced the photosynthesis performance (reflected in the $F_v/F_m$ ratio), therefore the ammonium uptake was reduced. In abundance of oxygen, there is a risk of nitrous oxide ($N_2O$) formation through oxidation of remainder ammonium (Sutka et al., 2006). Nitrous oxide is an important greenhouse gas (GHG) which is produced during incomplete nitrification and denitrification at WWTPs. This gas comprises approximately 14-26% of the total GHG emitted from the urban water infrastructure (Cruz et al., 2019; Kampschreur et al., 2009), therefore, a quick ammonium removal has magnificent environmental merits. Article 1 shows that in the beginning of every batch, when nutrients and light are abundant and the pH is in moderation, the treatment performance in considerably better than the second and third days. Bioavailability of the organic carbon for the heterotrophic bacteria could maximize the ammonium uptake through both bacterial and algal assimilation. Heterotrophic bacteria with maximum specific growth rate ($\mu_{max}$) of 4.0-8.0 $d^{-1}$ and microalgae with $\mu_{max}$ of up to 8.22±0.69

$d^{-1}$ could easily outperforms the nitrifying bacteria with $\mu_{max}$ between 0.6–0.8 $d^{-1}$ (Barbera et al., 2019; Metcalf & Eddy, 2003); therefore, the assimilation of nitrogen was considerably greater than conversion to nitrogen oxides on the first day. The results of Article one also exhibited that an extended treatment time does not necessarily translate into a more advanced nitrogen removal. Increase of the pH and FA can interrupt the MaB metabolic activities or even disintegrate them (Soroosh et al., 2022a).

## 2.2. Fate of Nitrogen and Phosphorus (Pretreated Wastewater)

An ideal MaB treatment does not entail autotrophic oxidation and reduction of ammonium (nitrification-denitrification process), our observation in Article 1 showed that nitrification was inevitably part of the ammonium uptake pathway as the DO concentration raised above the inhibitory levels (about 1 mg $L^{-1}$). Therefore, to reduce the nitrogen below the EUS91 level of 10 mg $L^{-1}$ removal of nitrogen in both forms of ammonium and nitrates is desired. When nitrogen is simultaneously available in both forms of total ammoniacal nitrogen (TAN: ammonia–N + ammonium–N) and nitrogen oxides, consumption of TAN is always prioritized by the algae as it requires less energy (Cai et al., 2013). Therefore, the simultaneous reduction of both nitrogenic compounds through biomass uptake is infeasible.

However, the nitrification on day 2 of each sequencing batch was intense thus, the amount of the leftover TAN after the second day was not remarkably high compared to the concentration of the NOx. Denitrification of the produced nitrate is not likely as the oxygen concentration was constantly in saturation and oversaturation levels during light hours after the first day. During the next dark periods when the dissolved oxygen (DO) was the lowest, deficiency of biodegradable organic carbon in addition to free gas exchange between the culture surface and ambient atmosphere through constant stirring of the culture with a low volume to surface ratio, the strict anoxic condition which is vital for denitrification is prevented. A DO concentration of 0.1 mg $L^{-1}$ can adversely influence denitrification because presence of only 0.5 mg $O_2$ $L^{-1}$ can halt it completely (Lie and Welander, 1994; Wang et al., 2006). Therefore, anerobic heterotrophic nitrogen removal was not a applicable post-treatment scenario for our MaB treatment.

The phosphorus removal was on the other hand, very promising as the pH raised but it was rather due to the chemical precipitation. The phosphorus removal was almost complete when the treatment with HRT of 3 days was applied. The amount of phosphorus after 2 days of treatment was slightly above the EUS91 limits. Although during the first 2 days of treatment the culture response in terms of nitrogen removal was significantly better than the third day, but the concentration of nitrogen in the effluent was unacceptable according to the European standards.

## 2.3. The MaB Treatment Function and the Inhibitors

From the results of Article 1 we learned that

1- The treatment objective of nitrogen uptake without extracellular oxidation could be realized on the first day of the treatment when the pH was not yet raised to inhibitory levels and the culture is self-sufficient in regard to balance its DO–$CO_2$, therefore no remarkable elevation of DO was measured on the first day.

2- In absence of DO on the first day, no distinguishable amount of nitrification was detected which in accord with the main purpose of MaB treatment which would require no specific post-treatment of the secondary pollutants (e.g. nitrite or nitrate).

3- The sequencing batch reactor (SBR) cultures of MaB in the real wastewater were healthier in the sense of photosynthesis and bacterial activities when the HRT was reduced from 3 days to 2 days.

4- The increase of the pH after the first day of treatment could cause a series of inhibition (including the inhibitions due to FA toxicity), among which, some were irreversible (Fig. 2). The intensity of these inhibitions reduced the measured $F_v/F_m$ ratio and the nitrification and heterotrophic activities when 3 days of HRT was exercised. This led to culture collapse after about 5 cycles of treatment, in the way that a considerable amount of the ammonium was washed out in the effluent untreated. Degradation of the microalgae and bacterial bounds was another side-effect of high pH toxicity. The disintegrated flocs would then suspend and washed out as the sequencing batches followed.

Fig. 2 exhibits the overall influence of high pH on MaB treatment. This diagram is available in Article 1.

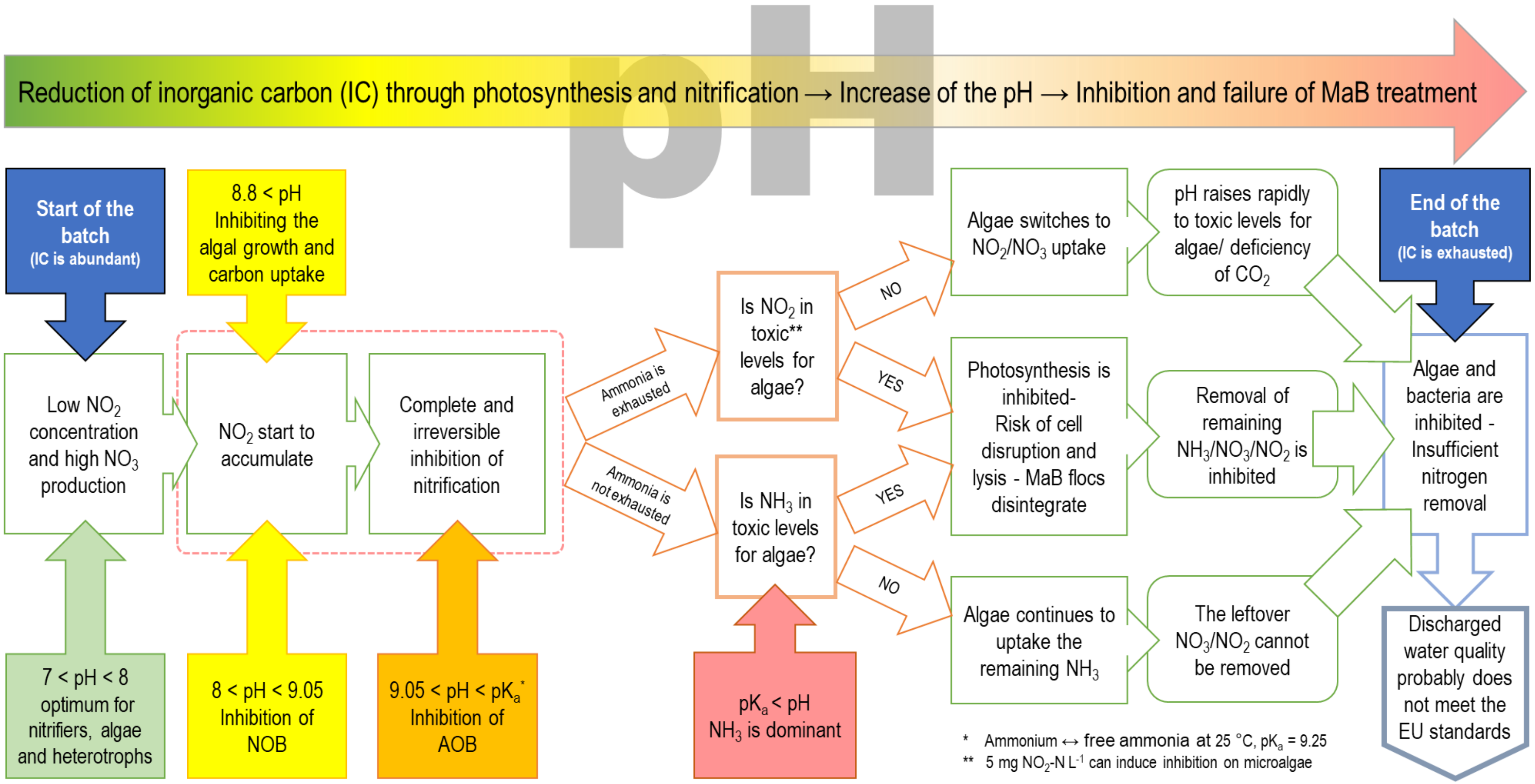


**Fig. 2** The chain of inhibitions that can initiate with as the pH levels increase due to photosynthesis.

Photosynthesis is not the only biological activity that impacts the pH. There are several biochemical pathways that could contribute to the raise of the pH in a MaB system. For instance, oxidation of ammonium by nitrifiers, algae and aerobic heterotrophs leads to release of proton and acidification. Table 2 summarizes the pH shifting reactions in a MaB culture. Some researchers have proposed interesting methods to regulate the pH by manipulating the nutrients and carbon in the medium instead of direct introduction of acid or base agents to the medium (Scherholz and Curtis, 2013; Uggetti et al., 2018)

| # | Reaction | Redox/stoichiometry formula | pH |
|---|---|---|---|
| RC | Aqueous $CO_2$* | $CO_2 + H_2O \rightleftarrows H_2CO_3$ [1,7] $\rightleftarrows HCO_3^- + H^+ (pK_a = 6.35)$ $\rightleftarrows CO_3^{2-} + 2H^+ (pK_a = 10.33)$ | ↓ |
| **Bacteria-based reactions** | | | |
| RB1 | Nitritation | $NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + H_2O + 2H^+$ [2] | ↓ |
| RB2 | Nitratation | $NO_2^- + 0.5\ O_2 \rightarrow NO_3^-$ [2] | – |
| RB3 | Nitrification | $NH_4^+ + 2\ O_2 \rightarrow NO_3^- + H_2O + 2H^+$ [3] | ↓ |
| RB4 | Heterotrophic | $\frac{5}{3}C_6H_{12}O_6 + 2NH_3 + 7O_2 \rightarrow \underbrace{C_5H_7O_2N}_{biomass} + 9H_2O + 5CO_2 + HNO_3 \rightleftarrows NO_3^- + H^+$ [4]<br>Respiration: $CH_2O + O_2 \rightarrow H_2O + CO_2 \rightleftarrows H_2CO_3 \rightleftarrows HCO_3^- + H^+$ [1,7] | ↓ |
| **Algae-based reactions** | | | |
| RA1 | Light reaction | $2NADP^+ + 3ADP + 3P + 2H_2O \xrightarrow{light} 2NADPH + 3ATP + O_2 + 2H^+$ [5]<br>Simplified: $2H_2O \xrightarrow{Light} 4H^+ + O_2$ | ↓ |
| RA2 | Dark reaction | $3CO_2 + 6NADPH + 9ATP + 6H^+ \rightarrow C_3H_6O_3 - phosphate + 3H_2O + 6NADP^+ + 9ADP + 8P$ [5]<br>Simplified: $CO_2 + 4H^+ + 4e^- \xrightarrow{2NADPH_2, 3ATP} (CH_2O)^{**} + H_2O$ [6] | ↑ |
| RA3 | Photosynthesis | $H^+ + HCO_3^- \xrightarrow{Light, 2NADPH_2, 3ATP} (CH_2O) + O_2$ [5] | ↑ |
| RA4 | $NO_3^-$ uptake | $NO_3^- + 4\ H_2O \rightarrow NH_4^+ + 7OH^-$ ($NH_4^+$ synthesis from $NO_3^-$ in the cell)<br>$16NO_3^- + 140H_2O + 106HCO_3^- + HPO_4^{2-} \xrightarrow{light} \underbrace{C_{106}H_{263}O_{110}N_{16}P}_{biomass} + 138O_2 + 124OH^-$ [7] or<br>$16NO_3^- + 122H_2O + 106HCO_3^- + HPO_4^{2-} + 124H^+ \xrightarrow{light} \underbrace{C_{106}H_{263}O_{110}N_{16}P}_{biomass} + 138O_2$ [8] | ↑ |
| RA5 | $NH_4^+$ uptake | $16NH_4^+ + 106H_2O + 106CO_2 + HPO_4^{2-} \xrightarrow{light} \underbrace{C_{106}H_{263}O_{110}N_{16}P}_{biomass} + 14H^+ + 106O_2$ [7] | ↓ |
| RA6 | Heterotrophic | $(1 + a)CH_2O^{**} + O_2 \rightarrow C(biomass) + (1 + a)H_2O + aCO_2$ [9] | ↓ |

**Table** 2 Stoichiometry of MaB biochemical ionic reactions and effects on the pH. Downward arrows (↓) indicate reduction and upward arrows (↑) imply rise of the pH. In the indexing, R stands for Reaction, C stands for Carbon, B stands for Bacteria, and A stands for Algae. For example, RA means it is a metabolic reaction in the algae.
* Evolution of $CO_2$ through MaB metabolism in the table impose effects on the $CO_2$-bicarbonate-carbonate equilibrium thus alkalinity.
** General formula for carbohydrates as source of organic carbon.
[1] Dahiya and Mohan, (2019); [2] Van Hulle et al., (2010); [3] Anthonisen et al., (1976); [4] Sykes, (1975); [5] Razzak et al., (2013); [6] Richmond, (2003); [7] Eze et al., (2018); [8] Müller et al., (2018); [9] Chojnacka and Marquez-Rocha (2004).

It is noteworthy that many algae strains including *Chlorella* species are capable to adopt concurrent autotrophic and heterotrophic metabolism which make them mixotrophic (Perez-Garcia et al., 2011; Zhang et al., 2016). Mixotrophic metabolism incorporates both reactions RA6 and RA3 in Table 2 simultaneously, therefore the overall influence of mixotrophic cultures on the pH is insignificant (Chojnacka and Marquez-Rocha, 2004). While the bacteria activity was inhibited by the high pH in our MaB culture, the organic carbon (OC) continued to be reduced but with a lesser intensity. This can indicate the mixotrophic metabolism by the algae strain(s), most likely, the *Chlorella* sp. However, during the latter batches of Exp.1B in Article 1, when the FA was in the inhibitory level for the algae, not only decline of OC was interrupted, but OC started to increase probably due to decay of algae cells and release of the organic compounds of the cytoplasm into the medium.

## 2.4. Dynamic of the pH in the MaB Systems

The $CO_2$ production and dissolution MaB cultures takes place through one of the nitrification (Eq. 1), heterotrophic bacteria activity (reaction RB4 in Table 2) and heterotrophic algae activity (RA6). When the produced $CO_2$ is less than the assimilated $CO_2$ through photosynthesis (RA3), bicarbonate ($HCO_3^-$) would be assimilated by algae as an alternative carbon source, leaving $OH^-$ behind that alkalinizes the medium as described in reaction (RC). On the other hand, assimilation of $NH_4^+$ as source of nitrogen (RA5), acidifies the medium by production of H+ in a higher magnitude resulting in overall acidification of algal culture (J. Wang et al., 2019). This was the case in the beginning of every batch when ammonium was not limited. Anyway, the rapid consumption of ammonium by microalgae, heterotrophic bacteria (PB4) and nitrification during day 1, and consumption of $NO_3^-$ (RA4) and carbonate by algae accelerates the $H^+$ removal and increases the pH drastically after the first few hours of illumination on the 2$^{nd}$ day of the batch. Therefore, the general increase of the pH in our experiments is caused by photosynthesis (RA3) when ammonium and $CO_2$ are depleted. This increase intensifies as the alkalinity depletes via photosynthetic and nitrification activities.

According to what explained above, high levels of pH was accountable for unsatisfactory nitrogen removal from municipal wastewater by the MaB. Self-regulation of pH biologically to avoid inhibitory thresholds is very difficult as many interrelated biotic and abiotic operators absorb or release protons in the medium. To avoid intense pH levels in microalgae cultures, a combination of nitrate and ammonium feeding can be used to reduce the pH through ammonium uptake and after that pH increase by means of nitrate uptake would keep the pH close to neutral (Scherholz and Curtis, 2013). This method is based on the fact that microalgae can metabolize both nitrate and ammonium as nitrogen source, leading to consumption and production of $H^+$ respectively (Table 2). However, what Scherholz and Curtis (2013) suggested cannot be applied in a large scale since ammonium is commonly the major source of nitrogen in the wastewater and manipulation of nitrogen composition exogenously is not viable for a large community. Besides, nitrogen uptake by the heterotrophic and autotrophic bacteria destabilizes the nitrogen balance for the algae.

The biological reactions presented in Table 2 affect each other not only through altering the pH, but competition over the nutrients can also cause a reaction to prevail or suppress other reactions. For example, both algae and nitrification consume alkalinity (Table 3) which subjects the culture to more abrupt fluctuation of the pH, whereas algae photosynthesis increases the pH, but nitrification acidifies the medium. Nitrification in absence of algae can drop the pH to below 6 while levels lower than 7.0 can severely depress the nitrification (Ahn, 2006). Algae on the other hand uptakes the carbonates and therefore can sustain the nitrification by maintaining the pH above the inhibitory levels. When the growth conditions are optimal for algae, intense ammonium removal can easily reduce the pH to below 5, while in contrast, nitrate uptake together with carbon fixation can increase the pH to above 10. Photoautotrophic algae like *Desmodesmus* sp. (one of the strains contributing in our microalgae stock) can raise the pH drastically to about 10.5 which can change the concentration of [$H^+$] in the culture intensively (Eze et al., 2018).

As the photosynthesis continues, pH can reach beyond tolerance of NOB and later AOB thus suppresses the nitrification. Since $CO_2$ can be used directly by the algae and form an equilibrium with bicarbonate, maintaining the $CO_2$ (either endogenously or exogenously) can provide buffer protection for extreme pH levels. **Table 3** summarizes the required resources and the products of the MaB process in **Table 2** which includes the metabolic activities that alter the $CO_2$ concentration.

| # | Process | $\mu_{max}$ [$d^{-1}$] | Consumables | Products |
|---|---|---|---|---|
| PC | Aqueous $CO_2$ * | | $CO_2$ | --- |
| **Bacteria-based reactions** | | | | |
| PB1 | Nitritation by AOB | 1.05< $\mu_{max}$[1]<1.14 | $NH_4^+$, Alkalinity, $O_2$ | $NO_2^-$, $CO_2$, $H_2O$ |
| PB2 | Nitratation by NOB | 0.91 < $\mu_{max}$[1]<1.31 | $NO_2^-$, $O_2$ | $NO_3^-$ |
| PB3 | Nitrification (overall) | 0.034 $h^{-1}$ [2]; 0.6-0.8 $d^{-1}$ [3] | $NH_4^+$, Alkalinity, $O_2$ | Biomass, $NO_3^-$, $H_2O$, $CO_2$ |
| PB4 | Heterotrophic | 0.62 $h^{-1}$ [2]; 4.0-8.8 $d^{-1}$ [3] | Organic carbon, $O_2$ | Biomass, $CO_2$, $H_2O$ |
| **Algae-based reactions** | | | | |
| PA1 | Light reaction | --- | $NADP^+$, ADP, P, $H_2O$ | NADPH, ATP, $O_2$ |
| PA2 | Dark reaction | --- | $CO_2$, NADPH, ATP | Glucose, $H_2O$, NADP, ADP, P |
| PA3 | Photosynthesis | --- | $CO_2$, $H_2O$ | Carbohydrate, $H_2O$, $O_2$ |
| PA4 | $NO_3^+$ uptake | | $NO_3^-$,$H_2O$, Alkalinity, $HPO_4^{2-}$ | Biomass, $O_2$ |
| PA5 | $NH_4^-$ uptake | 0.17 [4]; 0.68 [5]; 1.60 [7] | $CO_2$, $NH_4^+$, $H_2O$, $HPO_4^{2-}$ | Biomass, $O_2$ |
| PA6 | Heterotrophic | 0.13 $h^{-1}$ [8]; 1.2 $d^{-1}$ [9] | Organic carbon, $O_2$ | $CO_2$, $H_2O$ |

**Table** 3 MaB biological processes and their respective required resources and produced substances. In the indexing, P stands for Process, C stands for Carbon, B stands for Bacteria, and A stands for Algae.
[1] Munz et al., (2011); [2] Zhang et al., (2009); [3] Metcalf & Eddy, (2003); [4] Eze et al., (2018); [5] Samorì et al., (2013); [6] Krzemińska et al., (2014); [7] Xu and Boeing, (2014); [8] average μ, Heredia-Arroyo et al., (2011); [9] Martínez and Orús, (1991).

The maximum specific growth rate ($\mu_{max}$) of mixotrophic microalgae species is approximately equal to their maximum specific growth rates in heterotrophic ($\mu_{max,H}$) and autotrophic ($\mu_{max,A}$) metabolism combine (Martínez and Orús, 1991). For instance, the mixotrophic $\mu_{max}$ of *Chlorella vulgaris* is reported 0.198 $h^{-1}$, while $\mu_H$=0.098 $h^{-1}$ and $\mu_A$=0.110 $h^{-1}$. Maximum autotrophic specific growth rate of microalgae is usually less than the heterotrophic one (Richmond, 2003). Specific growth rates of the microorganisms in a MaB culture are important factors in the nutrients availability and the pH in a MaB treatment setting. In their comprehensive modeling of a monoculture of microalgae in wastewater supplemented with exogenous $CO_2$, Eze et al. (2018) could develop the equation to anticipate the pH. Regardless of the physicochemical constants, they could show that the pH has a negative correlation with the concentration of inorganic carbon [IC]. However, development of [IC] has a negative correlation with the concentration of microalgae [$M_b$] and concentration of orthophosphate, ammonium, and nitrate ([P],[$NH_4^+$],[$NO_3^-$] respectively). After all, the concentrations of microalgae, ammonium and nitrate have complex relationships with maximum specific growth rates when nitrate and ammonium are available as the source of nitrogen ($\mu_{max\,NO_3}$, $\mu_{max\,NH_4}$ respectively as presented in Table 3). Other algal growth, nutrients uptake, and nitrification kinetics add up to this complexity. However, the model that Eze et al. proposed provides a legitimate accuracy when the external carbon supply is available to balance the pH and provide the inorganic carbon for the microalgae. According to this model, it is suggested that addition of $CO_2$ is the best way to balance the pH by removing the extra $OH^-$ ions. Unfortunately, the kinetics of photosynthetic oxygenation for oxidation of organic carbon by heterotrophic bacteria is neglected in their model (Eze et al., 2018). Otherwise, effect of external $CO_2$ would be replaced with the consumption of organic carbon in the culture by the heterotrophic bacteria with the magnificent maximum specific growth rate (Table 3). However, it was a considerable hypothesis to investigate the effects of the external supplementation of the MaB treatment with $CO_2$ to regulate the pH and feed the algae in absence of enough organic carbon for the heterotrophic bacteria. This was a scenario which we could examine in the Article 2.

## 2.5. The Effects of Organic Carbon in MaB Treatment

Besides $CO_2$, addition of the organic carbon could activate more direct and controlled consumption of ammonium without involvement of nitrification. According to Table 3 (processes PB4 and PA5), the highest specific growth rates belong to combination of heterotrophic bacteria feeding on the organic carbon and ammonium uptake by the microalgae. Both metabolisms reduce the pH and can counter the effect of the light independent reactions (dark reaction, Table 2, RA2) in the algae.

The wastewater we used in Article 1 and Article 2 is considered weak in terms of the organic carbon content (Pereira et al., 2014). Some scholars have reported a remarkable nitrogen removal without any significant impact of nitrification (Pires et al., 2013; Praveen and Loh, 2015; Solimeno and García, 2019). It seems the C:N ratio of the wastewater used in those studies was higher than what used in this study. For example, the ratio of organic carbon to ammonium-nitrogen in Gutzeit's paper (Gutzeit et al., 2005), is 4.3±0.96 (w/w), whereas this ratio was 1.8±0.08 and 1.5±0.06 for the experiments of Article 1. Higher C:N ratio can promote heterotrophic respiration and $CO_2$

production that could lead to more ammonium uptake by algae and bacteria in an oxygen limited condition (Choul-gyun, 2002; X. Liu et al., 2019) in which nitrifying bacteria would be suppressed while the pH and free ammonia would be maintained below inhibitory levels for the MaB activities. When self-regulatory pH mechanisms lack, drastic increase of pH is observed in the MaB cultures with high ratio of inorganic to organic carbon (Van Den Hende et al., 2011).

It is reported that the COD:N ratio of 100:5 is favorable for heterotrophic ammonium oxidizers which have the fastest growth rates in MaB symbiosis (Yang et al., 2004). In Article 2, we showed that in a balanced oxygen production–consumption MaB system, the COD:N ratio (w:w) of about 16 is necessary to eliminate the effect of nitrification and assimilate all the ammonium into the algal and bacterial biomass. This would require introduction of organic carbon (glucose in our case) to the MaB culture. Therefore, Article 2 inspects the influence of exogenous inorganic carbon ($CO_2$) and exogenous organic carbon (glucose) on the nutrient removal capacity of the MaB biocenosis.

According to our results, when $CO_2$ was introduced to the culture to maintain the pH below 8.0, the ammonium uptake remained outstandingly high throughout the 6 sequencing batch reactors (SBR) with 2 days of HRT. Although photosynthesis could actively remove the ammonium, yet the oxygen elevation could let the nitrification progress. Hence, the EUS91 levels could not be achieved within 2 days because altering the nitrogen removal mechanism of the algae would require a longer time to acclimatize from the ammonium to nitrate consumption. Uptake of the nitrate however is an alkalinizing process which demand more $CO_2$ to neutralize its effect (Perez-Garcia et al., 2011)

After addition of supplementary glucose to the MaB culture in the pretreated municipal wastewater, we could observe that the culture conditions on the second day was similar to the first day of treatment when no extra carbon was introduced. That means that the DO concentration remained very low (nearly anoxic) as all the photosynthetic oxygen would be quickly consumed by the heterotrophic bacteria. Meanwhile, no significant production of NOx was measured as long as the concentration of COD remained above 200 mg $L^{-1}$. This could allow the algae and heterotrophs to assimilate the ammonium in the MaB biomass and therefore the concentration of the total suspended solids (TSS) was significantly higher than the samples from the control series. The ammonium was almost entirely consumed when the oxygen started to buildup and that left little room for the nitrification.

Adding organic exogenous organic carbon not only made it possible to achieve the primary objective of achieving the EUS91 within 2 days of treatment, but the removal rates could show that a complete removal of both phosphorus and nitrogen is feasible within 24 hours or even shorter. Fig. 3 demonstrates the overall performance of the MaB treatment and compares the daily removal rates as inorganic or organic sources of carbon are exogenously introduced in the culture. This graphic is also available as the graphical abstract of the Article 2.

| Characteristics of MaB treatment of real wastewater | | 0-24 hours (Day 1) | 24-48 hours (Day 2) | 48-72 hours (Day 3) | Overall treatment performance |
|---|---|---|---|---|---|
| Supplemented with inorganic carbon (ICS)<br><br>+ $CO_2$<br>pH < 8.0 | TN removal | ★★★☆☆ | ★☆☆☆☆ | ★★★★★ | ★★★☆☆* |
| | P removal | ★★★☆☆ | ★★☆☆☆ | ★★★★★ | ★★★★☆* |
| | Organic carbon | ★★☆☆☆ | ★☆☆☆☆ | ★☆☆☆☆ | *after 2 days<br>High DO conc. on day 2 promotes nitrification. Incomplete N removal |
| | DO concentration | ★☆☆☆☆ | ★★★★☆ | ★★★★★ | |
| | Nitrification | ★★☆☆☆ | ★★★★★ | ★☆☆☆☆ | |
| Supplemented with organic carbon (OCS)<br><br>+ Glucose<br>COD:N(ω)≈16 | TN removal | ★★★★★ | | | ★★★★★ |
| | P removal | ★★★★★ | | | ★★★★★ |
| | Organic carbon | ★★★★★ | When the organic carbon to nitrogen ratio is balanced in the wastewater, dissolved oxygen (DO) becomes the limiting factor for nitrification. Maximum biomass uptake of nutrients can make the EU standards of effluent attainable within 24 hours. | | |
| | DO concentration | ★☆☆☆☆ | | | |
| | Nitrification | ★☆☆☆☆ | | | |

**Fig. 3** A qualitative comparison of the MaB treatment in presence of supplementary inorganic and organic carbon. Number of the blue stars indicate the grade of the strength of the respective effect.

## 2.6. Optimizing the OC Supplemented MaB Treatment

As a rule of thumb, when the $F_v/F_m$ is in a healthy range (0.6-0.8) the amount of the added organic carbon should be enough inasmuch that the balanced oxygen consumption and production keeps the culture in anoxic condition. The oxygen should raise when the total nitrogen is below the EUS91 level of 10 mg N $L^{-1}$. In this condition, oxygen production (which is a function of light intensity) would be the main limiting factor for shortening the HRT of organic carbon supplemented MaB culture.

The high concentration of glucose (about 1 g $L^{-1}$) appeared to impose a temporary inhibitory effect on the photosynthesis performance (Liang et al., 2009) which was demonstrated in the measured $F_v/F_m$ ratio. Gradual addition of organic carbon could probably prevent this effect. However, it might be a feasible option of increase the initial algae to activated sludge ratio to generate more oxygen in the light hours. For example, as a practical approach, portions of the total OC supplement can be added intermittently throughout the treatment. The addition of OC portions can initiate once the DO start to elevate above the inhibitory level (about 0.4 mg $O_2$ $L^{-1}$) for the nitrifiers (Ma et al., 2009). Another hypothesis to investigate could be a boosting aeration in the early phase of the culture to initiate a strong cycle of $O_2$–$CO_2$ by letting the bacteria supply the algae with sufficient inorganic carbon. When the culture starts in anoxic condition, the entire pressure of starting the cycle is on the photoautotrophic activity of the microalgae to provide the oxygen for oxidation of the relatively large amount of organic carbon. It is noteworthy that heterotrophic activity of the microalgae can intensify the shortage of oxygen several folds (Flores-Salgado et al., 2021). The only available inorganic carbon for progressing the photosynthesis process is the existing but limited amount of the dissolved $CO_2$ and alkalinity of the wastewater. Therefore, a short but intense aeration of the MaB could activate the heterotrophic bacteria and supply the algae for their required $CO_2$. From the energy standpoint, the electricity for such boosting aeration is incomparable with the extensive energy input of the CAS treatment plants. Since this elevation of oxygen would be required in the beginning of the treatment, the aeration can be done using the gravity through making vertexes and cascades at the inlets of the MaB ponds (Rearte et al., 2021).

Alternatively, improving the light availability (e.g. using mirrors) or extended illumination hours can provide the energy for ammonium and phosphorus uptake through both photoautotrophic and heterotrophic activities. For example, employment of renewable energy (e.g. photovoltaic, wind or biogas from the harvested MaB biomass) to power the high performance light emitting diodes (LED) for illumination at nights can improve the removal rate and decrease the HRT (Borella et al., 2022).

## 2.7. A More Sustainable Approach for OC Supplementation

Application of pure glucose for treatment of wastewater is not an economic and sustainable approach as it would require competition with the food industry. Although the supplemented organic carbon would turn into the digestible and combustible biomass, it is crucial to find more sustainable and inexpensive substitutes for glucose. The sustainable alternatives for glucose should be waste based, highly degradable, and water-soluble material with low turbidity that contain a very high C:N ratio. The COD-rich wastewaters that we used in this research were mixed with the pretreated municipal wastewater to elevate the COD:N ratio to the optimum ratio (w:w) of about 16 as explained in the Article 2. The results of this investigation are not yet published but are presented here briefly.

There are diverse sources of organic waste with high carbon content (Al-Mallahi and Ishii, 2022; Gélinas et al., 2015) that can be used to balance a self-sufficient MaB treatment. We examined the effect of the lawn silage (LS) extract, wastewater from the lautering process of beer brewing (BBL) and glycerol as substitutes for glucose.

The LS is produced from filtration of the liquid which is extracted from the anaerobically digested lawn (silage) as described by Hertel et al. (2015). The BBL is the wastewater that is washed out from filtration of the solid residues of beer brewing. BBL can be reused for mashing the new material but it is normally discarded to avoid undesired decomposition and contamination of the process which would directly influence the taste of the final product (Narziß et al., 2017). The BBL for this experiment was collected from Kehrwieder Kreativbrauerei at Sinstorfer Kirchweg 74-92, 21077 Hamburg. Glycerol is a byproduct of biofuel industry and is normally delivered separably to the WWTP by tankers. The glycerin that was used in this test was collected from WWTP Köhlbrandhöft

(Köhlbranddeich 1) in Hamburg. Glycerin is a valuable substrate to improve the C:N ratio of the biogas slurries at the treatment plant and improves the biogas yield of the anaerobic digestion of the surplus activated sludge (Alves et al., 2020). Low concentrations of glycerol are proven to improve the biomass yield of the mixotrophic microalgae (Liang et al., 2009). Table 4 displays the concentration of the nutrients each wastewater-based OC supplement.

| OC supplement | TSS [mg $L^{-1}$] | VSS [mg $L^{-1}$] | COD [mg $L^{-1}$] | TN [mg $L^{-1}$] | $NH_4^+$-N [mg $L^{-1}$] | $PO_4^{3-}$-P [mg $L^{-1}$] | pH |
|---|---|---|---|---|---|---|---|
| LS | — | — | 71760 | 3411 | 1290 | — | — |
| BBL | 370 | 369 | 46200 | 398 | 18 | 77 | 5.3 |
| Glycerol | 1010 | — | 51500 | 1210 | 209 | 10800 | 3.1 |

**Table 4** The Characteristics of the waste substitutes for glucose as organic carbon supplement for the MaB treatment.

The COD:$NH_4$-N (w:w) of LS, BBL and glycerol are about 56, 2567, and 246 respectively. These ratios are much higher than what we found in the municipal wastewater (usually <10); therefore, they can be considered as candidates for organic carbon supplementation of MaB cultures. Because LS and glycerol contained a considerable amount of nitrogen, in calculation for balancing the COD:N ratio, their ammonium content was taken into consideration. Subsequently, the amount of the supplemented COD for the cultures with LS and glycerol were more than what would be required with glucose. Some details of the experiments to investigate the efficiency of these substitutes for glucose are available in Appendix 3 (section 4.3).

After calculation of required supplements, separate batches of MaB treatment with each substrate were carried out to test the general response of MaB culture to the new organic compounds. Despite other substrates, the primary tests with glycerol showed a sort of inhibition of microalgae with no considerable TAN, COD, or P removal in comparison with glucose (Patel et al., 2019). Besides, since the phosphorus content of the glycerol was notably high, a single step MaB treatment could not remove the excess phosphorus concentration and therefore, the primary objective of the MaB treatment as we aimed in this study could not be realized. Hence, to save time and laboratory resources, we excluded glycerol from further investigations. Testing the pretreated municipal wastewater with supplemented LSW and BBL took place in two batches. The measured treatment parameters are also exhibited in section 4.3.

According to Fig. 4, Fig. 5, Fig. 6, and Fig. 7, the treatment with LS would require a longer time as addition of lawn silage extract would undesirably elevate the nitrogen and phosphorus content of the medium and reaching the EUS91 limits would take a longer time which would negate the purpose of application of OC supplement. In the LS assay, none of the EUS91 thresholds were achieved.

Despite LS series, the BBL (Fig. 7) could showed a promising removal performance. In both batches, the nitrogen and phosphorus in the effluent reached the EUS91 limits before the experiment ended at hour 38., The wastewater from the lautering process of beer brewing (BBL) could remove the P and N about 5-10% slower in comparison with glucose which is a remarkable result to reduce the HRT to less than 2 days. The pH was remained under the inhibitory levels (as a result of quick ammonium uptake) and the settling characteristics of the MaB flocs was outstanding. More than 90% of the biomass was settled within 10 minutes of sedimentation which is comparable with the activated sludge.

The BBL supplement did not lead to any significant nitrification (averagely 0.11 mg $NO_2$–N $L^{-1}$ and 0.84 mg $NO_3$–N $L^{-1}$) in the effluent. This low level of nitrification took place during the last hours of the batch when the DO was elevated due to exhaustion of the organic carbon (COD<200 mg $L^{-1}$). This confirms the reliability of the stoichiometric balance of the C:N ratio that could be maintained through exogenous carbon supply (see Article 2 –section 4.2.2.3.3). Therefore, the wastewater from the beer industry can provide the extra organic carbon which is required to enhance the MaB treatment of the municipal wastewater without any external aeration or post treatment. The bioenergy value of the carbon-rich MaB sludge is out of the scope of this dissertation but it is undoubtedly an interesting subject for further studies.

## 3. Conclusion

Municipal wastewater treatment using microalgae-bacteria flocs has a remarkable potential to be introduced as a sustainable and profitable alternative for the conventional activated sludge process at the wastewater treatment plants. The self-sustaining capacity of the MaB flocs to provide the $CO_2$ for the microalgae is limited in the weak municipal wastewaters (COD<500 mg $L^{-1}$). HRT of 3 days can lead to culture collapse due to pH and free ammonia inhibitions while the HRT of 2 days would result in incomplete nitrified nitrogen removal. The balance of the $CO_2$–$O_2$ should be maintained as long as the nitrogen is above the EUS91. Addition of $CO_2$ could prevent the inhibitions but boosts the nitrification. Two days is not enough to remove the nitrogen content in form of ammonium and nitrates; hence, $CO_2$ supplementation of MaB treatment could not eliminate the nitrogen and phosphorus in less than 2 days but satisfactory results were achieved as $CO_2$ supplementation extended to the third day. Removal of surplus oxygen by heterotrophic activities is the key to eliminate the nitrification and minimize the treatment period. Supplementation of municipal wastewater with exogenous organic carbon can intensify and sustain the heterotrophic consumption of DO while ammonium can be directly employed for microalgae and heterotrophic bacteria biomass production. No carbon dioxide would be released in the atmosphere and no post-treatment of nitrogen or phosphorus would be necessary. The wastewater from the beer brewing industry can sustainably provide the sufficient organic carbon to reach the balanced COD:N ratio of 15.94 that is calculated from the stoichiometry of the balanced heterotrophic-photoautotrophic metabolisms within the MaB flocs.

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# 4. Appendix

## 4.1. Appendix 1, Article 1, Published in the Bioresource Technologies Reports (2022)

### Abbreviations

| | |
|---|---|
| AOB | ammonia oxidizing bacteria |
| CAS | conventional activated sludge |
| DO | dissolved oxygen |
| DON | dissolved organic nitrogen |
| DW | dry weight |
| ECDS91 | European Council Directive standard 91/271/EEC |
| EPS | extracellular polymeric substance |
| Exp.2d | A SBR of 6 batches with 2 days HRT |
| Exp.3d | A SBR of 5 batches with 3 days HRT |
| FA | free ammonia |
| GHG | greenhouse gas |
| HRT | hydraulic retention time |
| IC | inorganic carbon |
| MaB | microalgae-bacteria |
| NOB | nitrite oxidizing bacteria |
| NOx | nitrogen oxides ($NO_2$+$NO_3$) |
| OC | organic carbon |
| p.e. | population equivalent |
| PAM | pulse-amplitude modulated |
| PAR | photosynthetically active radiation |
| PS | photosystem |
| SBR | sequencing batch reactor |
| SD | standard deviation |
| SRT | sludge retention time |
| SVI | sludge volume index |
| TAN | total ammonia nitrogen |
| TC | total carbon |
| TIC | total inorganic carbon |
| TN | total (dissolved) nitrogen |
| TSS | total suspended solids |
| VSS | volatile suspended solids |
| WWTP | wastewater treatment plants |

# Influence of hydraulic retention time on municipal wastewater treatment using microalgae-bacteria flocs in sequencing batch reactors

Hadi Soroosh [a]*, Ralf Otterpohl [b], Dieter Hanelt [a]

**Abstract**

Real wastewater was treated with a consortium of indigenous microalgae and bacteria (MaB) in sequencing batch reactors (SBR) while the hydraulic retention time (HRT) reduced from of 3 days (5 batches) to 2 days (6 batches). Biomass uptake and nitrification were the main ammonium removal pathways (about 96% for both HRTs) and phosphate levels stayed within the European limit of 1 mg P $L^{-1}$. The alkalization due to lack of inorganic carbon triggered free ammonia inhibition and partial nitrification. Consequently, the total nitrogen removal could not satisfy the EU standard. Extreme pH levels (pH>9.5) occurred more frequently in the SBR with HRT of 3 days, leading to culture collapse and biomass washout. Maintaining the pH below 8.0 seems crucial for an efficient treatment. The pH of the SBR with HRT of 2 days was more moderate, making this HRT advantageous for the single-step MaB treatment of weak wastewaters.

**Keywords:** pH inhibition; ammonia inhibition; nitrite accumulation; inorganic carbon; nitrogen removal; nitrification

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### 4.1.1. Introduction

Municipal wastewater contains a variety of biotic and abiotic contaminants which are hazardous to the environment and public health. The European Council Directive standard 91/271/EEC (ECDS91) has set the limits for the concentration of the contaminants that are permitted to be discharged from wastewater treatment plants (WWTP) into the natural water bodies (The Council of the European Union, 1991). Among available technologies, the conventional activated sludge (CAS) process has been widely employed by the European WWTPs to meet these standards.

The CAS process heavily relies on aerobic bacteria activity, which claims 45%–75% of the electricity consumption by the treatment plants for the intense aeration of the activation sludge ponds (Longo et al., 2016). Furthermore, the contribution of CAS systems in global greenhouse gas (GHG) emission is considerable (Boonnorat et al., 2021).

As an alternative, microalgae treatment introduces a more sustainable approach to tackle the drawbacks of CAS treatment (Javed et al., 2019); Microalgae can utilize solar energy to uptake the nutrients in wastewater for photosynthesis and growth. Nevertheless, the degradation of organic matter by microalgae is not as efficient and separation of microalgal biomass from treated wastewater is challenging and economically nonviable (Gerardo et al., 2015).

Biocenosis of microalgae and bacteria (MaB) combines the advantages of microalgae and activated sludge technologies (Quijano et al., 2017). Photosynthesis oxygenation by microalgae eliminates the need for external aeration (Figure 1) and reduces the environmental impact of the entire treatment (Foladori et al., 2018).

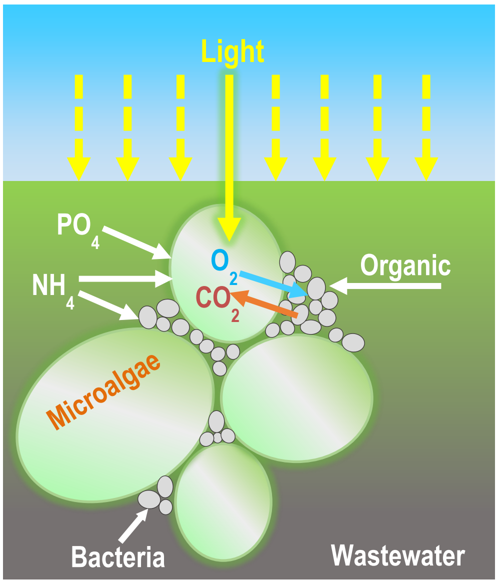


**Figure 1** Schematic of biocenosis of microalgae and bacteria (MaB) in wastewater.

Due to its superior nutrient removal capacity, negligible GHG emission, enhanced bio-flocculation characteristics, and potential for sustainable biofuel production, the MaB concept is receiving increased attention (Boonnorat et al., 2021; Qu et al., 2021).

MaB performance greatly relies on the available solar irradiation to the microalgae, often requiring ponds with relatively large surface areas. This is considered a disadvantage compared to the CAS treatment; therefore, finding solutions that minimize the hydraulic retention time (HRT) is crucial to limit the land use. Typically, CAS treatment requires 1 day of HRT, whereas attaining an adequate MaB treatment within this HRT remains a challenge (Foladori et al., 2018). Most of the available literature report an acceptable treatment performance in synthetic wastewater with HRTs of 3 days and longer. However, synthetic wastewater does not offer the actual biotic and abiotic characteristics of domestic wastewater. In this paper, single-step MaB treatment of real municipal wastewater is compared when the HRT is reduced 33% (from 3 days to 2 days). The primary goal is to use MaB photosynthetic oxygenation to remove nutrients through assimilation into the MaB biomass over a relatively shorter retention time (Olguín, 2012). The results are evaluated against the European standard of ECDS91. According to ECDS91, the limits for the nutrients in the effluent of European WWTPs with population equivalent (p.e.) of greater than 100,000 are 125 mg $L^{-1}$ for chemical oxygen demand (COD), 1 mg P $L^{-1}$, and 10 mg N $L^{-1}$ (The Council of the European Union, 1991).

### 4.1.2. Material and methods

#### 4.1.2.1. Preculture of MaB

##### 4.1.2.1.1. Algae preculture

Nutrients removal qualities and biomass yield of the MaB flocs can be improved when multiple strains of algae are co-cultured (Hena et al., 2015). To obtain indigenous microalgae strains, samples were collected from the activated sludge mixing basin and a secondary clarifier of Seevetal wastewater treatment plant, located south of Hamburg, Germany. Agar plates containing modified BG-11 (Rippka et al., 1979) supplemented with 100 mg $L^{-1}$ penicillin were used during the isolation process. The nutrients composition of the medium follows: citric acid (6 mg/l), ferric ammonium citrate (6 mg/l), EDTA (1 mg/l), $NaNO_3$ (1.5 g/l), $K_2HPO_4.3H_2O$ (41 mg/l), $MgSO_4.7H_2O$ (75 mg/l), $CaCl_2.2H_2O$ (38 mg/l), $Na_2CO_3$ (40 mg/l), HEPES-NaOH (0.5M, pH=7.5, 4.766 g/l) and trace elements of $H_3BO_3$ (2.86 mg/l), $MnCl_2.4H_2O$ (1.81mg/l), $ZnSO_4.7H_2O$ (222 μg/l), $Na_2MoO_4.2H_2O$ (39 μg/l), $CuSO_4.5H_2O$ (79 μg/l), $Co(NO_3)_2.6H_2O$ (49 μg/l).

The plates were kept in an ambient temperature of 19° C with light intensity of 40±5 μmol photons $m^{-2}$ $s^{-1}$. Through a combination of isolation techniques such as atomized cell spray, capillary transfer, and disinfection with antibiotics (Andersen, 2005), single strain axenic microalgae cultures were obtained. To compare the nutrients removal and growth rate performance of the collected algae, each strain was used separately to grow in the sterilized filtered wastewater (100 μm pore size, autoclaved, 121° C: 30 min) in tubular photobioreactors. Five of the most efficient strains in terms of nitrogen removal (> 20 mg N $L^{-1}$ $d^{-1}$) were chosen to contribute in MaB treatment. This was aligned with our goal to decrease the treatment period and to prevent or limit the presence of less productive strains which could increase the self-shading effect and reduce photosynthetic activities. Three strains of *Chlorella* sp. and 2 strains of *Desmodesmus* sp. were identified microscopically (Carl Zeiss MicroImaging, Germany). One strain of *Tribonema* sp. was also added to the consortium after microscopic imagery showed that this filamentous alga can act as a reliable skeleton to strengthen the microalgae and bacteria aggregates in an agitating culture setting.

A mixture of the selected strains with equal mass proportions were then added to sterilized sieved wastewater. The algae cocktail was preserved under a continuous supply of 4% $CO_2$ (0.4 vvm) and a gentle agitation rate of 100 rpm to avoid damage to filamentous cells (*Tribonema* sp.). The ambient temperature of 20° C and light intensity of 85 μmol photons $m^{-2}$ $s^{-1}$ (12h:12h light:dark photoperiods) were maintained.

#### 4.1.2.1.2. MaB stock culture

The ratio of which microalgae and activated sludge (bacteria) are mixed can impact the treatment performance substantially (Su et al., 2012). The MaB biocenosis can be sustained if the oxygen uptake rate by the heterotrophic bacteria is balanced with photosynthetic oxygen production rate by the algae. Their respective stoichiometric metabolisms are presented in equations 1 and 2 (Ebeling et al., 2006):

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^- + HPO_4^{2-} \xrightarrow{light} C_{106}H_{263}O_{110}N_{16}P + 106O_2 \quad \text{(Eq.4)}$$

$$NH_4^+ + 1.18C_6H_{12}O_6 + HCO_3^- + 2.06O_2 \rightarrow C_5H_7O_2N + 6.06H_2O + 3.07CO_2 \quad \text{(Eq.5)}$$

where $C_{106}H_{263}O_{110}N_{16}P$ and $C_5H_7O_2N$ represent the composition of algae (Sedlak, 1991), and heterotrophic bacteria biomass, respectively. After equating (Eq.4) and (Eq.5) based on the oxygen exchange, we have:

$$\frac{\underbrace{C_{106}H_{263}O_{110}N_{16}P}_{algal\ biomass}}{\underbrace{C_5H_7O_2N}_{Bacterial\ biomass}} = \frac{54.91}{33.52} = 1.64 \quad \text{(Eq.6)}$$

According to (Eq.6), the algae to heterotrophic bacteria mass ratio of 1.64 is needed to keep the MaB oxygen consumption and production in equilibrium. Given that the heterotrophic bacteria comprise 30-37% of the total organic matter of activated sludge (Torretta et al., 2014), the mass ratio of the algae to activated sludge in a MaB aggregate should range between 4.4 to 5.5. This ratio is close to what Flores-Salgado et al. (2021) found in their study. The maximum specific oxygen production rate of microalgae is 5.8 ± 3.8 times higher than the maximum specific $O_2$ uptake rate of heterotrophic bacteria. Therefore, the microalgae to activated sludge dried mass ratio of 5:1 was chosen to set up our experiment for optimal nutrient removal in domestic wastewater (Su et al., 2012).

Total suspended solids (TSS) of microalgae mix culture and the activated sludge (freshly collected from Seevetal WWTP) were measured separately using Sartorius infrared moisture analyzer (MA35, Germany). The required sludge to achieve 5:1 mass ratio was calculated and added to 500 ml of algal cocktail mixed in 500 ml of sieved pretreated wastewater. This MaB stock culture was kept in 20 °C, under light intensity of 85 µmol photons $m^{-2}$ $s^{-1}$ (12h:12h photoperiod) on a shaking bench (20 rpm). The MaB stock culture was harvested after 30 min of settlement and the medium was renewed weekly.

#### 4.1.2.2. Measurements

Dissolved oxygen (DO) concentration was measured using a digital oxygen meter (Oxi 323-B/SET, and $O_2$ sensor CellOx 325, WTW, Germany). The pH and temperature were measured with a digital meter (pH 323/SET WTW, Germany). Total suspended solids (TSS), and sludge volume index (SVI) were measured according to APHA (2005). A portable pulse-amplitude modulated (PAM) device (Junior-PAM, Walz, Germany) was used to measure the $F_v/F_m$ ratio, an indicator of the photosystem II (PS II) performance. To do so, measurements took place after the samples rested in darkness for 15 minutes. The average photosynthetically active radiation (PAR) was measured with a digital photometer (LI-250A, LI-COR).

##### 4.1.2.2.1. Chemical analysis

About 100 mL of the culture in each reactor was sampled, centrifuged (2700 rcf, 5 min), and vacuum filtered through nitrocellulose membrane filters (45 µm, MicronSep, Maine Manufacturing, USA) every day. The filtered water was preserved at -19±1 °C if immediate chemical measurements were not possible. Phosphate ($PO_4$-P), nitrite ($NO_2$-N), and nitrate ($NO_3$-N) were measured photometrically using a Hach photometer (DR3900) and the manufacturer's standard cuvettes (Hach, Germany). Total ammoniacal nitrogen (TAN) was measured according to German standard DIN 38 406–E5. Total carbon (TC), inorganic carbon (IC), organic carbon (OC, standard EN 1484) and total dissolved nitrogen (TN, standard EN 12260) were measured using autoanalyzer multi N/C 3000 (Analytik Jena, Germany).

The concentration of free ammonia (FA) was calculated according to Sinha and Annachhatre (2007):

$$\mathrm{FA} = [\mathrm{NH_3 - N}]_{\mathrm{free}} = [\mathrm{TAN}] \times \frac{10^{\mathrm{pH}}}{\exp\left(\frac{6344}{273 + \mathrm{T}}\right) + 10^{\mathrm{pH}}} \qquad \text{(Eq.7)}$$

where [TAN] is the concentration of total ammoniacal nitrogen and T is the temperature (°C).

Ammonium removal efficiency, nitrification rate, and dissolved organic nitrogen (DON) were calculated using the following equations:

$$\text{Ammonium removal efficiency [\%]} = 100 \times \left(1 - \frac{\mathrm{TAN_{end}}}{\mathrm{TAN_{initial}}}\right) \qquad \text{(Eq.8)}$$

$$\text{Nitrification rate [\%]} = 100 \times \frac{\left(\mathrm{NO_{x_{end}}} - \mathrm{NO_{x_{initial}}}\right)}{\mathrm{TAN_{initial}}} \qquad \text{(Eq.9)}$$

$$\mathrm{DON\ [mg\ N\ L^{-1}]} = \mathrm{TN} - \sum \mathrm{N_{inorganic}} = \mathrm{TN} - \left([\mathrm{TAN}] + [\mathrm{NO_2^- - N}] + [\mathrm{NO_3^- - N}]\right) \qquad \text{(Eq.10)}$$

where $NO_x=[NO_2^-]+[NO_3^-]$. $TAN_{initial}$ and $TAN_{end}$ are the concentration of TAN at the beginning and end of the measurement period, respectively. The amount of the free ammonia that strips off the culture is calculated based on what Metcalf & Eddy (2003) proposed:

$$[\mathrm{NH_3^{\uparrow} - N}] = \frac{[\mathrm{FA}]}{1 + \frac{[\mathrm{H^+}]}{\mathrm{K_a}}} \qquad [\mathrm{H^+}] = 10^{-\mathrm{pH}} \qquad \mathrm{K_a} = 10^{-\left(0.0897 + \frac{2729}{\mathrm{T[K]}}\right)} \qquad \text{(Eq.11)}$$

$[NH_3^{\uparrow}$-N] is the portion of free volatilizable ammonia that can leave the medium through off-gassing from the free surface of the culture.

#### 4.1.2.1. Statistical analysis

Real Statistics add-in (Release 7.7.2) for Microsoft Excel 365 was used for statistical analysis. To test the normality of our data, Shapiro-Wilk and d'Agostino-Pearson tests were performed. F-test was performed for homogeneity of variance. To investigate the significance of difference between pairs, based on the dataset conditions, either of *t*-test (parametric) or Mann-Whitney test for independent samples (non-parametric) were performed. To evaluate the correlation between two datasets, either of Pearson (parametric) or Spearman (non-parametric) coefficients were calculated. Two-tailed tests with alpha = 0.05 were implemented unless otherwise is stated. The uncertainties in the reported values are the standard deviation of the sample from its mean value.

#### 4.1.2.2. Experiments' design and setup

Cylindrical glass bioreactors (2L, d =130 mm, H=200 mm) were used in triplicates to treat 1.5 L of sieved (200 μm), pretreated municipal wastewater. The wastewater was collected from the effluent of primary clarifier of Seevetal WWTP. Initially, 1500 mL of sieved wastewater was inoculated with our MaB stock culture to obtain the biomass concentration of about 500 mg DW $L^{-1}$ in each reactor. The treatment was carried out in sequencing batch reactor (SBR) culture mode, consisting of a series of batches when the biomass from previous batch is used to inoculate the wastewater (as new medium) of the subsequent batch. The harvest was carried out using conical separatory glass funnels. After 30 minutes of gravity settlement, the biomass would be separated and used for the next batch. To investigate the influence of hydraulic retention time (HRT), two SBR experiments with HRT of 2 and 3 days were carried out which are called Exp.2d and Exp.3d, respectively. Exp.3d consist of 5 sequencing batches of 3 days each (15 days in total), and Exp.2d was conducted for 12 days, comprising an SBR of 6 batches of 2 days HRT.

Magnet stirrers with speed of 180 rpm were used to agitate the reactors. No external aeration nor $CO_2$ feeding were applied; therefore, pH could fluctuate biochemically. Irradiation of 140±3 μmol photons $m^{-2}$ $s^{-1}$ (from top) by means of 5×40W white fluorescent lamps (Pracht PPR SR E 100/3

Leuchte AVUS, Germany) was employed. The overall chemical properties of the wastewater used per experiment are presented in Table 1.

| Assay | HRT | pH | $PO_4$-P | $NO_3$ | $NO_2$ | $NH_4$ | TN | TSS | VSS | COD | TIC | TOC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | day | – | mg P $L^{-1}$ | mg N $L^{-1}$ | | | | g $L^{-1}$ | g $L^{-1}$ | mg $L^{-1}$ | mg C $L^{-1}$ | |
| Exp.3d | 3 | 7.09 | 7.55 | 0.24 | N.D. | 45.3±0.94 | 51.8 | 0.224 | 0.188 | 255 | 95.1±2.70 | 82.4±3.36 |
| Exp.2d | 2 | 7.12 | 7.26 | 0.31 | N.D. | 52.2±1.49 | 58.1 | 0.215 | 0.194 | 242 | 93.8±1.91 | 76.6±2.12 |

**Table 1** Wastewater characteristics for each experiment. N.D: Not detectable–below detection level.

### 4.1.3. Results and discussion

The assay with 2 days HRT (Exp.2d) was more successful in terms of nutrients removal rates and treatment stability compared to Exp.3d with 3 days of HRT. During Exp.3d, the culture quality degraded gradually to the collapse point after 12 days. After 4 batches, the flocs started to disintegrate and a considerable quantity of algae cells (about 140 mg TSS $L^{-1}$) was released, suspended, and washed out. The culture showed no meaningful sign of recovery, thus Exp.3d was terminated after 5 sequencing batches (15 days).

#### 4.1.3.1. Nitrogen removal

During the first 2 batches of Exp.3d, ammonium removal was complete, but it declined gradually to 91.8±2.2% with 4.0±0.8 mg $NH_4$-N $L^{-1}$ $d^{-1}$ in the effluent of the last batch. The concentration of ammonia and dissolved organic nitrogen increased as the abrupt breakage of aggregates was observed on the fifth batch (Table 2). The ammonium removal in Exp.2d was also complete during the first 3 batches and decreased slowly to 89.2±1.2% afterwards.

| | HRT | Daily removal | | | Average removal | | Final conc. |
|---|---|---|---|---|---|---|---|
| | [day] | Day1 [%] [mg N $L^{-1}$ $d^{-1}$] | Day 2 [%] [mg N $L^{-1}$ $d^{-1}$] | Day 3 [%] [mg N $L^{-1}$ $d^{-1}$] | [mg N $L^{-1}$ $d^{-1}$] | Percent | [mg N $L^{-1}$] |
| **Ammonium removal** | 3 | 46.1±3.3% | 33.4±4% | 16.5±3.1% | 45.8±0.5 | 96±2.1% | 1.9±0.8 |
| | | 22±1.6 | 15.9±1.7 | 7.9±1.2 | | | |
| | 2 | 51.9±3% | 44.3±3.3% | N/A | 46±0.6 | 96.2±1.4% | 1.8±0.6 |
| | | 24.9±1.4 | 21.2±1.5 | | | | |
| **Total nitrogen removal** | 3 | 38.2±3.3% | 16.4±4.4% | 9.2±4.2% | 35.8±1.6 | 63.9±3% | 20.2±1.5 |
| | | 21.4±1.8 | 9.2±2.5 | 5.2±2.3 | | | |
| | 2 | 40.4±4% | 18.1±4.8% | N/A | 33±1.6 | 58.5±3% | 23.4±1.6 |
| | | 22.8±2.3 | 10.2±2.7 | | | | |

**Table 2** Daily and overall ammonium and total nitrogen removal in Exp.3d (HRT=3 days) and Exp.2d (HRT=2 days). N/A: Not applicable.

In general, ammonium/ammonia (TAN) removals throughout both experiments were similarly high (about 96%) but the average daily ammonium removal rate in HRT=2d (23.0±5.4 mg TAN $L^{-1}$ $d^{-1}$) was more significant than the HRT=3d (16.3±6.7 mg TAN $L^{-1}$ $d^{-1}$, $p<0.001$). This indicates that the majority of the ammonium removal took place during the first 2 days of the batch, when the culture conditions were more favorable for microalgae-bacteria activities.

Like TAN removal, elimination of total nitrogen (TN) was higher in the beginning of both Exp.3d and Exp.2d and decreased as the experiment progressed; nevertheless, TN removal was not nearly as high as ammonium removal in either of the experiments. The untreated ammonia and organic nitrogen released from the dead cells could be responsible for the higher leftover TN during the last batches of 3 days of HRT in Exp.3d (Mitra et al., 2012). The average TN concentration in the effluents of SBRs with HRT of 3d and 2d were 22.3±1.4 and 22.9±1.2 mg N $L^{-1}$, respectively, which are above the limits demanded by the ECDS91 (Figure 2).

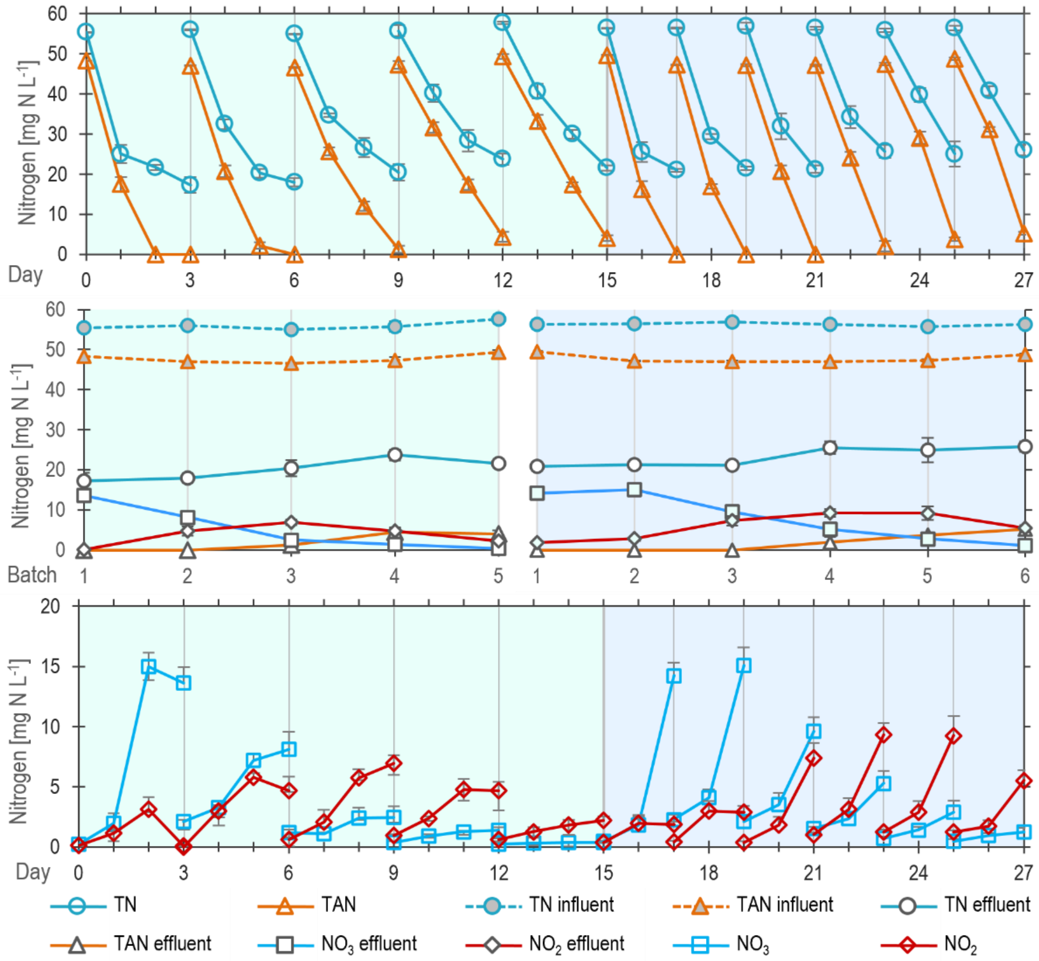


**Figure 2** Profiles of nitrogen. Light green shades belong to the SBR of 5 × 3 days (Exp.3d) and light blue shades belong to the SBR of 6 × 2 days (Exp.2d). Top: Profiles of total nitrogen (TN) and total ammoniacal nitrogen (TAN). Middle: Initial and final concentrations per batch. Bottom: variations of nitrite ($NO_2$) and nitrate ($NO_3$). Vertical lines indicate the batch boundaries. Error bars represent the standard deviation (N= ±SD).

The increasing concentration of nitrogen oxides ($NO_x$) in the effluent indicates that nitrification is accountable for the considerable difference between TAN and TN removals. Huang *et al.* (2015) reported that due to nitrification, their TN removal was limited to 40.7–45.4%. This rate is slightly less than what is achieved here. Nitrification by means of photosynthetic oxygenation can offer a sustainable alternative for mechanical aeration, but it would require denitrification as an extra treatment step to reach the ECDS91 levels. The scope of this study is to investigate the possibility of optimal nutrient removal through a single stage treatment. This would necessitate not the conversion to nitrates, but assimilation of ammonium into MaB biomass (Flores-Salgado et al., 2021).

As demonstrated in Figure 2, the rate and amount of nitrification were not persistent throughout the assays. Nitrite ($NO_2^-$) oxidation to nitrate ($NO_3^-$) was maximal during the first batch in both experiments but decreased as the culture condition changed adversely for the nitrite oxidizing bacteria (NOB). These conditions were more extreme when HRT was 3 days. As a result, the nitrite concentration increased gradually. The production of both nitrate and nitrite ($NO_x$) were relatively lower towards the end of the experiments (Figure 2), which shows that in addition to NOB, ammonia oxidizing bacteria (AOB) were also inhibited.

In presence of both ammonium and nitrates in the medium, microalgae prefer the ammonium as the source of nitrogen because ammonium metabolism requires less energy. Existence of ammonium inhibits transportation of nitrates into the algae cells; hence, it is only after the exhaustion of ammonium that algae can start to assimilate the products of nitrification (Scherholz and Curtis, 2013). In both assays, it took about 2 days for the ammonium to be consumed (equivalent to 45-50 mg $NH_4^+$-N $L^{-1}$). This was the reason that a significant part of the nitrogen in the effluents were in the form of nitrified ammonium before the inhibitions occurred (Figure 2).

#### 4.1.3.2. pH and ammonia

In the algal cultures with limited alkalinity and supply of $CO_2$, increase of the pH is inevitable. Assimilation of $CO_2$ through photosynthesis is known to be the major contributor in alkalization of the algal cultures. Ammonium removal helps the pH reduction, but its influence decreases as the ammonium uptake rate declines gradually. Elevation of pH stimulates the formation of FA in the equilibriums $NH_4^+ \rightleftarrows NH_3 + H^+$ with pKa of 9.25 at 25°C (J. Wang et al., 2019). Both pH and free ammonia (FA) can suppress MaB metabolism and activities (Chai et al., 2021).

In our experiments, the pH was raised to rather alkaline ranges which caused drastic variations in FA concentration. While the range of the pH on the first day of treatment stayed semi-neutral (between 7.5-8.0 mutually), the average pH raised to 8.97±0.20 and 8.89±0.15 on the second days of Exp.2d and Exp.3d, respectively (Figure 3). Extending the treatment to the third day (in Exp.3d), let the culture become even more alkalinized, with an average pH of 9.72±0.16. The pH of the SBR with HRT of 3 days was significantly higher than the assay with 2 days of HRT ($p$<0.001). Consequently, the overall FA concentration in Exp.3d was significantly higher (Figure 3) than the average in Exp.2d ($p$<0.05). The concentration of FA is calculated according to (Eq.7).

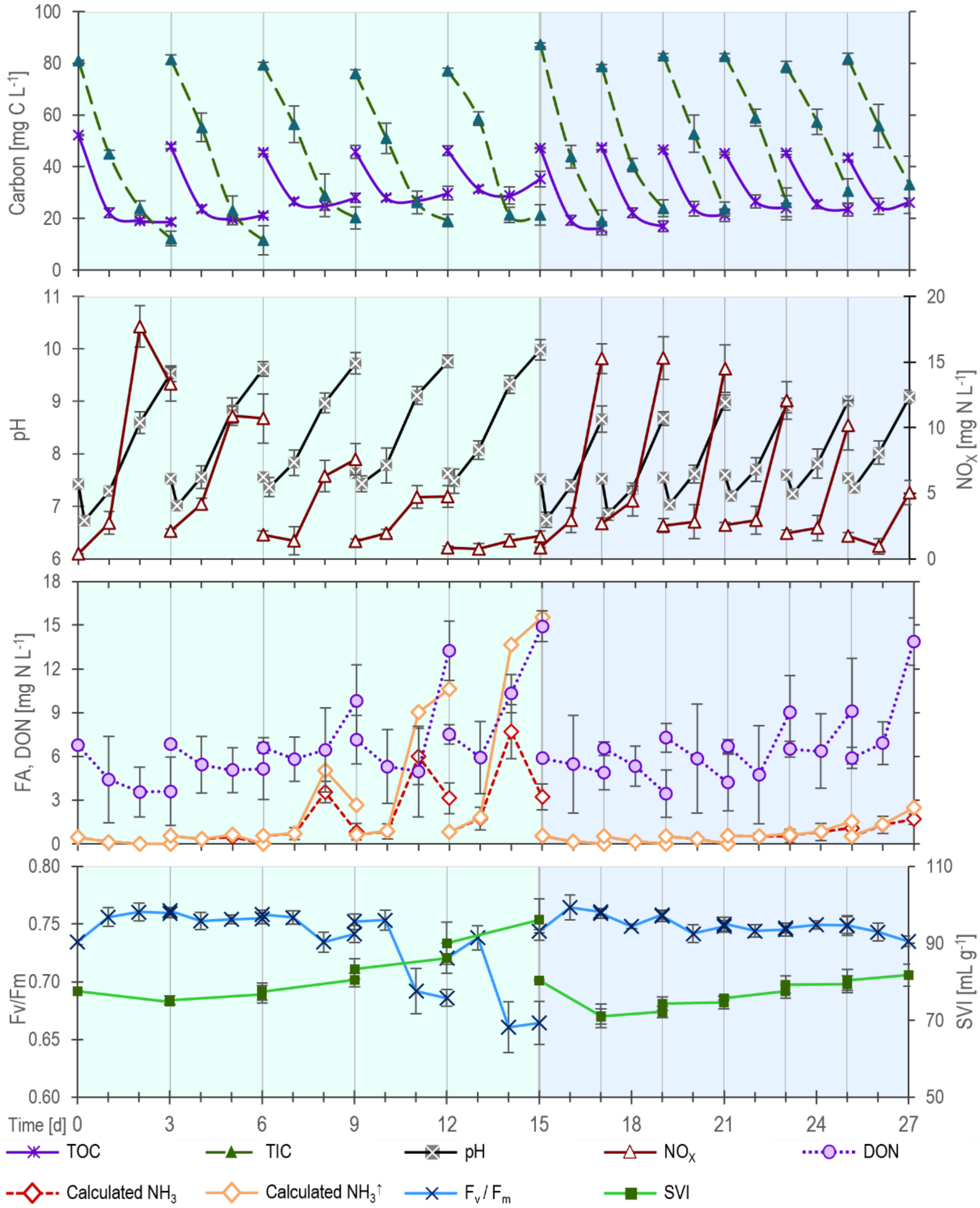


**Figure 3** The effects of pH and free ammonia (FA, $NH_3$) on carbon, nitrified ammonium ($NO_x$), dissolved organic nitrogen (DON), $F_v/F_m$, and the sludge volume index (SVI). Light green shades belong to the SBR of 5 × 3

days (Exp.3d) and light blue shades belong to the SBR of 6 × 2 days (Exp.2d). Vertical lines indicate batch boundaries. Error bars represent the standard deviation (N= ±SD).

In a MaB treatment, oxidation of organic carbon by the heterotrophs provides the required $CO_2$ for algae. Notwithstanding, the total organic carbon content of our wastewater was not relatively low (75–85 mg C $L^{-1}$). Moreover, 20–30% of the organic content of municipal wastewater is not biodegradable (Metcalf & Eddy, 2003). Low concentrations of biodegradable organic carbon can limit the respiration of the heterotrophic bacteria and also inhibit the $CO_2$ production in weak municipal wastewaters. About 90% of the consumed total organic carbon (TOC) per batch were consumed on the first day of treatment, comprising 44±5 and 48±5% of the TOC in the wastewater for Exp.2d and Exp.3d, respectively (Figure 3). As a result, the $CO_2$ supply was restricted after the first day. When $CO_2$ is consumed, bicarbonate ($HCO_3^-$) and carbonate ($CO_3^{2-}$) in the equilibrium make up the shortage by absorbing the hydrogen ion ($H^+$), thus the pH increases even to highly basic (pH>10) levels (Richmond and Hu, 2013). When the production of $CO_2$ is less than its consumption, increase of pH is expected. After the first day of treatment, pH was usually lower during the dark hours due to respiration, but it would increase rapidly during the illumination periods.

Beside $CO_2$ deficiency, the scarcity of alkalinity also facilitates the excessive increase of the pH in a MaB treatment. Alkalinity provides a buffer to moderate the drastic fluctuations of the pH. Extreme pH levels (as high as 11) can be found where this capacity is depleted (Richmond and Hu, 2013). Nitrifiers, heterotrophs, and microalgae compete over alkalinity. Among them, the autotrophic bacteria (nitrifiers) metabolism is more alkalinity demanding (Ebeling et al., 2006). To oxidize 1.0 g of $NH_4^+$–N, 7.05 g of alkalinity in form of $CaCO_3$ is required for nitrification, whereas this value is 3.13 g and 3.57 g for microalgae and heterotrophic bacteria, respectively. Therefore, the role of nitrification in alkalinization of MaB treatment is not neglectable.

Although formation of FA depends on the pH level, its concentration in the medium correlates also with the amount of untreated ammonium and temperature. Free ammonia is volatile and in a basic environment (pH>9), ammonia can leave the culture through stripping from the free surface of the reactor (Metcalf & Eddy, 2003). For example, more than 70% of the FA at the end of the last two batches of Exp.3d could be volatile (Figure 3). Therefore, part of the nitrogen removal in our treatment was through off gassing, which has severe environmental risks in a large scale treatment (Van Damme et al., 2018).

#### 4.1.3.3. Inhibitions

Different growth and metabolic inhibitions were observed as the pH and the FA concentration increased in the medium. In the last two batches of Exp.3d, a drastic drop of $F_v/F_m$ indicated that the PSII functionality was inhibited and therefore, photosynthesis was suppressed. This could be the reason for the low capability of the algae for nutrients uptake and biomass growth toward the end of the experiment. The $F_v/F_m$ and pH in Exp.3d showed a significant correlation (n= 60, rho= -0.37, $p$<0.01) but no such correlation was found in Exp.2d (Figure 3). However, the relationship between the increase of FA concentration and decline of $F_v/F_m$ was more considerable in both experiments (Exp.3d: n=60, rho= -0.69, $p$< 0.001; Exp.2d: n=54, rho= -0.40, $p$< 0.01). Free ammonia ($NH_3$) is toxic at relatively low concentrations. It has been shown that FA concentrations of 1.2 mM (16.8 mg $NH_3$-N $L^{-1}$) in cultures of green microalgae could lead to 50% and 90% inhibition of photosynthesis respectively (Azov and Goldman, 1982). FA can damage the photosystem I and II (PSI, PSII), electron transfer chain, oxygen evolution complex, and dark respiration (Markou et al., 2016). This effect seems to be irreversible as toxic levels of $NH_3$ can repress the synthesis of proteins which are necessary for repairing the damaged PSII (J. Wang et al., 2019). This can explain the poor biomass production after the FA increase during the late batches of Exp.3d.

The effects of increasing pH and FA concentration in the bacterial community was manifested in the earlier stages of the SBR. As the pH raised to about 8 (FA concentration about 0.5 mg $NH_3$–N $L^{-1}$), the NOB showed signs of inhibition. Meanwhile, the rate of ammonium oxidation (nitrite production) remained uninterrupted, therefore nitrite started to accumulate. Higher alkaline environment (pH>9.0) induced a total inhibition on nitrification. This accords with the account of Sinha and Annachhatre (2007) who stated that NOB inhibition occurs when pH is between 8.0-8.5

while on the contrary, AOB are robust toward this range of pH. Figure 2 shows that the pH–inhibited nitrifiers have a limited capacity for recovery (Lashkarizadeh et al., 2016). The inhibition was more dramatic as the alkaline condition persisted more frequently in Exp.3d with 3 days HRT. Shorter HRT of 2 days helped prevent extreme pH occurrences for a longer period. Since both intensity and extension of high pH during Exp.3d were significantly greater than Exp.2d, accumulation of $NO_2^-$ was more considerable when HRT was 3 days ($p<0.01$).

Similar to the pH toxicity, NOB and AOB were repressed as the FA concentration increased (Figure 3). Significant correlations between FA and $NO_x$ production rate were detected (for HRT=3d: $p<0.01$, rho=-0.33; HRT=2d: $p<0.001$, rho=-0.47). Free ammonia in the range of 10–150 mg $NH_3$–N $L^{-1}$ is inhibitory to AOB while only 0.1-1.0 mg $NH_3$–N $L^{-1}$ is enough to inhibit the NOB (Anthonisen et al., 1976). Different levels of sensitivity of nitrifiers against the pH and FA increase, leading to partial nitrifications which promotes the nitrite build up in the reactor.

Nitrite accumulation is associated with inhibitory effects on the nitrogen absorption by the algae. The amount of nitrite concentration and the nitrogen removal rate of the algae in the MaB cultures were correlated ($p<0.05$, R=-0.37 for HRT=3d and R=-0.36 for HRT=2d), which is in agreement with what González-Camejo et al. (2020) stated. In their report, up to 32%, 42% and 80% of the algal nitrogen removal decreased as the $NO_2^-$ concentration increased to 5, 10 and 20 mg N $L^{-1}$ respectively. In this study, the nitrite concentration was in range of 5–10 mg $NO_2^-$–N $L^{-1}$ on several occasions (Figure 2). Accumulated intracellular nitrite induce inhibitory effects on growth rate and photosynthesis of microalgae (Chen et al., 2009). Moreover, nitrite build up to above 5 mg $NO_2^-$–N $L^{-1}$can have a role in disruption of algae cell membrane and culture failure (W. Chen et al., 2011). During both experiments, lysis of cells (manifested in increase of TOC) was observed, but it was critical in the culture with HRT of 3 days with higher overall concentration of nitrite. Nitrite toxicity can be a major reason that the nitrified ammonium could not be efficiently removed by the microalgae, otherwise, reaching the ECDS91 threshold of 10 mg N $L^{-1}$ in the effluent would be easily feasible.

#### 4.1.3.3.1. Culture collapse

After 3 batches through Exp.3d (HRT of 3 days), the average pH was about 9.5-10 and the MaB aggregates began to disintegrate and wash out. The clear supernatant after the harvest of the early batches turned into a green cocktail of suspended single algae cells (mainly *Chlorella* sp.) during the last two batches (see the supplementary material). At the last batch, about 24% of the biomass remained suspended after 30 minutes of settlement and washed out. This value was about 6% at the end of the first batch.

The poor flocculation quality of MaB in high pH manifested itself in the increase of sludge volume index (SVI) which defines as the ratio of the sludge volume to the TSS. In both experiments, SVI increased as the pH raised to highly alkaline (pH>9.5), a characteristic of the treatment with HRT of 3 days. Significant correlations between the pH and SVI in Exp.3d ($p<0.05$, rho=0.42) and lack of such relationship in Exp.2d ($p>0.05$) confirms that the combination of high pH and increase of the free ammonia concentration (also associated with alkalinization) could reduce the settleability of the biomass considerably (Figure 3). The overall SVI in Exp.3d was significantly higher than Exp.2d ($p<0.01$).

Lower settleability, shortened the sludge retention time (SRT) from about 7.8 days in the beginning of the SBR, to about 4.1 days at the end of the last batch (47% more washout). This can be related to the amount of extracellular polymeric substances (EPS) in the culture. It is reported that a pH above 8.5, in combination with $CO_2$ scarcity weakens the EPS bounds and reduces the hydrophobicity of MAB aggregates leading to disintegration of MaB flocs (Arcila and Buitrón, 2017). This condition commonly occurred on the third days of treatment (Exp.3d). Correspondingly, when the FA builds up in the basic environments, the concentration of EPS and hydrophobicity of the aerobic bacteria decreases, which is associated with breakage of the bonds between the microalgae and bacteria (Lashkarizadeh et al., 2016; Y. Liu et al., 2019). The bacteria in MaB excrete EPS which has an essential role in formation of the MaB flocs. EPS functions as a protective layer for cell membrane-bonding enzymes. Degradation in EPS can cause disintegration of cell membrane, cell lysis and release of intracellular content.

An increasing amount of dissolved organic nitrogen (DON) was measured in the medium as the ammonia concentration increased above 3.5 mg $NO_3$-N $L^{-1}$ in Exp.3d (Figure 2 and Figure 3). The correlations between DON and FA in both experiments were significant (Exp.3d: n=60, rho=0.60, $p<0.001$; Exp.2d: n=54, rho=0.64, $p< 0.001$). Release of cell contents from dead MaB organisms reduced the TN removal as the FA concentration increased.

#### 4.1.3.3.2. Inhibition and treatment

According to our results, more than 2 days of MaB treatment of weak wastewaters does not promote a complementary nitrogen uptake by the algae. Instead, longer HRTs can alkalize the culture to critical points. High pH can initiate a domino of inhibitions which reduces the treatment capacity of the MaB culture considerably. Figure 4 summarizes these effects as the pH level increase.

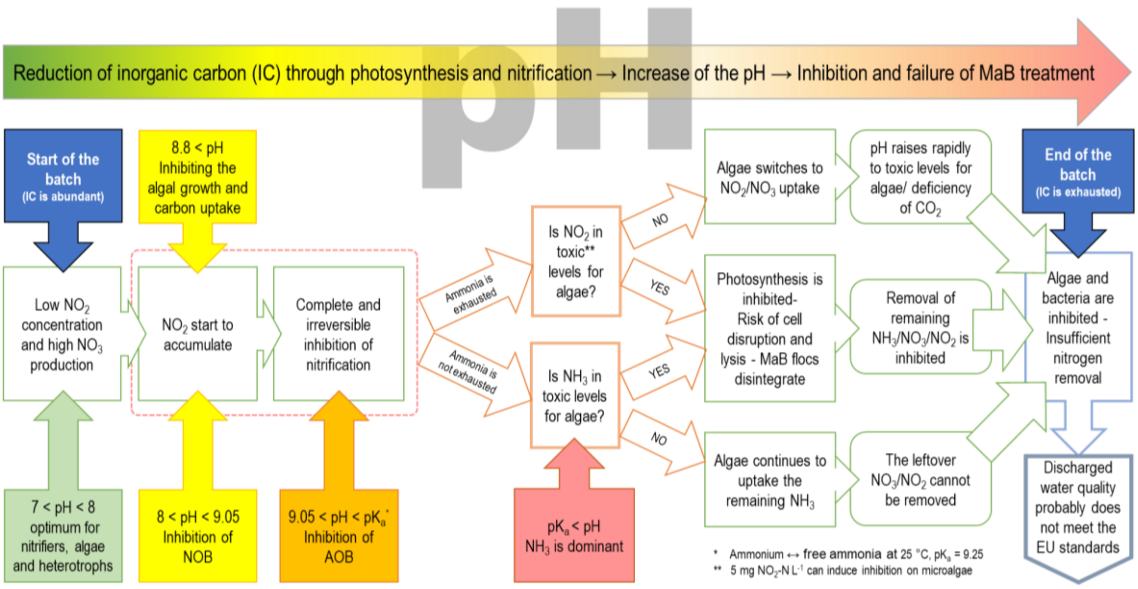


**Figure 4** The schematic diagram of inhibitory effects of rising pH on MaB treatment.

Keeping the pH below 8.0 seems to offer a safe margin for MaB organisms, enabling the treatment to meet the ECDS91 nitrogen limits withing 2 days or potentially a shorter period.

Hypothetically, these scenarios can reduce the nitrogen to ECDS91 levels within 2 days of HRT: a) the ammonia is completely assimilated by the photoautotrophic algae and heterotrophic bacteria, while the autotrophic nitrifiers are inhibited (e.g. via oxygen deficiency); or b) if nitrification is unavoidable, a rapid and complete oxidation of the remaining ammonium to nitrate enables the algae to uptake the produced nitrate in absence of ammonium and nitrite inhibition. Regulating the pH to below 8.0 by means of $CO_2$ injection can offer a meritorious sustainable solution with the latter approach (Chia et al., 2021). This will also prevent emission of ammonia in the atmosphere through off-gassing.

#### 4.1.3.4. Phosphorus removal

The phosphorus removal was almost complete due to both biomass uptake and precipitation as the pH raised (see the supplementary material). Phosphorus removals of 99±1% and 95±3% were achieved in Exp.3d and Exp.2d respectively. In total, the phosphorus concentration in the effluent of sequencing batches of Exp.3d (HRT= 3 days) and Exp.2d (HRT= 2 days) were 0.1±0.04 and 0.37±0.23 mg $PO_4$-P $L^{-1}$, which is below the limit set by the ECDS91 (1.0 mg P $L^{-1}$).

Biological phosphorus uptake seems to be the dominant pathway for the phosphate removal on the first day of every batch-treatment as the pH was close to neutral (average pH of 7.55). Nevertheless, elevation of pH level facilitates the pH removal through precipitation. A significant correlation between the pH and phosphorus content of both Exp.3d was detected (n=60, rho=-0.87, $p$<0.001) and Exp.2d (n=54, rho=-0.68, $p$<0.001). Our results confirm speciation and saturation-index (SI) formulation that demonstrates the relationship between the pH and phosphorus precipitation (Song et al., 2002). Dissolved phosphorus with concentrations above 0.29 mg P $L^{-1}$ at pH=8 and 0.06 mg P $L^{-1}$ at pH=9 is subjected to oversaturation and instant precipitation. Biological and chemical phosphorus removal over the first two days left little room for further removal on the third day in Exp.3d.

### 4.1.4. Conclusion

The European standard regarding TN could not be satisfied due to complete and partial nitrification. High pH and FA levels initiated a series of inhibitions in MaB community which eventually led to disintegration of the flocs in Exp.3d after 12 days. Compared to HRT=2 days, inhibitory impacts were more persistent in HRT=3 days. The inhibition of NOB resulted in nitrite accumulation which can inhibit the algae. We can conclude that HRT of 2 days is advantageous over 3 days of HRT in terms of pH regulation.

### 4.1.5. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### 4.1.6. Acknowledgement

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### 4.1.7. Supplementary material

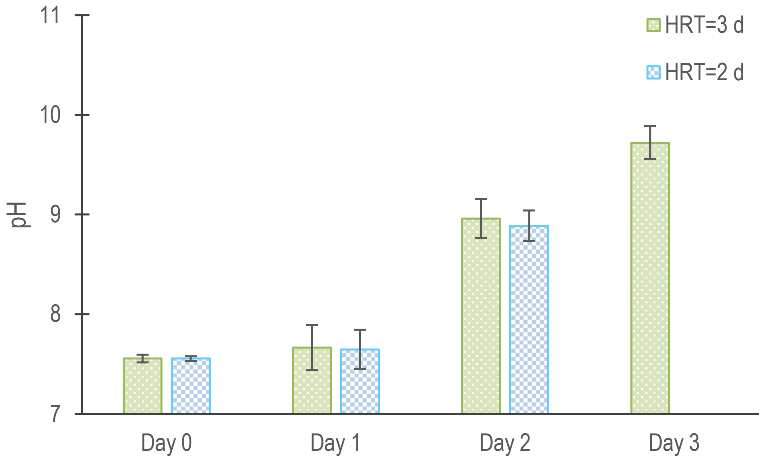


**Figure 5** Average pH in Exp.3d with HRT=3 days and Exp.2d with HRT=2 days. N= ±SD

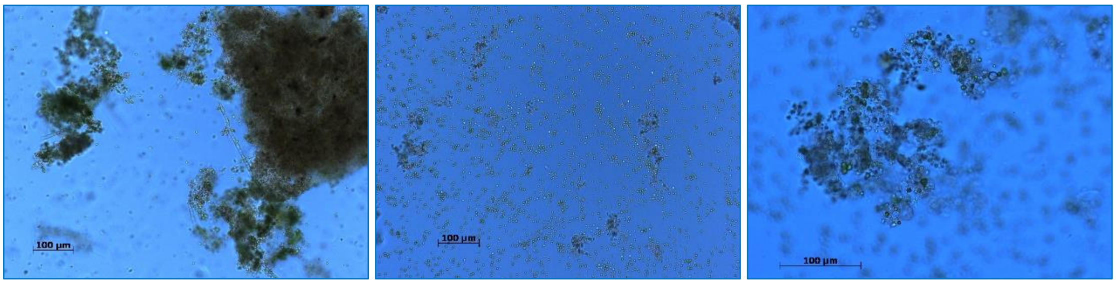


**Figure 6** Microscopic pictures of the cultures of MaB. At the end of the first batch of Exp.3d with 3 days of HRT (left); at the end of the 5th batch of Exp.3d (middle); at the end of the 6th batch of Exp.2d (right).

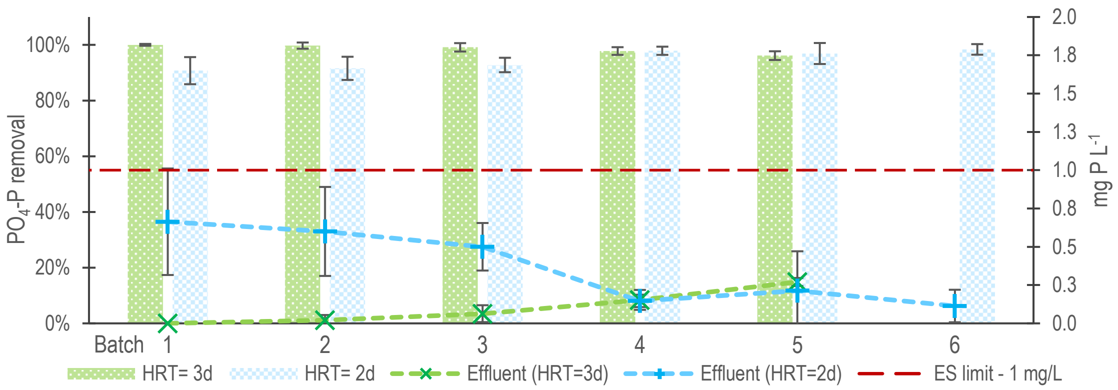


**Figure 7** Phosphorus removal performance of MaB treatment in Exp.3d and Exp.2d with HRT of 3 days and 2 days, respectively. ECDS91: The limit set by the European standard 91/271/EEC for the concentration of phosphorus in the effluent of WWTPs. N= ±SD

### 4.1.8. References


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## 4.2. Appendix 2, Article 2, Published in the Journal of Water Process Engineering (2022)

### Abbreviations

| | |
|---|---|
| AOB | ammonia oxidizing bacteria |
| CAS | conventional activated sludge |
| CS | control series |
| COD | chemical oxygen demand |
| DO | dissolved oxygen |
| DW | dry weight |
| EUS91 | European Council Directive standard 91/271/EEC |
| EPS | extracellular polymeric substance |
| FA | free ammonia |
| GHG | greenhouse gas |
| HRT | hydraulic retention time |
| IC | inorganic carbon |
| ICS | inorganic carbon supplement (experiment) |
| $ICS_{2d}$ | ICS experiment with 2 days of HRT |
| $ICS_{3d}$ | ICS experiment with 3 days of HRT |
| MaB | microalgae-bacteria |
| NOB | nitrite oxidizing bacteria |
| NOx | nitrogen oxides ($NO_2$+$NO_3$) |
| OC | organic carbon |
| OCS | organic carbon supplemented (experiment) |
| p.e. | population equivalent |
| PAM | pulse-amplitude modulated |
| PAR | photosynthetically active radiation |
| PS | photosystem |
| SBR | sequencing batch reactor |
| SD | standard deviation |
| SI | saturation index |
| SRT | sludge retention time |
| SVI | sludge volume index |
| TAN | total ammonia nitrogen |
| TC | total carbon |
| TIC | total inorganic carbon |
| TN | total (dissolved) nitrogen |
| TSS | total suspended solids |
| VSS | volatile suspended solids |
| WWTP | wastewater treatment plant |

# Influence of supplementary carbon on reducing the hydraulic retention time in microalgae-bacteria (MaB) treatment of municipal wastewater

Hadi Soroosh [a*], Ralf Otterpohl [b], Dieter Hanelt [a]

**Abstract**

Real municipal wastewater was treated using microalgae and bacteria (MaB) flocs with the goal to achieve the European standards (EUS91) in less than 2 days of hydraulic retention time (HRT). The pH in the control series (CS) was allowed to increase freely. In the inorganic carbon supplement (ICS) series, the pH was maintained below 8.0 by means of $CO_2$ injection. The presence of $CO_2$ regulated the nitrogen profiles, but the total removal did not improve. Nitrification was stronger and advanced more when $CO_2$ was applied during 2 days of HRT with an average ammonium oxidation rate of 40.5±4.9%. The residual phosphate and nitrogen oxides were assimilated on the third day of treatment. Balancing the organic carbon (COD:$NH_4$–N ratio of 15.9) suppressed the autotrophic activities (nitrification) and improved the biomass assimilation significantly. Batch experiments with balanced organic carbon could remove nitrogen and phosphorus with rates of 43.1±5.1 mg N $L^{-1}$ $d^{-1}$ (88.3±13.9%) and 6.21±0.07 mg $PO_4$–P $L^{-1}$ $d^{-1}$ (98.2±1.6%) after 28 hours. Our results show that balanced MaB culture can satisfy the EUS91 limits within approximately 24 hours of treatment.

**Keywords:** European standard; microalgae and bacteria; nitrification inhibition; organic carbon balance; pH regulation; wastewater treatment;

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### 4.2.1. Introduction

Efficient municipal wastewater treatment is one of the key elements to fulfill the sustainable development goals (SDG) in the United Nations' agenda for 2030 (UN Department of Economic and Social Affairs, 2016). While application of the conventional activated sludge (CAS) treatment technology is commonly practiced, microalgae biotechnologies offer remarkable advantages for sustainable development (Kanchanamala Delanka-Pedige et al., 2021). A coculture of microalgae and bacteria (MaB) in the wastewater can remove the organic and inorganic pollutants, reduce the greenhouse gas (GHG) footprint, save energy, and close the cycle of nutrients, carbon, and water (Chia et al., 2021; Yong et al., 2021). Photosynthesis in microalgae provides oxygen, which functions as an electron acceptor for the heterotrophic degradation of organic pollutants by the bacteria. In exchange, the excreted carbon dioxide and other inorganic/organic compounds from the bacteria sustain the photoautotrophic metabolism of the algae. MaB treatment has the capacity to remove carbon, nitrogen and phosphorus simultaneously without need for mechanical aeration; therefore, the tertiary and post treatment stages would be unnecessary (Flores-Salgado et al., 2021).

Large-scale exploitation of microalgae–bacteria treatment is not challenge-free. Photosynthesis under solar irradiation is the key element in MaB bioremediation which inevitably requires a large surface area for treatment in high-rate algal ponds (HRAP). Since the efficient depth of these ponds are limited by the light penetration, the only meaningful approach to reduce the amount of land required is to improve the treatment performance. The hydraulic retention time (HRT) can be shortened as the treatment performance is enhanced (Acién et al., 2016). Practically, an HRT of 2-8 days is suggested for MaB treatment of municipal wastewater (Anbalagan et al., 2016), whereas shorter HRTs are essential to make large-scale scenarios more feasible (Foladori et al., 2018).

In our earlier study, we observed that HRT has a significant impact on the pH level and buildup of inhibitors of MaB activities in 2 and 3 days of retention time (Soroosh et al., 2022a). It was observed that in weak municipal wastewaters, the cycle of $CO_2$ -$O_2$ between microalgae and heterotrophic bacteria became disrupted and a lack of $CO_2$ and an accumulation of oxygen disturbed nutrient removal as the main objective. The excessive amount of dissolved oxygen (DO) stimulates nitrification and therefore a significant amount of nitrogen was not assimilated but detected as nitrite and nitrate in the effluent. Nitrification consumes a great amount of alkalinity (Ebeling et al., 2006) which accelerates the increase of the pH due to photosynthesis. Although a shorter HRT could reduce the severity of the pH peaks, the pH on the second day of treatment still appeared to be high enough to trigger MaB inhibitions due to nitrite and free ammonia accumulation. This condition made acceptable nitrogen removal infeasible whereas the alkaline environment promoted nearly complete phosphorus removal (Soroosh et al., 2022a).

Restoring the balance of the $CO_2$-$O_2$ cycle by manipulating the carbon content is investigated in this study. Availability of inorganic carbon (IC) in form of $CO_2$, which forms $H^+$ and $HCO_3^-$ in neutral aqueous systems, can promote alkalinity and reduce the pH to prevent the pH inhibition chain (Anbalagan et al., 2016). Carbon dioxide is widely available and is known as a major contributor to climate change, therefore, sequestration of $CO_2$ in biomass is a meritorious sustainable approach (Chia et al., 2021). Carbon dioxide is a form of inorganic carbon which is produced and consumed during MaB metabolism. Eze et al. (2018) have developed a model which predicts the pH level based on the concentration of IC and the nutrients. This model suggests that the addition of $CO_2$ removes the extra $OH^-$ ions which is the superb way to balance the pH (Eze et al., 2018). Carbon dioxide can be delivered exogenously (e.g., through diffusion) or can be produced endogenously through heterotrophic consumption of the organic carbon (OC) in the wastewater. Direct application of $CO_2$ is recommended so that the emission of the greenhouse gases can be reduced while carbon-neutral biofuels are produced from the biomass. However, the supplementary organic carbon can also improve the nitrogen removal via mixotrophic uptake and heterotrophic nitrogen assimilation (Fan et al., 2020; X. Liu et al., 2019). Both methods are examined experimentally, and the results are compared with the European Council Directive standard of 91/271/EEC (referred to as EUS91 hereafter) which concerns urban wastewater treatment (The Council of the European Union, 1991).

This European standard (EUS91, Annex I), limits the concentration of the nutrients in the effluent of European wastewater treatment plants (WWTP) to 125 mg $L^{-1}$ for chemical oxygen demand (COD), 1 mg P $L^{-1}$, and 10 mg N $L^{-1}$ when the wastewater is collected from a large society with the population equivalent (p.e.) of greater than 100,000. These thresholds are less strict in smaller communities. The aim of this study is to investigate the feasibility of attaining the EUS91 limits within an HRT of 2 days or shorter with complete reliance on the photosynthetic oxidation capacity of MaB technology. The treatment performance of microalgae and bacteria in presence of exogenous carbon is measured and discussed in this research work. Sustainable methods to elude nitrification as the main element in extension of treatment beyond 2 days are described. The scalability and sustainability of the organic carbon supplementation which led to remarkably shorter HRTs are debated and some ideas for further research for optimization of MaB treatment are proposed.

### 4.2.2. Material and methods

#### 4.2.2.1. Microalgae-bacteria consortium

A mix-culture of six indigenous microalgae strains collected from Seevetal WWTP south of Hamburg, Germany, was used to form the microalgae-bacteria (MaB) aggregates. This algal cocktail consisted of three strains of *Chlorella* sp., two strains of *Desmodesmus* sp., and one strain of *Tribonema* sp. which were identified microscopically (Carl Zeiss MicroImaging, Germany). The activated sludge (suspended bacterial flocs) was collected from the concentration basin of the secondary clarifiers at Seevetal WWTP.

Settleability and nutrient removal of the MaB culture improves when the medium is inoculated with an algae to bacteria ratio (DW:DW) of 5:1 (Su et al., 2012). Stoichiometry of photoautotrophic algae and heterotrophic bacteria also confirms the validity of this ratio in an $O_2$ balanced MaB setting (Soroosh et al., 2022a). Thus, five portions of the algal cocktail were mixed with one portion of activated sludge to form the MaB culture in pretreated wastewater.

#### 4.2.2.2. Analytical methods

Dissolved oxygen (DO) concentration was measured using a digital oxygen meter (Oxi 323-B/SET, and $O_2$ sensor CellOx 325, WTW, Germany). The pH and temperature were measured with a digital meter (pH 323/SET WTW, Germany). Total suspended solids (TSS), dry weight (DW), and sludge volume index (SVI) were measured according to APHA (2005). A portable pulse-amplitude modulated (PAM) device (Junior-PAM, Walz, Germany) was used to measure the $F_v/F_m$ ratio, which is an indicator of the photosystem II (PSII) performance. To do so, measurements took place after the samples rested in darkness for 15 minutes. The average photosynthetically active radiation (PAR) was measured with a digital photometer (LI-250A, LI-COR).

#### 4.2.2.2.1. Chemical analysis

About 100 mL of the culture was sampled from each reactor, centrifuged (2700 rcf, 5 min), and vacuum filtered through nitrocellulose membrane filters (45 µm, MicronSep, Maine Manufacturing, USA) every day. The filtered water was preserved at -19±1 °C if immediate chemical measurements were not possible. Phosphate ($PO_4$-P), nitrite ($NO_2$-N), and nitrate ($NO_3$-N) were measured photometrically using a Hach photometer (DR3900) and the manufacturer's standard cuvettes (Hach, Germany). The total ammoniacal nitrogen (TAN) was measured according to German standard DIN 38 406–E5. Total carbon (TC), inorganic carbon, organic carbon (standard EN 1484) and total dissolved nitrogen (TN, standard EN 12260) were measured using an autoanalyzer multi N/C 3000 (Analytik Jena, Germany).

Ammonium removal efficiency, nitrification rate, disposed nitrate ratio and free ammonia concentration were calculated using the following equations:

$$\text{Ammonium removal efficiency [\%]} = 100 \times \left(1 - \frac{\text{TAN}_{\text{end}}}{\text{TAN}_{\text{initial}}}\right) \quad \text{(Eq.12)}$$

$$\text{Nitrification rate [\%]} = 100 \times \frac{(\text{NO}_{x_{\text{end}}} - \text{NO}_{x_{\text{initial}}})}{\text{TAN}_{\text{initial}}} \quad \text{(Eq.13)}$$

$$\text{Nitrate portion of discarded inorganic N [\%]} = 100 \times \frac{\text{NO}_{3_{\text{end}}}}{\text{TAN}_{\text{end}} + \text{NO}_{x_{\text{end}}}} \quad \text{(Eq.14)}$$

$$\text{FA} = [\text{NH}_3\text{-N}]_{\text{free}} = [\text{TAN}] \times \frac{10^{\text{pH}}}{\exp\left(\frac{6344}{273+\text{T}}\right) + 10^{\text{pH}}} \quad \text{(Eq.15)}$$

where $NO_x=[NO_2^-]+[NO_3^-]$. $TAN_{initial}$ and $TAN_{end}$ are the concentration of TAN at the beginning and end of the measurement period, respectively. T in FA formula is the temperature in °C (Sinha and Annachhatre, 2007).

#### 4.2.2.2.2. Statistical analysis

The Real Statistics add-in (Release 8.2) for Microsoft Excel 365 was used for statistical analysis. To evaluate the normality of our data, Shapiro-Wilk and D'Agostino-Pearson tests were performed. F-test was performed for homogeneity of variance. To investigate the significance of difference between pairs, based on the dataset conditions, either a *t*-test (parametric) or a Mann-Whitney test for independent samples (non-parametric) were performed. To evaluate the correlation between two datasets, either the Pearson (parametric) or the Spearman (non-parametric) coefficients were calculated. Two-tailed tests with alpha = 0.05 were implemented unless otherwise stated. The uncertainties in the reported values are within the standard deviation of the sample from its mean value.

### 4.2.2.3. Carbon supplementation

In this study, two experiments are presented which consist of MaB treatment of real wastewater with addition of IC in sequenced batch reactor mode, and treatment with supplementary OC in batch mode. The sequencing batch reactor (SBR) treatment includes using biomass from the previous batch to inoculate the wastewater (as a new medium) for the subsequent batch.

#### 4.2.2.3.1. Inorganic carbon supplementation (ICS) experiments

To study the influence of moderated pH, the MaB treatment was assessed with and without external $CO_2$. The control series (CS) was a MaB treatment in triplicate SBRs with an HRT of 2 days. No chemical pH adjustments or carbon supply took place during six sequencing batches (12 days) of CS.

In contrast, during the inorganic carbon supplemented (ICS) experiment, $CO_2$ (99.8% purity) was occasionally injected into the medium (1 vvm) to maintain the pH below the inhibitory level of 8.0 (Eze et al., 2018). The ICS experiment is comprised of two sub-experiments. One with an HRT of 2 days (hereafter $ICS_{2d}$), followed by two batches with an HRT of 3 days (hereafter $ICS_{3d}$). $ICS_{2d}$ consists of six batches. The first batch of $ICS_{3d}$ was created by extending batch six of $ICS_{2d}$ and a separate ICS batch with 3 days of HRT. Consequently, $ICS_{3d}$ is comprised of two sequencing batches of 3 days. The sludge retention time (SRT) of $ICS_{2d}$ and $ICS_{3d}$ are calculated to be 5.3 and 8 days, respectively.

#### 4.2.2.3.2. Organic carbon supplementation (OCS) experiments

After the ICS experiments, the OCS experiment was conducted in which real wastewater was supplemented with exogenous organic carbon. Glucose was chosen to elevate the OC content of real wastewater. This was done to observe the influence of easily biodegradable OC on the suppression of autotrophic bacteria activity, and therefore, eliminate the effects of nitrification on nitrogen assimilation (X. Liu et al., 2019). The concentration of the nutrients and other chemical properties of the medium were measured in intervals of 4–6 hours during the batch tests. In the first batch ($OCS_a$), 1000 mg of glucose per liter of wastewater was added regardless of the existing organic carbon content of the wastewater. For the second batch ($OCS_b$), the amount of supplementary OC (glucose) was optimized according to the stoichiometry of MaB symbiotic metabolism as described below.

#### 4.2.2.3.3. Balancing the organic carbon to nitrogen (C:N) ratio

The photoautotrophic and heterotrophic stoichiometric equations are presented in (Eq.4 and (Eq.5, respectively (Ebeling et al., 2006):

$$16NH_4^+ + 92CO_2 + 92H_2O + 14HCO_3^- + HPO_4^{2-} \xrightarrow{light} C_{106}H_{263}O_{110}N_{16}P + 106O_2 \qquad \text{(Eq.16)}$$

$$NH_4^+ + 1.18C_6H_{12}O_6 + HCO_3^- + 2.06O_2 \rightarrow C_5H_7O_2N + 6.06H_2O + 3.07CO_2 \qquad \text{(Eq.17)}$$

Where $C_{106}H_{263}O_{110}N_{16}P$ represents the stoichiometric composition of microalgae and $C_5H_7O_2N$ indicates the bacterial biomass when organic carbon (here glucose, $C_6H_{12}O_6$) is fed. After normalizing both equations based on the $O_2$ evolution/consumption in a MaB floc, we have:

$$\frac{16}{106}NH_4^+ + \frac{92}{106}CO_2 + \frac{92}{106}H_2O + \frac{14}{106}HCO_3^- + \frac{1}{106}HPO_4^{2-} \xrightarrow{light} \frac{1}{106}C_{106}H_{263}O_{110}N_{16}P + O_2 \qquad \text{(Eq.18)}$$

$$\frac{100}{206}NH_4^+ + \frac{118}{206}C_6H_{12}O_6 + \frac{100}{206}HCO_3^- + O_2 \rightarrow \frac{100}{206}C_5H_7O_2N + \frac{606}{206}H_2O + \frac{307}{206}CO_2 \qquad \text{(Eq.19)}$$

After adding (Eq.18 to (Eq.19:

$$\begin{aligned} &0.636\ NH_4^+ + 0.573\ C_6H_{12}O_6 + 0.618\ HCO_3^- + 0.009\ HPO_4^{2-} \\ &\qquad \rightarrow 0.009\ C_{106}H_{263}O_{110}N_{16}P + 0.485\ C_5H_7O_2N \qquad \text{(Eq.20)} \\ &\qquad + 2.074\ H_2O + 0.622\ CO_2 \end{aligned}$$

Equation (Eq.2 shows that in a MaB culture, where all the produced oxygen is utilized for the heterotrophic bacterial growth, oxidizing 1 mol of glucose by the heterotrophic bacteria requires 1.11 mols of ammonium (0.863 mol $NH_4^+$-N). Given that the glucose and nitrogen molar masses are 180.16 and 14.01 g mol$^{-1}$, the w:w ratio of the glucose to ammonium-nitrogen in a balanced culture can be calculated as follows:

$$\frac{1\ mol\ Glucose}{0.863\ mol\ NH_4^+} = 1.159\frac{180.16\ g\ Glucose}{14.01\ g\ NH_4^+ - N} = 14.9\ \frac{g\ Glucose}{g\ NH_4^+ - N} \qquad \text{(Eq.21)}$$

With synthetic wastewaters we can easily manipulate the medium composition to achieve the mass ratio of 14.9 (w:w) glucose to nitrogen, but in real wastewaters, the amount of organic carbon usually fluctuates and is presented as the COD value. To have a more practical approach to determine the amount of the OC in the wastewater, the glucose in (Eq.21 was replaced with its equivalent COD value:

$$\begin{aligned} &C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + H_2O \Rightarrow COD = \frac{\Delta\ (O_2)}{\Delta\ (C_6H_{12}O_6)} \\ &\qquad = \frac{6\ (32\ g\ O_2\ mol^{-1})}{180.16\ g\ glucose\ mol^{-1}} \Rightarrow \frac{g\ COD}{g\ Glucose} = 1.07 \end{aligned} \qquad \text{(Eq.22)}$$

This ratio was confirmed using stock glucose solutions ($R^2$=1.0, $p$ <0.001).

By combining (Eq.21 and (Eq.22, it is expected that 14.9×1.07= 15.94 mg COD would be needed to eliminate 1 mg $NH_4^+$–N in a balanced MaB biocenosis.

The COD:N (w:w) ratio of the wastewater that was used in the $OCS_b$ experiment was only about 4.6 (62.6 mg $NH_4^+$–N L$^{-1}$ and 287 mg COD L$^{-1}$). The MaB organisms would need about 998 mg COD to exhaust the ammonium nitrogen while the biodegradable COD content of the wastewater provides a small portion of it. The deficit COD would need to be supplied externally. External supply of organic carbon provides extra electrons for heterotrophic activities. Increasing the OC/IC ratio is expected to improve the nitrogen removal in weak wastewaters (Van Den Hende et al., 2011).

To estimate the amount of the biodegradable COD in the wastewater, a batch culture of MaB supplemented with $CO_2$ was carried out before the $OCS_b$ experiment. The residual COD after 3 days of treatment was 77 mg $L^{-1}$ which is presumably nonbiodegradable. Therefore, the removed COD was considered biodegradable, thus it was deducted from the required 998 mg COD $L^{-1}$. The result (788 mg COD $L^{-1}$) returns the amount of equivalent supplementary glucose. Hence, about 736 mg of glucose was added to every liter of wastewater to satisfy the C:N ratio in (Eq.2. This amount of glucose was about 26% less compared to $OCS_a$.

#### 4.2.2.4. Experimental set-up

Cylindrical glass bioreactors (2L, d =130 mm, H=200 mm) were used in triplicate to treat 1.5 L of sieved (200 μm) pretreated municipal wastewater. The wastewater was collected from the effluent of the primary clarifier at Seevetal WWTP. Initially, 1500 mL of sieved wastewater was inoculated with our MaB stock culture to obtain the biomass concentration of 500–600 mg DW $L^{-1}$ in each reactor. The harvest was conducted using conical separatory glass funnels. After 30 minutes of gravity settlement, about 600 mg of the biomass was separated and used for the next batch.

To keep the pH <8.0 throughout the ICS experiments, an AT control system (Aqua Medic, Germany), its respective sensors, and shut-off magnetic valves, were used. A diagram of the setup that was used for both $ICS_{2d}$ and $ICS_{3d}$ is shown in Fig. 1.

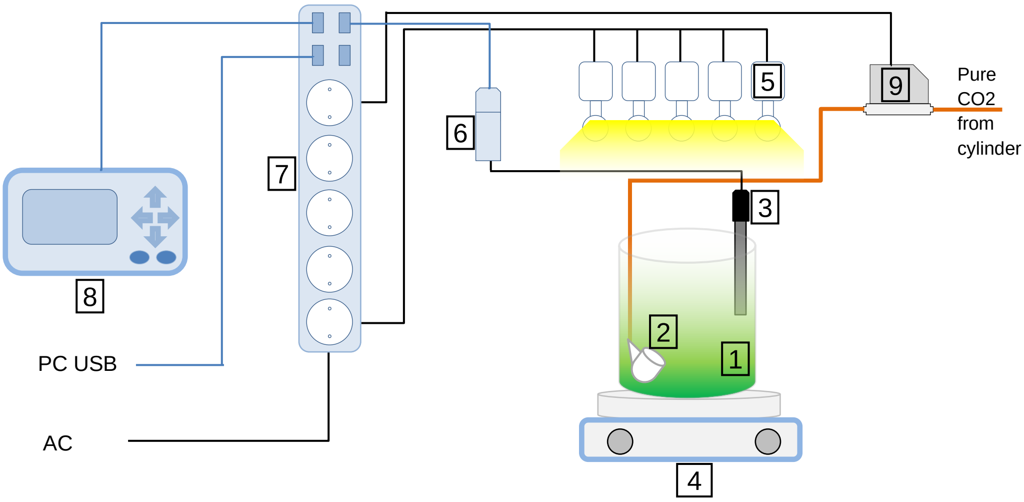


**Fig. 1** Setup of MaB reactors with controlled pH (Exp.ICS). 1: Reactor; 2: $CO_2$ diffuser; 3: pH probe; 4: Magnet stirrer; 5: Fluorescent tubes; 6: pH interface; 7: Power box and USB interfaces; 8: AT controller and user interface; 9: Electromagnetic $CO_2$ valve (M-ventil).

Magnet stirrers with a speed of 180 rpm were used to agitate the medium and limit the shear stress on filamentous algae *Tribonema* sp. in the MaB consortium. Irradiation of 140±3 µmol photons $m^{-2}$ $s^{-1}$ (from top) by means of 5×40W white fluorescent lamps (Pracht PPR SR E 100/3 Leuchte AVUS, Germany) was employed in 12:12 hours (light:dark) photoperiods. During the $OCS_b$ experiment, an extra source of light (Philips Scheinwerfer RVP351 HPI-TP250W KA) was employed which increased the light intensity on the surface of the reactors by 65±3 µmol photons $m^{-2}$ $s^{-1}$.

The OCS batches ended when the nitrogen and phosphorus were below the EUS91 limits (TAN<10 mg $L^{-1}$ and $PO_4$-P <1 mg $L^{-1}$). The chemical characteristics of the used wastewaters are presented in Table 1.

**Table 1** Wastewater characteristics for each experiment: CS: control series, ICS: Inorganic carbon supplemented, OCS: organic carbon supplemented experiment. (n.d.): not detectable–below detection level.

| Assay | pH | $PO_4$-P | $NO_3$ | $NO_2$ | $NH_4$–N | TN | TSS | VSS | COD |
|---|---|---|---|---|---|---|---|---|---|
| | – | mg P $L^{-1}$ | | mg N $L^{-1}$ | | | g $L^{-1}$ | g $L^{-1}$ | mg $L^{-1}$ |
| CS | 7.12 | 7.26 | 0.31 | (n.d.) | 52.2 ± 1.4 | 58.1 | 0.215 | 0.194 | 242 |
| ICS | 7.11 | 7.48 | 0.19 | (n.d.) | 53.6±0.3 | 59.8 | 0.211 | 0.178 | 260 |
| $OCS_a$ | 7.06 | 8.42 | (n.d.) | (n.d.) | 73.4±0.3 | 78.2 | 0.252 | 0.179 | 247 |
| $OCS_b$ | 7.07 | 8.05 | (n.d.) | (n.d.) | 62.6 | 68.8 | 0.154 | 0.114 | 287 |

### 4.2.3. Results and discussion

#### 4.2.3.1. Treatment in controlled pH (ICS)

Treatment with a controlled pH eliminates the concerns regarding the inhibitory effects of high pH, FA toxicity, and nitrite accumulation (Soroosh et al., 2022a). Additionally, the bioavailability of $CO_2$ to microalgae enhances the photosynthetic performance of the algae and prevents photorespiration (Richmond and Hu, 2013). The ICS experiment has shown that in a controlled pH setting, the behavior of the MaB culture is remarkably more regular and predictable.

##### 4.2.3.1.1. Culture characteristics

The sludge volume index (SVI) can indicate the settleability of the flocs and therefore the harvest performance of the culture. The higher the SVI, the lower the density of the flocs and lower settleability. A sludge with SVI≥150 is classified as poorly settleable (Jin et al., 2003). It has been shown that the raised levels of pH and FA can decrease the hydrophobicity of aerobic granules and MaB flocs, and therefore, increase the SVI (Soroosh et al., 2022a; Yang et al., 2004). The average SVI in ICS (67.5±1.7 ml $g^{-1}$) was significantly less than the CS (76.5±2.1, $p<0.001$) which can be associated with the higher levels of free ammonia and pH in the CS.

The oxygen concentration followed a similar pattern during the SBRs. The heterotrophic respiration caused the DO to drop to a nearly anoxic condition in the culture, inhibiting the nitrifying bacteria activity; and therefore, uptake in the MaB biomass was responsible for most of the ammonium removal on the first day (Fig. 2 and Fig. 3). Not only could the microalgae freely utilize the ammonium, but the abundance of biodegradable OC made active heterotrophic bacteria metabolism possible (Boelee et al., 2014). The average measured DO after 2.5 hours through SBRs were 0.46±0.12 mg $L^{-1}$ and 0.95 ±0.53 mg $L^{-1}$ for ICS and CS, respectively. However, DO started to build up rapidly after the first dark period because the oxygen generation of the microalgae surpassed the respiration rate of heterotrophic bacteria (Flores-Salgado et al., 2021).

Oxygen concentration reached saturation and supersaturation at the end of both the control and ICS batches. This signifies the inactivity of the heterotrophic bacteria when the OC was exhausted. However, the $F_v/F_m$ ratio showed no significant drop as the oxygen built up in the reactors ($p<0.001$, $n=54$; see Fig. 2) which shows no considerable effects of photorespiration (Rearte et al., 2021). The $F_v/F_m$ ratio of the $ICS_{3d}$ did not change in comparison to $ICS_{2d}$ (mean 0.77±0.02). The rate and intensity of oxygenation during the last batches of the control series were higher than the ICS. One reason for this could be that the higher pH level of the CS increased the FA concentration (up to 2.5 mg $NH_3$–N $L^{-1}$), inhibiting the nitrite oxidizing bacteria (NOB) as the main oxygen consuming agents in that phase of the culture (Azov, 1982). It is expected that the $CO_2$ diffusion in the ICS motivate the off-gassing of the supersaturated DO and increases the $CO_2$:$O_2$ ratio in favor of photosynthesis. It is the same reason that adding one day to HRT in $ICS_{3d}$ did not influence the DO concentration on the third day of the batch. The average oxygen concentration was slightly higher on the third day but remained below 16 mg $L^{-1}$.

We observed that a sort of bacterial inhibition is generally at work when the DO is unreasonably higher than usual. For example, the results in Fig. 2 and Fig. 3 reveal that the DO in the CS increases as the COD consumption and nitrification decline. The average concentration of DO during the CS experiment was significantly higher than ICS ($p<0.05$). This can indicate that limiting the pH to 8.0 could prevent the long-lasting impacts of pH inhibition on the heterotrophic and autotrophic bacteria as the main consumers of photosynthetically produced oxygen. However, the rise in DO concentration in ICS was slower but inevitable because the maximum specific oxygen production rate of microalgae is 5.8 ± 3.8 times higher than the specific $O_2$ uptake rate of heterotrophic bacteria (Flores-Salgado et al., 2021). This situation provides a great opportunity for oxidation of ammonium (nitrification) by the autotrophic bacteria (Fig. 2 and Fig. 3).

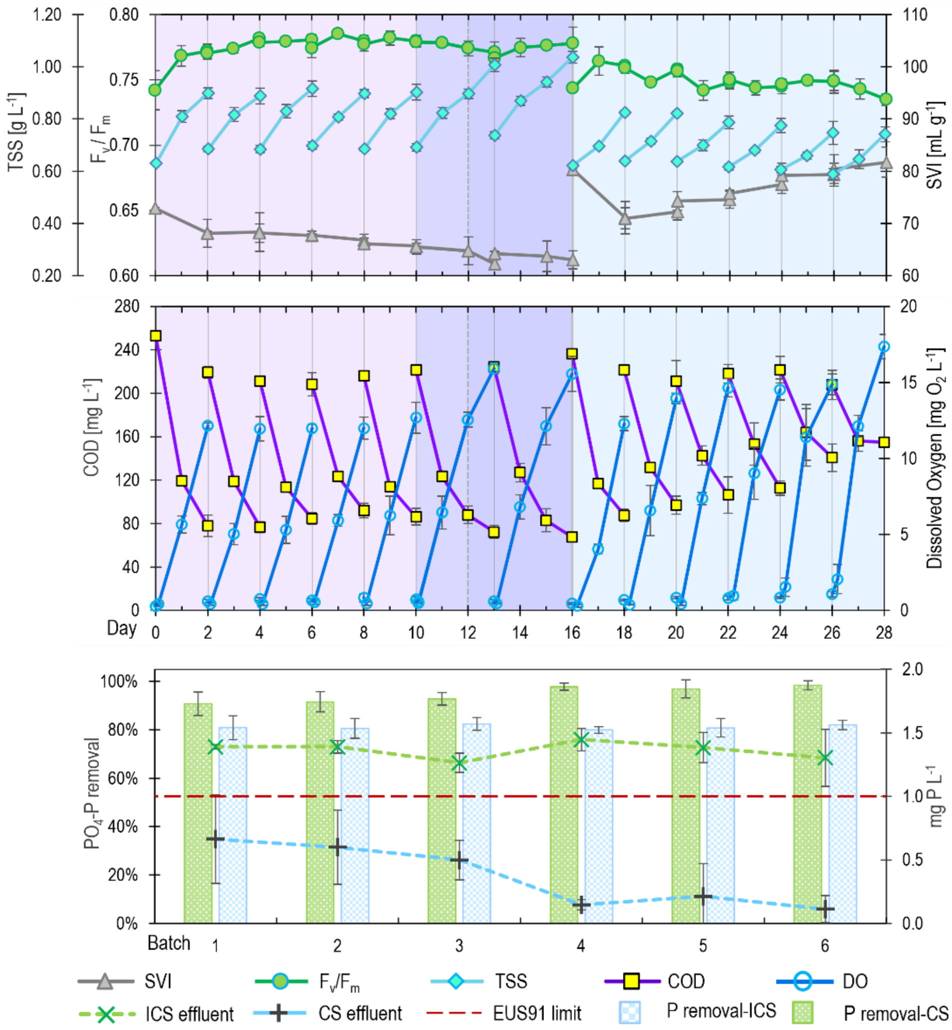


**Fig. 2** Culture characteristics of the control series (CS) and inorganic carbon supplemented (ICS) series. The pink and purple shaded areas represent the ICS experiment in 2 days and 3 days of hydraulic retention time (HRT) respectively. The blue shaded area indicates the IC with 2 days HRT. SVI: Sludge volume index; TSS: total suspended solids; COD: chemical oxygen demand; DO: dissolved oxygen; EUS91 limit: The maximum allowed phosphorus concentration in the effluent of wastewater treatment plants (1 mg P L-1) according to European Council Directive standard 91/271/EEC.

Photosynthetic oxygenation is associated with the uptake of the dissolved $CO_2$, leading to an increase in the pH which was neutralized by injecting $CO_2$ into the ICS experiment. The average pH of the CS (8.03±0.15) was significantly higher than the controlled pH of the ICS with an average measured value of 7.44±0.13 ($p$<0.001). The pH level on the second day of the batch signifies the fate of the treatment. The average measured pH of the ICS was 7.30±0.27 on the second day while the CS was considerably more alkaline with an average pH of 8.89±0.15. A pH over 8.0 initiates the pH, ammonia, and nitrite inhibitions which can leave irreversibly impact the MaB functions. This may be the reason that despite an increasing trend in the DO, the COD levels near the end of the CS could not match the levels in the earlier batches.

The phosphorus removal was correlated with the pH level in the CS (rho = 0.68, $p < 0.001$). The effect of the elevated pH on stimulation of phosphorus precipitation could help the phosphate level fall below the EUS91 threshold (Xia et al., 2021). The saturation index (SI) of the average pH in the CS experiment (11.9) was considerably more than the SI in $ICS_{2d}$ (5.2) (Song et al., 2002). This difference indicates that the microalgae biomass uptake has a stronger role in phosphorus removal in the ICS experiment. Nevertheless, the total amount of this removal was less than CS but this difference was not significant ($p > 0.05$). The phosphorus concentration in the effluent of ICS could not reach the 1 mg P $L^{-1}$ limit as mandated by the EUS91.

#### 4.2.3.1.2. Nitrogen removal

Total ammoniac nitrogen (TAN) removals were efficient in both assays. Control series removed the TAN almost entirely, and $ICS_{2d}$ reduced the ammonium up to 95.3±1.7%. Similar to the CS, nitrification was responsible for the removal of a considerable amount of ammonium in the ICS series. Our nitrification results are contrary to the findings of Huang et al. (2015) wherein it was reported the inhibitory effects of microalgae on nitrifying bacteria and bioactivity of granules in their MaB cultures.

In our experiments, the overall conversion patterns were different because the pH and the ammonium inhibitions were irrelevant in the ICS experiments. The OC content in the CS series limited the respiratory $CO_2$ production and this influenced the $O_2$ evolution and the pH balance. Lack of oxygen for the first hours of every batch before the OC was exhausted, suppressed the nitrification. Meanwhile, algae and heterotrophic bacteria faced no limitation or competition for nutrients to attain exponential growth. Due to the abundance of $CO_2$, algae and nitrifying bacteria experienced more competition over TAN uptake; this can explain the higher TAN removal during the first days of $ICS_{2d}$ series with 33.9±1.5 mg TAN $L^{-1}$ compared with the 24.9±1.4 mg TAN $L^{-1}$ for the CS. Therefore, the amount of the nitrogen removed through assimilation into the MaB biomass was higher on the first day of ICS (26.4±2.1 mg $L^{-1}$) compared to the CS (22.8±2.3 mg $L^{-1}$) on the first day. Gradual increase of DO during the last hours of the first day may initiate the nitritation and later nitration. In the beginning of the second day, both nitrite and nitrate were detected in the culture in comparable concentrations (Fig. 3). Remarkable production of nitrate after the first day of treatment in the ICS series exhibits that regulation of the pH through elevation of $CO_2$ could prevent inhibition of nitrite oxidizing bacteria (NOB) because alkalinity, pH, and DO were in favor of NOB activities. Hence, nitrite accumulation and its inhibitory impact on microalgae would be prevented when the culture is supplemented with IC while the negative effects of high nitrate concentration are also avoided (Chen et al., 2009; González-Camejo et al., 2020).

Nitrification was more advanced in ICS as the ratio of nitrate to total inorganic nitrogen (TAN+NOx–N) in the effluent increased from 47.9±8.0% in CS to 89.0±13.5% in $ICS_{2d}$. Meanwhile, the average $NO_2$-N concentration in the effluent of the SBR of the CS dropped from 6.1±1.0 mg N $L^{-1}$ to 1.7±1.1 and 0.0±0.1 mg N $L^{-1}$ in $ICS_{2d}$ and $ICS_{3d}$, respectively. Another distinct difference between the nitrification in the CS and ICS was the predictability of the culture behavior in the absence of pH and FA inhibition. The fluctuation of the nitrification rates in ICS were negligible compared to the CS which can introduce the supplementary addition of $CO_2$ to MaB treatment which is a reliable and sustainable alternative to conventional nitrification with no need for a significant amount of electricity to intense mechanical aeration (Akizuki et al., 2021; Vargas et al., 2016). However, the remaining TN in the effluent of both CS and $ICS_{2d}$ were above the 10 mg N $L^{-1}$ limit introduced by the EUS91; therefore, denitrification as a separate post–treatment would be necessary to remove the excess of nitrogen oxides (Carvalho et al., 2021). Since the MaB treated water is very rich in oxygen, denitrification would be inhibited unless it is done during the night when the bacterial and algal respiration would eliminate the DO (Boelee et al., 2014; Flores-Salgado et al., 2021).

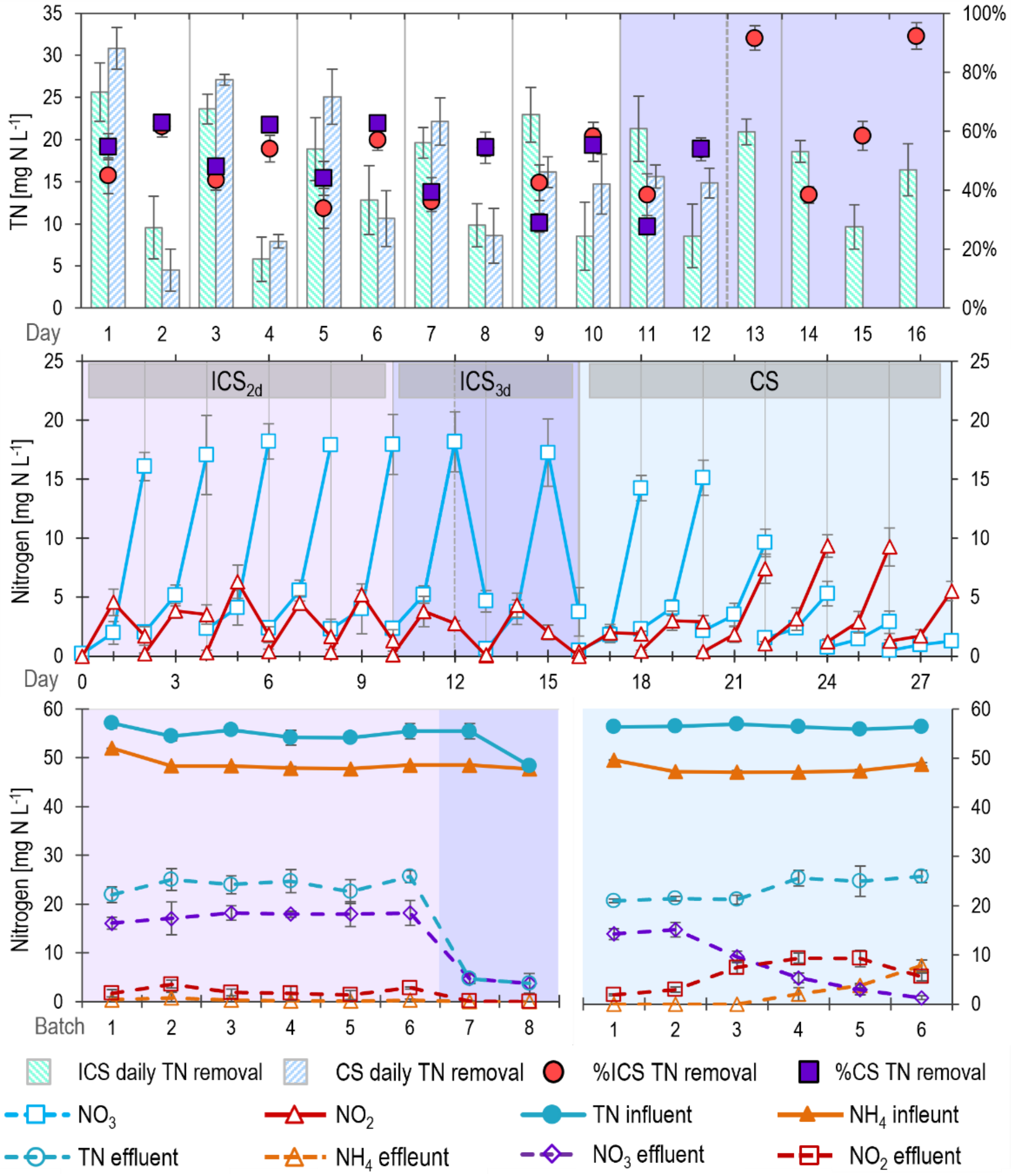


**Fig. 3** The nitrogen profile in inorganic carbon supplemented (ICS) and control series (CS) experiments. The pink and purple shaded areas represent the ICS experiment in 2 days and 3 days of hydraulic retention time (HRT) respectively. The blue shaded area indicates the IC with 2 days HRT. Top: initial and final concentrations per batch. Middle: daily TN removal. Bottom: profiles of nitrification products ($NO_2$ and $NO_3$)

The nitrate assimilation by the algae can take place only after the complete exhaustion of TAN (Chen and Wang, 2020). The algal cells can utilize ammonium as their source of nitrogen, nitrate has to be reduced to nitrite and then to ammonium prior to the incorporation in the glutamine synthesis (X. Liu et al., 2019). Therefore, the produced was did not begin to be assimilated before the ammonium was consumed entirely. Switching the nitrogen source from ammonium to nitrate is normally associated with a lag (acclimatation) phase. This can explain the difference between the amount of the removed TN on the first and the second days (26.5±2.2 and 8.8±2.4 mg N $L^{-1}$ for $ICS_{2d}$). Therefore, some nitrified TAN remained at the end of all batches of both ICS and CS. The nitrification was stronger in ICS as the pH stayed in the safe margin for the nitrifiers. Up to 40.5±4.9% of TAN in the wastewater was converted into nitrogen oxides (NOx) in $ICS_{2d}$ while 29.5±3.1% of the inlet ammonium was nitrified at the end of CS averagely. This ratio was 8.8±3.3% for $ICS_{3d}$. The other difference was that the NOB could function significantly better in the ICS series. Nitrate comprised up to 89±13.5% of the leftover inorganic nitrogen (NOx+TAN) in the effluent, while in the CS series, only 47.9±8.0% of the inorganic nitrogen in the effluent was detected in form of $NO_3^-$. Thus extending the treatment period could lead to an acceptable TN removal with limited risk of nitrite inhibition (González-Camejo et al., 2020). To examine this hypothesis, we extended the fifth batch of ICS to a third day. The nitrogen content of the medium after 3 days was 4.4±0.8 mg N $L^{-1}$ which complies with the EUS91 limits. Repeating the SBR batch with supplementary $CO_2$ (regulated pH) with 3 days of HRT ($ICS_{3d}$) has confirmed that the MaB can assimilate the TAN and oxidized ammonium into the biomass to attain the European standard (Boelee et al., 2014). Although extending the treatment to 3 days with the presence of exogenous $CO_2$ seems promising in terms of water quality, it negates our primary objective of reducing the HRT to less than 2 days as described in the introduction.

The removal performance and MaB growth rate on the first day of both CS and ICS was significantly more than the second day. The reason for this is that on the first day, both algae and heterotrophic bacteria could grow within the abundance of nutrients. The organic carbon and ammonium could boost the heterotrophic growth which was associated with intensive consumption of oxygen. This made it possible for algae to remove ammonium without experiencing critical competition with the nitrifiers. But as the oxygen evolution exceeded the oxygen consumption by OC–deprived heterotrophic bacteria, the nitrifying bacteria made the ammonium unavailable to the algae and induced the lag phase which is initiated by switching to nitrate for the photoautotrophic metabolism.

##### 4.2.3.1.3. Phosphorus profile

During ICS, the culture was not limited by either nitrogen, or phosphorus because regulating the pH caused orthophosphate ($PO_4^{3-}$) to remain bioavailable throughout the treatment as the saturation-index (SI) remained under the saturation point and crystallization was prevented (Song et al., 2002).

On the second day of CS, when the average pH was 8.89±0.15, the amount of dissolved phosphorus (0.37±0.23 mg P $L^{-1}$) was significantly less than the phosphorus content of ICS (1.36±0.12 mg P $L^{-1}$, $p<0.01$) which is strongly correlated with the alkaline nature of the CS experiment. The amount of phosphorus removal on the first day in $ICS_{2d}$ was on average 3.2±0.3 mg P $L^{-1}$ (57±7% of SBR total P removal) whereas, on the second day, limiting ammonium and moderate pH did not allow the same amount of removal through the biomass uptake, or the precipitation. Extending the ICS to 3 days could provide the opportunity to remove the remainder of the phosphorus via biomass assimilation (more than 98% of $PO_4$-P was removed in 2 batches $ICS_{3d}$ with 0.10±0.15 mg P $L^{-1}$ in the effluent).

##### 4.2.3.1.4. Suppression of nitrification

From the results of the ICS series, suppression of nitrification is a key element to achieve the European standard of EUS91 within an HRT of shorter than 2 days. In other words, if we can extend the conditions of the first hours of MaB treatment –a treatment without nitrification– we can reach the European limits in a remarkably shorter period. One way to eliminate nitrification is the application of inhibitors. Addition of  chemical agents like 2-chloro-6-(trichloromethyl) pyridine (TCMP) and allylthiourea (ATU) can prevent ammonium oxidation but these can cause secondary contamination and suppression of the heterotrophic bacteria (Yang et al., 2022). Nitrifiers, particularly ammonia oxidizing bacteria (AOB), are sensitive to light intensity (Zhang et al., 2009); however, as the density of the biomass in the medium increases, the inhibitory effects of light on the AOB diminishes. In order to have high sunlight penetration, the depth of the medium would need to decrease which would require a very larger land area, which is incongruent with the goal of reducing the HRT.

Extending the conditions of the early hours of the MaB batches by supplying external organic carbon is a meaningful approach in terms of the controllability and predictability of the treatment while the nitrification is simply inhibited due to lack of oxygen. The maximum growth rate of the autotrophic nitrifying bacteria is far less than those which are heterotrophic; therefore, in the absence of limiting factors (e.g. DO, OC, N), the heterotrophs can outcompete the autotrophs (Ebeling et al., 2006). According to Monod equation, when the amount of C (g) is greater than 0.81+0.59N (g) in the culture, the heterotrophic bacteria growth overcomes the nitrifiers; thus, nitrification would be negligible as we approach the C:N ratio of 10 (Van Hulle et al., 2010; Zhang et al., 2009). In stoichiometric balance of the oxygen evolution and consumption in MaB flocs in a MaB symbiosis (see Material and methods), a C:N ratio of about 6.4 is required to keep the culture anoxic and metabolically balanced (Eq.2.

#### 4.2.3.2. MaB treatment in elevated organic carbon (experiment OCS)

Compared to ICS assays, the pattern and intensity of nitrogen removal were considerably different in the OCS batch experiments. Nitrification could not progress as long as (exogenous) organic carbon was abundant in the wastewater. Improved biomass growth in a moderate pH level led to superior settleability (SVI=47±5 ml $g^{-1}$ in $OCS_b$) compared to ICS and CS experiments. Fig. 4 shows microscopic imagery of the flocs in OCS experiment which could reach to several millimeters.

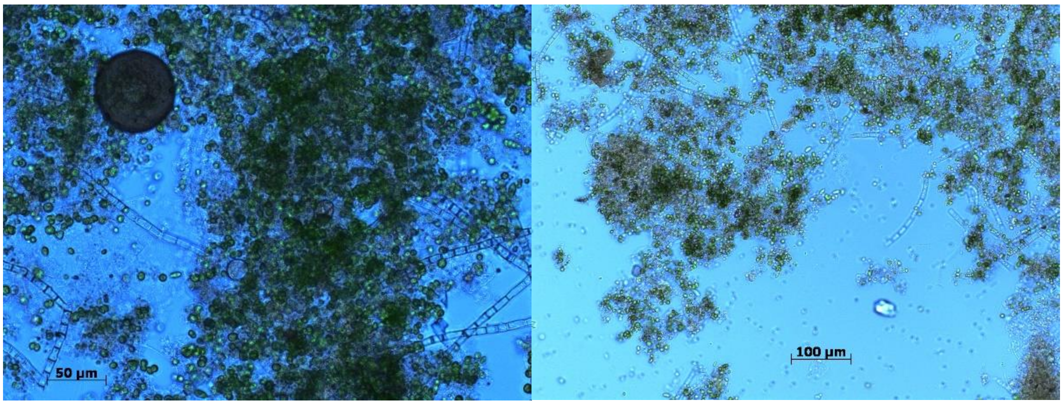


**Fig. 4** Microscopic view of the MaB flocs in OCS experiments. Filamentous algae *Tribonema* sp. and the bacterial community could form consistent structures to form larger aggregate.

##### 4.2.3.2.1. Heterotrophic activity inhibits the nitrification

Despite the ICS series, the TN and TAN curves followed a similar declining course in OCS experiments. Between 24-28 hours through both batches of OCS, both TN and TAN reached the nitrogen standard threshold of 10 mg N $L^{-1}$ according to EUS91 concurrently. This was achieved about 60% faster than in the ICS (in absence of additive organic carbon). The outstanding heterotrophic activity of the bacteria and mixotrophic nitrogen consumption by the algae left no oxygen for the autotrophic nitrifiers. Therefore no NOx was produced before the EUS91 limits were achieved (Van Hulle et al., 2010). The oxygen concentration remained essentially anoxic (DO<0.25 mg L$^{-1}$) while nitrification is completely inhibited when DO<2 mg L$^{-1}$. Inhibition of nitrification makes it impossible to credit denitrification as a major nitrogen removing mechanism.

The oxygen depletion persisted as long as the COD remained above 200 mg L$^{-1}$, implying that the heterotrophic uptake could consume the entire photosynthetic oxygen produced by the algae (see Fig. 5), therefore the nitrification was eliminated, and the heterotrophic uptake was limited by photosynthesis.

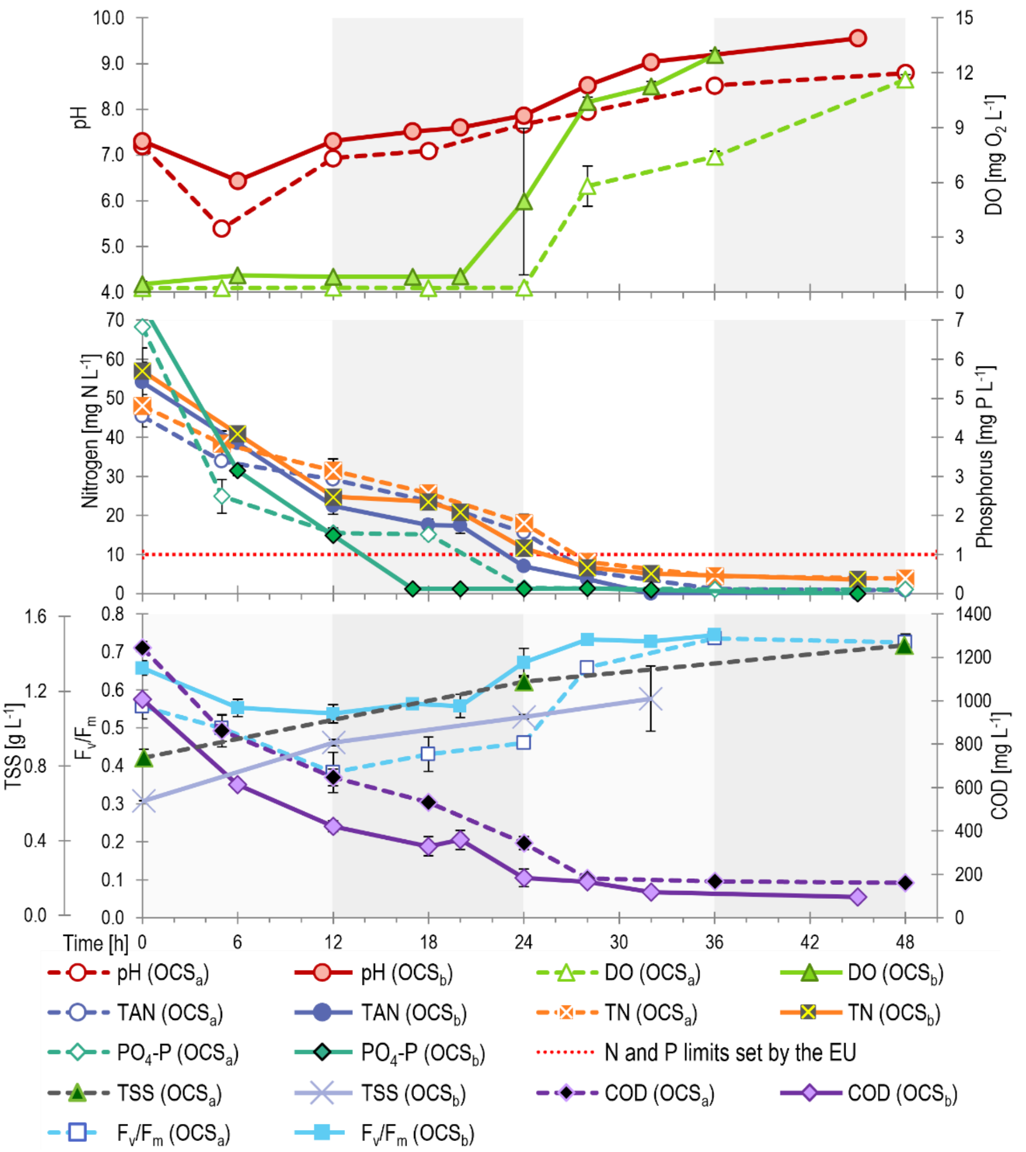


**Fig. 5** Chemical and biological characteristics of 2 batches of municipal wastewater MaB treatment with organic carbon supplement (OCS). OCSa is supplemented with 1000 mg of glucose and the added glucose to OCSb is proportional to its nitrogen content. Darker bands represent the dark hours. TSS: total suspended solids; COD: chemical oxygen demand; Error bars represent the standard deviation (N= ±SD).

The pH and especially DO increase drastically as the second illumination period began which can be interpreted as an indicator for the completion of ammonium removal (Foladori et al., 2018). The relatively lower $F_v/F_m$ throughout the anoxic phase shows that the PSII reaction centers were stressed, and the light energy could not promote oxidation of water (oxygen evolution) as efficiently as in ICS assays. Liang et al. (2009) showed that high concentrations of glucose can decrease the efficiency of photosynthesis. In their study, the inhibitory effects were severe when glucose concentration was more than 1% (w/v) which is much higher than the concentration of glucose in the OC experiments. It is beneficial to supplement the culture gradually to obviate the inhibitory effects on PSII. As the COD drops below 200 mg $L^{-1}$, no considerable inhibition was observed ($r_{OCSa}$ = -0.61 and $r_{OCSb}$ =-0.58, $p$<0.01 in both batches).

#### 4.2.3.2.2. Treatment characteristics with supplementary organic carbon

In MaB treatment with an unregulated pH (Experiment CS), ammonium uptake and respiration are the major elements that counter the alkalinization effects of photosynthesis. This effect diminishes as the organic carbon content depletes. In the OCS experiment, an abundance of organic carbon skewed the culture pH to acidic for a major part of the light period (the first 12 hours) which was associated with the ammonium uptake rate of up to 3.4±0.4 mg $NH_4$-N $L^{-1}$ $h^{-1}$ in $OCS_b$. Meanwhile, COD removal of 76.8±15.9 COD $L^{-1}$ $h^{-1}$ (34.1±5.7 OC $L^{-1}$ $h^{-1}$) and 65.7±3.1 COD $L^{-1}$ $h^{-1}$ (16.2±2.7 OC $L^{-1}$ $h^{-1}$) were achieved during the first 5 hours for $OCS_a$ and $OCS_b$, respectively. It seems that during the first 24 hours, a shortage of photosynthetic oxygenation was the limiting factor in removing the ammonium-N. The increase of light intensity in $OCS_b$ by about 46% increased the ammonium removal of $OCS_a$ from 29.9±3.8 to 47.1±5.2 mg N $L^{-1}$ $d^{-1}$ in $OCS_b$ (approximately a 57% increase).

During the first illumination period, the pH slowly increased to the neutral zone as the COD declined. Extra light application in $OCS_b$ provided a slightly more alkaline environment in compared to the OCSa experiment which is proportional to the PSII inhibition in the samples with higher glucose concentration. Evidently, not only higher light intensity did not cause photoinhibition, but it also limited the suppressive effect of the glucose on photosynthesis which accelerated the COD reduction through stronger oxygen evolution. A strong and positive correlation between the pH and $F_v/F_m$ confirms the association of the increased pH and photosynthesis (r= 0.77 in OCSa and r=0.74; $p$<0.001 for both).

During the first light period, removal rates were outstandingly high. The average TN and COD removal rates of $OCS_a$ during the first 12 hours of illumination were 36.2±13.5 mg N $L^{-1}$ $d^{-1}$ and 1304±102 mg COD $L^{-1}$ $d^{-1}$, respectively. These values were 70.3±13.4 mg N $L^{-1}$ $d^{-1}$ and 1277±62 mg COD $L^{-1}$ $d^{-1}$ for $OCS_b$. The phosphorus removal rates were 11.51±0.28 and 12.84±0.21 mg PO4-P $L^{-1}$ $d^{-1}$ in this period for the $OCS_a$ and $OCS_b$, respectively.

**Table 2.** Removal rates through different light cycles of MaB treatment with organic carbon supplement (OCS).

| | | $OCS_a$ | | | $OCS_b$ | | |
|---|---|---|---|---|---|---|---|
| | | TN | COD | $PO_4$–P | TN | COD | $PO_4$–P |
| Initial conc. [mg $L^{-1}$] | | 48.0±5.3 | 1245±27 | 6.83±0.02 | 57.0±6.0 | 1007±13 | 7.38±0.08 |
| Removal [%] | First light period (12 hrs) | 34.5±13.4% | 48.0±8.1% | 77.2±2.4% | 56.5±12.3% | 58.1±5.1% | 79.8±1.9% |
| | Dark period | 27.9±19.4% | 24.4±9.5% | 20.7±2.5 | 23.0±18.4% | 23.7±7.4% | 18.5±2.5% |
| | 4 hrs through the second light period (hrs 24-28) | 20.8±20.3 | 13.1±6.5% | 0.1±0.9% | 8.7±19.6% | 1.8±6% | -0.1±2.2 |
| 28 hours of MaB treatment: | | | | | | | |
| Removal [%] | | 83.2±14.8% | 85.4±3.4% | 98.0±0.7% | 88.3±13.9% | 83.5±2.6% | 98.2±1.6% |
| Removal rate [mg $L^{-1}$ $d^{-1}$] | | 34.3±4.7 | 912±23 | 5.74±0.04 | 43.1±5.1 | 721±16 | 6.21±0.07 |
| *Concentration [mg $L^{-1}$] | | 8.1±1.5 | 181±3 | 0.14±0.04 | 6.7±0.2 | 166±14 | 0.14±0.01 |

* The concentration in the medium after 28 hours of treatment.

Table 2 depicts that the majority of the nutrients were removed during the first light period which shows the effectiveness of both photosynthetic aeration and photoautotrophic assimilation in the MaB flocs. However, the removal through the dark hours were not insignificant. When the nitrogen and carbon are balanced, any amount of exogenously absorbed or endogenously produced oxygen will be used to break up the organic matter and sustain the symbiotic cycle of nutrients within the MaB aggregates and therefore, this eliminates any chance for autotrophic bacteria activity (nitrification). The concentration of nitrite and nitrate detected in the first 24 hours of treatment in both OCS assays steadily remained nearly zero. Only after the COD concentration fell below 200 mg

$L^{-1}$, did the oxygen and the pH start to rise which creates opportunity for nitrification. Since both nitrogen and COD were removed proportionally (Table 2), the remaining amount of ammonium for nitrification was already too low (about 10 mg $L^{-1}$) to promote any considerable influence on the TN concentration. The first signs of nitrification (0.37±0.03 mg $NO_2$-N in $OCS_a$ and 0.26±0.22 mg $NO_2$-N $L^{-1}$ in $OCS_b$) were detected in the samples taken 36 hour and 24 hours after the start of $OCS_a$ and $OCS_b$, respectively. This shows that higher COD in $OCS_a$ could delay the buildup of DO to beyond the inhibitory threshold for AOB. Therefore, the maximum amount of NOx was slightly higher but insignificant in $OCS_b$ (2.69±0.27 mg NOx–N $L^{-1}$ after 32 hours) versus 1.18 mg NOx–N after 48 hours of $OCS_a$. However, the water quality can meet the EUS91 requirements in less than 28 hours. It is expected that this time can easily fall below 24 hours if the illumination period is extended. This condition is naturally available in the summer season, especially in northern of Europe.

The nutrients and carbon removal rates decreased significantly during the dark period as photosynthesis was absent and mechanical mixing in free-surface bioreactors could maintain a limited gas exchange with the atmospheric oxygen. It has however been shown that the heterotrophic uptake of organic carbon by the algae is not necessarily correlated to the availability of the oxygen (Fan et al., 2020). The heterotrophic metabolism in this period could claim a 304±48 and 238±48 mg COD $L^{-1}$ decrease for $OCS_a$ and $OCS_b$, respectively. This amount of glucose removal was associated with the recovery of the photosynthesis activity after the dark period as the $F_v/F_m$ increased. It is noteworthy that the inhibitory effects of calculated glucose on photosynthesis in the balanced culture of $OCS_b$ with 46% higher light intensity was milder than $OCS_a$ according to their average $F_v/F_m$ values. This difference was statistically insignificant ($F_v/F_m$ of $OCS_b$ = 0.63±0.08 versus 0.56±0.13 for $OCS_a$, $p$=0.06). It could be that PSII underwent less pH stress due to milder acidification of the $OCS_b$ during the first light period (Zhao et al., 2021). After the first light-dark cycle (24h), the concentration of the nutrients was either in or close to the EUS91 limits in both assays. These values for $OCS_a$ were 18.04±2.32 mg N $L^{-1}$ (62.5±22.4% removal), 344±29 mg COD $L^{-1}$ (72.4±4.9% removal), and 0.15±0.03 mg P $L^{-1}$ (97.9±0.6% removal). Measurement of these parameters in $OCS_b$ showed a better treatment performance with 11.63±1.95 mg N $L^{-1}$ (79.6±17.3% removal), 184±41 mg COD $L^{-1}$ (81.8±5.4% removal) and 0.13±0.01 mg P $L^{-1}$ (98.3±1.6% removal). After only 28 hours

EUS91 limits for N and P were satisfied but COD fell below 125 mg $L^{-1}$ after about 31 hours (Fig. 5).

These results demonstrate that higher light intensity could efficiently improve the nutrients uptake through both heterotrophic and photoautotrophic metabolisms. According to our results, it is plausible to achieve a standard treatment in long summer days (especially in northern of Europe) in less than one day of retention time as the long summer daylight can provide the energy for meaningful photosynthetic aeration of the MaB flocs.

There are industrial wastewaters with high biodegradable carbon content which can supply the required organic carbon to balance the C:N ratio in the MaB treatment. This offers the advantage of reducing the load on existing conventional wastewater treatment plants in a given region. For example, the wastewater from alcohol, cheese, and starch and corn processing could be evaluated for this purpose (Al-Mallahi and Ishii, 2022; Gélinas et al., 2015).

It is noteworthy that having a surplus of organic carbon slightly greater than what the forementioned equations suggest, can be beneficial as the heterotrophic bacteria can continue producing $CO_2$ while ammonium is already depleted. The combination of nitrogen shortage and $CO_2$ abundance promotes intracellular lipid accumulation in the algae which can improve biodiesel yield from the harvested biomass, promoting further economic processing of the evolving biomass (Arguelles and Martinez-Goss, 2021; Ma et al., 2022).

### 4.2.4. Conclusion

The MaB technology can be reliably used to substitute mechanical aeration for wastewater treatment. Regulating the pH with supplemental $CO_2$ could prevent the inhibitions in 2 and 3 days of HRT. Nitrification hindered attaining the EUS91 limits after 2 days, but 3 days of treatment was successful in terms of water quality. Balancing the organic carbon exogenously could lead to a single-stage nitrogen and phosphorus removal in about 24 hours. More studies are needed to investigate the influence of gradual increase of organic carbon on the inhibition of photosynthesis. Sustainable alternatives for glucose in this scenario should be examined.

### 4.2.5. Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could influence the work reported in this paper.

### 4.2.6. Acknowledgement

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## 4.3. Appendix 3, Substitutes for Glucose to Elevate the COD:N Ratio

The pre-treated wastewater was collected from the Seevetal WWTP. The mixtures with the organic carbon supplements were prepared to achieve the COD:N (w:w) ratio of about 16. Illumination with photoperiod of 14:10 hours (light:dark) was applied. For each supplementation, 2 batches of treatment of the municipal wastewater (see Table 5) were conducted. Except the control series (with no OC supplement), other assays were conducted for 38 hours which includes 2 illumination periods of 14 hours that were separated by 10 hours of dark period. This would provide enough time to understand if the treatment qualities of the supplemented MaB cultures were comparable with glucose. Some of the chemical and physical properties of the collected municipal wastewaters in these assays are presented per type of the supplement:

| | Municipal Wastewater characteristics | | | | | |
|---|---|---|---|---|---|---|
| **Supplement** | TSS [mg $L^{-1}$] | VSS [mg $L^{-1}$] | COD [mg $L^{-1}$] | TN [mg $L^{-1}$] | $NH_4^+$–N [mg $L^{-1}$] | $PO_4^{3-}$–P [mg $L^{-1}$] |
| Control | 158 | 154 | 358 | 60 | 52 | 4.6 |
| Glycerol | 132 | 126 | 258 | 57 | 52 | 3.2 |
| LSW | 203 | 168 | 276 | 63 | 57 | 6.1 |
| BBL | 148 | 139 | 216 | 60 | 59 | 5.5 |

**Table** 5 The characteristics of the municipal wastewaters which were used for supplementation with the respective substrates. The wastewater composition varied as some samples were more diluted due to rainy weather (the wastewater collection system is a combined one).

To avoid  possible inhibitions due to high concentration of the supplements and a better chance for acclimatization of the MaB with the new medium, the batches started without addition of the supplementary organic carbon substrates. After 4-5 hours, about 40%–50% of the calculated amount of substrate was added and culture continued until the measured oxygen would show a spike which would mean that the organic carbon is consumed and the risk of nitrification would increase. Therefore, the rest of the organic carbon supplement was introduced into the culture. The results of the wastewater treatment without supplementation (Control series), with Glucose (G), with lawn silage extract (LS), and the wastewater from the beer brewing lautering (BBL) are shown below.

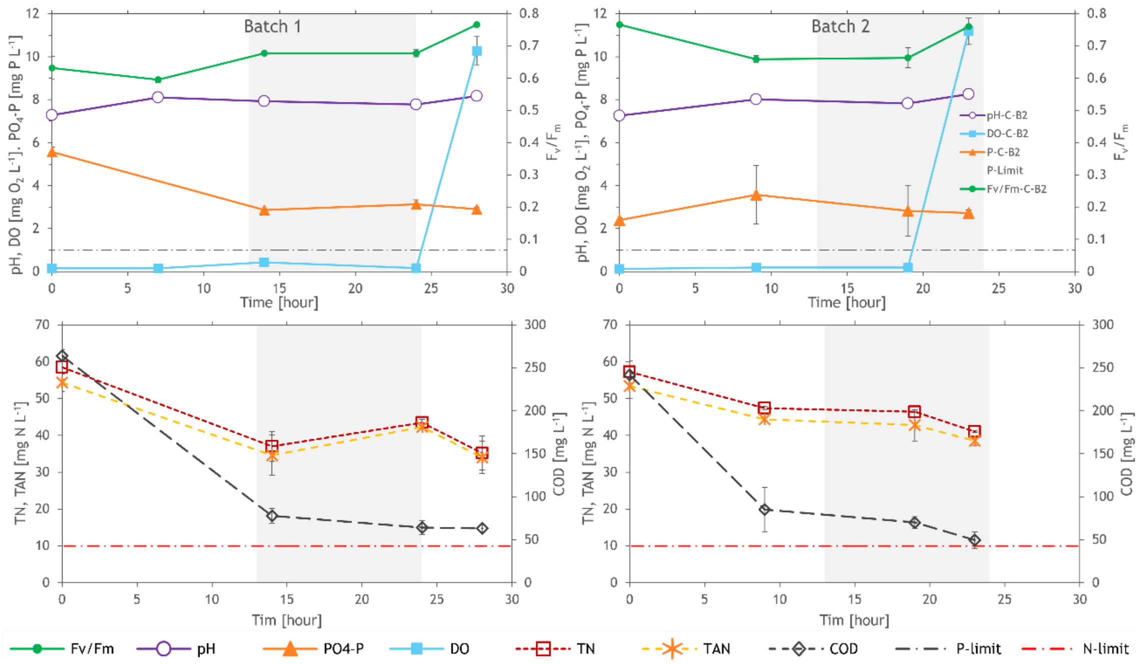


**Fig. 4** Treatment of municipal wastewater in the MaB culture without supplementation (Control).

General note for the charts: DO: dissolved oxygen, TN: total nitrogen, TAN: total ammoniacal nitrogen, COD: chemical oxygen demand; The P-limit and N-limits lines are the maximum allowed concentration of the phosphorus and nitrogen in the effluent by the European tandard (EUS91). These limits are 1 mg P L$^{-1}$ and 10 mg N L$^{-1}$ respectively. The shaded area represents the dark period. The error bars indicate the standard deviation (N=±SD)

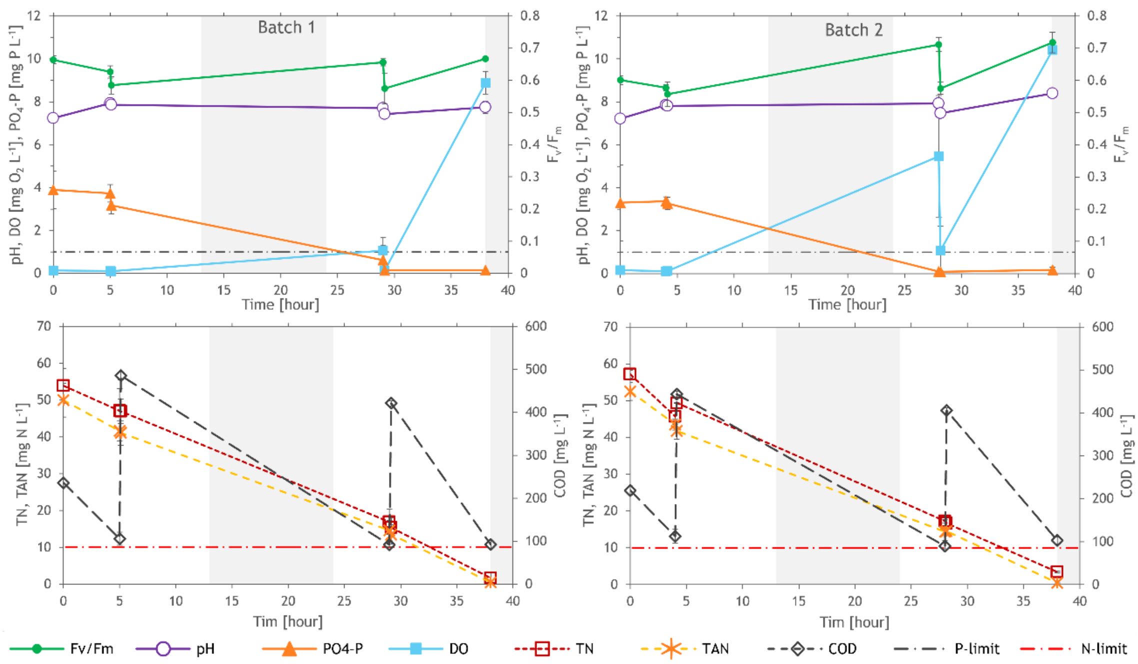


**Fig. 5** Treatment of municipal wastewater in the MaB culture supplemented with glucose as the external source of organic carbon.

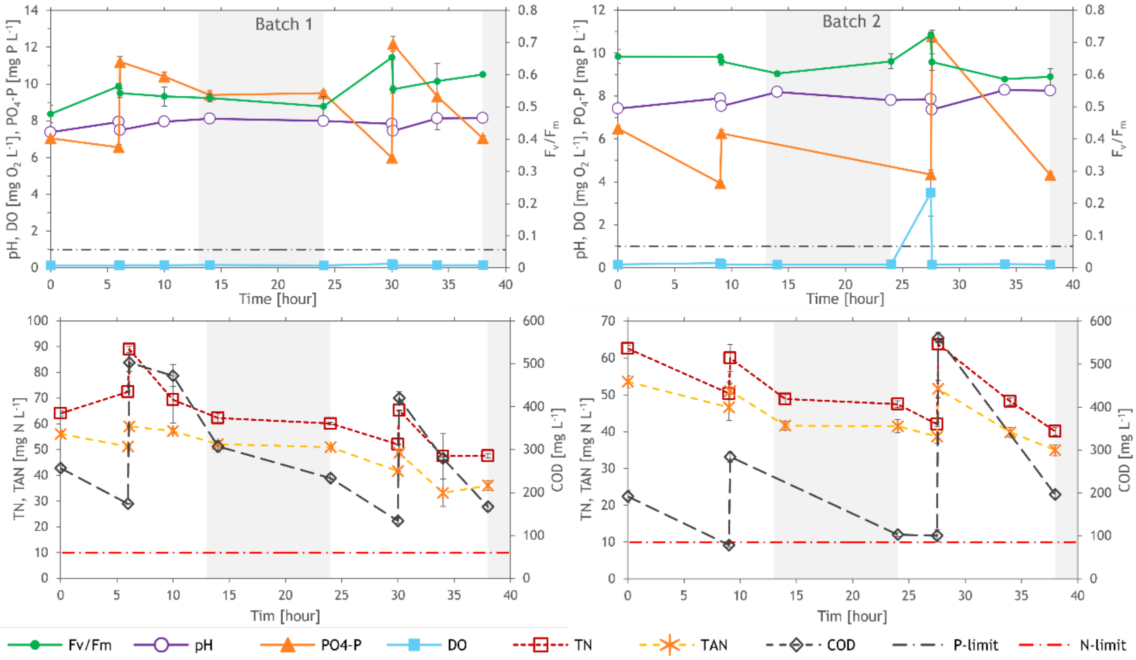


**Fig. 6** Treatment of municipal wastewater in the MaB culture supplemented with lawn silage (LS) extract as the external source of organic carbon.

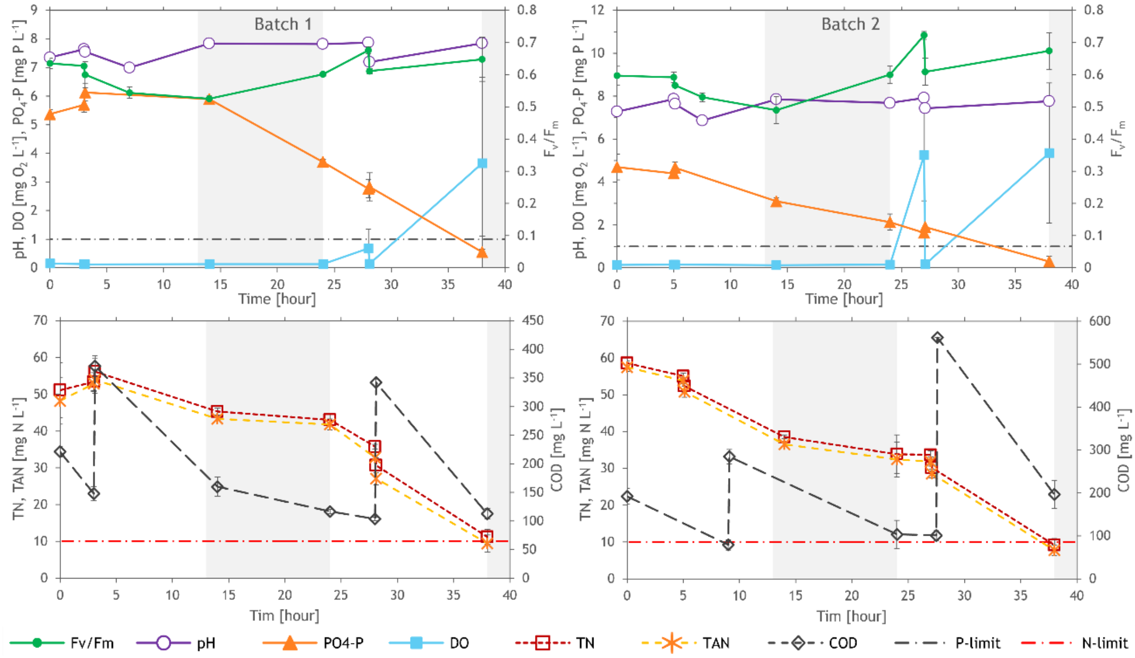


**Fig. 7** Treatment of municipal wastewater in the MaB culture supplemented with the wastewater from the lautering process of beer brewing (BBL) as the external source of organic carbon.