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Research article

Evaluating CO_2 emissions from continuous flow and batch growth systems under autotrophic mode: Implications for GHG accounting of biological nutrient removal

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ABSTRACT

The oxidation of ammonia by autotrophic bacteria is a central part of the nitrogen cycle and a fundamental aspect of biological nutrient removal (BNR) during wastewater treatment. Autotrophic ammonia oxidation produces protons and results in net-CO_2 production due to the neutralizing effect of bicarbonate alkalinity. Attention must be paid to the propensity for this produced CO_2 to be transferred to the atmosphere where it can act as a greenhouse gas (GHG). In the context of BNR systems, bicarbonate-derived CO_2 emissions should be considered distinct from the biogenic CO_2 that arises from cellular respiration, though this distinction is not made in current GHG accounting practices. The aim of this study was to evaluate the performance of two experimental systems operated under autotrophic mode and buffered with bicarbonate, to investigate the relationship between ammonia removal and gaseous CO_2 emissions. The first system consisted of continuously aerated lab-scale batch reactors, which were effective in demonstrating the important link between ammonia oxidizer activity, pH, and gaseous CO_2 production. Depletion of the buffer system always led to a rapid decline in system pH and cessation of CO_2 emissions when the pH fell below 7.0. The second system was a tubular continuous-flow biofilm reactor which permitted comparison of ammonia removal and CO_2 emission rates. A linear relationship between ammonia removal and CO_2 emissions was demonstrated and the quantified CO_2 production was relatively close to that which was predicted based on the stoichiometry of nitrification, with this CO_2 being detected in the gas phase. It was apparent that this system offered minimal resistance to the mass transfer of CO_2 from the liquid to gas, which is an important factor that determines how much of the bicarbonate-derived CO_2 may contribute to greenhouse gas emissions in engineered systems such as those used for BNR.

1. Introduction

Disruption of the global carbon cycle by anthropogenic inputs of carbon dioxide (CO_2) to the atmosphere has led to increasing global temperatures, since Earth's climate has long been regulated by the partial pressure of this gas (Martin, 2017). In the absence of serious intervention, CO_2 concentrations will continue to rise and could double by the end of the 21st century (Beaulieu et al., 2012). Collectively, the microbial processes involved in wastewater treatment represent a significant source of greenhouse gasses (GHG), including CO_2, nitrous oxide (N_2O), and methane (CH_4) (Zang et al., 2015; Mannina et al., 2016; Chen et al., 2018; Gallego-Schmid and Tarpani, 2019). While voluntary reporting of gaseous emissions from municipal wastewater treatment plants is currently uncommon, future regulations could mandate it, like the reporting required for the treatment of many industrial wastewaters (Environment and Climate Change Canada, 2019). Regardless of potential regulatory obligations, the broader societal progression towards sustainability and climate change mitigation, and the desire for more accurate GHG accounting protocols, highlight the need for a thorough understanding of all possible GHG sources during biological wastewater treatment.

An increasing number of treatment systems have adopted biological nutrient removal (BNR) to mitigate the deleterious effects of excess nitrogen and phosphorous in receiving waters. A fundamental aspect of nitrogen removal, whether based on conventional nitrification-denitrification or more efficient alternatives such as partial nitritation-

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ANAMMOX, is the oxidation of ammonia by chemolithoautotrophic bacteria under aerobic conditions (Winkler and Straka, 2019). Ebeling et al. (2006) devised the overall stoichiometric equation for the oxidation of ammonia to nitrate during autotrophic nitrification, using half-reaction relationships and taking into account the inorganic carbon requirement for cell synthesis by ammonia and nitrite oxidizing bacteria (Equation (1)). Each mole of ammonia is almost entirely consumed for energy generation, with only a small fraction of the ammonia-nitrogen being used for biomass growth, resulting in the production of 0.977 mol of nitrate and 1.95 mol of protons (H^+) (Ebeling et al., 2006). In systems with insufficient buffering capacity, the accumulation of protons can cause the pH to drop and ultimately inhibit nitrifier activity, leading to insufficient effluent quality (Hu et al., 2015). The alkalinity content in BNR systems is therefore an important factor to ensure optimal nitrogen removal performance, though the contribution of this proton neutralization to potential GHG emissions from BNR systems is often not considered.

$$NH_4^+ + 1.83O_2 + 0.094CO_2 + 0.024HCO3_3^- \rightarrow 0.024C_5H_7O_2N + 0.977NO_3^- + 0.953H_2O + 1.95H^+ \quad (1)$$

In geographical regions rich in limestone and dolomite, the carbonate content of the source water in BNR systems can provide enough alkalinity to ensure that ammonia limits are achieved, however hydroxides and other wastewater components can also contribute to pH buffering (Shanahan and Semmens, 2015). In other regions, it is sometimes necessary to supplement the alkalinity via chemical addition, which can involve the use of carbonate-based compounds such as sodium carbonate or sodium bicarbonate. The carbonate system includes the carbonate ion, bicarbonate ion, and carbonic acid in a pH-dependent equilibrium (Equation (2)), with carbonic acid forming its own pH-independent equilibrium with CO_2 (approximately 99.8% as CO_2 at 25 °C) (Equation (3)) (Shanahan and Semmens, 2015; Martin, 2017). At the pH range in BNR aeration tanks where ammonia oxidation occurs (i.e. pH 6.8–8.0), the bicarbonate ion is the dominant species of the carbonate system and provides buffering against acidification by absorbing protons at a one to 1 M ratio (Shanahan and Semmens, 2015).

Ebeling et al. (2006) extended the stoichiometry of autotrophic nitrification to also consider the interaction with the carbonate equilibrium (Equation (4)). Evidently, for BNR systems in which the alkalinity requirement is entirely fulfilled by the carbonate system, as is the case at many treatment plants, each mole of ammonia can be expected to result in the production roughly 1.86 mol of CO_2 from the neutralization of protons by the bicarbonate ion (Ebeling et al., 2006). The propensity for this produced CO_2 to be transferred to the atmosphere is highly dependent on many factors such as reactor operation (e.g. batch versus continuous-flow) and the mechanism of CO_2 transfer (e.g. air-stripping versus diffusion), both which were explored in this study. Other important parameters that can affect the mass transfer coefficient of CO_2 in BNR systems include the bubble size during aeration, operating temperature, and wastewater characteristics (Amaral et al., 2019).

$$H_2CO_3 \rightarrow H^+ + HCO_3^- \rightarrow 2H^+ + CO_3^{2-} \quad (pK_{a1} = 6.35;\ pK_{a2} = 10.33) \quad (2)$$

$$CO_2 + H_2O \rightarrow H_2CO_3 \quad (3)$$

$$NH_4^+ + 1.83O_2 + 1.97HCO_3^- \rightarrow 0.02444C_5H_7O_2N + 0.97\,NO_3^- + 2.90H_2O + 1.86\,CO_2 \quad (4)$$

Ammonia oxidation is the largest consumer of alkalinity during BNR processes, since it results in an ever-growing pool of protons that must be neutralized. Despite the metabolic requirement for inorganic carbon, the potential for autotrophic ammonia oxidation to result in net-CO_2 emissions deserves attention in the context of GHG accounting. It is common to attribute all direct CO_2 emissions generated during aerobic treatment processes to cellular respiration (Willis et al., 2016), however CO_2 produced from the neutralization of protons by bicarbonate alkalinity could be considered as a distinct source, given the different timescales involved in the cycling of biogenic versus inorganic carbon. The phenomenon in question could be particularly relevant for BNR systems treating high strength ammonia wastewater and therefore should be studied empirically to inform and validate related modelling approaches.

On a fundamental level, CO_2 production during autotrophic ammonia oxidation is generally well understood (Ebeling et al., 2006), though greater focus on the practical implications as they relate to reactor design and operating conditions is required, especially considering ongoing GHG mitigation efforts. The objective of the present study was therefore to evaluate the performance of continuously aerated batch reactors and continuous-flow biofilm reactors under autotrophic mode, in order to investigate the relationship between ammonia removal and gaseous CO_2 emissions in both systems. The biofilm reactor involved the use of a continuous-flow CO_2 evolution measurement system (CEMS) which was originally developed in-house as a tool for tracking the metabolic activity of heterotrophic biofilms (Kroukamp and Wolfaardt, 2009). The experiments described herein represent the novel use of this experimental system to investigate CO_2 emissions arising indirectly from ammonia oxidation.

2. Materials and methods

2.1. Cultivation of nitrifying biomass in a fixed-film continuous-flow bioreactor

A fixed-film continuous-flow bioreactor (Fig. S1) was operated autotrophically in order to cultivate and maintain an actively nitrifying microbial community, such that biomass from this bioreactor could be routinely sampled and inoculated into experiments with online monitoring of CO_2 flux. The same autotrophic bioreactor was previously used in a study that tested the acclimation of nitrifiers under increasing ammonia loads (Aqeel and Liss, 2020). The microbial community analysis performed in that study suggested that the bioreactor (therein referred to as "BioCord SBR") already contained a community of nitrifying bacteria, including ammonia oxidizers, at the onset of the present study. The bioreactor had a working volume of 2.2 L and contained approximately 0.25 m of BioCord™ (Bishop Water Technologies, Renfrew, ON), a rope-like material which provided approximately 0.6 m^2 of surface area for microbial attachment and biofilm formation. The use of BioCord™ helped to ensure the development of a fixed biofilm which minimized washout of the slow-growing autotrophic biomass, since the dilution rate (roughly 1 d^{-1}) was higher than the maximum specific growth rate (μmax) values previously reported for ammonia and nitrite-oxidizing bacteria (e.g. 0.54 d^{-1} and 0.67 d^{-1}, respectively, in Blackburne et al., 2007).

The bioreactor was originally seeded as per the method described by Aqeel and Liss (2020), which involved the use of activated sludge from a municipal wastewater treatment plant (Cataraqui Bay Wastewater Treatment Plant, Kingston, Ontario, Canada) as the seed biomass.

Following the conclusion of that study, the bioreactor was carefully transported to a new location and re-started without reseeding after approximately 4 h of downtime. To minimize the loss of any sloughed biomass, the reactor was first operated for 24 h with no wasting, after which it received a continuous flow of autotrophic synthetic wastewater medium for the duration of the study. The medium was delivered via a peristaltic pump (Watson-Marlow, Concord, ON) to the bottom of the bioreactor while a second pump (MilliporeSigma, Burlington, MA) continuously drained effluent at the 2.2 L mark, establishing a hydraulic retention time of 24 h. The bioreactor was continuously aerated with 0.2 μm-filtered ambient room air, delivered through an airstone using a standard aquarium pump, which facilitated efficient mass transfer of oxygen while also providing mixing. Periodic checks with a dissolved oxygen probe (Fisher Scientific, Ottawa, ON) indicated that the dissolved oxygen concentration was maintained near saturation (7–9 mg/L). The pH of the bioreactor was also monitored in situ via a pH probe (Cole-Parmer, Montreal, QC) to ensure the pH was maintained at 7.5 ± 0.3.

2.2. Autotrophic synthetic wastewater medium

An autotrophic synthetic wastewater medium modified from Hoang et al. (2014) was used as the feed for the fixed-film continuous-flow bioreactor. It consisted of $FeSO_4 \cdot 7H_2O$ (1.80×10^{-2} mM), KH_2PO_4 (0.59 mM), $CaCl_2 \cdot 2H_2O$ (0.20 mM), $MgSO_4 \cdot 7H_2O$ (0.29 mM), $Na_2MoO_4 \cdot 2H_2O$ (4.13×10^{-5} mM), $CuSO_4 \cdot 5H_2O$ (1.56×10^{-3} mM), $CoCl_2 \cdot 6H_2O$ (1.72×10^{-3} mM), and $ZnSO_4 \cdot 7H_2O$ (1.53×10^{-3} mM). It also contained $(NH_4)_2SO_4$ (0.79 mM) as the source of ammonia and $NaHCO_3$ (3.5 mM) as the source of bicarbonate alkalinity. The same medium was also used for subsequent batch and continuous-flow growth experiments with CO_2-monitoring, though the concentrations of ammonia and bicarbonate were varied to study the impact on CO_2 flux. Each component of the medium was maintained as a 1000x stock solution and diluted as necessary, except for $(NH_4)_2SO_4$ and $NaHCO_3$, which were prepared from powder. Notably absent from the medium was any form of organic carbon, which was meant to limit heterotrophic growth during the enrichment of autotrophic nitrifiers (Hoang et al., 2014). The pH of the medium was typically not adjusted prior to use, except in experiments involving short duration feeding with different compositions of the medium. In those cases, the pH was standardized to 7.5 in order to minimize the incidental effect of variable influent pH.

2.3. Determining ammonia removal performance

The performance of the fixed-film continuous-flow bioreactor was assessed using a method like that employed by Aqeel and Liss (2020). Briefly, 0.01 L samples of liquid effluent were collected every five days and immediately filtered (0.2 μm) to prevent further biological activity. The concentration of nitrogen as ammonia, nitrite, and nitrate was then quantified based on single samples using test kits from Hach (London, ON), employing the salicylate method (Hach Method 10,031), diazotization method (Hach Method 10,019), and the chromotropic method (Hach Method 10,020), respectively. Absorbance values were obtained using a bench-top spectrophotometer (VWR International, Mississauga, ON) rather than the prescribed handheld instrument, and subsequently converted to concentrations using standard curves that were constructed for each nitrogen species. When a sample concentration exceeded the detection limit of the kit, the sample was appropriately diluted and re-analyzed, with this dilution accounted for in subsequent calculations. Ammonia removal in the batch and continuous-flow growth experiments with CO_2 monitoring was also determined using the test kit, though this involved comparing single initial and end-point samples (batch) and time-resolved influent and effluent samples (CEMS). In the latter case, the flow rate of the medium was used to express ammonia removal as a rate (mmol/h) to allow for a more suitable comparison to the CO_2 emission rate.

2.4. Preparation of inocula for CO_2 flux experiments

The fixed-film continuous-flow bioreactor provided a consistent source of inocula for experiments studying the flux of CO_2 during autotrophic ammonia removal. At the onset of each experiment, biomass was first sampled from the fixed-film bioreactor by gently cutting and removing two short biofilm-colonized segments of the BioCord™ material (approximately 3 cm each). This was carried out after first halting aeration in order to minimize incidental sloughing of the biomass when the BioCord™ was handled. The sampled segments were then placed in a 0.05 L culture tube to which 0.04 L of fresh medium was added, and vortexed at maximum speed for 1 min to transfer the attached biomass into suspension. The concentration of suspended solids was then determined via dry weight, which provided a simple method of standardizing the amount of biomass that was used for inoculation.

2.5. CO_2 flux during autotrophic ammonia removal in batch reactors

The performance of small-volume batch reactors (Fig. S2) was investigated when operated in autotrophic mode, with the aim to compare ammonia removal and CO_2 emission characteristics under different bicarbonate loads. The set-up was similar to the flowing gas, static liquid configuration for respirometry measurements in the gas phase (GFS) described by Spanjers and Vanrolleghem (2016), though adapted here to permit measurement of headspace CO_2 rather than O_2. The reactors consisted of 0.25 L Erlenmeyer flasks with constant mixing via a stir bar and were sealed using a rubber stopper through which two 20-gauge needles were inserted to facilitate continuous aeration of the medium and sampling of headspace gas. The pH of each batch reactor was also monitored and logged at 1.5 h intervals using a pH probe (Cole-Parmer, Montreal, QC), ensuring that an airtight seal was maintained so that headspace CO_2 measurements were not affected by fluctuations in the composition of ambient indoor air. The reactors were therefore continuously aerated with a 0.2 μm-filtered gas mixture with CO_2 concentration fixed at approximately 9 mM (Messer, Whitby, ON). The aeration gas also contained enough oxygen to maintain the dissolved oxygen concentration near saturation (7–9 mg/L). Mass flow controllers (Aalborg, Orangeburg, NY) were used to deliver the aeration gas through airstones at a flow rate of 0.02 L/min.

Batch reactors were filled with 0.20 L of autotrophic synthetic wastewater medium and then inoculated with 0.01 L of biomass suspension that was appropriately diluted so that an initial suspended solids concentration of roughly 350 mg/L was achieved in the reactors. This was in the range used by Blackburne et al. (2007) in similar batch nitrification experiments with CO_2 monitoring (i.e. 270-360 mg/L). After briefly mixing, 0.01 L samples were filtered and removed to confirm the initial ammonia concentration, where after the reactors were sealed and incubated at room temperature. The headspace of each reactor was connected to a non-dispersive infrared CO_2 analyzer (LI-COR Biosciences, Lincoln, NB), which logged the parts per million CO_2 concentration every minute. Time-resolved CO_2 concentration data was then used to calculate the CO_2 emission rate (mmol of CO_2/h) using the ideal gas law as well as the flow rate of the aeration gas (Bester et al., 2010). Each batch reactor was operated in parallel with an uninoculated control reactor to ensure that changes in CO_2 emissions could be attributed to microbial activity and not abiotic factors. When CO_2 emissions from the inoculated reactors returned to baseline, the experiment was considered complete, and the reactors were opened and sampled to determine the total ammonia removed during the incubation period.

2.6. CO_2 flux during autotrophic ammonia removal in a continuous-flow biofilm reactor

A continuous-flow CO_2 evolution measurement system (CEMS) was used to investigate CO_2 emissions during ammonia removal by

autotrophic biofilms. This system was originally developed as a means of tracking whole-biofilm metabolism (Kroukamp and Wolfaardt, 2009) and has since received extensive use as a tool to measure the responses of heterotrophic biofilms to perturbations such as antibiotic exposures (e.g. Jackson et al., 2019). A recent study has also adapted the system to investigate CO_2 uptake by photoautotrophic microalgal biofilms (Ronan et al., 2020), however the present study represents the novel use of the CEMS for studying CO_2 flux during autotrophic ammonia removal. The CEMS was 1.5 m in length and consisted of a tube-within-a-tube, whereby a biofilm was grown under plug flow conditions inside a silicone tube (ID 1.57 mm; OD 2.41 mm; VWR International, Mississauga, ON) that was housed within a larger diameter Tygon™ tube (ID 4.76 mm; OD 7.94 mm; VWR International, Mississauga, ON) (Fig. S3). The total volume and surface area of the biofilm-colonizable region was 0.0029 L and 0.0074 m^2, respectively. Given the high gas permeability of silicone and the comparatively lower permeability of Tygon™, the CO_2 concentration within the interstitial space reflected the flux of CO_2 during biofilm growth. Online monitoring of this flux was achieved using a CO_2-free sweeper gas (Messer, Whitby, ON), which flowed through the interstitial space at 0.01 L/min via a mass flow controller (Aalborg, Orangeburg, NY) and into a non-dispersive infrared CO_2 analyzer (LI-COR Biosciences, Lincoln, NB).

The CEMS was sterilized by passing a 20% solution of commercial bleach through for a minimum of 6 h, followed by rinsing with sterile dH_2O for a minimum of 4 h. Flow of autotrophic synthetic wastewater medium was then initiated at 0.015 L/h using a peristaltic pump (Watson-Marlow, Concord, ON), which resulted in a dilution rate (approximately 5.17 h^{-1}) that was considerably higher than the expected μmax for autotrophic nitrifiers. The medium reservoir was kept sealed and connected to a gas bag inflated with 9 mM CO_2 gas to mimic an atmosphere with fixed CO_2 concentration. The CEMS was inoculated by injection of approximately 0.003 L of biomass suspension. After injection, flow of the medium was halted for 1 h to allow initial attachment to the inner walls of the CEMS. Growth curves of the autotrophic biofilms were created, and experiments were subsequently carried out using mature biofilms (>21 days old) to ensure that stable operating conditions were first reached and maintained for several days. The aim of these experiments was to evaluate the utility of the CEMS under autotrophic mode with variable ammonia and bicarbonate loading, by quantifying and comparing the rates of ammonia removal and CO_2 emissions. An additional experiment was also performed to test the stability of the system when the medium was supplemented with organic carbon in the form of sodium acetate (1.78 mmol/h) for a period of 24 h.

3. Results and discussion

3.1. Performance of the fixed-film continuous-flow bioreactor

Interest in fixed-film technologies such as BioCord™ has grown in recent decades, especially as a method of improving the nitrification capacity of existing wastewater treatment systems, since retention of autotrophic biomass is usually a limiting factor for efficient BNR (Gan et al., 2018; Skoyles et al., 2020). The fixed-film bioreactor was operated in a manner that was meant to support the growth of nitrifying microbes with autotrophic metabolism, since the medium lacked organic carbon. Previous use of the autotrophic bioreactor in a study that investigated ammonia removal under increasing ammonia loads required approximately 45 days after seeding until stable operating conditions were achieved, which was not surprising given the relatively slow growth rate of autotrophs (Aqeel and Liss, 2020). At the conclusion of that study, the bioreactor was transported to a new location and the present study was initiated without any biomass wasting, which contributed to the comparatively shorter time required to once again reach stable operation.

Ammonia removal efficiency greater than 97% was consistently achieved, except for one instance when it dropped to roughly 93% on day 51 (Fig. 1). Influent and effluent measurements showed that on average, effluent nitrate-N accounted for roughly 95% of the influent ammonia-N, which was only slightly lower than the expected value (97.7%) based on the stoichiometry of autotrophic nitrification (Ebeling et al., 2006). This suggested that denitrification was minimal under these operating conditions. The oxidation of ammonia to nitrate was presumably via nitrite, though the effluent concentration of nitrite was usually below detection. The use of BioCord™ was effective at maintaining appreciable biomass in the continuous-flow bioreactor (Fig. S4), though the average suspended solids concentration in the effluent was found to be 9.12 ± 11.05 mg/L, which suggested that biomass sloughing did occasionally occur. Nonetheless, the performance of the fixed-film bioreactor indicated the continued presence of autotrophic ammonia oxidizers, and so biomass from this bioreactor was regularly sampled and used in experiments to study CO_2 flux during autotrophic ammonia removal.

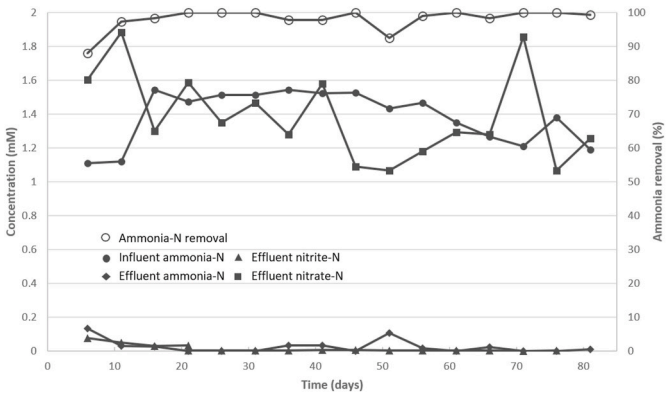

Fig. 1. Influent and effluent ammonia-N, nitrite-N, and nitrate-N concentrations were measured in the fixed-film continuous-flow bioreactor, as well as the overall ammonia removal efficiency.

3.2. CO_2 flux during autotrophic ammonia removal in batch reactors

CO_2 flux during autotrophic ammonia removal was investigated using small volume batch reactors inoculated with nitrifying biomass from the fixed-film continuous-flow bioreactor. The batch reactors were intentionally prepared to be alkalinity-limited so that the effect of buffer depletion could be observed. Ammonia removal and CO_2 flux was examined in autotrophic synthetic wastewater medium as described in Table 1. Not surprisingly, batch reactors with lower initial bicarbonate content (Fig. 2A) experienced a pH limitation much sooner than those with more bicarbonate (Fig. 2B), which was signified by a steep decline in the slope of the pH curves. This also resulted in less ammonia being removed (Table 1) and much narrower CO_2 emission curves, with less total CO_2 being produced and transferred to the gas phase. However, regardless of the initial reactor conditions, CO_2 emissions rapidly declined and ultimately ceased when the bulk pH fell below 7.0, indicating that ammonia oxidizer activity was inhibited below this point.

The simple batch reactor set-up provided an effective platform to assess the potential of microbial activity to contribute to CO_2 evolution and greenhouse gas emissions during the aerobic stages of BNR. This

Table 1
Ammonia removal performance of batch reactors.

Medium	Replicate	Ammonia provided (mmol)	Bicarbonate provided (mmol)	Ammonia removed (mmol)
A	**1**	2.52	1.41	0.70
	2	2.52	1.41	0.52
B	**1**	2.52	2.82	1.08
	2	2.52	2.82	1.07

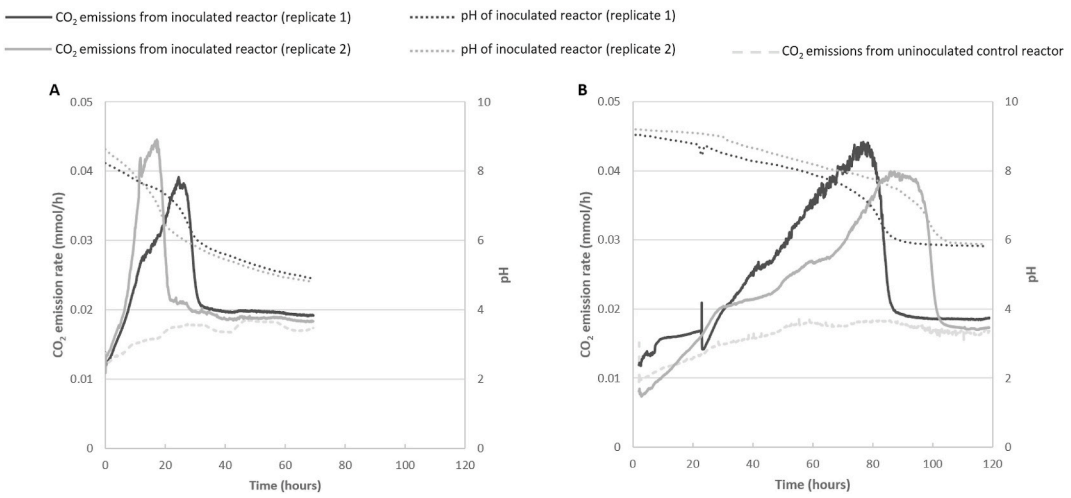

Fig. 2. pH and CO_2 emission profiles were measured for duplicate batch reactors and an uninoculated control reactor established using different compositions of the autotrophic synthetic wastewater medium (A and B), as outlined in Table 1.

study and results are important to the assessment of full-scale systems but require consideration of site-specific differences in the mass transfer coefficient for CO_2. Nonetheless, the common practice of attributing all direct CO_2 emissions during aerobic treatment stages to respiration by heterotrophic microorganisms (Mannina et al., 2016) could overlook the potential for a portion of the emitted CO_2 to have arisen from the neutralization effect of bicarbonate alkalinity.

3.3. CO_2 flux during cultivation of autotrophic biofilms in CEMS

The requirement for an airtight seal in the batch reactors limited analyses of ammonia removal to only initial and endpoint samples. To further investigate the relationship between autotrophic ammonia removal and CO_2 flux, a CEMS was used to facilitate time-resolved monitoring of both the rates of ammonia removal and CO_2 emission. The CEMS also allowed for the exclusive study of autotrophic biofilms and their contribution to CO_2 emissions, since non-sessile cells and flocs were removed with the flow of medium due to a high dilution rate that greatly exceeded the expected growth rate of autotrophs. Unlike for the batch reactors, the autotrophic medium used for cultivating biofilms in CEMS provided ammonia rather than bicarbonate as the limiting component so that the specific relationship between CO_2 emissions and ammonia removal could be elucidated when alkalinity was in excess. Growth curves of the autotrophic biofilms prepared in triplicate with medium that supplied ammonia and bicarbonate at 0.09 and 0.21 mmol/h, respectively, showed lag phases of varying duration (approximately 8–15 days), which was considerably longer than those previously reported for heterotrophic biofilms cultivated using CEMS (e.g. less than 24 h in Jackson et al., 2019). Given the relatively high initial pH measured in the effluent (>9), it is likely that free ammonia inhibition exacerbated the already slow growth rate of the autotrophic biofilms. After an acclimation period, the biofilms ultimately achieved stable ammonia removal of 55–65%, which corresponded to a decrease in the bulk pH and rapid increase in CO_2 emissions (Fig. 3).

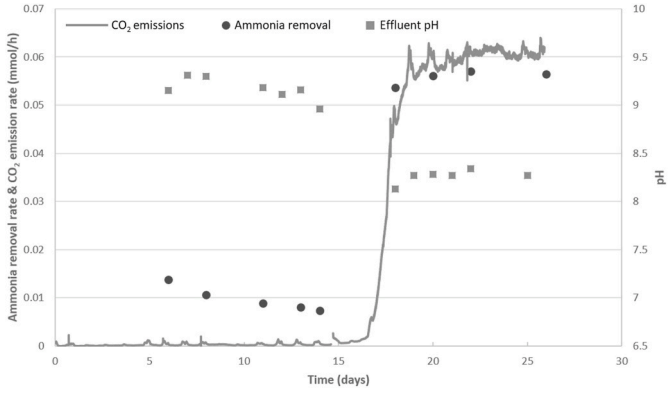

Fig. 3. CO_2 emission rate and ammonia removal rate were measured during cultivation of an autotrophic biofilm starting from the time of inoculation.

3.4. Influence of alkalinity source on CO_2 emissions during autotrophic ammonia removal in CEMS

Although the time required for the autotrophic biofilms to achieve stable operating conditions was somewhat variable, the platform provided an ideal tool for investigating the effect of nutrient changes on overall system performance. In duplicate experiments, the rates of ammonia removal and CO_2 emission were compared when alkalinity was primarily provided by bicarbonate, as well as when a non-carbonate source of alkalinity was used. It was hypothesized that if the main source of CO_2 emissions was the depletion of bicarbonate alkalinity, these emissions should cease when bicarbonate was removed from the medium. Autotrophic biofilms were grown for approximately 21 days using medium which provided ammonia and bicarbonate at 0.09 and 0.21 mmol/h, respectively. The medium was then switched to one in which bicarbonate was replaced with the biologically inert zwitterionic sulfonic acid buffer HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), which was adjusted to pH 7.5 and supplied in excess at 0.38 mmol/h.

Though HEPES has received widespread use in microbiological research, including in the cultivation of autotrophic ammonia oxidizing microbes (Bollmann and Laanbroek, 2001; French et al., 2012), its use as source of alkalinity during aerobic wastewater treatment is uncommon. It was nonetheless used here to test whether CO_2 emissions would persist during ammonia removal in the absence of bicarbonate alkalinity. Transition from feeding with the bicarbonate-buffered medium to one buffered with HEPES was marked by a rapid decline in the rate of CO_2 emissions, and when flow of the bicarbonate-buffered medium was resumed after 24 h, CO_2 emissions quickly returned to the previously established rate (Fig. 4). Ammonia removal persisted throughout these changes, although its rate was reduced by approximately 40% during feeding with the HEPES-buffered medium. Despite this, the dramatic decline in CO_2 emissions when bicarbonate was removed from the medium supported the notion that virtually all the gaseous CO_2 emitted from the CEMS under autotrophic mode could be attributed to the

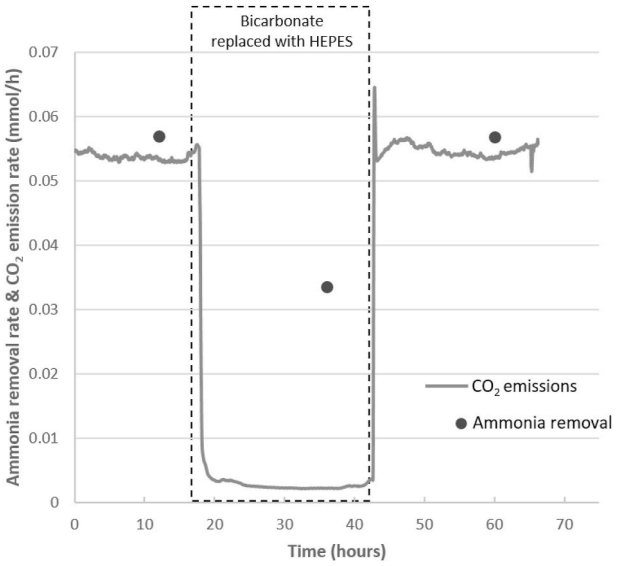

Fig. 4. CO_2 emission rate and ammonia removal rate exhibited by a mature autotrophic biofilm were measured when the bicarbonate content of the autotrophic synthetic wastewater medium was replaced with HEPES buffer.

neutralization effect of bicarbonate alkalinity.

3.5. Influence of organic carbon on CO_2 emissions during autotrophic ammonia removal in CEMS

It is possible for heterotrophic microbes to persist within completely autotrophic nitrification systems, as they are capable of surviving on the organic soluble microbial products of the autotrophic biomass (e.g. Kindaichi et al., 2004; Ni et al., 2011). Indeed, Aqeel and Liss (2020) showed that the biomass within the fixed-film bioreactor that was used as the source of inocula for the CEMS did previously include some heterotrophs despite the lack of organics in the feed. They attributed this heterotrophic growth to endogenous decay in the autotrophic system

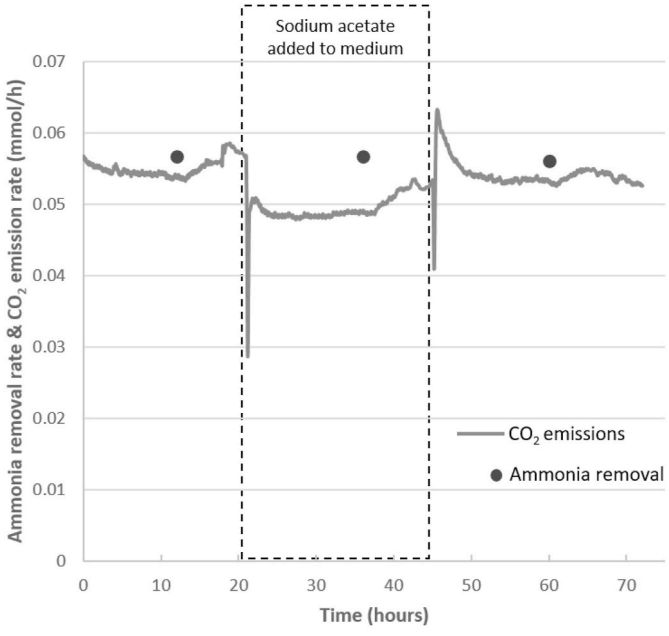

Fig. 5. CO_2 emission rate and ammonia removal rate exhibited by a mature autotrophic biofilm were measured when the autotrophic synthetic wastewater medium was temporarily supplemented with sodium acetate.

(Aqeel and Liss, 2020). While most of the CO_2 emitted from the CEMS could presumably be attributed to bicarbonate depletion, the potential contribution of heterotrophic respiration to overall CO_2 emissions during autotrophic growth is also an important consideration. The stability of the system was tested when the medium was supplemented with organic carbon to see if this would lead to increased CO_2 production from heterotrophic growth. The autotrophically-cultivated biofilm was subjected to 24 h of feeding with medium that supplied ammonia, bicarbonate, and sodium acetate at rates of 0.09, 0.21, and 1.78 mmol/h, respectively. The downshift in CO_2 emission rate that resulted (Fig. 5) may not have been a biological effect but could be due to slight differences in the influent pH during the medium changes, since the ammonia removal rate exhibited by the biofilm appeared to be unaffected. The fact that CO_2 emissions did not rapidly increase above the previously established baseline when organic carbon was added to the feed, suggested that the heterotrophs in the biofilm were not readily able to utilize this labile carbon source, and likely entered a lag phase upon the change in nutrient status. This does not preclude the presence of heterotrophs, as some are likely able to survive on the organic carbon from endogenous decay (Aqeel and Liss, 2020). Rather, the results suggest that under completely autotrophic conditions in CEMS, the CO_2 arising from heterotrophic activity was minimal compared to the CO_2 arising from bicarbonate depletion. This is in agreement with the estimation made by Blackburne et al. (2007), that heterotrophic oxidation of decayed or lysed cell material accounted for only 2% of the CO_2 produced during nitrification in autotrophic batch reactors.

3.6. Influence of variable ammonia and bicarbonate on CO_2 emissions during autotrophic ammonia removal in CEMS

The CEMS was used to elucidate the relationship between the rates of CO_2 emission and ammonia removal under variable ammonia and bicarbonate feeding rates. According to the stoichiometry of autotrophic nitrification, each mole of ammonia removed can be expected to result in the production of 1.86 mol of CO_2 (Ebeling et al., 2006), however the propensity for this bicarbonate-derived CO_2 to transfer to the gas phase is often not considered in the context of GHG accounting. An autotrophic biofilm was once again grown to stable operating conditions with medium that provided ammonia and bicarbonate at 0.09 and 0.21 mmol/h, respectively. The biofilm was then fed three different compositions of the medium in 130-min intervals, during which the ammonia feeding

Table 2
Impact of variable influent ammonia and bicarbonate on ammonia removal and CO_2 emissions from nitrifying biofilms. CO_2 emission rate is expressed as an average with standard deviations, based on instantaneous rates determined each minute.

Medium	Ammonia feeding rate (mmol/h)	Bicarbonate feeding rate (mmol/h)	Measured ammonia removal rate (mmol/h)	Estimated bicarbonate depletion rate* (mmol/h)	Measured CO_2 emission rate (average) (mmol/h)
I	0.19	0.42	0.08	0.16	0.13 ± 0.0008
II	0.09	0.42	0.05	0.10	0.09 ± 0.0007
III	0.02	0.42	0.01	0.02	0.03 ± 0.0007
IV	0.09	0.85	0.06	0.12	0.09 ± 0.0010
V	0.09	0.42	0.05	0.10	0.09 ± 0.0004
VI	0.09	0.21	0.05	0.10	0.09 ± 0.0005

*Estimation is based on the stoichiometric equation for autotrophic nitrification elucidated by Ebeling et al. (2006), which assumes the consumption of 1.97 mol of bicarbonate per mol of ammonia removed.

rate was varied while the feeding rate of all other components, including bicarbonate, remained fixed (Table 2: I-III). The short duration of feeding was chosen so that the contribution of biomass growth to changes in system performance could be minimized, and the pH of the influent medium was fixed at 7.5 throughout the changes. The average CO_2 emission rate during each of the three feeding scenarios was determined based on the instantaneous rates that were calculated for each minute of feeding and were subsequently expressed with standard deviations. The ammonia concentration within CEMS effluent samples collected during the final 30 min of feeding with each medium was compared to that of the influent and used to calculate ammonia removal rate (Table 2: I-III) by taking into account the flow rate of the medium. Not surprisingly, the CO_2 emission rate decreased proportionally with decreasing rate of ammonia removal (Fig. 6A).

It was apparent that variability in the ammonia feeding rate affected the ammonia removal rate in the CEMS, which in turn led to proportional changes in the rate of CO_2 emissions. Another test was performed to determine the effect of variability in the bicarbonate feeding rate. It was hypothesized that if bicarbonate were always provided in excess, changes to the bicarbonate feeding rate would not affect the ammonia removal and CO_2 emission rates. Here, the bicarbonate feeding rate was varied while the feeding rate of all other components remained constant (Table 2: IV-VI). Though the biofilm was originally cultivated using medium without any pH adjustment, the three formulations tested here had their pH adjusted to 7.5 prior to feeding, which was the likely cause of the initial increase in CO_2 emissions after the first medium change (Fig. 6B). The rates of ammonia removal and CO_2 emission during each of the two subsequent changes appeared to remain relatively stable (Table 2: IV-VI), further emphasizing the important relationship between these two parameters.

Taken together, the rates of ammonia removal and CO_2 emission observed during each of the six feeding scenarios (Table 2: I-VI) showed linearity in the relationship between ammonia removal and CO_2 emissions ($R^2 = 0.9523$) (Fig. S5). In each case, the amount of gaseous CO_2 produced was very close to the total amount of CO_2 predicted based on the stoichiometry of autotrophic nitrification. That is, while 1.86 mol of CO_2 can be expected to be produced per mole of ammonia removed (Ebeling et al., 2006), these experiments showed that on average, the removal of each mole of ammonia resulted in 1.92 ± 0.54 mol of CO_2. This was significant not only due to the fact the results agreed with stoichiometry, but rather that all this produced CO_2 was detected in the gas phase. Indeed, 91.52 ± 13.59% of the depleted bicarbonate was subsequently detected as gaseous CO_2. This would suggest that there was minimal resistance to the mass transfer of CO_2 from the liquid to gas, which was not surprising given the ample surface area available for diffusion of CO_2 through the permeable membrane. Any extrapolation of these results to full-scale would therefore need to consider the possibility of lower mass transfer coefficients experienced in real BNR systems, wherein continuous aeration rather than membrane diffusion is the major driver of CO_2 mass transfer. In this sense, the results of the CEMS experiments may be representative of extreme cases that demonstrate the importance of considering mass transfer of CO_2 in the design and operation of BNR systems, since failure to do so could lead to a portion of the bicarbonate alkalinity being converted to CO_2 and released to the atmosphere.

3.7. Implications of bicarbonate-derived CO_2 for GHG accounting

Though modelling approaches have been useful in assessing the mass-transfer of CO_2 and other gasses between phases during wastewater treatment (Mannina et al., 2016), further in situ studies would be beneficial to appreciate the scale at which bicarbonate-derived CO_2 emissions might occur at real BNR plants. According to the stoichiometry of autotrophic nitrification, 1.86 mol of CO_2 are produced per mole of ammonia removed, corresponding to 5.85 g of CO_2 per g of ammonia-N (Ebeling et al., 2006). The CEMS experiments supported this stoichiometric prediction, with all the produced CO_2 being detected in the gas phase. Domestic wastewater produced annually is estimated to comprise approximately 20 million tonnes of ammonia (Cruz et al., 2019) and this amount is expected to reach approximately 35 million tonnes by 2050 (Bodirsky et al., 2014). In a worst-case scenario, if all this ammonia is removed via autotrophic nitrification in BNR systems that are completely buffered by bicarbonate, and all of the resulting CO_2 is transferred to the atmosphere, this could result in an annual emission of roughly 90 million tons of bicarbonate-derived CO_2, with this increasing to 159 million tons by 2050. This estimation provides a possible upper limit that can be useful to understand the significance of this GHG emission source. For context, in 2016, wastewater treatment systems were said to be responsible for 1.3% of the 49.4 billion tonnes of CO_2eq emitted to the atmosphere, amounting to 642.2 million tonnes of CO_2eq (Ritchie and Roser, n. d.). However, this value only accounts for the direct N_2O and CH_4 emissions arising during wastewater treatment processes. The omission of bicarbonate-derived CO_2 from this tally could therefore be a significant oversight and warrants the further development and implementation of more sustainable BNR solutions that could avoid these CO_2 emissions. An example is the anammox process, which according to stoichiometry does not require alkalinity, since protons are consumed rather than produced (Strous et al., 1998).

With respect to GHG accounting of BNR systems, the focus has mainly been on N_2O emissions, however the present study demonstrated

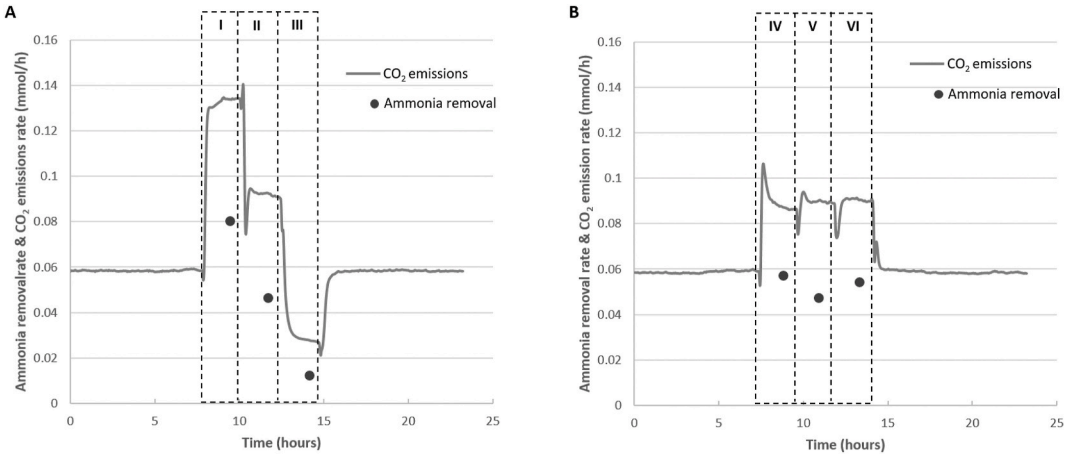

Fig. 6. CO_2 emission rate and ammonia removal rate exhibited by mature autotrophic biofilms were measured when fed different compositions of the autotrophic synthetic wastewater medium to achieve variable ammonia feeding rate (A) and variable bicarbonate feeding rate (B), as outlined in Table 2.

the potential for bicarbonate depletion to contribute to CO_2 emissions during autotrophic ammonia oxidation. In a critical review of full-scale N_2O monitoring campaigns for BNR systems, Vasilaki et al. (2019) reported that N_2O was released at a ratio of 0.017 mol per mol of influent-N (median). Comparing this to the stoichiometry of autotrophic nitrification and the results of the present study, it is evidently possible for much more bicarbonate-derived CO_2 than N_2O to be emitted from BNR systems. Notwithstanding the greater global warming potential of N_2O versus CO_2, this should warrant further investigation, taking into account the impact of variability in design and operating conditions on the mass transfer of CO_2 from liquid to gas, as well as the unique alkalinity profile of the BNR source water.

It is common practice to attribute all direct CO_2 emissions during aerobic wastewater treatment processes, including those occurring during BNR, to respiration by heterotrophs (Ashrafi et al., 2015; Mannina et al., 2016; Chen et al., 2018; Gallego-Schmid and Tarpani, 2019). However, future research should focus on elucidating the relative contributions of bicarbonate depletion and cellular respiration to overall CO_2 emissions. Heterotrophic respiration is thought to involve short-term recycling of biogenic carbon between the atmosphere and biosphere, and so the CO_2 released from aeration tanks is usually not included in GHG accounting (Willis et al., 2016). Not only does this approach fail to recognize the potential for some of the organic load in wastewater to be of fossil origin, as noted by Law et al. (2013) and Griffith et al. (2009), it also overlooks the possibility that some of the emitted CO_2 came from the neutralization effect of bicarbonate alkalinity during ammonia oxidation. Considering the longer timescale involved in the formation and subsequent dissolution of carbonate rock, the CO_2 emitted in this process should be considered distinct from the CO_2 arising from respiration.

In some respects, the emission of bicarbonate-derived CO_2 during wastewater treatment is analogous to the limestone-derived CO_2 emitted during cement manufacturing, which is widely recognized as a major emitter of GHGs (Benhelal et al., 2021). In both processes, CO_2 emissions can be interpreted as the anthropogenic transmutation of “old” carbon from carbonate sources in the lithosphere and hydrosphere, to the atmosphere. While it is typical for CO_2 from limestone decomposition to be included in GHG accounting of the cement manufacturing industry (Benhelal et al., 2021), it is uncommon for bicarbonate-derived CO_2 emissions to be included in GHG accounting of wastewater treatment systems (Willis et al., 2016). CO_2 production and subsequent emission arising from the neutralizing effect of bicarbonate alkalinity deserves careful consideration for integration into future GHG accounting protocols for BNR systems.

4. Conclusion

Notwithstanding the autotrophic nature of nitrifiers, CO_2 emissions can arise from the depletion of bicarbonate alkalinity caused by acidification during ammonia oxidation. Experiments with lab-scale autotrophic batch reactors inoculated with nitrifying biomass demonstrated the important link between ammonia oxidation, pH, and bicarbonate-derived CO_2 emissions. Depletion of the buffering capacity in the batch reactors led to a rapid decline in the system pH, and cessation of CO_2 emissions when the pH fell below 7.0. The performance of nitrifying biofilms in a CO_2-evolution measurement system suggested linearity in the relationship between ammonia removal rate and CO_2 emission rate, which demonstrated the stability of the system under autotrophic running mode. The ratio of ammonia removal to CO_2 production was similar to that which was predicted based on the stoichiometry of autotrophic nitrification, with all of this produced CO_2 detected in the gas phase. An estimation of bicarbonate depletion based on the stoichiometry of ammonia oxidation suggested that approximately 90% of the depleted bicarbonate was released as gaseous CO_2. Any direct extrapolation of the results to full-scale treatment plants must take into account site specific differences in reactor configuration, operating conditions, and other wastewater characteristics such as the alkalinity profile (e.g., bicarbonate content). Overall, the study demonstrated that a potentially significant portion of the bicarbonate alkalinity in BNR systems may be depleted and converted to gaseous CO_2. It is therefore important to recognize this mechanism as a possible source of GHG emissions from BNR systems, as this CO_2 could be considered distinct from the biogenic CO_2 that arises from heterotrophic degradation of organic matter.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.112928.

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