Ammonia removal from swine wastewater by simultaneous nitrification-denitrification processes within a photobioreactor

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Abstract
The interaction between naturally occurring anaerobic biodigestion effluent wastewater bacteria and *Chlorella* sp. during nitrogen removal by de-/nitrification processes within a photobioreactor was studied. qPCR assays were used to quantify the abundance of total bacteria (*16S* rDNA), nitrifyers (*amoA*) and denitrifyers (*nirS*). Ammonia removal coincided with the increasing growth of total bacteria (1.2×10^{10} copies μL^{-1} at 48 h) and accumulation of NO_3 and NO_2 intermediates. Low oxygen concentrations prevailed (< 0.2 mg/L) during dark periods and low microalgae biomass (up to 48 h) thus stimulating denitrifying bacteria growth (1.8×10^{6} copies μL^{-1} at 48 h). NO_3 accumulation and N_2O production coincided with denitrification inhibition by the low C/N ratio. The oxidative-reductive environment encountered in a non-sterile photobioreactor can benefit swine wastewater nutrient removal by the simultaneous enhancement of de-/nitrification processes.

Introduction
Swine breeding wastewaters presents a global environmental concern because of the problems associated with soil acidification, water eutrophication and atmospheric ammonia emissions. These environmental problems are mostly due to the high nitrogen content from swine waste, where up to 70% of the nitrogen present in liquid manure is composed of ammonium (≅180 mM). Among several wastewater biological treatment processes used to remove nitrogen, strategies that are based on the growth of microalgae are currently been considered worldwide as alternative to produce valuable feedstock to renewable biofuels.
Photosynthetic batch reactors are able to completely remove ammonium from swine wastewater, converting 25 to 100% of nitrogen and 70 to 90% of phosphate into biomass [1]. Nitrogen removal rates from photobioreactors inoculated with acclimated bacteria from nitrification/denitrification sludge settler tank were comparable to conventional denitrification-nitrification activated sludge configurations [1]. During nitrification processes, ammonia (NH_4^+) is oxidized to nitrate (NO_3^-), which is the form of nitrogen that favors nitrogen assimilation for plant growth, though microalgae (*Chlorella vulgaris*) also grow well on either NO_3^- or NO_2^- [2]. Nonetheless, little is known about the applicability of non-inoculated photobioreactors to remove nitrogen in carbon-limiting effluent from anaerobic swine wastewater biodigestion. Moreover, a few studies have observed N_2O and fugitive methane emissions associated with microalgae cultures, which is critical to be eliminated in order to achieve a favourable life cycle balance for GHG emissions [3,4].
This study addresses the potential of a mixotrophic photobioreactor to remove ammonia from swine wastewater derived from anaerobic biodigestion and the interactions between microalgae and microbial communities during wastewater treatment within the photobioreactor. A non-sterile batch photobioreactor was considered to better mimic conditions that are likely to prevail at field scale applications. Emphasis was placed on the analysis of functional genes involved in the biological nitrogen cycle in order to elucidate the role of microbial-mediated biodegradation processes within the photobioreactor. The concentration of nitrifying and denitrifying bacteria was correlated with nitrogen species and N_2O produced over time within the photobioreactor.

Material and Methods
Experimental Setup
A non-sterile 9-L glass bottle reactor was utilized as a photobioreactor. The swine wastewater was collected from an upflow anaerobic sludge blanket (UASB) effluent reactor with the following

characteristics (g L^{-1}): pH 7.9, 3–8 TSS, 1.5–6.5 TOC, 2.5–4.5 BOD5, 5–8 $CaCO_3$ alkalinity, 1.5–2 TN, 0.900–1.5 NH_3-N. Diluted wastewater (2:5 tap water) was inoculated with 30% v/v microalgae (10 g L^{-1} dry weight of *Chlorella vulgaris*). The reactor was closed, continuously stirred and maintained at room temperature (21±1°C) with a photoperiod of 12h.

Analytical Procedures

Samples were collected daily and analyzed parameters included pH, dissolved oxygen (DO), temperature, chlorophyll, and nitrogen forms. NO_2, NO_3, and NH_3 were determined according to [5]. TN and TOC were measured using a TOC analyser (Multi C/N 2100, Analytik Jena). N_2O, NH_3 and CH_4 gases were continuously monitored throughout the whole experiment with a photoacoustic infrared spectroscopy equipment (INOVA 1122, Lumasensetm Technologies inc., USA). Microalgae growth was measured by chlorophyll extraction using 100% methanol. Tubes containing 1.5 mL samples were centrifuged at 16,000g for 10 min, pellet was ressuspended in methanol, vortexed and placed overnight in the dark at 4°C. The absorbance of the green supernatant was measured at two wavelengths, 650 and 665 nm. Chlorophyll content was calculated using equations described by [6].

DNA Extraction, Production of Standard Curves and qPCR Data Analysis

Total bacteria (*16S* rDNA), nitrifying (*amoA*) bacteria and denitrifying (*nirS*) bacteria were estimated by real-time quantitative PCR (qPCR) analysis with primers and conditions described in Table 1. DNA was extracted according to MoBio UltraClean Microbial DNA kit following manufacturer's instructions. Standard curves were prepared (10^9 to 10^1 gene copies μL^{-1} of *nirS* or *16S* gene copies) by amplification of *nirS* fragments and insertion into pCR® 2.1-TOPO® vector (Invitrogen, USA) then further transformed into DH5α Escherichia coli competent cells [7]. Clones were grown in Luria-Bertani medium plates supplemented with ampicillin (50 mg mL^{-1}). Colonies were chosen and the plasmidial DNA was extracted by alkaline method [7]. The presence of the inserted sequence in plasmid DNA was confirmed by conventional. SYBR green kit was used to quantify DNA with qPCR temperature conditions for targeting *16S, amoA* and *nirS* as previously demonstrated [11-13].

Results

Microalgae Growth

Chlorella vulgaris was able to grow in the photobioreactor fed diluted swine wastewater from anaerobic biodigestion effluent with a specific exponential growth rate (μ) of 0.06 h^{-1} (Figure 1). The production of microalgae was not affected by the diluted swine wastewater and the lag phase of algal growth was obtained after 72 h (Figure 1).

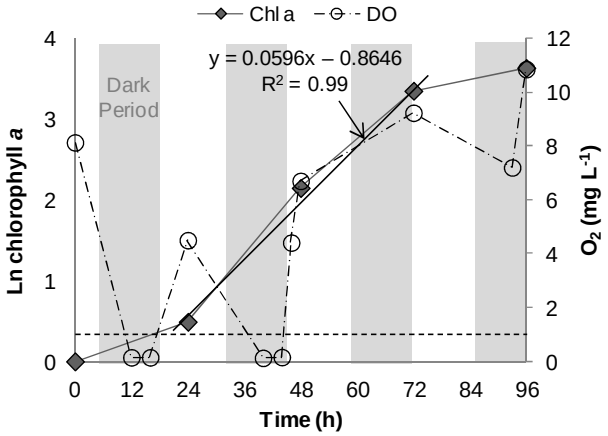

Figure 1. Chlorophyll a and dissolved oxygen concentrations profile. Dashed line represents minimum oxygen concentration required to support nitrification. Denitrification occurs at DO<0.2 mg $L^{-1.}$ Linear regression represents microalgae growth rate (μ).

Production of Standard Curves and Quantification of 16S and nirS genes

Standard curves for quantitative PCR were obtained by preparing 10-fold dilutions of genomic DNA from *E. Coli* (ATCC 35218) for *16S* analysis and from plasmids containing either *nirS* or *amoA* fragment amplified with primers [8-10]. Both standard curves showed high correlation efficiencies

with R2 > 0.99 and the *16S*, *amoA* and *nirS* genes PCR amplification was 96%, 97% and 87% efficient, respectively (data not shown).

Nitrification Processes within the Photobioreactor

NH3-N concentration steadily decreased from 430 mg L^{-1} to 235 mg L^{-1} reaching a removal efficiency of 45% after 96 h of treatment (Figure 2). The increasing bacteria (*16S rDNA*) concentration (from 1.5×10^4 at 0 h to 1.2×10^{10} at 48 h) provided circumstantial evidence to support nitrification/denitrification processes. NO_3^- (50 mg L^{-1}) and NO_2^- (30 mg L^{-1}) accumulation served to further support the occurrence of nitrification during the initial stages of the biodegradation process (up to 48 h). These results support the notion that photobioreactor inoculation with acclimated bacteria from nitrification/denitrification activated sludge may not be necessary in order to accomplish satisfactory nitrogen removal.

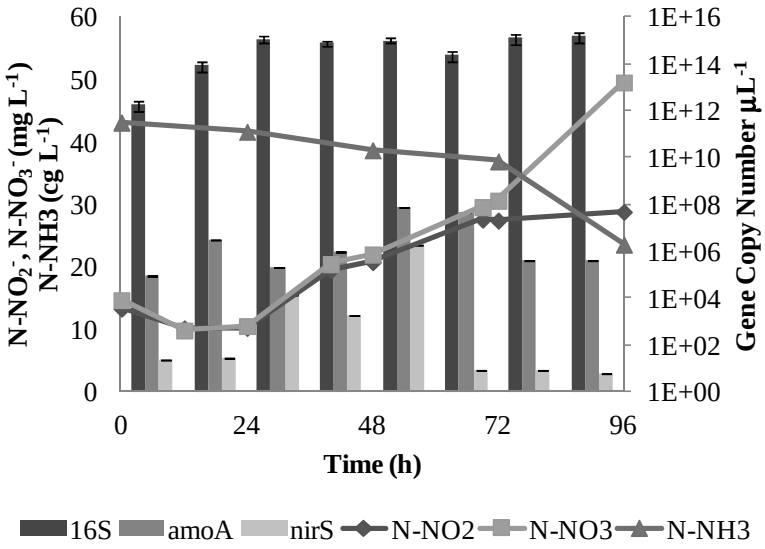

Figure 2. Average ammonia, nitrate, nitrite, total bacteria (*16S* rDNA), nitrifying (*amoA*) and denitrifying (*nirS*) bacteria concentration profile within the photobioreactor.

Denitrification Processes within the Photobioreactor

Low DO values (< 0.2 mg L^{-1} up to 48h) observed during dark periods and low microalgae biomass (Figure 1) served to stimulate the growth of anoxic denitrifying *nirS*-harboring bacteria. The *nirS* gene was detected in high concentrations up to 48-hours of experiment (1.87×10^4) and it strongly supported the hypothesis of N_2O production by denitrifying bacteria (Figure 2). These results corroborate with other studies that quantified N_2O emissions from microalgae culture under laboratory conditions, suggesting that GHGs were produced by denitrifying bacteria within the culture [3,4].

N_2O Emissions

Figure 3 shows N_2O production and consumption through time in two distinct mixotrophic photobioreactors. N_2O emissions peaked at dark conditions where anoxic conditions are favoured, reaching concentrations between 120 and 140 ug L^{-1} after 48 hours of experiment (Figure 3, left side). Laboratory and full-scale studies have suggested that elevated nitrite concentrations and low chemical oxygen demand (COD) to nitrogen ratio can also increase N_2O emissions in the system [11,12]. Between 48 and 96 hours nitrate and nitrite concentrations increased, which may have contributed to N_2O production in the system. It was also likely that the low C/N ratio found (data not shown) was the main factor that contributed to N_2O emissions (Figure 3, right side). To test this hypothesis, sodium acetate was added and N_2O decreased instantly, thus allowing complete denitrification processes within the photobioreactor.

Conclusion and perspectives

This work was conducted to demonstrate the interaction between bacteria-microalgae within a mixotrophic photobioreactor simulating ammonia bioremediation from swine wastewater. Microalgae growth promoted the establishment of simultaneous oxidative-reductive environments and nitrification and denitrification processes. The role of microalgae was particularly important to aerobic ammonia

removal contributing with adequate levels of oxygen needed to warrant complete bacterial nitrification. In this regards, engineered photobioreactors can be built to minimize the costs and technical difficulties associated with the implementation of external oxygen supplies. In a nutshell, naturally-occurring bacteria-microalgae interactions in photobioreactors can provide an attractive polishing step to effectively remove ammonia (and perhaps other nutrients such as phosphorus) from swine wastewater previously treated by anaerobic digestion.

Though microalgae cultivation systems are associated to the benefits of CO_2 gases sequestration, it was observed that N_2O was produced by denitrifying bacteria present in the photobioreactor, which was caused by the low C/N ratio. Closed photobioreactors have the advantage of retaining N_2O in the system, which could be further eliminated by changing conditions to allow a complete denitrification process, such as the addition of a carbon source in this case.

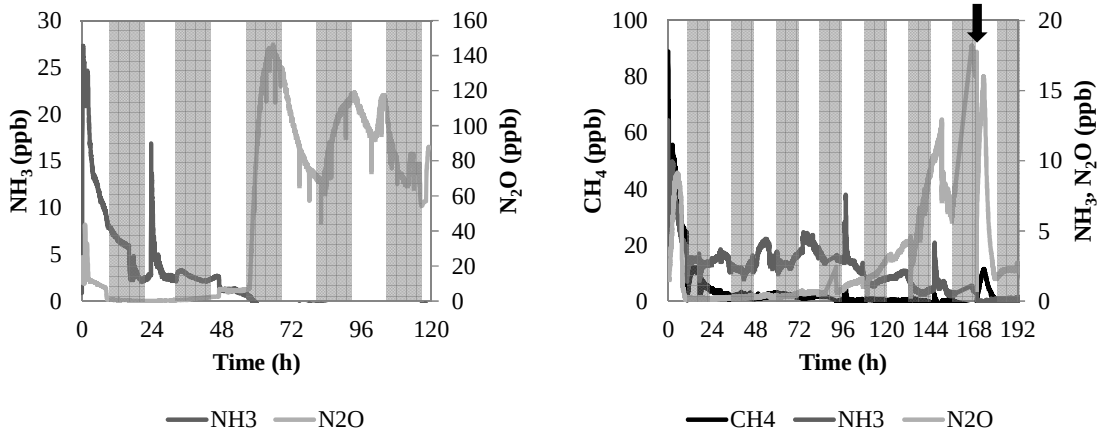

Figure 3. N_2O, NH_3 and CH_4 absolute concentrations measured by photoacustic spectroscopy (INNOVA) in two independent photobioreactor studies. Arrow in left picture indicates addition of a carbon source. Dark cycles are represented by dark columns.

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