



## Anammox enrichment and constructed wetland inoculation for improvement of wastewater treatment performance

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### Original Article

#### Abstract

This study contributes to the improvement of low-cost biotechnology for wastewater treatment in constructed wetlands (CWs). Constructed wetlands are energy efficient engineered systems that mimic the treatment processes of natural wetlands, removing polluting organic matter, nutrients, and pathogens from water. The aim of this study was to investigate the advisability of the inoculation of horizontal subsurface flow constructed wetlands with the enriched biomass of anaerobic ammonium oxidation (anammox) bacteria to enhance nitrogen removal. Contaminants removal in constructed wetlands occurs mainly due to the biological transformations caused by indigenous water-borne microorganisms. However, the role of different microbial mechanisms is still unknown. To estimate the role of the anammox process in wetlands the laboratory-scale fixed bed reactor planted with *Juncus effusus* was inoculated with enriched biomass of anammox bacteria and fed with synthetic wastewater containing ammonium-nitrogen as the main contaminant. In order to obtain the active enriched culture of anammox bacteria, an upflow anaerobic fixed bed reactor inoculated with activated sludge from a municipal wastewater treatment plant was run. The reactor was fed with enrichment medium containing ammonium and nitrite in high concentrations. After 270 days of operation, nitrite was not found in measurable levels, the concentration of ammonium had slightly increased, and the concentration of nitrate in the reactor had significantly dropped compared to its level at the initial phase. The microbial association, which had developed in the enrichment reactor, allowed continuous removal of ammonium and nitrite. The anammox bacteria abundance in the reactor accounted for approximately 95% of total biomass.

**KEYWORDS:** Wastewater, Wetlands, Bioreactors, Nitrogen, Ammonium, Bacteria

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#### Introduction

Water is essential to human life, and therefore, water resources must be protected from

contamination. Discharge of untreated wastewater into the natural water bodies is one of the main causes of water pollution. However, accurate wastewater treatment is a serious challenge for many developing countries due to the high construction and exploitation costs of

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the treatment systems. This problem can be solved by the implementation of inexpensive competitive alternatives to conventional treatment technologies.

Constructed wetlands (CWs) are energy-efficient, chemical-free, and easy-to-operate robust wastewater treatment systems. The design of CWs utilizes the principles and properties of natural wetlands, but provides better control over the treatment process. The horizontal subsurface flow (HSSF) constructed wetlands are one of the most widespread and reliable types of near-natural treatment technologies used around the world.<sup>1</sup> Specified CWs comprise macrophytes that are introduced into porous medium (gravel, sand, and etc.) and wastewater that flows under the surface of the bed mainly in a horizontal direction. Horizontal flow constructed wetlands efficiently remove organic matter, suspended solids, and pathogenic microorganisms from sewage. However, nutrient (nitrogen and phosphorous) removal rates in these systems are considerably low.<sup>2</sup> In particular, nitrogen removal efficiencies have been reported as only between 30% and 50% in long term studies.<sup>3-5</sup>

It was clarified that if the loading of ammonia to a wetland exceeds the growth requirements of the plants, bacteria, and algae, microbial transformation of nitrogen to its gaseous forms is generally the dominant N removal process in CWs.<sup>5-7</sup> Moreover, Kadlec<sup>8</sup> has stated that the loading limit for bacterial transformation to predominate in CW is equal to the loading rate of approximately 120 g-N m<sup>-2</sup> yr<sup>-1</sup>. However, the function of emergent water plants is to provide favourable conditions and habitat for the microbes, and they can enhance nitrogen removal mainly via indirect effects on the physicochemical and microbial processes.<sup>7</sup> Harvesting the above ground biomass of macrophytes in HSSF CWs can contribute to a less than 10% removal of nitrogen, as compared to the inflow load.<sup>9</sup> In addition, it should be considered that elevated pH values promote

ammonia volatilization from the system; however, nitrogen removal through this process is generally insignificant if the pH is below 8.0.<sup>5,7</sup>

As was explained, researchers have stated that the major mechanism of nitrogen removal from wastewater in CWs is biological transformation by indigenous water-borne microorganisms. Nevertheless, there is no "conventional wisdom" that explains the low efficiency of nitrogen removal in horizontal flow CWs, because present understanding of the relative importance of different microbial pathways in wetlands is very poor. Therefore, nitrogen removal is the issue that receives probably the most attention today from researchers who work in the field of constructed wetlands. Still, it should be taken into account that constructed wetlands are very complex bioreactors, where numerous physical, chemical, and biological processes caused by plants, microorganisms, and contaminants take place simultaneously. Thus, they are difficult to study.<sup>6,10</sup>

Estimated oxygen fluxes into HSSF wetlands are generally insufficient to supply conventional nitrification-denitrification; nevertheless, microbial denitrification is still considered to be the primary nitrogen removal mechanism.<sup>7</sup> Moreover, the denitrification process was rarely measured directly in treatment wetlands. Therefore, recently discovered alternative microbial pathways of nitrogen transformation common for low-oxygen aquatic environments are considered to play an important role in the nitrogen cycling process in treatment wetlands.<sup>11</sup> A few studies have been conducted to investigate the newly discovered anammox process in constructed wetlands.<sup>12-15</sup> Thus, the goal of the present study was to inoculate the model horizontal subsurface flow constructed wetland with enrichment culture of anammox bacteria in order to enhance removal efficiency of nitrogen.

## Materials and Methods

The enrichment culture of anaerobic ammonium oxidation (anammox) bacteria was cultivated in

an anaerobic upflow fixed bed reactor (henceforth referred to as anammox-reactor). Figure 1 shows the principal configuration of the experimental setup. The experimental setup consisted of the following major components:

- Anammox-reactor [(1) in Figure 1];
- A peristaltic pump for continuous recirculation of the reactor medium;
- A water-lock [(2) in Figure 1] for elimination of the formed  $N_2$  gas;
- A water heating device [(3) in Figure 1] with a circulation pump;
- The trace mineral solution (TMS) storage vessel [(4) in Figure 1] connected to a gas bag [(7) in Figure 1];
- Storage vessels [(5) and (6) in Figure 1] for two enrichment half-media, which were also connected to the gas bag;

- Magnetic stirrer [(8) in Figure 1] for vessel 5;

- Two peristaltic pumps for feeding of the enrichment media and TMS.

The reactor itself was a vertical glass column (0.15 m diameter), which was wider in the upper end (0.2 m diameter) in order to promote the gas separation. The reactor was equipped with a peripheral twin-wall enclosure (thermostatic jacket) and operated at the temperature of 30 °C. Warm water (34 °C) from the heating device circulated inside the enclosure. Total volume of the reactor was equal to 25 l and volume of the liquid medium was 20 l (fill factor = 0.85). The anammox-reactor was completely filled with Kaldnes type K1 polyethylene biofilm carriers (density = 0.95 g/cm<sup>3</sup>, void fraction = 95%).<sup>16</sup>

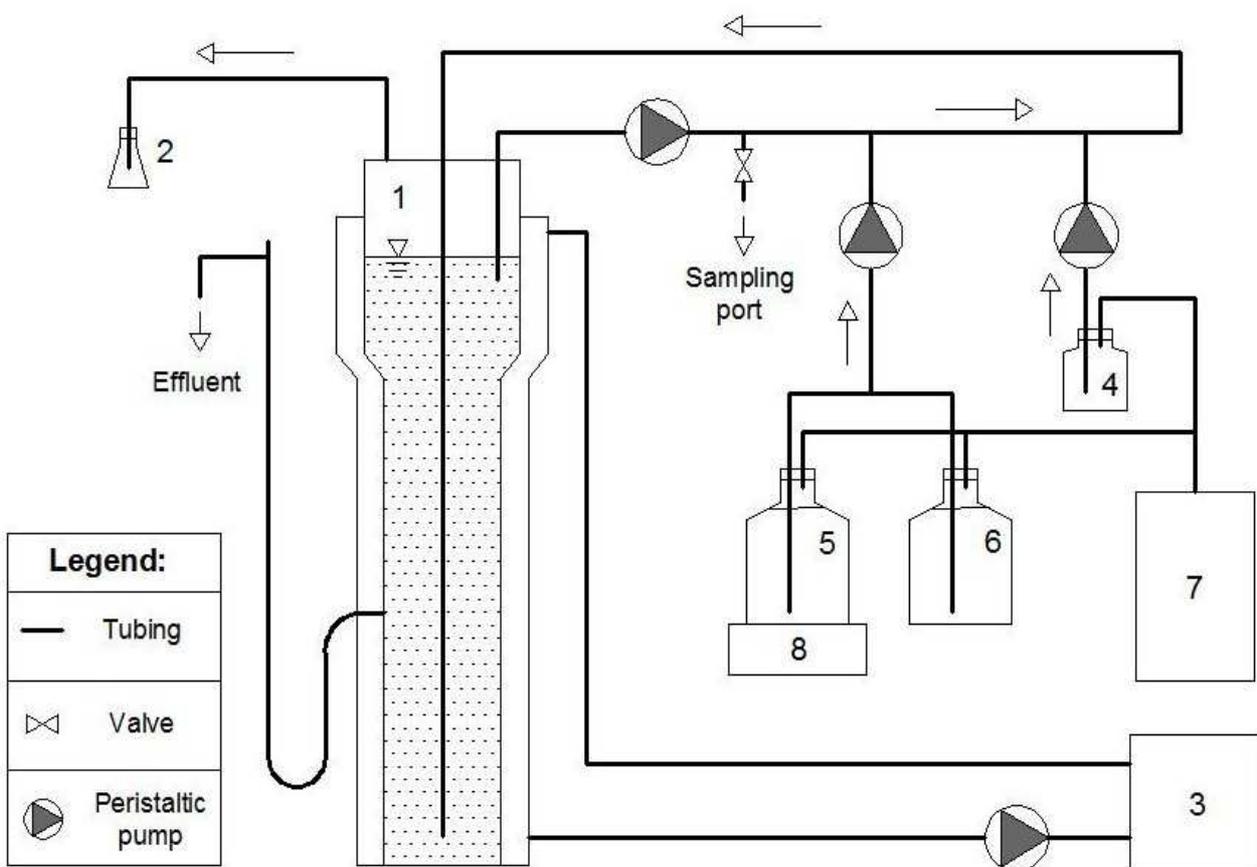


Figure 1. Schematic diagram of the lab-scale setup of anammox-reactor

The reactor was filled with enrichment liquid medium of a given composition which permits preferential emergence of anammox bacteria. The reactor was inoculated with pre-enriched activated sludge from the Leipzig-Rosenthal municipal wastewater treatment plant [440000 population equivalent (PE)]. The highly concentrated medium (henceforth referred to as end-medium) was separated into 2 half media (Table 1), which were stored in separate 5 l Schott bottles, in order to prevent precipitation, and kept in anaerobic conditions to prevent oxygen input into the reactor. The concentrations of  $\text{NaNO}_2$  and  $\text{NH}_4\text{Cl}$  in the end-medium (which was formed after unification of the streams from 2 storage tanks) were initially set to 10 mM. The media were supplied periodically by one peristaltic inflow pump with 2 channels. During start-up period, 32 ml of the end medium was supplied twice a day (two cycles), resulting in 20 mg-N day<sup>-1</sup>. The ammonia and nitrite concentration in the end-medium were increased gradually to 18 mM each. Number of feeding cycles was gradually increased to 12 per day and feeding volume was increased to 48 ml per cycle, resulting in 290 mg-N day<sup>-1</sup> or 14.5 g-N m<sup>-3</sup> reactor day<sup>-1</sup>. TMS, containing (mg l<sup>-1</sup>) NaEDTA, 15.0 g;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.43 g;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.24 g;  $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.81 g;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.25 g;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.22 g;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.19 g;  $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$ , 0.11 g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g; and  $\text{H}_3\text{BO}_3$ , 0.07 g, was supplied with a separate peristaltic pump in amount of 1 ml per liter of end-medium.

**Table 1. Enrichment medium composition**

Component	Concentration in the end-medium (g l <sup>-1</sup> )
1 <sup>st</sup> half-medium (N and C supply)	
$\text{NaNO}_2$	1.240
$\text{NH}_4\text{Cl}$	0.960
$\text{KHCO}_3$	1.500
2 <sup>nd</sup> half-medium (Minerals supply)	
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.110
$\text{KH}_2\text{PO}_4$	0.014
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.012
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.300
$\text{Na}_2\text{SO}_4$	0.200

To emulate the horizontal subsurface flow constructed wetland, planted fixed bed reactor (PFR) with continuous recirculation (providing free from macro-gradient conditions within the root bed) developed by Kappelmeyer et al.<sup>17</sup> was run. PFR was operated under the defined conditions of an average summer day in a moderate climate in a greenhouse (the Phytotechnicum of the Helmholtz Centre for Environmental Research). The plastic PFR with a diameter of 0.3 m and a height of 0.28 m was filled with 17 kg of gravel (particle size 4-8 mm) and planted with *Juncus effusus*. The pore water volume was equal to 14 L with hydraulic retention time (HRT) approximately 7 days. The PFR was fed with synthetic wastewater containing ammonium-nitrogen as the main contaminant (50 mg-NH<sub>4</sub><sup>+</sup> l<sup>-1</sup>). Composition of the wastewater is presented in table 2. TMS (with the same composition as that for anammox-reactor) was supplied in 1.5 ml per liter of the synthetic wastewater. Wastewater inflow rate was equal to 1.85 l d<sup>-1</sup>.

**Table 2. Synthetic wastewater composition**

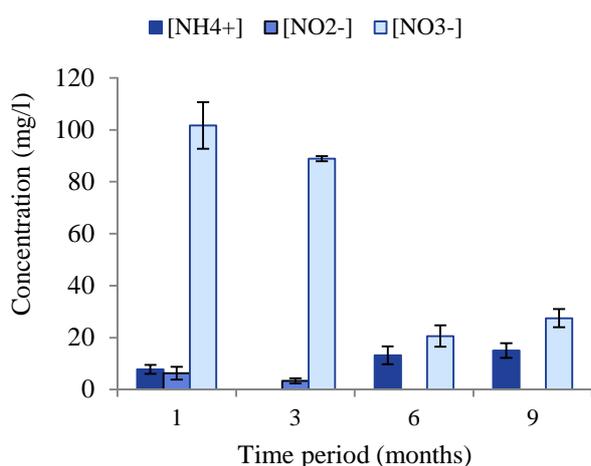
Component	Concentration (g l <sup>-1</sup> )
$\text{NH}_4\text{Cl}$	0.150
$\text{KHCO}_3$	1.180
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$	0.110
$\text{KH}_2\text{PO}_4$	0.014
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.012
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.300

After the enrichment of bacterial culture in the anammox-reactor the biofilms were mechanically transferred from the plastic carriers to the liquid phase, and then, the liquid phase was homogenized by high rate recirculation. Then, 3 l of the liquid enriched culture were anaerobically taken into 1 L glass bottles. Inoculation of the PFR was performed using a metal needle of a large diameter and a peristaltic pump. The enriched culture was slowly introduced into the PFR. After the inoculation, the feeding of the PFR was paused for 48 hours, while the recirculation was run.

To analyze treatment performance of the PFR, different parameters were monitored, including temperature, pH, redox potential (Eh), dissolved oxygen, pore volume, inorganic nitrogen concentrations, and microbial community development. The analytical methods were based on the publication by Wiessner et al.<sup>18</sup> Real-time quantitative polymerase chain reaction (RT-PCR)<sup>19</sup> with non-specific (bacterial) and group-specific PCR oligonucleotide primers were used for microbial community analysis. Total DNA was isolated from enrichment culture samples (40 ml) using DNeasy® Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) and the FastPrep® Instrument (MP Biomedicals, Irvine, CA, USA). Group-specific primer set targeting 16S ribosomal RNA gene sequences of anammox bacteria (AMX) was used.

## Results and Discussion

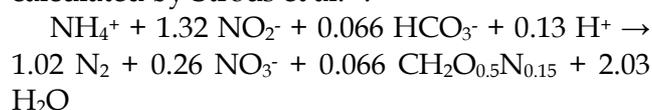
As can be observed in figure 2, during the first month of the operation, nitrate-nitrogen was strongly prevalent among nitrogen species in the anammox-reactor. This could be a result of nitrifying bacteria (Nitrosomonas and Nitrobacter), dominant in the reactor at the initial phase.



**Figure 2. Nitrogen species composition dynamics in anammox-reactor**

After 3 months, the concentrations of both

nitrate and ammonium decreased (Figure 2), while the inflow load was considerably increased. This could be evidence, to some extent, of nitrogen removal via the anammox pathway by direct oxidization of ammonium by nitrite to dinitrogen gas. After 6 months of operation, considerable elevation in activity was observed (Figure 2). The concentration of nitrite had dramatically dropped, but nitrite was not measurable; however, the concentration of ammonium had increased. This confirmed the possibility of the anammox activity, due to the following facts: nitrate was no longer produced in high quantities, while ammonium was present in higher amounts in the system, meanwhile nitrite was completely removed, while ammonium was still present. The presence of ammonium can be explained by the ammonium to nitrite ratio in the feeding medium, which was equal to 1:1. However, stoichiometric ratio amounted to 1:1.32 as calculated by Strous et al.<sup>20</sup>:



Experimental ammonium to nitrite ratio of 1:1 was used in order to prevent accumulation of nitrite in the system, which is toxic to living organisms in high concentrations. The stoichiometry of the anammox process can also explain the accumulation of ammonium in the system with increasing of the nitrogen load to the system.

After 9 months of operation under anaerobic conditions, the microbial association, which has developed in the anammox-reactor, allowed continuous removal of ammonium and nitrite in the ratio that was similar to one of the anammox processes. After 270 days of operation, the liquid samples of the enrichment culture from the anammox-reactor were taken for genetic analysis using molecular biological techniques. Since attached-growth biomass has grown on the surfaces of support media, the biofilm was previously mechanically transferred to the liquid phase.

**Table 3. Real-time quantitative polymerase chain reaction (RT-PCR) results for the water samples from anammox-reactor**

Sample	Genes copy number $\pm$ SD (copies $\mu\text{l}^{-1}$ )		Relative abundance of anammox bacteria (%)
	Total prokaryotes	Anammox	
AMX1	$9.26\text{E} + 6 \pm 7.70\text{E} + 5$	$8.82\text{E} + 6 \pm 6.64\text{E} + 5$	95.3
AMX2	$9.12\text{E} + 6 \pm 6.33\text{E} + 5$	$8.77\text{E} + 6 \pm 8.58\text{E} + 5$	96.1
AMX3	$1.11\text{E} + 7$	$1.04\text{E} + 7 \pm 2.65\text{E} + 5$	93.7

SD: Standard deviation; AMX1: Anammox reactor

Table 3 presents results of the real-time PCR analysis of 3 water samples (AMX1, AMX2, and AMX3) taken from the anammox-reactor. Results have therefore provided evidence of anammox bacteria dominance in the developed microbial association.

At the same time, the model planted fixed bed reactor system was operated. The PFR reached generally stable physicochemical conditions and removal efficiencies. In particular, the total nitrogen (TN) removal efficiencies were equal to about 50%. Furthermore, residual nitrogen was present only in the form of ammonium and nitrate. The redox potential (Eh) during daylight hours amounted to  $300 \pm 50$  mV. However, it should be considered that CWs, are interpreted as redox multi-gradient systems, with both reducing and oxidizing conditions occurring at the same time in different zones (root surfaces, gravel bed, microbial biofilms). These factors have created favourable living conditions for both aerobic and anaerobic organisms.

To estimate the role of the anammox process in nitrogen transformations and its influence on the treatment performance of CWs, the PFR was inoculated with obtained enrichment culture of anammox bacteria. Inoculation is essentially important for the study, because anammox bacteria are slow growers (cells double only once per 11-20 days). The changes in nitrogen removal rates and microbial community composition will be further determined, while the achievement of higher nitrogen removal rates can be expected. When anammox bacteria adapt to the environmental conditions of CWs, the main limitations for the classic process of nitrogen

removal (not enough oxygen and organic carbon) could become an advantage for anammox.

### Conclusion

The anammox bacteria abundance in the anammox-reactor accounted for approximately 95% of the total microbial biomass. This is a suitable amount for inoculation of the experimental planted-fixed bed reactor, which was used as a model constructed wetland system. The results showed that upflow anaerobic fixed bed reactor filled with plastic carriers can be used for anammox enrichment. The obtained bacterial association is capable of anaerobic ammonium oxidation and can more efficiently remove high concentrations of nitrogen. Therefore, the inoculation of constructed wetlands with enrichment culture of anammox can improve poor performance of the system in terms of nitrogen removal.

### Conflict of Interests

Authors have no conflict of interests.

### Acknowledgements

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