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ScienceDirect
Systematic and Applied Microbiology 31 (2008) 114–125
SYSTEMATIC AND APPLIED MICROBIOLOGY
www.elsevier.de/syapm

Physiological and phylogenetic study of an ammonium-oxidizing culture at high nitrite concentrations ☆

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Received 22 August 2007

Abstract

Oxidation of high-strength ammonium wastewater can lead to exceptionally high nitrite concentrations; therefore, the effect of high nitrite concentration (>400 mM) was studied using an ammonium-oxidizing enrichment culture in a batch reactor. Ammonium was fed to the reactor in portions of 40–150 mM until ammonium oxidation rates decreased and finally stopped. Activity was restored by replacing half of the medium, while biomass was retained by a membrane. The ammonium-oxidizing population obtained was able to oxidize ammonium at nitrite concentrations of up to 500 mM. The maximum specific oxidation activity of the culture in batch test was about 0.040 mmol $O_2\,g^{-1}$ protein min^{-1} and the K_s value was 1.5 mM ammonium. In these tests, half of the maximum oxidation activity was still present at a concentration of 600 mM nitrite and approximately 10% residual activity could still be measured at 1200 mM nitrite (pH 7.4), or as a free nitrous acid (FNA) concentration of 6.6 mg l^{-1}. Additional experiments showed that the inhibition was caused by nitrite and not by the high sodium chloride concentration of the medium. The added ammonium was mainly converted into nitrite and no nitrite oxidation was observed. In addition, gaseous nitrogen compounds were detected and mass balance calculations revealed a nitrogen loss of approximately 20% using this system. Phylogenetic analyses of 16S rRNA and ammonium monooxygenase (*amoA*) genes of the obtained enrichment culture showed that ammonium-oxidizing bacteria of the *Nitrosomonas europaea*/*Nitrosococcus mobilis* cluster dominated the two clone libraries. Approximately 25% of the 16S rRNA clones showed a similarity of 92% to *Deinococcus*-like organisms. Specific fluorescence in situ hybridization (FISH) probes confirmed that these microbes comprised 10–20% of the microbial community in the enrichment. The *Deinococcus*-like organisms were located around the *Nitrosomonas* clusters, but their role in the community is currently unresolved.

Keywords: Nitrification; High nitrite concentration; Ammonium oxidation; *Nitrosomonas*; *Deinococcus*

Abbreviations: AOA, ammonium-oxidizing archaea; AOB, ammonium-oxidizing bacteria; FISH, fluorescence in situ hybridization; FNA, free nitrous acid concentration; NOB, nitrite-oxidizing bacteria; SBR, sequencing batch reactor.

☆ *Nucleotide sequence accession numbers*: The sequences obtained in this study are available under accession numbers EU017376–EU017378 and EU221320–EU221324 in GenBank.

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doi:10.1016/j.syapm.2008.01.002

Introduction

The elimination of soluble nitrogen compounds plays an important role in the treatment of wastewater [19], because these compounds can contribute significantly to the pollution and eutrophication of aquatic environments. Ammonium is the main nitrogen component of untreated wastewater and it is removed using biological treatment systems, such as activated sludge or biofilm systems. Autotrophic ammonium-oxidizing bacteria (AOB) and ammonium-oxidizing archaea (AOA) convert ammonium via hydroxylamine to nitrite [5], whereas nitrite is converted to nitrate by nitrite-oxidizing bacteria (NOB). Nitrite and nitrate are reduced to gaseous nitrogen compounds via nitric oxide (NO), to nitrous oxide (N_2O), or dinitrogen gas (N_2) at the expense of organic or inorganic electron donors in processes such as denitrification or anammox, respectively [19].

The intermediate nitrite has several adverse effects on various nitrogen cycle bacteria. At concentrations of 30 mM, nitrite showed inhibitory effects on the AOB *Nitrosomonas* sp. [35]. Elevated nitrous acid concentrations were also toxic for AOB in activated sludge [3]. It has been shown that the effect of nitrite on AOB is caused by free nitrous acid (FNA) [3]. FNA also had inhibitory effects on the ammonium monooxygenase enzyme of *Nitrosomonas europaea* [45].

Nitrite also has a substrate inhibitory effect on the NOB *Nitrobacter* [3,51] and anammox bacteria [47]. FNA had an inhibitory effect on NOB in the presence of carbon dioxide, but not without carbon dioxide. Therefore, the effect of FNA was assumed to be on the anabolism and not on the catabolism of NOB [51]. This difference was not observed for an AOB enrichment of *Nitrosomonas* [50]. The anammox bacteria were not inhibited by ammonium or nitrate at high concentrations of 70 mM for a week in a sequencing batch reactor (SBR). In contrast, nitrite at concentrations higher than 7 mM at pH 7.0 completely inactivated the anammox bacteria. Surprisingly, this nitrite inhibition could be overcome through the addition of trace amounts of either hydrazine or hydroxylamine, which most probably generated the energy production in the organisms [47]. In batch tests, with a *Kuenenia stuttgartiensis* enrichment culture, 12 mM nitrite at pH 7.0 caused only a temporary inhibition. After 3 days, the activity resumed at the same rate as with 5 mM. However, at 18 mM the anammox activity was completely lost [12].

Recently, a new group of AOA was discovered in marine ecosystems. Molecular surveys showed that AOA were also present in various soils, wastewater treatment plants and marine ecosystems [29,36] The isolated organism *Nitrosopumilus maritimus* SCM1 was the first chemolithoautotrophic ammonium-oxidizing microbe in the domain of the Archaea [26]. It has been hypothesized that these marine Crenarchaeotes are responsible for maintaining the low marine ammonium concentration. *N. maritimus* was inhibited by very low concentrations of organic compounds and ammonium oxidation was completely inhibited at concentrations of 0.35 mM nitrite [26]. An overview of the influence of nitrite, as a substrate and product, on the AOB, AOA, NOB and anammox bacteria is summarized in Table 1.

Nitrite is not only toxic for nitrogen cycle organisms but also for many other bacteria and the processes influenced by nitrite. Nitrite has a negative influence on the growth of *Acinetobacter* sp. [54] and acts as a bactericide for *Clostridium sporogenes*, a food spoilage bacterium [9]. Nitrite also inhibits aerobic and anoxic phosphate uptake by phosphate-accumulating organisms [39]. Furthermore, nitrite is also toxic for anaerobic organisms involved in processes such as denitrification [16], sulfate reduction [34] and methanogenesis [25,34].

Several new wastewater treatment concepts based on AOB and anammox bacteria, such as SHARON, OLAND, CANON and ANAMMOX [17,27,44,52], have been developed for the treatment of high-strength ammonium wastewater. Application of these concepts results in significant reduction of oxygen demand and COD usage. SHARON is based on decoupling the AOB and NOB by selection of AOB using solid retention times (thus growth rates) at temperatures higher than 20 °C [17]. The SHARON effluent is ideal as influent for the ANAMMOX process and consists of almost equimolar concentrations of ammonium and nitrite [52], albeit at quite high concentrations (above 35 mM). The reactor choice for the ANAMMOX process is essential for eliminating the toxicity of nitrite [46]. CANON and OLAND processes are both based on (micro)aerobic systems that combine both AOB and anammox bacteria [27,44].

Nitrification systems that treat high-strength ammonium wastewater may experience conditions with high nitrite concentrations. Therefore, our aim was to investigate the effect of the produced nitrite on an enriched and mixed AOB population in a batch reactor system. The ecophysiological parameters, as K_s, V_{max} and K_i of nitrite, were determined and the bacterial composition was analyzed by 16S rRNA and *amoA* gene sequencing and in situ hybridization.

Materials and methods

Batch reactor and operational conditions

A 10 l batch reactor was stirred at 150 rpm and operated at room temperature. The reactor was inoculated with 5 l of activated sludge from the b-stage, which is the basin mainly responsible for nutrient removal in the Dokhaven wastewater treatment plant