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RESEARCH ON THE CULTIVATION OF NITRIFYING BACTERIA AND POSSIBILITY OF THE BIOMASS STORAGE

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Abstract
The efficiency of nitrification has a great influence on the effect of removing nitrogen from wastewater. Problems with the process are associated with reducing the age of activated sludge, lower temperatures of wastewater in aeration chambers, rapid changes in the amount and composition of wastewater containing toxic substances or inhibiting the oxidation of ammonia nitrogen. The research concerned the possibility of cultivation and storage of the nitrifying bacteria biomass, which could be added to the activated sludge in case of a drop of nitrification efficiency. The study showed that even a four-year-old storage of nitrifying bacteria does not result in their complete destruction.

Streszczenie
Sprawność procesu nitryfikacji ma duże znaczenie dla efektu usuwania związków azotu ze ścieków. Problemy z prawidłowym przebiegiem nitryfikacji podczas oczyszczania ścieków pojawiają się przy skróceniu wieku osadu czynnego, niskich temperaturach ścieków w komorach napowietrzania, gwałtownych zmianach składu i ilości ścieków dopływających do oczyszczalni oraz dopływem ścieków zawierających substancje toksyczne lub inhibitujące proces utleniania azotu amonowego. Podjęto badania nad sprawdzeniem możliwości hodowli i przechowywania biomasy bakterii nitryfikacyjnych, którą można byłoby dodawać do osadu czynnego w sytuacjach spadku sprawności nitryfikacji. Przeprowadzone badania wykazały, że nawet 4-letni okres przechowywania bakterii nitryfikacyjnych nie powoduje ich zaniku.

Keywords: **AOB; NOB; FISH.**

1. INTRODUCTION

Bacteria oxidizing ammonium nitrogen to nitrate were first described by Winogradsky in 19th century [1]. During long-term research different types of nitrification bacteria oxidizing ammonia nitrogen and nitrite were distinguished. Ammonia oxidizing bacteria (archea) (AOB and AOA) constitute e.g.: *Nitrosomonas europaea* / *Nitrosococcus mobilis*, *Nitrosomonas communis*, *Nitrosomonas marina, Nitrosomonas oligotropha*, *Nitrosomonas cryotolerans, Nitrosomonas sp., Nitosococcus sp.*, *Nitrocistis oceanus* and to nitrite oxidising bacteria belong *Nitrobacter sp., Nitrococcus sp., Nitrospira sp.*, *Nitrospina sp*. [1]. Until now, no bacteria have been able to carry out completely the two processes of nitrification.

Bacteria of the Nitroso- (*Nitrosospira sp., Nitrosolobus sp., Nitrosovibrio sp*. and *Nitrosococcus sp*.) genus

belong to aerobic which do not produce spores. However, they are resistant to harsh and dry conditions and for many years are able to go into a state of suspended animation. They belong to chemolithoautotrophs and their basic feature is to create a colony on the border of two phases, e.g. on the edge of sand or soil grains. In the laboratory conditions, the bacteria can adhere to the glass walls and pieces of chalk added to neutralize the acid reaction that appears during nitrification. Additionally, bacteria of the *Nitrosomonas genus* (*Nitrobacter sp., Nitrospira sp., Nitrococcus sp*.) are characterized by growing on the surface of sand or soil grains [2, 3, 4]. The processes of nitrification can also be carried out by heterotrophic organisms such as fungi: *Aspergillus flavus, Penicillium, Cephalosporium* [1, 2, 5].

Nitrification takes place both in the aquatic and soil environment. In the activated sludge, on most wastewater treatment plants (WWTP), *Nitrosomonas sp*. and *Nitrobacter sp*. are detected [1].

During the reduction conditions Anaerobic ammonia oxidation bacteria (*Anammox bacteria*) described for the first time by Mulder et al., can occur [6]. Anammox bacteria use the nitrite for the reduction of ammonia. The bacteria include such genera as: *Brocadia, Kuenenia, Scalindua, Anammoxoglobus and Jettenia*.

In recent years, there has been a growing interest in cultivation of nitrifying bacteria for biomass production which would increase the rate of nitrification in periods of a drop of biochemical activity mainly due to the decreasing temperature of the treated wastewater [7]. The study aimed at demonstrating the ability of the biomass storage and determining their biochemical activity after a long period of storage.

2. MATERIALS AND METHODS

2.1. Cultivation of nitrifying bacteria

The inoculum of nitrifying bacteria was cultured during the pilot-scale experiment. This concerned ammonia removal from the air coming from livestock buildings ventilation system. The pilot-scale system consisted of a water tank, blower and tube diffuser (Fig. 1). The amount of air injected to the tap water was controlled by a flow meter whereas the concentration of ammonia in the air, blown into and out of the container, was measured by Pro GasBadge of Industrial Scientific detector. In addition, concentration changes of ammonia, nitrites and nitrates were controlled in the water. The results of these studies were presented in the previous work by Frąszczak and Łomotowski [8].

The research was conducted from May 2009 to September 2011 on a pig farm in Łosice (Fig. 2). The study started on 2nd May, 2009 showed that it took 80 days to grow nitrifying bacteria. After this period nitrification process was observed. Due to a drop of

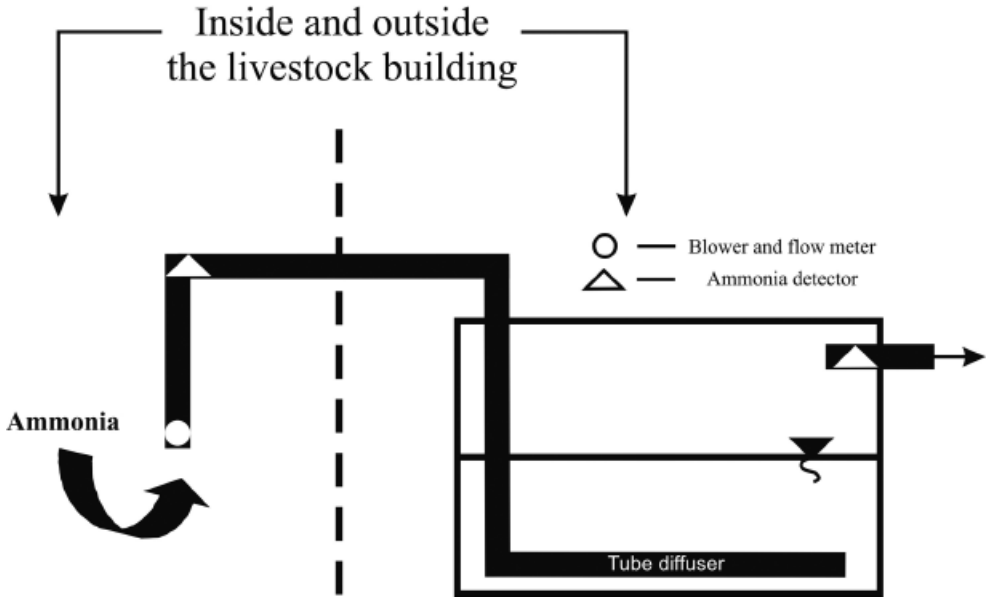

Figure 1.
Pilot-scale experiment

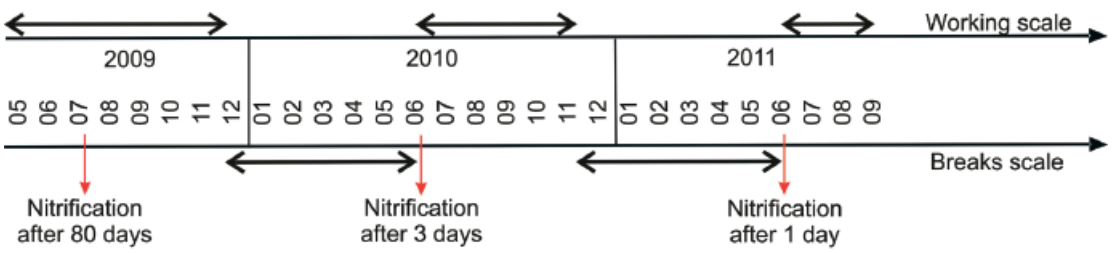

Figure 2.
The phases of the first experiment and the start time of nitrification

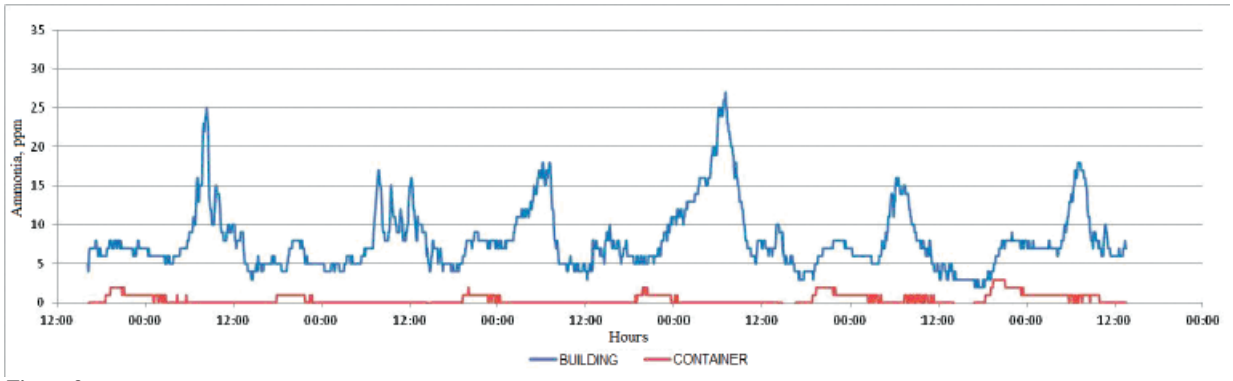

Figure 3.
The changes in the concentration of ammonia in the air coming from livestock building into and out of the container filled in nitrifying bacteria biomass in the days of 20th-25th June 2010

temperature below zero, the research was stopped on 7th December 2009 and the biomass of nitrifying bacteria was left in the container. As a result of low temperature for a long time, the water with nitrifying bacteria biomass accumulated in the container froze. On 17th June, 2010 after filling the container with water the ammonia in the air was being controlled again. Nitrification was observed after 3 days from blowing the air from livestock building to the container. The ammonia was completely removed from the blown air (Fig. 3). On 25th November, 2010 after more than 5 months of observations, due to weather conditions, the system of blowing air was turned off once again. The aeration system was restarted on 15th June 2011. Immediately after blowing air with the increased content of ammonia, nitrification and denitrification were observed.

Research work on the removal of ammonia from the air coming from livestock buildings completed in September 2011. After the research the nitrifying bacteria biomass was collected in plastic containers and stored in a refrigerator.

2.2. After storage of nitrifying bacteria biomass

After a two-year-old storage, the research on the presence of nitrifying bacteria in the nitrifying bacteria biomass was carried out with fluorescence in situ hybridization (*FISH*) according to the procedure described in publications [9-11]. Nso1225 probe was used to indicate *Betaproteobacterial ammonia-oxidizing bacteria* whereas to identify *Nitrosospira spp*. and *Nitrobacter spp*., Nsv443 and NIT3 were applied respectively. The sludge samples were fixed in 4% paraformaldehyde. The following probes were labelled with Oregon green for Nso1225 and Nsv443 and with CY3 for NIT3. Hybridization was performed with different stringency at 35% for Nso1225, 30% for Nsv443, and 40% for NIT3. The hybridization solution contained 30%, 35% or 40% formamide, 0.9 mM NaCl, 20 mM Tris-HCl (pH 7.4), and 0.01% sodium dodecyl sulfate. Fluorescence labelled probes were mixed with the hybridization solution and added to each sample well and incubated in a hybridization chamber at 46°C for 3 h in the dark. After the hybridization step all slides were gently rinsed with pre-warmed washing buffer in the dark at 48°C for 15 min. All slides were air-dried in the

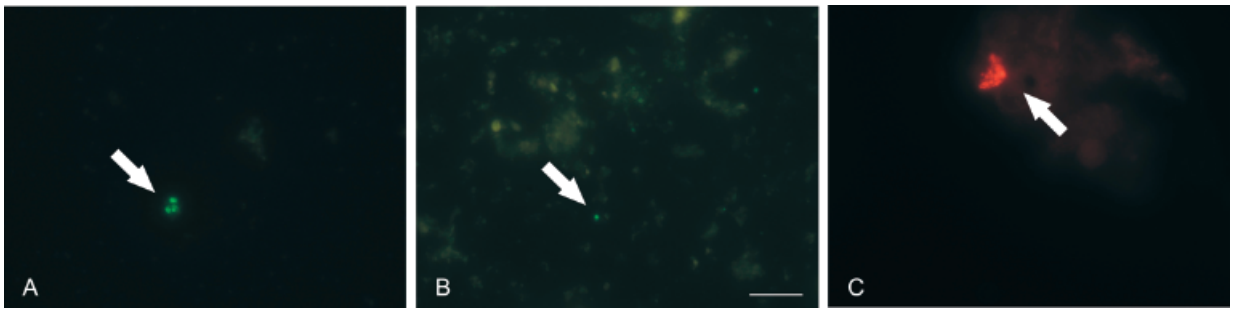

Figure 4.
***FISH* analysis for determination of nitrifying bacteria in the bioflocs taken from the two-year-old nitrifying bacteria biomass. Hybridization with the Nsv443 for *Nitrosospira spp*. (A), Nso1225 for *Betaproteobacterial* ammonia-oxidizing bacteria (B) and NIT3 for *Nitrobacter spp*. (C). Scale bar, 10 µm**

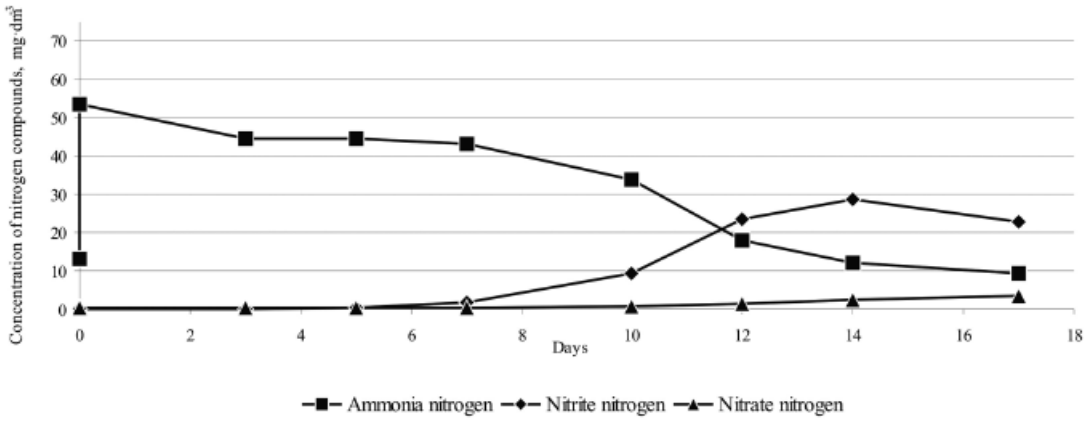

Figure 5.
Changes in the concentration of ammonia, nitrates and nitrites in the sample of the two-year-old nitrifying bacteria biomass

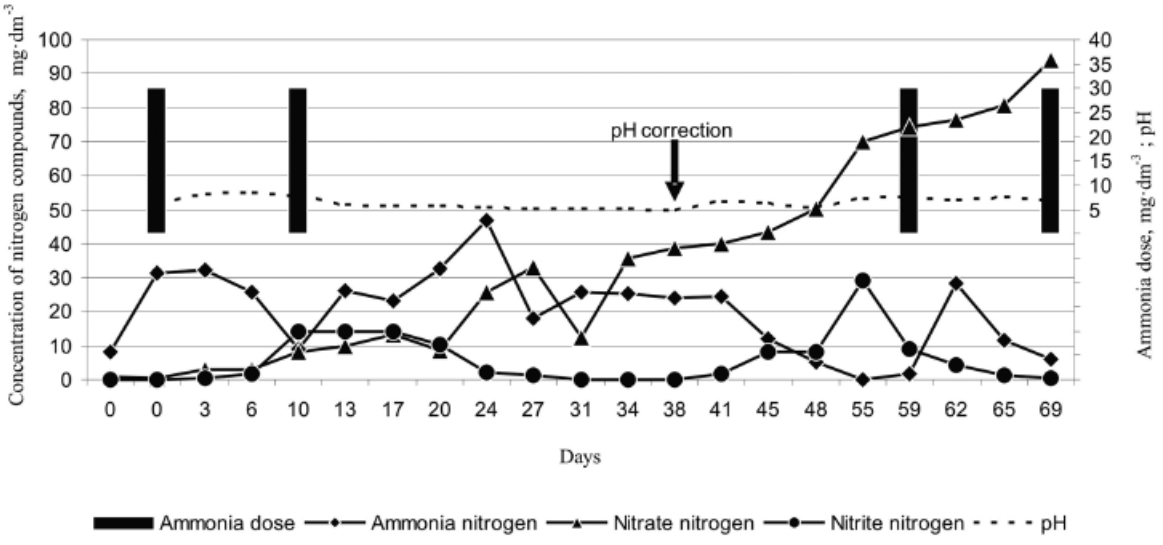

Figure 6.
Changes in the concentration of ammonia, nitrates, nitrites and reaction in the sample of the four-year-old nitrifying bacteria biomass

dark. For total cell staining DAPI was added in a final concentration of 1 μg/cm^3. Slides were examined using epifluorescence microscopy (Nicon Eclipse LV100). The results of these tests are presented in Fig. 4.

In September 2013, under laboratory conditions the following experiment was carried out. To the two-year-old nitrifying bacteria biomass, ammonium chloride was added as a source of ammonia nitrogen and subsequently aeration was started. The experiment was designed to show whether after two years of storage in the nitrifying bacteria biomass there are nitrifying bacteria able to conduct the nitrification process. A similar experiment was carried out after four years. In the experiment the dose of ammonium chloride was lower to check if it is sufficient to start the nitrification. The optimal pH range for nitrifying bacteria growth is between 6.5-8.5 [12]. When the pH decreased below 6, pH correction was conducted. The growth of nitrifying bacteria biomass was not controlled during the whole experiment because of little and nearly constant volume of the biomass. The results of the experiments are presented in Fig. 5 and 6.

In both experiments, after two and four years of storage, the biomass behaved similarly. After few days after the addition of ammonium chloride and the start of aeration, decreasing in ammonia and increasing in nitrite and then in nitrate, were observed. Bacteria cumulated in the biomass carry out the nitrification process properly as long as the ammonia concentration, oxygen and pH control are provided.

3. DISCUSSION

There are some periods of a drop of biochemical activity of bacteria responsible for nitrification process on biological sewage treatment plants, mainly due to the decreasing temperature of the wastewater. To counteract the breakdown of nitrification process there is a need to complete the biomass in a tank. Some companies offer products containing nitrifying bacteria but it costs a lot [13]. The research on the air purification coming from the livestock building showed that there is a possibility of removing ammonia from the air and cultivating the nitrifying bacteria which can improve the nitrification process at sewage treatment plants. The experiments carried out on the farm in Łosice showed that nitrifying bacteria are resistant to freezing. It is commonly known that nitrifying bacteria are soil bacteria and are adapted to changing environmental conditions during the year. In winter soil is frozen for many weeks therefore soil microorganisms must be adapted to changing temperatures as well as oxygen and humidity conditions. Long term research of Garbosky and Giambiagi confirmed resistance of nitrifying bacteria to changing conditions in soil [14]. In a period of strong soil humidity e.g. after rainfall, in the soil profile anoxic conditions could be observed. Nitrifying bacteria are classified to facultative anaerobes, which allows them to survive in anaerobic conditions [15]. This feature of the nitrifying bacteria is used in systems suitable for biological removal of carbon, nitrogen and phosphorus from wastewater. Ability of nitrifying bacteria to survive at low temperatures and anaerobic conditions can be used for their storage.

There are results of research concerning cultivation and short-term storage of nitrifying bacteria but mainly in controlled conditions to examine the influence of temperature and ph on cell activity. Grunditz and Dalhammar research on pure cultures of *Nitrosomonas* and *Nitrobacter* isolated from activated sludge proved slight decrease of cell activity during storage [16]. It is a novelty as nobody has concentrated on long-term storage of biomass for later use in aquatic solutions.

The study showed that even a 4 year-period of storage of the nitrification bacteria does not lead to their complete destruction. Favorable conditions cause immediate biochemical activation of nitrifying bacteria.

4. CONCLUSION

In conclusion, the study demonstrates that:

1. Nitrifying bacteria cultures can be stored for several years at low temperatures. They are resistant to freezing.
2. The biomass of nitrifying bacteria can be produced during the purification of air coming from the livestock buildings, as well as from wastewater containing high concentrations of ammonia nitrogen.
3. The nitrifying bacteria, from the several-year biomass storage, could carry out the nitrification process properly on condition that the oxygen, ammonia concentration and pH control are provided. Therefore, the biomass could be used to support the nitrification process on wastewater treatment plants.
4. It is advisable to continue the research on the cultivation of nitrifying bacteria and implementation of technologies for controlling the biomass content of nitrificants in the activated sludge.

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