DOI: http://dx.doi.org/10.18524/2307-4663.2019.1(45).155333

UDC 579.2.606:628

M.M. Chaban, T.V. Gudzenko
Odesa I.I. Mechnykov National University,
Dvoryanska str., 2, Odesa, 65082, Ukraine,
e-mail:chaban.nik@onu.edu.ua

ANAMMOX BACTERIA DETERMINATION IN THE PHARMACEUTICAL PRODUCTION WASTEWATER

Summary
Anammox bacteria are found in sewage treatment systems, as well as in the other ecological niches, where there are anaerobic conditions. The ***aim*** *of the study wasto establish the ANAMMOX bacteria (****An****aerobic* ***AMM****onium* ***OX****idation) presence and their systematic affiliation in active sludge samples.* ***Methods.*** *The ammonium, nitrite and nitrate concentration determination in the samples was obtained using spectrophotometric reactions. The ANAMMOX process presence was determined by the active sludge incubation method with mineral nutrient medium under anaerobic conditions. The anammox bacteria presence and genus affiliation were determined by FISH (fluorescence hybridization in situ) technique using specific tagged primers:the universal Tamra-Amx-0368, and also Fam-Amx-0820 and Fam-Kst-1 275.* ***Results.*** *For the anammox bacteria synthetic nutrient medium preparation there were determined the ammonium and nitrite ions concentration in the experimental test. The nutrient mineral medium was analyzed for the residual ammonium and nitrite ions concentration after cultivation. For further visual examination of the ANAMMOX bacteria, FISH reaction and the samples microscopy there were performed. Three tagged primers were used in the reaction: the universal Tamra-Amx-0368, and also Fam-Amx-0820 and Fam-Kst-1 275.* ***Conclusions.*** *The decrease of the concentration of ammonium ions by 0,0395 g / l and ions nitrites at 0.0179 g / l in a synthetic nutrient medium in anaerobic conditions with gas release indicates the presence of microorganisms responsible for the ANAMMOX process.The hybridization results indicated Can. Brocadia and Can. Kuenenia presence in the sludge and water sample, in the range of8 to 10 microcolonial units per 50 µl, as well as Kuenenia stuttgartiensis, but in smaller number, 3 to 4, of microcolonial units.*

Key words: wastewater, anammox bacteria, sludge.

About 20 years have passed since the discovery of the ANAMMOX microorganisms responsible for Anaerobic AMMonium OXidation process –anaerobic ammonium oxidation with the release of nitrogen gas (N_2) and nitrate into the environment [8]. From the beginning of the ammonium anaerobic oxidation study, these microorganisms were found in sewage treatment systems, and subsequently began to be found in other ecological niches where there were anaerobic conditions [10, 6, 4]. To date, thanks to molecular genetic studies of anamox microorganisms, it became possible to distinguish them into five genera, which were assigned to the

newly created order *Candidatus Brocadiales* of the class *Planctomycetia* [7, 12, 13]. The study of the reaction of anamox bacteria to changes in various physical factors showed their ability to adapt to temperature and pH [11, 2].

The aim of this work was to analyze a sample of pharmaceutical wastewater for the presence of the ANAMOX process and the possible presence of anamox bacteria responsible for this process.

Materials and methods

The study material was a sample of sewage and sludge from pharmaceutical production. The sample was stored at + 3 °C, sample pH was 7.6. All studies were performed in triplicate.

Chemical analysis of the NH_4^+, NO_2^-, NO_3^- ions concentration in the test sample. To determine the concentration of ions that are key to the cycle of Nitrogen (NH_4^+, NO_2^-, NO_3^-), the sample was pre-filtered by a pump through a filter with a pore diameter of 0.3 μm, the sample was then divided into three parts, each part was introduced appropriate reagent to the determination of specific ions (Nessler's reagent, Griss's reagent and phenolsulfidic acid) [3]. To determine the optical density a Biorad Smart spec Plus spectrophotometer was used, with a wavelength (λ) 425 nm to react to ammonium and 530 nm to a nitrite reaction.

Anamox process analysis. To determine the ANAMOX process presence in the obtained sample a nutrient mineral medium was used, where the concentration of ammonium salts and nitrite was at the data level obtained by chemical analysis of the test sample. The composition of the nutrient medium (g/l): $(NH_4)_2SO_4$ – 0.0514; $NaNO_2$ – 0.024; $NaNO_3$ – 0.010; $KHCO_3$ – 1.248; NaH_2PO_4 – 0.05; $CaCl_2$ – 0.3; $MgSO_4$ – 0.2; $FeSO_4$ – 0.006; EDTA – 0.006 [9]. A 500 ml chemical flask was filled with 350 ml of a nutrient mineral medium, and added 70 ml of the liquid sludge from the sample, then the tube was closed with a gas outlet. The resulting suspension was then purged for 20 minutes with Ar / CO2 gas (95% / 4.5%) to remove dissolved oxygen and obtain anaerobic conditions. The flask was placed in a thermostat at a temperature of 32 °C until gas bubbles appearance [9, 1]. The pH was kept at 7.6 using 1M NaOH and 1% $H2SO_4$ with anaerobic conditions maintenance and restoring.

FISH microscopy. For the visual detection of the anamox bacteria presence the FISH method of microscopy was applied using specific labeled probes [5] with some modifications in the labeled probes hybridization method (Table). In particular, to increase the quality of fluorescent probes hybridization with target cells, we used 1.5 ml Eppendorf tubes as a miniature closed chamber with the humid conditions required for the hybridization process. Cell fixation was performed in 2% paraformaldehyde on ice at + 3 °C for 2 hours. Washing the sample was carried out twice with a suitable buffer for 15 minutes at 48 °C (Table). The Biokom thermostat for Eppendorf type tubes (Thermo 48) was used for hybridization. Zeiss fluorescence microscope was used to analyze the results with a Sony RX-100 IV camera used for the results fixation.

Table

Fluorescent probe for FISH microscopy

Labeled probe	Specificity	Sequence 5' – 3'	Formamid/ NaCl, mM
Tamra-Amx-0368	All anamox bacteria	CCTTTCGGGCATTGCGAA	15/338
Fam-Amx-0820	*Can. Brocadia* i *Can. Kuenenia*	AAAACCCCTCTACTTAGTGCCC	40/56
Fam-Kst-1275	*Kuenenia stuttgartiensis*	TCGGCTTTATAGGTTTCGCA	25/159

Results and discussion

In the pharmaceutical production sample obtained the concentration of ammonium ions $NH_4^+$was 0.052 g/l and nitrite ions $NO_2^-$was 0.024 g/l. Subsequently, the obtained concentrations values of these ions were used to prepare a synthetic nutrient medium to detect the ANAMOX process.

A synthetic nutrient medium with concentrations of ammonium salts $(NH_4)_2SO_4$) 0.052 g/l and nitrite ($NaNO_2$) 0.024 g/l, as was determined in the sample, was used in the study. In the process of the sludge sample incubation in the synthetic medium on the second day the small gas bubblesallocation was determined near the sludge particles, and the sludge itself under the action of bubbles rose to the liquid surface.

After the extracting small gas bubbles active process ending (4–5 days) the nutrient mineral medium was analyzed for the residual ammonium and nitrite ions concentration. As a result of chemical analysis, we found that the concentration of ammonium ions decreased from 0.052±0.5 g/l to 0.0125±0.7 g/l, the concentration of nitrite ions – from 0.024±0.6 g/l to 0.0061±0.75 g/l. The decrease in the ammonium and nitrite ions concentration in the synthetic nutrient medium under anaerobic conditions with the release of gas indicates the presence of microorganisms responsible for the ANAMOX process.

For further visual examination of the bacteria responsible for the ANAMOX process and the confirmation of their presence, FISH reaction and the samples microscopy were performed. The sludge from the main tank was used to determine the presence of the bacteria responsible for the ANAMOX process in the analysis. Three labeled probes were used in the reaction (Table). Initially, the presence of all anamox bacteria in the sample was analyzed using the Tamra-Amx-0368 universal labeled probe. Figure 1 presents the results of hybridization with the microcolonies of anamox bacteria marked.

A 50–60 μl sample was used for hybridization. Analysis of this sample volume revealed the presence of a small number of anamox bacteria microcolonies. The total number of microcolonies per hybridization volume was 10–12 units.

The sample was then analyzed using the Fam-Amx-0820 and Fam-Kst-1275 labeled probes (Table). Figure 2 shows the results of hybridization with the Amx-0820 labeled probe, and Figure 3 shows the Kst-1275 labeled probe.

The hybridization results shown in the pictures indicate the presence of *Can. Brocadia* and *Can. Kuenenia* genera representatives (Fig. 2) and *Kuenenia stutt-*

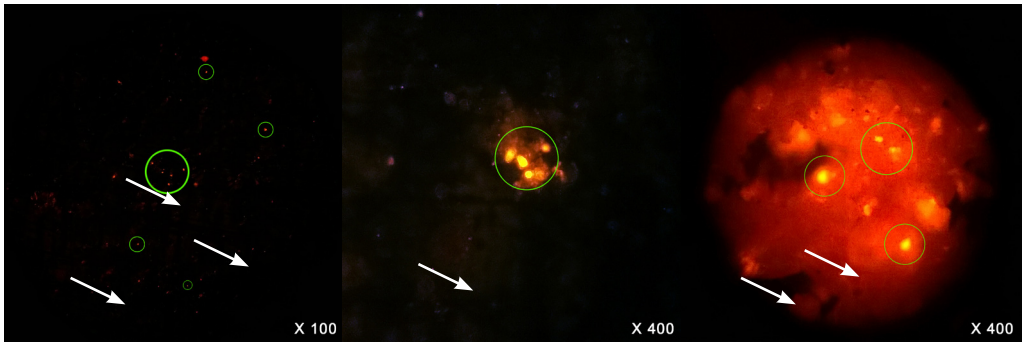

Fig. 1. Colonies of anammox microorganisms with increase of 100 and 400 times: hybridization of probe Amx-0368 with anammox bacteria

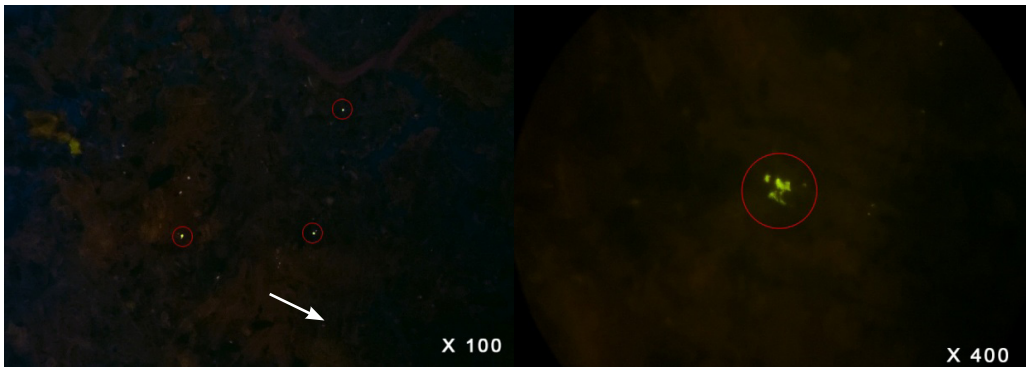

Fig. 2. Colonies of anammox bacteria with increase of 100 and 400 times: hybridization of probe Amx-0820 with anammox bacteria

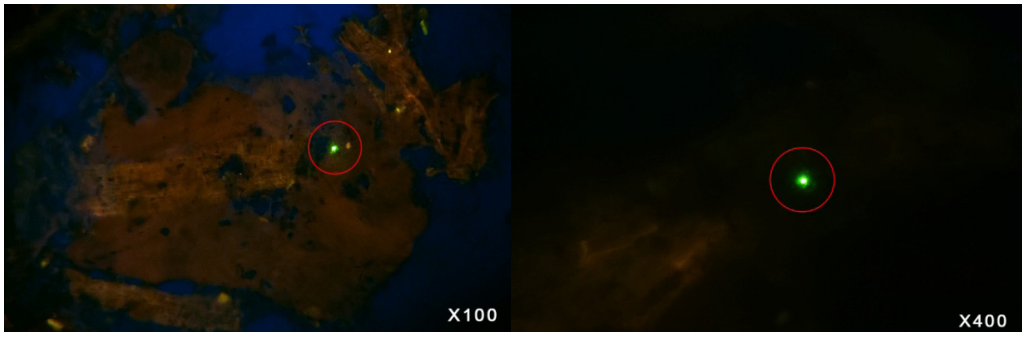

Fig. 3. Colonies of anammox bacteria with increase of 100 and 400 times: hybridization of probe Kst-1275 with anammox bacteria

gartiensis representatives (Fig. 3) in the sludge and water sample. In the visual examination of the hybridization results 8 to 10 microcolonies units of *Can. Brocadia* and *Can. Kuenenia* representatives were established, and the presence in a smaller number, from 3 to 4 microcolonies, of *Kuenenia stuttgartiensis* representatives per sample volume of 50 μl.

The results of the research have confirmed the ANAMOX process presence in sewage sludge from pharmaceutical production and the bacteria responsible for it. It has also been found that the number of microcolonies of *K. stuttgartiensis* is approximately twice less than that of *Can. Brocadia* and *Can. Kuenenia* in the fields of view analyzed. There were found 8–10 microcoloniesof *Can. Brocadia* and *Can. Kueneniain* a 50 μl volume. Representatives of *Kuenenia stuttgartiensis* were found in a number of 3–4 microcolonies in a 50 μl volume. This may indicate the adaptation of specific representatives of anamox bacteria to certain conditions in these wastewater probes.

References

1. Gvozdyak PI, Sapura OV. Simple method of detection and intensivity estimation of anaerobic processes accompanied by gas release. Microbiology and Biotechnology. 2009;4(8):53–57 (In Ukrainian).

2. Malevannyj AM, Yatchyshyn JJ, Malevannyj MS. Estimation of factors influencing the specific activity of the anammox process. Bulletin of the National University "Lviv Polytechnic". 2010;(677): 285 – 289 (In Ukrainian).

3. Novikov YV, Lastochkina CO. Methods of water quality study of reservoirs. Moscow: Medicine. 1990. 236 (In Russian).

4. Shandrovich VT, Malevannyj MS, Malevannyj AM. Application of the ANAMMOX process for purifying sewage from nitrogen compounds. Bulletin of the National University "Lviv Polytechnic". 2014;(787): 352 – 357 (In Ukrainian).

5. Baeten JE., Batstone DJ. Modelling anaerobic, aerobic and partial nitritation-anammox granular sludge reactors – A review. Water Research.2018;(149):322–341.

6. Bagchi S. Metatranscriptomics reveals the molecular mechanism of large granule formation in granular anammox reactor. Scientific Reports. 2016;(6):28327, available at: https://www.nature.com/articles/srep28327

7. Chen H., Jin R. Summary of the preservation techniques and the evolution of the anammox bacteria characteristics during preservation. Appl. Microbiol Biotechnol. 2017;101(11):4349–4362.

8. Kartal B. Anammox Biochemistry: a Tale of Heme c Proteins – A review. Cell Press. 2016;(41):998–1011 Doi: 10.1016/j.tibs.2016.08.015

9. Kocamemi BA., Dityapak D. Anammox start-up strategies: the use of local mixed activated sludge seed versus Anammoxseed. Water Science & Technology. 2018;78(9):1901–1915.

10. Sonthiphand P. Biogeography of anaerobic ammonia oxidizing (anammox) bacteria. Frontiers in Microbiology. 2014;(5):399. Doi: 10.3389/fmicb.2014.00399

11. Zhang Z., Liu S. Hot topics and application trends of the anammox biotechnology: a review by bibliometric analysis. SpringerPlus.2014;(3):220. Doi: 10.1186/2193-1801-3-220

12. Zhang L., Okabe S. Rapid cultivation of free-living planktonic anammox cells.Water Research. 2017;(127):204–210.

13. Zhu G., Wang S. Resuscitation of anammox bacteria after >10,000 years of dormancy. ISME journal. 2018. doi: 10.1038/s41396-018-0316-5

Received 28.01.2019.