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The use of Archaea in the bioaugmentation of activated sludge as a method for the biological removal of nitrogen compounds

Wykorzystanie archaea w bioaugmentacji osadu czynnego jako metody biologicznego usuwania związków azotu

Abstract

This paper examines the effect of Archaea on wastewater treatment in sequencing biological reactors (SBR). The research was carried out in two SBR reactors: a reactor with activated sludge bioaugmented with Archaea (microorganisms which constitute a third domain besides Bacteria and Eukaryotes); a reactor with conventional activated sludge was used as a control. Archaea were incubated in laboratory conditions as recommended by Archaea Solutions Inc. The research revealed that the time period required for the acclimation of the activated sludge in the presence of Archaea was twice as long as in the case of regular nitrifying activated sludge. The observed nitrogen and phosphorous removal from wastewater was achieved to a higher extent in sludge with Archaea and the sludge itself settled faster. The required concentration of oxygen in the reactor with Archaea was lower than in the classic set-up – this resulted in lowering the operating costs of the treatment plant. Furthermore, the denitrification process was significantly shorter and did not require nitrate nitrogen (V).

Keywords: Archaea, nitrogen removal, bioaugmentation, SBR, Anammox

Streszczenie

W pracy przebadano wpływ archeanów na proces oczyszczania ścieków w cyklicznych reaktorach biologicznych. Badania przeprowadzono w dwóch reaktorach typu SBR, z których jeden był poziomem odniesienia (oczyszczanie w warunkach klasycznych), a w drugim oczyszczano ścieki osadem czynnym poddanym bioaugmentacji archeanami, mikroorganizmami stanowiącymi trzecią domenę obok bakterii i eukariontów. Archeany były inkubowane w warunkach laboratoryjnych wg metody zalecanej przez ArchaeaSolutions, Inc. Badania wykazały, że adaptacja osadu czynnego do pracy w obecności archeanów wymaga dwa razy dłuższego czasu, niż zwykły osad czynny, zdolny do efektywnej nitryfikacji. Efektywność usuwania związków azotu i fosforu ze ścieków w obecności archeanów jest większa, a osad czynny szybciej sedymentuje. Wymagane stężenie tlenu w reaktorze z archeanami jest mniejsze niż w układzie klasycznym, co zmniejsza koszty eksploatacji oczyszczalni. Proces denitryfikacji jest znacznie skrócony i nie wymaga obecności azotanów(V).

Słowa kluczowe: archeany, usuwanie azotu, bioaugmentacja, SBR, Anammox

1. Introduction

The development of new techniques and technologies causes an increase in the various anthropogenic pollutants.

At the same time, tighter restrictions concerning the quality of the wastewater treatment plant effluent require the use of biological methods, which are more efficient than chemical or mechanical treatments. However, these approaches are sometimes insufficient; therefore, new methods to enhance biological processes are being sought. Substances such as nitrogen and phosphorous compounds are a particularly big problem. Another important factor which effects the operating costs of wastewater treatment plants is energy consumption – this is mainly as a result of wastewater aeration. This is why innovative new biological methods have been developed such as ANAMMOX, modified SHARON, CANON, OLAND and SNAP. Bioaugmentation of activated sludge is also one of the most well-known methods for intensifying the biological treatment process.

The oxidation of ammonia nitrogen and the removal of nitrogen compounds are important issues in the global circulation of nitrogen. Nitrogen compounds can be removed through the use of methanogenic microorganisms (Archaea) – these constitute a third domain alongside Bacteria and Eukaryotes. Archaea are diversified microorganisms, which are genetically separate from the two other domains. The Woese hypothesis on this topic was confirmed by research carried out in 2000. On the basis of the latest research, three groups of Archaea can be distinguished – facultative, anaerobic, and aerobic. It is currently believed that two groups of microorganisms are responsible for nitrogen removal – chemolithotrophic ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing *Archaea* (AOA) [1, 2]. In the anaerobic condition of seawater, anaerobic ammonium-oxidizing (Anammox) bacteria and Archaea [3, 4] are mainly responsible for nitrogen transformation. The process is autotrophic and bacteria need bicarbonates and CO_2 for the biosynthesis of cells. The research shows that most frequently, Archaea are single-cell, non-pathogenic organisms. Taxons of Archaea identified in recent years are Euryarchaeota, Crenarchaeota, Thamarcheaota, Korarchaeaota, Aigarchaeota and Nanoarcheota. It is currently believed that the transformation of nitrogen compounds in ground and water systems is mainly performed by *Crenarchaeota* [1, 3] – of the aforementioned taxons these are the most diverse and common found in the environment [1]. An increasing number of new research results are continually published in this area. Phylogenetic analysis of Archaea in 16S rRNA (16SrDNA) sequencing has shown that run-off from landfills had distinct Archaea populations, consisting mainly of two taxons – Euryarchaeota and Crenarchaeota [5]. However, the presence of Archaea in activated sludge presents new possibilities to determine their role in the process of the removal of ammonium ions. It has been shown that Archaea contain homologues of a gene for methyl reductase of coenzyme M (MCR) and other enzymes involved in the production of methane from CO_2 [6, 7, 8]. The presence of AOA was also detected in systems of biological wastewater treatment [9].

The constant presence of Archaea in the activated sludge of municipal wastewater treatment plants (WWTPs) has been well known since the beginning of twenty-first century [10, 11]. Over that time studies have been performed, either in the laboratory or at actual WWTPs on

the role of Archaea in oxidation of ammonia and removal of nitrogen compounds [12, 13]. However, significant differences in sludge biocoenosis and a large genetic diversity of both AOA and AOB were observed among the WWTPs [10, 14, 15]. Therefore, as AOA must be considered as ammonia-oxidizers, the relationship between AOA and AOB needs to be clarified as does the relationship between AOA population and nitrogen load [15, 16]. Therefore, the authors decided to use Archaea cultured under laboratory conditions on an organic growth medium, as recommended by Archaea Solutions Inc., to augment the activated sludge.

2. Material and methods

The research was carried out in two identical SBRs (see Fig. 1) – one dosed with Archaea (reactor A) and the another was used as a control for reference purposes (reactor B). The cycle time was twelve hours and it was divided into phases, as presented in Fig. 2. The reactor was supplied with synthetic sewage prepared according to Polish standard (PN-72/C-04550.09). Non-chlorinated tap water, after being left to settle for twenty-four hours, was dosed with the following substances: dry stock cube 152mg/L; casein peptone 339mg/L; ammonium chloride (NH_4Cl) 20mg/L; sodium chloride 7mg/L; calcium chloride ($CaCl_2 \times 6H_2O$) 7.5mg/L; magnesium sulphate ($MgSO_4 \times 7H_2O$) 2mg/L; monobasic potassium phosphate (KH_2PO_4) 16mg/L, dibasic potassium phosphate (K_2HPO_4) 40mg/L; and mechanical wasterwater (10 mL/L) from Płaszów WWTP (Kraków) to introduce micro and macro elements. The characteristics of the raw wastewater are presented in Table 1 – these characteristics full within the range of what would be typically expected of municipal wastewater. The ratio of BOD/COD was 1/1.3 and this was lower than typical ratios (1/2) observed in municipal wastewaters. Chemical analyses were performed in accordance with the applicable standards.

Table 1. Physical-chemical characteristics of raw wastewater

	TS	Spp.	Spo.	BOD_5	COD	$P\text{-}PO_4^{3-}$	$P\text{-}P_{tot}$	Alkalinity	pH
	mg/L	mg/L	mg/L	mgO_2/L	mgO_2/L	mgP/L	mgP/L	mval/L	-
Mean	690.0	296.5	393.5	425.0	557.0	14.3	17.2	4.8	7.1

	$N\text{-}N_{Kj}$	$N\text{-}NH_4^+$	$N\text{-}NO_2^-$	$N\text{-}NO_3^-$	$N\text{-}N_{tot}$	TC	TOC	TNC
	mgN/L	mgN/L	mgN/L	mgN/L	mgN/L	mgC/L	mgC/L	mgC/L
Mean	66.08	21.86	0.01	0.16	66.25	217.70	160.00	57.70

TS – total solids; Spp. – dry residue; Spo. – dry residue after ignition; BOD_5 – biochemical oxygen demand; COD – chemical oxygen demand; PPO_4^{3-} – phosphate phosphorus; $P\text{-}P_{tot}$ – total phosphorus; NN_{Kj} – Kjeldahl nitrogen; NNH_4^+ – ammonia nitrogen; NNO_2^- – nitrite nitrogen; NNO_3^- – nitrate nitrogen; NN_{tot} – total nitrogen; TC – total carbon; TOC – total organic carbon; TNC – total inorganic carbon

Assuming a slow growth of *Archaea*, the age of the sludge used in the process was forty days for both reactors. The volume of the twice-daily decanted wastewater was 25% of the reactor volume (V_{cr} = 20L) in a single cycle. The temperature of the sewage was 25°C. The reactors

operated at different average concentrations of dissolved oxygen; the oxygen concentration was controlled through the use of oxygen probes coupled with fans. Reactors used fine bubble aeration with membrane diffusers located at the bottom of the reactor. Process parameters such as: pH, redox potential and oxygen concentrations were constantly monitored and registered by a computer. In reactor A (with Archaea), the oxygen concentration was 1.3mg/L while in reactor B, it was 3.0mg/L. The oxygen concentrations had to be kept different since oxygen concentrations above 2.0mg/L are toxic for facultative Archaea. Therefore, lower oxygen concentrations generated lower energy costs for reactor A.

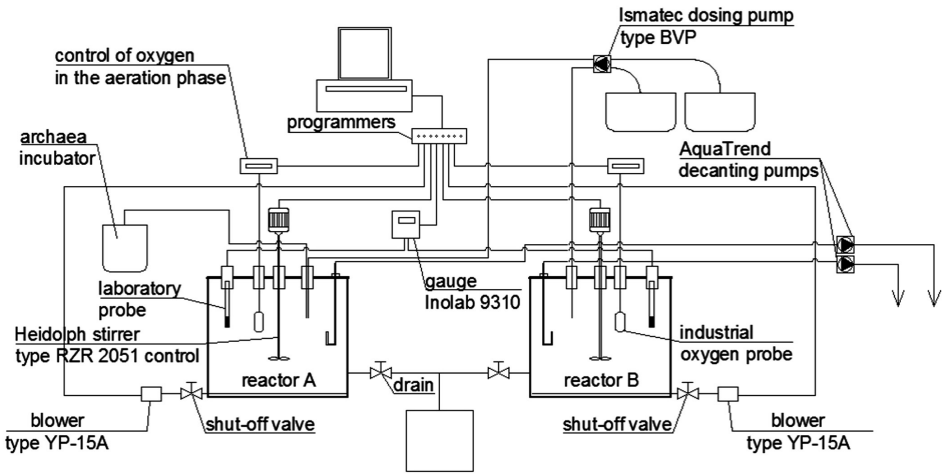

Fig. 1. Experimental stand in the laboratory

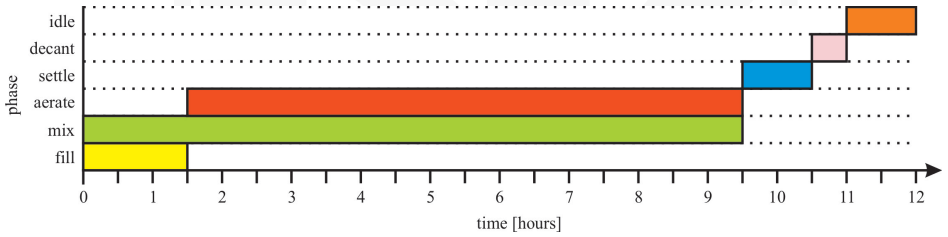

Fig. 2. Phases in the 12-hour SBR cycle

Archaea were incubated on a substrate purchased from Archaea Solutions Inc (US), in the dark at 28°C. They were then rinsed out of the incubator by a stream of dechlorinated tap water at a rate of 1L/h (Archaea dry solids 0.19345 mg/day). The incubator solution contained Archaea and small amounts of fungi and bacteria, as well as organic and inorganic impurities washed off the substrate. The average concentration of dry solids in the incubator effluent was 1.125g/L, while the concentration of non-volatile solids after drying at 550°C was approximately 0.1065g/L. The biomass rinsed out from the incubator and fed to reactor A was 1.02g/L. The presence of AOA in the incubator effluent was analysed on the basis of

the presence of the DNA of microorganisms; genes of archaeal ammonia monooxygenase α subunit (AOA amoA) and the same bacterial gene (AOB amoA) in the polymerase chain reaction (PCR) were detected. Additionally, the identification of archaeal 16S rRNA and bacterial 16S rRNA was performed as a control and reference procedure.

Wastewater treated in reactors A and B was subjected to a qualitative analysis (Fig. 4 and Fig. 5). Chemical analyses were performed in accordance with the applicable standards.

A solution sample of 5L was taken from the incubator and filtered through an MCE membrane of 3.0μm. The accumulated pellets were then mechanically collected, rinsed twice with 0.9% NaCl, suspended in 100μl of sterile 0.9% NaCl solution and used to isolate the DNA. The DNA was isolated with the GeneMATRIX Soil DNA Purification Kit® (EURx, Poland) according to the producer's protocol. The authors decided to modify the protocol and extended the homogenisation time up to twenty minutes. The PCR reaction took place in a MasterCycler® thermocycler (Eppendorf, Germany) with a pair of starters, as described in Table 2. The reaction profile was as follows: initial denaturation 95°C/3min; 35 cycles including phases 94°C/45s, 53°C/45s, 72°C/60s; final extension 72°C/10min. Recombinant polymerase of DNA (Biotools, Spain) was used and the composition of the PCR mixture was set-up in accordance with the producer's recommendations. The products were separated in 1.5% agarose gel using SYBR Gold dye.

Table 2. Sequence of the pair of starters used in the work

	Sequence	Reference
AOA-amoA	F: 5'-sTAATGGTCTGGCTTAGACG R: 5'-GCGGCCATCCATCTGTATGT	[17]
AOB-amoA	F: 5'- GGGGTTTCTACTGGTGGT R: 5'- CCCCTCkGsAAAGCCTTCTTC	[18]
archaeal 16S rRNA	F: 5'- ACkGCTCAGTAACACGT R: 5'- yCCGGCGTTGAmTCCAATT	[19, 20]
bacterial 16S rRNA	F: 5'- CCTACGGGAGGCAGCAGT R: 5'- CGTTTACGGCGTGGACTA	[21]

3. Results and discussion

The growth medium in the Archaea incubator was changed every two weeks. During the incubator's period of operation, the solution fed to reactor A was constantly monitored with regard to the detection of Archaea DNA, particularly that which is able to oxidize ammonia (presence of the amoA gene was detected). It was found that ammonia-oxidizing Archaea were present in the incubator's effluent almost throughout the whole period of operation. Immediately after the first full day of incubation, the presence of a small quantity of AOA-amoA signal was observed; however, the incubator's operation soon stabilized and stayed this way until the end of the fourteen-day cycle (Fig. 3A/B). It was not confirmed whether the incubator emitted a detectable amount of ammonia-oxidizing bacteria to reactor A, although the composition of the incubator substrate showed the presence of such bacteria (Fig. 3C, sewage K).

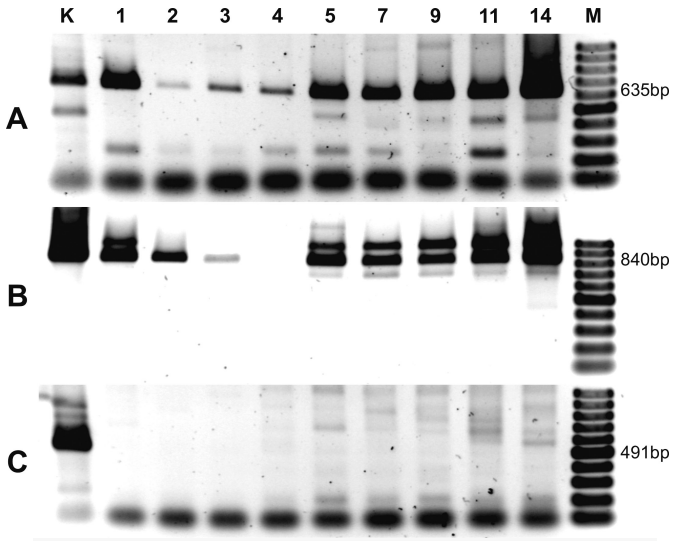

Fig. 3. Changes in the amount of *Archaea* in the incubator effluent. Results of the PCR reaction for: **A** – AOAamoA; **B** – 16S rRNA Archaea; **C** – AOBamoA. The numbers show the day following a start-up of the Archaea incubator. **K** – reaction control (incubator substrate), **M** – ladder 1kb. 1.5% agarose

Acclimation of the activated sludge in the control condition (reactor B), i.e. without Archaea and at an oxygen concentration of 3.6mg O_2/L (Fig. 4), lasted for approximately thirty days. After a certain period of time, the stable removal of nitrogen compounds was observed in the reactor. In the first thirty days, a decrease of nitrate nitrogen(III), ammonia nitrogen, Kjeldahl nitrogen and total nitrogen was noted; however, the concentration of nitrate nitrogen(V) increased to approximately 14mg N/L.

The results of the research show that sludge biomass in reactor A was slightly higher than in reactor B. Furthermore, the removal of inorganic substances was more effectively in reactor A (with Archaea) while organic substances were more effectively removed in reactor B. This could be explained by the halophilic nature of Archaea [12]. The start-up of the reactors was carried out by inoculation with sewage sludge from the Płaszów WWTP. The results indicate the complete transformation of the nitrogen compounds in the reactor. In the presence of oxygen, the biological ammonification and nitrification to nitrate nitrogen(III) takes place as follows [12, 13]:

- bacteria oxidizing ammonia AOB produce nitrites

$$NH_3 + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+ \quad (1)$$

- bacteria oxidizing nitrites to nitrates NOB participate in the transformation of nitrite

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (2)$$

Denitrification of nitrate nitrogen(V) and nitrate nitrogen(III) to molecular nitrogen N_2 then takes place. Nitrate nitrogen(III) is a transient form and was therefore only found in small amounts in the final effluent; however, nitrate nitrogen(V), as a nitrification end product, predominated in the effluent. In the system that was not acclimated to the effluent, the amounts of nitrate nitrogen(III) and nitrate nitrogen(V) were small; however, ammonium nitrogen appears in large amounts. The treatment process in reactor A (Fig. 5) went differently. Here, the adaptation time for activated sludge was over seventy days, i.e. over twice as much

as in reactor B. During acclimatisation of the activated sludge, a systematic decrease of all forms of nitrogen in the effluent was observed and only nitrate nitrogen(III) increased. The concentration of nitrate nitrogen(V) was small to the point of being practically negligible.

The average concentrations of nitrogen and phosphorous compounds in the reactor effluents after sixty-seven days of operation (i.e. once treatment efficiency in reactors A and B was stable) are presented in Table 3 and in Figs. 6 & 7.

A simplified reaction of the ammonification and shortened denitrification which took place in reactor A (with Archaea) can be presented as follows:

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \quad (3)$$

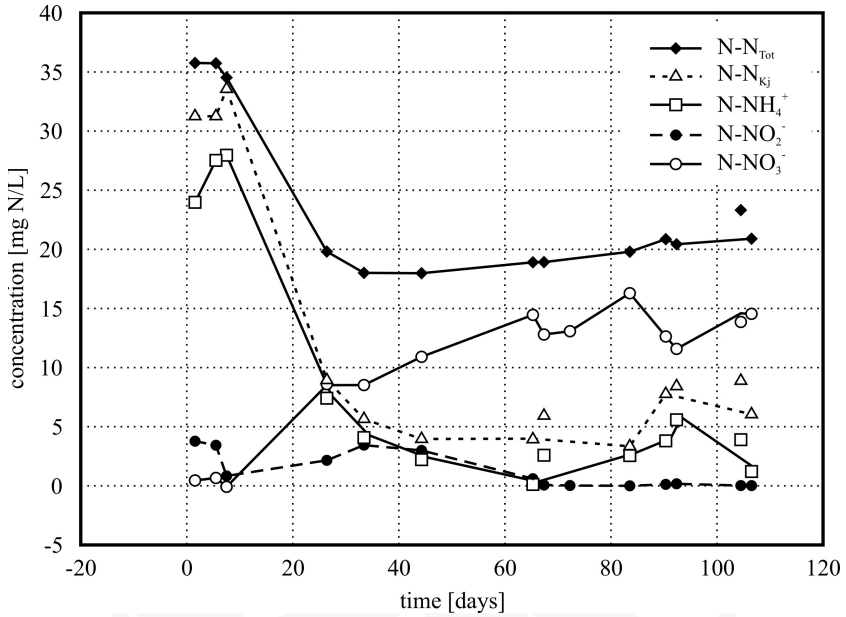

Fig. 4. Transformation of nitrogen in wastewater in reactor B

Archaea was found to accelerate the removal of nitrogen compounds and neither ammonium nor nitrate nitrogen was found (reactor A). There is no second phase of nitrification in which nitrate nitrogen is formed. At the same time, the effective removal of total nitrogen takes place with a higher level of efficiency than in reactor B [12, 13].

Ammonification and denitrification of ammonia nitrogen and nitrites take place in reactor at a significantly lower oxygen concentrations. Nitrites are not found in raw wastewater since they are the product of biochemical transformations. Nitrates do not participate in these reactions since they are not present in raw wastewater nor are they produced during the treatment process as a result of transformation; there are also hardly present in the effluent.

The process differs from the SHARON and Anammox processes, which have been used in numerous industrial applications. The SHARON (single-reactor, high-activity, ammonia removal over nitrite) process is carried out in aerobic conditions by bacteria oxidizing NNH_4 to NNO_2^-, which is then reduced to N_2 in anoxic conditions. The process conditions are a

temperature of 35°C, a pH of 7 and separate aerobic and anoxic tanks. The process requires the addition of methanol. The Anammox process occurs in anaerobic environments, at oxygen concentrations lower than 0.3 mgO_2/L, pH levels of 6.5-9 and with preliminary nitrification. A high concentration of ammonia ions is required (>0.2g NNH_4/L) and therefore the process has become an option for the treatment of wastewater, which meets these specific characteristics. During the process, the complete conversion of ammonia ions to gaseous nitrogen takes place with no external source of organic carbon present. The highest levels of efficiency of the process are observed for a NNO_2/NNH_4 ratio within the range of 1-1.5 [22].

The transformation of different ammonia compounds during the Anammox process can be described with a stoichiometric relationship [23] – this demonstrates that nitrates are produced as a by-product of the reaction [4. 21]:

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow$$
$$\rightarrow 1.0N_2 + 0.26NO_3^- + 0.066CH_2O_{0,5}N_{0.15} + 2.03H_2O \quad (4)$$

In the Anammox process, a higher heterotrophic denitrification bacteria growth is observed at the total carbon to nitrogen ratio of C/N=1 this is the ratio that is recommended by the authors [4]. The process where the molecular nitrogen N_2 is generated by Anammox requires neither complete oxidization (nitrification) nor electron donors, such as methanol in both classic denitrification and SHARON. The process is, to a large extent, similar to the process involving Archaea; however, nitrates are observed here which are not necessary for Archaea. The concentration of ammonia nitrogen is about 20mg/L, the value typical for municipal sewage. The NNO_2^-/NNH_4 ratio is lower than $5x10^{-4}$ since there is not much NNO_2 in raw sewage. The process proceeds at an oxygen concentration below 1.5 mgO_2/L.

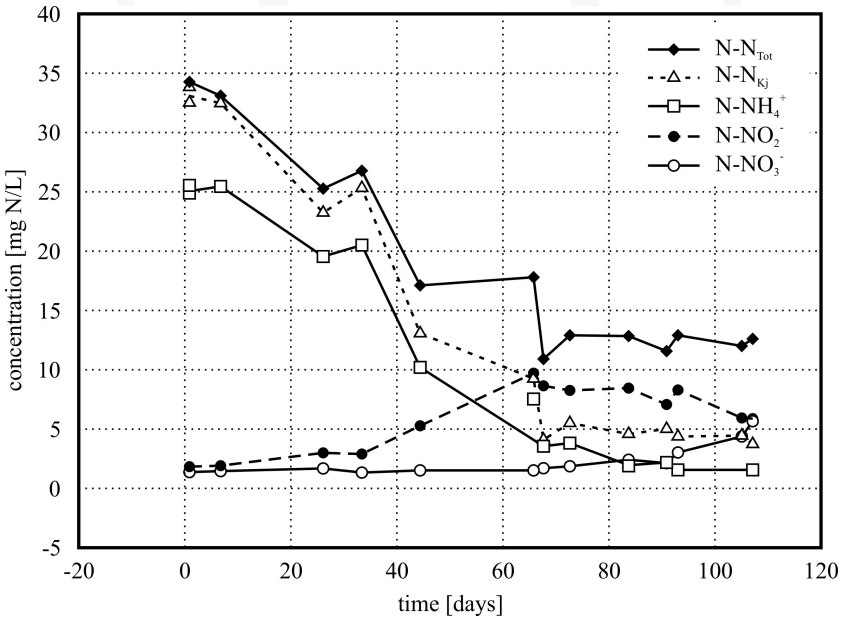

Fig. 5. Transformation of nitrogen in wastewater in reactor A

Table 3. The average effluent values from reactors A and B (67-120 days of rector operation)

	$P\text{-}PO_4^{3-}$ mgP/L	$P\text{-}P_{tot}$ mgP/L	$N\text{-}N_{Kj}$ mgN/L	$N\text{-}NH_4^+$ mgN/L	$N\text{-}NO_3^-$ mgN/L	$N\text{-}NO_2^-$ mgN/L	$N\text{-}N_{tot}$ mgN/L	COD mgO_2/L	BOD_5 mgO_2/L
A	8.82	10.28	4.72	1.495	2.697	9.39	16.80	28.93	5.23
B	10.5	10.88	6.77	3.328	13.87	0.097	20.74	18.20	6.75

BOD_5 – biochemical oxygen demand; COD – chemical oxygen demand; PPO_4^{3-} – phosphate phosphorus; P-`P_{tot} – total phosphorus; NN_{Kj} – Kjeldahl nitrogen; NNH_4^+ – ammonia nitrogen; NNO_2^- – nitrite nitrogen; NNO_3^- – nitrate nitrogen; NN_{tot} – total nitrogen

The results of research presented in Table 3 and in Figures 6 and 7 show that the efficiency of nutrient removal in reactor A is greater than in reactor B; the denitrification process is much shorter and does not require the presence of nitrate nitrogen(V). Reactors A and B operated with the same model wastewater; therefore, Table 3 demonstrates only the final effluent quality rather than wastewater treatment efficiency. When comparing the quality of the final effluent, it can be concluded that the total nitrogen removal efficiency in reactor A (with *Archaea*) was 20% higher than in reactor B; the removal of BOD_5 was >7% while the removal of NO_3 was >80.5%. These are important differences, which may be even greater at higher concentrations of *Archaea*. Moreover, a lower level of energy consumption was observed in reactor A.

The higher efficiency of the process is true for both nitrogen and phosphorous compounds. The results of the research agree, to a certain extent, with the results reported by other researchers [3]. However, the authors did not manage to maintain a stable treatment process and observed a lower production of activated sludge [3].

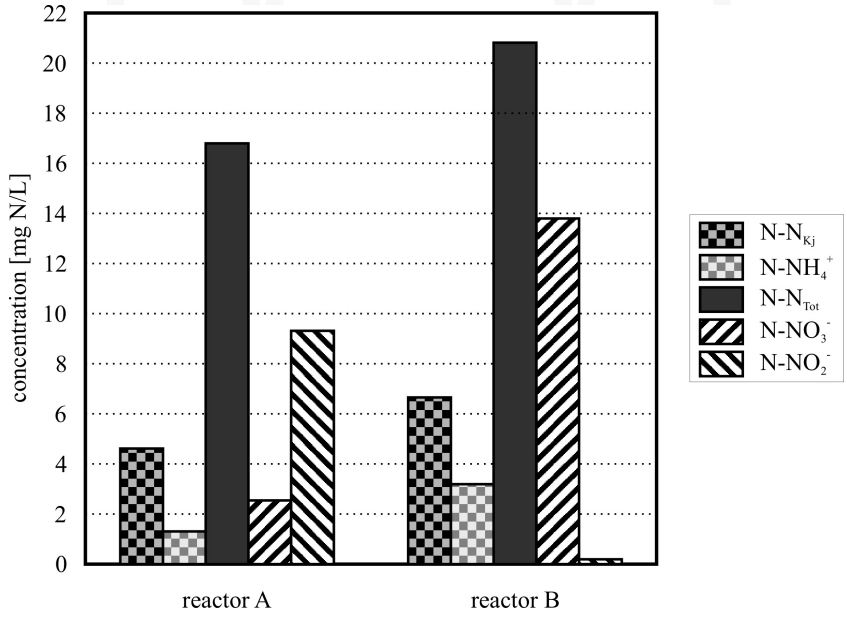

Fig. 6. Concentration of nitrogen in reactors A and B after sludge acclimatisation. Transformation of nitrogen in wastewater

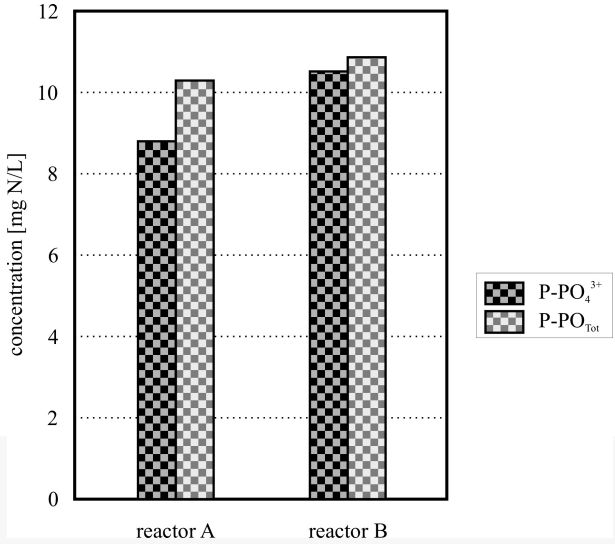

Fig. 7. Concentration of phosphorus in reactors A and B after sludge acclimatisation

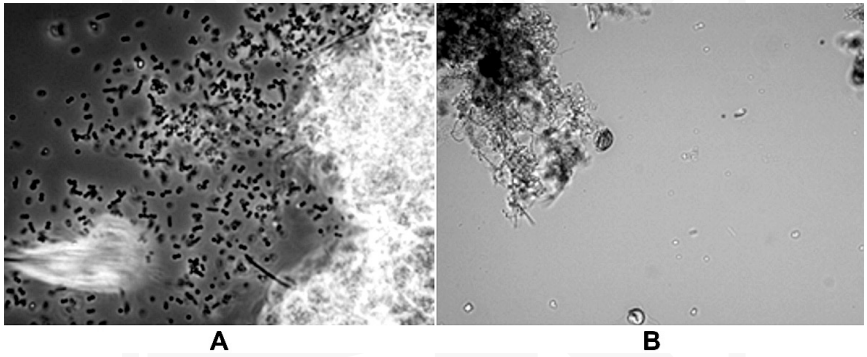

Fig. 8. Sludge after 51 days of process operation. A – sludge flock in reactor A; 400x+ phase contrast, B – sludge flock in reactor B; 200x. (photo by Wojciech Brągiel)

The sludge concentrations in both reactors were similar throughout the research period. A lower amount of activated sludge in reactor A was only observed in the early period of operation, when a proportion of sludge was in suspension. At that time, a higher concentration of suspended solids was observed in the effluent. The concentrations of activated sludge in reactors A and B were 1.7674g of dry solids per litre and 1.6726g of dry solids per litre, respectively. The inorganic fraction in sludge from reactor A (0.3176g/L) was by 3.6% lower than in reactor B (0.36g/L) on average – this could indicate halophilicity of the sludge from reactor A.

The photographs of activated sludge after fifty-one days of process operation in reactors A and B also show some differences. Large aggregates of slow-flowing microorganisms are visible over the sludge from reactor A and in its void spaces. On the other hand, the sludge flocks from reactor B are dense, without the sheath of slow-flowing microorganisms – this is typical for an acclimated activated sludge that functions properly.

4. Conclusions

The research studies carried out in two systems – a classical aerated system and a system with activated sludge bioaugmented with Archaea – lead to the following conclusions, which can serve as guidelines for the operation of municipal wastewater treatment systems with SBRs:

- Activated sludge can be bioaugmented with Archaea. The sludge acclimation time is over sixty days,
- Treatment of wastewater with Archaea occurs at lower oxygen concentrations (<1.3mg O_2/L) – this provides great energy savings at the treatment plant,
- When comparing the quality of the final effluent, it can be concluded that the total nitrogen removal efficiency in reactor A (with Archaea) was 20% higher than in reactor B; the removal efficiency of BOD_5 was >7%, and the removal efficiency of NO_3 was >80.5%. These are important differences which may be even greater at higher concentrations of Archaea,
- The images of activated sludge from the two reactors are different; sludge with Archaea has numerous slow-flowing microorganisms flowing between flocks and above the surface of the active sludge,
- The presence of Archaea improves the efficiency of nutrient removal,
- The denitrification process was significantly shorter and did not require nitrate nitrogen(V).

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