Letter to Editor

Accumulation of Phosphorus by Filamentous Microorganisms

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Abstract

Many microorganisms accumulate phosphor as polyphosphates. This paper highlights the great importance of filamentous microorganisms. So far the ability to store phosphorus in volutin granules by filamentous microorganisms has not been evaluated. The aim of this research was to prove the ability to store phosphorus in volutin granules as well as to show the meaning of potassium and magnesium ions in the phosphorus uptake by filamentous microorganisms that dominate the floating scum over the bioreactors liquid surface and monocultures of bacteria.

Electron microscopy and energy dispersive X–ray analysis was used to assess the composition of polyphosphate granules in filamentous bacteria.

Keywords: filamentous microorganisms, phosphorus, polyphosphates

Introduction

Microorganisms assimilate phosphorus, which enters into the composition of several macromolecules in the cell [1, 2]. Some microorganisms have the ability to store phosphorus as polyphosphates in special granules (volutin granules). Polyphosphate is a linear polymer of residues linked together by high-energy phosphoanhydride bonds and may account for up to 10–20% of cellular dry weight [3, 4]. Polyphosphate can be observed under bright-field or phase-contrast microscopy. Neisser's stain is used to observe these granules under a bright-field microscope. Nuclear magnetic resonance (NMR) has also been used recently to detect polyphosphate granules in wastewater microorganisms [5, 6]. Phosphorus accumulation by non-filamentous bacteria is at present well-known and has often been confirmed.

Filamentous microorganisms are normally a component of activated sludge microflora [7]. The filamentous microorganisms are responsible for foam formation and activated sludge bulking. Foaming is a common problem encountered in many wastewater treatment plants worldwide [8-12]. This article highlights the great importance of filamentous microorganisms in phosphorus uptake.

Microorganisms require various forms of cations. They constitute approximately 1% of dry weight of the microbial cell, as the cofactors for some enzymes. Potassium and magnesium stimulates enzyme reactions associated with the synthesis of cell materials [13]. The importance of potassium and magnesium for microorganisms responsible for phosphorus uptake is even more significant [13, 14]. Potassium defines cell membrane permeability [15] and plays a major role in the phosphate transport between the surrounding environment and the cell. Moreover, this cation is an essential counterion for polyphosphate in the cell, and is general an important factor in the cell's energy generation [16, 17].

An enzyme, polyphosphate kinase, catalyzes polyphosphate biosynthesis in the presence of magnesium ions by transferring the terminal phosphoryl group from ATP

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to the polyphosphate chain [18]. Polyphosphate degradation is driven by several enzymes to depend on inorganic cations. Magnesium acts as an important counterion of polyphosphates too. It is taken up and released simultaneously with phosphate. Consequently these cations (potassium and magnesium) are necessary for polyphosphate accumulation in microorganisms cells.

Materials and Methods

The investigated samples were taken from several municipal enhanced biological nutrient removal (EBNR) wastewater treatment plants. Samples of foam floating on the surface of bioreactors were analyzed.

The investigations included determination of filamentous microorganism species present in the foam. For identification of the species, seeding, morphological and biochemical testes were done.

The following mediums for cultivation of filamentous poly-P bacteria were used:

- Medium Rouf and Stockes, liquid and solid [19]
- Medium MSV, liquid and solid [20]
- ATCC Medium 25 – broth and constant, Product Information Sheet for ATCC® 27809
- Kim and Pagilla medium [21]
- Modified R2A medium [11]
- Slijkhuis medium modified by Foot, liquid and solid [22, 23]
- Blood agar also was used in the investigations.

Sample were incubated at 15°C, 20°C, 26°C, 30°C and 37°C for 1 and 21 days. Every day the morphology of grown colonies (on fixed media) was determined.

The morphological determinations concerned:

1. Mixed culture of foam microorganisms (also of activated sludge) – direct samples,
2. Mixed culture growing on liquid media (in the experiments the adaptation ability to the cultivation conditions was evaluated, because in laboratory condition the filamentous bacteria do not always produce threads, like *Sphaerotilus natans*),
3. Cultivation of monocultures on fixed media were prepared, determined in the case the morphology of grown cultures and samples for determination of the shape, outside structure, length and width of the threads as well as for determination of the ability to form granules of volutine.
4. sulphur test

For *in vitro* diagnosis of some filamentous poly-P bacteria, the API ZYM tests (of BioMerieux) which are a semi quantitative micro-method, serving for assessment of the enzymatic activity were used. Because the API ZYM test can not be considered a tool for identification of the bacteria genus or species, the analysis of obtained results was based on the criteria established by Bergey's Manual of Determinative Bacteriology [24], on the purchased products of culture collection ATCC and also on published results. The products of American Type Culture Collection have been used as a morphological and biochemical features model of poly-P filamentous microorganisms, for microorganisms diagnosis by API ZYM tests.

Also, the investigation aimed at determining the presence in the foam of non-filamentous microorganism species. For identification tests API 20E, API Staph, API Strep, API Coryne, and API NE were used.

For microscope morphological analyses a bright field and contrast phase microscope coupled with a camera for observations was used. The microscope used (Nikon Alphaphot – 2 YS coupled with camera Panasonic GP – KR 222) allowed size measurements by the *Lucia* – ScMeans Verion 4.51 programme. Samples for microscopic investigations were stained according to the Neisser method.
The clusters containing volutin granules were examined by a scanning electron microscope equiped with an energy-dispersive analysis of X-ray attachment (EDX). Samples of 500 cm^3 were filtrated for about 6 hours under vacuum on the Buchner funnel. The retained sludge was next dried for 5 hours at 105°C. The dry matter was disintegrated in an agate mortar.

For SEM (Scannig Electron Microscope) measurements aluminum plates with a diameter of about 1 cm were used. First they were covered with a special glue that conducts electricity and then the powdered samples were put onto plates. The plates with the powder were heated for about 15 minutes using an infrared lamp and then a thin layer of gold was spread. The images of prepared samples and the content of elements were evaluated. The spectrum of the gold was omitted.

The foam samples also were analyzed chemically. Samples were analyzed for pH, temperature, redox (Oxidation Reduction Potential – ORP), phosphates, total phosphorus, the various nitrogen forms – nitrates, nitrites and ammonium ($N - NO_3$, $N - NO_2$ and $N - NH_4$), COD, BOD and dissolved oxygen. Determinations were carried out in accordance with the Standard Methods for the Examination of Water and Wastewater (17th edition) [25]. For colorimetric analyses a HACH DR 400 spectrophotometer was used. The concentration of potassium, magnesium and calcium were determined on an atomic absorption analysis instrument – AAnalyst 100 Perkin Elmer.

Changes of phosphate concentrations in identified monoculture microorganisms concerned sterilized wastewater samples. The process of sterilization was carried out at 121°C and at a pressure of 0.15 MPa. Reactionary chambers were filled with sterilized wastewaters and monoculture microorganisms were added.

Results and Discussion

The isolated species of filamentous microorganisms had similar morfological characteristics. In all of the examined foam samples, among the filamentous microorganisms there was a domination of unbranched forms, while the branched "tree-like" and dichotomic forms were less frequently present (Photo1.)

Among the isolated bacteria and *Actinomycetales*, based on the cultivated, morfological and biochemical tests, the following species have been distinguished: *Microthrix parvicella* (*M. parvicelal* was not identified by biochemical tests), *Nocardia amarae*, *Nocardia sp.*(J – 27)*, Nocardia pinensis – like bacteria*, *Sphaerotilus natans* (established by Bergey's Manual of Determinative Bacteriology), *Rhodococcus chubuensis* (*Gordonia sputi*) and sulphur compounds oxidizing filamentous bacteria (Photos 2- 7).

Based on microbiological analysis of floating foam on the surface of bioreactors, the presence of other heterotrophic bacteria species (which are not forming threads) were also determined. Among the more than 120 species of heterotrofic bacteria not forming threads, *Acinetobacter calcoaceticus* was diagnosed (forming occasionally a chain of cells) by many authors made responsible for phosphorous removal from sewage [26, 27]. The diagnosis calls attention to limited qualitative variation of bacterial biocenosis. This is the result of the ability of adaptation to sewage conditions only by a limited number of species. Sewage can be considered an open system in which both the abiotic environment and living organisms are interacting. Sewage is an environment in which xenobiotics are commonly present, and could be considered as a blocked system affecting the changes in bacteria population quality, biotic composition, changes of living microorganism metabolism as well as the rate of supplied organic substrate biodegradation. These conditions determine the transformation of sewage microorganism populations.

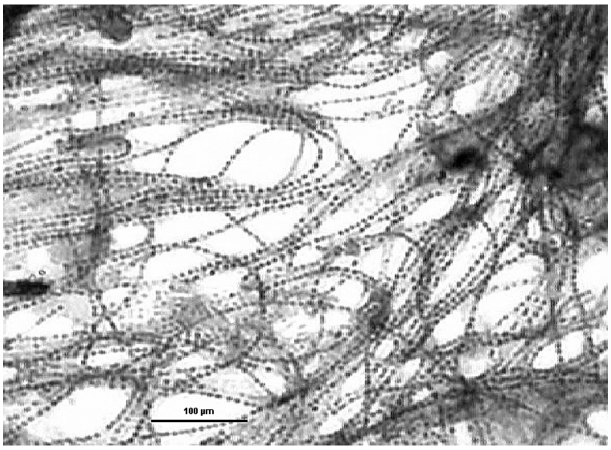

Photo 1. Filamentous microorganisms in foam – bright field microscope.

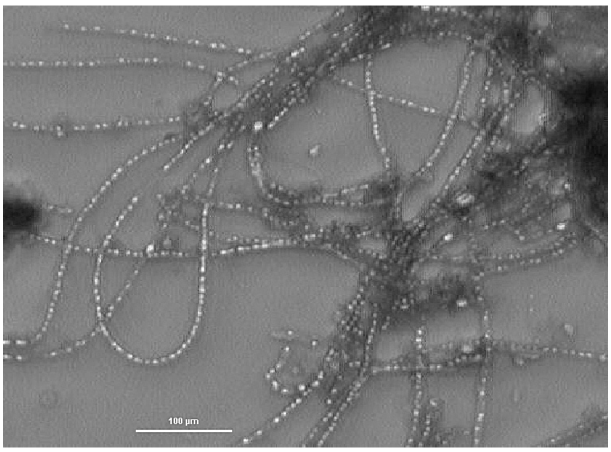

Photo 2. *Microthrix parvicella* – Neisser's stain – contrast phase microscope.

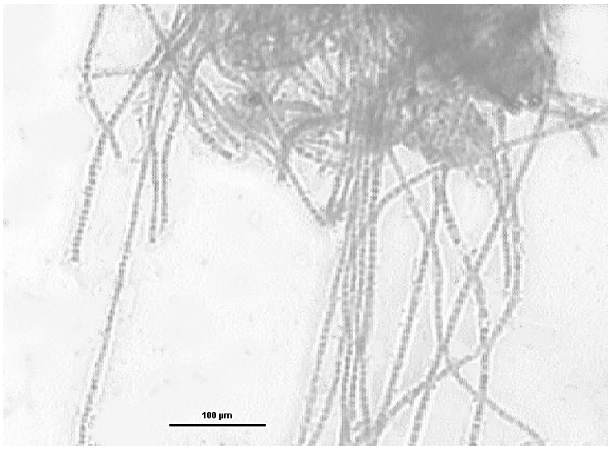

Photo 3. *Sphaerotilus natans* – Gram's stain – bright field microscope.

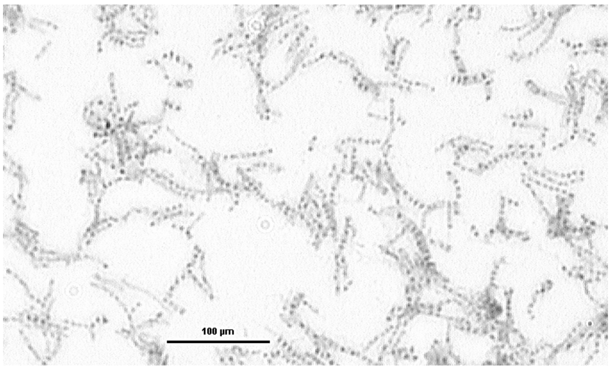

Photo 4. *Nocardia amarae* – Neisser's stain – bright field microscope.

The achieved results by the intermediary cultivation method and the biochemical method do not necessarily reflect the real composition of present species. The reason is a lack of universal nutrient media on which all species of all microorganisms actually present in the specific environment could grow. With the used nutrient media, colonies of only those bacteria, which are able to grow in the specific conditions appear.

Microbiological analysis of foam collected from wastewater treatment plants in the Silesia region of Poland have shown that the dominantly present filamentous microorganisms is *Microthrix parvicella* [28, 29]. The microorganism commonly present in the foam however, lacks the ability of phosphorous accumulation in the form of volutin granules *Sphaerotilus natans* [7, 30].

The results presented here show clearly that the fila-

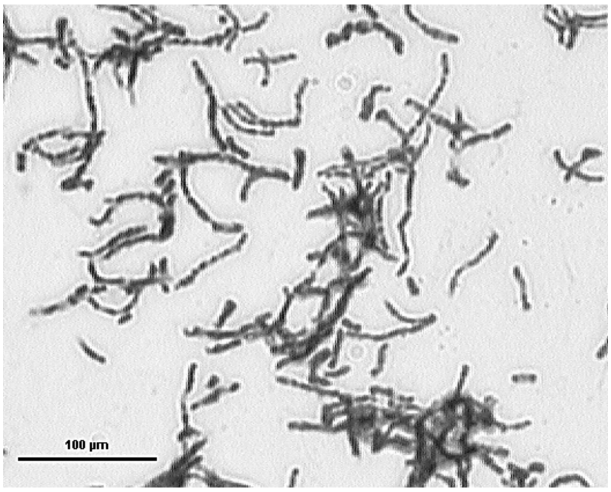

Photo 5. *Nocardia* sp. (J – 27) – Gram's stain bright field microscope.

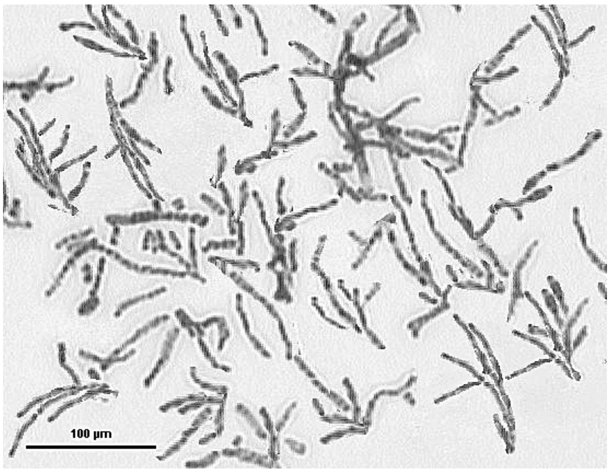

Photo 6. *Nocardia pinensis – like bacteria* – Gram's stain – bright field microscope.

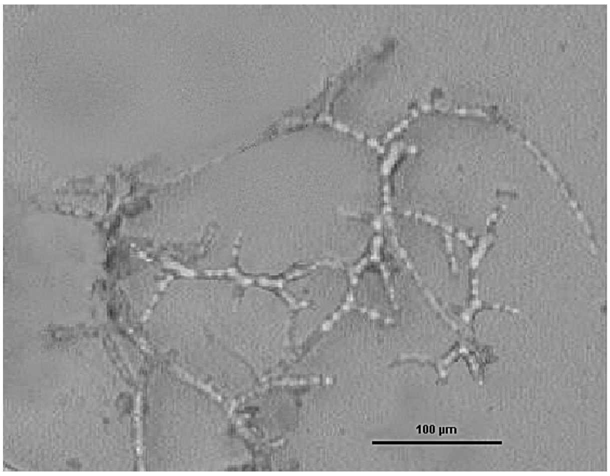

Photo 7. *Rhodococcus chubuensis* – Neisser's stain – contrast phase microscope.

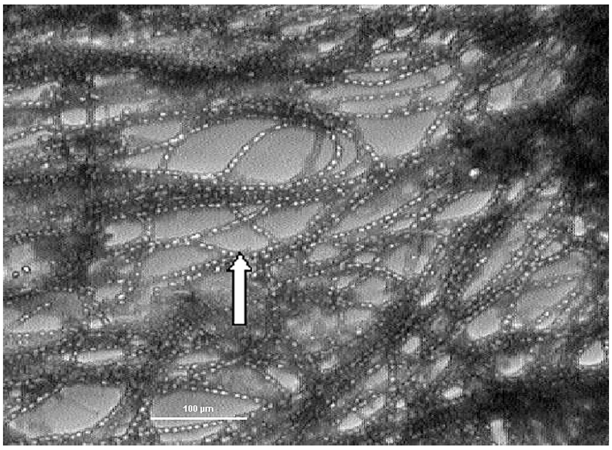

Photo 8. Polyphosphate granules in the filamentous microorganisms – contrast phase microscope (arrow indicate the volutin granules).

mentous microorganisms have a capacity of phosphorous uptake and accumulation. The determined ability to store phosphorus in volutin granules was supported with the results of microscopic observations of bacteria stained according to Neisser tests (Photo 8).

This observation suggested that phosphorus uptake was above the required for biomass synthesis, and a substantial part was stored within the microorganism cells as large polyphosphates polymers.

The microscopic observations of polyphosphate granule growth within the bacteria cells were confirmed with measured uptake of phosphorus from liquid by filamentous microorganisms (Fig.1).

The identified monoculture microorganisms removed different amounts of phosphates from wastewater of an initial concentration of 59.8 mg/Po_4^{3-}/dm^3. The decrease of phosphate concentrations in the liquid due to accumulation by monoculture microorganisms was shown in Fig.2.

The results show that *Microthrix parvicella* has the capacity of distinct phosphorus removal from wastewater (Fig. 2). The measured effect of phosphates removal from the liquid by *Microthrix parvicella* was similar to that by *Acinetobacter calcoaceticus.*

The results obtained in this study showed that uptake and accumulation of phosphates in aerobic conditions was in correspondence to the potassium and magnesium ions removed from the foam liquid (Figs.3, 4).

Monoculture microorganisms caused a decrease of concentration of K^+ and Mg^{2+} ions in relation to initial values which were: 35.6 mg K^+/dm^3 and 19.0 mg Mg^{2+}/dm^3 (Fig. 5).

To obtain more detail of the morphology of phosphorus-accumulating filamentous microorganisms using a scanning electron microscope equipment with an energy dispersive analysis of X–ray attachment (EDX) was used. These analyses, combined with electron diffraction studies, confirm that these deposits are polyphosphates. Quantitative analyses indicated that the inclusion could contain above 25% phosphorus, above

12% potassium, above 4% magnesium and above 9% calcium (Fig.6).

The ratio of elements located within the phosphorus–rich inclusion were determined for several samples with the SEM-EDX system, which indicated a K/P ratio of 0.48, Mg/P ratio 0.16 and Ca/P ratio 0.35. These ratios correspond accurately with the literature for microorganisms in wastewater [14, 31]. It was conformed that the effects of biological phosphorus uptake by filamentous bacteria also depend on the presence of potassium, magnesium and calcium ions.

Conclusions

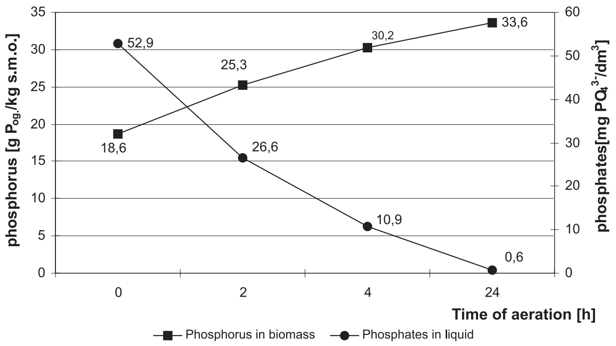

Fig. 1. Increase of stored phosphorous by polyculture filamentous microorganisms in parallel to phosphates decrease in liquor (under aerobic conditions).

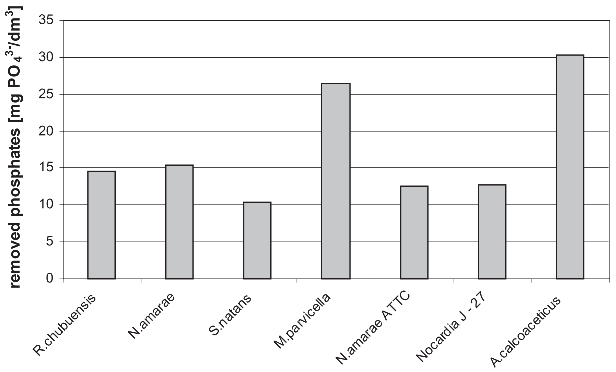

Fig. 2. Phosphates removed from waste water by the various species of filamentous microorganisms.

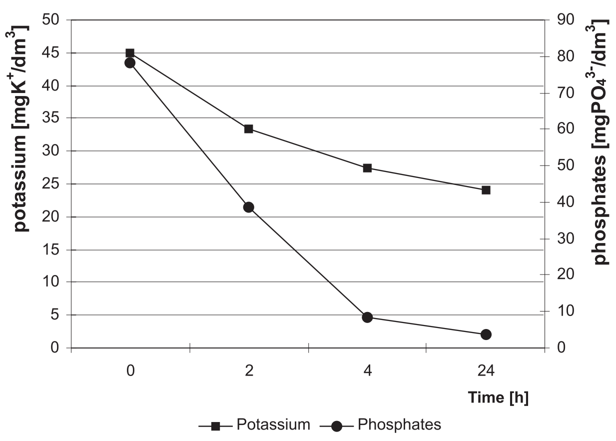

Fig. 3. Changes of phosphates and potassium concentration in foam liquid with time aeration, by polyculture of filamentous microorganisms.

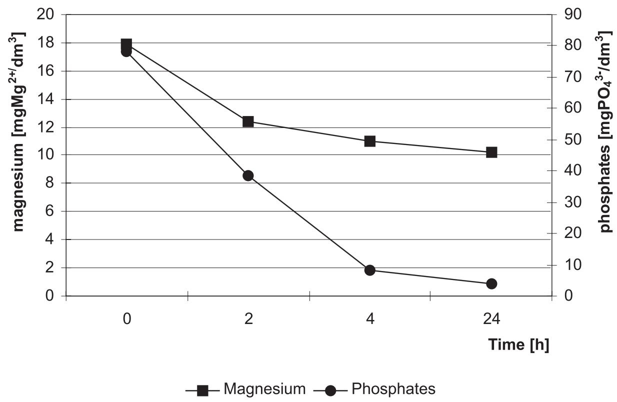

Fig. 4. Changes of phosphates and magnesium concentration in foam liquid with time aeration, by polyculture of filamentous microorganisms.

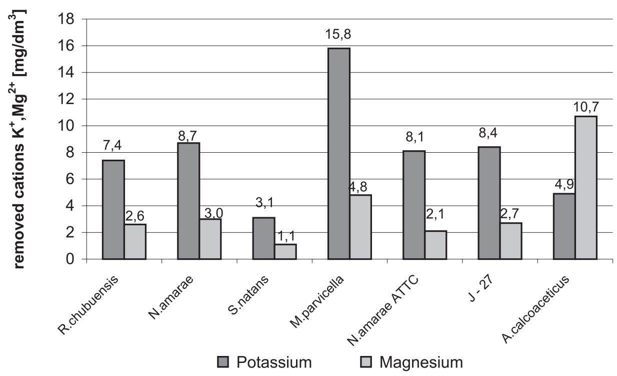

Fig. 5. Cations removed from wastewater by the various species of filamentous microorganisms.

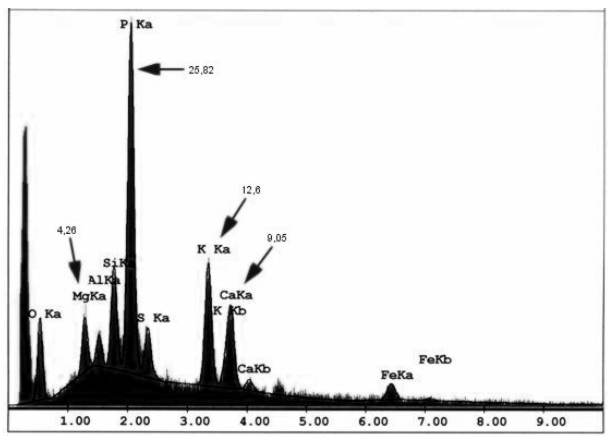

Fig. 6. Quantification (Diffraction pattern).

1. Microbiological analysis of the foam collected from several enhanced biological wastewater treatment plants in the Silesia region of Poland have shown that the dominantly present filamentous microorganism is *Microthrix parvicella.*
2. Filamentous bacteria dominantly present in the floating scum over the bioreactor liquid surface have a capacity for phosphorus uptake and accumulation.
3. Scanning electron microscope equipment with an energy dispersive analysis of X–ray attachment (EDX) conclusively confirmed that the granules were rich in phosphorus.
4. The effects of biological phosphorus removal depended on the presence of potassium and magnesium ions.
5. The clusters also contained significant amounts of potassium, magnesium and calcium. On the basis of SEM – EDX system analysis it was possible to determine that the volutin granules are potassium polyphosphate, magnesium polyphosphate or calcium polyphosphate.
6. The presented results of investigations have explicitly demonstrated that filamentous bacteria such as *Microthrix parvicella* have a significant potential to contribute to the process of phosphorus removal from wastewater.

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