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FILAMENTOUS MICROORGANISMS OCCURRING IN FOAM OF BIOLOGICAL WASTEWATER TREATMENT PLANTS AND POSSIBILITIES OF THEIR IDENTIFICATION

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MIKROORGANIZMY NITKOWATE WYSTĘPUJĄCE W PIANIE W BIOLOGICZNYCH OCZYSZCZALNIACH ŚCIEKÓW I MOŻLIWOŚCI ICH IDENTYFIKACJI

W wielu oczyszczalniach ścieków występują uciążliwości wynikające z tworzenia się piany na powierzchni ścieków w urządzeniach do ich biologicznego oczyszczania. Powstawanie piany jest związane z charakterystyką ścieków oraz stosowanymi parametrami procesu osadu czynnego, co sprzyja rozwojowi specyficznych mikroorganizmów nitkowatych, pianotwórczych. O ile w spuchniętych osadach czynnych można spotkać około 30 gatunków mikroorganizmów nitkowatych to w pianie nie więcej niż około 10 gatunków. Podstawową metodą wykorzystywaną do identyfikacji mikroorganizmów nitkowatych występujących w pianie są obserwacje mikroskopowe, które prowadzić można zarówno w preparatach bezpośrednich, nieutrwalonych i niebarwionych tzw. przyżyciowych z zastosowaniem kontrastu fazowego jak również preparatach utrwalonych po zastosowaniu różnych metod barwienia zarówno w technice jasnego jak i ciemnego pola. W krajowych oczyszczalniach ścieków najczęściej występuje kilka gatunków, takich jak: promieniowce nocardiopodobne, *Microthrix parvicella, Nostocoida limicola, Typ 021N/Thiothrix sp.*

Summary

In many wastewater treatment plants (WWTPs) inconveniences resulting from foam formation on the surface of activated sludge wastewater treatment devices appear. Foaming phenomenon is related to the characteristics of raw sewage and applied technological parameters of activated sludge process which promote the development of specific foam-forming filamentous microorganisms. In bulking activated sludges there are about 30 species of filamentous microorganisms and in the foam not more than about 10 species. Basic method of identification of filamentous microorganisms present in foam are microscopic investigations which can be performed both in vivo by direct observation of no stained, so called living smears, with contrast – phase device and in stained smears after different stain methods in direct light or dark field. In domestic WWTPs the following species commonly occur: *nocardioform actinomycetes, Microthrix parvicella, Nostocoida limicola, Type 021N/Thiothrix sp.*

FILAMENTOUS MICROORGANISMS IN FOAM AND AGENTS PROMOTING THEIR DEVELOPMENT

In many activated sludge plants especially these with nitrogen and phosphorous removal [1, 13, 19] difficulties and operational problems appear, such as foam formation, mostly in brown shades, so-called biological foam [8]. The foam appears on the surface of sewages in biological wastewater treatment devices, such as aeration tanks and secondary settling tanks [3, 6, 13]. Majority of authors are of the opinion that foaming phenomenon is of physico-chemical and biological character and consists of three phases: air – sewage – cells of foam-forming microorganisms. The occurrence in sewage of hydrophobic compounds and filamentous microorganisms, which contain in their cell walls hydrophobic matter, in presence of air bubbles, is responsible for the foaming phenomenon on sewage surface [7, 8, 10–12, 20]. Soddell and Seviour [15, 16] think that presences in foam of branched filaments of nocardioform actinomycetes which form characteristic net between flocs of activated sludge, so-called bridging, create possibilities of mechanical capture of air bubbles from sewage, what can simplify flotation of the whole flocs or their fragments. Together with flocs and air particles, hydrophobic substances such as lipids, oils and greases appear in treated sewages outflow up to the surface of sewage. Several authors suppose that certain filamentous microorganisms can synthesize and discharge outside hydrophobic metabolic substances, such as: lipids, lipopeptides, proteins and carbohydrates. These substances gathered on the surface of air bubbles can cause their joining together and absorption of some microorganism's cells. Therefore aggregates are created which simultaneously outflow up to the surface of sewage and form the foam. Additionally, the presence in sewage of surface active substances, create foaming conditions on sewage surface in wastewater biological devices. Out of 30 different filamentous microorganisms occurring in bulking activated sludges, only about 10 species and/or morphological types according to Eikelboom are capable to create foam [14, 19, 22]. Results of investigations conducted in different climatic zones indicate that the same or similar filamentous microorganisms occur in foams. From foam are mostly isolated: *Microthrix parvicella, nocardioform actinomycetes, Nostocoida limicola,* called as *N.* limicola like group or *N. limicola* like organisms), and Eikelbooms morphological types: 0041/9675, 1851, 0092 [14]. Results of investigations conducted in Poland on twelve activated sludge WWTPs [8] indicate that the *nocardioforms* were dominating group which occurred in 67% analyzed samples alone or with others species of filamentous microorganisms. Another group was *M. parvicella* which occurred in 57% investigated samples alone or with other following species*: N. limicola, Type 021N/ Thiothrix sp.* Numerous literature data concerning *M. parvicella* indicate the unusual function of these bacteria in foaming process and their physiological distinctness in the food chain of microorganisms occurring in activated sludge biocenosis. These results convinced some investigators to recognize these microorganisms as actinomycetes not generating branches (so called Candidatus *M. parvicella*) [9, 15, 16].

Referring to the investigation results of different authors, Kunst *et all.* [10] suggest to classify all filamentous microorganisms occurring in foam, on the basis of their metabolic processes and chemotaxonomic property of cell walls into 3 following groups:

- bacteria S (*Thiothrix sp., Beggiatoa sp., Type 021N, Type 0914*),
- Gram-negative bacteria (*Sphaerotilus natans, Haliscomenobacter hydrossis, Type 021N, Type 1863*),

– Gram-positive bacteria (*Microthrix parvicella, nocardioforms, Nocardia sp., Type 0092, Type 1851, Nostocoida limicola).*

However, from the point of view of the practical possibility of identification of filamentous microorganisms and frequency of their occurrence in foam, these same authors categorize them in 2 groups. The first includes dominant and most common filamentous microorganisms such as: *Microthrix parvicella, nocardioform actinomycetes, Type 021N, Thiothrix sp., Haliscomenobacter hydrossis, Sphaerotilus natans*. Whereas the second group incorporates rarely occurring microorganisms such as: *Type 0092, Type 0041/0675, Nostocoida limicola, Beggiatoa sp.*

Investigations of Davenport and Curtis [2] indicated that it is mainly microorganisms containing alpha branched, beta hydroxy fatty acids, so called mycolic acids in cell walls, that participate in foaming phenomenon. Therefore the above microorganisms including such compounds are defined as „mycolata". Mycolic acids contained in filamentous microorganisms such as *Nocardia, Rhodococcus and Gordona* are also present in rod and coccoid-shaped microorganisms such as *Caseobacter, Corynebacterium* and *Mycobacterium*. The investigation results indicate that among „mycolata" coccoidal and rod forms constitute the biggest part accounting about 80%.

In accordance with Kunst and co-workers [10] excessive growth of filamentous microorganisms in activated sludge devices and foaming events can be expected when:

– activated sludge loading is 0.05–0.15 kg BOD/kg d,
– recirculation of the foam removed from aeration tanks or secondary settling tanks to the wastewater treatment system is applied,
– lipids (mainly long chain fatty acids (LCFA)) are present in high concentration in sewages,
– dissolved oxygen concentration in aeration zone is below 2 mg/dm^3
– ammonia compounds are in high concentration,
– application of alternate oxic-anoxic conditions are applied with time contact of sewage in anoxic zone of above 1 hour.

According to Westlund *et al.* [21] even only one of the above factors may be the reason of exploitation difficulties as a result of the growth of filamentous microorganisms and consequent foam formation.

MICROSCOPIC EXAMINATIONS OF FILAMENTOUS MICROORGANISMS

The main morphological features useful and important in identification of filamentous microorganisms are:

– filaments shape and spatial structure,
– filaments width and length,
– presence or absence of a sheath or a slime coating,
– branching,
– motility.

Many authors provide other attributes. There are: intracellular granules, incisions, septa between cells, attached growth or other forms on surface of trichomes.

Basic method used in identification of filamentous microorganisms are microscopic examinations, which may be conducted both in vivo with contrast phase as well as after application of different stain methods in direct light or in dark field.

CONTRAST PHASE DEVICE APPLICATION

The contrast phase method is very useful in observations and investigations of all transparent microobjects loosely distributed in a thin liquid layer. Suitably diluted sample of foam fulfils those requirements. In the performance of microscopic analyses in contrast phase method very efficient illumination system is necessary as well as careful preparation of microscopic slides, basic and cover (clean, unfattened). All contaminations such as: pollens, dust, fats blots, blots due to inaccurate washing or drying of slides, intensively giving way and dispersing light have negative effect on quality and contrast.

The commonly used staining methods are: crystal violet stain, Gram stain and Neisser stain.

CRYSTAL VIOLET STAIN

Staining is carried out applying aqueous 0.2% solution of crystal violet in the following way. A drop of diluted foam sample is put on a microscopic slide and mixed with a drop of crystal violet solution. After covering of microscopic slide and removing excess of liquid, observations are be conducted in dark field or with assistance of contrast phase device at 200–400x magnification. Trichomes of filamentous microorganisms are visible brightly shining on the dark background. Activated sludges flocs and other organic or mineral matter concentrations occurring in the foam are also stained but are of different color. This staining can be used not only to detect the presence of sheath or other tubing forms on trichomes but it is also useful to evaluate the abundance category of filamentous microorganisms in foam.

GRAM STAIN

Thin smears from about 10-fold diluted foam sample, fixed by air drying at room temperature undergo staining. Smear fixing by heating or drying is not advised. Thin dry smear is covered with alcoholic solution of crystal violet and left for 90 sec. Afterwards the excess of stain is carefully removed by rinsing with small volume of water, then smear is covered with Lugol solution and left for 60 sec. After removal of the excess of stain and rinsing the smears with small volume of water, the slide is covered with 95% ethanol with 2% of acetone. Afterwards, by 20–30 sec of gentle movements unattached colorful complex is removed. This step of the method is extremely important and should be performed very precisely because extension of time can cause the complete decolorisation of the specimen. After rinsing of smear with large volume of water the microscopic slide is covered additionally with contrast stain safranine or fuchsine and left for 60 sec. After removal of stain and rinsing with water, the smear is then air dried at room temperature.

The Gram stain procedure is applied for chemotaxonomic determination of microorganisms on the basis of their cell walls relation to the used stains. In the result of above staining method Gram-positive bacteria are blue-violet, whereas Gram-negative are orange or red, depending on the contrast stain. The stained smears should be examined under direct illumination at 200–400x magnification or under oil immersion at 1000x magnification. Among filamentous microorganisms occurring in foam Gram-negative organisms are dominant. Only *Nocardia* and *nocardioforms* are always Gram-positive. Up

till now, it had been assumed that the second Gram-positive microorganism is *Microthrix parvicella*, however last investigations showed that it can be both Gram-positive and Gram-negative [5, 19].

NEISSER STAIN

Thin smears from about 10-fold diluted foam sample, fixed by air drying at room temperature undergo staining. Staining can be performed by using kit of reagents, available as commercial product, composed of 3 stains: Neissers IA and IB and II (chrysoidine). However, it is important that stains are always freshly prepared. Staining consists of two stages. First of all, immediately before staining dying mix is prepared including reagents IA and IB in 2:1 proportion. The smear on microscopic slide done from 10-fold diluted suspension of foam is covered with previously prepared dying mix and left for 10–15 sec. After removal of the excess of stain and precise rinsing with water, the microscopic slide is then covered with Neissers reagent II for about 45 sec. After removing stain and precise rinsing of smear with large volume of water, the smear is ready for examination.

The above method of staining is applied for identification of several microorganisms which can intensively accumulate granules of polyphosphates (volutine) in cells. These granules can be stained by anilic dye in acid environment. They do not get discolored after rinsing with water whereas protoplasm of cells of filamentous microorganisms gets discolored in these conditions. Three kinds of results of the Neisser stain can be observed two of which are positive. The whole trichome appears to be blue-violet or only blue-violet metachromatic polyphosphate granules inside the cells are visible after staining (for example *M. parvicella, Thiothrix sp., Beggiatoa sp., Nocardia sp.*, and several Types: *0042, 0675, 021N, 0914, 1863*). Without staining these granules is poorly visible, however after the test they are clearly noticeable from protoplasm. In the case when the result of staining is negative trichomes of filamentous microorganisms are light brown or yellowish. The microorganisms which after the test are blue-violet or grey-blue are considered as Neisser-positive, and light brown or yellowish are Neisser-negative. Examination should be conducted in direct light at 200–400x magnification or under oil immersion at 1000x magnification.

ABUNDANCE CATEGORY OF FILAMENTOUS MICROORGANISMS

In scientific literature different counting methods of abundance degree of filamentous microorganisms in the activated sludge were described [4, 6, 10, 19]. Recently, it concerns also the microorganisms present in foam. Older methods concerning mainly investigations of activated sludge are time- and labor consuming. The most simple and rapid method, modified from older methods [4, 6], has been elaborated by Kunst and co-workers [10]. In this a qualitative method the abundance of filamentous microorganisms is classified by the symbolic scale from (0–1) – none to (6–7.7) – excessive. Counting analysis of filamentous microorganisms can be performed both in living smears in contrast phase and after staining by crystal violet at 200-400x magnification.

PROPERTIES OF MOST COMMON FILAMENTOUS MICROORGANISMS IN FOAM

The most frequent filamentous microorganisms observed in foam of domestic WWTPs are: *nocardioform actinomycetes, Microthrix parvicella, Nostocoida limicola, Thiothrix sp., Type 021N.* Their morphological characteristic is given below.

Nocardioform actinomycetes

Term of *nocardioforms or nocardioform actinomycetes* is used to describe filamentous microorganisms which create irregular system of filaments (hyphae, trichomes) similar to mycelium strains of *Fungi (Molds*). Characteristic feature of their identification is the system of short threads of 10–30 μm length and about 1.0 μm in diameter, without sheaths possessing real branching septa between cells and incisions. *Nocardioform actinomycetes* do not accumulate in stock materials; however occasionally in cells protoplasm granules which stain in blue by Neissers method. They are Gram-positive and Neisser-negative.

Microthrix parvicella

Recognition of this filamentous microorganism is relatively easy on the basis of characteristic system of trichomes, called „coiled", composed of numerous loops strongly curved often hooked trichomes of about 0.6–0.8 μm of width. The characteristic features are numerous empty places in trichomes probably as a result of lisa of several cells. It is assumed that relation of *M. parvicella* to reagents used in Neisser stain is constant, Neisser-negative. During Gram stain *M. parvicella* is Gram-positive or Gram-negative. It depends on cells age because young cells which often occur one by one and mature cells which create well formed trichome are stained in different ways. Trichomes which are not branched have no sheaths. Septa between cells and incisions are difficult to observe. In terms of morphology properties of *M. parvicella* are very similar to the system of *Nostocoida limicola*. These bacteria can occur both as individual cells and as shorter, (50–100 μm) or longer (200 μm) trichomes. The length of trichomes of *M. parvicella* depends on season. During summer when foaming is poor, threads of *M. parvicella* are shortest, about 50–200 μm, while in winter and spring when foaming is more intense trichomes are significantly longer and reach even 500 μm.

Nostocoida limicola

The system of threads of this filamentous microorganism is very similar to *M. parvicella. Nostocoida limicola* can occur in three kinds of morphological types I, II and III and are insignificantly different mainly in trichomes diameters.

In type I cells are more or less spherical (oval) 0.6–0.8 μm diameter, without sheaths, without branching and without attached growths. Protoplasm does not contain granules of spare materials. Trichomes are Gram-positive and Neisser-positive and their length is predominantly about 100-200 μm.

In type II trichomes are thicker, the cell diameter is 0.8–1.4 μm and their length most frequently is 100–200 μm. Septa between cells are poorly visible; cells are spherical or have a disk shape.

In type III trichomes are long and coiled (200–300 μm), septa between cells are well visible. Cells have shape of disks of 1.5–2.0 μm diameter.

In practice of routine investigations we can assume that all types can occur in the

same foam and are very difficult to differentiate by conventional methods. In recent references filamentous microorganisms morphologically similar to *N. limicola* are colectively defined as „*N. limicola*-like organisms". Recognition of *N. limicola* from *M. parvicella* can be done by Neisser stain. Sometimes in smears stained by Neisser stain method we can observe brighter protoplasm in cells than septa between cells.

Thiothrix sp

As for as shape and length of trichome are concerned, two morphological types I and II are often distinguished in literature. They are characterized by simple or slightly curved, immovable trichomes. Besides they have sheaths and are without branching and have different lengths from 50 to 500 μm and are outstanding radiantly from the flocs. Cells have rectangular form of about 0.7–2.5 x 3–5 μm diameter and young cells are thinner than the older ones. The incisions are present but usually visible at the end of trichomes. These bacteria depose sulfur in the form of granules between protoplasm and cellular wall. In addition bacteria fabricate poly hydroxy butyrate acid (PHB) and polyphosphates as spare substances. *Thiothrix sp* is usually Gram-negative and Neisser-negative, however big gathering of granules of sulfur may give false positive result.

Type 021N

A big similarity in morphology of trichomes and their spatial structure and the same results of staining make it very difficult to distinguish trichomes *Type 021N* from *Thiothrix sp.* on the basis of microscopic observations. The presence of the sheath enabling the recognition of trichomes of *Thiothrix sp.* is very difficult to observe in microscopic examination. For the above-mentioned reasons in scientific literature we can find *Thiothrix sp./Type 021N*. Besides, both *Thiothrix sp.* and *Type 021N* create system of simple or slightly crooked trichomes about 100 to 500 μm long and 1–2 μm wide. Trichomes of *Type 021N* are usually longer than *Thiothrix sp.* Trichomes are Gram-negative and Neisser-negative. However, it happens that due to the particularly big gathering of sulfur in cells, some trichomes can be Gram-positive and Neisser-positive what makes their identification more difficult.

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