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Original Research

Amplified Ribosomal DNA Restriction Analysis (ARDRA) as a Screening Method for Normal and Bulking Activated Sludge Sample Differentiation

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Abstract

In the activated sludge process, the share of filamentous bacteria is crucial for proper settlement and high sewage treatment efficiency. Disequilibrium of the microbial community may be a reason for the appearance of bulking episodes. During 19 months (August 2007-February 2009) activated sludge samples were collected and analyzed by physicochemical, microscopic, and molecular methods. Amplified ribosomal DNA restriction analysis was chosen to detect molecular changes between normal and bulking sludge samples, which may be markers, informing about disadvantageous changes in the microbial population. Quantity and length of restriction products were the basis to create dendrograms representing the phylogenetic relationship of activated sludge samples. The most suitable restriction enzymes for finding molecular differences between normal and bulking sludge were *Msp*I and *Hha*I.

Keywords: activated sludge, bulking sludge, ARDRA, 16S rRNA, dendrograms

Introduction

Almost a hundred years after development, the activated sludge process is still one of the most popular and the widest used microbiological technology. The possibility to remove organic compounds, phosphorous, and nitrogen pollution from wastewater quickly and with high efficiency accounts for its utility. The activated sludge is a small but complex ecosystem, composed of bacteria, protozoa, fungi, algae, metazoa, and viruses. Each group of this organisms plays an important role in whole process, but bacteria are responsible for the chemical conversions [1-3]. They constitute about 95% of the microbial population and form flocs, whose structure and compaction determine treatment quality [1, 3-5]. The proper size and architecture of flocks are assured by filamentous bacteria. However, when they have been growing extensively, coming out of flocks and bridging between them (Fig. 1), problems with a good separation of activated sludge are observed, which may result in poor effluent quality [3, 5, 6]. Filamentous bacteria are not the major group in this microbial community, but changes in their growth influence the whole process intensively. The reason for bulking is still unknown. It is supposed that the substrate concentrations in sewage and micro-gradients of nutrients in flocks may play a key role [3, 6]. Bulking sludge is an unfavorable process, occurring

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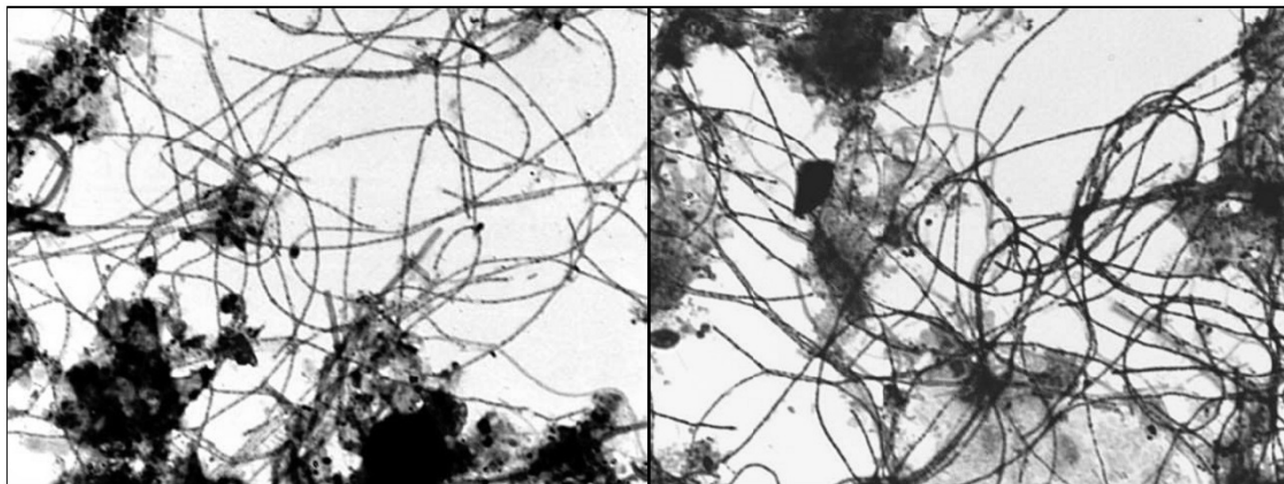

Fig. 1. Neisser and Gram stain of bulking activated sludge from WWTP "Wschód" in Łaziska Górne.

periodically in many wastewater treatment plants (WWTPs) in Poland and around the world [1, 3, 7-9]. More than 30 types of filamentous bacteria are considered to contribute to bulking sludge [1, 7, 8]. The main organisms causing this process can differ between influent type or location of wastewater treatment plants – climatic zone, country, and even region, but there are still a few characteristic bulking types, like *Microthrix parvicella*, 021N Type, or 0041 Type [1, 3, 7, 8, 10]. In Poland, *M. parvicella*, *Actiomycetes,* and 021N Type are observed most frequently [9].

The microscopic observation of filamentous bacteria with the use of an identification key developed by Eikelboom [1, 7, 8] allows us to determine their type based on morphological properties. However, even identical filaments can be phylogenetically distinct, or the same type of bacteria can change morphologically under different environmental conditions [3, 11]. A good solution for reliable bulking sludge studies seems to be molecular methods, based on DNA or RNA analysis [12]. They allow us to perceive slight molecular differences – not appearing morphologically – between bacteria and detect even low numbers of microorganisms in a sample without need for cultivation [2, 12, 13]. Using *in situ* techniques (for example FISH – fluorescent *in situ* hybridization) makes it possible to study a bacteria's morphology in their natural environment [5, 14].

The whole microbial community of activated sludge is often characterized on the basis of 16S rRNA gene analysis [2, 10-12, 15-21]. This gene, encoding rRNA, an element of a small subunit of ribosome (SSU), contains both highly variable and conserved fragments that enable us to analyze all organisms in the community as well as differentiate and classify them. Knowing each species occurring in biocenosis is not necessary to distinguish their characteristic attributes [12, 13, 16, 18]. One of these methods is the amplified ribosomal DNA restriction analysis (ARDRA). Although it gives no information about types of microorganisms in the sample, it allows us to assess genetic changes, quickly compare different communities, or study the influence of environmental conditions or chemicals on biocenosis richness [16, 18]. In our study we attempted to apply ARDRA to monitor activated sludge conditions in foaming and non-foaming phases of the operation of the wastewater treatment plant "Wschód" in Łaziska Górne, Poland. Normal and bulking sludge present differences in microbial community composition, but it is not clear which species and strains of filamentous are significant for the bulking episodes or whether their presence has an effect on the flocks-form microorganisms. Different product profiles, obtained from digestion by means of endonuclease, are characteristic for a microbial community under the given conditions. A genetic marker for the bulking sludge – that restricts the DNA fingerprinting pattern – results from ARDRA analysis and could be a useful tool for recognizing early signs of disadvantageous bulking.

Experimental Procedures

Sample Collection

From September 2007 to February 2009 an investigation of sewage and sewage sludge was carried out. A place for activated sludge sampling was the municipal wastewater treatment plant "Wschód" in Łaziska Górne, a twenty-thousandth town in southern Poland. Its effluent amounts to 2161±218 m^3 monthly.

The samples were collected 1-2 times per month. Sludge temperature and environmental conditions (air temperature and pressure) on sampling time were reported. Altogether 30 samples of activated sludge were collected.

Conventional monitoring of activated sludge and sewage, according to the Polish Standards for water and wastewater, was performed at WWTP "Wschód." For molecular analysis, the samples of activated sludge, filling 1/3 volume of the transport bottle (the rest filled with air), at ambient temperature, were immediately delivered to Sosnowiec to the Department of Biotechnology and Genetic Engineering Medical University of Silesia, Poland.

Physicochemical Analysis

After collection in the on-site laboratory, sludge samples were analyzed using conventional techniques as follows: sludge and air temperature, sludge volume index (SVI) [22], sedimentation time [23], and suspended matter concentration [24]. Biochemical oxygen demand (five-day) (BOD_5) [25], chemical oxygen demand (COD) [26], and total suspended solids [24] in influent and effluent were reported too. The effluent quality was proven by measurement nitrates [27], nitrites [28], ammonia [29], sulphur [30], and chloride [31] concentrations. Together with microscopic analysis, these results were grounds to divide division sludge samples into two groups: normal and bulking sludge.

Microscopic Analysis

Every microscopic preparation done from freshly collected activated sludge samples were observed in the on-site laboratory using a light microscope. The general number of organisms in activated sludge and occurrence frequency of filamentous bacteria were assayed according to a key described by Michałkiewicz [32]. Special attention was paid to estimate the number of filamentous bacteria.

DNA Isolation

The 45 ml of collected sludge sample were pelleted by centrifuge for 5 minutes at 10.000 x g and frozen at -80ºC untill further analysis. After thawing, 250 mg of wet weighed pellets were placed in an appropriate tube and resuspended with 625 μl of DNA extraction buffer (100 mM Tris-HCl pH 8.0, 100 mM sodium EDTA pH 8.0, 100 mM sodium phosphate pH 8.0, 1.5 M NaCl, 1% cetyl trimethylammonium bromide – CTMB). Genomic DNA isolation was performed using a previously described method [20]. The pellet was finally resuspended in 200 μl of TE (10:1) buffer, pH 8.5. The extracts were left overnight at 4ºC to dissolve. The quality and quantity of isolated DNA were determined specrophotometrically (BioPhotometer Eppendorf, Germany) by measuring the optical densities at 260 and 280 nm.

Amplification of 16S rRNA Genes

The 16S rRNA fragments from activated sludge were amplified by PCR with universal primers 27F (5'-AGA GTT TGA TCA TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT T-3') [2, 19], where Y means C or T. The primers were synthesized at the Institute of Biochemistry and Biophysics, Polish Academy of Science, Warsaw, Poland. Before adding to the reaction mixture, DNA extracts were diluted in bideionized water to the final concentration 15 ng/μl. PCR was carried out in 50 μl volumes containing: 10 x *Taq* Buffer, 125 mM of $MgCl_2$, 10 mM of each dNTPs, 5.0 μM of each primers, 1.5 units of *Taq* polymerase (Fermentas, Lithuania) and 30 ng of genomic DNA as a template. The PCR thermal profile used was an initial denaturation at 98ºC for 2 min, 30 cycles of denaturation at 93ºC for 1 min, annealing at 60ºC for 45 sec, and extension at 72ºC for 2 min. A final extension of PCR product was performed at 72ºC for 10 minutes. The amplification was conducted on a Mastercycler thermal cycler (Eppendorf, Germany). The presence and yield of specific PCR products of approximately 1.500 bp were controlled by electrophoresis on 1.2% agarose gel containing ethidium bromide (0.5 ng/μl). An aliquot of each reaction was collected and frozen at -20ºC for further analysis.

Restriction Analysis

Five endonucleases *Bsu*RI, *Hha*I, *Hinf*I, *Mbo*I, and *Msp*I (Fermentas, Lithuania) were chosen for further study of 16S rRNA amplimers. For each of them, the recognition nucleotide sequence of DNA is different. These were frequently used enzymes in restriction analysis, given the most significant differences between species [15, 17-19, 33]. Amplified DNA was digested for 8 h at 37ºC in thin-wall tubes. The volume of the reaction mixture amounted to 20 μl and it contained 10 μl of PCR product, 10 U of restriction enzyme and an appropriate buffer (10 x Tango or Red Buffer). Restriction was stopped by the 20-minutes thermal inactivation at 65ºC or 80ºC (60ºC for *Hha*I, *Mbo*I, *Msp*I and 80ºC for *Bsu*RI, *Hinf*I) according to the producer's recommendation. Restriction fragment patterns were analyzed by electrophoresis on 8% polyacrylamide gel stained with ethidium bromide solution (0.5 ng/μl).

Computer Analysis of Restriction Fragment Patterns

Gels were photographed under UV illumination and digitalized with a gel documentation system: LabWorks™ Version 4, Ultra-Violet Products, BioImaging Systems (Cambridge, UK). The band patterns on each sample and band size evaluation on each gel were estimated based on simultaneous pUC Mix Marker 8 (Fermentas, Lithuania) – DNA molecular weight standard – gel separation. Pairwise comparison of the band patterns and cluster analysis of average linkages of the ARDRA fingerprinting patterns was performed using Ward's method. The dissimilarity measure used in Ward's algorithm was the Euclidean distance.

Statistical analysis was conducted using STATISTICA v. 6.0 (StatSoft, Poland). In order to compare both groups of activated sludge (normal and bulking sludge), we used Student's t-distribution (normal distribution) and Mann-Whitney U test. $P \leq 0.05$ was considered statistically significant.

Results

Differences between Sludge Samples from the Wastewater Treatment Plant "Wschód"

The restriction-site frequency for each used enzyme was different, which took the effect of receiving sets of DNA fragments – restriction reaction products. Electrophoresis profiles for *Hha*I restriction (Fig. 2) are

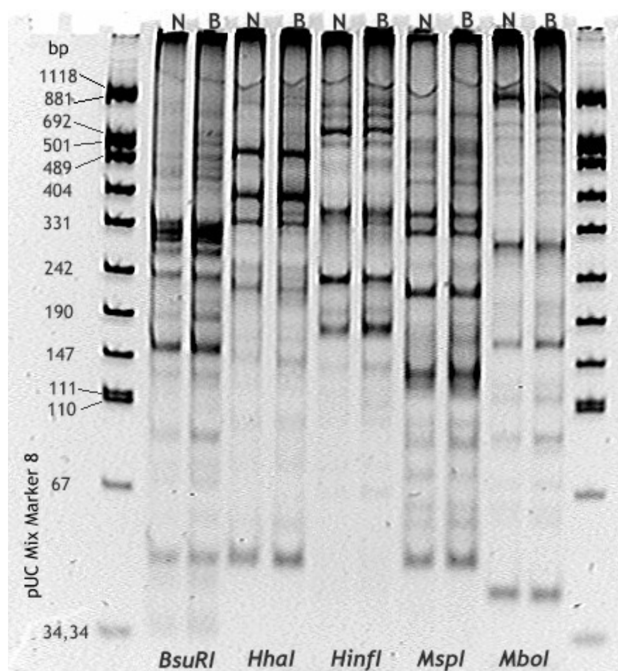

Fig. 2. DNA fingerprint patterns of activated sludge samples – normal "N" (6. 08. 2007) and bulking "B" (28. 01. 2008) sludge samples – after *Bsu*RI, *Hha*I, *Hinf*I, *Msp*I, and *Mbo*I restriction analysis.

composed of at least 20 bands ranging from 51 to 1.138 bp, and there are some characteristic only for bulking sludge samples. Almost all samples from September 2007 to February 2008 (classified as the bulking sludge samples, based on microscopic and physicochemical analysis) show restriction hydrolysis product – weakly visible on gel band – sized as ~820 bp length DNA fragment. A fragment ~260 bp length is present in these sample profiles, but it appears also for sludge samples from September to December 2008, primarily recognized as normal sludge. This may suggest that, although the results for microscopic and physicochemical analysis are regular, the imperceptible microbial community changes occur and may further lead to the bulking episodes. It also may implicate that the composition of sludge biocenosis has changed seasonally, but not every change has to be disadvantageous for treatment efficiency. This problem needs closer examination.

A similar situation relates to *Msp*I restriction, where profiles of samples from September to December 2007 and 2008 have common bands, not found in the rest samples. The tiny but noticeable bands correspond with the restriction products of about 290 and 600 bp. Two restriction profile characteristics for normal (6. 08. 2007) and bulking (28. 01. 2008) sludge samples are present in Fig. 2. The lines of their densitogram show maxima by different lengths of DNA fragments. Some bands occur in all restriction profiles for bulking sludges from August 2007 to February 2008, but not for samples from August 2008 to February 2009. It may implicate, that in spite of the same season, the microbial richness varies, dependent on year.

There are no significant differences between *Hinf*I restriction patterns for normal and bulking sludge (Fig. 2) from the wastewater treatment plant "Wschód". The plurality of bands was conspicuous, but finding even one characteristic for all bulking or normal samples was impossible.

The restriction with a use of *Bsu*RI has no significant discriminatory effects. Lane profiles are similar, the bands are common for all sludges (Fig. 2). Only one band distinguishes the samples. Hardly visible one – 810 bp length – occur for some bulking sludges. However, it is not specific enough to be a genetic marker for bulking activated sludge.

The *Mbo*I enzyme does not allow us to differentiate the normal and bulking sludge, too. Quantity and length of digestion products are comparable (Fig. 2). For some samples, additional (weakly intensive) products are observed, but it may be caused by diverse PCR efficiency. These bands are present only in lanes where all bands show high luminescence, much higher than in other lanes.

Dendrograms

Numbers and position of bands obtained from 16S rDNA restriction analysis of each activated sludge sample were the basis for creating dendrograms. Intracluster similarities and distances between isolates depict their genetic relationships.

The *Bsu*RI restriction product profiles were clustered into three major groups: A, B, and C (Fig. 3). Group C includes all typically bulking samples (from December 2007 to February 2008), drawing slight distinctions. The remaining samples of bulking sludge (from August to September 2007) were classified in group A together with samples from April to the begining of June 2008. The distance between bulking sludge samples from different seasons is long. The third group (B) clusters sludges in good condition – from June to December 2008. All fingerprints show demonstrate high intracluster similarities.

A deep-branching dendrogram for *Hha*I restriction was obtained (Fig. 3). Almost all normal sludge samples are grouped in cluster B together, only four samples (one from September 2007, April, October, and December 2008) were affiliated to the "bulking" group A, although other samples from these months are classified to the "normal" (B). High similarity demonstrate August and September samples – both normal and bulking sludge as opposed to February (2008) samples, belonging to different clusters.

Two (A and B) major branches of dendrogram from *Hinf*I restriction reaction product profiles were obtained. The distance between them is relatively long as opposed to intracluster distances. Cluster B groups the most normal activated sludge samples and one bulking sample as well. Within cluster A almost all bulking sludge and a few normal sludge samples are gathered and its inner branch groups only bulking sludge samples. The division into two groups (bulking and normal sludge samples) is noticeable, with not every sample joined to the "bulking" cluster (A) being collected from bulking activated sludge. Only restriction analysis by means of *Hinf*I endonuclease lets us demonstrate differences between two samples from

December 2008 – 4.12. and 11.12. (restriction analyses with remaining enzymes classify these samples as nearly identical).

The dendrogram created on the basis of *Mbo*I restriction reaction products did not join the normal or bulking sludge samples together. Even samples from one month – February 2008 (typically bulking sludge) were affiliated to every one of three groups – A, B, and C. Some samples from the same month or season are assembled to the smaller sub-groups, but more distinct clustering is not noticeable.

High distances are characteristic for *Msp*I restriction reaction products dendrogram (Fig. 3). Group A includes bulking samples (August 2007, January-February 2008) together with April or May (2008) samples, primarily recognized as normal sludge. Remaining bulking samples belong to a distant cluster C, also with summer samples (2008). The inner branch of the *Msp*I dendrogram (cluster B) groups normal sludge samples from April to December 2008. Two samples collected the same day – 17. 02. 2009 belong to different clusters. The activated sludge sample is joined to the “bulking” group (C), whereas the foam sample to the “normal” (B).

Physicochemical and Microscopic Analysis

During two years of activated sludge sample collection, heightened results of Sludge Volume Index were observed permanently. The average value of SVI was 178 ml/g, which indicates – according to Eikelboom [1] – the bulking activated sludge. Notwithstanding, problems with flocs sedimentation from April 2008 to February 2009 were seldom reported. This observation has been supported by microscopic analysis. In a given period, the number of filamentous bacteria was also higher but still regular (data not shown). The increase of SVI value entails decreasing efficiency of sewage treatment, expressed by percentage BOD_5 reduction (Fig. 4a). Even with SVI value higher than 100 or even 150 ml/g (from May to November 2008), activated sludge is still capable of efficient organics removal (~95% BOD_5 reduction). A significant decrease of wastewater treatment quality (observed from December 2007 to the begining of April 2008) may be caused by excessive growth of filamentous bacteria.

Similar dependence refers to COD (chemical oxygen demand) values (Fig. 4b) and amount of total suspended solids (Fig. 4c). In the time of filamentous bacteria exces-

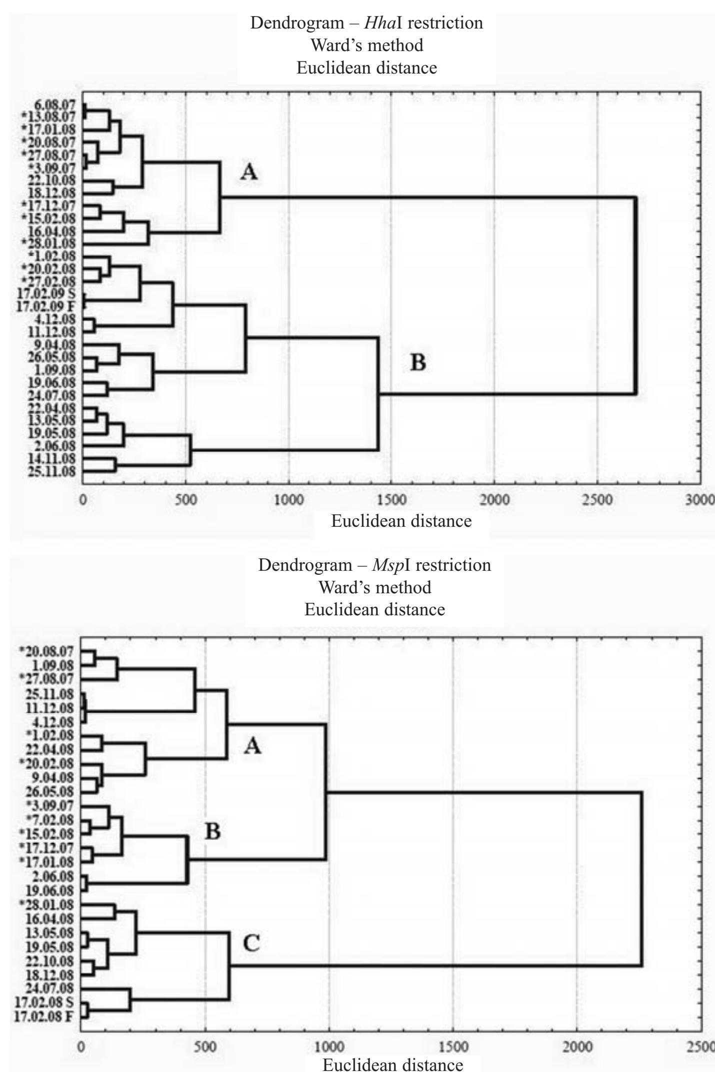

Fig. 3. The relationship of activated sludge community determined by restriction analysis with use of *Hha*I and *Msp*I. Dates with a mark “*” stand for the bulking sludge samples.

sive growth (November 2007 – February 2008) decrease in treatment efficiency is observed, but high SVI in March and April 2008 do not have a bad effect on this process.

Statistical Analysis

The sewage treatment process results in loss of organic mass and change of pollution indicators. These conversions decide about activated sludge properties and purification quality. Determination of correlation between each parameter may be the basis for evaluation of activated sludge process efficiency. Values of SVI, BOD_5, COD, and TSS for bulking and normal sludge were compared. No statistically significant differences were found. Negative correlation between SVI values and proportional COD reduction were noted (r=-0.4853; p=0.041) (Fig. 5). Worse COD reduction during activated sludge process accompanied to higher values of SVI. Similar correlation was not observed in the case of BOD and TSS parameters.

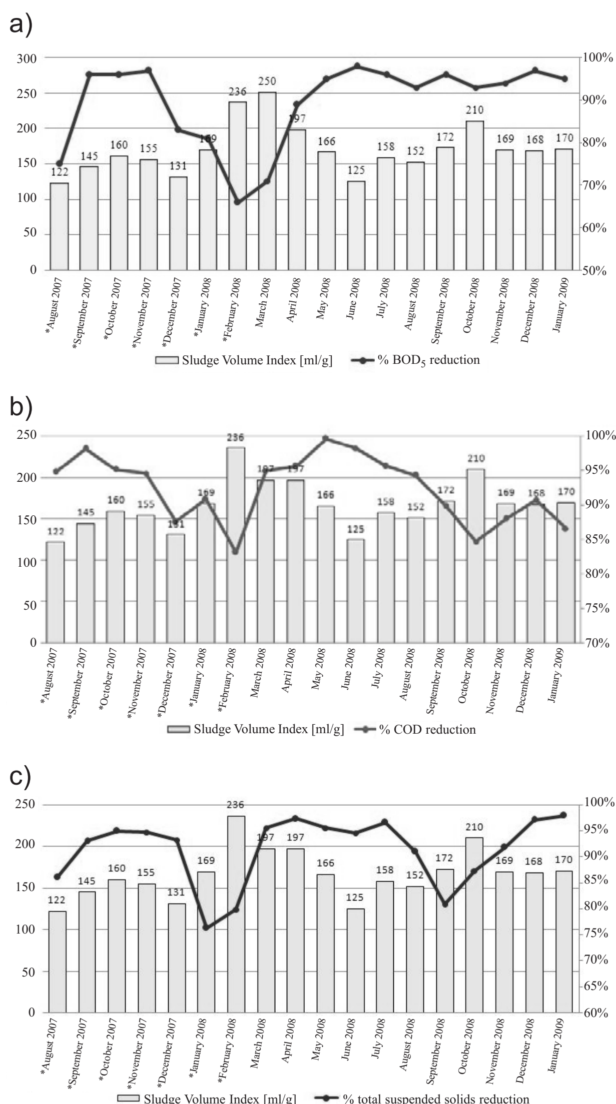

Fig. 4. The dependence of SVI values and treatment efficiency, expressed by percentage BOD_5 (a), COD (b), and total suspended solids (c) reduction.
Months with a mark "*" stand for the bulking sludge samples.

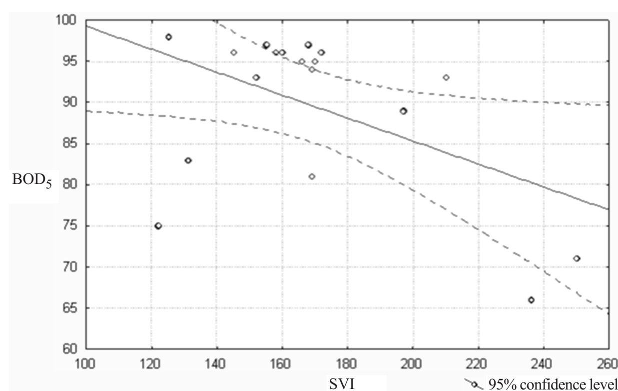

Fig. 5. Diagram of scatter SVI vs. COD values (r=-0.4853; p=0.041).

Discussion of Results

Amplified rRNA restriction and confirmation analysis is a useful method for confronting isolates undergoing condition changes or samples from different environments [12, 18, 34]. The use of five restriction enzymes has caused obtainment of different restriction product profiles for each of them. Some, like for *Mbo*I or *Bsu*RI digestions, were nearly identical for all samples – both normal and bulking sludges. It does not mean, though, that this method is useless. The most important is to choose an enzyme, proper for differentiating the biocenosis on a required level – strain, species or group of microorganisms. The *Mbo*I enzyme is more suitable for differentiation within the species [15]. The richness of sludge species may be too high to notice the differences between each strain. The absence of differences between sludge patterns does not ensure that the composition of sludge biocenosis is exactly the same. Slight but visible differences were observed after *Hha*I or *Msp*I restriction.

The common bands (290 and 600 bp) obtained after *Msp*I restriction for all autumnal samples (but not for the other samples) suggest the occurrence of similar microbial composition and may be characteristic for samples from this season. But a fragment closely related to bulking was 440 bp long. It could be an indicator for bulking sludge symptoms. Further study may be focused on its nucleotide sequence, to create a probe for hybridization. This could be used as a widely available test for bulking detection.

Variations in the number and length of restriction fragments for each enzyme implicate that the microbial community of activated sludge is still in transformation. Species compete with each other for nutrients and influence [1, 3]. The environmental conditions like temperature, humidity, or substrate concentration in sewage play a key role in these actions. The dependence on SVI and BOD_5, COD or TSS values was observed, but only for SVI vs. COD was the correlation of statistical significance found. The composition of sludge biocenosis changes seasonally, but most distinctly at the turn of winter and spring, summer and autumn, which may favour bulking episodes [3, 9]. This theory is

supported by our results – restriction profiles show the biggest differences between samples from January/February and April/May. However, not all changes mean undesirable consequences. *Hha*I restriction has classified all samples from August to February from 2007/08 and 2008/09 as similar (but different from samples March-July), although after *Msp*I digestion they showed differences. From April 2008 to February 2009 bulking episodes were not observed. These changes were not dissimilar to the "bulking" changes, but kept the sludge in good condition and did not make effluent quality worse. Similar profiles obtained for all samples after restriction by each endonuclease may be evidence of high similarity of the community composition for normal and bulking sludge. It was even suggested that the participation of filamentous bacteria in bulking sludge biocenosis is too little for detection by denaturing gradient gel electrophoresis [3]. Since there are many reasons for bulking occurrence [4], the aim of this study was to find molecular differences between normal and bulking activated sludge, not necessarily related to filamentous bacteria. The development and use of modern technologies that use PCR amplification or DNA microarrays has recently introduced the possibility of fast examination and screening of activities as well as the composition of microbial communities. ARDRA, as a simple method based on restriction endonuclease digestion of the amplified fragment of bacterial 16S rDNA, gives only a little information about the type of microorganisms present in the sample, but allows to search for changes in the community over time, or even to compare those changes to current environmental conditions [18].

Dendrograms obtained in the base of the number and length of restriction reaction products show considerable differences for each of five used endonucleases. Some – like for *Mbo*I or *Msp*I restriction – group together both normal and bulking activated sludge samples and do not distinguish them. In another dendrogram – after *Bsu*RI restriction – normal and bulking sludge samples are joined to separate clusters, but surprisingly the two "bulking" groups show high distances and simultaneous low intracluster differences. It may suggest that the microbial community of normal or bulking sludge is similar under given conditions (for example during the month or season), but within a longer period of time undergoes changes, whereby undesirable episodes (like bulking) may occur. Creating dendrograms also lets us apprehend differences imperceptible on restriction product profiles. Though just-noticeable differences in the number and length of *Hha*I digestion products, samples of normal and bulking activated sludge are joined together in separate clusters.

The ARDRA method is able to detect differences between sludge samples. However, ARDRA patterns are revealing dominant genera and are not always capable of detecting existing community changes, which may be crucial for keeping the balance of microbial populations [35]. Two enzymes – *Msp*I and *Hha*I were the most suitable tools for in situ differentiation of normal and bulking sludge, clustering and indicating their genetic correlations. A way to increase the resolution of ARDRA is using the six-base cutting enzyme, which may lead to a decrease in the number of restriction products [36]. According to Gich [18], simultaneous application of double restriction endonuclease is also more effective and allows the discovery of important community changes. *Hha*I was also chosen as the most efficacious restriction enzyme at detecting and differentiating 16S rRNA genes [19, 33]. It is recommended for screening of microbial diversity [33]. Together with *Msp*I, *Hha*I restriction could lead to finding more genetic markers of bulking sludge or follow genetic changes of microbial biocenosis in response to environmental conditions.

Conclusions

The study of composition and changes of microbial communities from activated sludge is essential to understand and assure the proper working of treatment systems. The ARDRA-based approach with cluster analysis of DNA fingerprinting patterns applied in our study has been demonstrated to be effective in possessing new data concerning the activated sludge dynamics regarding the bulking process.

- The activated sludge bulking process impacts negatively on treatment efficiency.
- Biocenosis of activated sludge shows seasonal changes (expressed by different DNA fingerprints after restriction analysis).
- Not all changes in the biocenosis are related to the bulking process.
- *Hha*I and *Msp*I are the most suitable enzymes for restriction analysis of genomic DNA from activated sludge and finding the molecular marker of bulking symptoms.

Although limitations exist, like difficulty on band visualization, the probability of the incidence of artifact bands due to inhibition of endonuclease hydrolysis by PCR reaction components, or inhibition of PCR due to the presence of trace pollutants in DNA extracts, should all be taken into consideration. Nevertheless, a genetic marker for the bulking sludge – restriction DNA fingerprinting pattern- result of ARDRA analysis, emphasizes the ability of molecular activated sludge monitoring and could be a useful tool for recognizing early signs of the bulking process.

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