Evaluation of the accuracy of two simple methods for microscopic activated sludge analysis

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ABSTRACT

Biological microscopic analysis is a popular method employed in wastewater treatment plants worldwide for evaluating activated sludge condition. However, many operators still have reservations regarding its reliability. In this study, we evaluated and compared two methods of microscopic sludge investigation: the sludge index (SI) and the Eikelboom–van Buijsen method (EB). We investigated 79 activated sludge samples from nine treatment plants located in southern Poland over a 1-year period. For each sample, sludge volume index values were calculated and compared with the results of evaluation made on the basis of microscopic analysis. Additionally, the effluent quality was analysed in 45 of 79 cases, including investigation of suspended solids, biochemical oxygen demand, chemical oxygen demand, total nitrogen and total phosphorous. The sign test and Wilcoxon matched pairs test showed that a significant difference existed between the two investigated methods. General conclusions from both methods do not provide reliable information concerning nitrogen and phosphorus removal. The EB method had a tendency to be more conservative in its general conclusions than the SI method. Both are highly reliable for estimating activated sludge quality and solid separation properties.

Key words | activated sludge, Eikelboom and van Buijsen method, microscopic analysis, sludge index

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INTRODUCTION

Most biological wastewater treatment plants (WWTPs) face problems associated with the foaming and bulking of activated sludge when the balance between microorganisms in the sludge is disturbed. Microscopic observations are helpful for defining causes of the sedimentation problems (e.g. overgrowth of filamentous bacteria, dispersed flocs, turbid effluent) but also provide information about sludge age and toxic substances that can flow into the plant. Thus, microscopic sludge investigation is an important part of controlling the activated sludge process. However, if methods regarding sludge investigation are to be helpful to plant operators, the method must be quick, simple and cheap and should provide additional information that is complementary to the results of chemical analyses.

A manual for general microscopic sludge investigation was first proposed by Eikelboom and van Buijsen in 1981, but the method (EB) was actually developed around 1975. The technique described in that manual (as well as its sequel, published in 2000) has become a standard procedure in biological treatment plants around the world. Similar methods were also developed in other countries. The German Bayerisches Landesamt für Wasserwirtschaft (Bavarian State for Water Management) developed and described a method (Großmann *et al.* 1999) widely used in Germany. In the United States the first edition of a manual by Jenkins, Richard and Daigger was published in the early 1990s, and later on the third edition was published in 2003 (Jenkins *et al.* 2003). Another method of microscopic sludge investigation, called the sludge index (SI), was developed and proposed by Grupo Bioindicacion Sevilla in 2001, and it is popular both within and beyond Spain.

The primary advantage of the aforementioned methods is that they are simple and do not require species determination

doi: 10.2166/wst.2018.487

or time-consuming counting of protozoans, which is required for the sludge biotic index (SBI) (Madoni 1994).

In the SI method, an investigator assigns points (0–100) for macroscopic and microscopic features. The characteristics that are used in this method are presented in Table 1. Floc size is estimated on the basis of direct measurement of at least 15 randomly selected flocs (Rodriguez *et al.* 2004; Arregui *et al.* 2012). The SI method expresses sludge quality on a five-level scale (Table 1).

In the EB method, an investigator estimates the number of different microorganisms within a two-scale range. For filamentous bacteria, a scale called the Filament Index (FI) has values between 0 (absent) and 5 (numerous filaments). Scores are assigned on the basis of reference photographs. For other microorganisms such as *Zoogloea*, polyphosphate-accumulating organisms (PAOs), spirils, spirochaetes, protozoans and metazoans, the points are assigned on a scale of 0 to 3 (indicating from zero to numerous cell/colonies per slide). The same scale (0–3) is used for free-living bacteria cells, but in this case, an index of 3 indicates hundreds of cells within a field of view. Estimated values for all microorganisms are then integrated to draw a final conclusion. The final results of the EB method are used to assess sludge quality, which is expressed as 'poor', 'moderate' or 'good'. Criteria for establishing the sludge quality on the basis of the EB method are shown in Table 2.

The goal of this study was to evaluate and compare two methods of microscopic sludge investigation, the EB method and the SI method. These two methods were chosen because they are currently the most popular microscopic methods used in Europe. Additionally, the method that was developed in the United States by Jenkins and coworkers is similar to the EB method. The comparison of the two methods enables determination of their compatibility and stresses the strengths and weaknesses of each method.

MATERIAL AND METHODS

We investigated 79 activated sludge samples over a 1-year period between April 2015 and April 2016. Sludge samples were obtained from nine wastewater treatment plants located in Małopolska voivodship (southern Poland). Treatment plants varied in their population equivalent (PE) (765 to 780,000) and capacity of sewage flow (93 to 165,000 m^3/day), as well as their technological design: O (only aeration tank), A/O (anaerobic/aerobic), A2/O (anaerobic/anoxic/aerobic), and three-stage Bardenpho with predenitrification. General information for each treatment plant is presented in

Table 1 | Macro- and microscopical parameters and numerical values considered to determine sludge index (SI) (according to Arregui *et al.* (2012))

Macroscopical characteristics		
Turbidity	High	0
	Medium	4.5
	Low	9
Suspended floc	High	0
	Medium	4.5
	Low	9
Decantability	High: V30 pouring of within the first 10 min	9
	Medium: V30 pouring of between 10 and 20 min	4.5
	Low: V30 pouring of after 20 min	0
Odour	Correct	3
	Incorrect	0
Microscopical characteristics		
Flocs		
Morphology	Regular	4
	Irregular	0
Size	Large >500 μm	4
	Medium 150–500 μm	7
	Small <150 μm	0
Structure	Compact	18
	Intermediate	9
	Open	0
Texture	Strong	4
	Fragile	0
Cover	<10%	0
	10–50%	7
	>50%	3.5
Filamentous bacteria within floc	>20 filaments per floc	0
	5–20 filaments per floc	7
	<5 filaments per floc	14
Suspended filamentous bacteria	High	0
	Low	3
Protists diversity	>7 species	13
	4–7 species	7
	<4 species	0
Range of total point values in SI methods and their sludge quality equivalents		
Total points	**Sludge quality**	
0–19	very bad	
20–39	bad	
40–79	medium	
80–89	good	
90–100	excellent	

Table 2 | Criteria for establishing the sludge quality (according to Eikelboom (2000))

	Good	Moderate	Poor
Filament Index	<3	3 to 4	4 to 5
Free-living cells	0 to 1	2 to 3	≥3
Spirils	0	1	≥2
Ciliates/Testate amoeba	≥1	<1	0
Flagellates/Amoeba	0	1 to 2	≥3
% flocs >25 μm	>80 to 90	>50 to 70	<50
Floc structure	compact	open	–
Floc strength	robust	weak	–
Floc shape	rounded	irregular	–
Floc size			
Small	<25 μm		
Medium	25–250 μm		
Large	>250 μm		

Tables 3 and 4. For each sample, the SI was calculated according to Rodriguez et al. (2004) with some modifications as proposed by Arregui et al. (2012), and sludge quality was estimated according to specifications in Eikelboom (2000).

Each sludge sample was investigated immediately following its delivery to the laboratory. Microscopic investigation was performed with Olympus IX 71 and Nikon Eclipse 80i microscopes with 10× and 20× objectives. For each method, at least two homogeneous subsamples (25 μL) of activated sludge were analysed. Additionally, for the EB method, Gram and Neisser staining of the sludge smear was completed according to instructions in the Eikelboom manual (2000). Gram and Neisser staining are essential for determining types of filamentous bacteria, but Neisser staining is also helpful for estimating the density of PAOs (Čech et al. 1994).

For each of 79 samples sludge volume index (SVI) after 30 minutes of settling and mixed liquor suspended solids

Table 3 | Main characteristics of investigated WWTPs

WWTP code	Technology	PE	Average sewage flow [m^3/day]	Aeration tank volume [m^3]	% industrial waste	N and P elimination
Pl	Bardenpho	780,000	165,000	5 × 27,000	10	Yes
Sk	A2/O	100,569	6,390	2 × 6000	30	Yes
Np	A2/O	38,475	5,270	2 × 4320	35–40	Yes
Bl	A2/O	4,700	730	2 × 209	0	No
Ng	A2/O	4,133	550	209	0	No
Rd	A/O	765	93	123.6	0	No
Zl	O	1,075	95	84	0	No
Ch	A2/O	51,286	15,140	2 × 2849	15–20	Yes
Si	A2/O	7,462	925	1940	0	No

Table 4 | Effluent and operational parameters (mean ± standard deviation)

WWTP code	SS [mg/L]	BOD_5 [mg/L]	COD [mg/L]	N_{total} [mg/L]	P_{total} [mg/L]	MLSS [g/L]	SVI [mL/g]
Pł	–	–	20.3 ± 2.1	8.6 ± 3.7	0.2 ± 0.2	4.8 ± 0.4	183 ± 13
Sk	5.5 ± 2.0	4.7 ± 1.2	49.8 ± 9.3	11.4 ± 2.3	0.4 ± 0.4	5.6 ± 1.3	130 ± 16
Np	11.8 ± 9.4	8.2 ± 8.1	39.7 ± 15.3	17.0 ± 12.8	2.2 ± 4.4	4.5 ± 0.7	107 ± 29
Bl	9.0 ± 4.8	9.8 ± 3.9	47.4 ± 10.6	41.6 ± 18.1	6.3 ± 1.2	2.9 ± 0.6	222 ± 61
Ng	17.7 ± 6.5	15.6 ± 4.1	60.7 ± 7.2	51.0 ± 10.8	3.0 ± 1.0	5.9 ± 1.7	141 ± 31
Rd	4.9 ± 0.8	5.6 ± 3.0	26.4 ± 4.9	25.3 ± 7.3	3.9 ± 1.2	3.4 ± 1.4	234 ± 147
Zl	9.1 ± 7.5	4.9 ± 2.1	43.3 ± 22.6	61.2 ± 15.4	9.7 ± 6.8	8.9 ± 3.1	95 ± 42
Ch	21.5 ± 17.0	8.7 ± 6.0	30.7 ± 5.0	8.3 ± 2.1	0.5 ± 0.2	4.7 ± 0.6	213 ± 26
Si	2.3 ± 0.3	3.7 ± 1.2	26.7 ± 5.1	18.3 ± 2.5	6.0 ± 0.5	4.0 ± 0.1	211 ± 20

(MLSS) were determined. Then SVI values were calculated and compared with conclusions drawn from microscopic analysis. Additionally, in 45 out of the 79 cases, the effluent quality was analysed in context of the obtained results. The concentration of suspended solids (SS), biochemical oxygen demand (BOD_5), chemical oxygen demand (COD), total nitrogen (N_{tot}), and total phosphorus (P_{tot}) concentrations in the effluent were measured according to APHA (2005) methods (Table 4). Effluent samples from WWTPs Pł, Sk, Np, Ch and Si were analysed in treatment plant laboratories, whereas WWTPs Bl, Ng, Zl and Rd were analysed in the university laboratory.

Evaluation of microscopic analysis methods

Because the values for floc diameter categorized as small, medium and large differ significantly in the two methods (Tables 1 and 2), we used floc classes from the SI method because they are also consistent with the classes proposed by Jenkins *et al.* (2003).

To unify the number of assessment levels in the EB and SI methods, we reduced results from the SI method to three levels. Specifically, we integrated the levels of 'very bad' with 'bad', and 'good' with 'excellent' (i.e. we extended the point range for 'bad sludge' to 0–39 and the point range for 'good sludge' to 80–100). In this way we obtained unified categories for both methods: 'bad', 'medium' and 'good' which were then compared with the help of a non-parametric sign test and Wilcoxon matched pairs test.

For the SI method, we used non-parametric Spearman rank order correlation method and parametric general regression model (GRM) to determine the relationship between: (i) total points and SVI, (ii) floc points and SVI, and (iii) total points and SS, BOD_5 and COD.

For 'floc points' we determined subgroups extracted from the microscopic characteristics related to sludge floc attributes: morphology, size, structure, texture, cover, suspended filamentous bacteria and filamentous bacteria within the floc. For analysis, we log-transformed our dependent variable (SVI) to meet the assumption for the analyses of a normal distribution of residuals.

For the EB method, we correlated the FI values with SVI values. In order to estimate the content of PAOs, from each well-mixed sample we transferred 50 μL of the sludge onto a microscopic slide, prepared a smear, and then stained them with the Neisser method. Then, the abundance of PAOs colonies was estimated according to the EB method. We analysed the relationship between the estimated content (index) of PAOs in sludge and the level of total phosphorus reduction. For analysis in the GRM, we only used samples from treatment plants that did not use chemicals for phosphorus precipitation ($N = 26$).

All statistical analyses were performed using the Statistica 13 package (TIBCO Software Inc. 2017).

RESULTS

The sign test and Wilcoxon matched pairs test showed that there was a significant difference in the results obtained from the two methods ($Z = 2.971$, $p = 0.003$ and $Z = 2.757$, $p = 0.006$). Within the sample suite, 29 of 79 samples had different results, but none of the investigated samples were determined to be 'good' by one method and simultaneously determined to be 'bad' by the second method. The SI method classified more samples as 'bad' (12) and, at the same time, classified fewer samples as 'good' (12) compared to the EB method, which classified five samples as 'bad' and 20 as 'good'. In both methods, the majority of samples were classified as 'medium': 54 and 53 accordingly in the SI and EB method (Figure 1).

In both methods, sludge samples that were classified as 'good', 'medium' and even 'bad' also showed high levels of organic matter reduction and low concentrations of SS, BOD_5 and COD in the effluent. In none of the investigated treatment plants the Polish Water Law limits (for PE = 2000–15,000, SS = 35 mg/L, BOD_5 = 25 mg/L, COD = 125 mg/L) of the above-mentioned parameters were exceeded during the monitoring period. Based on the SI method, four samples of the 45 in which effluent parameters were measured were classified as 'good'. At the same time the EB method classified 10 samples of the same 45 as 'good' ones. Total N and P concentration exceeded the limit in all four samples classified as 'good' in SI methods and in six of the 10 classified in the EB method. Only one

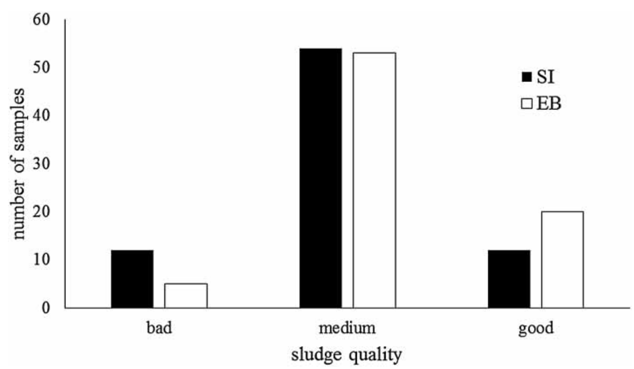

Figure 1 | Sludge samples evaluation according to two microscopic methods.

sample classified as 'good' by the SI method, as well as four 'good' samples according to EB specifications had an SVI higher than 150.

In the SI method log-transformed SVI values had stronger negative correlation with floc points ($F_{(1,77)} = 56.3$, $p < 0.001$, $r^2 = 0.422$) than with the total points ($F_{(1,77)} = 34.1$, $p < 0.001$, $r^2 = 0.307$) (Figure 2).

Floc points, as mentioned in the 'Materials and methods' section, are the sum of points representing floc attributes, including morphology, size, structure, texture, cover, and filamentous bacteria growing within and outside flocs. Our analysis showed that the microscopically observed floc attributes explained 42.2% ($r^2 = 0.422$) of the variance in SVI values between samples. Also non-parametric Spearman rank correlation analysis showed significant relationship between raw SVI values and total SI points and floc points (Table 5).

In the EB method, FI showed a weak correlation with SVI values ($F_{(1,77)} = 24.154$, $p < 0.001$, $r^2 = 0.239$) (Figure 3)

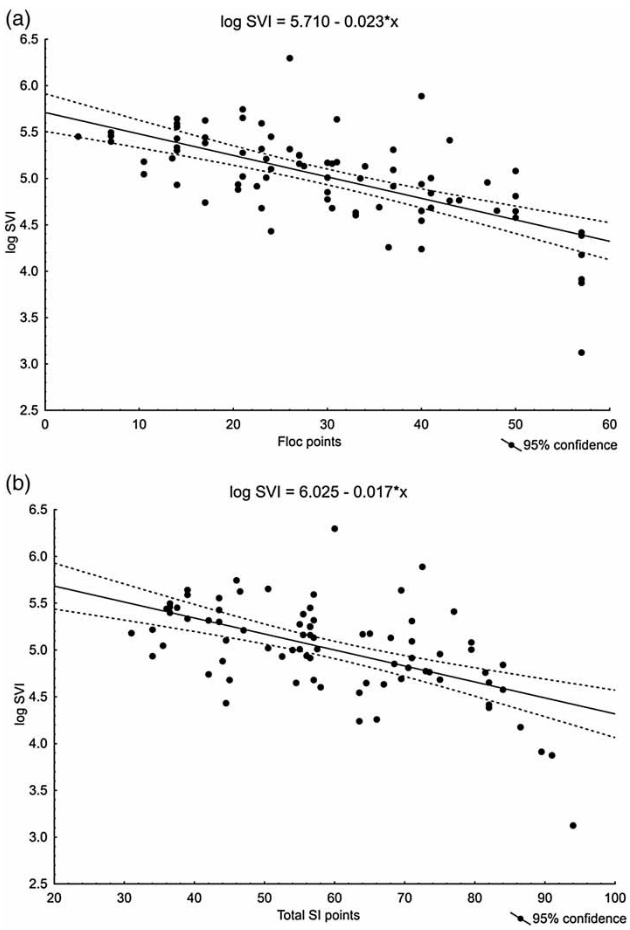

Figure 2 | Relation between log SVI and floc points (a) and total SI points (b) in SI method.

Table 5 | Spearman rank order correlations between different parameters

Parameters	Number of cases	Spearman R	p
Total SI points and SVI	79	−0.522	<0.001
Floc SI points and SVI	79	−0.641	<0.001
PAOs and P_{tot} reduction	26	0.532	0.005
Total SI points and SS	40	−0.375	0.017
Total SI points and BOD_5	40	−0.351	0.027
Total SI points and COD	45	−0.294	0.051

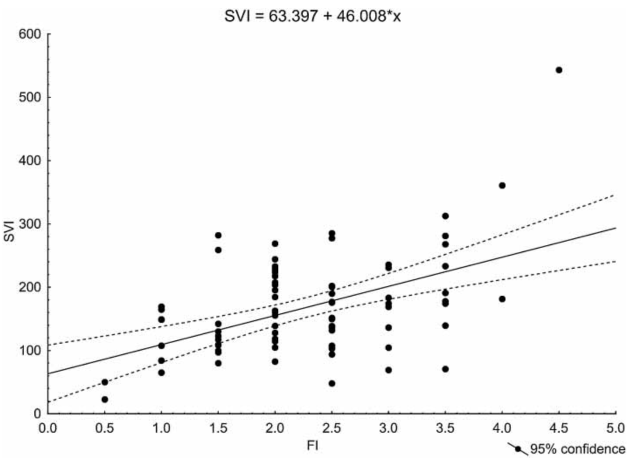

Figure 3 | Relation between SVI and FI in EB method.

and only 23.9% of the variance in the SVI values was explained by FI values. In the EB method, a higher estimation of PAOs is correlated with a higher level of total phosphorus reduction (Table 5). However, this relationship was quadratic (Figure 4). Interestingly, the research showed that, contrary to predictions, the most effective

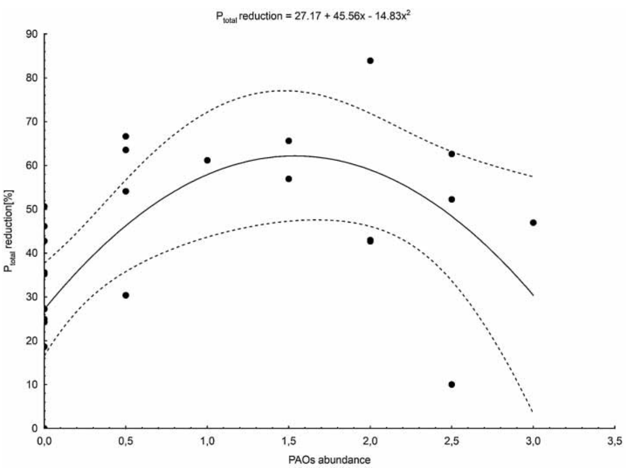

Figure 4 | Relation between P_{total} reduction and PAOs abundance.

total phosphorus reduction was not performed by the sludge with the highest PAOs abundance.

In both methods, sludge that was evaluated as 'good' and 'moderate' showed high level of organic matter reduction and low concentrations of SS, BOD_5 and COD in the effluent. The Spearman rank correlation analysis showed slightly negative correlation between total SI points and SS and BOD_5 concentration in effluent. The correlation between total SI points and COD was on the statistical significance limit (Table 5). The GRM analyses showed that higher values of total SI points had slight negative correlation with all three parameters. SS and BOD_5 concentration were log-transformed to meet GRM assumptions. Total SI points had negative correlation with log-SS ($F_{(1,38)} = 5.78$, $p = 0.021$, $r^2 = 0.131$), log-BOD_5 ($F_{(1,38)} = 4.85$, $p = 0.034$, $r^2 = 0.113$), and COD ($F_{(1,43)} = 4.25$, $p = 0.045$, $r^2 = 0.09$) concentration in the effluent (Figure 5). None of the investigated treatment plants exceeded the above-mentioned limits, even when the sludge quality was evaluated as 'bad'. This fact indicates that even activated sludge in bad condition could result in effluent of acceptable quality.

DISCUSSION

Our research showed the usefulness of both methods for activated sludge quality assessment, despite differences between them. These differences may arise from the fact that the SI method puts a greater emphasis on floc attributes than does the EB method. The EB method, on the other hand, focuses on the diversity and density of different groups of microorganisms. Moreover, the SI method takes into account macroscopic observation of sedimentation properties whereas the EB method does not.

Our investigation showed that estimation of filamentous bacteria density (FI) explained 23.9% of the variance in the SVI values. When additional floc attributes, such as morphology, size, structure, texture and cover, were taken into account, the explanation of variance increased almost two times to 42.2%. These results showed that the density of filamentous bacteria was the most influential factor for SVI values, but floc attributes are also very important and should be taken into consideration during microscopic investigation. Ganczarczyk (1994) showed that the size of the sludge flocs is an important parameter related to settling properties. Jin et al. (2003) found that the floc size, FI and fractal dimension were major parameters associated with SVI. Additionally, according to Jin et al. (2003), the

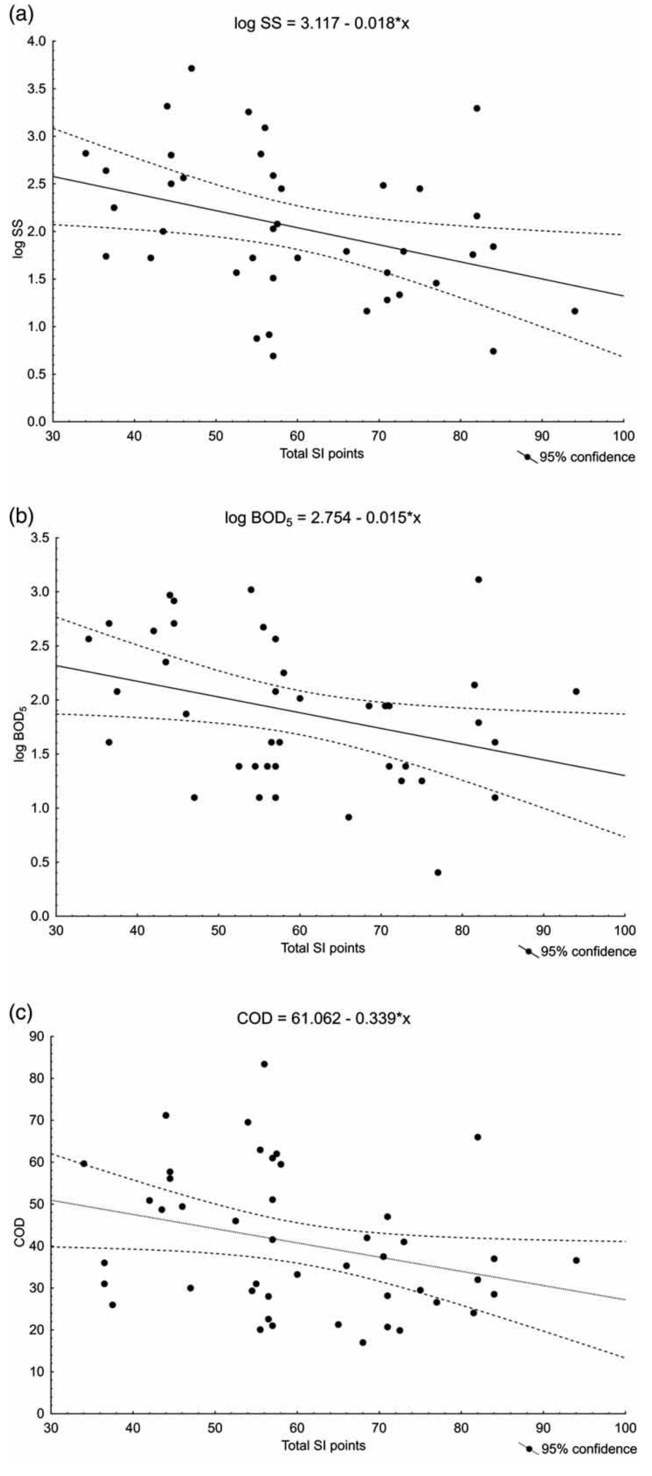

Figure 5 | Relation between log SS (a), log BOD_5 (b), COD (c) and total points in SI method.

morphological and physical properties of the sludge flocs had more influence on the compressibility and settleability than did the chemical properties. On the other hand, Yu et al. (2016), who collected samples from 20 WWTPs, did not find a relationship between the particle size and SVI, especially for samples where SVI was lower than 200 mL/g.

Amaral & Ferreira (2005) used image analysis to monitor WWTP performance and found a strong correlation between the proportion of total filament length to total suspended solids (TL/TSS), aggregates content (TL/TA) and SVI values. Total filamentous bacteria length (TL) was the sum of all the filaments' skeleton pixels within an image multiplied by a factor 1.2222. Total aggregates area (TA) was the sum of all aggregate pixels within the image. Consistent results of the relation between filamentous bacteria abundance and SVI were obtained in our research based on the simple and cheap EB method where filamentous bacteria abundance is estimated by comparison of microscopic observations with reference images (Figure 3).

Yu *et al.* (2016) demonstrated that it could be difficult to find a simple and apparent relationship between floc size and SVI because the latter is governed by a number of different factors such as MLSS, temperature, and floc structure. Our research based on the SI method, where the floc's size and structure were assigned a numerical value (the higher the value – the more compact and robust the flocs) clearly showed the negative correlation between floc points and SVI (Figure 2(a)).

A study by Burger *et al.* (2017) showed that floc stability depends on both the abundance of filamentous bacteria and the balance between them and floc-forming bacteria. Instead of determining the FI or counting filaments within flocs, Salvadó (2016) proposed another method that could be used to calculate total filament length expressed as m/mg. This method is also quick and precise, and for some operators, more transparent. Some researchers have proposed sophisticated image analysis methods (Amaral & Ferreira 2005; Mesquita *et al.* 2009a, 2009b, 2011; Liwarska-Bizukojc *et al.* 2014), but the most considerable disadvantage of these methods is the high cost of equipment (digital camera and image analysis programs). Most WWTPs are not equipped with such devices, and thus the analyses cannot be performed on the spot. Our results showing the relation between FI and SVI proved sufficient accuracy of the EB method in estimation of filamentous bacteria abundance.

Pedrazzani *et al.* (2016) used the SI, SBI and filamentous bacteria analysis to monitor the performance of treatment plants that receive swine slaughterhouse sewage. Their case study showed that the investigated methods are also applicable for monitoring industrial plants even though the changes in microfauna and physico-chemical parameters were too subtle to detect any correlations. Nevertheless, SI and SBI values pointed at stability of the process. In our study the sum of total points assigned by the SI method was negatively correlated with SS, BOD_5 and COD concentration values in the effluent. These results indicate that the SI method not only is reliable for estimating the quality of activated sludge in the context of sedimentation properties, but to some extent could also help to predict the efficiency of SS, BOD_5 and COD removal.

It should be emphasized that the methods of microscopic sludge analysis that were tested in this study were designed primarily to detect biological disturbances caused by toxic influent or filamentous bacteria overgrowth in the wastewater treatment process, rather than predicting the quality of effluent based on microbial communities composition. Our results confirmed that these methods cannot be used as a reliable tool to evaluate N and P removal as the concentrations of the total nitrogen and phosphorous exceeded the limits in effluent even when the sludge was classified as 'medium' or 'good'. However, it turned out that the relation between the density of PAO-like bacteria and the level of total phosphorus reduction can be described by a quadratic model (Figure 4). The model allows approximate estimation of phosphorus removal efficiency when the EB method is applied.

Although Neisser and Gram staining methods are not as accurate for identifying PAOs and filamentous bacteria as molecular methods (e.g. fluorescence *in situ* hybridization), they are sufficient for routine biological analysis that is required for appropriate WWTP management. As Salvadó (2016) noted, the application of epifluorescence and molecular methods in a WWTP is not an easy task because it requires special equipment and highly trained staff. Moreover, most WWTPs do not have the financial resources to perform molecular analysis in their laboratories.

One of the most important advantages of the tested methods is their accessibility to WWTP staff. The person performing the analysis needs neither extensive biological knowledge nor an ability to recognize different species of protozoans. Arregui *et al.* (2012) showed that the number of protist species and their density has the largest value of variation coefficients between different laboratories.

The SI method has one advantage over the EB method. The SI method is based on points, so the results can be easily used in reports and record sheets and operators can easily follow changes in macro- and microscopic characteristics of the sludge.

Our study was the first attempt to check the suitability of microscopic sludge investigation methods on such a large scale. We performed our investigations on nine full-scale municipal WWTPs over the period of 1 year.

To summarize, the results clearly indicate that the routine microscopic sludge investigation is a useful tool to evaluate sludge condition. However, as each of the tested methods puts emphasis on other aspects of the activated sludge and often provides complementary information, it would be advisable to develop a new, more appropriate protocol combining elements of already existing methods (e.g. Eikelboom–van Buijsen method, SI and the sludge biotic index).

CONCLUSIONS

1. The comparison of both methods shows significant differences between them.
2. The EB method showed a tendency to be more conservative in general conclusions than the SI method.
3. Both methods took into account similar floc attributes, but the EB method was more descriptive and took higher note of activated sludge microorganisms than did the SI method.
4. The SI method is reliable for estimating activated sludge quality in the context of sedimentation properties and, to some extent, could also help to predict SS, BOD_5 and COD removal efficiency.
5. Both methods are quite fast (one analysis lasts less than 1 hour) and provide general information about liquid–solid separation ability of activated sludge.
6. Microscopic sludge investigation methods are very useful in long-term or seasonal observations for finding general trends or causes of perturbations.
7. Due to demonstrated differences between the methods, developing a new protocol of sludge assessment, combining elements of already existing methods, seems to be a necessity.

ACKNOWLEDGEMENTS

We would like to thank Anna Kubicka and Katarzyna Pawlik for help with chemical analysis, and Dr Wojciech Fiałkowski and two anonymous reviewers for valuable comments on earlier version of the manuscript. This research was funded by the National Centre for Research and Development and the National Fund for Environmental Protection and Water Management Grant GEKON1/O3/214361/8/2014 and Jagiellonian University Funds DS/MND/WBiNoZ/INoS /20/2016.

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First received 22 May 2018; accepted in revised form 16 November 2018. Available online 3 December 2018