Acta Bot. Croat. 68 (1), 57–66, 2009

CODEN: ABCRA 25
ISSN 0365–0588

Plate method for counting proteolytic sulphide-producing bacteria

BOŽIDAR STILINOVIĆ, JASNA HRENOVIĆ*

University of Zagreb, Faculty of Science, Division of Biology, Rooseveltov trg 6, 10000 Zagreb, Croatia

The proteolytic sulphide-producing bacteria (PSPB) are widely distributed in water and sediment and are a good indicator of the ecological status of ecosystems. From the ecological point of view it is important to distinguish the physiological group of PSPB from other bacteria producing H_2S from sulphate, sulphite or thiosulphate. A new medium named peptone-cysteine-ammonium iron citrate agar (PCA) was developed and tested. The medium regularly gave a higher number of colony forming units than the control media for detection of sulphide formation, indole production and motility (Merck) and iron sulphite agar (Oxoid). For the enumeration of PSPB from the environment, the simultaneous incubation of samples in aerobic and anaerobic conditions is recommended and a higher number should be taken for interpretation.

Keywords: Isolation, bacteria, counting, sediment, sulphide, peptone, proteins

Abbreviations: CFU – colony forming units; **PCA** – peptone-cysteine-ammonium iron citrate agar; **PSPB** – proteolytic sulphide-producing bacteria; **SIM** – commercial medium for detection of sulphide formation, indole production and motility (Merck); **ISA** – commercial iron sulphite agar, Oxoid

Introduction

The processes of the decomposition of proteins by bacteria play an important role in the mineralization of organic matter. Many bacteria decompose protein compounds to end products such as ammonia, hydrogen sulphide (H_2S) and mercaptans. Mineralization of proteins occurs in the water mass and in benthic sediments under aerobic and anaerobic conditions. The decomposition of putrescible organic matter by heterotrophic bacteria, both in water and sediment ecosystems, is an important step in the cycling of sulphur in the nature. During putrefaction of proteins, a particular amount of H_2S originating from the sulphur-containing amino acids (organic sulphur) is liberated. Under aerobic conditions the organic sulphur compounds are mineralized to sulphate, and in anaerobic conditions the result is the production of H_2S (FENCHEL et al. 1998).

* Corresponding author, e-mail: jasnah@zg.biol.pmf.hr

There are many methods and formulations of culture media used for enumeration of classical sulphate-reducing bacteria, which reduce sulphate to H_2S during anaerobic respiration (DE BRUYN and CLOETE 1993, SKJERDAL et al. 2004). In addition to the classical sulphate-reducing bacteria, a physiological group of sulphide-producing bacteria that produce H_2S from sulphite or thiosulphate has been much investigated (STILINOVIC and HRENOVIC 2004a). However, some bacteria do not produce H_2S from sulphate, sulphite or thiosulphate but only from the sulphur-containing amino acids (GRAM et al. 1987, SKJERDAL et al. 2004). The physiological group of sulphide-producing bacteria, which produce H_2S from proteins, is less often referred to in the recent literature. From the ecological point of view it is important to distinguish the physiological group of proteolytic H_2S producing bacteria (PSPB) from other bacteria that produce H_2S from sulphate, sulphite or thiosulphate.

The bacterial production of H_2S is generally detected in/on nutrient media by observing the blackening which it produces in the presence of salts of certain metals (such as lead, iron, bismuth) owing to the dark colour of the sulphide of these metals. Standard nutrient media for the enumeration of H_2S-producing bacteria contain sulphate, sulphite, thiosulphate or cysteine, and bacteria capable of forming H_2S from either source of sulphur would appear as black colonies. It must be noted, that there is no generally accepted medium for the isolation and enumeration of the PSPB. The goal of this study was to design an original medium for the isolation and enumeration of PSPB from environmental samples. On the basis of previous investigations (STILINOVIĆ and FUTAČ 1990), we started with the fact that the anaerobic condition will be effectuated in the middle of all bacterial colonies, growing aerobically on the surface of the solid nutrient medium during the incubation period. In the middle of the H_2S producing colony, after a necessary time of incubation on the appropriate medium rich in amino acids containing sulphur, a black colour in the centre of a bacterial colony will appear soon. Therefore, it will not be necessary to incubate the inoculated nutrient medium in anaerobic conditions.

Materials and Methods

Environmental samples

In total, 56 different environmental samples from the Zagreb region were tested. Of these, 28 samples were of fresh water from natural and artificial lakes, streams and rivers and 28 samples were of sediments from the corresponding fresh water. The subsurface water samples (10 cm) and corresponding surface layer of sediment (5 cm) were collected in sterile bottles, stored at 4 °C and analysed in the laboratory within 2 h of collection. The pH-values and dissolved oxygen concentration of water and interstitial water in sediment were measured with WTW 330 pH-meter and WTW Oxi 330i oxygen meter in situ.

Media

The medium peptone-cysteine-ammonium iron citrate agar (PCA) was evaluated for the isolation and enumeration of PSPB. The composition of PCA medium was (in g L^{-1} of distilled water): peptone bacteriological (Biolife) 5.0; proteose peptone (Biolife) 5.0; L-cysteine (Fluka) 0.25; NaCl (Kemika) 5.0; ammonium iron (III) citrate (Sigma-Aldrich) 1.0; K_2HPO_4 (Kemika) 0.3; agar (Biolife) 15.0; pH 7.4±0.2.

The Merck commercial medium for the detection of sulphide formation, indole production and motility (SIM) and the Oxoid Iron sulphite agar (ISA) were used as standard media for comparison with the obtained results. The composition of SIM medium was (in g L^{-1} of distilled water): peptone from casein 20.0; peptone from meat 6.6; ammonium iron (III) citrate 0.2; sodium thiosulfate 0.2; agar 15.0; pH 7.3±0.2. The composition of ISA medium was (in g L^{-1} of distilled water): tryptone 10.0; sodium sulphite 0.5; iron (III) citrate 0.5; agar 15.0; pH 7.1±0.2. The pH of the medium was adjusted with 1 mol L^{-1} HCl or 1 mol L^{-1} NaOH. The media were autoclaved (121 °C/20 min) and poured into Petri plates.

Experimental methods

Fresh samples were analysed within 2 h of collection. Serial dilutions (10^{-1} to 10^{-4}) of one millilitre of water or one g of sediment were prepared. Dilutions (0.1 mL) were plated by a spread plate method (APHA 1995) in triplicate onto PCA, SIM and ISA. These triplicates were directly incubated at 26±0.1 °C in order to obtain the number of aerobically grown PSPB. Another triplicate of inoculated PCA, SIM and ISA was incubated (26±0.1 °C) in Anaerocult A (Merck) in order to obtain the number of anaerobically grown PSPB. The number of developed black colonies after 3 d of incubation did not increase by incubation up to 5 d. Therefore, the counted number of all developed colonies after 3 d of incubation was taken as final and CFU (colony forming units) of PSPB per one mL of water or one g of sediment were calculated.

As many different colony types of PSPB as were visually distinguishable grown on PCA, SIM and ISA were isolated on nutrient agar (Biolife). Pure cultures of PSPB were stained by the Gram method and examined in a light microscope (Olympus, BX51) under the immersion objective at the magnification of 1000×. The isolates were identified according to the general characteristics given in Bergey's Manual of Determinative Bacteriology (HOLT et al. 1994) and biochemical characteristics using the API 20E and API 50CH commercial kit.

Data analysis

The statistical analyses were done using the computer program Statistica (STATSOFT INC. 2006). The results were set up as CFU of PSPB obtained on PCA versus CFU of PSPB obtained on SIM or ISA medium. Data of this type are independent, and therefore the ordinary Student's t-test was performed. The null hypotheses tested by the analysis were: PCA, SIM and ISA medium showed no difference in the final CFU. Results were taken to be significant at the 5 % level ($p=0.05$). The correlation between variables was estimated using the Pearson linear correlation.

Results

After the inoculation of PCA the first blackening of the colonies appeared after 24 h of incubation and increased by incubation up to 3 d, but did not increase further by incubation up to 5 d. SIM and ISA media showed the same trend in the development of black colonies. Therefore, the final results considered for PCA, SIM and ISA were 3 d after the sample inoculation. In all cases on PCA, SIM and ISA media besides PSPB (black colonies) the ac-

companied heterotrophic non-PSPB were observed (pale colonies, Fig. 1). These non--PSPB bacteria constituted about 98% of total population, but did not disturb the recognition and enumeration of PSPB. The non-PSPB correlated significantly positively with the number of PSPB on all three media ($r=0.59$, $p<0.05$). The CFU of PSPB correlated significant positively with the CFU of total developed CFU on all media ($r=0.62$, $p<0.05$).

Fig. 1. Black colonies of proteolytic sulphide-producing bacteria (PSPB) and pale colonies of non- proteolytic sulphide-producing bacteria, cultivated on the Peptone-cysteine-ammonium iron citrate agar medium.

The results of the evaluation of PCA compared to the control SIM medium for the isolation and enumeration of PSPB from different environmental samples are shown in figures 2 and 3. A higher CFU of PSPB was obtained by cultivation on PCA than on SIM. In water samples (Fig. 2A) the CFU of aerobically grown PSPB were on average 1.5 times higher ($p>0.05$) when cultivated on PCA (310 mL^{-1}) than on SIM (210 mL^{-1}). In the water samples (Fig. 2B) the CFU of anaerobically grown PSPB were on average 1.2 times higher ($p>0.05$) when cultivated on PCA (149 mL^{-1}) than on SIM (125 mL^{-1}). In the sediment samples (Fig. 3A) the CFU of aerobically grown PSPB were 2.9 times higher ($p<0.05$) when cultivated on PCA (12,314 g^{-1}) than on SIM (4,286 g^{-1}). In the sediment samples (Fig. 3B) the average values of anaerobically grown CFU of the PSPB were 3.2 times higher ($p<0.05$) when cultivated on PCA (36,244 g^{-1}) than on SIM (11,165 g^{-1}).

The results of the evaluation of PCA compared to the control ISA medium for the isolation and enumeration of PSPB are shown in figures 4 and 5. When testing different environmental samples, a higher CFU of PSPB was always obtained by cultivation on PCA than on ISA medium. In the water samples (Fig. 4A) the CFU of aerobically grown PSPB were on average 2.6 times higher ($p<0.05$) when cultivated on PCA (1,921 mL^{-1}) than on ISA (728 mL^{-1}). In the water samples (Fig. 4B) the CFU of anaerobically grown PSPB were on average 1.9 times higher ($p>0.05$) when cultivated on PCA (85 mL^{-1}) than on ISA (44 mL^{-1}). In the sediment samples (Fig. 5A) the CFU of aerobically grown PSPB were 3.2 times higher ($p<0.05$) when cultivated on PCA (2,349 g^{-1}) than on ISA (730 g^{-1}). In the sediment samples (Fig. 5B) the average values of anaerobically grown CFU of the PSPB were 3.6 times higher ($p<0.05$) when cultivated on PCA (26,690 g^{-1}) than on ISA (7,388 g^{-1}).

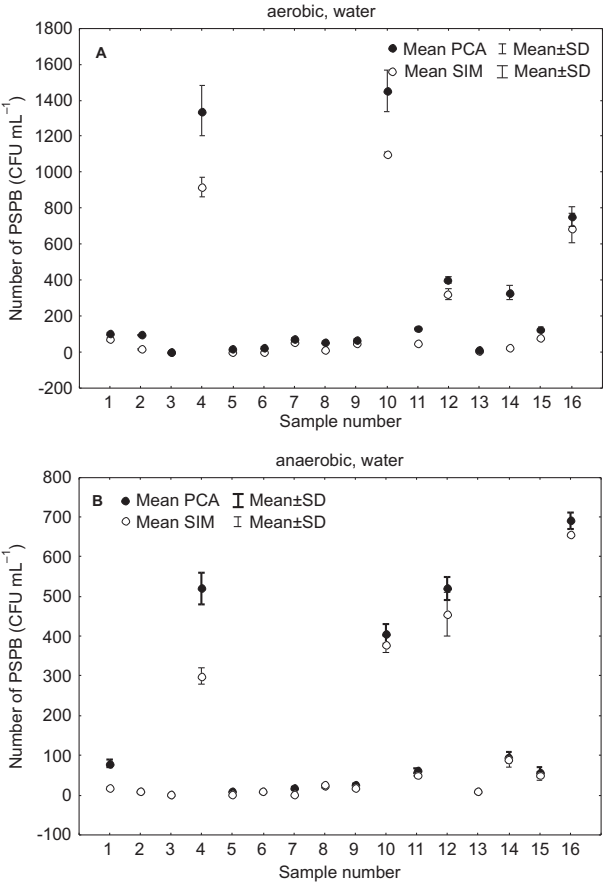

Fig. 2. Colony forming units (CFU mL^{-1}) of aerobically (A) and anaerobically (B) grown proteolytic sulphide-producing bacteria (PSPB) isolated from the samples of water on the PCA and SIM medium.

In the samples of water the numbers of PSPB cultivated aerobically were higher than those cultivated anaerobically (2.1 and 1.7 times higher on PCA and SIM, respectively, $p>0.05$ and 22.6 and 16.5 times higher on PCA and ISA, respectively, $p<0.05$). In the samples of sediment the numbers of anaerobically cultivated PSPB were higher than those cultivated aerobically (2.9 and 2.6 times higher on PCA and SIM, respectively, $p<0.05$ and 11.4 and 10.1 times higher on PCA and ISA, respectively, $p<0.05$). This affinity of PSPB population to grow in aerobic or anaerobic conditions is explained with the adaptation to the environmental conditions. Namely, the concentration of dissolved oxygen was higher in water samples (6.9–9.1 mg O_2 L^{-1}), while the sediment samples were anoxic (0.3–2.0 mg O_2 L^{-1}). The average pH values of sediment samples (7.71±0.28) were significantly ($p<0.05$) lower than pH of the corresponding water samples (8.26±0.27).

When summarising all results, the CFU of the PSPB were significantly ($p<0.05$) higher when isolated on PCA than on the SIM or ISA media. The average values of CFU of PSPB were 3.1 times higher when cultivated on PCA ($12\pm23 \times 10^3$) than on SIM ($4\pm8 \times 10^3$) and

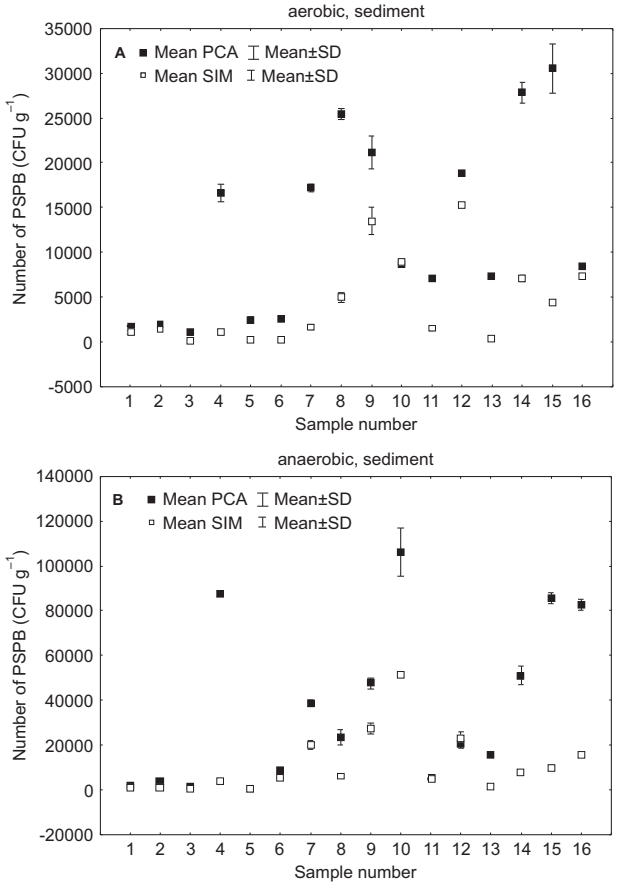

Fig. 3. Colony forming units (CFU g^{-1}) of aerobically (A) and anaerobically (B) grown proteolytic sulphide-producing bacteria (PSPB) isolated from the samples of sediment on the PCA and SIM medium.

3.5 times higher when cultivated on PCA ($8\pm23 \times 10^3$) than on ISA ($2\pm5 \times 10^3$). The correlation between PCA and SIM ($r=0.72$, $p<0.05$) and PCA and ISA ($r=0.78$, $p<0.05$) was significantly positive, which indicated that higher CFU were not the result of false positive results. The coefficient of variation of results on PCA averaged 9.4%, which is not higher than for SIM (10.4%) or ISA (13.5%) and is satisfactory for a biological method such as CFU determination.

The 64 isolated pure cultures of PSPB belonged to the common microflora of freshwater and sediments. There was no appreciable difference in bacterial genera isolated on PCA, SIM and ISA. Microscopic examination of the isolated colonies of aerobically grown PSPB confirmed Gram-negative non-spore forming rod shaped cells and Gram-positive sporogenic rod shaped cells in all cases. The common feature of isolates was gelatinolytic activity and facultative anaerobic type of metabolism. Isolates mainly belonged to the genera *Aeromonas*, *Shewanella*, *Proteus, Bacillus* and in few cases *Erwinia* and *Citrobacter.*

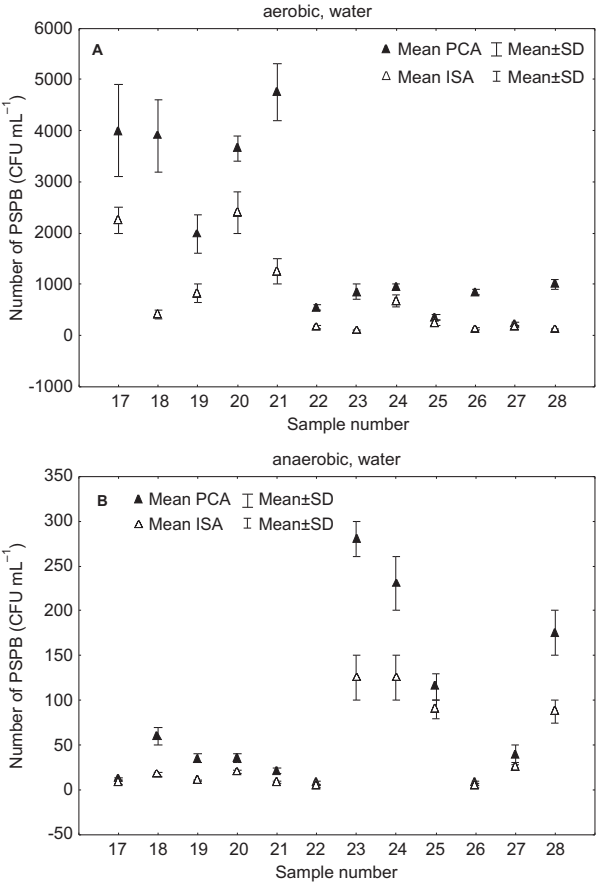

Fig. 4. Colony forming units (CFU mL^{-1}) of aerobically (A) and anaerobically (B) grown proteolytic sulphide-producing bacteria (PSPB) isolated from the samples of water on the PCA and ISA medium.

On anaerobically grown plates, beside facultative anaerobes, the obligate anaerobic bacteria from the genus *Clostridium* were isolated.

Discussion

The compound H_2S is toxic to animals and plants and its formation in the environment occupies a great attention. The H_2S in environmental samples could be the product of bacterial anaerobic decomposition of organic matter containing amino acids with sulphur such as methionine, cysteine and cystine (assimilatory sulphate reduction) or the product of reduction of sulphate, sulphite or thiosulphate (dissimilatory sulphate reduction) (BITTON 2005). On the standard media for the detection of H_2S-producing bacteria it is not possible to distinguish whether H_2S is the product of the degradation of proteins or the reduction of thiosulphate (in the case of SIM) or sulphite (in the case of ISA). Cysteine possesses the ad-

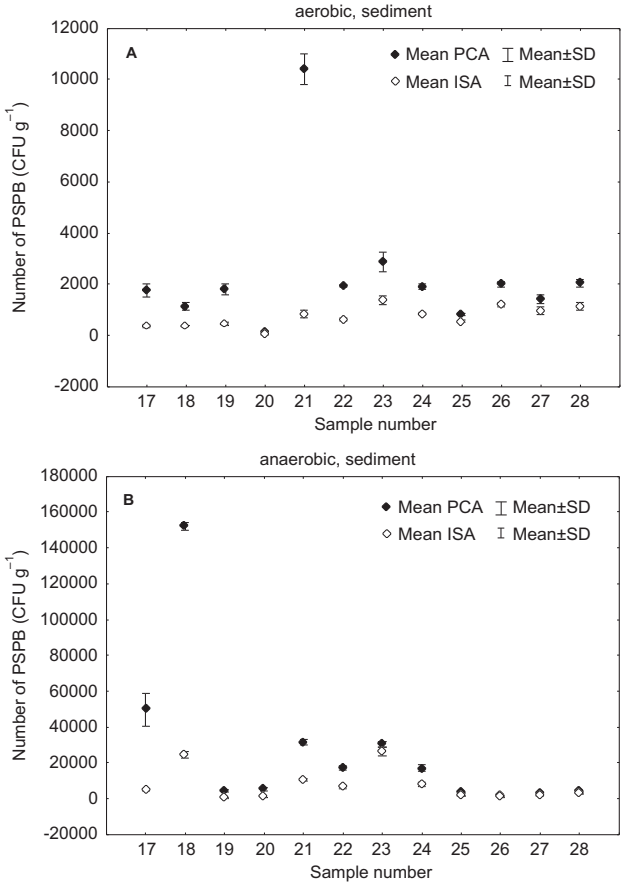

Fig. 5. Colony forming units (CFU g^{-1}) of aerobically (A) and anaerobically (B) grown proteolytic sulphide-producing bacteria (PSPB) isolated from the samples of sediment on the PCA and ISA medium

vantage that it may be attacked with the liberation of H_2S by certain bacteria which do not reduce sulphate, sulphite or thiosulphate. With this aim, in this study a medium containing only proteins as a source of sulphur has been designed.

The designed PCA medium always gave a higher number of PSPB than the standard SIM and ISA media. Since all media tested in this study (PCA, SIM and ISA) contained proteins in the form of peptones, the sodium thiosulphate or sodium sulphite as supplements in SIM and ISA media can be eliminated in the explanation of the difference of obtained CFU of PSPB. The higher CFU of PSPB obtained on PCA than on SIM or ISA media were probably the result of the composition of the peptones used (KAHN 1924) and the initial cysteine content. The suitability of tested media for the isolation and enumeration of PSPB can be given in order: PCA, SIM and ISA.

All the pure cultures of PSPB isolated on PCA grew and produced black colonies on SIM and ISA media. Therefore, it can be summarised that the higher numbers of PSPB on

PCA is rather the result of better recovery of PSPB from environmental samples than the result of isolation of more bacterial species. All the isolated pure cultures of PSPB possessed the ability to liquefy gelatine. Here it should be mentioned that at the same time the gelatinolytic activity (STILINOVIĆ and HRENOVIĆ 2004b) does not imply that they are PSPB.

The PSPB may have facultative anaerobic or obligate anaerobic type of metabolism. Therefore the question is raised in which condition of oxygen supply the environmental samples should be incubated. The designed medium in this study inoculated with the same samples was incubated in both aerobic and anaerobic conditions. The results showed that a higher CFU of PSPB was grown in aerobic conditions of incubation in the water samples which had a higher dissolved oxygen concentration. In the anoxic sediment samples a higher CFU of PSPB was grown in anaerobic than in aerobic conditions of incubation. Therefore, for the enumeration of PSPB from environmental samples the simultaneous incubation of the same samples in aerobic and anaerobic conditions is recommended and a higher number should be taken for interpretation.

Besides the lower dissolved oxygen concentration, the examined sediment samples had a lower pH values than the corresponding water samples, which indicated a higher intensity of the process of putrefaction. The processes of decomposition of organic matter in sediment are more intensive than in the water column, and this explains the higher numbers of PSPB obtained in the sediment than in the water samples. The PSPB could always be found in water known to receive sewage, human or animal wastes or stools (THOMPSON 1921). Therefore the isolation of PSPB from environmental samples can be associated with the quality of the water and presence of readily biodegradable organic matter.

Conclusions

The presented PCA allows the isolation of higher CFU of PSPB than the standard SIM or ISA media. The PCA can be incubated in aerobic or anaerobic conditions in order to count most real CFU of PSPB from environmental samples. The presented method could find an application in the detection of the consortia of PSPB in the environment where the degradation of proteins and H_2S production play an important role.

Acknowledgements

This research was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (project no. 119-1191155-1203). Special thanks to R. Horvat and V. Ramljak for technical assistance during field and laboratory work.

References

APHA, 1995: Standard methods for the examination of water and wastewater, APHA--AWWA-WPCF, New York.

BITTON, G., 2005: Wastewater microbiology. John Wiley and Sons, Inc., New Jersey.

DE BRUYN, E. E., CLOETE, T. E., 1993: Media for the detection of sulphide-producing bacteria in industrial water systems. Journal of Microbiological Methods 17, 261–271.

FENCHEL, T., KING, G. M., BLACKBURN, T. H., 1998: Bacterial biogeochemistry: the ecophysiology of mineral cycling. Elsevier, Amsterdam.

GRAM, L., TROLLE, G., HUSS, H. H., 1987: Detection of specific spoilage bacteria from fish stored at low (0 °C) and high (20 °C) temperatures. International Journal of Food Microbiology 4, 65–72.

HOLT, J. G., KRIEG, N. R., SNEATH, P. H. A., STALEY, J. T., WILLIAMS, S. T., 1994: Bergey's manual of determinative bacteriology. Williams and Wilkins, Baltimore.

KAHN, M. C., 1925: Hydrogen sulphide production by anaerobic spore-bearing bacteria. Journal Bacteriology 10, 439–447.

SKJERDAL, O. T., LORENTZEN, G., TRYLAND, I., BERG, J. D., 2004: New method for rapid and sensitive quantification of sulphide-producing bacteria in fish from arctic and temperate waters. International Journal of Food Microbiology 93, 325–333.

STATSOFT INC., 2006: Statistica (data analysis software system) version 7.1.

STILINOVIĆ, B., FUTAČ, N., 1990: Methods of study the bacterial production of hydrogen sulphide from proteins. Biologia (Bratislava) 45, 229–234.

STILINOVIĆ, B., HRENOVIĆ, J., 2004a: Rapid detection of sulfide-producing bacteria from sulfate and tiosulfate. Folia Microbiologica 49, 513–518.

STILINOVIĆ, B., HRENOVIĆ, J., 2004b: Percentage of gelatinolytic bacteria among heterotrophic bacteria as indicator of water qualty. Folia Microbiologica 49, 53–58.

THOMPSON, L. S., 1921: The group of sulphide producing bacteria. The Journal of Medical Research 42, 383–389.