REVISTA DE LA SOCIEDAD VENEZOLANA DE MICROBIOLOGIA

RSVM

Revista de la Sociedad Venezolana de Microbiología 2012; 32:121-125

Artículo original

Methodological aspects for the culture and quantification of heterotrophic sulfate-reducing bacteria

Alejandra Carolina Zamora*, Nora Malaver

Laboratorio de Microbiología Ambiental, Instituto de Zoología y Ecología Tropical, Facultad de Ciencias, Universidad Central de Venezuela. Caracas, Venezuela.

Recibido 21 de noviembre de 2011; aceptado 20 de mayo de 2012

***Abstract*:** This paper presents a comparison of various methods for culture, quantification, and maintenance of sulfate-reducing bacteria (SRB) under laboratory conditions, using liquid and semisolid media for water and soil samples. Starkey, Postgate B, API and modified Baars media were used with an incubation time of 21 days in a GasPack™ anaerobic jars type. The modified Baars medium was more efficient for the quantification of SRB in both liquid and semisolid media when compared with other culture media tested, detecting differences of three orders of magnitude in soil samples and in two orders for water samples at 8 days of incubation. The semisolid modified Baars medium in Petri dishes allowed the isolation of pure cultures of SRB by the streak plate method. It was found that strains in liquid modified Baars medium remain viable for up to three months, while in the same semisolid medium were kept only one month.

Keywords: hydrogen sulfide, sulfate-reducing bacteria, culture media, culture methods, SRB quantification.

Aspectos metodológicos para el cultivo y la cuantificación de bacterias heterotróficas sulfato-reductoras

***Resumen*:** Este trabajo presenta una comparación de diversos métodos para el cultivo, cuantificación, y mantenimiento de bacterias sulfato-reductoras (BSR) en condiciones de laboratorio, utilizando medios líquidos y semisólidos para muestras de agua y suelo. Se utilizaron los medios de cultivo de Starkey, Postgate B, API y Baars modificado con un tiempo de incubación de 21 días en jarras de anaerobiosis tipo GasPack™. Se determinó que para la cuantificación de SRB, tanto en medio líquido como en semisólido, el medio Baars modificado es más eficiente comparado con los demás medios de cultivo probados, detectando diferencias de tres órdenes de magnitud en muestras de suelo y de dos órdenes de magnitud en muestras de agua a los 8 días de incubación. El medio Baars modificado semisólido servido en placas de Petri permitió el aislamiento de cultivos puros de BSR mediante siembra por agotamiento. Se encontró que en el medio modificado de Baars líquido las cepas se mantienen viables hasta por tres meses mientras que en el mismo medio semisólido sólo se mantienen durante un mes.

Palabras clave: sulfuro de hidrógeno, bacterias sulfato-reductoras, medios de cultivo, métodos de cultivo, cuantificación de SRB.

* Correspondencia:
E-mail: alejandra.zamora@gmail.com

Introduction

The sulfate-reducing bacteria (SRB) are strict anaerobic microorganisms; however, they can be found in aerobic environments, specifically in anoxic micro-zones [1]. They constitute a diverse morphological group including cellular forms such as cocci, bacillus, cellular aggregates and multi-cellular filaments.

The heterotrophic SRB obtain their energy for cell synthesis and growth from oxidation of organic compounds. This process is coupled to the reduction of sulfate to hydrogen sulfide, through several metabolic pathways, but the most common in natural environments is the dissimilatory reduction of sulfate to sulfide. This sulfide can be released as hydrogen sulfide or precipitate in the soil as ferrous sulfide (FeS) in a process linked to the oxidation of organic matter [2,3].

The hydrogen sulfide (H_2S) released by the SRB is a strong reducing agent, highly toxic to other organisms. The SRB have economic importance for the industries, due to corrosion and contamination problems that cause the production of H_2S [4,5]. The H_2S reacts with water to form

sulfuric acid, which deteriorates the metal pipes and concrete structures [6,7] in a process known as microbiologically influenced corrosion (MIC) [8]. The MIC occurs due to the aggressive attack of the metabolites produced during the adhesion of microorganisms to the metal (pitting corrosion) [9] or concrete surfaces (biocorrosion).

However, the activity of the SRB has great ecological importance because of their involvement in the mineralization of organic matter in anaerobic environments [10]. The SRB are responsible for degrading about 53% of the organic matter in marine and coastal environments, where sediments have low redox potential and high sulfate content, limiting the oxidation of organic substrates [11]. They also have been used in bioremediation of toxic pollutants to the environment, such as toluene and xylene [12]. This process is possible because the SRB are capable of breaking the ring structure of some organic compounds, and consequently, these are mineralized [13].

Therefore, the development of techniques for detection, quantification and isolation are necessary to understand the role of SRB in different industrial and ecological processes. Considerable efforts have been oriented to develop fast and reliable methodologies for detection and quantification of SRB in natural and artificial environments [14]. In general, the methods used to enumerate these bacteria can be divided in two different categories: (i) direct detection and (ii) culture methods.

Direct detection of SRB is mostly used in current research involving these bacteria. This method has been developed in the last few years and includes various techniques such as the use of cultivate antibodies of SRB [15], immunodetection of the enzyme adenosine-5'-phosphosulfate (APS) reductase [16] and sequence analysis of genes encoding 16S rRNA [17,18], particularly the analysis of the dissimilatory sulfite reductase genes (*dsrAB*) [19].

Culture methods for quantification of SRB based on the most probable number (MPN) technique has been widely used for a long time [5,10,17,20-22]. Up to this point, a great variety of culture media have been developed for specific environments, including activated sludge, marine sediments and samples from the oil industry [1,2,5,10,20,22,23]. All these culture media contain lactate as carbon and energy source, and the presence of SRB is always assessed by the formation of a black color precipitate of FeS.

However, traditional techniques based on selective microbial cultures offer a limited utility for quantification and characterization of the SRB isolated from environmental samples. Most strains do not grow up *in vitro* either because the culture media do not have the specific growth conditions or, in other cases, different strains of SRB are interdependent with other microorganisms [24]. For this reason, the number of SRB detected by culture methods may be underestimated [25]. Thus, the isolation of pure cultures and maintenance of the strains in the laboratory are very complicated tasks. They demand great efforts and time because these bacteria lose viability in a short time.

Real-time PCR is a sensitive and rapid molecular method, which permits detection and quantification of bacterial populations through DNA technology. This approach does not require culture of the target organisms and is therefore ideally suitable to studies [25,26]. However, these techniques involve high costs and specialized equipment, often not available if the studies are not associated with scientific research.

Considering these issues, we propose a methodology that allows the culture isolation and maintenance of SRB strains in the laboratory. This technique differs from the conventional method and does not require the use of chambers under N_2, CO_2 or H_2 atmospheres. It also has the versatility to allow the quantification of colony forming units (CFU) in Petri dishes with semisolid medium or the enumeration of SRB in liquid media using the most probable number (MPN) technique. In this regard, this paper is a contribution to the knowledge of different microbiological techniques, to facilitate the easy and fast detection of SRB from environmental samples.

Materials and methods

Specimen collection: It were used two specimen types: (a) a water specimen taken from a refinery effluent, intended to come into contact with hydrocarbons; (b) a sediment specimen collected by obtaining three cores from shallow permanently water-covered soil (approximately 10 cm depth) from the Lower Orinoco region, Anzoátegui State, Venezuela. These samples were stored in sterile containers at 4 °C until processing in the laboratory.

SRB activation in enriched cultures: To detect the presence of SRB, collected samples were previously activated in Starkey liquid medium as described by Toerien *et al.* [20]. This medium was prepared by initially heating the components and subsequently 95 mL was served in bottles under a N_2 atmosphere and hermetically sealed. It was sterilized in an autoclave at 121 ºC with 15 pounds per square inch of pressure for 20 minutes. Five (5) mL of the water specimen were directly inoculated in the medium using a sterile syringe. For the determination of SRB in the sediment, 10 gr of the sediment were placed inside an Erlenmeyer containing 90 mL of 0.85% NaCl solution and a paraffin layer of 3 mm to create anaerobic conditions. The Erlenmeyers were shaked during 8 hours for the SRB activation and 5 mL of this solution were inoculated into the bottles containing Starkey liquid medium. Bottles were incubated in darkness for 21 days at 25 ºC. The presence of SRB was determined by the formation of a FeS black precipitate on the bottom of the bottle.

Enumeration of SRB: The enumeration of SRB was performed using the Baars's modified medium [27], in which the calcium sulfate was substituted by sodium sulfate and calcium chloride, and a 50% sodium lactate solution was used instead of a 70% solution as a carbon source (Table 1). The medium was prepared by dissolving the salts in distilled water and autoclaving for 20 minutes at 121 ºC

Table 1. Base medium used for enumeration and isolation of pure cultures of sulfate-reducing bacteria(*).

Basal medium	Quantity (g/L)
Dipotassium phosphate (K_2HPO_4)	0.5
Ammonium Chloride (NH_4Cl)	1
Calcium Chloride† ($CaCl_2$)	0.5
Sodium sulfate† (Na_2SO_4)	0.5
Magnesium sulfate heptahydrate ($MgSO_4.7H_2O$)	2
Sodium lactate 50%‡ ($C_3H_5NaO_3$)	5
Distilled water	1 liter
Pure Agar§	8
Complementary solution	
Ferrous ammonium sulfate hexahydrated $(NH_4)_2SO_4.FeSO_4.6H_2O$	Solution at 1%

(*) Baars´s modified medium (1930). (†) The original Baars´s medium contains calcium sulfate at a ratio of 1 g/L. ‡ The Baars´s medium (1930) contains sodium lactate 70%. (§) For semisolid medium.

with 15 pounds per square inch of pressure. Separately, the complementary solution was prepared and sterilized by filtration (Millipore® membrane HA type) and was added to the medium base at 5% (v/v). The semisolid medium was prepared adding 8 gr. l^{-1} of purified agar (55% w/v) in aerobic conditions.

Serial dilutions up 10^{-4} were made in 0.85% NaCl solution to determine the MPN of SRB in the samples. They were inoculated in test tubes with Baars's modified medium using the MPN technique in series of 5 tubes, adding into each one a paraffin layer of 3 mm to create anaerobic conditions. This proceeding was made by triplicate. The test tubes were kept in darkness at 25 °C for 21 days. Standardized tables of MPN with 95% confidence intervals were used to estimate SRB density. Simultaneously, another inoculation was performed by triplicate, using the deep planting method with the semisolid medium in Petri dishes, for SRB colony forming units count (CFU/mL). The Petri dishes were incubated in anaerobic jars under an oxygen-free atmosphere generated by Anaerocult A (Merck®) at 25 °C during 21 days.

To compare the efficiency of the tested culture medium, the quantification of SRB was carried out in Postgate´s B and Starkey´s liquid and semisolid media [21,22] as well as in API medium (Himedia ®) incubated under the same conditions.

SBR isolation from pure cultures of SRB: The isolation and purification of the strains was performed following the streak plate method directly from growth on the agar surface, using Baars´s modified semisolid medium [27].

Statistical analysis: A Student *t* test was used to determine whether MPN and CFU values obtained with different media were significantly different. Differences were considered significant at the 95% confidence level.

Results and discussion

SRB enumeration in water and sediment specimens: Figure 1 shows the number of SRB quantified in the water specimen. MPN values that fluctuated between $1.8x10^3$ and $2.4x10^4$ at 8 days of incubation followed the same trend of the CFU in semisolid medium (from $1.6x10^3$ to $1.6x10^4$) using Baars´s modified medium [27]. Differences of one to two orders of magnitude were found for water specimens (Figure 2) when compared Baars´s modified medium with Postgate´s B and Starkey's media (Anova, $p<0.05$).

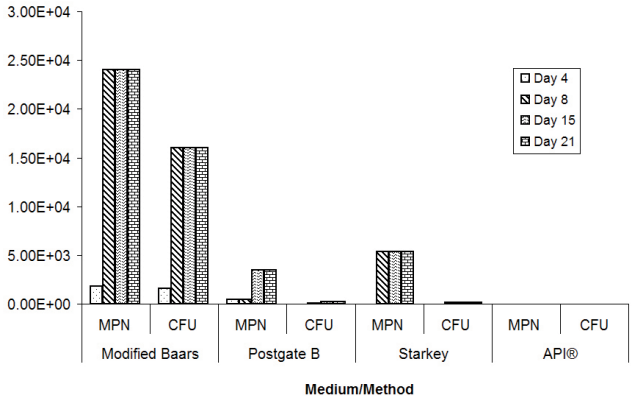

Figure 1. SRB enumeration in water samples using different culture media and counting techniques.

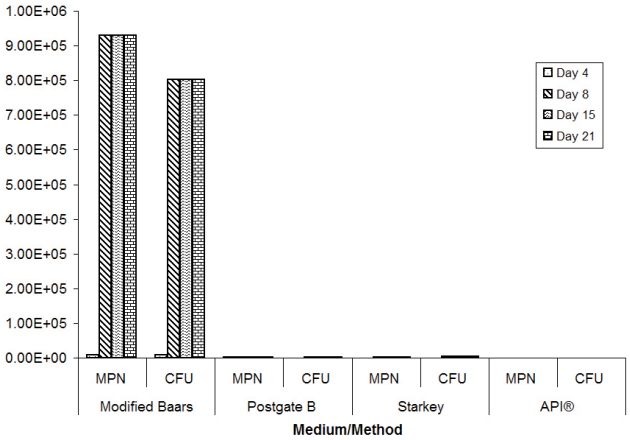

Figure 2. SRB enumeration in soil samples using different culture media and counting techniques.

The SRB density in the API synthetic medium underestimated the number of CFU, since only were detected after 21 days of incubation, showing differences of more than three orders of magnitude compared with the specimens grown on other culture media (Figure 1). It was also found that Starkey and Postgate B semisolid media required an incubation period of 15 days.

A similar pattern was reported by Jain [2] who determined that use Postgate´s B semisolid medium requires less incubation time (7 days) compared with synthetic media. A similar result was obtained by Vester & Ingvorsen [10] who

compared different culture media for SRB enumeration through the MPN technique, finding that synthetic media significantly underestimate the SRB number. These saline media containimg lactate as unique electron donor, while the API synthetic medium has yeast extract. Regarding the latter idea, Gibson *et al.* [28] argued that a large number of SRB natural populations were physiologically different from laboratory strains, and were not able to grow in media containing high levels of organic substrates.

Figure 2 shows the SRB concentration in sediment specimens with the different culture media used. Like for water specimens, the MPN values in the sediment increased from $9.3x10^3$ to $9.2x10^5$ after incubation of 7 days using Baars´s modified medium, while the bacterial numbers counted in Starkey, Postgate B and API mediums were significantly lower. Differences up to two orders of magnitude at 8 days in liquid and solid media were found between the media tested. The same trend was observed for CFU count on semisolid medium.

The sediment specimens (Figure 2) showed a SRB concentration which only required 8 days of incubation, whereas the API medium required an incubation time of at least 21 days. The differences in the SRB counts determined on the tested media could be related to the procedures used for activating the sediment into the liquid medium using a 0.85% NaCl solution, because the concentration of salts, particularly the NaCl, should change the bacterial concentration in the specimen being enumerated [29]. Also the absence of a reducing agent in the medium (e.g. cystein, rezarsurin) could be important.

It is interesting to note the presence of sulfate-reducing bacteria in the upper section of sediment. In this aspect, it has been recently demonstrated that SRB were present in oxygenated and non-oxygenated environments, especially those close to surface sediments [30]. This might explain the presence of SRB on sediment and water specimens studied.

The results shown on figures 1 and 2 indicate that the number of viable SRB was greater when using saline mediums (Baars, Postgate B and Starkey) when compared to the synthetic media tested (API). In this regard, Jain [2] showed that with the API medium [23] the values of MPN were significantly lower than those determined by the Postgate B and Baars media. Furthermore, Tanner [29] used API-38 commercial medium suggested by the American Petroleum Institute [23], and determined that SRB density detected by the commercial media was lower compared to Baars and Postgate B media. Moreover, their study found that the addition of ammonium salts, calcium and phosphorus improved the sensitivity of the API medium. According to this author, the data presented on figures 1 and 2 indicate that saline medium were more efficient for the SRB enumeration. Moreover, the number of SRB quantified in this work was similar to that reported by Tanner [29] for sediment specimens at the same incubation time (from 10^2 to 10^3 MPN).

Starkey and Postgate B media did not show any results using conventional anaerobic techniques (GasPak™ jars with anaerobic generator Anaerocult A), whereas when the flasks were gassed with N_2 there were SRB expression. This pattern could be attributed to the culture media preparation conditions and inoculation technique, because these media are frequently used with techniques in which the atmosphere on the incubation chamber is purged of oxygen using anoxic gases, such as N_2 or a mixture of N_2:CO_2 as a redox agent with an 80:20 ratio [31]. In this sense, Tanner [29] suggested that anaerobic conditions and a low redox potential are important for the estimation of SRB, especially from environmental specimens.

It is important to note that in media containing lactate as electron donor, the *Desulfovibrio* or *Desulfococcus* groups prevailed in SRB counting [32]. This approach is limited because the SRB diversity is broadest. However, the use of this methodology for preliminary detection on SRB in environmental and industrial samples will enhance the monitoring of these microorganisms.

SRB isolation and maintenance from pure cultures: The Baars´s medium or similar ones have been used a long time for the maintenance of SRB pure culture [26,29]. SRB colonies showed a blackening of the medium allowing for quantification and subsequent isolation using routine microbiological techniques. The strains grown after 4 days of incubation and remained viable up to 30 days in the semisolid medium, whereas in the liquid medium were viable until 90 days.

Another aspect to consider during the isolation of SRB is temperature, because temperature seems to affect the viability of these bacteria. During the tests, it was determined that the platinum loop affects the survival of SRB cells, causing not growth in culture medium. For this reason, sterile wooden sticks were used, resulting in successfully pure strain isolations.

Conclusions

The standardization of a methodology that allows the use of conventional techniques to study SRB are of great applications in microbial ecology.

Modification of the Baar's medium (1930) that can be used in liquid phase to estimate MPN and on semisolid phase for direct counting of CFU for SRB, proved to be efficient for environmental specimens (e.g., soil) and specimens of industrial origin (water effluent), finding great differences when compared with Starkey, Postgate B and commercial API synthetic media.

It has the advantage of being a simple method that requires no special techniques for the preparation of culture media and inoculation, and reduces significantly the incubation time to 8 days compared to other culture media tested. Moreover, Baars´s medium allows the isolation and maintenance of pure viable strains for up to 90 days.

Acknowledgements

The authors thank to Jorge Pérez-Eman for his helpful comments and critical review of this manuscript. We also thank to Susana Marin for improving the English language, and to the three anonymous reviewers who enriched this paper with their critics.

References

1. Widdel F, Bak F. Gram-negative mesophilic sulfate-reducing bacteria. En: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH, editores. The Prokaryotes. 2nd edition. New York: Springer Verlag; 1992. pp. 3352-78.
2. Jain DK. Evaluation of the semisolid Postgate's B medium for enumerating sulfate-reducing bacteria. J Microbiol Methods. 1995; 22:27-38.
3. Atlas R, Bartha R. Ecología Microbiana y Microbiología Ambiental. 4ta Edición. España: Editorial Addison Wesley; 2002.
4. Hamilton WA. Sulphate-reducing bacteria and anaerobic corrosion. Ann Rev Microbiol. 1985; 39:195-217.
5. Postgate JR. Versatile medium for the enumeration of sulphate-reducing bacteria. Appl Microbiol. 1963; 1:265-7.
6. Luptakova A, Macingova E, Harbulakova V. Positive and negative aspects of sulphate-reducing bacteria in environment and industry. Nova Biotechnol. 2009; 9:147-54.
7. Torrado-Rincón JR, Calixto-Gómez DM, Sarmiento-Caraballo AE, Pankeva-Álvarez JH. Evaluación del molibdato y nitrato sobre bacterias sulfato-reductoras asociadas a procesos de corrosión en sistemas industriales. Rev Arg Microbiol. 2008; 40:52-62.
8. Remazeilles C, Dheilly A, Sable S, Lanneluc I, Neff D, Refait P. Microbiologically influenced corrosion process of archaeological iron nails from the sixteenth century. Corros Eng Sci Technol. 2010; 45:388-94.
9. Lim C-P, Zhao D, Takase Y, Miyanaga K, Watanabe T, Tomoe Y, Tanji Y. Increased bioclogging and corrosion risk by sulfate addition during iodine recovery at a natural gas production plant. Appl Microbiol Biotechnol. 2011; 89:825-34.
10. Vester F, Ingvorsen K. Improved most-probable-number method to detect sulfate-reducing bacteria with natural media and radiotracer. Appl Environ Microbiol. 1998; 64:1700-07.
11. Torres-Alvarado MR, Fernández F, Barriga-Sosa IA, Ramírez-Vives F. Dinámica de las bacterias anaeróbicas en las fases terminales de la mineralización de la materia orgánica en el sedimento de los ecosistemas Carretas-Pereyra y Chantuto-Panzacola. Hidrobiol. 2006; 16:183-96.
12. Edwards EA, Wills LE, Reinhard M, Garbic-Galic D. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl Environ Microbiol. 1992; 58:794-800.
13. Muyzer G, Stams AJM. The ecology and biotechnology of sulfate-reducing bacteria. Nat Rev Microbiol. 2008; 6:441-54.
14. Tatnall RE, Stanton KM, Ebersole RC. Methods of testing for the presence of sulfate-reducing bacteria. Corrosion 88. Houston: NACE International; 1998.
15. Lillebæk R. Application of antisera raised against sulfate-reducing bacteria for indirect immunofluorescent detection of immunoreactive bacteria in sediment from the German Baltic Sea. Appl Environ Microbiol. 1995; 61:3436-42.
16. Odom JM, Jessie K, Knodel E, Emptage M. Immunological cross-reactivities of adenosine-59-phosphosulfate reductases from sulfate-reducing and sulfide-oxidizing bacteria. Appl Environ Microbiol. 1991; 57:727-33.
17. Teske A, Wawer C, Muyzer G, Ramsing N. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. Appl Environ Microbiol. 1996; 62:1405-15.
18. Stubner S. Quantification of Gram-negative sulphate-reducing bacteria in rice field soil by 16S rRNA gene-targeted real-time PCR. J Microbiol Methods. 2004; 57:219-30.
19. Leloup J, Quillet L, Berthe T, Petit F. Diversity of the dsrAB (dissimilatory sulfite reductase) gene sequences retrieved from two contrasting mudflats of the Seine estuary, France. FEMS Microbiol Ecol. 2006; 55:230-8.
20. Toerien DF, Thiel PG, Hattingh MM. Enumeration, isolation and identification of sulphate-reducing bacteria of anaerobic digestion. Water Res. 1968; 2:505-13.
21. Hardy JA. The enumeration, isolation and characterization of sulphate-reducing bacteria from North Sea waters. J Appl Bacteriol. 1981; 51:505-16.
22. Fedorak PM, Semple KM, Westlake DW. A statistical comparison of two culturing methods for enumerating sulfate-reducing bacteria. J Microbiol Methods. 1987; 7:19-27.
23. American Petroleum Institute. API Recommended Practice for the Biological Analysis of Subsurface Injection Waters. API RP 38. 2nd Edition. New York: American Petroleum Institute; 1965.
24. Wagner M, Amann R, Lemmer H, Schleifer K. Probing activated sludge with oligonucleotides specific for proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. Appl Environ Microbiol. 1993; 59:1520-5.
25. Ritz K. The plate debate: cultivable communities have no utility in contemporary environmental microbial ecology. FEMS Microbiol Ecology. 2007; 60:358-62.
26. Dorador C, Castillo G, Witzel KP, Vila I. Bacterial diversity in the sediments of a temperate artificial lake, Rapel reservoir. Rev Chil Hist Nat. 2007; 80:213-24.
27. Baars J. Over sulfaatreductie door Bacterien. Dissertion, W.D. Meinema, N.V. Delft. Holland. 1930.
28. Gibson GR, Parkers RJ, Herbert RA. Evaluation of viable counting procedures for the enumeration of sulfate-reducing bacteria in estuarine sediments. J Microbiol Methods. 1987; 7:201-10.
29. Tanner RS. Monitoring sulfate-reducing bacteria: comparison of enumeration media. J Microbiol Methods. 1989; 10:83-90.
30. Dolla A, Fourner M, Dermoun Z. Oxygen defense in sulfate-reducing bacteria. J Biotechnol. 2006; 126:87-100.
31. Macy JM, Snellen JE, Hungate RE. Use of syringe methods for anaerobiosis. Am J Clin Nutr. 1972; 25:1318-23.
32. Karnachuk OV, Pimenov NV, Yusupov SK, Frank YA, Frank YA, Kaksonen AH, Puhakka JA, Ivanov MV, Lindström EB, Tuovinen OH. Sulfate reduction potential in sediments in the Norilsk mining area, Northern Siberia. Geomicrobiol J. 2005; 22:11-25.