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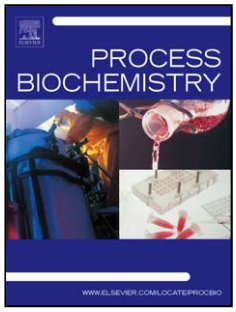


Title: Microbial Processes and Bacterial Populations Associated to Anaerobic Treatment of Sulfate-Rich Wastewater

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data with the results of molecular biology in an attempt to suggest potential metabolic routes at different operational conditions.

In the presence of sulfate, sulfite or thiosulphate, sulfate-reducing bacteria (SRB) can grow heterotrophically or lithotrophically on different substrates. SRB are important members of the microflora of a typical anaerobic digester [8]. The electron donors for sulfate-reducing microorganisms in anaerobic reactors include a variety of low-molecular mass organic compounds, such as mono- and dicarboxylic aliphatic acids, alcohols, polar aromatic compounds and even hydrocarbons. Oxidation of organic compounds may be incomplete, with acetate (often simultaneously with $CO_2$) as a potential by-product; or it is a complete oxidation, leading to the final production of $CO_2$. Dissimilatory biological sulfate reduction is a process carried out by many bacteria and some archaea [9]. In such environment, simple compounds (ethanol, methanol, acetate and $H_2/CO_2$) seem to be preferred over complex substances [10]. Ethanol was found to be a suitable carbon and energy source for sustaining sulfate reduction in such anaerobic reactors. The use of ethanol in sulfate–reducing systems has already been applied in full-scale plants [11, 4] in lab-scale UASB [12] and in a fluidized bed reactor [13].

The aim of this study was to characterize the microbial biofilm community colonizing a mineral coal substrate inside an ASBBR pilot-plant treating a sulfate-rich wastewater from an industrial sulfonation process. In addition, the microbial characterization was further associated to a simplified mathematical model analysis contextualizing the experimental data in order to suggest potential metabolic pathways under different conditions of COD/Sulfate ratios.

## 2. Materials and Methods

*Influent Wastewater*

Sulfonated oils are produced from the reaction of vegetal oils with sulphuric acid and liquid ammonia in a batch reactor operated under controlled temperature. The industrial wastewater (Table

1) containing high sulphate concentration, which originated from washing the products of this process, was collected in plastic vessels and transported to the laboratory for feeding the rector.

*ASBBR reactor*

The 1.2 $m^3$ ASBBR reactor was constructed in "Fiberglass" and it was filled with 500 kg of irregular pieces of mineral coal (40 to 80 mm of diameter) occupying a volume of 1.0 $m^3$ (porosity = 0.5). The treatment volume available by cycle or batch mode was 0.6 $m^3$ .The outlet biogas tube from head-space (0.2 $m^3$) was immersed in a hydraulic seal (100 l) containing an alkaline solution (NaOH) for $H_2S$ removal.

The influent wastewater was pumped from a storage tank (0.6 $m^3$) to a circular perforated tube located at the reactor's bottom for achieving a better liquid distribution. Mixing was provided by liquid recirculation (up-flow) by means of a centrifuged pump (Jacuzzi-model 5JL15) connected to the inflow distribution system. The cycle time was of 48 h, including the steps of feeding (1 h), reaction with liquid recirculation (46 h) and discharge (1 h). The reactor was operated at ambient temperature (31±2$^0$C) in the Laboratory of Biological Processes (Universidade de São Paulo, São Carlos-Brasil). A scheme of the experimental set-up is presented in Figure 1.

*Operational conditions (ASBBR)*

Initially, the ASBBR was inoculated with 0.2 $m^3$ of anaerobic sludge taken from a full-scale UASB treating domestic sewage. The reactor was operated during 30 batch cycles under increasing influent sulfate concentrations of 0.25 and 0.5 $gSO_4^{-2}$ $l^{-1}$ for inoculum acclimatization. Domestic sewage (Table 2) at different volumes depending on the desired influent sulfate concentration was used to dilute the sulfate-rich industrial wastewater. In this phase, domestic sewage was the only electron donor used for sulfate reduction and the COD/sulfate ratio were 2.13±0.35 and 1.89±0.65, respectively.

Afterwards the reactor was fed with 1.0 (20 cycles), 2.0 (8 cycles) and 3.0 $gSO_4^{-2}$ $l^{-1}$ (12 cycles) corresponding to sulfate loading rates of 0.65 to 1.90 $kgSO_4^{-2}$/cycle during 40 cycles. Recirculation was provided by a centrifugal pump with capacity for 3.5 $m^3$ $h^{-1}$.Ethanol was used as the main

electron donor for sulfate reduction. The added volume varied according to the sulfate removal efficiencies obtained for the different COD/sulfate ratios applied aiming at maximizing the sulfate reduction efficiency. The COD/sulfate ratios applied in the ASBBR reactor were 1.77±0.26 (1 $gSO_4^{-2}$ $l^{-1}$), 1.64±0.40 (2$gSO_4^{-2}$ $l^{-1}$) and 1.50±0.25 (3$gSO_4^{-2}$ $l^{-1}$).

*Reactor monitoring*

Sulfate and COD removal efficiency were monitored during 40 cycles. Temporal profiles for the three different operational conditions (1.0, 2.0 and 3.0 $gSO_4^{-2}$ $l^{-1}$) were carried out after the ASBBR reactor had achieved stability, keeping the high sulfate reduction efficiency of approximately 99%. Samples for analyses were taken from the suction pipe of the recirculation pump during 48 h (cycle time).

Analysis of sulfate, COD (total), pH, total dissolved sulfide (TDS) were performed according to the Standard Methods [14] during temporal profiles. Volatile fatty acids (VFA) as acetic acid and ethanol concentrations were determined by gas chromatography in equipment HP 6890, with a HP-INNOVAX (30m x 0.25mm x 0.25µm) column and $H_2$ as the carrier gas [15] to get the temporal profile data.

*Mathematical model*

The generation of simplified mathematical model (Figure 2) involved five reactions (1, 2, 3, 4 and 5) representing the main metabolic pathways for acidogenesis, methanogenesis and sulphidogenesis in presence of ethanol as organic source.

$$Ethanol \xrightarrow{k_{1A}} Acetate + H_2 \ (acidogenesis) \quad (1)$$

$$Acetate \xrightarrow{k_{1M}} CH_4 + CO_2 \ (methanogenesis) \quad (2)$$

$$Sulfate + Ethanol \xrightarrow{k_{1S}} Acetate + H_2S \ (sulphidogenesis) \quad (3)$$

$$Sulfate + Ethanol \xrightarrow{k_{2S}} H_2S + CO_2 \ (sulphidogenesis) \quad (4)$$

$$Sulfate + Acetate \xrightarrow{k_{3S}} H_2S + CO_2 \ (sulphidogenesis) \quad (5)$$

Differential equations (6, 7 and 8) were proposed to represent first order kinetic model of these metabolic pathways based in the simplified mathematical model. The differential equations were

solved by applying the Method of Runge-Kutta (4th order) using the program developed in Microsoft Excel® [16]. The first order kinetic parameters were obtained by minimizing the differences between the theoretical and experimental data (sulfate, ethanol and acetic acid).

$$\frac{d[Sulfate]}{dt} = -k_{1S}.[Sulfate] + k_{2S}.[Sulfate] + k_{3S}.[Sulfate^{-}] \quad (6)$$

$$\frac{d[Ethanol]}{dt} = -k_{1A}.[Ethanol] + k_{1S}.[Sulfate] + k_{2S}.[Sulfate^{-}] \quad (7)$$

$$\frac{d[Acetic]}{dt} = +k_{1A}.[Ethanol] + k_{1M}.[Acetic] + k_{3S}.[Sulfate] + k_{1S}.[Sulfate^{-}] \quad (8)$$

The theoretical data were used to adjust in experimental profiles of sulfate, ethanol and acetic acid obtained from the operational conditions in the ASBBR (1.0, 2.0 and 3.0 $gSO_4^{2-}\ l^{-1}$). The kinetic parameter obtained from the mathematical model can predict the metabolic routes developed by sulfate-reducing bacteria (SRB), acidogenic bacteria (AB) and methanogenic archaea (MA) in the several sulfate concentration applied. If any kinetic parameter value was equal to zero, the corresponded metabolic route was disconsidered.

*Molecular analysis*

Microbial diversity was assessed using 16S rRNA clone library obtained from biofilm samples collected inside the reactor subjected to influent sulfate concentrations of 1.0, 2.0 e 3.0 $gSO_4^{2-}\ l^{-1}$ at the end of the respective trial. Microbial biomass was obtained by washing the mineral coal matrices with PBS-buffer and total bacterial DNA was then extracted as described elsewhere [17]. PCR amplification were carried out using the universal bacterial primes 27 f ( 5`AGA GTT TGA TCC TGG CTC AG 3`) and 907r (5`CCG TCA ATT CCT TTG AGT TT 3`) [18] and archaeal primers 1100f (5´ AAC CGT CGA CAG TCA GGY AAC GAG CGAG 3`) and 1400R (5`CGG CGA ATT CGT GCA AGG AGC AGG GAC 3`) [19]. The 16S rRNA fragments were cloned into the plasmid pCR 2.1 TOPO-TA easy vector system, and transformed into *E. coli* DHα5 as suggested by the manufacturer (Invitrogen®). Clones were randomly selected from original 300 colonies for bacteria and archaea and they were screened for positive inserts with M13 primers

according to the manufacturer instructions. A total of 100 randomly chosen positive clones, for each operational condition, were sequenced in ABI 377 DNA Sequencer (Perkin-Elmer) using M13 primers (forward and reverse, separately). The resultant nucleotide sequences were assembled, checked for potential chimerical sequences and compared with the electronic database in order to identify the closest matches (Ribosomal Database Project) [20]. The ribossomal 16S DNA fragments were grouped to produce a phylogenetic tree for the domain bacteria showing the evolutionary distance between the cloned sequences and sequences of type category downloaded from the Ribosomal Database Project (RDP). The tree was constructed using PAUP and Kimura 2-parameter algorithm. Bootstraps values higher than 90 were not displayed in the tree. Shannon and evenness indexes were calculated using observed frequencies within the clone library [21].

## 3. Results and discussion

During start-up, which consisted of an acclimation period of 30 cycles, ASBBR showed averages of sulfate reduction efficiencies of 97 and 99% at the initial concentrations of 0.25 and 0.50 $gSO_4^{-2}.l^{-1}$, respectively. After this period, the reactor was monitored during 40 cycles under influent sulfate concentration of 1.0, 2.0 and 3.0 $gSO_4^{-2}$ $l^{-1}$. The maximum sulfate removal rate (SRR) was of 1.6 $kgSO_4^{2-}$/cycle at the sulfate loading rate (SLR) of 1.9 $kgSO_4^{2-}$/cycle (Figure 3). Sulfate reduction efficiencies were very high (about 99%) for the concentrations of 1.0, 2.0 and 3.0 $gSO_4^{-2}$ $l^{-1}$, respectively. Effluent sulfate concentrations were never recorded exciding the value of 5 $mgSO_4^{-2}$ $l^{-1}$. On the other hand, a gradual decrease of COD removal efficiencies was observed along the experiments. The mean of removal efficiencies decreased from 70% to 41% for organic loading rates (OLR) ranging from 1.4 to 3.0 kgCOD/cycle (Figure 3). The mean values of COD concentrations were 0.72 g $l^{-1}$ (1.0 $gSO_4^{-2}$ $l^{-1}$), 1.45 g $l^{-1}$ (2.0 $gSO_4^{-2}$ $l^{-1}$) and 3.01 g.$l^{-1}$ (3.0 $gSO_4^{-2}$ $l^{-1}$) for sulfate concentration of 1.0, 2.0 and 3.0 $gSO_4^{-2}$ $l^{-1}$, respectively. In average, and with a different tendency than efficiency, organic removal rates (ORR) increased from 0.95 to 1.75 kgCOD/cycle in the trial with 1.0 and 2.0 $gSO_4^{-2}$. However, ORR decreased to 1.20 kgCOD/cycle in the trial with

3.0 $gSO_4^{-2}$. The mean value of the effluent pH also decreased from the beginning (7.1) to the end (6.7) of the period the reactor was subjected to 3.0 $gSO_4^{2-}$ $l^{-1}$.

A first order kinetic model was adjusted to the temporal profiles of sulfate, ethanol and acetic acid at the operating conditions of 1.0; 2.0 and 3.0 $gSO_4^{2}$ $l^{-1}$, respectively (Figure 4). Figure 2 shows a schematic map of potential metabolic routes within the ASBBR and Table 3 summarizes the first-order kinetic parameters which were used as input data to describe such idealized model. A direct comparison of the schematic pathways shown in Figure 2 with the kinetic parameters of Table 3 formed the basic rationale used to predict the preferential pathways. For instance, at high sulfate concentration the mathematical model suggested that acidogenesis and methanogenesis ($k_{1A}$ and $k_{1M}$ = zero, Table 2) were inhibited by sulfate concentrations and that sulfate reduction was mainly performed by incomplete sulfate-reducing activity ($k_{1S}$ = 0.0594 $h^{-1}$). According to such rational, the results obtained with the kinetic model suggested that ASBBR operated exclusively under sulphidogenic condition and, therefore, the effluent showed high concentrations of acetic acid (equation 3). On the other hand, values of non ionized sulfide concentration were below the reported inhibitory values of 50-250 mg/L, for methanogens and 50-550 mg/L for SRB [22]. Additionally, Celis-García et al. [23] reported that a sulfidogenic biofilm could operate under total sulfide concentrations as high as 1,200 mg/L and any toxic effect due to sulfide concentrations at the prevailing pH values obtained seemed to be unlikely for SRB or methanogens. On the other hand, the former authors report experiments carried out in continuous-flow reactors without coal as support material. In this work, the operating system using batch reactor with recirculation may be the cause of inhibitory effect at lower sulfide concentration. Recirculation in batch systems increases the risk of sulfide toxicity, particularly at the adopted high up-flow velocity of 22 m $h^{-1}$.

Microbial analysis identified 32 distinct operating taxonomic units (OTUs) retrieved from a clone library containing 100 entries for each operating condition of sulfate loading rates (1.0, 2.0 and 3.0 $gSO_4^{2}$ $l^{-1}$, respectively) and only one recurrent clone of a methanogenic archaea was observed in the treatments with initial concentrations of 1.0 and 2.0 $gSO_4^{2}$ $l^{-1}$. Figure 5 shows OTUs

phylogenetic associations and Figure 6 shows their frequencies in their respective clone libraries; which were obtained from the reactor's biomass sampled at the end of each trial separately (1.0, 2.0 and 3.0 $gSO_4^{-2}$ $l^{-1}$ respectively). The majority of the OTUs were either associated to the group I (non-acetate oxidizers) or group II (acetate oxidizers) of the sulfate-reducing bacteria.

The molecular inventory suggests a decrease of the microbial diversity with the increase of sulfate concentration. The values of Shannon and Evenness indexes were of 1.09 and 0.39; 1.20 and 0.38, and 0.79 and 0.35 for the sulfate concentrations of 1.0, 2.0 and 3.0 $gSO_4^{2}$ $l^{-1}$, respectively. Although sulfate removal efficiencies were high (about 99%) during the trials, variations in the OTUs frequencies suggested a significant shift in bacterial species.

Considering the operational condition of 1.0 and 2.0 $gSO_4^{2}$ $l^{-1}$, the predominant bacterial groups were affiliated with *Beta-proteobacteria* (*Aminomonas* spp. and *Thermanaerovibrio* spp.) and *Delta-proteobacterias* (*Desulfovibrio* spp. and *Desulfomicrobium* spp.). In such operational conditions, it was also observed the presence of methanogenic archaea (99% of similarity with *Methanosaeta* sp. NCBI AY 454768). Possible combinations between physiologies of these groups suggest a potential syntrophic interaction between some species of sulfate-reducing bacteria and methanogenic archaea in the trials at initial concentrations of 1.0 and 2.0 $gSO_4^{2}$ $l^{-1}$. Such organisms are commonly associated to the group of "Synergists" [24]. The contribution of synergists in the performance of anaerobic reactors has not been fully explored [25]. Some species are capable of growing on amino acids (*Aminomonas paucivorans* [26], *Thermanaerovibrio acidovorans* [27], *Aminobacterium mobile* [28] and *Aminobacterium colombiense* [29]); and others (*Thermanaerovibrio velox* [30] and *Anaerobaculum* sp.) may show preferences for carbohydrates [31]. Despite the fact that those distinct species show different metabolic preferences at certain conditions, their presence and frequency in the sample (Figure 6) correlate with the degradation pathway suggested by coefficient $k_1a$ (0.206 $h^{-1}$) which reflect the conversion of the ethanol to acetic acid at high rates in Period I by such type of acidogenic organisms (Table 3).

Temporal profiling of acids during each batch cycle suggested a higher rate of acetate accumulation in the first hours of the cycle against their overall consumption rates (500 and 1200 mg $l^{-1}$) for the initial concentrations of 1.0 and 2.0 $gSO_4^2$ $l^{-1}$, respectively, (Figure 4a and 4b). The kinetic coefficient $k_{1M}$ for the respective former initial sulfate concentrations (0.0637 and 0.0378 $h^{-1}$, Table 3), may be associated to the activity of methanogenic species such as *Methanosaeta* spp.; which were organisms identified in the respective samples.

It is known that syntrophic acidogenic bacteria benefit from the activity of SRB that oxidize hydrogen, once the former assist on the maintenance of low partial pressure of this gas within the reactor. Several of the observed OTUs matched organisms that are capable of hydrogen oxidation (*Desulfovibrio* spp. and *Desulfomicrobium* spp). Furthermore, syntrophic acidogenic bacteria may use ethanol as electron donors and such activity could result in the accumulation of acetate. The potential existence of such metabolic pathway in the trials was explored by the results obtained with coefficient $k_{1S}$ (Table 3 and Figure 2). For instance, the coefficient $k_1a$ of 0.2064 $h^{-1}$ for concentration of 1.0 $gSO_4^{2-}$ $l^{-1}$ may refer to $H_2$ consumption by litotrophic bacteria [32]. *Desulfomonile* spp. OTUs were observed at initial concentration of 2.0 $gSO_4^{2-}l^{-1}$. These types of organisms are acetate oxidizers and such metabolic pathway was described by coefficient $k_{3S}$; which was significantly higher in the phase 1 (Table 3). In the same way, higher frequency of *Desulforhabdus* spp in clone library 2 may correlate to the complete oxidation of ethanol [9] which was at peak on Period II (coefficient $k_{2S}$, Table 3).

At initial concentrations of 3.0 $gSO_4^2$ $l^{-1}$ the influent showed significant increase in TDS and in the concentration of volatile acids (as acetic acid) (Figure 4c) and as methanogenesis was inhibited methanogenic organisms were not detected in the clone library. The decrease in the organic matter removal rates were probably related to the accumulation of acetic acid in the reactor [33, 34] caused primarily by inhibition of methanogenesis. Inhibition was a result of the increase in the concentration of non-ionized sulfide ($H_2S$) from 55 to 177 mg $l^{-1}$ (TDS: 132 to 287 mg $l^{-1}$) during treatment of high sulfate concentrations. Furthermore, it has been reported that the outcome of

sulfide inhibition depends not only on the pH, which is directly related to the $H_2S$ concentration, but also on the TDS concentration and the biomass characteristics [35]. This suggests that both TDS and $H_2S$ may promote an inhibitory effect on the organisms (SRB and MA). The decrease of pH values in the effluent at initial concentration of 3.0 $gSO_4^{2-}$ $l^{-1}$ (from 6.7 to 5.6) suggests the presence of non-dissociated $H_2S$ as the main form of sulfur. Figure 4d shows the variations in the $H_2S$ for the trial with initial concentrations of 1.0, 2.0 and 3.0 $gSO_4^{-2}$ $l^{-1}$ and maximum of $H_2S$ concentration were of 115 mg $l^{-1}$ ($H_2S$/TDS=0.51), 138 mg $l^{-1}$ ($H_2S$/TDS=0.45) and 194 mg $l^{-1}$ ($H_2S$/TDS=0.88), respectively. Therefore, the inhibitory concentration of methanogenesis (3.0 $gSO_4^{-2}$ $l^{-1}$), was coincidental with the predominance of non-dissociated $H_2S$ (profile-88%), but it did not correlate with the TDS concentration, as it was also observed elsewhere [36]. This suggests that $H_2S$ exerted higher inhibitory effect on the methanogenic organisms than on the sulfate-reducing bacteria and this result was observed at sulfate concentrations equal or higher than 2.0 $gSO_4^{-2}$ $l^{-1}$.

It is known that the competition between SBR and MA is determined by the COD/sulfate ratios. According to some authors, the boundary determining a truly sulfate-reducing environment occurs on a COD/sulfate ratio of 0.67 [37]. On the other hand, there is some controversy between the applications of such COD/sulfate ratios when applied to biological treatment of complex wastes. The combination of organic substrates and of microbial species may affect the final ratios of COD to sulfate concentration. It was observed a significant shift in the microbial species in the different treatments (Figure 6). The frequency and diversity of moderate thermophilic fermentative bacteria decreased with increasing sulfate concentrations. Although *Dysgonomonas* spp. and *Coprotermobacter* spp. are associated to fermentative bacteria [38, 39], the mathematical model showed that their potential activity did not affected the main metabolic pathways described by coefficient $k_{1S}$ (0.0594 $h^{-1}$) and $k_{3S}$ (0.01357 $h^{-1}$), respectively (Table 3). The absence of acetoclastic methanogenic organisms and the accumulation of acetate, highlighted by the coefficient $k_{1S,}$ can be associated to the appearance of *Desulfovibrio* spp., which are acetate producing bacteria. On the other hand, the appearance of *Desulfurella* can be also associated with

the complete oxidation of acetate as highlighted by the coefficient $k_{3S}$. This genus include acetate moderate thermophilic oxidizer which are capable of completely mineralize ethanol, lactate [40] and acetate as sole carbon.

## 4. Conclusions

These experiments showed that full-scale ASBBR reactors filled with mineral coal is an effective alternative for treating sulphate-rich wastewater. The reactor achieved significant sulfate reduction efficiencies (99%) in a short period of operation at different sulphate initial concentrations (1.0 to 3.0 $gSO_4^{2-}\ l^{-1}$). Mineral coal was an effective inert support for biomass attachment, In addition, it can be concluded that this reactor configuration and operating parameters favour removal of sulphate at high rates. The kinetic parameters of the adopted model described the experimental data and, in conjunction to the microbial characterization, they suggested that at initial concentrations of 1.0 and 2.0 $gSO_4^{2-}\ l^{-1}$ both sulphidogenesis and methanogenesis may have occurred simultaneously. The observed kinetic parameters used for considering metabolic relations among acidogenic bacteria, acetoclastic methanogenic archaeas and sulfate-reducing bacteria of the group I (non acetate oxidizers) and of the group II (acetate oxidizers) showed significant parallel and potential for physiological interaction which culminated with high rates of sulfate removal. However, high concentrations of reduced sulfur compounds (TDS) and high residual COD were observed at influent sulfate concentrations higher than 2.0 $gSO_4^{2-}\ l^{-1}$. Under such conditions, methanogenesis was inhibited by high concentrations of undissociated $H_2S$ formed during the sulfate reduction process. At 3.0 $gSO_4^{2-}\ l^{-1}$, the microbial diversity index was lower than in other tested concentrations indicating a significant shift in the community occurred as a result. On the other hand, as no decrease of sulfate removal efficiencies was observed, it is possible that the selected organisms were metabolically adapted which helped to sustain the functional stability of the system.

The bacterial populations and the patterns of the main metabolic pathways observed in this work shown that the functional stability in terms of sulfate reduction does not imply microbial

community stability. It was observed that a dynamic community was able to remove sulfate at high rates and at high initial concentrations.

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**Table1.** First-order kinetic constants obtained with the model proposed for metabolic pathways in several periods (I - 1.0 $gSO_4^{2-}.l^{-1}$, II - 2.0 $gSO_4^{2-}.l^{-1}$ and III-3.0 $gSO_4^{2-}.l^{-1}$).

| Constants ($h^{-1}$) | Period I | Period II | Period III |
|---|---|---|---|
| $k_{1S}$ | 0,000437 | 0,095578 | 0,059420 |
| $k_{2S}$ | 0,000235 | 0,009219 | 0,000000 |
| $k_{3S}$ | 0,062894 | 0,010000 | 0,013569 |
| $k_{1A}$ | 0,206439 | 0,000000 | 0,000000 |
| $k_{1M}$ | 0,063724 | 0,037798 | 0,000000 |

## Figure captions

**Figure 1.** Schematic representation of operation ASBBR containing biomass immobilized in mineral coal.

**Figure 2.** Figure 2: Schematic model proposed for metabolic pathways.

Where:

$k_{1S}$ - first-order kinetic constant that represents the reduction of sulfate by microorganisms reducing sulfate using ethanol (partial oxidation) and generating acetic acid and $H_2S$;

$k_{2S}$ - first-order kinetic constant that represents the reduction of sulfate by microorganisms reducing sulfate using ethanol (complete oxidation) and generating $CO_2$ and $H_2S$;

$k_{3S}$ - first-order kinetic constant that represents the reduction of sulfate by microorganisms reducing sulfate using acetic acid (complete oxidation) and generating as final products $CO_2$ and $H_2S$;

$k_{1A}$ - first-order kinetic constant that represents the conversion of ethanol to acetic acid and $H_2$ by the acidogenic bacteria;

$k_{1M}$ - first-order kinetic constant that represents the conversion of acetic acid to $CO_2$ and $CH_4$ by methanogenic archaea.

**Figure 3.** Mean values of SLR (Sulfate loading rate), SRR (Sulfate removal rate), OLR (Organic loading rate) and ORR (Organic removal rate) by cycle in several periods (I -1.0 $gSO_4^{2-}\ l^{-1}$, II - 2.0 $gSO_4^{2-}\ l^{-1}$ and III-3.0 $gSO_4^{2-}\ l^{-1}$).

**Figure 4.** Temporal profiles of sulfate (●), ethanol (■) and acetic acid (▲) obtained experimentally for sulfate concentration of 1.0 $gSO_4^{2-}\ l^{-1}$ (a), 2.0 $gSO_4^{2-}\ l^{-1}$ (b), 3.0 $gSO_4^{2-}\ l^{-1}$ (c) by first order model adjusted (-·-·-·- sulfate, ▬ ethanol and ····· acetic acid) and sulfide concentration (1.0 $gSO_4^{2-}\ l^{-1}$ (▲), 2.0 $gSO_4^{2-}\ l^{-1}$ (■), 3.0 $gSO_4^{2-}\ l^{-1}$ (●)).

**Figure 5.** Phylogenetic tree of distance showing similarities between the assessed OTUs (about 880 pb) for domain *Bacteria* and some sequences of type category (full name) downloaed from the ribosomal database project website. The numbers beside clones (1, 2 and 3) refers to the operational conditions ((1.0, 2.0 e 3.0 $gSO_4^{-2}\ l^{-1}$) respectively. The tree was constructed using PAUP and Kimura 2-parameter algorithm (bootstraps values higher than 80 were not shown). The scale bar represents 5% changes per nucleotide.

**Figure 6.** Histogram showing the frequencies (%) of the cloned 16S rRNAs obtained from the bacteria biomass sampled at the end of the trial (1.0, 2.0 and 3.0 $gSO_4^{-2}\ l^{-1}$) and the affiliations with

the sequences of type category (full name) downloaded from the Ribosomal Database Project.

Figure 1

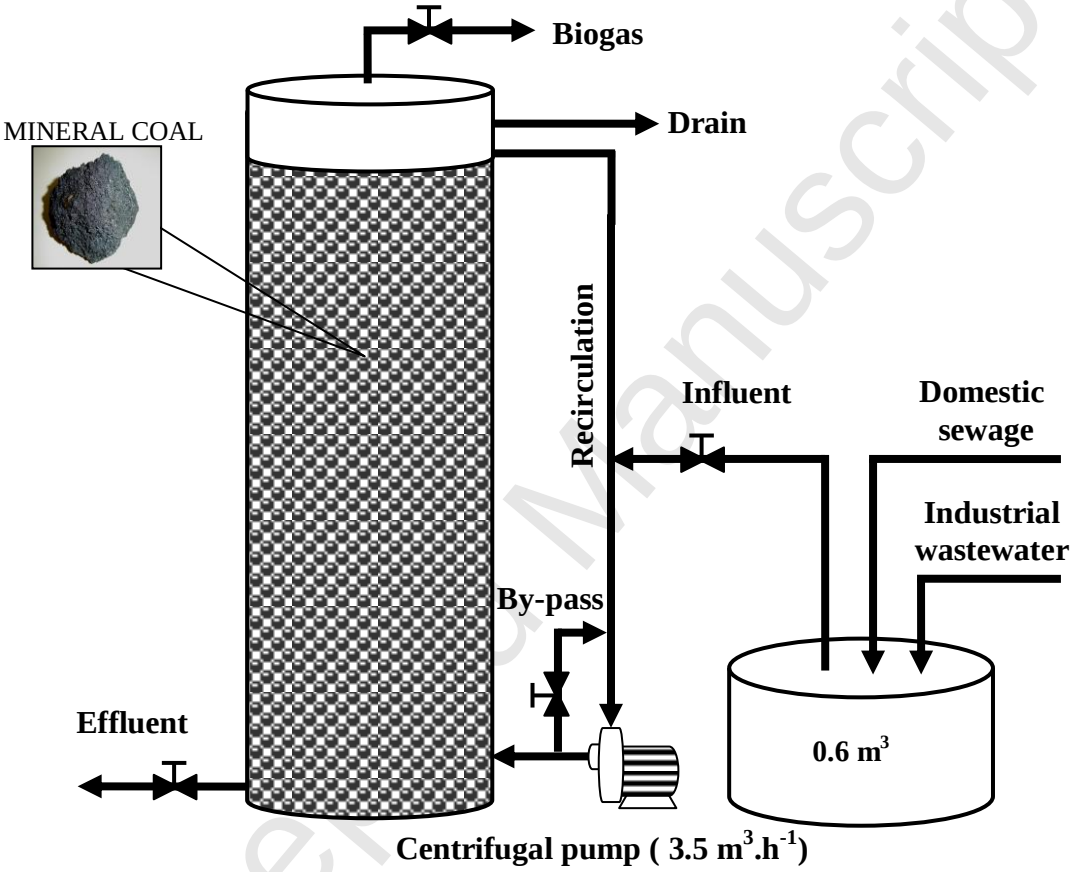


Figure 2

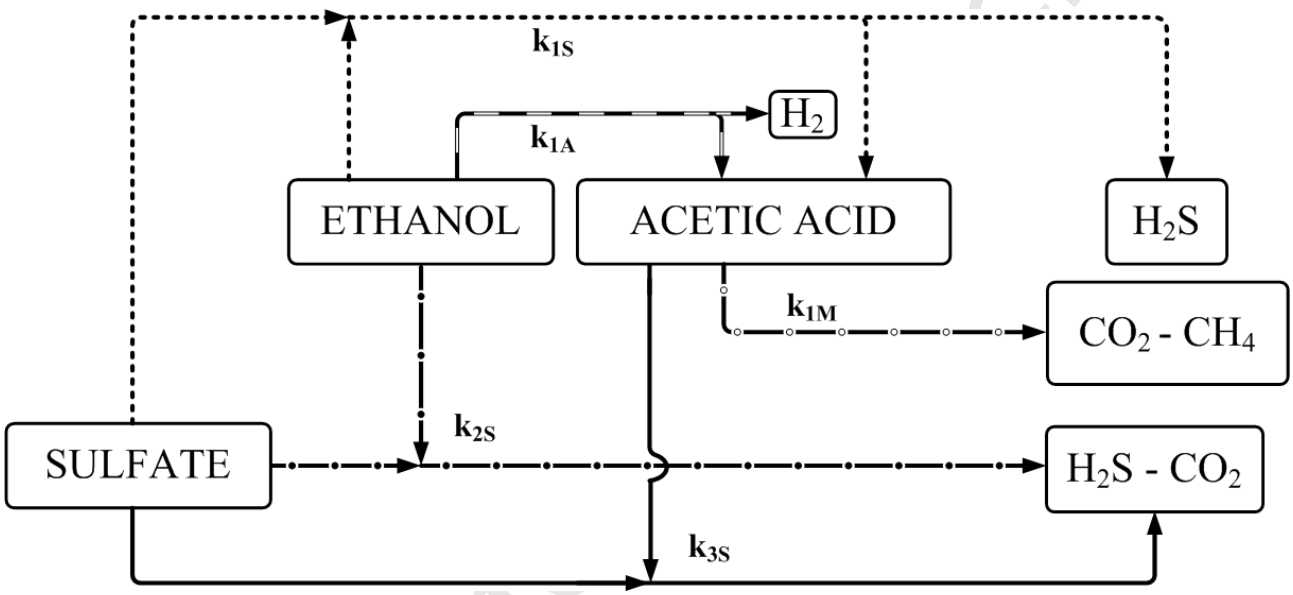
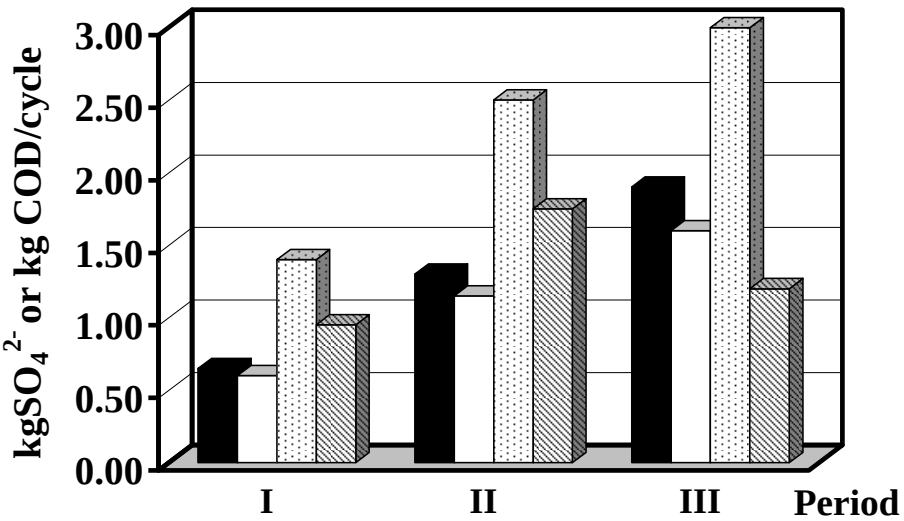
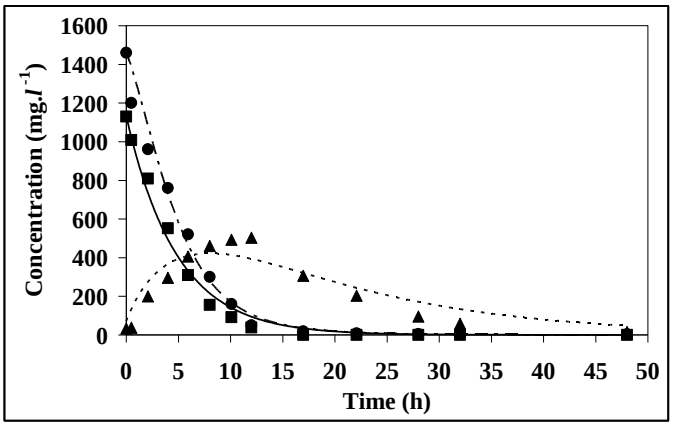


Figure 3

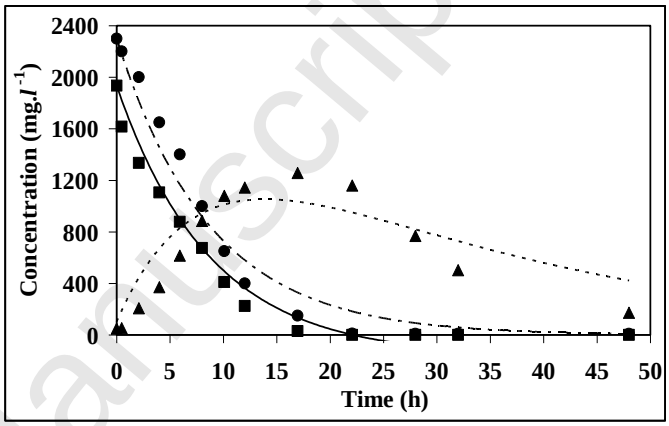



| | I | II | III |
|---|---|---|---|
| SLR | 0.65 | 1.30 | 1.90 |
| SRR | 0.60 | 1.15 | 1.60 |
| OLR | 1.40 | 2.50 | 3.00 |
| ORR | 0.95 | 1.75 | 1.20 |

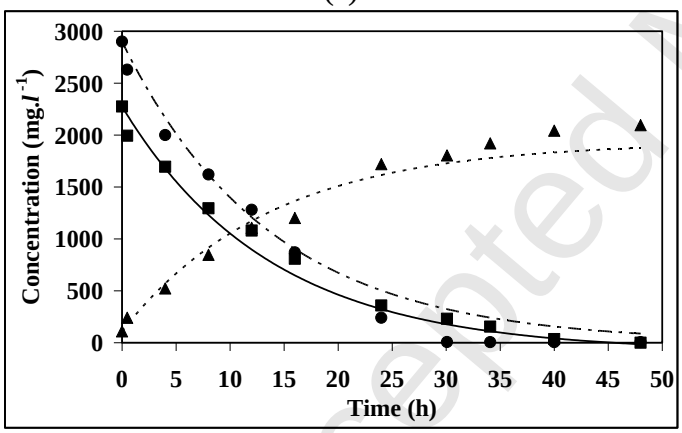
Figure 4



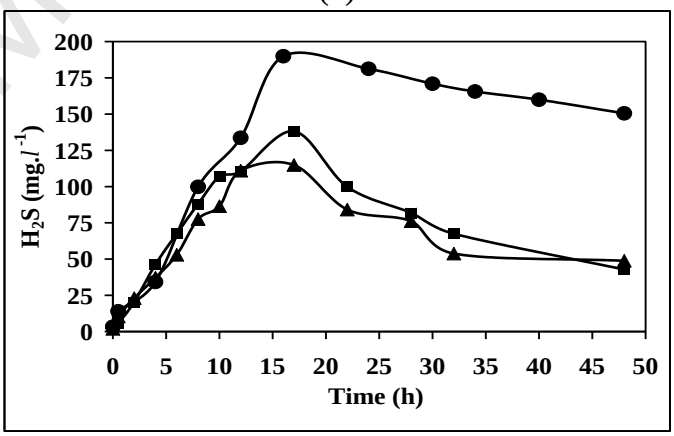

(a)




(b)




(c)




(d)

Figure 5

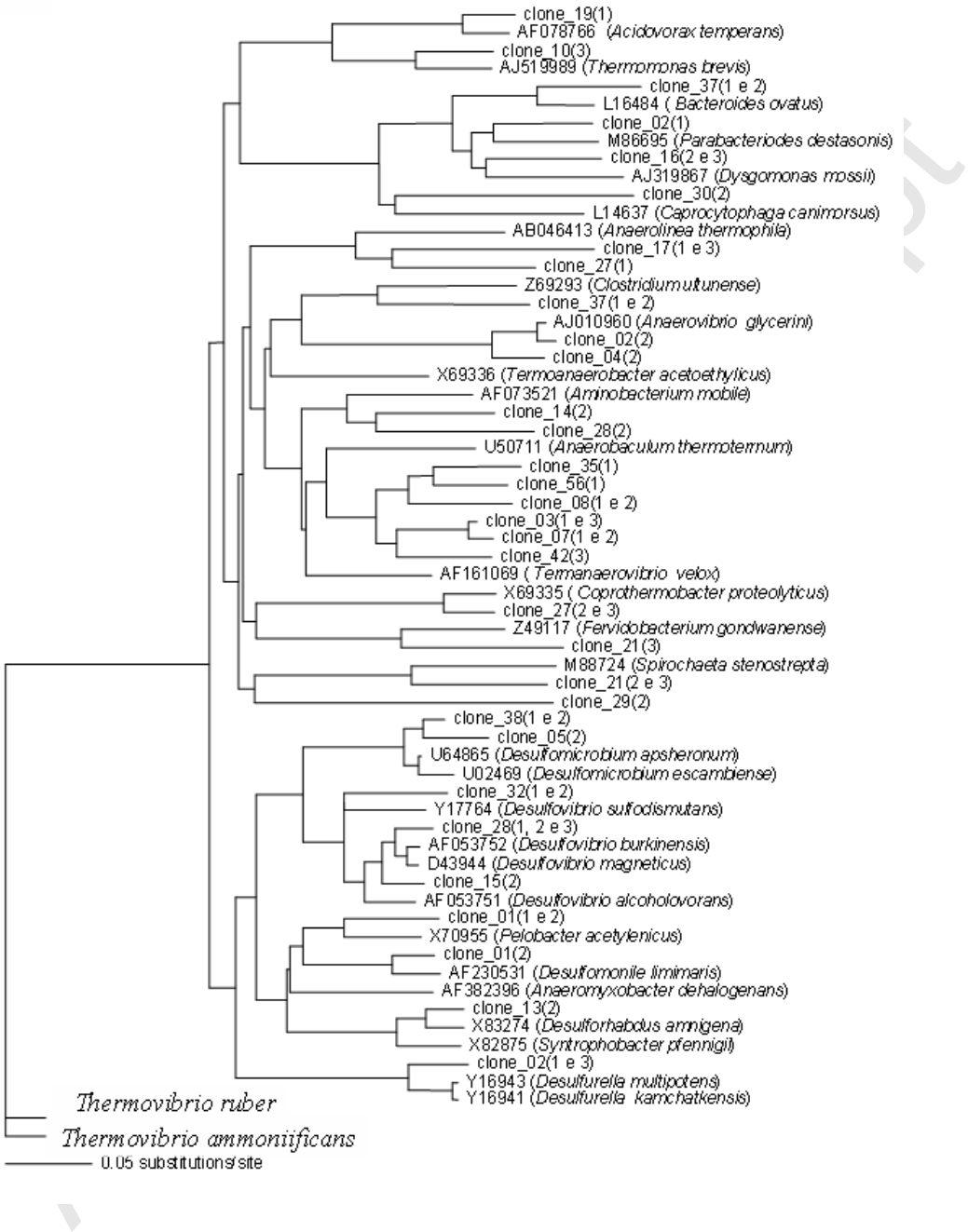


Figure 6

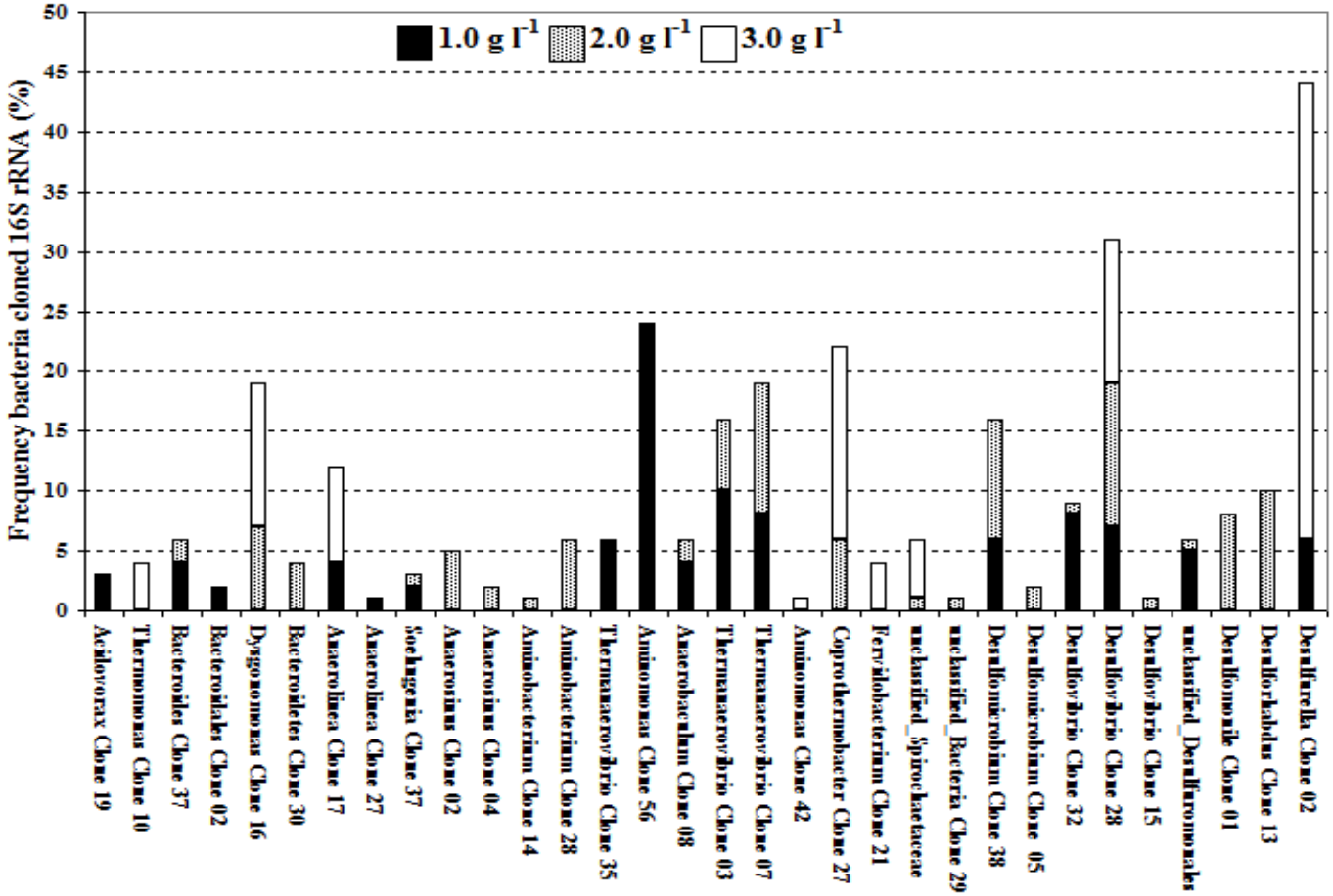


**Table1.** Characteristics of the industrial wastewater (20 samples)

| Variables | Minimum | Maximum | Mean |
|---|---|---|---|
| pH | 2.31 | 3.25 | - |
| $COD_{Total}$ ($g.l^{-1}$) | 9.24 | 15.43 | 13.7±4.1 |
| $COD_{Filtered}$ ($g.l^{-1}$) | 8.98 | 10.90 | 10.6±1.3 |
| $NH_4^+$ ($g.l^{-1}$) | 1.32 | 1.87 | 1.52±0.5 |
| $SO_4^{-2}$ ($g.l^{-1}$) | 183 | 284 | 201±35 |

**Table2**. Characteristics of the domestic sewage (50 samples)

| **Variables** | **Minimum** | **Maximum** | **Mean** |
|---|---|---|---|
| Temperature (ºC) | 15 | 25 | 21±2 |
| pH | 6.6 | 7.7 | - |
| BA ($mgCaCO_3^{-2}.l^{-1}$) | 84 | 206 | 130±24 |
| VFA ($mgHac.l^{-1}$) | 25 | 59 | 45±13 |
| $COD_{Total}$ ($mg.l^{-1}$) | 406 | 860 | 569±112 |
| $COD_{Filtered}$ ($mg.l^{-1}$) | 173 | 307 | 243±33 |
| $N_{Total}$ ($mg.l^{-1}$) | 18 | 66 | 41±5 |
| $PO_4^{-2}$ ($mg.l^{-1}$) | 12 | 19 | 14±2 |
| $SO_4^{-2}$ ($mg.l^{-1}$) | 10 | 31 | 24±8 |
| TSS ($mg.l^{-1}$) | 83 | 269 | 131±44 |
| VSS ($mg.l^{-1}$) | 68 | 209 | 105±34 |

**Table3.** First-order kinetic constants obtained with the model proposed for metabolic pathways in several periods (I - 1.0 $gSO_4^{2-}.l^{-1}$, II - 2.0 $gSO_4^{2-}.l^{-1}$ and III-3.0 $gSO_4^{2-}.l^{-1}$).

| Constants ($h^{-1}$) | Period I | Period II | Period III |
|---|---|---|---|
| $k_{1S}$ | 0,000437 | 0,095578 | 0,059420 |
| $k_{2S}$ | 0,000235 | 0,009219 | 0,000000 |
| $k_{3S}$ | 0,062894 | 0,010000 | 0,013569 |
| $k_{1A}$ | 0,206439 | 0,000000 | 0,000000 |
| $k_{1M}$ | 0,063724 | 0,037798 | 0,000000 |