

**Potential of industrial by-products and wastes from the Iberian Peninsula as
carbon sources for sulphate reducing bacteria**

Short Title: Potential of industrial by-products and wastes as carbon sources for SRB

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9 **for sulphate reducing bacteria**
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12 **Abstract**

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14 Industrial by-products and wastes from Portugal and Spain were tested for the first time as carbon
15 sources/electron donors for sulphate reducing bacteria. Cultures in mineral medium supplemented with the tested
16 substrates were monitored and sulphate reduction efficiency is discussed in light of substrates compositions, dosages and
17 corresponding chemical oxygen demand/[SO₄²⁻] ratios. The use of doses targeting a ratio of 1.5 was a good strategy to
18 optimize sulphate reduction activity. As expected, this activity was faster for substrates that have in their composition
19 simple compounds such as low chain alcohols and organic acids and/or compounds that can be rapidly degraded such as
20 sugars, though it also occurred in a longer-term perspective with substrates composed mainly of slowly degradable
21 compounds such as cellulose and lignin. Among eighteen tested substrates, six supported high sulphate reduction
22 efficiency during incubation periods varying between two and four weeks (sugared water from a factory of candies,
23 beetroot molasses, olive mill wastewaters not decanted and decanted, orange molasses without conservative and
24 municipal wastewater from Mina de São Domingos, Portugal), while eight substrates sustained moderate sulphate
25 reduction efficiency during periods from three to seven weeks (water from washing beetroots, Carbocal®, orange
26 molasses with conservative, liquor extracted from orange peels, orange peel fragments, water from washing industrial
27 equipments used to produce orange juice, pine nut shells and pine cone fragments). Nevertheless, after four months of
28 incubation, total sulphate removal was observed with three of the solid substrates tested (orange peels, pine nut shells and
29 pine nut cones).
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42 **Keywords**

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44 Chemical oxygen demand/[SO₄²⁻] ratio; electron donors; organic substrates; sulphate reduction
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48 **Introduction**

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50 Sulphate reducing bacteria (SRB) comprises a diverse group of microorganisms with the ability to obtain energy
51 through anaerobic respiration by dissimilatory sulphate reduction, in which sulphate is the oxidising agent (electron
52 acceptor) for the oxidation of certain compounds (electron donors), resulting in the release of sulphide. The resistance of
53 SRB to extreme conditions and the fact that sulphide reacts with several metals forming precipitates have made these
54 microorganisms known for their potential in bioprocesses to treat waters contaminated with metals and sulphate, such as
55 acid mine drainage (AMD) (*e.g.* Costa et al. 2017; Dev et al. 2017; Miran et al. 2017; Muhammad et al. 2017). However,
56 these waters are usually poor in compounds used by SRB as carbon sources and electron donors to obtain energy, thus
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1 their biological treatment requires addition of such products (e.g. Lefiticariu et al. 2015; Vasquez et al. 2016). Therefore,
2 the efficiency and economic viability of a SRB based bioremediation process depends largely on the substrate used as
3 carbon source and/or electron donors to feed bioreactors. The substrate must have compounds that can be directly used in
4 the anaerobic respiration of SRB and/or others that despite not being immediately viable can be relatively rapidly
5 decomposed into suitable ones.
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8 Organic compounds resulting from fermentation processes, such as ethanol and methanol, as well as others such
9 as volatile fatty acids (formic, acetic, propionic, butyric) and short chain fatty acids (lactic, pyruvic, malic) or just
10 hydrogen or monoxide carbon, can be used as carbon sources and/or electron donors by SRB (e.g. Liamleam and
11 Annachatre 2007; Parshina et al. 2010). However, the high costs associated to purchasing and handling such compounds
12 in pure forms hamper their use in large scale operating treatment processes. The chemical reactions involved in the
13 metabolic transformation of compounds that can be used as energy sources by SRB have been described for example by
14 Cao et al. (2012) and benefits and drawbacks for known possible electron donors in biological wastewater treatment
15 processes based on sulphate reduction were reviewed by Hao et al. (2014).
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21 Over the years several low-cost industrial by-products rich in carbon compounds have been successfully tested
22 as sources of electron donors for SRB, as for example sugar cane molasses and bagasse from the sugar processing
23 industry (e.g. Michailides et al. 2015; Hussain and Qazi 2016). On the other hand, the use of zero-cost wastes to sustain
24 SRB bioremediation processes is even more interesting from the economic point of view. Moreover, the use of wastes to
25 treat waters contaminated with metals is also particularly interesting from an environmental point of view since it may
26 constitute an integrated system to treat simultaneously different pollutants. Different types of wastes rich in carbon
27 compounds, such as for example winery wastes, animal manure and grass cuttings (Martins et al. 2009; Zhang and Wang
28 2014; Mulopo 2016), have already been reported as valuable carbon sources/electron donors for SRB. A different
29 approach is the use of SRB for co-treatment of different wastes. According to Li et al. 2017, AMD was used as a source
30 of sulphate to strengthen the anaerobic treatment of landfill leachate. The results obtained showed high removal
31 efficiency of chemical oxygen demand (COD), methane production and also heavy metals removal.
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38 The Iberian Pyrite Belt (IPB), located in the south-west of the Iberian Peninsula (Figure 1), is one of the largest
39 metallogenetic provinces of massive polymetallic sulphides in the world. The mining activity in this region goes back
40 thousands of years and has been very intense in the last two centuries, having produced considerable amount of residues
41 which deteriorate the environment of the mining areas and surroundings, mainly due to AMD. This is an extremely acid
42 leachate with high contents of sulphate and metals produced when the piles of mine tailings, particularly those from
43 metallic sulphides extraction, are exposed to air and water (Lowson 1982). The impact of AMD on the environment in
44 the IPB region is still huge nowadays, especially in large mines left abandoned after the exploration period, such as the
45 case of a mine in a small village named Mina de São Domingos, located in Southeast Portugal on the left margin of
46 Guadiana River, near the Spanish frontier (Figure 1). In this mine site, as in others of the IPB, the AMD is not only
47 confined to the pit lake, but also affects several water bodies nearby the mining area, highlighting the need of
48 intervention aiming the remediation of AMD in those sites (Pereira et al. 2004).
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55 While it is known that many organic substrates may sustain directly or at some stage of their degradation
56 sulphate reducing activity, as said above, in practice prior to testing them is not known whether any industrial by-product
57 or waste has toxic compounds for SRB. On the other hand, an organic substrate of a type already tested but from a
58 different local or industry and/or the use of different sulphate reducing bacteria communities does not guarantee similar
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1 results to the previously reported in literature. Thus, in the present work several low-cost industrial by-products and
2 wastes generated in the Iberian Peninsula (Table 1) were tested for the first time to our knowledge as sources of carbon
3 and electron donors for the anaerobic respiration (sources of energy) of SRB. The tested products were selected based on
4 their availability, on the fact that other products with similar composition were previously successfully tested for the
5 same purpose, and/or on reported evidences of the presence of SRB in those substrates (see table 1).
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8 The research was carried out at the laboratory of Environmental Technologies of the Ecology and Restoration of
9 Riverine, Estuarine and Coastal Habitats (ECOREACH) research group of Centre of Marine Sciences, located at the
10 University of Algarve, in Portugal during 2015 and 2016. The achievements of the present study can be used for the
11 development and implementation of economically viable treatment plants for the bioremediation of AMD in the IPB
12 region, as well as in other regions where these products may also be available. In addition, the achievements obtained can
13 encourage further studies aiming the co-treatment of AMD and some of the tested wastes.
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19 **Material and Methods**

20 Substrates tested

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22 The list of tested substrates and summarized information about their compositions, obtained from published
23 literature and/or from the respective suppliers, is presented in table 1.
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26 The ratio of COD to $[\text{SO}_4^{2-}]$ in the feed is a key parameter in determining whether SRB would compete with
27 methanogens (*e.g.* Annachhatre and Suktrakoolvait 2001; Lu et al. 2017; Kiyuna et al. 2017; Jiang et al. 2018). Thus, the
28 COD/ $[\text{SO}_4^{2-}]$ ratio is commonly used to calculate the quantity of organic substrates supplied to feed bioreactors with SRB
29 (Vela et al. 2002; Neculita and Zagury 2008; Kiran et al. 2017). According to the stoichiometry of the process and
30 assuming that all the COD is from compounds that can be utilised by SRB, waters with a COD/ $[\text{SO}_4^{2-}]$ ratio of 0.67
31 contain enough sulphate to completely remove the organic matter via sulphate reduction (Vela et al. 2002). However,
32 usually not all carbon compounds present in the organic substrates are suitable for SRB. Moreover, several other factors
33 can influence the competition between SRB and other microorganisms. Therefore, when testing/selecting new substrates
34 to feed SRB based processes it is important to establish their respective optimal COD/ $[\text{SO}_4^{2-}]$ ratios (Prasad et al. 1999).
35 Taking this into account, the COD values and $[\text{SO}_4^{2-}]$ were measured in all substrates tested as carbon sources/electron
36 donors. In addition, the tested substrates were analyzed for the concentrations of phosphorous and nitrogen, two
37 important nutrients also essential for SRB growth, and for pH, a chemical parameter critical for biologic activity. For the
38 liquid substrates, dilutions in distilled water were made when necessary to fit the quantification ranges of the analytic
39 methods for COD, $[\text{SO}_4^{2-}]$, [N] and [P]. For the solids, measurements were made in samples of the aqueous phase
40 obtained after homogenization of 10% (w/v) substrate in distilled water and 24 hours leaching without stirring.
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51 Experimental design

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53 The activity of SRB using the different organic substrates as carbon sources/electron donors was tested in
54 duplicates of anaerobic batch reactors inoculated with a SRB enriched culture as follows: (1) positive controls were made
55 in original Postgate B, a medium for SRB in which lactate is the carbon source and electron donor; (2) the tests were
56 made using different quantities of each substrate added to a modified Postgate B medium prepared without lactate and (3)
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1 the negative controls were prepared in this modified medium without any carbon source/electron donor added. Moreover,
2 some tests and controls were carried out with calcite tailings as a buffering agent and others without it: after a first
3 experiment with a few substrates, in which high sugared contents caused pH drops to values not suitable for SRB, all
4 further tests and controls were carried out with supplements of calcite tailings to prevent acidification. To monitor the
5 batch reactors, 5 mL initial culture media and 5 mL of samples periodically collected with a syringe were analysed, after
6 centrifugation at 4000 rpm during 5 minutes at room temperature. Redox potential (Eh) and soluble $[\text{SO}_4^{2-}]$ and $[\text{S}^{2-}]$
7 were monitored as indicators of SRB activity. The pH was monitored due to its importance as a limiting factor for SRB
8 growth (O'Flaherty et al. 1998; Willow and Cohen 2003). Cultures were monitored weekly until $[\text{SO}_4^{2-}]$ stabilized, which
9 varied from approximately two weeks and nine weeks. Three of the tests with solid substrates (Orange peels, Pine nut
10 shells and Pine cone fragments) were additionally monitored after 4 months. A schematic representation of the
11 experiments performed is provided in figure 2.
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19 Source and enrichment of SRB community

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21 The SRB community used in these experiments was obtained from sludge collected in the first lagoon of the
22 waste water treatment plant (WWTP) passive lagoon system Faro-Olhão, Portugal. The bacteria were harvested by
23 centrifugation at 2500 g for 10 min at room temperature ($25^\circ\pm 3^\circ\text{C}$), washed with Postgate B medium, harvested again by
24 centrifugation in the same conditions and grown in Postgate B medium in anaerobic batch flasks at room temperature
25 before use in this work.
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31 Batch tests

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33 Experiments were performed in anaerobic batch reactors inoculated with 5% (v/v) of the SRB enriched culture
34 and incubated at room temperature ($25^\circ\pm 3^\circ\text{C}$). In order to achieve the anaerobic conditions: (i) the medium was purged
35 with nitrogen gas before bacterial inoculation, (ii) 5% (v/v) of liquid paraffin was added to the medium after inoculation
36 to eliminate oxygen diffusion and (iii) finally the bottles were sealed with butyl rubber stoppers and aluminium crimp
37 seals. Resazurin was added as an indicator to confirm the absence of oxygen. Tests with liquid organic substrates were
38 performed using 120 ml glass bottles containing 100 ml of medium and tests with solid substrates were done in 250 glass
39 bottles containing 200 ml of medium. Media and laboratory glass material used in the batch experiments were sterilized
40 by autoclaving. Moreover, as described in the experimental design section, some batch reactors were supplemented with
41 10% (w/v) of a neutralizing/buffering material: a powder residue from a marble stone cutting and polishing industry
42 mainly composed of magnesium calcite (*89%), quartz (*11%) and traces of illite mineral, as shown by X-ray diffraction
43 analysis (Martins et al. 2009).
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53 Substrates dosing

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55 Several studies have shown that when the COD/ $[\text{SO}_4^{2-}]$ ratio is below 1.7 the SRB prevail over the methanogens
56 (e.g. Wolicka and Borkowski 2009). Thus, it is generally accepted that in mixed populations when this ratio is lower than
57 the mentioned value the SRB have good conditions to succeed. In this work, since the tests were carried out with the
58 substrates diluted in modified (without lactate) Postgate B medium, whenever possible at least one of the tested dilutions
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of each substrate was prepared to obtain a COD/[SO₄²⁻] ratio similar to that one calculated for the original Postgate B medium (Postgate, 1984) based on its [lactate (C₃H₅O₃)] and [SO₄²⁻], which are 2.8 and 1.7 g/L, respectively.

As demonstrated by van Haandel and van der Lubbe (2007), equation 1 can be used to calculate the theoretical chemical oxygen demand (COD_t) of a compound with a structural formula C_xH_yO_z.

$$(1) \quad \text{COD}_t = 8 \cdot (4x + y - 2z) / (12x + y + 16z) \text{ grams of oxygen per gram of } C_xH_yO_z$$

Calculated with this equation, the COD_t for lactate is 0.99 g O/g C₃H₅O₃. Therefore, the COD_t for the original Postgate B medium, estimated based on its lactate concentration, is 0.99*2.8 = 2.77 g O/L. With this value the COD/[SO₄²⁻] ratio of Postgate B medium is calculated as 2.77/1.7 = 1.63 (rounded to 1.5 for simplicity).

Liquid substrates

For the liquid organic substrates, the percentage (v/v) of each substrate used to make the dilution in modified Postgate B (without lactate) in order to obtain a COD/[SO₄²⁻] ratio of 1.5 was calculated using both the COD and [SO₄²⁻] values measured for each substrate at the time of the experiment and the [SO₄²⁻] of Postgate B medium (1700 mg/L).

Trying to avoid major dilutions in the [SO₄²⁻] that would be adverse for the activity and proliferation SRB, the maximum percentage of liquid substrate tested was in general 20% (w/v), even for the cases in which the respective estimated COD/[SO₄²⁻] ratios were below 1.5 (except in the case of municipal wastewater for which a 50% (v/v) dose was tested).

Solid substrates

The quantity of solid substrates tested was decided based on COD/[SO₄²⁻] ratios calculated with the [SO₄²⁻] in Postgate B medium (1700 mg/L) and the COD and [SO₄²⁻] values measured in the initial characterization of the substrates (using 10% (w/v) of solid substrate as described above). For example, if the COD/[SO₄²⁻] ratio calculated by this way for a certain solid substrate was 3, it was assumed that the percentage of that substrate needed to make a COD/[SO₄²⁻] of 1.5 would be 5% (w/v). The maximum percentage of solid tested was 10% (w/v), even for the cases in which the respective estimated COD/[SO₄²⁻] ratios were below 1.5.

Analytical Methods

Redox potential (Eh) and pH were determined using a pH/E Meter (GLP 21, Crison). The [SO₄²⁻] and [S²⁻] were quantified by molecular UV/visible spectrophotometry using the SulfaVer4 (8051, Hach-Lange) and methylene blue (8131, Hach-Lange) methods, respectively, using a DR2800, Hach-Lange spectrophotometer. This equipment was also used to determine COD with cuvette tests for the dichromate method (LCK 514, Hach-Lange) with 2h digestion at 148°C. To quantify total nitrogen (N) and total phosphorous (P) concentrations, the persulphate acid digestion method (10072, Hach-Lange) and the molybdovanadate with persulphate acid digestion method (10127, Hach-Lange) were used, respectively. The analytical procedures for the Hach-Lange methods were those indicated by the manufacturer. As the number of independently repeated experiments is small (n = 2), rather than showing the results with error values and statistics, the means are presented with the distance to individual data points.

Results and discussion

Initial characterization of substrates

The initial characterization of the tested substrates is shown in table 2. The pH varied from acid (4.03) to alkali (9.58), highlighting the need of neutralizing the medium pH after mixing with the substrates.

In what concerns the COD, the high range variation in the tested substrates (from 190 to $\sim 1.2 \cdot 10^6$ mg/O₂/L) suggests major differences on their composition in organic compounds. This idea is reinforced by a COD increase in the substrates accompanied with an increase in their [N] and [P] (from <10 to 51136 mg/L and from <2 to 17379 mg/L, respectively), two major elements in the composition of cells and thus in biological materials. When comparing the COD and the [N] and [P] measured on the tested substrates (Table 2) with the information for these parameters obtained from the literature or from the suppliers (Table 1), in general a concordance in the magnitude of values is seen. The case that deserves comment is the COD in beetroot molasses since the measured value is much higher (more 576730 mg/O₂/L) than the value obtained from the information sent by the supplier. However, it has to be noted that the value presented in table 1 is a theoretical estimation (COD_t) based on the composition of beetroot molasses described by the supplier and it is possible that this substrate has more organic compounds than those reported, which could justify the discrepancy between the estimated COD_t and the measured COD.

Regarding the [SO₄²⁻], the variation among the tested substrates is not so wide: eleven substrates have [SO₄²⁻] below 500 mg/L, six substrates between 500 and 1000 mg/L and just one is above 1000 mg/L (2290 mg/L). Therefore, the possibility of having COD/[SO₄²⁻] ratios of 1.5 when diluting the substrates in Postgate B medium without lactate was mainly depending on the COD values of substrates.

Souring problem and pH control

In a first round of tests with beetroot molasses as the carbon source/electrons donor, in which the initial pH was neutralized with sodium hydroxide but none buffering agent was added to the cultures, in all dilutions tested the pH decreased to values (pH < 5.5) that inhibited SRB activity. This is shown by the low percentages of sulphate removal (< 10%) and low production of sulphide (< 10 mg/L) achieved in those cases (tests N.^{ers} 7 to 10 in table 3 **and in graphs in supplementary material**). That also occurred with the sugared water from the factory of candies, though only in the dilution with 20% of this substrate. In this case, the removal of sulphate was just 20% and the [S²⁻] reached was only 39 mg/L (test N.^{er} 3 in table 3 **and in graphs in supplementary material**). This phenomenon was expected for the sugar rich substrates. For example, Cao et al. (2012) reported as a major drawback of using sucrose the high content of volatile fatty acids (formic acid, lactic acid and butanedioic acid) resulting from fermentation, which create a souring problem that impacts negatively on the growth of SRB and results in lower sulphate removal rates. Indeed, **although SRB bioreactors operating under acidic conditions have already been reported (e.g. at pH 4.5, 4.0, 3.5 and 3.25 in Elliott et al. 1998), the optimum pH for the growth of most SRB has long been known to be between pH 5 and 9 (Postgate 1984). In this particular case, results previously reported indicate that SRB communities obtained from WWTP sludge collected at Faro, Portugal, as those used in this work, are active in neutral pH but loose activity in media with pH values lower than 5.5 (Martins et al. 2009). Nevertheless, the souring problem was solved maintaining the pH between 6 and 8 by adding 10% (w/v) of calcite tailings to cultures in additional tests with beetroot molasses from the sugar factory and with**

sugared water from the candies factory, as well as in all further tests with the other organic substrates (except for Carbocal® because it is a lime rich product capable of neutralizing pH by itself).

Sulphate reduction efficiency

General analysis

The results obtained with the control cultures prove the success of the followed strategy to test the substrates as carbon sources/electron donors for SRB by using Postgate B medium lacking lactate. In the positive controls with the original Postgate B medium (with lactate) the high removals of sulphate (87 and 92%) together with the high $[S^{2-}]$ reached (259 and 304 mg/L) just in 14 days, suggest high activity and proliferation of SRB, while in the negative control with the modified Postgate B (without lactate), the low sulphate removal (25%) and the low $[S^{2-}]$ achieved (9 mg/L) indicate low activity and weak or no proliferation of SRB (tests N.ºs 47 to 49 in table 3 and in graphs in supplementary material). With this in mind and considering the cultures in which the pH remained neutral throughout the incubation time, it is possible to say that the sulphate reduction efficiencies, shown by the evolution of $[SO_4^{2-}]$ and $[S^{2-}]$, varied depending on the doses and composition of the tested substrates. The cultures reached levels of sulphate reduction that were classified as high ($\geq 70\%$ sulphate removal and $\geq 100\text{mg/L}$ sulphide produced); moderate (≥ 30 and $< 70\%$ sulphate removal and $\geq 30\text{mg/L}$ sulphide produced) and low ($< 30\%$ sulphate removal and $< 30\text{mg/L}$ sulphide produced).

For the liquid substrates it was possible to calculate doses to obtain COD/ $[SO_4^{2-}]$ ratios of 1.5 for almost all the cases (Table 2). Doses close to those estimates were tested for substrates with relatively high COD values: beetroot molasses, orange molasses with conservative and without conservative, liquor extracted from orange peels, olive mill wastewater (OMW) decanted and not decanted and sugared water from the factory of candies, by descending order of COD values. For the liquid substrates with relatively low COD values (waters from washing the beetroots, waters from washing the orange juice processing equipments, all the wastewaters from the paper pulp and paper factories and the municipal wastewater from the WWTP of Mina de São Domingos) the relatively high doses calculated were not tested since they would have caused major dilutions in the $[SO_4^{2-}]$, deteriorating the optimal conditions for SRB. For the solid substrates the maximum dose tested was 10% (w/v). However, just for orange peel fragments the estimated quantity of product needed for a 24h leachate yielding a COD/ $[SO_4^{2-}]$ ratio of 1.5 was lower than 10% (w/v).

In general, high or moderate sulphate reduction efficiencies (as defined above) were achieved in the cultures with doses of substrates estimated to yield COD/ $[SO_4^{2-}]$ ratios of 1.5 based on their initial characterizations, demonstrating that this is a good strategy to follow when testing new carbon sources/electron donors for SRB. Nevertheless, when the COD and the $[SO_4^{2-}]$ measured in the beginning of experiments were used to calculate this ratio, its value was in most cases lower (between 0.37 and 0.95) than the targeted 1.5 (Table 3). The reason for this is that the COD values were in most cases lower than the expected according to the initial characterization of substrates, probably because they were stored at room temperature and some degradation of their organic compounds occurred between the initial characterization and the tests. It is worth to note that the possibility of storing the organic substrates to be used as carbon sources/electron donors at room temperature, avoiding the high energy costs associated with refrigeration, would be a huge advantage in favour of implementing SRB based bioremediation processes.

1 The bacterial populations in the inoculum were adapted to the carbon source/electron donor lactate that was
2 available in Postgate B, the growth media used for the SRB enrichment, which was also used in the positive controls.
3 Previous phylogenetic analysis of *dsr* gene sequences from a consortium also enriched from WWTP sludge collected at
4 Faro, Portugal, using Postgate B, revealed three species of genus *Desulfovibrio* (*D. desulfuricans*, *D. aminophilus* and *D.*
5 *fructosovorans*) (Martins et al. 2009), which have better growths in media with lactate than with other carbon
6 sources/electron donors. *D. desulfuricans* was described for the first time as *Spirillum desulfuricans* more than 100 years
7 ago by Martinus Beijerinck and was later reclassified (Muyzer and Stams 2008). Nutritional studies on this species have
8 shown that growth on other organic substances was poorer than on lactate (MacPherson and Miller 1963). *D.*
9 *aminophilus* was discovered on a dairy wastewater treatment plant and has also optimum growth on a medium containing
10 lactate and sulphate, despite being able to use several electron donors with sulphate as an electron acceptor, (Baena et al.
11 1998). *D. fructosovorans* was isolated for first time from estuarine sediments and differed from all other described
12 *Desulfovibrio* species by the ability to degrade fructose (Ollivier et al. 1988). Thus, the longer times to achieve the
13 maximum sulphate removals in the cultures with the substrates tested as carbon sources/electron donors, when compared
14 to the positive controls, indicate that none of those substrates is rich in lactate. In fact, although carbon sources/electron
15 donors that are directly used by SRB have been reported in the composition of some of the tested substrates, their
16 quantities are small when compared to other organic compounds present in those substrates. For example, several short-
17 chain fatty acids (lactic, formic, acetic, and propionic acids) known to be suitable as electron donors for SRB (Cao et al.
18 2012) are approximately 7% of the composition of beetroot molasses, while sugars, which are first fermented into
19 simpler products (Maree et al. 1987; Cao et al. 2012), are approximately 47% of its composition (Table 1). Hence, the
20 inoculated SRB community had to adapt to different carbon sources/electron donors and/or to new bacterial equilibria
21 and mutualistic relationships resulting from the proliferation of species capable of transforming compounds present in the
22 tested substrates into other products suitable for the SRB present in the consortium. For most of the tested substrates, the
23 graphs showing the evolution of the monitored parameters reveal stair profiles with periods of high and low (or no)
24 sulphate reduction activity along the incubation time, which clearly suggests shift points in the respective microbial
25 populations (supplementary material). The test with 5% (v/v) of orange molasses without conservative is an elucidative
26 example (Figure 3). The exceptions are sugared water from the factory of cadies and beetroot molasses, which supported
27 more continuous SRB activities and therefore faster removals of sulphate. In all cultures with these substrates high
28 removals of sulphate were reached in two weeks. In the other cases in which high removals of sulphate were also
29 achieved, that was observed just after three weeks (Table 3 and supplementary material).

46 *Liquid by-products*

48 Exceptionally, for sugared water from the factory of cadies and for beetroot molasses, the COD values measured
49 in the beginning of the experiments were higher than expected. Substrates dilutions in modified Postgate B (v/v) with
50 20% of the former and 0.25% of the second were estimated to yield a COD of 3000 mgO₂/L, although the measured
51 COD values were 3488 and 4035 mgO₂/L, respectively. This together with the [SO₄²⁻] somewhat lower than the expected
52 for Postgate B medium, resulted in COD/[SO₄²⁻] ratios close to 3 instead of 1.5, which did not prevent high SRB activity
53 (values of 79 % and 71 % sulphate reduction were obtained, respectively). In fact, for beetroot molasses a 0.5% dilution
54 (v/v) with a COD/[SO₄²⁻] ratio of 4.88 revealed an even higher sulphate reduction efficiency (85%). Initial higher COD
55 values and lower [SO₄²⁻] than expected were also measured in the positive controls, originating COD/[SO₄²⁻] ratios

1 around 2.5, but not affecting the SRB activity which remained very high in those cases. These results obtained with
2 sugared water from the factory of candies and with beetroot molasses are consistent with results obtained by other
3 authors with sugar rich substrates. For example, Wang et al. (2008) studied SRB activities using molasses wastewater
4 from a beetroot sugar refinery as the carbon source at varying COD/[SO₄²⁻] ratios. They observed that at a critical ratio
5 of 2.7, neither COD nor sulphate were in excess for a high sulphate reduction (88%), while at lower ratios of 1.9 and 0.9
6 the percentage of sulphate removal decreased to 65% and 39%, respectively. At even higher ratios of 4.5 and 3.6 high
7 removals of sulphate were still observed (>95%), though excessive production of ethanol or acetate was detected.
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10 For the other molasses tested in this work (orange molasses) the dilution supporting the highest sulphate
11 reduction activity (70%) was also 0.5% (v/v) as for beetroot molasses. However, in this case, the analysis performed in
12 the beginning of the tests indicated a much lower COD/[SO₄²⁻] ratio (<1). This may be related to the fact that when the
13 tests started this substrate had the classical odour of alcohol, indicating that some fermentation had already occurred. If
14 so, at that time the relative amount of simplest organic molecules to the amount of more complex organic molecules was
15 relatively higher than at the time of the initial characterization of this substrate. This can justify the much lower COD
16 values than expected (around 400 instead of around 1800 mgO₂/L) and consequently the lower COD/[SO₄²⁻] ratios in the
17 beginning of these tests. Moreover, this is in agreement with the fact that sulphate reduction was not inhibited since it is
18 known that SRB use small simple organic molecules to obtain energy rather than large complex ones. When orange
19 molasses with conservative CetoSTAT® TMR was used, there was also an odour of alcohol and the initial COD values
20 were also much lower than the expected based on the initial characterization of substrates. Nevertheless, in this case, the
21 sulphate reduction achieved was just moderate (49%), contrasting with the higher value (70%) obtained with orange
22 molasses without any conservative. It is evident that the conservative must have inhibited some sort of biological activity
23 that has affected the sulphate reduction efficiency.
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33 In the cultures with the liquor extracted from orange peels (a sugar rich extract used to produce the orange
34 molasses), the COD values measured when the tests started were also lower than expected based on the product
35 characterisation. Due to that, in this case, the assumed optimum dosage (corresponding to a COD/[SO₄²⁻] ratio of 1.5) of
36 this substrate was not tested. For dilutions with 1.25% and 2.5% (v/v) of this substrate, having initial COD/[SO₄²⁻]
37 ratios lower than 1, sulphate reductions were about 35%, while for dilutions with 10% substrate and with an initial COD/[SO₄²⁻]
38] ratio of 3.18, the removal of sulphate was only 11%. This suggests that in the cultures with less amount of substrate the
39 lack of organic compounds to feed the SRB prevented higher sulphate reduction efficiencies. On the other hand, when
40 more quantity of substrate was tested the SRB activity may have been inhibited by compound(s) with a putative toxic
41 effect on SRB. Eventually an intermediate substrate concentration between 2.5% and 10% will result in higher sulphate
42 reduction efficiencies.
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49 It is known for a long time that in mixed bacterial cultures the sugars can be quickly fermented and the resulting
50 products, such as ethanol and short-chain fatty acids, can then be used by SRB (Maree et al. 1987; Cao et al. 2012). Thus,
51 it is most likely that the sulphate reduction activities here discussed for the cultures with the sugar rich by-products were
52 the result of such biological dynamics. Indeed, the souring problem and pH control discussed above corroborate this idea.
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57 *Solid by-products*

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1 With Carbocal®, the initial COD and [SO₄²⁻] almost did not varied despite the wide different doses tested,
2 making COD/[SO₄²⁻] ratios ranging only from 0.73 to 0.97. Nevertheless, for this substrate the SRB activity increased as
3 the tested doses increased and a maximum of 53% sulphate removal was achieved in 40 days with the highest dose tested
4 of 10% (w/v). This can be justified by the presence of organic compounds in the solid particles of this substrate which
5 need time to be degraded into simpler molecules suitable as carbon sources/electron donors for SRB. With the three
6 tested substrates composed of relatively large solids (orange peels, pine nut shells and pine nut cones), the high sulphate
7 reductions (> 90%) achieved after four months of incubation confirm that with enough time the slowly degradable
8 organic solids are probably converted into compounds suitable for the anaerobic respiration of SRB. It has to be said that
9 in such cases dosing the substrate as a function of COD measured in the liquid phase of the medium has probably a
10 limited utility, counting mostly for the initial SRB activity based on the already solubilised compounds available.

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16 The tests with 1% and 4% (w/v) of orange peels, with initial COD/[SO₄²⁻] ratios of 0.85 and 0.94 respectively,
17 revealed high sulphate removals (>90%). However, when 8 % (w/v) of this substrate was used, yielding a COD/[SO₄²⁻]
18 ratio of 1.13, the removal of sulphate was very low (6%), indicating that in this case some compound(s) leached to the
19 medium reach a toxic level for SRB. This idea is supported by the similar behavior observed when liquor extracted from
20 orange peels was used. Orange peels are rich in sugars and have also organic acids (Table 1), one of which, malic acid,
21 already reported as suitable for SRB (Cao et al. 2012). Despite that, with this substrate just a moderate removal of
22 sulphate (47%) was observed during one month, even if almost total removal (99%) was detected after four months. This
23 occurred because this substrate is composed of solid tissues with cellulose, hemicelluloses and lignin (Table 1), which
24 are slowly and gradually degraded, allowing the organic compounds to be dissolved and available for the SRB just after
25 relatively long periods. The results obtained in this work together with the results published by Hussain and Qazi (2012),
26 who reported a sulphate reduction of 69% after fifteen days in Postgate B medium using watermelon rind powder (2%)
27 instead of lactate, indicate that fruit peels have high potential as carbon source/electron donors for medium/long term
28 remediation processes. Such processes may be useful in passive systems for the decontamination of large water bodies.
29 Indeed, in passive systems the most efficient carbon sources/electron donors are mixtures usually containing relatively
30 easily biodegradable substrates (with soluble sugars, starch, amino acids, and proteins) and recalcitrant ones (with
31 cellulose, hemicelluloses and lignin) (Neculita et al. 2007). Such passive medium/long term bioremediation processes
32 with SRB are useful for example in pit lakes with acidic sulphate rich waters formed by open-pit mining operations
33 (Castro and Moore 2000).

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44 In the case of pine cone fragments and pine nut shells, the high potential for passive medium/long term
45 bioremediation processes is even more evident as they are almost totally composed by the recalcitrant organic
46 compounds cellulose, hemicelluloses and lignin (Table 1). These complex organic polymers cannot be consumed directly
47 by known SRB; however they can be converted into simpler molecules suitable to SRB (Logan et al. 2005). Cellulosic
48 substrates have been tested as sources of organic compounds in SRB bioreactors for more than 20 years (Bechard et al.
49 1994) and the use of this type of substrates is still a focus of research. For example, Choudhary and Sheoran (2011)
50 observed relatively low sulphate removal efficiencies (25%) when testing wood chips and sawdust in synthetic AMD
51 water using retention times up to 10 days. More recently, Zhang and Wang (2014) tested the treatment of synthetic AMD
52 water amended with sawdust during 15 days, but again no significant sulphate reduction was observed in this case. Yet
53 these authors also tested this substrate as the sole carbon source for SRB in a modified Postgate C medium during 35
54 days and in this case a sulphate reduction of 50% was achieved at the end. This is in accordance with the results obtained
55 in this work when using 10% (w/v) pine cone fragments and 10% (w/v) pine nut shells in a similarly modified medium:
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1 moderate removals of sulphate (39 and 36%) were achieved after a month and high removals (97 and 99%) were
2 observed after four months (Tests Ners 45 and 46 in table 3 and in graphs in supplementary material). An interesting
3 observation done in this work is that the $[S^{2-}]$ measured in the cultures with these cellulosic substrates was always
4 relatively low (between 7 and 20mg/L), even after four months of incubation when total removal of sulphate was
5 achieved. A probable explanation for this can be the adsorption of sulphide to these substrates. Cellulose, as a natural or
6 modified cellulosic material, can be employed as adsorbent for various substances, including organic compounds, metal
7 ions, dyes, etc., and many papers have been published discussing the importance of cellulose-based adsorbents in water
8 pollution control (e.g. Hokkanen et al. 2016). Particularly regarding the adsorption of hydrogen sulphide, it is well known
9 that activated carbon prepared from cellulosic compounds has industrial relevance. Another possible justification for the
10 low $[S^{2-}]$ detected despite the high sulphate removal in the tests with the cellulosic substrates, could be the proliferation
11 of sulphidotrophic microorganisms. Perhaps it is worth to explore in future research what happened in this case, as it may
12 lead to the development of biological processes to treat waters contaminated with sulphate without an excessive
13 production of sulphide, which can be very important from an environmental point of view. In fact, in several wastewater
14 treatment plants it is necessary to install systems to capture hydrogen sulphide avoiding the release of this toxic gas to the
15 atmosphere.
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25 *Wastewaters*

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27 In the case of water used to wash the equipments from the orange juice factory, considering that its organic
28 compounds are remains of orange juice and extract from orange peels, both rich in sugars, it was expected to support
29 some SRB activity. However, since it is a highly diluted substrate it was predictable that sulphate reduction efficiency
30 would not be very high. The very low initial COD/ $[SO_4^{2-}]$ ratio of 0.37 in the beginning of the experiment suggests that
31 the lack of nutrients was the cause for just a moderate sulphate reduction efficiency.
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35 The low percentages of sulphate reduction achieved in the cultures supplemented with 20% (v/v) of any of the
36 tested wastewaters from the paper pulp and the paper factories, all with very low initial COD/ $[SO_4^{2-}]$ ratios (< 0.25),
37 reinforce the idea that low values of this ratio indicate insufficient nutrients for the reduction of all sulphate available.
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40 With water from washing beetroots, the maximum sulphate removal of 31%, achieved for cultures with 20% of
41 this substrate in approximately one month, also suggests insufficient nutrients available for SRB. However, the
42 hypothesis that more time would have been necessary for the degradation of part of the organic matter present this
43 substrate, namely as slowly degradable solid particles from the materials used to fertilize the beetroot fields and/or
44 beetroot fragments (Table 1), should be considered. This hypothesis is supported by the fact that similar initial COD and
45 $[SO_4^{2-}]$ values were measured despite the different doses used, resulting in similar COD/ $[SO_4^{2-}]$ ratios (0.77 and 0.79),
46 together with the fact that the higher quantity of substrate used the higher SRB activity was achieved, as it also happened
47 in the tests with Carbocal® (discussed above).
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55 The best sulphate reduction activities achieved in one month when using OMW as carbon source/electrons
56 donor were obtained with percentages approximately 3 times greater than the percentages calculated to make a
57 COD/ $[SO_4^{2-}]$ ratio of 1.5. This suggests that just part of the organic compounds present in this substrate is used by SRB
58 during this incubation period. In OMW most part of organic matter corresponds to oils and fats (1.67 to 8.16%);
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however, it also contains sugars (0.1 to 0.9 %), polyalcohols (~1%) and volatile organic acids (~0.1 %) (Table 1). Focusing on sugars, which can be used by SRB after relatively rapid fermentation, and comparing the tested dilutions of OMW with the tested dilutions of the two substrates richest in sugars (beetroot and orange molasses), it can be seen that: (1) The best dilution for both molasses was 0.5% (v/v) and these substrates had approximately 45% sugars in their composition, which means that there was around 0.22% of sugars in the best cultures containing these substrates; (2) The best dilution for sulphate reduction with OMW corresponds to a concentration of 20% (v/v). Thus, supposing that this substrate had 0.44% of sugars in its composition (half the maximum value described in table 1), the best cultures with it would have 0.088% of sugars. This percentage is almost half the quantity of sugars present in the best cultures with molasses. Therefore, it can be said that probably sugars have played a major role in supporting the SRB activity in the cultures with OMW. In addition, it is known that alcohols and volatile organic acids can be directly used by SRB. Thus, based on a typical composition of OMW (Table 1) it can be estimated that approximately 0.2% of polyalcohols and 0.02% of volatile organic acids probably contributed for the SRB activity in the cultures with 20% OMW. Moreover, polyphenols are also important in OMW (0.0002 to 1.5%). It has been discovered that one of its most abundant phenolic compounds (1,4-tyrosol) can be oxidized by a SRB (*Desulfovibrio marrakechensis*) isolated for the first time from OMW in Morocco (Chamkh et al. 2009) and identified by 16S rRNA gene sequence analysis in OMW from Greece after treatment tests using amendments of laccase and cellobiose dehydrogenase (Prasetyo et al. 2015). This raises the hypothesis that SRB with this metabolic pathway may exist as well in OMW ponds in Portugal. If so, the bacterial communities in cultures with this substrate could have used this compound for sulphate reduction. This speculative analysis about which compounds of this substrate can support the biological activity of SRB communities is just a starting point for further research work aiming to explore this issue. Nevertheless, these results, proving that OMW is a good carbon source/electrons donor for SRB, open the way for the development of what would be a very interesting bioremediation process for this type of wastes, which are generated in high amount during the olive oil extraction process and have become an important challenge since its direct disposal in nature causes extremely negative impacts (Amaral et al. 2008).

In municipal wastewater (sewage), the organic pollutants are mainly proteins, saccharides and aliphatics (at least 78% of the total COD), most of them macromolecules that constitute slowly biodegradable organic matter, though rapidly biodegradable organic components and readily dissolved compounds such as micromolecular organic acids and alcohols are also present (e.g. Zhang et al. 2016). The reduction of sulphate in the tests with municipal wastewater from Mina de São Domingos started just one week after inoculation and continued during about one month while sulphate was present (graphs for tests N^o 43 and 44 in supplementary material). This suggests that probably the readily and rapidly degradable dissolved compounds had been consumed during the transportation and storage and that the SRB activity might have been sustained by slowly biodegradable organic components after their transformation into simplest products. The municipal wastewater used in this work was collected, immediately transported at 25^o±3^oC during three hours and then stored at 4^oC for three days before its use in the experiments. These results seem to contradict the work published by Kumar et al. (2011), which suggests that the presence of rapidly biodegradable and readily dissolved organic components in sewage is critical for an effective SRB activity. These authors reported that though sewage stored at 4 °C had retained a high total organic carbon equivalent to prior storage, it failed to increase dissolved organic carbon and failed to stimulate SRB activity. After addition of labile organic carbon consisting of lactic acid and ethanol to the microcosm, effective removal of sulphate was observed, indicating biological activity of SRB. However, it should be noted that the duration of storage used by these authors was 3 years, a much longer period than the three days storage in this work.

1 Probably, during those three years the slowly degradable organic compounds with potential to sustain SRB activity were
2 consumed due to some biological activity occurring at 4°C. All these findings prove that municipal wastewater can be
3 used as carbon source/electrons donor for SRB based processes, provided that it is immediately used and not after
4 storage.
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7 8 **Conclusions** 9

10 This work demonstrates the high potential of several industrial by-products and wastes as carbon
11 sources/electron donors for SRB, which being available in the Iberian Peninsula can be easily used in bioremediation
12 processes to treat AMD in the IPB region.
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15 Moreover, it reports the approximate time needed to achieve high sulphate reduction with these substrates and
16 the required *doses* of substrate (% v/v or % w/v, depending if the substrate is liquid or solid) and respective *COD/[SO₄²⁻]*
17 *ratios* that favour SRB activity in sulphate rich aqueous environments with neutral pH.
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20 Some substrates support high SRB activity in relatively short periods of two to four weeks: sugared water from
21 the factory of candies (20 % (v/v) *dose*, 3.10 *ratio*); beetroot molasses (0.25 to 0.5 % (v/v) *dose*, 3.17 to 4.88 *ratio*);
22 OMW not decanted (20 % (v/v) *dose*, 1.08 *ratio*); OMW decanted (20 % (v/v) *dose*, 1.63 *ratio*); orange molasses without
23 conservative (0.5 % (v/v) *dose*, 0.61 *ratio*) and municipal wastewater from a small village near an inactive mine in the
24 IPB (20 to 50 % (v/v) *dose*, 0.90 to 2.34 *ratio*).
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28 Others substrates require longer periods, up to four months, to sustain high sulphate reduction efficiencies:
29 orange peel fragments (1 to 4 % (w/v) *dose*, 0.85 to 0.94 *ratio*); pine nut shells (10 % (w/v) *dose*, 0.92 *ratio*) and pine
30 cone fragments (10 % (w/v) *dose*, 0.90 *ratio*).
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34 35 **Supplementary data** 36

37 Supplementary data associated with this article can be found in the online version: graphs showing the evolution
38 of measured parameters along the experiments.
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56 processing plant Azucarera (Eng. Maria Hernandez Garcia), Olive mill Lagar Santa Catarina (Eng. Renato Rocha),
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1 Águas Públicas do Alentejo, WWTP Mina de São Domingos (Eng. Olga Martins), Paper and paper pulp factories
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31 **Figure captions**

32 **Figure 1** - Iberian Pyrite Belt geological map indicating the most important mining sites (adapted from Relvas et al.
33 2006).

34 **Figure 2** - Schematic representation of the experimental design.

35 **Figure 3** - Evolution of parameters measured in the cultures with 5% (v/v) of orange molasses without conservative.
36 Values are averages \pm mean absolute deviations (in some points bars are smaller than the line width, thus not visible)

47 **Table captions**

48 **Table 1** - Typical compositions of tested substrates and justifications to be tested

49 **Table 2** - Characterization of the tested substrates in terms of pH, COD, [SO₄²⁻], [N] and [P] plus the percentages of
50 substrates estimated to obtain COD/[SO₄²⁻] ratios of 1.5 when diluted in Postgate B medium without lactate

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Table 3 - Summarized results for measured parameters: initial (first day of experiment before inoculation with sulphate reducing bacteria) and values achieved during incubation

Tables

Table 1

Substrates and their suppliers	Typical composition	Source of composition information	Why tested?
<i>Candy factory Dulciora in Valladolid, Spain:</i>			
- Sugared water (from an homogenization tank)	Water with sucrose. COD = 5 to 11 g O ₂ /L	Supplier	Foreseeable success in the light of works already published with other by-products from candy factories (<i>e.g.</i> sweetmeat waste fractions (Das et al. 2013)).
<i>Sugar from beetroot processing factory Azucarera located in Jerez de la Frontera, Spain:</i>			
- Beetroot molasses	Sucrose = 45.5 % (w/v) Glucose = 0.14 % (w/v) Fructose = 0.45 % (w/v) Rafinose = 1.3 % (w/v) Lactic acid = 3.2 % (w/v) Formic acid = 0.53 % (w/v) Acetic acid = 0.69 % (w/v) Propionic acid = 2.5 % (w/v) COD _(theoretical for these compounds) = 615.27 g O ₂ /L	Supplier	Foreseeable success, in the light of the works already published with other type of molasses (<i>e.g.</i> sugarcane molasses (Michailides et al. 2015)).
- Water from washing the beetroots	Water contaminated with soil and agricultural fertilizers. COD = 2.5 g O ₂ /L.	Supplier	After detection of sulphidric gas in this substrate, the presence of SRB was confirmed by counts with the method of the most probable number using Postgate E as growth medium (unpublished data).
- Carbocal®	A lime rich powder with precipitated non sugared beetroot compounds. Organic matter = 9.7 % (w/w) Humidity = 32 % (w/w) Calcium oxide = 34 % (w/w) Magnesium oxide = 0.8 % Nitrogen = 0.28 % (w/w) Phosphorus = 0.36 % (w/w)	Supplier	Already used as a neutralisation agent of acidic mining lakes (Koschorreck et al. 2007). Although having organic compounds in its composition, it was never tested to our knowledge as source of carbon/electron donor for SRB.
<i>Olive oil Press Lagar de Santa Catarina, located in Santa Catarina da Fonte do Bispo, Portugal:</i>			Portugal and Spain are among the five largest producers of olive oil in the world and the extraction process generates high

<i>Paper pulp and paper factories Navigator Company, located in Setubal, Portugal:</i>	The wastewaters from this industry contain a variety of organic and inorganic contaminants that mostly originate from tannins, lignins, resins, and chlorine compounds.	(Buzzini and Pires 2007)	
- Pulp factory alkaline wastewater	COD = 1.3 g O ₂ /L Phosphorus = 0.003 g/L Nitrogen = 0.003 g/L Sodium sulphate = 2.8 g/L	Supplier	In Portugal, near the western beginning of the IPB, is located one of the world's major industrial complexes producing paper pulp and paper (Navigator Company, formerly Portucel Soporcel), which produces large amounts of wastewaters. Biological sulphate reduction was successfully tested to treat the primary clarifier effluent from a paper mill with a simultaneous high-rate removal of sulphate and COD (Chen and Horan 1998), which suggests the presence of sources of carbon /electron donors suitable for SRB in paper industries wastewaters.
- Pulp factory neutral wastewater	COD = 1.9 g O ₂ /L Phosphorus = 0.009 g/L Nitrogen = 0.004 g/L Sodium sulphate = 3.8 g/L	Supplier	
- Paper factory wastewater	COD = 1.0 g O ₂ /L Phosphorus = 0.001 g/L Nitrogen = 0.007 g/L	Supplier	
- Final mixed treated effluent	COD = 0.3 g O ₂ /L Phosphorus = 0.003 g/L Nitrogen = 0.002 g/L Sodium sulphate = 2.3 g/L	Supplier	
<i>WWTP lagoon system in Mina de São Domingos, Portugal:</i>	Dissolved organic matter in raw wastewater or biologically treated wastewater consists of different molecular weight (MW) fractions ranging from low MW substances (e.g. amino acids, carboxylic acids, alcohols, aldehydes, etc.) to high MW compounds such as humic substances, polysaccharides, and proteins.	(e.g. Raunkjaer et al. 1994; Zhang et al. 2016)	Aiming to encourage the implementation of SRB biological processes to treat the AMD in small villages, such as Mina de São Domingos. Previous works have reported that municipal wastewater can be used as source of carbon/electron donors for SRB (e.g. Costa and Duarte 2005), thus having potential to sustain SRB biological processes in remote locals.
- Municipal wastewater from the first lagoon with dissolved and suspended solids	COD = 0.55 ± 0.28 g O ₂ /L Oils and fats = 0.041 ± 0.018 g/L Phosphorus = 0.011 ± 0.004 g/L Nitrogen = 0.102 ± 0.046 g/L	Supplier	
<i>Pine nuts industry in Pedrajas de San Sebastian, Spain:</i>			In Europe, Portugal and Spain are the main producers of pine nuts and this activity generates large amounts of pine nut shells and pine cones, which by their availability have potential to be an important source of cellulosic compounds. Other types of cellulosic compounds have
- Pine nut shells	Cellulose = 33.3 % Hemicellulose = 32.1 % Lignin = 32.4 % Extractives (essential oils, resins, etc.) = 1.7 % Nitrogen = 0.4 %	(Kim et al. 2014)	

- Pine cone fragments

Cellulose = 43.8 %
Hemicelulose = 27.2 %
Kalsol lignin = 21.5 %
Acid soluble lignin = 0.6 %
Extractives (essential oils, resins, etc.) = 5.2 %
Nitrogen = 0.27 %

(Rambabu et al. 2016)

(Almendros et al. 2015)

already been reported as suitable sources of carbon/electron donors for SRB (*e.g.* wood chips and sawdust (Choudhary and Sheoran 2011; Zhang and Wang 2014)).

Table 2

Substrates and their origins	pH	COD (g O₂/L)	[SO₄²⁻] (g/L)	[N] (g N/L)	[P] (g P/L)	Estimated dose for COD/[SO₄²⁻] = 1.5^a (%)^b
<i>Candy factory:</i>						
- Sugared water (from an homogenization tank)	4.44	14.8	0.05	0.29	0.12	14.73
<i>Sugar processing factory from beetroot:</i>						
- Beetroot molasses	7.64	1192	2.3	51.1	17.3	0.21
- Water from washing the beetroots	7.31	1.1	0.011	0.38	<0.002*	70.47
- Carbocal® ^c	9.58	1.1	0.096	<0.01*	<0.002*	72.36
<i>Olive oil Press:</i>						
- Olive mill wastewater not decanted	4.48	39.9	0.46	2.4	0.18	6.11
- Olive mill wastewater decanted	4.73	44.8	0.53	2.2	0.13	5.48
<i>Orange juice factory:</i>						
- Orange molasses with conservative (CetoSTAT® TMR)	4.20	724.0	0.68	43.9	0.17	0.35
- Orange molasses without conservative	5.35	707.0	0.98	21.4	0.40	0.36
- Liquor extracted from orange peels	4.03	150.4	0.26	3.9	2.6	1.67
- Orange peel fragments ^c	5.19	7.0	0.027	0.053	0.18	26.86
- Water from washing the equipments	6.46	4.4	0.41	0.29	<0.002*	40.00
<i>Paper pulp and paper factories:</i>						
- Pulp factory alkaline wastewater	9.35	1.6	0.82	<0.01*	0.0034	88.97
- Pulp factory neutral wastewater	7.80	1.0	0.82	<0.01*	0.0095	I
- Paper factory wastewater	7.31	0.33	0.154	<0.01*	<0.002*	96.34
- Final mixed treated effluent	7.49	0.19	0.56	<0.01*	0.0034	I
<i>WWTP at "Mina de São Domingos":</i>						
- Municipal waste water from the first lagoon	7.65	0.46	0.060	0.099	0.0088	87.27
<i>Pine nuts industry:</i>						
- Pine nut shells ^c	5.88	0.30	0.016	<0.01*	<0.002*	90.07
- Pine cone fragments ^c	5.95	0.41	0.018	<0.01*	0.010	86.94

a) Calculated as described in materials and methods for dilutions in Postgate B (1.7g/L SO₄²⁻) without lactate (assuming its COD is zero).

b) v/v for liquids and w/v for substrates.

c) To characterize solid substrates, measurements were made in samples after homogenization of 10% (w/v) substrate in water and 24 hours lixiviation without agitation.

* **Limit of detection (LOD)**

i = impossible (for substrates that just allow making dilutions in Postgate B without lactate with COD/[SO₄²⁻] ratios lower than 1.5).

Table 3

Test N ^{er}	Substrates	Initial Values						Values achieved									
		Tested dose ^a (%)	pH	Redox Potent. Eh (mV)	COD (mgO ₂ /L)	[SO ₄ ²⁻] (mg/L)	COD / [SO ₄ ²⁻] ratio	Redox Potent. Eh (mV)		pH		Maxim. [S ²⁻] (mg/L)		Maxim. SO ₄ ²⁻ removal (%)		Time to reach maxim. SO ₄ ²⁻ removal (days)	Time to reach 90% of maxim. SO ₄ ²⁻ removal (days)
								Aver.	MAD ^b	Aver.	MAD ^b	Aver.	MAD ^b	Aver.	MAD ^b		
<i>Candy factory:</i>																	
1		2	6.44	-177	1265	1451	0.87	-92	56	6.89	0.03	15	1	24	0	17	17
2	Sugared water (from an homogenization tank)	10	6.67	-79	2640	1338	1.97	-199	7	5.92	0.02	65	3	39	1	17	17
3		20	6.25	-50	3488	1200	2.91	-125	31	5.15	0.17	39	20	20	9	29	17
4		5	7.29	-48	1967	1266	1.55	-348	3	7.02	0.00	33	2	47	4	26	26
5	Sugared water (from an homogenization tank) + calcite ^c	10	7.25	-68	2640	1279	2.06	-350	2	6.83	0.02	79	2	57	1	26	14
6		20	7.15	-53	3488	1125	3.10	-314	38	6.79	0.02	142	11	79	4	40	14
<i>Sugar processing factory from beetroot:</i>																	
7		0.25	6.69	86	4035	1523	2.65	-52	7	5.13	0.00	9	0	5	0	17	17
8	Beetroot molasses	0.5	6.68	29	6580	1550	4.25	13	14	4.29	0.00	4	1	5	1	17	17
9		1.33	6.86	-71	13619	1559	8.74	84	1	4.25	0.01	2	1	6	2	17	17
10		10	7.41	-177	122000	1910	63.87	-29	1	5.18	0.05	3	2	3	0	17	17
11		0.25	7.46	86	4035	1273	3.17	-344	172	6.96	0.08	138	14	71	2	26	14
12	Beetroot molasses + calcite ^c	0.5	7.36	29	6580	1348	4.88	-301	61	7.00	0.08	192	24	85	6	40	26
13		1	7.37	-71	11920	1398	8.53	-262	57	6.51	0.08	46	3	30	3	40	40
14		2	7.31	-165	23840	1493	15.97	-114	25	6.46	0.00	4	1	-7	4	40	40
15	Water from washing the beetroots	10	7.42	-286	954	1238	0.77	-361	7	7.67	0.03	27	3	17	1	29	29
16		20	7.51	-285	886	1120	0.79	-359	14	7.42	0.01	74	1	31	7	49	49
17		0.1	7.36	-176	1079	1476	0.73	-58	6	7.25	0.03	7	0	18	0	17	17
18	Carbocal® ^d	1	8.07	-229	1240	1552	0.80	-49	9	7.85	0.02	1	1	21	1	29	17
19		10	8.14	-197	1544	1589	0.97	-418	1	7.85	0.00	195	2	53	1	40	26
<i>Olive oil press:</i>																	
20		4	7.34	60	461	1062	0.43	-343	10	7.40	0.04	44	1	55	14	28	28
21	Olive mill wastewater (OMW) not decanted + calcite ^c	7	7.20	60	695	1044	0.67	-352	6	7.26	0.04	59	7	69	27	28	28
22		20	7.07	55	1142	1056	1.08	-332	50	7.28	0.06	149	49	95	2	28	28
23		3.5	7.45	63	428	1053	0.41	-295	53	7.35	0.01	45	0	42	2	28	28
24	Olive mill wastewater (OMW) decanted + calcite ^c	6	7.43	57	627	998	0.63	-330	28	7.47	0.11	52	9	64	8	28	28
25		20	7.00	59	1357	835	1.63	-325	38	7.24	0.04	226	39	93	2	28	28

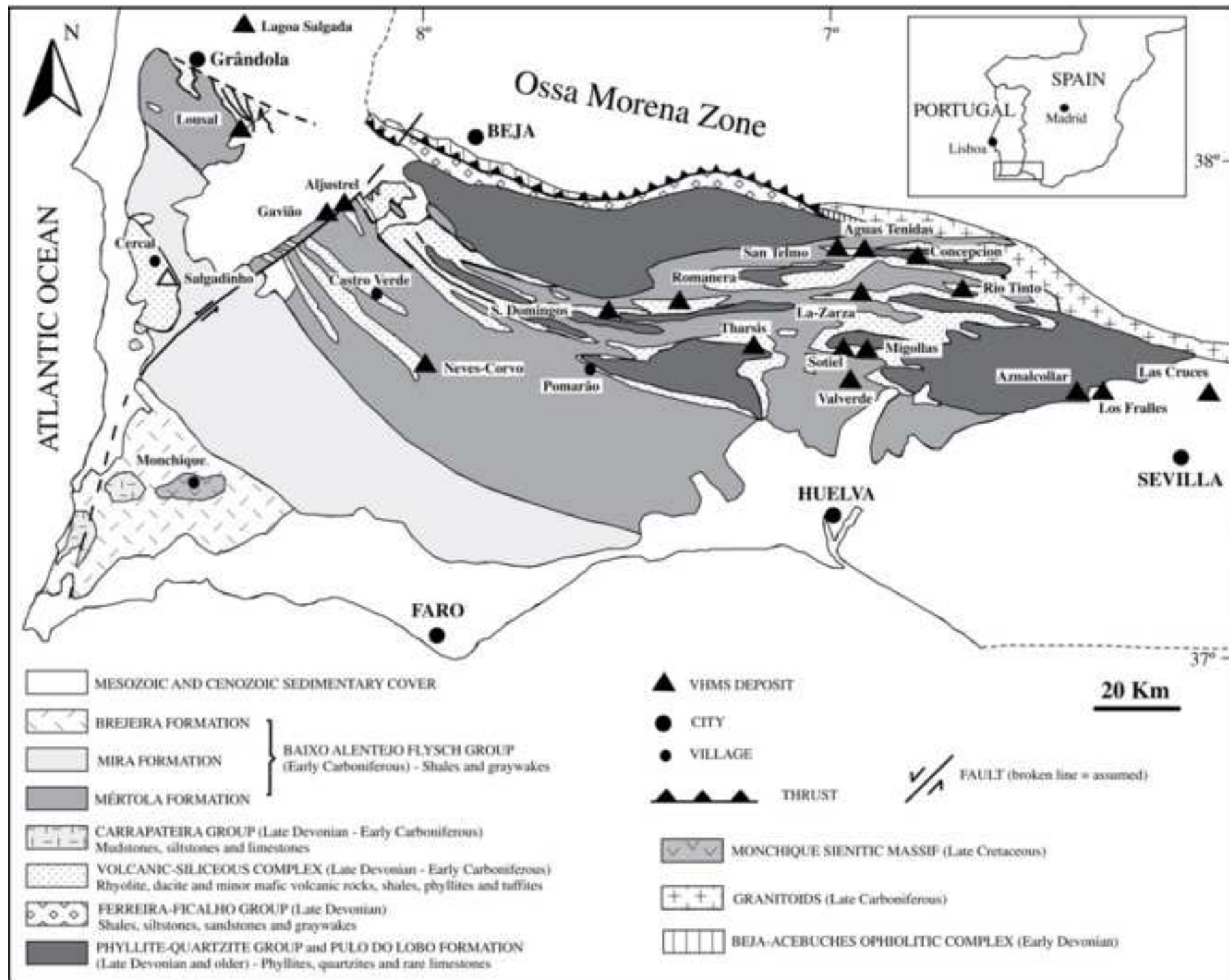
<i>Orange juice factory:</i>																	
26		0.25	7.58	23	384	932	0.41	-304	59	7.01	0.01	48	2	22	1	28	28
27	Orange molasses with conservative (CetoSTAT® TMR)	0.5	7.41	31	760	844	0.90	-298	71	6.83	0.01	36	9	49	1	42	42
28	+ calcite ^c	1	7.37	80	1359	1037	1.31	-225	31	6.39	0.06	56	37	18	15	42	42
29		0.25	7.63	53	448	1202	0.37	-303	57	6.95	0.00	49	10	51	3	28	28
30	Orange molasses without conservative + calcite ^c	0.5	7.57	64	741	1222	0.61	-344	1	6.84	0.08	106	12	70	3	28	28
31		1	7.30	57	1062	1190	0.89	-233	13	6.42	0.04	83	14	46	0	28	21
32		1.25	7.39	26	577	1254	0.46	-225	53	6.85	0.09	21	27	37	1	28	21
33	Liquor extracted from orange peels	2.5	7.26	26	877	1256	0.70	-202	52	6.50	0.11	39	38	34	13	28	21
34	+ calcite ^c	10	6.52	60	3475	1093	3.18	-112	7	5.94	0.08	5	0	11	0	14	7
35		1	7.31	42	963.5	1138.5	0.85	-363	1	7.26	0.08	106	45	91	9	121	121
36	Orange peel fragments ^d + calcite ^c	4	7.33	59	1146	1224	0.94	-258	22	6.21	0.04	95	8	99	1	121	121
37		8	7.02	35	1320	1164	1.13	-110	11	6.10	0.17	2	1	6	3	28	28
38	Water from washing the equipments + calcite ^c	20	7.37	81	349	935	0.37	-314	67	7.30	0.08	49	16	53	12	21	21
<i>Paper pulp and paper factories:</i>																	
39	Pulp factory alkaline wastewater + calcite ^c	20	7.59	70	221	1001	0.22	-226	8	7.41	0.01	17	1	25	6	14	14
40	Pulp factory neutral wastewater + calcite ^c	20	7.69	67	250	870	0.29	-93	23	7.43	0.04	4	1	11	5	21	21
41	Paper factory wastewater + calcite ^c	20	7.55	53	212	1111	0.19	-209	181	7.29	0.01	29	2	29	2	14	14
42	Final mixed treated effluent + calcite ^c	20	7.69	65	223	933	0.24	-49	15	7.52	0.01	23	9	27	2	28	28
<i>WWTP at "Mina de São Domingos":</i>																	
43	Municipal waste water from the first lagoon + calcite ^c	20	7.23	48	743	830	0.90	-251	175	7.42	0.23	218	16	82	2	28	28
44		50	7.44	44	1099	469	2.34	-197	133	7.30	0.27	153	60	81	18	28	28
<i>Pine nuts industry:</i>																	
45	Pine nut shells ^d + calcite ^c	10	7.14	27	877	951.5	0.92	-28	13	7.41	0.01	7	2	99	0	121	121
46	Pine cone fragments ^d + calcite ^c	10	7.07	41	911.5	1007.5	0.90	-124	36	7.19	0.11	18	1	97	0	121	121
<i>Controls:</i>																	
47	Positive 1: Postgate B	-	6.63	184	4112	1462	2.81	-397	1	7.23	0.02	304	13	92	0	14	7
48	Positive 2: Postgate B + calcite ^c	-	7.07	183	4112	1664	2.47	-399	1	7.27	0.03	259	9	87	0	14	7
49	Negative: Postgate B without lactate + calcite ^c	-	7.33	33	876	1089.5	0.80	-202	59	7.57	0.10	9	5	25	10	28	28

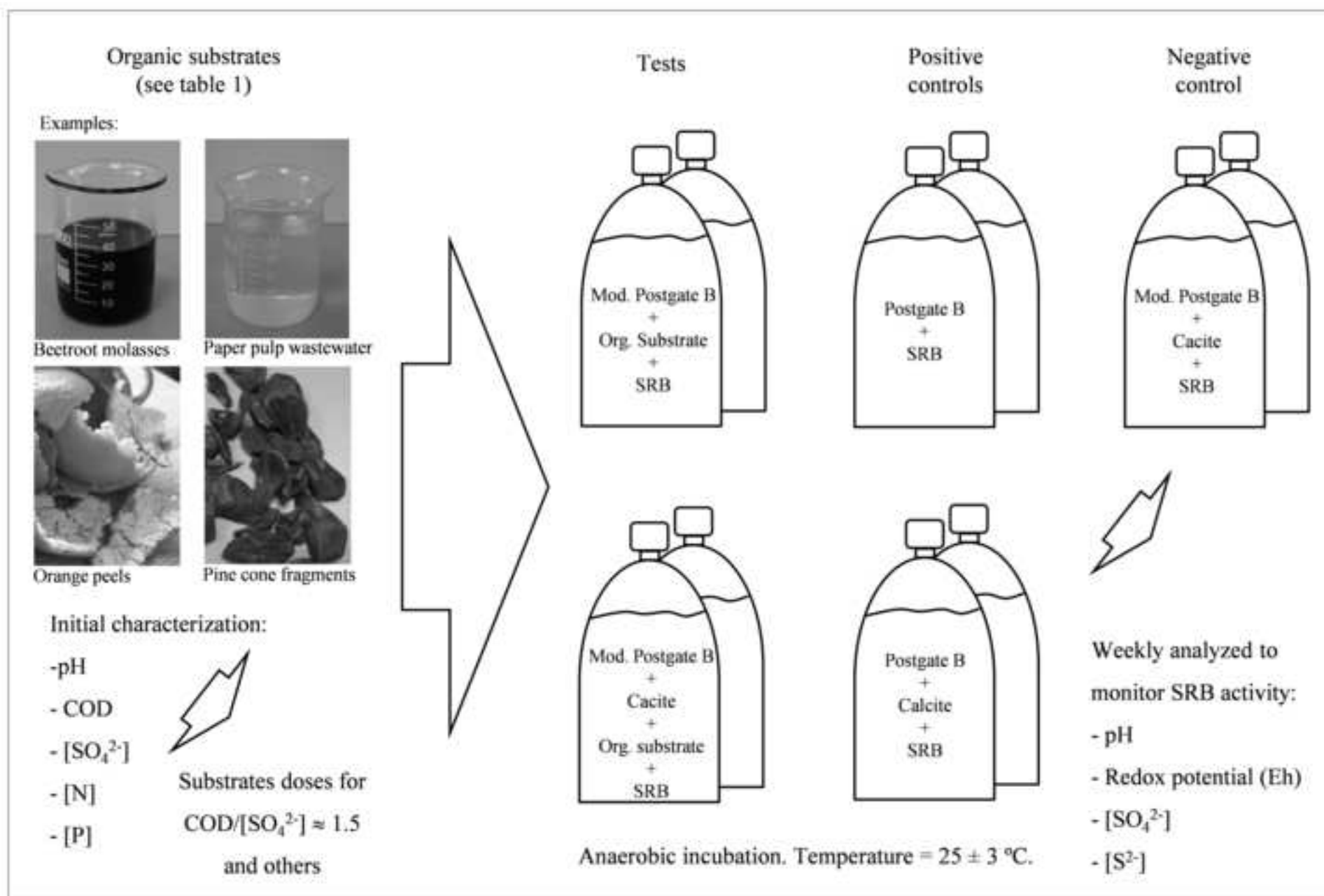
a) Substrate percentages tested in Postgate B medium without lactate (v/v for liquids and w/v for solids).

b) Aver. = Average; MAD = mean absolute deviation (the average distance between each data value and the mean - in this case, with n=2, it is the real distance to individual data points).

c) 10% (w/v) of calcite tailings powder.

d) In the cultures with the solid substrates Carbocal®, orange peel fragments, pine nut shells and pine cone fragments, the parameters were measured in the aqueous phase of medium 24 hours after mixing the substrates.





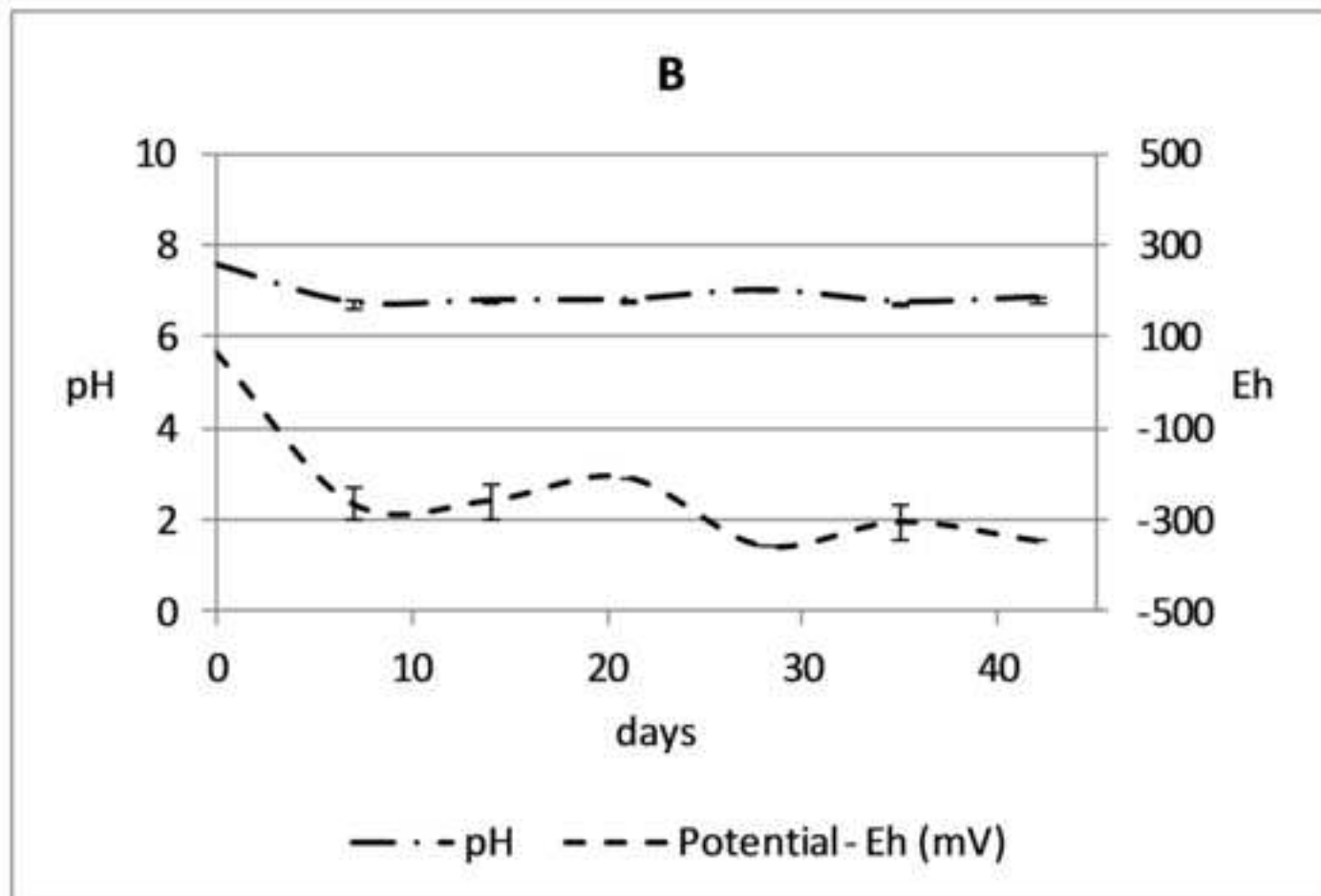
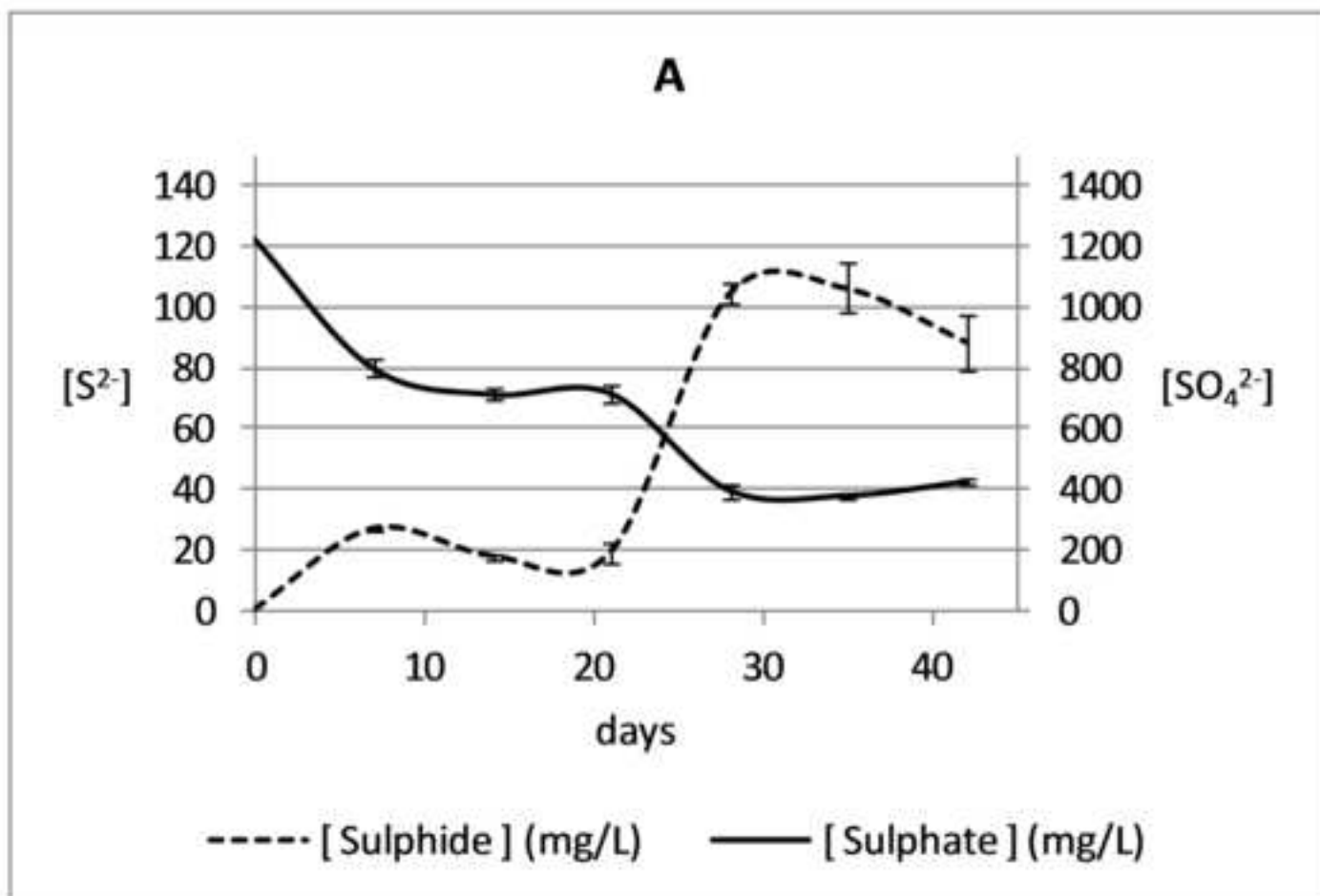
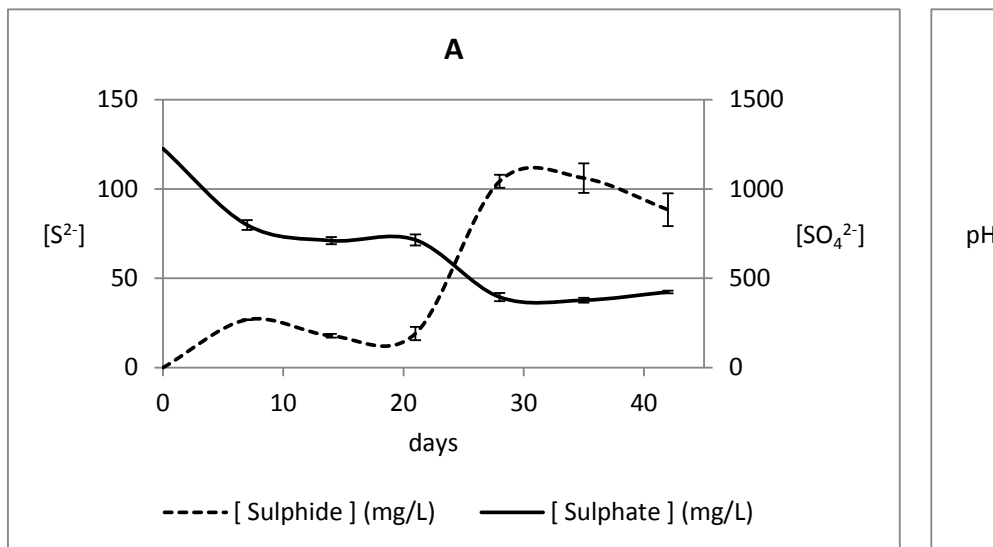
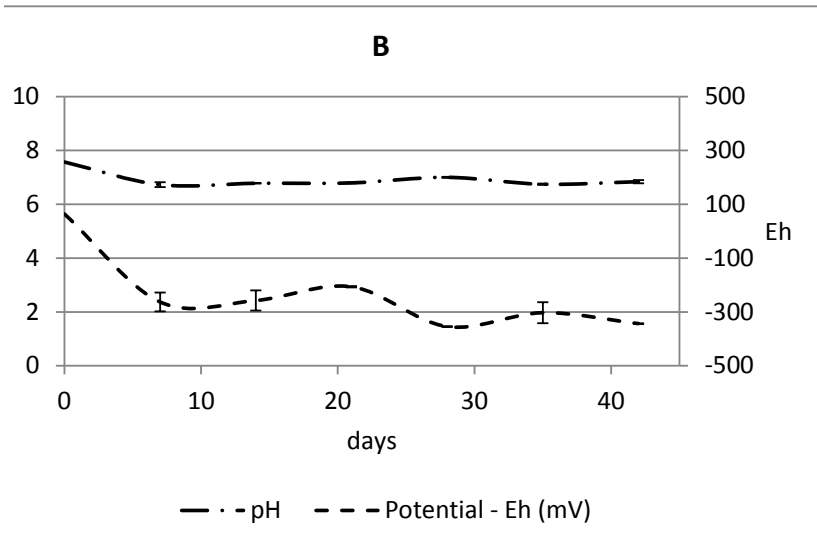


Fig. 3



Experiment		date	2/11,
Test N.er		Substrates	initial n
30		Days	
Orange molasses without conservative - 0.5% (v/v)		Potential - Eh (mV)	
calcite tailings - 10% (w/v)		pH	
		[Sulphide] (mg/L)	
		[Sulphate] (mg/L)	



/2015	2/19/2015				2/26/2015			
	replicates		average	MAS	replicates		average	MAS
	1	2			1	2		
0	7				14			
64	-228	-298	-263.00	35.00	-295	-220	-257.50	37.50
7.57	6.82	6.64	6.73	0.09	6.77	6.79	6.78	0.01
0	27.03	26.71	26.87	0.16	16.81	18.88	17.85	1.04
1226	771	826	798.50	27.50	691	731	711.00	20.00

3/4/2015				3/11/2015			
replicates		average	MAS	replicates		average	MAS
1	2			1	2		
		21				28	
-209	-204	-206.50	2.50	-353	-356	-354.50	1.50
6.81	6.78	6.80	0.01	7.02	6.98	7.00	0.02
15.36	22.81	19.09	3.73	100.68	107.97	104.33	3.65
684	746	715.00	31.00	372	418	395.00	23.00

3/18/2015			
replicates		average	MAS
1	2		
		35	
-342	-264	-303.00	39.00
6.72	6.77	6.75	0.02
114.36	97.82	106.09	8.27
364	391	377.50	13.50

25-03--2015			
replicates		average	MAS
1	2		
		42	
-343	-344	-343.50	0.50
6.9	6.78	6.84	0.06
79.24	97.6	88.42	9.18
416	431	423.50	7.50



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