Systematic and Applied Microbiology 42 (2019) 125998

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: http://www.elsevier.com/locate/syapm

Enhanced aniline degradation by *Desulfatiglans anilini* in a synthetic microbial community with the phototrophic purple sulfur bacterium *Thiocapsa roseopersicina*

Xiaoman Xie[a,b], Nicolai Müller[a,*]

[a] Department of Biology, University of Konstanz, Constance, Germany
[b] Konstanz Research School Chemical Biology, Constance, Germany

ARTICLE INFO

Article history:
Received 18 February 2019
Received in revised form 23 June 2019
Accepted 26 June 2019

Keywords:
Aniline
Desulfatiglans anilini
Sulfide
Thiocapsa roseopersicina
Carboxylation

ABSTRACT

Desulfatiglans anilini is a sulfate-reducing bacterium (SRB) capable of oxidizing aniline, although growth and aniline turnover rates are slow, making it difficult to analyze the metabolism of the strain. Therefore, this study was designed to investigate the effect of sulfide on growth of *D. anilini* cultures, in order to improve its growth and aniline turnover rates, and study the biochemical mechanisms of sulfide inhibition. Hydrogen sulfide was found to inhibit growth of *D. anilini*, regardless of whether the strain was grown with aniline or phenol, and complete inhibition was observed at 20 mM hydrogen sulfide. For improving the growth of *D. anilini* with aniline, the sulfide-consuming phototrophic bacterium *Thiocapsa roseopersicina* was co-cultured in a synthetic microbial community with *D. anilini* using a co-cultivation device that continuously removed hydrogen sulfide from the culture. The doubling time of *D. anilini* with aniline was 15 days in the co-cultivation device, compared to 26 days in the absence of a sulfide-oxidizing partner. Moreover, the aniline degradation rate was significantly increased by a factor of 2.66 during co-cultivation of *D. anilini* with *T. roseopersicina*. The initial carboxylation reaction during aniline degradation was measured in cell-free extracts of *D. anilini* with carbon dioxide (CO_2) as a co-substrate in the presence of aniline and ATP. The effects of hydrogen sulfide on this aniline carboxylating system and on phenylphosphate synthase activity for phenol activation were studied, and it was concluded that hydrogen sulfide severely inhibited these enzyme activities.

Introduction

Aniline is a toxic aromatic compound mainly used in the industrial production of dyes, rubbers, pharmaceuticals, plastics and paint, and the extensive use of aniline in industry has led to serious pollution problems in waters and soils as aniline is hardly degraded in nature [33]. In addition to physicochemical methods (for example, photodecomposition [33], ozone oxidation [18] and electro-Fenton [3]), bioremediation is an attractive treatment strategy for removing environmental contaminations as it can completely mineralize organic pollutants and can be applied in *in situ* processes [14]. In the past few decades, numerous studies have paid attention to the biodegradation and bioremediation of aromatic compounds. Efforts have been spent on the optimization of bioremediation methods and biodegradation conditions, elucidation of biodegradation pathways of aromatic compounds and characterization of degradation in pure or enrichment cultures. Therefore, the removal of aromatic compounds from contaminated sites by bioremediation may be eventually achieved [8].

Some toxic aromatic compounds can be eliminated by novel aerobic bioremediation techniques. For example, mixtures of benzene, toluene, and *p*-xylene can be removed at high and sustained rates by a constructed microbial consortium immobilized on a biofilter [31], while phenol and 4-chlorophenol can be biotransformed by *Pseudomonas putida* immobilized on polystyrene beads or hollow fiber [24,25]. Likewise, anaerobic bacteria also play a very important role in bioremediation of aromatic compounds. By using nitrate as an electron acceptor, 78% and 68% of the total petroleum hydrocarbons and polynuclear aromatic hydrocarbons in sediments of the Dofasco Boatslip, Hamilton Harbour were biodegraded in 197 days [30]. Sulfate-reducing bacteria (SRB) have already been used to treat contaminated environmental sites. It was demonstrated that by sulfate injections into an aquifer contaminated with petroleum, degradation of benzene by sulfate reducing

* Corresponding author.
E-mail address: Nicolai.Mueller@uni-konstanz.de (N. Müller).

https://doi.org/10.1016/j.syapm.2019.06.003
0723-2020/

bacteria was stimulated, resulting in removal of benzene along the groundwater flow path [2]. An integrated bioremediation process, by combining sulfate reduction-denitrification with alkaganeite, was successfully used for total petroleum hydrocarbons removal and odor mitigation from contaminated marine sediment [51].

Analogously, microorganisms capable of degrading aniline would be useful for developing bioremediation techniques applied to aniline-contaminated sites. To date, it has been well documented that aniline can be removed efficiently by a few aerobic aniline degraders [4,5,21,32,41,50]. An aerobic aniline-degrading bacterium, strain HY99, was found to degrade aniline under either aerobic or nitrate-reducing conditions [19]. *Desulfatiglans anilini* is the only bacterium known to degrade aniline under strictly anoxic sulfate-reducing conditions [38,39], which is the typical mode of anaerobic respiration in marine sediments. Nonetheless, there are some limitations to the application of bacteria such as *D. anilini* in bioremediation processes, as growth and aniline turnover rates are slow and the metabolic pathway of aniline degradation is not sufficiently understood. Moreover, growth is inhibited at concentrations above 1–2 mM aniline [39]. Thus, growth stimulating factors or inhibiting compounds need to be identified in order to optimize the growth of *D. anilini* with aniline, and for developing efficient cultivation methods under these conditions. Some factors that may affect the growth of bacteria are generally the pH of the medium, growth factors like metal ions, incubation temperature, and growth inhibitors. In sulfate-reducing bacteria, sulfate is reduced to hydrogen sulfide, which was found to inhibit growth at elevated concentrations [16,27,34]. This inhibition is due to direct toxicity of hydrogen sulfide to the cell rather than complexation and, hence, unavailability of free ionic iron by sulfide [34]. It is assumed that sulfide inhibits certain enzymes in SRB. Cytochrome c_3, which functions as a sulfur reductase in *Desulfovibrio vulgaris* strain Hildenborough, is especially inhibited by hydrogen sulfide. Most likely, inhibition is caused by accumulation of sulfide as the end-product of the sulfur reductase reaction [12]. It is worth speculating that SRB have evolved mechanisms that alleviate the inhibitory effects of hydrogen sulfide. At least in one case of a naturally occurring microbial consortium, which is dominated by SRB (PB-SRB1) and phototrophic sulfide-oxidizing purple sulfur bacteria (PB-PSB1), sulfur cycling on the microscale level between the two types of organisms appears to facilitate sulfate reduction through constant removal of the end-product hydrogen sulfide [42,47]. Syntrophic cooperation between aniline-degrading SRB and sulfide-oxidizing phototrophs in synthetic communities, hence, could possibly enhance aniline degradation.

Aniline was proposed to be carboxylated to 4-aminobenzoate with CO_2, as concluded from experiments with dense cell suspensions [39], and 4-aminobenzoate would then be activated to 4-aminobenzoyl-CoA. The latter is then reductively deaminated to benzoyl-CoA (Fig. S3A). Activities of 4-aminobenzoyl-CoA ligase and benzoyl-CoA synthase could be measured in cell-free extracts of *D. anilini* [39]. However, there is no strong evidence for the reactions proposed for the conversion of aniline to 4-aminobenzoate. Carboxylation as an initial step in degradation of aromatic compounds has so far been observed in phenol degradation by nitrate-reducing bacteria [7,22,36,40,45], an iron-reducing bacterium [35], and the sulfate-reducing bacterium *D. anilini* [48]. Carboxylation of phenol requires the expenditure of two ATP equivalents that can easily be afforded by nitrate-reducing bacteria (Fig. S3B). The β-phosphate is covalently bound to phenol, and the γ-phosphate is released as inorganic phosphate [36]. However, *D. anilini* decomposes one mole of aniline to three moles of acetyl-CoA, which are terminally oxidized to CO_2 via the Wood-Ljungdahl pathway, yielding approximately one ATP equivalent per acetyl moiety [43]. Therefore, sulfate-reducing bacteria can hardly spend two ATP equivalents on aniline activation. In a study on acetone degradation by sulfate-reducing bacteria, formate was found to be the co-substrate for activating acetone via formylation [1,15], thus providing a novel mechanism for the activation of organic compounds in sulfate-reducing bacteria. To date, the exact mechanism of aniline activation, especially the cofactor used for the substitution of aniline, is still unknown. Earlier studies indicated a dependence of aniline degradation on CO_2, but carboxylation was never measured in *in-vitro* enzyme assays [39].

Therefore, the objectives of this study were to investigate the effect of sulfide on the growth of *D. anilini*. Furthermore, improvement of *D. anilini* growth by co-cultivating the strain with the sulfide-oxidizing purple sulfur bacterium *Thiocapsa roseopersicina* and investigating the initial activation reaction of aniline, as well as identifying possible targets of sulfide inhibition in *D. anilini*, were within the scope of this study.

Materials and methods

Bacterial strains and culture media

D. anilini DSM 4660 was isolated from marine sediments as a sulfate-reducing bacterium oxidizing aniline [38]. *T. roseopersicina* (DSM 217) and *D. anilini* (DSM 4660) were both purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ).

D. anilini was grown in bicarbonate-buffered (30 mM) and sulfide-reduced (2 mM) brackish water medium [37] with sulfate as the electron acceptor, as previously described [38]. The basal medium was autoclaved at 121 °C with 1 bar overpressure and cooled to room temperature under a stream of N_2/CO_2 (80% N_2, 20% CO_2). Then, 30 mM $NaHCO_3$, 1 mL selenite-tungstate solution/L and 1 mL seven vitamins solution/L [46] were added to the medium. A stock solution of a trace element mixture (SL 13) [29] was prepared, sterilized by autoclaving, and added to the basal medium (1:1000 v/v). Resazurin (0.4 mg L^{-1}) was added as a redox indicator. Sodium sulfide was added from anoxic stock solutions sterilized by autoclaving at a final concentration of 2 mM. The pH of the medium was adjusted to 7.2. *T. roseopersicina* was cultured in the same bicarbonate-buffered and sulfide-reduced brackish water medium. The medium used for the co-culture experiments of *D. anilini* and *T. roseopersicina* was the same bicarbonate-buffered and sulfide-reduced brackish water medium as described above.

Cultivation conditions

Pure cultures of *D. anilini* were prepared in triplicate by inoculating approximately 0.5 mL of a pre-culture (exponential phase) into 10 mL medium in rubber-stoppered Hungate tubes with N_2/CO_2 in the headspace. 1 mM aniline (or 2 mM phenol) and 5 mM (or 8 mM) sodium sulfate were added from 1.0 M sterile stock solutions. To study the influence of sulfide and metal ions on growth, various concentrations of sodium sulfide (0 mM, 2 mM, 5 mM, 10 mM, 15 mM or 20 mM) or different metal ions (50 μM $ZnCl_2$, 50 μM $MnCl_2$ or 25 μM $CoCl_2$) were added to the basal cultures of *D. anilini* from individual sterile stock solutions. Cultures were incubated at 30 °C in the dark.

Pure cultures of *T. roseopersicina* were prepared in triplicate by inoculating approximately 0.5 mL of a pre-culture (exponential phase) into 10 mL basal brackish medium in rubber-stoppered Hungate tubes with N_2/CO_2 in the headspace. Co-cultures were prepared by adding *D. anilini* to the pure culture of *T. roseopersicina* with additional 1 mM aniline and 5 mM sodium sulfate. Pure cultures of *T. roseopersicina* and co-cultures were incubated at 30 °C under constant illumination at a distance of 300 mm from a 25 W tungsten bulb. Growth in the test tubes was monitored at 600 nm

by using a tube spectrophotometer (Camspec M107 visible spectrophotometer, Leeds, LS25 1DX, UK).

The co-cultivation device for allowing the exchange of H_2S between the pure cultures of *D. anilini* and *T. roseopersicina* was made from regular laboratory bottles with a GL45 opening and a glass outlet at the bottom and were purchased from Ochs Glasgerätebau (Bovenden-Lenglern, Germany). Butyl rubber stoppers for GL45 openings were from the same source. The glass outlets of the bottles were connected with butyl-rubber tubing (isoversinic) and an outlet valve to allow for pressure compensation when the bottles were filled with media. A glass tube containing cotton wool as a sterile filter was added to the tube connection. Rubber-stoppered 1 L bottles were flushed with a mixture of N_2/CO_2 (80%/20%) and autoclaved. Each bottle in this device was filled by syringes with 500 mL medium.

Preparation of cell-free extracts

Cells grown with aniline were harvested at an optical density of 0.2 at 600 nm (OD_{600}). All experiments with cell-free extracts were undertaken under strictly anoxic conditions inside an anoxic glove box (Coy Laboratory Products, Michigan, USA). Cells were centrifuged at 20,300 x g at 4 °C for 30 min (Dupont Sorvall, Midland, Canada). The resultant pellet was washed at least twice with 50 mM potassium phosphate (KPP) buffer, pH 7.5, supplemented with 3 mM dithiothreitol (DTT) as a reducing agent. Cell-free extracts were prepared in the same KPP buffer, pH 7.5, with 3 mM DTT and 0.5 mg DNaseI/mL, by passaging the cells through a French pressure cell (SLM Aminco, Cat. No. FA003, Urbana, Illinois, USA) three times at a pressure of 137 MPa, followed by centrifugation (30 min, 30,300 x g, 4 °C, Optima™ TL Ultracentrifuge, Beckman Coulter, Brea, California, USA) to remove cell debris.

Co-substrate assay

Aniline degradation activity was measured in a 2 mL assay mixture containing KPP buffer (3 mM DTT, pH 7.5), 0.5-0.6 mg protein, 0.5 mM aniline, 2 mM ATP, 2 mM $MgCl_2$, and 30 mM $NaHCO_3$ at 30 °C. Control assays did not contain ATP. The gas phase was N_2. Samples (200 μL) were taken at different time intervals during a 3 h incubation period. 200 μL acetonitrile were added to the samples, followed by centrifugation at 11,700 x g for 10 min to stop the enzyme reaction and remove proteins. The supernatant was used for determination of aniline concentrations by HPLC (see analytical methods).

The effect of sulfide on enzyme activity

For detecting the effect of sulfide on enzymatic activity of the aniline-carboxylating enzyme system, 2 mM or 5 mM sodium sulfide from a 500 mM sodium sulfide stock solution were added to the above-mentioned standard enzyme assay mixture. To detect the effect of sulfide on enzymatic activity of phenylphosphate synthase in cell free extracts of phenol-grown cells, 2 mM or 5 mM sodium sulfide from a 500 mM sodium sulfide stock solution were added to the standard assay mixture, as previously described [36]. Samples (200 μL) were taken at different time intervals during an incubation period of several hours. 200 μL acetonitrile was added to the samples, followed by centrifugation at 11,700 x g for 5 min to stop the enzyme reaction. The supernatant was used for determination of aniline or phenol concentrations by HPLC (see analytical methods).

Analytical methods

Samples were taken periodically using sterile syringes and needles. For the determination of growth and the concentrations of aniline, sulfate, and sulfide in the co-cultivation device, three samples were taken at the same time: one sample was used immediately to measure the optical density at 600 nm by using a cuvette spectrophotometer (6300 visible spectrophotometer, Jenway, Staffordshire, UK). The second sample was centrifuged at 11,700 x g for 10 min and the supernatant was used for aniline and sulfate measurement. The third sample was used immediately to measure sulfide concentrations (see below).

Concentrations of aniline, phenol or 4-aminobenzoate were measured with a Shimadzu UPLC (Shimadzu, Kyoto, Japan) system equipped with a UV–vis diode array detector and a reversed-phase 4 μm Max-RP 80 Å LC column (250*4.6 mm, Synergi, Phenomenex, Torrance, California, USA). Eluents were prepared with filtered ultrapure water with 0.1% H_3PO_4 (buffer B), and acetonitrile with 0.1% H_3PO_4 (buffer A). A decreasing gradient of buffer B from 70% to 50% was used at a flow rate of 1 mL min^{-1}. Samples of 50 μL each were injected into the column. Peaks were identified by comparison of retention times and UV spectra to standard samples. Sulfate was analyzed with the barium chloride method [44], and sulfide in the liquid phase was quantified photometrically by the methylene blue method [10]. For higher sulfide concentrations, samples were diluted by adding 0.1 M NaOH. Protein concentrations in cell-free extracts were estimated with the Bradford assay [6].

Chemicals

All standard chemicals were of analytical quality and were obtained from Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany) or Sigma (St. Louis, USA). Gases were purchased from Messer-Griesheim (Darmstadt, Germany) and Sauerstoffwerke Friedrichshafen (Friedrichshafen, Germany).

Results

Effect of sulfide and metal ions on the growth rate of D. anilini

In pure cultures of *D. anilini*, according to the reaction stoichiometry, 3.75 mol of sulfide were formed by sulfate reduction when 1 mol aniline was oxidized to carbon dioxide [38]. To investigate the impact of sulfide on growth of *D. anilini*, growth was monitored via OD_{600} measurements in six cultures (with 1 mM aniline as substrate) in triplicate with various concentrations of sodium sulfide. The results shown in Fig. 1 indicate inhibition of growth in the presence of additional sodium sulfide. The growth rate decreased with increasing sodium sulfide concentrations, and complete inhibition was observed at a concentration of 20 mM sodium sulfide. When 5 mM sodium sulfide was added, growth of *D. anilini* decreased by 26.8%. Similar results were obtained using 2 mM phenol as substrate (Fig. 2).

The effects of three metal ions (Co^{2+}, Mn^{2+}, Zn^{2+}) on the growth of *D. anilini* were also studied. Growth experiments were performed in basal medium with four treatments in triplicate, as follows: the control culture without addition and the three test cultures with the elevated concentration of one trace element each. Optical densities were analyzed in the mid-exponential growth phase 48 days after inoculation (Fig. S1). The single addition of Mn^{2+} reduced the growth rate of *D. anilini* compared to the non-amended culture and the final OD_{600} was 0.112. Addition of Co^{2+} to the pure culture had no significant effect on the growth rate and the final OD_{600}. When Zn^{2+} was added, the growth rate of *D. anilini* increased slightly by 7.5% and the final OD_{600} (0.135) was slightly higher compared to

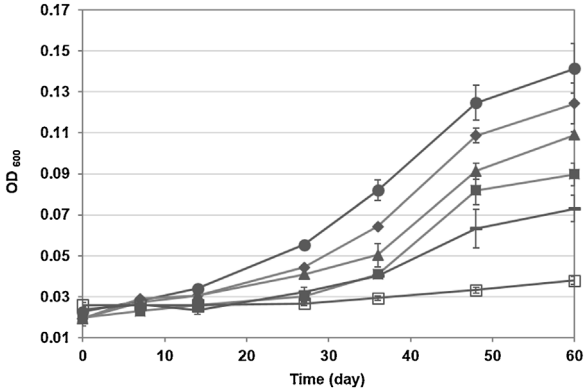

Fig. 1. Effect of sodium sulfide on the growth of *D. anilini* with aniline (1 mM) and sulfate (4 mM). Sodium sulfide (in addition to the sulfide present in the medium as a reducing agent) was added to the medium before inoculation, as follows: none (solid circle); 2 mM sodium sulfide (solid rhombus); 5 mM sodium sulfide (solid triangle); 10 mM sodium sulfide (solid square); 15 mM sodium sulfide (short line); 20 mM sodium sulfide (empty square).

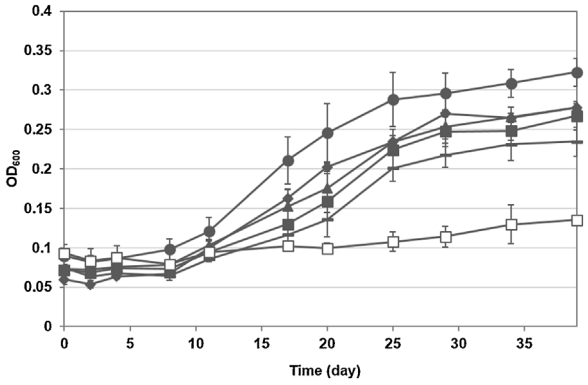

Fig. 2. Effect of sodium sulfide on the growth of *D. anilini* with phenol (2 mM) and sulfate (8 mM). Sodium sulfide (in addition to the sulfide present in the medium as a reducing agent) was added to the medium before inoculation, as follows: none (solid circle); 2 mM sodium sulfide (solid rhombus); 5 mM sodium sulfide (solid triangle); 10 mM sodium sulfide (solid square); 15 mM sodium sulfide (short line); 20 mM sodium sulfide (empty square).

the control (Fig. S1). None of the above metal ions caused a significant increase in the growth rate of *D. anilini*. Similar results were observed when lower concentrations (5 μM) of the metal ions were employed.

Growth in co-culture

Since sulfide inhibited the growth of *D. anilini*, a co-culture with a sulfide-consuming bacterium, *T. roseopersicina*, was established. *T. roseopersicina* is a purple sulfur bacterium belonging to the family *Chromatiaceae* and is capable of photolithoautotrophic growth under anoxic conditions in the light with sulfide as an electron donor [13]. Growth curves of the cultures and aniline concentrations in pure and co-cultures are shown in Fig. 3. The pure culture of *D. anilini* grew very slowly with a doubling time of 20 days. The pure culture of *T. roseopersicina* stopped growing when the electron donor sulfide added as a reductant was completely consumed. *T. roseopersicina* in the co-culture grew continuously with the sulfide provided by *D. anilini*. The rate of aniline consumption by *D. anilini* in co-culture was much higher with 0.016 mM per day compared to the pure culture of *D. anilini* with only 0.006 mM per day, and thus aniline degradation during co-cultivation occurred 2.66 times faster compared to the *D. anilini* pure culture. Since *T. roseopersicina* was unable to utilize aniline (Fig. 3), the higher aniline consumption rate in the co-culture must have resulted from increased activity of *D. anilini*.

To investigate the individual growth rate of *D. anilini* and *T. roseopersicina*, and the profiles of sulfide, sulfate and aniline by each strain, a co-cultivation device (Fig. S2) was designed that connected the headspaces of the pure cultures of *D. anilini* and *T. roseopersicina* in order to allow hydrogen sulfide exchange through the gas phase. As shown in Fig. 4, 1.01 mM aniline was completely consumed in 33 days together with 3.89 mM sulfate reduction to sulfide, which did not accumulate in the *D. anilini* culture but was taken up by *T. roseopersicina* leading to the formation of sulfate and growth of the *T. roseopersicina* culture. After 33 days, 2.34 mM sulfate was formed by *T. roseopersicina*. The milky appearance of the culture and the presence of bright refractive round particles visible in the cells by phase contrast microscopy, suggested that most of the 6 mM sulfide (2 mM was present in the medium as a reductant and 4 mM was formed by the *D. anilini* culture) consumed by *T. roseopersicina* was oxidized to elemental sulfur. The dissolved sulfide concentration in the *D. anilini* culture was constant across a range of 2 mM during the co-cultivation process, which was lower than for the pure culture (4.52 mM dissolved sulfide in the pure *D. anilini* culture in the stationary phase). The doubling time of *D. anilini* in the co-culture decreased significantly to 15 days. Obviously, *D. anilini* benefitted from the lower toxicity of hydrogen sulfide in the co-culture compared to the pure culture.

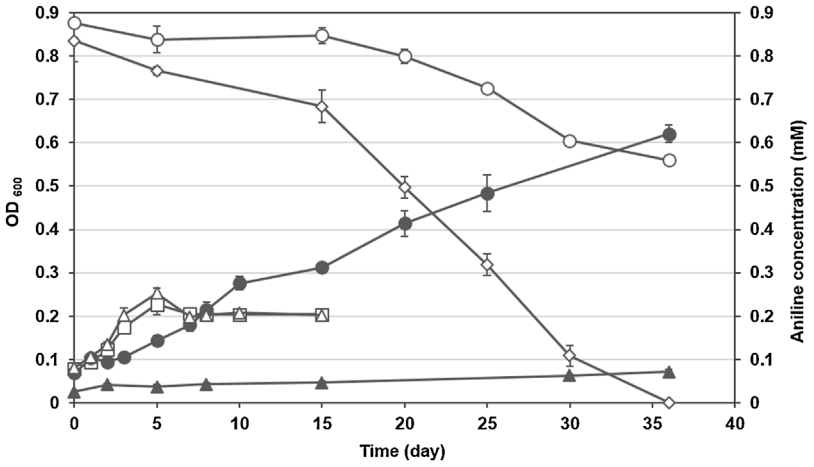

Fig. 3. Growth and aniline degradation in pure cultures (*D. anilini* or *T. roseopersicina*) and co-culture (*D. anilini* plus *T. roseopersicina*). Growth of *D. anilini* (solid triangle); growth of *T. roseopersicina* (empty triangle); growth of *T. roseopersicina* with 1 mM aniline (empty square); growth of co-culture including *D. anilini* and *T. roseopersicina* (solid circle); aniline degradation in co-culture (empty rhombus); aniline degradation in pure culture of *D. anilini* (empty circle). Values are means ± standard deviation (n = 3).

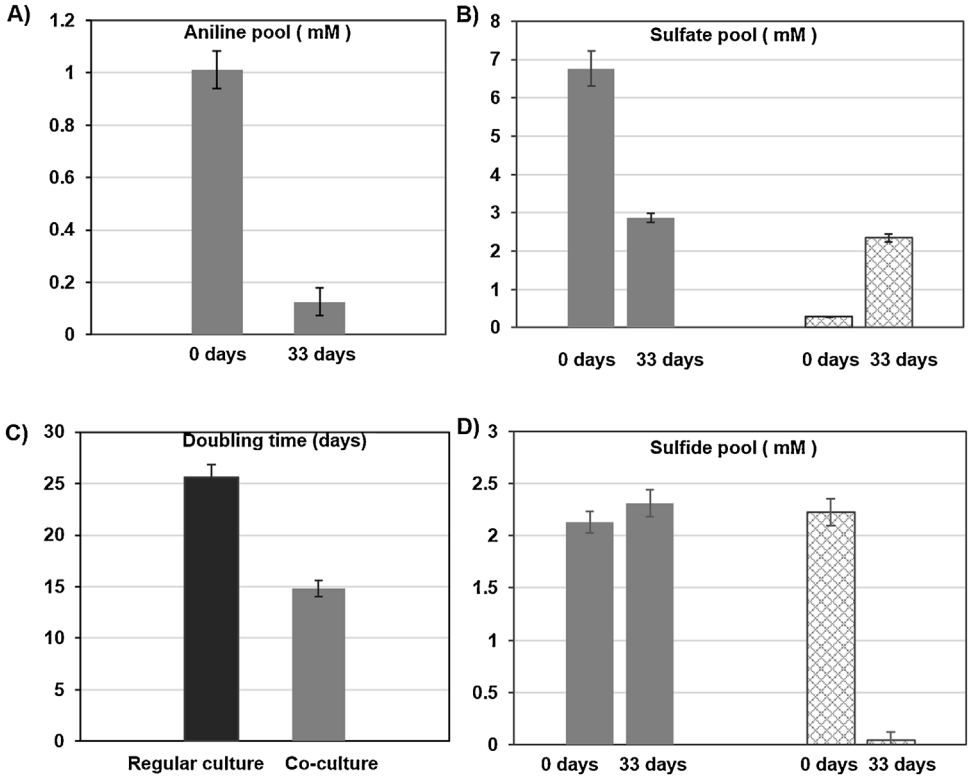

Fig. 4. Changes of aniline, sulfate, and sulfide in a co-cultivation setup after 33 days. (A) Aniline concentration in the bottle with a *D. anilini* culture; (B) Sulfate concentration in both cultures; (C) Comparison of the doubling time of *D. anilini* in the co-cultivation setup and pure culture; (D) Sulfide concentration in both cultures. *Desulfatiglans anilini* in co-culture (solid grey); *Desulfatiglans anilini* in pure culture (solid dark grey); *Thiocapsa roseopersicina* (pattern fill). Values are means ± standard deviation (n = 3).

Co-substrate for aniline activation

To understand the mechanism of sulfide inhibition on the growth of *D. anilini*, the aniline activation reaction was investigated (Fig. S3A). It has been demonstrated in dense cell suspensions that CO_2 is required for aniline activation [39]. Therefore, this dependency on CO_2 was also tested in cell-free extracts of *D. anilini* in order to strengthen the carboxylation hypothesis. The activity of aniline utilization was tested in cell-free extracts of aniline-grown *D. anilini* cells by measuring the depletion of aniline in the presence of ATP and CO_2 (Fig. 5). Fig. 1 shows that in the presence of CO_2 as a co-substrate, the rate of aniline degradation was 9 nmol/(min x mg protein) within 2 h incubation, and practically no aniline was utilized in the absence of CO_2. ATP is an essential co-factor for aniline utilization. Formate was also tested as a co-substrate to replace CO_2, but no aniline was depleted in the presence of formate (data not shown). No end product of the reaction was detected in the assay system by HPLC.

Effect of sulfide on aniline carboxylation and phenylphosphate synthase

The influence of sulfide on the enzyme activity of aniline carboxylation in *D. anilini* was tested (Fig. S3A). The activity in cell-extracts was reduced by half when 5 mM sodium sulfide was added to the assay system (Table 1). The pH of the enzyme assay system was 7.5, 7.7 or 7.9 in the presence of 0 mM, 2 mM or 5 mM sodium sulfide, respectively, and showed no significant differences in pH due to the addition of sodium sulfide, thus indicating that sulfide rather than pH influenced the enzyme activity.

Since sulfide also inhibited the growth of *D. anilini* on phenol, and to test whether the effect of sulfide on enzyme activity was only specific for the aniline-carboxylating enzyme system, the influence of sulfide on the activity of phenylphosphate synthase, the enzyme catalyzing the conversion of phenol to phenylphosphate in the presence of ATP, was also measured (Fig. S3B). The activity of phenylphosphate synthase was measured with phenol and ATP as substrates in cell-free extracts of phenol-grown *D. anilini* cells [48]. The activity of phenylphosphate synthase was assayed as the rate of phenol consumption (Table 1). 2 mM or 5 mM sodium sulfide were added to the enzyme assay system to measure the effect of sodium sulfide on the activity of phenylphosphate synthase. Table 1 shows that the activity of phenylphosphate synthase decreased by 60% after the addition of 2 mM sodium sulfide, and nearly 90% of phenylphosphate synthase activity was inhibited by 5 mM sodium sulfide. From these findings, it was concluded that sulfide negatively affected *D. anilini* aniline and phenol activation by inhibiting the enzyme activity of the aniline-carboxylating enzyme system and phenylphosphate synthase (Fig. S3).

Discussion

Hydrogen sulfide is formed by sulfate reduction in the sulfate-reducing strain *D. anilini* and it inhibited growth of *D. anilini* (Figs. 1 and 2), which is in accordance with previous studies on the inhibition of growth by hydrogen sulfide in sulfate-reducing bacteria (SRB) [16,27,34]. Inhibition of SRB due to hydrogen sulfide was found to be direct and reversible [34]. By co-culturing with *T. roseopersicina*, a phototrophic sulfide consuming bacterium, a significant increase in the growth rate of *D. anilini* could be achieved, as well as an increase in the aniline consumption rate of *D. anilini*. During the course of co-cultivation in a co-cultivation device, the concentration of sulfide in the liquid phase was continuously quantitatively recorded. Compared to a previous growth

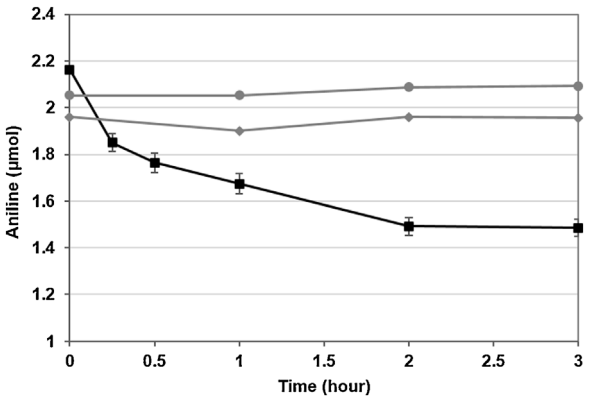

Fig. 5. Aniline consumption by cell-free extracts of *D. anilini* with CO_2 as a co-substrate. For black circles, black squares and grey circles, values are means ± standard deviation (n = 3). Standard assay with CO_2 (black squares); standard assay with N_2 (grey circles); control assay without ATP with CO_2 (rhombus).

study performed at pH 6.2 and pH 6.6 [34], more gaseous H_2S was converted to water-soluble HS^- in our culture (pH 7.2), according to a sulfide solubility chart at varying pH [17]. At the same time, a certain amount of sulfide was always left in the liquid phase and could not be removed efficiently by *T. roseopersicina.* By lowering the cultivation pH further, the equilibrium of hydrogen sulfide concentrations between the gaseous and liquid phases could be shifted towards gaseous hydrogen sulfide release. However, at more acidic pH, *D. anilini* did not grow any further, since the pH growth range lies between 6.0 and 8.0 [38]. In the co-cultivation device used in the present study, the gaseous H_2S in the cultivation vessel of *D. anilini* was driven into the vessel of *T. roseopersicina* due to the increased partial pressure generated by continuous hydrogen sulfide formation through *D. anilini* and the low partial pressure resulting from H_2S consumption by *T. roseopersicina*. This experimental setup was chosen to assess properly the contribution of each individual strain to biomass production and aniline degradation. The system could be improved further by cultivating both strains directly in one cultivation vessel, such that communicating gas phases would not be necessary anymore and the sulfide concentrations during growth could be kept at lower levels. However, aniline degrading SRB-like *D. anilini* would still need a certain amount of sulfide left in the cultivation media to maintain a sufficiently low redox potential. In the current study, replacement of sulfide as a reducing agent by titanium-NTA was attempted, however, growth of *D. anilini* did not improve under these conditions (data not shown). Testing other potential reducing agents, such as palladium/hydrogen or thiol-compounds that are less toxic compared to sulfide (dithiotreitol, thioglycolate) will be the subject of further investigation. It is proposed that anaerobic aniline degradation for bioremediation purposes could be improved by employing consortia consisting of aniline degrading SRB and phototrophic purple sulfur bacteria. The fact that phenylphosphate synthase is also sensitive to elevated concentrations of sulfide indicated that anaerobic phenol degradation by *D. anilini* could also be improved by maintaining low sulfide concentrations. While aerobic microorganisms (e.g. *P. putida*) are more efficient at degrading aromatic pollutants, anaerobic aniline or phenol degraders could also be of importance at contaminated sub-surface sites, where pump-and-treat procedures cannot be employed [20].

However, since the system used in this study was rather artificial, it is questionable whether such consortia of aniline-degrading SRB and phototrophic purple sulfur bacteria actually occur in nature. Consortia of SRB and sulfide-oxidizing purple sulfur bacteria have already been described to occur naturally in so-called "pink berries" present in the Sippewissett salt marsh and a syntrophic sulfur-cycling system between both types of organisms was described [42,47]. The studies on the pink berry consortia showed that the strictly anaerobic SRB also occurred at the sediment-water interface of intertidal pools and not only in sulfate-rich, anoxic sediments as in the case of *D. anilini* [38,42,47]. Furthermore, the example of the pink berries shows that sulfur-cycling between SRB and phototrophic sulfide-oxidizers can occur at the micromolar scale (sulfide concentrations of 0–500 µM) and improvement of growth of such consortia, as well as other sulfide-sensitive SRB in the laboratory, could possibly be accomplished by reducing the amount of sulfide and/or sulfate added to such cultures to less than 1 mM [47].

D. anilini was isolated from marine sediments of the North Sea coast, but also grows well in brackish water medium [38]. Marine sediments, especially in coastal areas, are known for increased activities of SRB and thus for their high sulfide concentrations [28]. *Thiocapsa* species are also known to be present in marine systems [9,11] and aniline-degrading consortia of the type described in this study could therefore possibly also occur in natural environments. However, aniline as a mainly man-made aromatic compound is unlikely to be present in non-contaminated environmental systems, which suggests that co-operations between SRB and phototrophic purple sulfur bacteria may play a significant role mainly during breakdown of naturally occurring aliphatic or aromatic compounds. Since aniline may occur both in polluted marine or freshwater ecosystems or contaminated soils, application of the marine strain *D. anilini* as a bioremediation agent in low-salinity environments would need artificial growth conditions, while at the same time infusing the culture with contaminated low-salinity liquids, which could theoretically be accomplished by providing the strain in membrane-separated bioreactors together with high molecular weight osmolytes that mimic high salinity. Such membrane-based bioreactors have already been employed in model systems treating phenol with the fungus *Neurospora crassa* [26], as well as in field-scale setups for aerobic treatment of groundwater contaminated with gasoline [49].

In the present study, inhibition of the degradation of aniline by sulfide under sulfate-reducing conditions was investigated in *D. anilini*. The experiments with cell-free extracts confirmed that CO_2 was needed as a co-substrate in order to activate aniline, as observed previously in dense cell suspension [39]. In this reaction, ATP is required and is most likely hydrolyzed to ADP or AMP and inorganic phosphate, such that a phosphate-aniline ligation product can be formed as an intermediate, analogous to the formation of phenylphosphate as an intermediate in phenol degradation. This intermediate could then be carboxylated to form 4-aminobenzoate (Fig. S3). The effects of sulfide on the aniline-carboxylating enzyme system and on phenylphosphate synthetase were investigated. In the presence of sulfide, the activities of the aniline-carboxylating enzyme system and phenylphosphate synthase were inhibited in

Table 1
Effect of sulfide on the activity of the aniline-carboxylating enzyme system and phenylphosphate synthase.

	Aniline-carboxylating enzyme system			Phenylphosphate synthase		
Sodium sulfide (mM)	0	2	5	0	2	5
Activity (nmol/min × mg)	13	11	7	25	7	2.5
Percentage (%)	100	84.6	53.8	100	28	10

cell-free extracts of *D. anilini*. Hydrogen sulfide is known to inhibit enzyme reactions by modifying the catalytic centers of metalloproteins, for example, in angiotensin-converting enzyme. This enzyme contains zinc, and hydrogen sulfide may react with zinc and thus inhibit enzyme activity [23]. In a recent study investigating phenol degradation in *D. anilini*, it was reported that phenylphosphate synthase activity depended on manganese, magnesium and potassium ions as co-factors, and the most likely candidate genes responsible were annotated as pyruvate-water dikinase [48]. For the *in-vitro* reaction of aniline activation tested in this study, addition of magnesium was essential, and probably acted as a chelating cofactor for ATP, as observed previously in other kinase reactions. However, manganese, magnesium and potassium ions react poorly with sulfide in aqueous solution, which suggests that these enzyme cofactors are most likely unaffected by sulfide. In a study on cytochrome c_3 as a sulfur reductase in various SRB, sulfide was demonstrated to inhibit enzyme activity by end-product inhibition rather than iron depletion [12]. However, sulfide is not part of the enzyme reaction of the aniline and phenol activating enzyme systems in *D. anilini*, indicating that the inhibition in this case was of an allosteric nature or due to cofactor modification. The genes coding for enzymes involved in aniline activation by anaerobic bacteria have not yet been identified, and the metal co-factors of the aniline-carboxylating enzyme system are still unknown.

The addition of trace elements, including cobalt, manganese or zinc, to the basal medium did not enhance the growth of *D. anilini*, indicating that these trace elements were not crucial limitation factors for growth of *D. anilini* and that the co-factor responsible for activation or carboxylation of aniline is possibly different from the trace elements tested, or can be exchanged with other metals. It appears that inhibition of *D. anilini* growth with aniline by sulfide is most likely due to a combination of end-product inhibition during sulfate reduction, as well as allosteric inhibition or cofactor modification of the enzymes involved in aniline degradation.

Conclusion

D. anilini is the only known sulfate-reducing bacterium (SRB) that can degrade aniline, a toxic aromatic compound, under completely anoxic conditions. Increasing concentrations of sulfide in *D. anilini* cultures in the presence of aniline led to inhibition of growth with complete growth inhibition at sulfide concentrations of 20 mM. A co-cultivation device, in which both growth rate as well as the rate of aniline consumption by *D. anilini* were effectively increased, was established, thus providing a novel synthetic consortium of SRB and phototrophic purple sulfur bacteria that has the potential to serve as an agent for efficiently remediating aniline contaminated sites. It was demonstrated by *in-vitro* enzyme activity assays that CO_2 was a co-factor for the activation of aniline. The aniline activating enzyme activity was inhibited by sulfide and it is concluded that the growth inhibiting effect of sulfide can at least be partially explained by the inhibition of this enzyme system.

Acknowledgements

X. X. is grateful to the China Scholarship Council (CSC) for providing a PhD scholarship. The authors thank Bernhard Schink for critically reading the manuscript, and Antje Wiese for preparing growth media.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.syapm.2019.06.003.

References

[1] Acosta, O.B.G., Hardt, N., Schink, B. (2013) Carbonylation as a key reaction in anaerobic acetone activation by *Desulfococcus biacutus*. Appl. Environ. Microbiol. 79, 6228–6235.
[2] Anderson, R.T., Lovley, D.R. (2000) Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer. Environ. Sci. Technol. 34, 2261–2266.
[3] Anotai, J., Lu, M.C., Chewpreecha, P. (2006) Kinetics of aniline degradation by Fenton and electro-Fenton processes. Water Res. 40, 1841–1847.
[4] Aoki, K., Ohtsuka, K., Shinke, R., Nishira, H. (1984) Microbial-metabolism of aromatic-amines. 4. Rapid biodegradation of aniline by *Frateuria* species ANA-18 and its aniline metabolism. Agric. Biol. Chem. 48, 865–872.
[5] Bachofer, R., Lingens, F., Schafer, W. (1975) Conversion of aniline into pyrocatechol by a *Nocardia* sp.; incorporation of oxygen-18. FEBS Lett. 50, 288–290.
[6] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
[7] Breinig, S., Schiltz, E., Fuchs, G. (2000) Genes involved in anaerobic metabolism of phenol in the bacterium *Thauera aromatica*. J. Bacteriol. 182, 5849–5863.
[8] Cao, B., Nagarajan, K., Loh, K.C. (2009) Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl. Microbiol. Biotechnol. 85, 207–228.
[9] Caumette, P., Guyoneaud, R., Imhoff, J.F., Suling, J., Gorenko, V. (2004) *Thiocapsa marina* sp. nov., a novel, okenone-containing, purple sulfur bacterium isolated from brackish coastal and marine environments. Int. J. Syst. Evol. Microbiol. 54, 1031–1036.
[10] Cline, J.D. (1969) Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14, 454–458.
[11] Dewit, R., Vangemerden, H. (1987) Chemolithotrophic growth of the phototrophic sulfur bacterium *Thiocapsa roseopersicina*. FEMS Microbiol. Ecol. 45, 117–126.
[12] Fauque, G., Herve, D., Le Gall, J. (1979) Structure-function relationship in hemoproteins: the role of cytochrome c_3 in the reduction of collodial sulfur by sulfate-reducing bacteria. Arch. Microbiol. 121, 261–264.
[13] Guyoneaud, R., Suling, J., Petri, R., Matheron, R., Caumette, P., Pfennig, N., Imhoff, J.F. (1998) Taxonomic rearrangements of the genera *Thiocapsa* and *Amoebobacter* on the basis of 16S rDNA sequence analyses, and description of *Thiolamprovum* gen. nov. Int. J. Syst. Bacteriol. 48, 957–964.
[14] Head, I.M. (1998) Bioremediation: towards a credible technology. Microbiol-UK 144, 599–608.
[15] Heider, J., Schuhle, K., Frey, J., Schink, B. (2016) Activation of acetone and other simple ketones in anaerobic bacteria. J. Mol. Microbiol. Biotechnol. 26, 152–164.
[16] Hilton, B.L., Oleszkiewicz, J.A. (1990) Sulfide-induced inhibition of anaerobic digestion. J. Environ. Eng. ASCE 116, 1007–1008.
[17] Holmer, M., Hasler-Sheetal, H. (2014) Sulfide intrusion in seagrasses assessed by stable sulfur isotopes—a synthesis of current results. Front. Mar. Sci. 1, 1–12.
[18] Jing, Z.Q., Cao, S.W., Yu, T., Hu, J. (2015) Degradation characteristics of aniline with ozonation and subsequent treatment analysis. J. Chem. N. Y.
[19] Kahng, H.Y., Kukor, J.J., Oh, K.H. (2000) Characterization of strain HY99, a novel microorganism capable of aerobic and anaerobic degradation of aniline. FEMS Microbiol. Lett. 190, 215–221.
[20] Khan, F.I., Husain, T., Hejazi, R. (2004) An overview and analysis of site remediation technologies. J. Environ. Manage. 71, 95–122.
[21] Konopka, A., Knight, D., Turco, R.F. (1989) Characterization of a *Pseudomonas* sp. capable of aniline degradation in the presence of secondary carbon-sources. Appl. Environ. Microbiol. 55, 385–389.
[22] Lack, A., Fuchs, G. (1994) Evidence that phenol phosphorylation to phenylphosphate is the first step in anaerobic phenol metabolism in a denitrifying *Pseudomonas* sp. Arch. Microbiol. 161, 132–139.
[23] Laggner, H., Hermann, M., Esterbauer, H., Muellner, M.K., Exner, M., Gmeiner, B.M.K., Kapiotis, S. (2007) The novel gaseous vasorelaxant hydrogen sulfide inhibits angiotensin-converting enzyme activity of endothelial cells. J. Hypertens. 25, 2100–2104.
[24] Li, Y., Loh, K.C. (2006) Activated carbon impregnated polysulfone hollow fiber membrane for cell immobilization and cometabolic biotransformation of 4-chlorophenol in the presence of phenol. J. Membr. Sci. 276, 81–90.
[25] Li, Y., Loh, K.C. (2006) Continuous cometabolic transformation of 4-chlorophenol in the presence of phenol in a hollow fiber membrane bioreactor. J. Environ. Eng. ASCE 132, 309–314.
[26] Luke, A.K., Burton, S.G. (2001) A novel application for *Neurospora crassa*: progress from batch culture to a membrane bioreactor for the bioremediation of phenols. Enzyme Microb. Technol. 29, 348–356.
[27] McCartney, D.M., Oleszkiewicz, J.A. (1991) Sulfide inhibition of anaerobic degradation of lactate and acetate. Water Res. 25, 203–209.
[28] Mudryk, Z.J., Podgorska, B., Ameryk, A., Bolalek, J. (2000) The occurrence and activity of sulphate-reducing bacteria in the bottom sediments of the Gulf of Gdańsk. Oceanologia 42, 105–117.
[29] Müller, N., Griffin, B.M., Stingl, U., Schink, B. (2008) Dominant sugar utilizers in sediment of Lake Constance depend on syntrophic cooperation with methanogenic partner organisms. Environ. Microbiol. 10, 1501–1511.
[30] Murphy, T., Moller, A., Brouwer, H. (1995) *In situ* treatment of Hamilton Harbour sediment. J. Aquat. Ecosyst. Stress Recov. 4, 195–203.

[31] Oh, Y.S., Bartha, R. (1997) Construction of a bacterial consortium for the biofiltration of benzene, toluene and xylene emissions. World J. Microbiol. Biotechnol. 13, 627–632.
[32] Parales, R.E., Ontl, T.A., Gibson, D.T. (1997) Cloning and sequence analysis of a catechol 2,3-dioxygenase gene from the nitrobenzene-degrading strain *Comamonas* sp. JS765. J. Ind. Microbiol. Biotechnol. 19, 385–391.
[33] Qi, X.H., Zhuang, Y.Y., Yuan, Y.C., Gu, W.X. (2002) Decomposition of aniline in supercritical water. J. Hazard. Mater. 90, 51–62.
[34] Reis, M.A.M., Almeida, J.S., Lemos, P.C., Carrondo, M.J.T. (1992) Effect of hydrogen-sulfide on growth of sulfate reducing bacteria. Biotechnol. Bioeng. 40, 593–600.
[35] Schleinitz, K.M., Schmeling, S., Jehmlich, N., von Bergen, M., Harms, H., Kleinsteuber, S., Vogt, C., Fuchs, G. (2009) Phenol degradation in the strictly anaerobic iron-reducing bacterium *Geobacter metallireducens* GS-15. Appl. Environ. Microbiol. 75, 3912–3919.
[36] Schmeling, S., Narmandakh, A., Schmitt, O., Gad'on, N., Schuhle, K., Fuchs, G. (2004) Phenylphosphate synthase: a new phosphotransferase catalyzing the first step in anaerobic phenol metabolism in *Thauera aromatica*. J. Bacteriol. 186, 8044–8057.
[37] Schmidt, A., Frensch, M., Schleheck, D., Schink, B., Müller, N. (2014) Degradation of acetaldehyde and its precursors by *Pelobacter carbinolicus* and *P. acetylenicus*. PLoS One 9, e115902.
[38] Schnell, S., Bak, F., Pfennig, N. (1989) Anaerobic degradation of aniline and dihydroxybenzenes by newly isolated sulfate-reducing bacteria and description of *Desulfobacterium anilini*. Arch. Microbiol. 152, 556–563.
[39] Schnell, S., Schink, B. (1991) Anaerobic aniline degradation via reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. Arch. Microbiol. 155, 183–190.
[40] Schuhle, K., Fuchs, G. (2004) Phenylphosphate carboxylase: a new C-C lyase involved in anaerobic phenol metabolism in *Thauera aromatica*. J. Bacteriol. 186, 4556–4567.
[41] Schukat, B., Janke, D., Krebs, D., Fritsche, W. (1983) Cometabolic degradation of 2-chloroaniline and 3-chloroaniline because of glucose-metabolism by *Rhodococcus* sp. An-117. Curr. Microbiol. 9, 81–86.
[42] Seitz, A.P., Nielsen, T.H., Overmann, J. (1993) Physiology of purple sulfur bacteria forming macroscopic aggregates in Great Sippewissett Salt Marsh, Massachusetts. FEMS Microbiol. Ecol. 12, 225–236.
[43] Spormann, A.M., Thauer, R.K. (1988) Anaerobic acetate oxidation to CO_2 by *Desulfotomaculum acetoxidans*. Demonstration of enzymes required for the operation of an oxidative acetyl-CoA/carbon monoxide dehydrogenase pathway. Arch. Microbiol. 150, 374–380.
[44] Tabatabai, M.A. (1974) Rapid method for determination of sulfate in water samples. Environ. Lett. 7, 237–243.
[45] Tschech, A., Fuchs, G. (1989) Anaerobic degradation of phenol via carboxylation to 4-hydroxybenzoate: *in vitro* study of isotope exchange between $^{14}CO_2$ and 4-hydroxybenzoate. Arch. Microbiol. 152, 594–599.
[46] Widdel, F., Pfennig, N. (1981) Studies on dissimilatory sulfate-reducing bacteria that decompose fatty-acids. 1. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments: description of *Desulfobacter postgatei* gen. nov., sp. nov. Arch. Microbiol. 129, 395–400.
[47] Wilbanks, E.G., Jaekel, U., Salman, V., Humphrey, P.T., Eisen, J.A., Facciotti, M.T., Buckley, D.H., Zinder, S.H., Druschel, G.K., Fike, D.A., Orphan, V.J. (2014) Microscale sulfur cycling in the phototrophic pink berry consortia of the Sippewissett Salt Marsh. Environ. Microbiol. 16, 3398–3415.
[48] Xie, X., Müller, N. (2018) Enzymes involved in the anaerobic degradation of phenol by the sulfate-reducing bacterium *Desulfatiglans anilini*. BMC Microbiol. 18, 93.
[49] Zein, M.M., Suidan, M.T., Venosa, A.D. (2006) Bioremediation of groundwater contaminated with gasoline hydrocarbons and oxygenates using a membrane-based reactor. Environ. Sci. Technol. 40, 1997–2003.
[50] Zeyer, J., Wasserfallen, A., Timmis, K.N. (1985) Microbial mineralization of ring-subsituted anilines through an *ortho*-cleavage pathway. Appl. Environ. Microbiol. 50, 447–453.
[51] Zhang, Z., Lo, I.M.C., Yan, D.Y.S. (2015) An integrated bioremediation process for petroleum hydrocarbons removal and odor mitigation from contaminated marine sediment. Water Res. 83, 21–30.