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Stimulation of *in vitro* anaerobic oxidation of methane rate in a continuous high-pressure bioreactor

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ABSTRACT

Anaerobic oxidation of methane coupled to sulphate reduction (SR-AOM) prevents oceanic methane emissions and is considered as a major environmental process in the deep-sea sediments. To stimulate *in vitro* SR-AOM activity, we designed a (continuous) high-pressure bioreactor system. Fed-batch mode incubations showed that the elevated methane pressure stimulated SR-AOM activity: when the methane pressure increased from 1 to 4.5 to 8 MPa, the initial SR-AOM activity increased from 3.46 to 8.64 to 9.22 μmol sulphide production/g_{dw}/day; the apparent affinity (K_m) for methane was 37 mM. However, in each fed-batch mode incubation, there was an inhibitory effect observed after 2 days, probably due to the accumulation of sulphide and the increase of pH. When the reactor was operated in a continuous mode, the SR-AOM activity was constantly increasing within 18 days at both 1 and 8 MPa pressures.

1. Introduction

Anaerobic oxidation of methane coupled with sulphate reduction (SR-AOM) is a major sink in the oceanic methane budget. Beneath the sea floor, every year there is 75–320 Tg of methane generated, and only a small fraction (5–20 Tg/year) escapes from oxygen-depleted marine sediments, while most of which has been oxidized anaerobically by the microorganisms using sulphate as electron acceptor (Boetius et al., 2000; Nauhaus et al., 2002; Reeburgh, 1976; Valentine, 2002). The net stoichiometry of this reaction is (Reeburgh, 2007):

$$CH_4 + SO_4^{2-} \rightarrow HCO_3^- + HS^- + H_2O \quad \Delta G^{\circ\prime} = -16.6 \text{ kJ/mol } CH_4 \tag{1}$$

Many research groups have demonstrated the presence of anaerobic methanotrophic archaea (ANME) and sulphate reducing bacteria (SRB) in SR-AOM active sediments (Boetius et al., 2000; Girguis et al. 2005; Hinrichs et al., 1999; Knittel et al., 2005; Orphan et al., 2001). However, due to their extreme low growth rates (doubling times between 2 and 7 months) (Girguis et al., 2005; Krüger et al., 2008; Meulepas et al., 2009; Nauhaus et al., 2007), the biochemistry of methane oxidation and assimilation of methane into anaerobic methanotrophs is poorly understood and the knowledge on the regulating factors is very limit.

It is necessary to well study SR-AOM process, because: firstly, methane is a powerful greenhouse gas: one molecule of methane is 25 times more potent than one molecule of CO_2 (DeLong, 2000). Secondly, in the anaerobic layer of the sediment, methane is the major carbon and energy source driving the overall ecology. The energy gained from SR-AOM supports the growth of the microorganisms, which are playing an important role in the cold seep marine ecosystem and the foodweb there. The carbon dioxide produced from SR-AOM (Eq. (1)) precipitated with metals (Ca, Mg, etc.) to build up the sea floor landscape. In this way, the carbon dioxide is immobilized without diffusing into the atmosphere as greenhouse gas.

Some researchers focus on *in situ* analysis with geochemical and molecular techniques, while the others succeeded to restore SR-AOM activity *in vitro* using different reactor technologies (Girguis et al., 2005; Meulepas et al., 2009; Nauhaus et al., 2007). This research focused on the *in vitro* study of SR-AOM, especially on the reactor technology aspect, mainly considering: (1) high methane partial pressure: according to Eq. (1), the concentration of aqueous methane is expected to be a key variable for the reaction thermodynamics and kinetics. The poor solubility of methane (1.3 mM in sea water at ambient pressure at 15 °C, Yamamoto et al., 1967) demands a high methane partial pressure to stimulate SR-AOM reaction. (2) Continuous flow: another major concerning is that according to Le Châtelier's Law, the accumulated metabolites such as CO_2 and sulphide should be removed sufficiently before the SR-AOM reaction is slowed down. Especially the accumulated sulphide needs to be removed since it has toxic effect on sulphate reducing bacteria (Rabus and Heider, 1998). Based on the above

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reasons, the technology to combine high-pressure and continuous flow into one reactor system is highly demanded in order to stimulate *in vitro* SR-AOM activity. Moreover, SR-AOM hot spots were commonly found beneath hundreds to thousands meters of water depth, while a continuous pore-water flux was observed. Therefore for a better simulation of *in situ* SR-AOM active site environment, the high-pressure and continuous flow are both necessary.

In this paper, we report a novel continuous high-pressure bioreactor system developed to study SR-AOM process. The objective of applying this reactor system was to simulate diverse methane-rich deep-sea environments; to stimulate *in vitro* SR-AOM activity; and to get more information on the kinetics of SR-AOM process.

2. Methods

2.1. Origin and storage of the sediment samples

Sediment samples from Capt Aryutinov Mud Volcano (MV) (coordinates: 35:39.700/07:20.012, Gulf of Cadiz, Atlantic Ocean, 1200 m water depth) were collected on 30th April 2006 during a Maria S. Merian 1/3 cruise. For the sampling, a plastic liner was inserted into a Box Core (40 cm deep) that contained pieces of gas hydrate (0.5–5 cm long). The core was immediately caped and stored at 4 °C in trilaminate PEI aluminium bag (KENOSHA C.V., Amstelveen, The Netherlands) under nitrogen atmosphere.

2.2. Activation of the sediment at ambient pressure

All manipulations were carried out under an anoxic atmosphere of N_2 using either Hungate technique (Miller and Wolin, 1974) or an anoxic glove box (Concept 1000, L.E.D. Techno NV, Belgium). Original sediment was 2 times diluted with artificial seawater medium. Every liter of the basal medium consisted of: NaCl 26 g, $MgCl_2{\cdot}6H_2O$ 5 g, $CaCl_2{\cdot}2H_2O$ 1.4 g, Na_2SO_4 1.3 g, NH_4Cl 0.3 g, KH_2PO_4 0.1 g, KCl 0.5 g, a bicarbonate solution 30 ml, a trace element solution 1 ml, a vitamin mixture solution 1 ml, a thiamine solution 1 ml, and a vitamin B_{12} solution 1 ml. The bicarbonate solution, the trace element solution and the vitamin solutions were made according to Widdel and Bak (1998). The pH of the medium was adjusted to 6.8 by adding sulphuric acid. The headspace was flushed and filled with methane at 0.2 MPa absolute pressure. The incubation was performed at 10 °C in dark. After 43 days the active slurry was flushed with methane gas to remove sulphide accumulated, and further diluted with fresh medium up to 4 times dilution. Sulphuric acid was added to adjust pH back to 7.

To ensure that the sulphate reduction is solely coupled with methane oxidation, a control experiment without methane supplying was performed. A volume of 25 ml sediment slurry was transferred through a needle (18G 1 1/2″, 1.2 × 40 mm, BD Microlance, Ireland) into a 50 ml brown serum bottle. The serum bottle containing slurry was flushed with N_2 and placed vacuum before being incubated at 0.2 MPa absolute pressure of N_2 as headspace.

2.3. Scheme of the high-pressure bioreactor system

The high-pressure bioreactor system includes three main parts: the conditioning vessel, the high-pressure pump, and the incubation vessel (Fig. 1).

The conditioning vessel (Parr, USA) has a volume of 1 l and is made of T316 stainless steel. It is directly connected to a liquid pipette (to charge liquid medium at high-pressure) and a gas pipette (to charge methane gas at high-pressure). The methane gas bottle pressurizes both the liquid pipette and the gas pipette. In this vessel, artificial seawater medium is saturated with methane at a determined pressure (up to 8 MPa), and further transferred to the incubation vessel by a high-pressure pump (HPLC pump series III, SSI, USA). In the conditioning vessel, the pressure was built up solely by gas pressure, which determines the amount of methane saturated into medium and available as substrate. A motor-mixer ran at 40 rpm to achieve a homogeneous dissolved methane concentration in the whole vessel.

The high-pressure pump is piston based with a separated chamber to rinse the piston while it is moving. This self-flushing function allows us to feed salty water without damage the piston and its seals. The flow rate can be varied between 0.1 and 40 ml/min.

The incubation vessel (Parr, USA) has a volume of 0.6 l and is made of titanium to prevent potential microbial corrosion. It is placed inside a thermal regulated incubator (5–55 °C) (Tintometer, Germany) to control the incubation temperature. The pressure inside this incubation vessel can be elevated up to 16 MPa, and is regulated by the pump flow and a backpressure regulator followed (Swaglok, Switzerland).

There are two sampling ports: one is placed between the conditioning vessel and the high-pressure pump to take influent samples; the other is placed between the incubation vessel and the backpressure regulator to take effluent samples.

2.4. Chemical and biological analysis

Dissolved sulphide was analyzed by using LCK 653 Sulphide Cuvette and a Dr. Lange Xion 500 model LPG-385 photo-spectrometer (Hach Lange GMBH, Düsseldorf, Germany). To measure sulphate and chloride concentrations, 0.11 ml sample was firstly diluted with 0.99 ml zinc acetate solution (5 g/l) and then centrifuged at 13,200*g* for 3 min to remove insoluble zinc sulphide. A sample of 1 ml new supernatant was diluted with 9 ml mannitol solution (20 mM), and filtrated through 0.45 μm membrane filter (Millipore, NYSE:MIL, USA). Finally the samples were measured on an IC system (761 Compact IC, Metrohm, Switzerland). The pH value was read by putting a drop of supernatant from slurry on pH indicator paper (pH 5.5–9.0, Merck, Germany). The short chain fatty acid analysis was performed as described before (Nollet et al., 1997).

The dry weight (dw) and the volatile suspended solid (VSS) of the sediment were measured according to the standard methods (APHA, 1995). To check weather there is a significant washing out of biomass, the total amount of bacteria and archaea in the effluent samples was quantified by using Flow Cytometry and ATP quantification (Wang et al., 2007, 2008).

2.5. Calculations

When the methane pressures were lower than 2.5 MPa, the dissolved methane concentrations were calculated according to Henry's Law, taking temperature and salinity into account (Yamamoto et al., 1967). When the methane pressures were higher than 2.5 MPa, the dissolved methane concentrations were calculated according to Duan's models (Duan and Mao, 2006).

The rate of the specific SR-AOM activity was given in the unit of μmol sulphide production/g_{dw}/day. For the continuous mode incubation, the loss of sulphide due to continuous washing out from the effluent and sampling was taken into account, and the trend lines were added to show the increase of specific activity over time.The Gibbs free energy was calculated based on the following formula:

$$\Delta G = \Delta G^{\circ\prime} + R \cdot T \cdot \ln\left(\frac{[HS^-] \cdot [HCO_3^-]}{[CH_4] \cdot [SO_4^{2-}]}\right) \quad (2)$$

where R is the gas constant (8.314 J/mol/K), T is the absolute temperature. Concentrations of HS^- and SO_4^{2-} were from the measurement.

Since the bicarbonate production was negligible compared with 30 mM HCO_3^- in the medium as a buffer, 30 mM was used as bicarbonate concentration for all the calculations in this paper. $[CH_4]$ is the calculated dissolved methane concentration in aqueous phase.

The SR-AOM kinetics can be described by Michaelis–Menten kinetics

$$r = \frac{V_{max}[CH_4]}{K_m + [CH_4]} \quad (3)$$

where r is the SR-AOM rate, V_{max} is the maximum SR-AOM rate, K_m is the Michaelis–Menten (or half-saturation) constant, and $[CH_4]$ is the dissolved methane concentration. K_m is an affinity constant and is given as the CH_4 concentration where the SR-AOM rate is half of the maximum SR-AOM rate, V_{max}. According Eq. (3), the K_m was calculated based on fed-batch incubations at different methane pressure (1, 4.5, and 8 MPa).

3. Results

3.1. Batch incubations at ambient pressure

In a first set of experiments the anaerobic oxidation of methane couple with sulphate reduction was tested in batch at ambient pressure. Sulphide production can be sensitively quantified despite the pressure drop in the headspace due to the sampling; therefore it was monitored as the activity indication. An increase of sulphide concentration has been observed rapidly at ambient pressure incubation. After about 40 days, the pH increased to 8.5, the sulphide concentration increased up to 4 mM and stopped. To avoid the inhibitory effect of sulphide and to adjust the pH back to 7, sulphuric acid was added at day 43, the slurry was diluted with fresh medium as a ratio of 1:1 (V/V) and flushed with methane gas (Fig. 2). After the dilution, a steep increase of the sulphide concentration has been observed again (Fig. 2). The sulphide production after 4 times dilution (130.1 μM/day) was as fast as at 2 times dilution (131.3 μM/day). Control experiments with the same activated slurry but under N_2 atmosphere instead of methane showed no sulphate reduction or sulphide production after 26 days, showing that sulphate reduction was exclusively coupled with methane oxidation (Fig. 2). No short chain fatty acids accumulation was observed during the whole incubation time (data not shown). The sulphide production is a direct proof of SR-AOM activity, and sulphide production rate directly indicates for SR-AOM rate.

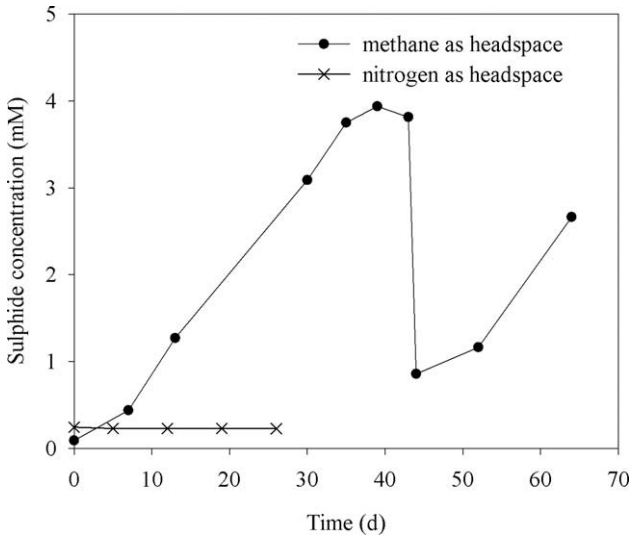

Fig. 2. The accumulative sulphide concentrations during the ambient pressure incubations under methane or nitrogen as headspace.

3.2. Reactor design and operation

When the reactor system was operated continuously, there was a constant depressurization in the conditioning vessel due to: the lost of mass in the conditioning vessel because the liquid and the dissolved gas were transferred to the incubation vessel; and the volume of the headspace increased in the same rate as pump flow. To slow down this depressurization, the connection between the gas charge pipette and the conditioning vessel was always open, which means there was always an extra 300 ml volume (from this gas charge pipette) used as headspace. Besides, the gas charge pipette was frequently filled up to keep the pressure variation less than 0.2 MPa during all the experimental time.

In the incubation vessel, there was no headspace. The pressure inside was solely built up by hydraulic pressure – the accumulation of liquid containing saturated methane. Because of the effluent samplings, the pressure in the incubation vessel dropped by suddenly losing liquid. This pressure drop resulted in an immediate liquid flow from the conditioning vessel to the incubation vessel. Although the pressure drop could be recovered immediately, the

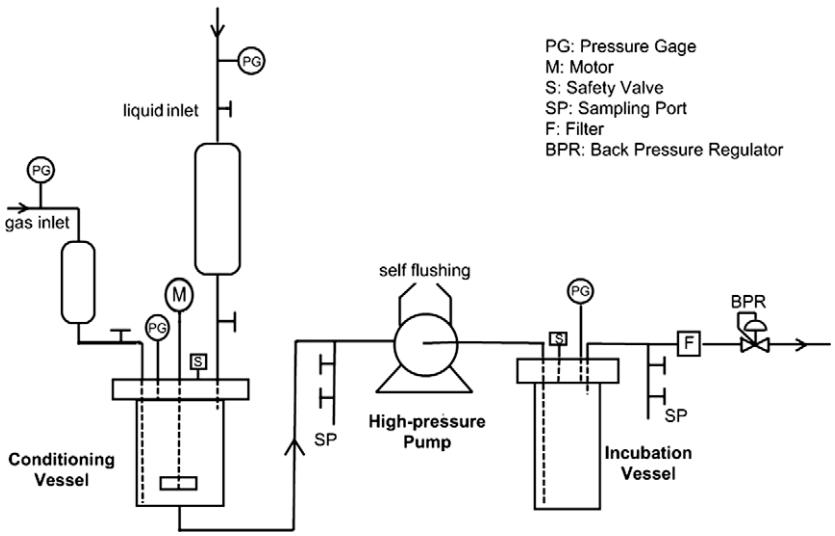

Fig. 1. The continuous high-pressure bioreactor system. PG: pressure gage; M: motor; S: safety valve; SP: sampling port; F: filter; BPR: back pressure regulator.

fast liquid flow may: (1) induce the mixing inside the incubation vessel; (2) disturb the biofilm formation. To overcome this pressure drop, different sampling volumes were examined. When the volume of the sampling port was reduced from 5 to 2 ml (0.3% of the incubation vessel volume), the pressure drop could be reduced considerably. During the pressure drop, the dissolved gas got released and took the volume of the sampling port. Therefore the volume of the sampling port can not be less than 2 ml otherwise there is not enough liquid left for analysis.

3.3. Fed-batch incubation experiments at high-pressure

To examine the effects of the pressure and the batch mode separately, in a first set of experiments, the reactor was run at high-pressure in fed-batch mode. A sample of 200 ml 4-times diluted active slurry from ambient pressure incubation was transferred into the special designed high-pressure bioreactor (Fig. 1) and further diluted with fresh medium. The final dilution was 12 times. For the fed-batch mode incubations, every day 10% liquid from incubation vessel was taken out for analysis and was replaced by fresh medium from conditioning vessel. Three different methane pressures were tested in a series: 1 MPa, 4.5 MPa, and 8 MPa. At all 1, 4.5, and 8 MPa methane pressures, sulphide production was observed rapidly. However, after 50 h, the accumulated sulphide started to inhibit SR-AOM activity (Fig. 3). Therefore initial SR-AOM activity was calculated based on the first 4 data points. When the methane partial pressure was increased from 1 to 4.5 to 8 MPa, the initial specific SR-AOM activity was increased from 3.46 to 8.64 to 9.22 μmol sulphide production/g_{dw}/day (Table 1). The apparent affinity (K_m) for methane was 37 mM, which was calculated using data from high-pressure incubations (1, 4.5, and 8 MPa).

3.4. Continuous incubation experiments at high-pressure

In order to keep sulphide below inhibitory concentration, the high-pressure bioreactor was operated in a continuous mode. The incubations were performed at 1 and 8 MPa methane pressure. At each pressure, the flow rate was set at 6 ml/h, which represents 4.2 days HRT (hydraulic retention time). The flow cytometry and ATP both read between 10^9 and 10^{10} cell/l in the effluent, which corresponded to about 1.3 mg/day biomass in the effluent. In the incubation vessel, there was 4.9 g biomass in total (according to VSS analysis), which means there was 0.5% biomass was washed out during the total experimental period in case no growth was expected. Therefore the loss of biomass due to washing out was negligible in this research.

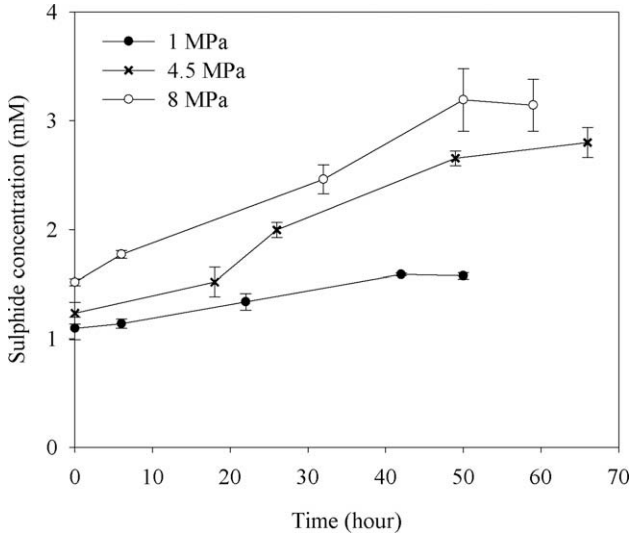

Fig. 3. Accumulative sulphide concentrations during fed-batch mode incubations at different methane pressures: 1 MPa, 4.5 MPa, and 8 MPa. The standard deviation was calculated based on 3 samplings.

Table 1
Specific SR-AOM activity compared with *in situ* data and fed-batch mode incubation at different pressures.

Incubation condition	Initial SR-AOM activity (μmol sulphide production/g_{dw}/day)	Gibbs' free energy gained from SR-AOM reaction (kJ/mol CH_4)
Ex situ	0–0.01[a]	27.9[a]
0.2 MPa batch	0.18[b]	30.1[b]
1 MPa fed-batch	3.46[c]	34.2[c]
4.5 MPa fed-batch	8.64[c]	37.8[c]
8 MPa fed-batch	9.22[c]	44.7[c]

[a] Calculation based on the data from Niemann et al. (2006).
[b] Calculation based on the data from ambient pressure incubation.
[c] Calculation based on the data from high-pressure incubations.

During the incubations under 1 MPa pressure (13 mM saturated methane, 100 m water depth), the methane loading rate was 31 μmol/g_{dw}/day, and the sulphate loading rate was 24 μmol/g_{dw}/day. The sulphide concentration in the effluent increased from 0.12 mM to 0.70 mM within 18 days incubation (Fig. 4A). When the methane partial pressure and incubation pressure both increased to 8 MPa (90 mM saturated methane, 800 m water depth), the flow rate was kept at 6 ml/h (HRT was 100 h), the methane loading rate and the sulphate loading rate were 216 and 24 μmol/g_{dw}/day. The sulphide concentration started to increase from the very beginning (Fig. 4B).

The specific SR-AOM activities slightly increased during incubations at both 1 MPa and 8 MPa pressures, and the increasing rates were comparable: 0.079 (μmol sulphide production/g_{dw}/day) per day at 1 MPa and 0.078 (μmol sulphide production/g_{dw}/day) per day at 8 MPa (Fig. 4).

4. Discussion

4.1. The rapid recovery of SR-AOM activity at ambient pressure incubation

After one year storing under anaerobic condition in cold, the sediment from Captain Aryutinov MV showed rapid sulphate reduction activity when methane and sulphate were supplied. This rapid recovery of SR-AOM activity demonstrated that the active sediment could be long-term stored under anaerobic atmosphere and at low temperature without losing SR-AOM activity completely. Moreover, this sulphate reduction activity was only present when methane was supplied as carbon source and electron donor (Fig. 2). Therefore the sulphide production monitored in this study is sufficient for monitoring SR-AOM activity.

At ambient pressure incubation, 4 mM sulphide concentration exhibited inhibitory effects on the SR-AOM activity (Fig. 2). Indeed, according to Eq. (2), when the sulphate concentration decreased from 10 mM to 6 mM and the sulphide concentration increased from 0.09 to 4 mM, the Gibbs free energy gained from the reaction decreased from 16.6 to 10.1 kJ/mol CH_4. The reduced energy supply for microorganisms is limiting the microbial activity. Besides, it has been reported that a high hydrogen sulphide concentration is toxic to sulphate reducing bacteria (Rabus and Heider, 1998). On the

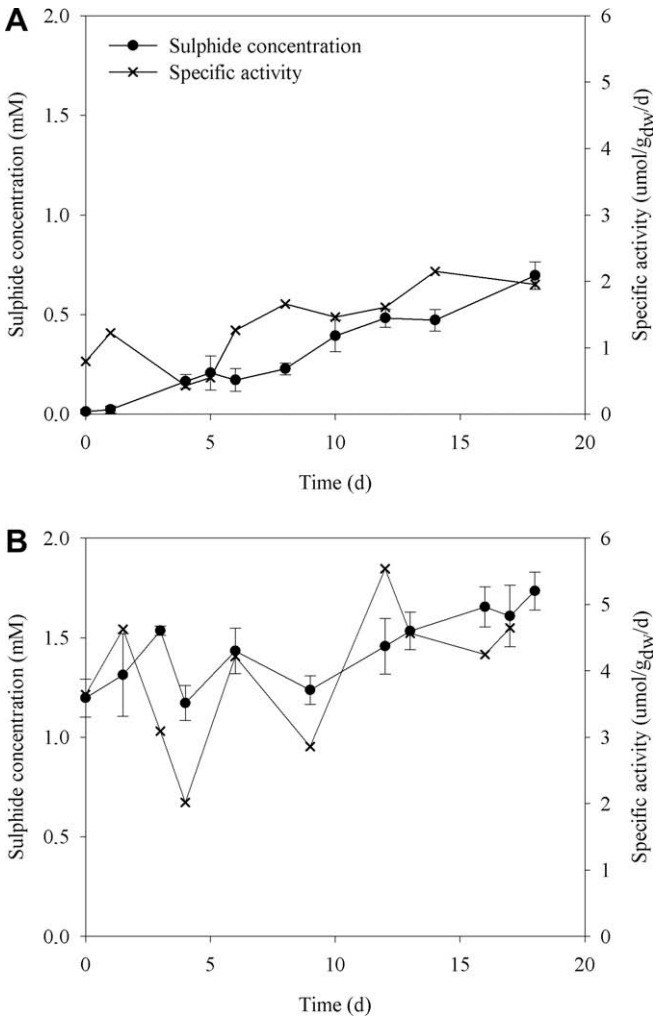

Fig. 4. Effluent sulphide concentrations and the specific AOM activities from incubations with continuous flow at different methane pressures: 1 MPa (A) and 8 MPa (B). The standard deviation was calculated based on 3 samplings.

other hand, this inhibitory effect can also be caused from the elevated alkalinity due to the production of sulphide and carbon dioxide (Eq. (1)). Nauhaus et al. (2005) have reported the optimum pH for SR-AOM activity from Hydrate Ridge and Black Sea sediments was around 7. At higher pH, more hydrogen sulphide and carbon dioxide are dissolved. Nevertheless, this inhibitory effect was reversible. When the hydrogen sulphide was stripped out, the slurry was refreshed, the SR-AOM activity recovered immediately (Fig. 2).

4.2. Effect of methane pressure on AOM activity

The results in this paper have clearly demonstrated that when the methane pressure was increased, the SR-AOM activity was enhanced (Table 1). Previous studies have shown that the SR-AOM activity and the ANME growth rate were almost linearly dependent on the methane pressures in the range up to 1.4 MPa (Nauhaus et al., 2007; Thauer and Shima, 2008). Our work has showed the first time SR-AOM activity is not necessarily linearly dependent on the methane pressure when the pressure is over 4.5 MPa (Table 1). This indicated for our sediment that (1) the dissolved methane concentration at 4.5 MPa (57 mM) was already across the apparent affinity constant (K_m) for methane; (2) the initial SR-AOM activity at 4.5 MPa (8.64 μmol sulphide production/g_{dw}/d) is above the half maximum rate ($\frac{1}{2}V_{max}$). The K_m for methane calculated using data from the high-pressure incubations in this paper (1, 4.5, and 8 MPa), was 37 mM. This in accordance with what Thauer calculated from lower-pressure (from 0 to 1.4 MPa) incubations of Hydrate Ridge sediment, which is also ANME-2 dominant: a K_m for methane above 10 mM (Nauhaus et al., 2007; Thauer and Shima, 2008). Sediments, dominated by different types of ANME, may have significant different K_m value: Girguis et al. (2005) had evidence that ANME-1 could outcompete ANME-2 for resources. More research is needed here to examine if the K_m is depending on the ANME type. Compared with aerobic methane oxidation bacteria (MOB), which showed K_m in the order of nM to μM depending on the types (Arcangeli and Arvin, 1999; Dunfield et al., 1999), the anaerobic methanotrophic archaea have a much lower affinity. This can be related to the natural environment where they are from: MOB is normally found in the soil and shallow water where the saturated methane concentration is hundreds to thousands times lower than the deep sea where ANME was normally found. Due to the low affinity, a high-pressure bioreactor system is necessary to elevate the SR-AOM activity.

The SR-AOM activity was not liner correlated to the Gibbs free energy gained from the reactions (Table 1). It seems that not only the high-pressure methane, but also the high-pressure incubation was promoting the SR-AOM activity. Little has been known about the effect of pressure on ANME and SRB though. This high-pressure bioreactor system can help us to get a closer look at the process in the future.

4.3. The comparison of fed-batch mode with continuous mode incubations

For the short-term incubations, fed-batch mode gave promising results: when the reactor was operated under the same methane pressure, the initial SR-AOM activity from fed-batch mode incubation (with HRT of 50 days) was higher than that from the continuous incubation (with HRT of 4.2 days) (Fig. 3, Table 1). After 2 days the SR-AOM activities in the fed-batch mode incubations were inhibited at all the tested methane pressures, while the SR-AOM activities in the continuous mode incubations were increasing constantly within 18 days. The continuous system promoted the advective mass transport, especially highly saturated methane transport, and therefore promoted the metabolism (Girguis et al., 2003). Besides, during the advective mass transfer, the sulphide can be sufficiently washed out before causing inhibitory effect. However, in this study the continuous system was only run for 18 days at each pressure for a demonstration. For a long-term operation, while the specific SR-AOM activity is getting higher and higher, there is a chance that the sulphide concentration will reach the level to be toxic. In this case, one solution is to dilute the biomass in order to lower the overall SR-AOM activity, while another solution is to reduce the HRT in order to sufficiently wash out the sulphide. However, the SR-AOM process may not be in favour of a low HRT. Low HRT introduced high shearing force to the microorganisms, which they rarely see in the *in situ* environment. Although not fully understood, SR-AOM is commonly interpreted as a syntrophic conversion, while ANME and SRB form consortia and share intermediate (DeLong, 2000). If this is the case, the high shearing force will break the consortia and make the intermediate difficult to reach by the syntrophic partners. Besides, the ANME cells may get washed out easier under high shearing force compared with other microorganisms forming biofilm inside the reactor.

4.4. The comparison of ex situ AOM activity with in vitro incubations at high-pressures

SR-AOM activity was measured in the Captain Aryutinov MV crater centre station, close by the origin of the inoculum of this

study. The values observed during the high-pressure incubation represented a 900-fold increase compared to the *ex situ* rates measured by Niemann et al. (2006) (Table 1). These discrepancies between *ex situ* rates and *in vitro* high-pressure incubation rate can be explained by several factors: (1) the possibility of different conditions between the two stations cannot be discarded, especially since the inoculum used in this study came from a gas hydrate rich site. It has been commonly addressed that the heterogeneity in activity is substantial in all the marine sediments, even when the sampling pots are within low spatial distance; (2) in spite of the high *in situ* hydrostatic pressure, the *in situ* methane concentration may not correspond to the saturation conditions used in this study, and the SR-AOM rate would actually be lower in sediments than in our *in vitro* incubations. Although the methane concentration may largely be underestimated due to depressurization, the data from Niemann and co-workers still pointed only about 3 mM dissolved methane in the pore water (Niemann et al. 2006); (3) Up to 5 mM *in situ* sulphide concentration had been observed (Niemann et al., 2006), which could have given considerable inhibitory effect on *in situ* SR-AOM activity.

The high-pressure bioreactor described in this study is able to mimic deep-sea environment to a certain extend. The significant difference between *in vitro* SR-AOM activity observed from this reactor and the *in situ* SR-AOM activity should be well considered. It gave a hint that the estimation of the SR-AOM activity under *in situ* conditions should also rely on methane measurements in sediment and geochemical modelling of methane flux but not only based on the turnover rate.

4.5. The high-pressure bioreactor system

The continuous high-pressure bioreactor system developed in this work has the advantage to simulate the majority of *in situ* deep-sea environment by controlling the following parameters: (1) the substrate concentration which presents the pore water composition including ion concentration (mainly sulphate) in the medium and dissolved methane concentration; (2) the medium flow rate which represents the flux; (3) incubation pressure for microorganisms; (4) continuous/batch feeding mode which represents the seep or non-seep environments; (5) incubation temperature.

One major concerning on the technology aspect of restoring AOM activity *in vitro* is how to supply sufficient methane to microorganisms. In accordance with Girguis et al. (2003), the procedure applied in this paper also introduces a continuous advective flow containing saturated methane to feed the microorganisms; only Girguis' reactor was operated at low methane pressure. Nauhaus et al. (2007) found it is essential to elevate methane pressure in order to elevate SR-AOM activity, therefore they introduce 1.4 MPa methane into a steel cylinder containing sediment to increase the dissolved methane concentration; this experiment was set up in a semi-continuous mode for two years and growth of ANME-2 has been observed meanwhile. Nauhaus's experiments for the first time proved the essential of high methane pressure. However, the diffusion-based set up has disadvantage of limiting mass transfer. Meulepas et al. (2009) supplied methane by bubbling it through the sediment for almost 3 years and the enrichment of ANME-2 has been observed. In all the systems described before, the amount of methane passing by the microorganisms is more than they can consume, but the dissolved methane concentration is not enough at the low-pressure systems due to the high K_m value (37 mM dissolved methane). This information is essential to design a suitable reactor to restore SR-AOM activity and to study its mechanism. The continuous high-pressure reactor system is a good solution for this sufficient methane supply. This was also confirmed by Deusner and his colleagues (Deusner et al., 2009).

5. Conclusions

A continuous high-pressure bioreactor to simulate the deep-sea environment, especially for *in vitro* study on SR-AOM process, has been designed and validated. The high methane pressure, indicating the high methane bio-availability, is necessary to elevate SR-AOM activity due to the high K_m value for methane (37 mM). The continuous flux, indicating the constant substrates supply and products removal, promotes a long-term high SR-AOM activity. Therefore, with the help of this reactor system, the test time to study kinetics and metabolic pathway of SR-AOM can be significantly reduced. Furthermore, long-term continuous high-pressure incubation is strongly recommended for active biomass enrichment.

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