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# Methanogenic System of a Small Lowland Stream Sitka, Czech Republic

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Additional information is available at the end of the chapter

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## 1. Introduction

Methane (CH<sub>4</sub>) is an atmospheric trace gas present at concentration of about 1.8 ppmv, that represents about 15% of the anthropogenic greenhouse effect (Forster et al. 2007). The atmospheric CH<sub>4</sub> concentration has increased steadily since the beginning of the industrial revolution (~ 0.7 ppmv) and is stabilized at ~1.8 ppmv from 1999 to 2005 (Forster et al. 2007). An unexpected increase in the atmospheric growth of CH<sub>4</sub> during the year 2007 has been recently reported (Rigby et al. 2008), indicating that the sources and sinks of atmospheric CH<sub>4</sub> are dynamics, evolving, and not well understood. Freshwater sediments, including wetlands, rice paddies and lakes, are thought to contribute 40 to 50 % of the annual atmospheric methane flux (Cicerone & Oremland 1988; Conrad 2009).

The river hyporheic zone, volume of saturated sediment beneath and beside streams containing some proportion of water from surface channel, plays a very important role in the processes of self-purification because the river bed sediments are metabolically active and are responsible for retention, storage and mineralization of organic matter transported by the surface water (Hendricks 1993; Jones & Holmes 1996, Baker et al. 1999, Storey et al. 1999, Fischer et al. 2005). The seemingly well-oxygenated hyporheic zone contains anoxic and hypoxic pockets („anaerobic microzones“) associated with irregularities in sediment surfaces, small pore spaces or local deposits of organic matter, creating a ‘mosaic’ structure of various environments, where different microbial populations can live and different microbially mediated processes can occur simultaneously (Baker et al. 1999, Morrice et al. 2000, Fischer et al. 2005). Moreover, hyporheic-surface exchange and subsurface hydrologic flow patterns result in solute gradients that are important in microbial metabolism. Oxidation processes may occur more readily where oxygen is replenished by surface water infiltration, while reduction processes may prevail where surface-water exchange of oxygen

is less, and the reducing potential of the environment is greater (Hendricks 1993). As water moves through the hyporheic zone, decomposition of the organic matter consumes oxygen, creating oxygen gradients along the flow path. Thus, compared to marine or lake surface sediments, where numerous studies on  $O_2$  profiles have showed that  $O_2$  concentrations become zero within less than 3 mm from the surface, the hyporheic sediment might be well-oxygenated habitats even up to the depth of 80 cm (e.g. Bretschko 1981, Holmes et al. 1994). The extent of the oxygen gradient is determined by the interplay between flow path length, water velocity, the ratio of surface to ground water, and the amount and quality of organic matter. Organic matter decomposition in sediments is an important process in global and local carbon budgets as it ultimately recycles complex organic compounds from terrestrial and aquatic environments to carbon dioxide and methane. Methane is a major component in the carbon cycle of anaerobic aquatic systems, particularly those with low sulphate concentrations. Since a relatively high production of methane has been measured in river sediments (e.g. Schindler & Krabbenhoft 1998, Hlaváčová et al. 2005, Sanders et al. 2007, Wilcock & Sorrell 2008, Sanz et al. 2011), we proposed that river sediments may act as a considerable source of this greenhouse gas which is important in global warming (Hlaváčová et al. 2006).

Breakdown of organic matter and gas production are both results of well functioned river self-purification. This degrading capacity, however, requires intensive contact of the water with biologically active surfaces. Flow over various morphological features ranging in size from ripples and dunes to meanders and pool-riffle sequences controls such surface-subsurface fluxes. Highly permeable streambeds create opportunities for subsurface retention and long-term storage, and exchange with the surface water is frequent. Thus, study of the methane production within hyporheic zone and its subsequent emission to the atmosphere can be considered as a measure of mineralization of organic matter in the freshwater ecosystem and might be used in evaluation of both the health and environmental quality of the rivers studied.

Methane ( $CH_4$ ) is mostly produced by methanogenic archaea (Garcia et al. 2000, Chaban et al. 2006) as a final product of anaerobic respiration and fermentation, but there is also aerobic methane formation (e.g. Karl et al. 2008). Methanogenic archaea are ubiquitous in anoxic environments and require an extremely low redox potential to grow. They can be found both in moderate habitats such as rice paddies (Grosskopf et al. 1998a,b), lakes (Jürgens et al. 2000, Keough et al. 2003) and lake sediments (Chan et al. 2005), as well as in the gastrointestinal tract of animals (Lin et al. 1997) and in extreme habitats such as hydrothermal vents (Jeanthon et al. 1999), hypersaline habitats (Mathrani & Boone 1995) and permafrost soils (Kobabe et al. 2004, Ganzert et al. 2006). Rates of methane production and consumption in sediments are controlled by the relative availability of substrates for methanogenesis (especially acetate or hydrogen and carbon dioxide). The most important immediate precursors of methanogenesis are acetate and  $H_2/CO_2$ . The acetotrophic methanogens convert acetic acid to  $CH_4$  and  $CO_2$  while the hydrogenotrophic methanogens convert  $CO_2$  with  $H_2$  to  $CH_4$  (Conrad 2007).

Methane oxidation can occur in both aerobic and anaerobic environments; however, these are completely different processes involving different groups of prokaryotes. Aerobic methane oxidation is carried out by aerobic methane oxidizing bacteria (methanotrophs, MOB), while anaerobic methane oxidizers, discovered recently, thrive under anaerobic conditions and use sulphate or nitrate as electron donors for methane oxidation (e.g. Strous & Jetten 2004). MOB are a physiologically specialized group of methylotrophic bacteria capable of utilizing methane as a sole source of carbon and energy, and they have been recognized as major players in local and global elemental cycling in aerobic environments (Hanson & Hanson 1996, Murrell et al. 1998, Costelo & Lidstrom 1999, Costelo et al. 2002, McDonald et al. 2008). Aerobic MOB have been detected in a variety of environments, and in some they represent significant fractions of total microbial communities (e.g. Henckel et al. 1999; Carini et al. 2005, Trotsenko & Khmelenina 2005, Kalyuzhnaya et al. 2006). However, the data on the diversity and activity of methanotrophic communities from the river ecosystems are yet fragmentary. Methanotrophs play an important role in the oxidation of methane in the natural environment, oxidizing methane biologically produced in anaerobic environments by the methanogenic archaea and thereby reducing the amount of methane released into the atmosphere.

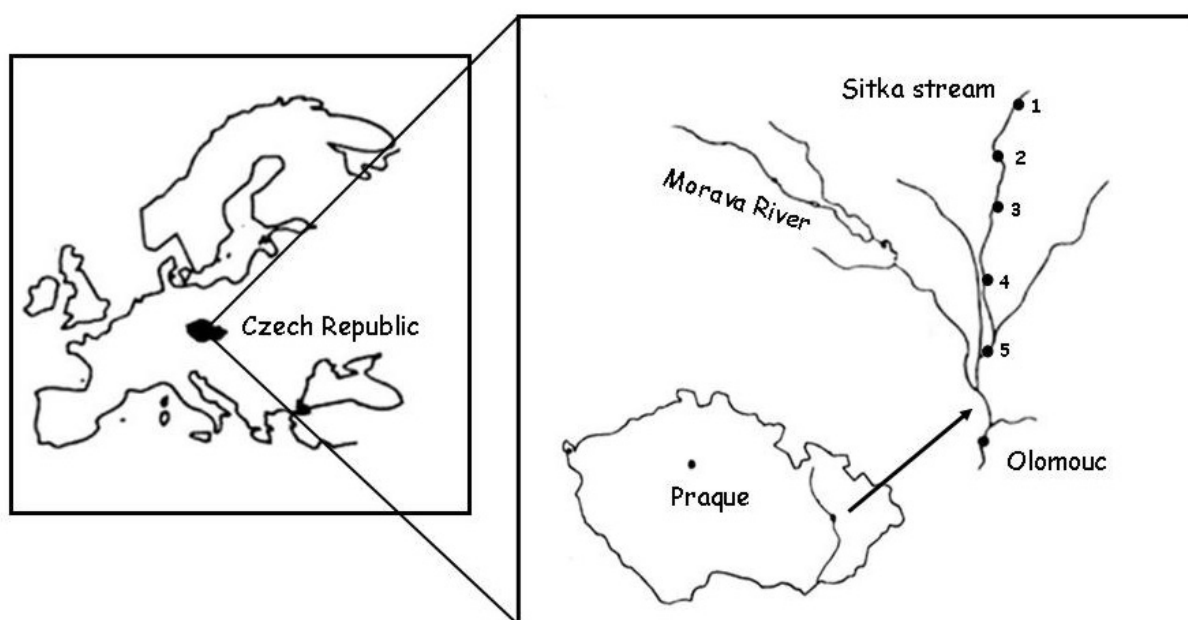
The present investigation is a part of a long-term study focused on organic carbon and methane dynamics and microbial communities in hyporheic zone of a Sitka, small lowland stream in Czech Republic. The overall purpose of this research was to characterize spatial distribution of both methanogens and methanotrophs within hyporheic sediments and elucidate the differences in methane pathways and methane production/consumption as well as methane fluxes and atmospheric emissions at different sites along a longitudinal profile of the stream.

## 2. Material and methods

### 2.1. Study site

The sampling sites are located on the Sitka stream, Czech Republic (Fig. 1). The Sitka is an undisturbed, third-order, 35 km long lowland stream originating in the Hrubý Jeseník mountains at 650 m above sea level. The catchment area is 118.81 km<sup>2</sup>, geology being composed mainly of Plio-Pleistocene clastic sediments of lake origin covered by quaternary sediments. The mean annual precipitation of the downstream part of the catchment area varies from 500 to 600 mm. Mean annual discharge is 0.81 m<sup>3</sup>.s<sup>-1</sup>. The Sitka stream flows in its upper reach till Šternberk through a forested area with a low intensity of anthropogenic effects, while the lower course of the stream naturally meanders through an intensively managed agricultural landscape. Except for short stretches, the Sitka stream is unregulated with well-established riparian vegetation. River bed sediments are composed of gravels in the upper parts of the stream (median grain size 13 mm) while the lower part, several kilometres away from the confluence, is characterised by finer sediment with a median grain size of 2.8 mm. The Sitka stream confluent with the Oskava stream about 5 km north of Olomouc. More detailed characteristics of the geology, gravel bar, longitudinal

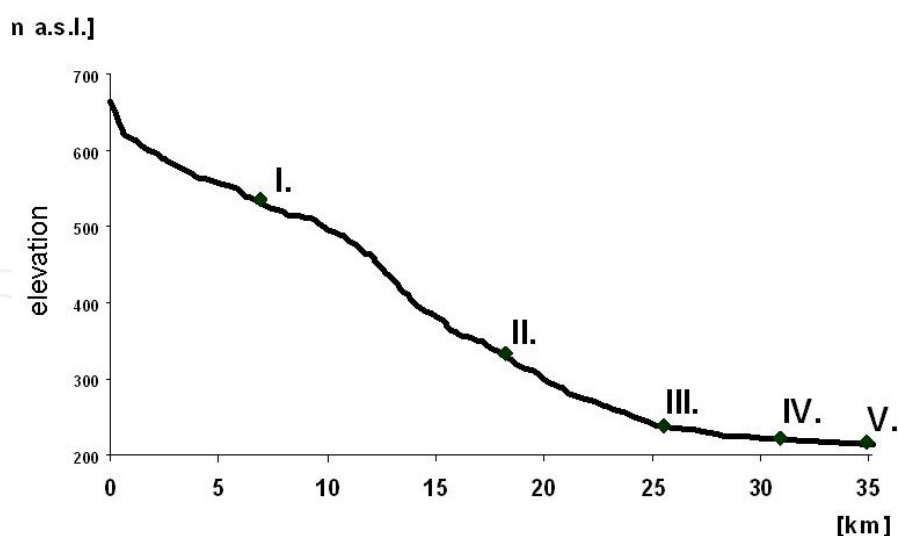
physicochemical (e.g. temperature, pH, redox, conductivity,  $O_2$ ,  $CH_4$ ,  $NO_3$ ,  $SO_4$ ) patterns in the sediments and a schematic view of the site with sampling point positions have been published previously (Rulík et al. 2000, Rulík & Spáčil 2004). Earlier measurements of a relatively high production of methane, as well as potential methanogenesis, confirmed the suitability of the field sites for the study of methane cycling (Rulík et al. 2000, Hlaváčová et al. 2005, 2006).



**Figure 1.** A map showing the location of the Sitka stream. Black circles represent the study sites (1-5)

## 2.2. Sediment sample collecting and sample processing

Five localities alongside stream profile were chosen for sampling sediment and interstitial water samples based on previous investigations (Figure 2, Table 1). Hyporheic sediments were collected with a freeze-core using  $N_2$  as a coolant (Bretschko & Klemens 1986) throughout summer period 2009-2011. At each locality, three cores were taken for subsequent analyses. After sampling, surface 0-25 cm sediment layer and layer of 25-50 cm in depth were immediately separated and were stored at a low temperature whilst being transported to the laboratory. Just after thawing, wet sediment of each layer was sieved and only particles  $< 1$  mm were considered for the following microbial measurements and for all microbial activity measurements since most of the biofilm is associated with this fraction (Leichtfried 1988).



**Figure 2.** Graphic depiction of the thalweg of the Sitka stream with sampling localities. The main source of pollution is an effluent from Šternberk city sewage water plant, located just in the middle between stretch II and III.

Variable/ Locality	I.	II.	III.	IV.	V.
elevation above sea-level [m]	535	330	240	225	215
distance from the spring [km]	6,9	18,2	25,6	30,9	34,9
channel width [cm]	523	793	672	444	523
average flow velocity [m.s <sup>-1</sup> ]	0,18	0,21	0,46	0,42	0,18
stretch longitude [km]	12,6	9,3	6,3	4,7	2,3
stretch surface area [km <sup>2</sup> ]	0,043	0,06	0,043	0,024	0,012
stretch surface area (%)	24	32	24	13	7
dominant substrate composition	gravel	gravel	gravel	silt-clay	gravel-sand
grain median size [mm]	12,4	12,9	13,2	0,2	5,4
surface water PO <sub>4</sub> <sup>3-</sup> [mg L <sup>-1</sup> ]	0,15	0,24	7,0	2,6	1,8
surface water N - NO <sub>3</sub> <sup>-</sup> [mg L <sup>-1</sup> ]	0,01	0,21	1,2	0,5	0,18
surface water N - NH <sub>4</sub> <sup>+</sup> [mg L <sup>-1</sup> ]	0,39	0,26	0,66	0,72	0,61
surface dissolved oxygen saturation [%]	101,7	110,0	105,8	108,5	103,5
surface water conductivity [μS.cm <sup>-1</sup> ]	107,5	127,5	404,8	394,0	397,7
hyporheic water conductivity [μS.cm <sup>-1</sup> ]	115,3	138,3	414,5	506,5	416,2
surface water temperature [°C]	8,1	9,7	10,7	11,1	8,9
surface water DOC [mg L <sup>-1</sup> ]	2,47	0,81	2,62	2,69	3,74
hyporheic water DOC [mg L <sup>-1</sup> ]	2,05	1,31	2,71	5,76	2,62

**Table 1.** Longitudinal physicochemical patterns of the Sitka stream (annual means). Hyporheic water means mix of interstitial water taken from the depth 10 up to 50 cm of the sediment depth

A few randomly selected subsamples (1 mL) were used for extraction of bacterial cells and, consequently, for estimations of bacterial numbers; other sub-samples were used for



measurement of microbial activity and respiration, organic matter content determination, etc. Sediment organic matter content was determined by oven-drying at 105 °C to constant weight and subsequent combustion at 550 °C for 5 hours to obtain ash-free dry weight (AFDW). Organic matter values were then converted to carbon equivalents assuming 45 % carbon content of organic matter (Meyer et al. 1981). Sediment from another freeze-core was oven-dried at 105 °C and subjected to granulometric analysis. Grain size distribution and descriptive sediment parameters were computed using the database SeDi (Schönbauer & Lewandowski 1999).

### 2.3. Water samples and analysis of methane

Surface water was collected from running water at a depth of 10 cm below the surface level in autumn 2009 at each study site. Interstitial water samples were collected using a set of 5–6 minipiezometers (Trulleyová et al. 2003) placed at a depth of about 20–50 cm randomly in sediments at each study site. The initial 50–100 mL of water was used as a rinse and discarded. As usual, two subsamples of interstitial water from each minipiezometer were collected from a continuous column of water with a 100 mL polypropylene syringe connected to a hard PVC tube, drawn from a minipiezometer and injected into sterile, clear vials (40 mL) with screw-tops, covered by a polypropylene cap with PTFE silicone septa (for analysis of dissolved gasses) and stored before returning to the laboratory. All samples were taken in the morning between 9 a.m. and 12 noon. All measurements were done during the normal discharge levels (i.e. no spates or high flood levels were included). Interstitial water temperature, dissolved oxygen ( $\text{mg L}^{-1}$  and percent saturation) and conductivity were measured in the field with a portable Hanna HI 9828 pH/ORP/EC/DO meter. Dissolved organic carbon (DOC) was measured by Pt-catalysed high temperature combustion on a TOC FORMACS<sup>HT</sup> analyser. Long term observation of interstitial water temperature was carried out using temperature dataloggers Minikin (EMS Brno, Czech Republic) buried in the sediment depth of 25–30 cm for a period of one year. Dissolved ferrous iron ( $\text{Fe}^{2+}$ ) concentration was measured using absorption spectrophotometry after reaction with 1,10-phenanthroline. Concentrations of organic acids were measured using capillary electrophoresis equipped with diode array detector HP 3D CE Agilent (Waldbronn, Germany). Limits of detection for particular organic acids were set as following: LOD (acetate) =  $6,2 \mu\text{mol L}^{-1}$ ; LOD (propionate) =  $4,8 \mu\text{mol L}^{-1}$ ; LOD (butyrate) =  $2,9 \mu\text{mol L}^{-1}$ ; LOD 32 (valerate) =  $1,8 \mu\text{mol L}^{-1}$ .

Concentrations of dissolved methane in the stream and interstitial water were measured directly using a headspace equilibration technique. Dissolved methane was extracted from the water by replacing 10 mL of water with  $\text{N}_2$  and then vigorously shaking the vials for 15 seconds (to release the supersaturated gas from the water to facilitate equilibration between the water and gas phases). All samples were equilibrated with air at laboratory temperature. Methane was analysed from the headspace of the vials by injecting 2 mL of air sub-sample with a gas-tight syringe into a CHROM 5 gas chromatograph, equipped with the flame ionization detector ( $\text{CH}_4$  detection limit =  $1 \mu\text{g L}^{-1}$ ) and with the 1.2m PORAPAK Q column (i.d. 3 mm), with nitrogen as a carrier gas. Gas concentration in water was calculated using

Henry's law. The saturation ratio ( $R$ ) was calculated as the measured concentration of gas divided by the concentration in equilibrium with the atmosphere at the temperature of the water sample using the solubility data of Wiesenburg & Guinasso (1979).

## 2.4. Methanogenic potential and methanotrophic activity

The rate of methane production (methanogenesis) was measured using the PMP method (Segers 1998). C-amended solutions (flushed for 5 minutes with  $N_2$ ) with acetate  $Ca(CH_3COO)_2$  (100 mg C in the incubation flask) were used for the examination of methanogenic potential. All laboratory sediment incubations were performed in 250-mL dark glass flasks, capped with rubber stoppers, using approximately 100 g (wet mass) of sediment (grain size < 1 mm) and 180 mL of amended solution or distilled water. The headspace was maintained at 20 mL. Typically, triplicate live and dead (methanogenesis was inhibited by addition of 1.0 mM chloroform) samples from each depth were stored at 20°C in the dark and the incubation time was 72 hours; however, subsamples from the headspace atmosphere were taken every 24 hours. Gas production was calculated from the difference between final and initial headspace concentration and volume of the flask; results are expressed per volume unit of wet sediment ( $CH_4$  mL<sup>-1</sup> WW hour<sup>-1</sup>) or per unit dry weight of sediment per one day ( $\mu$ g  $CH_4$  kg<sup>-1</sup> DW day<sup>-1</sup>). Rate of potential methane oxidation (methanotrophy) was measured using modified method of methane oxidation in soil samples from Hanson (1998). Briefly, 50 mL of methane was added by syringe to the closed incubation flask with the sieved sediment and then the pressure was balanced to atmospheric pressure. All laboratory sediment incubations were performed in 250-mL dark glass flasks, capped with rubber stoppers, using approximately 100 g (wet mass) of sediment (grain size < 2 mm). Typically, triplicate live and dead (samples killed by  $HgCl_2$  to arrest all biological activity) samples from each depth were stored at 20°C in the dark, and incubation time was 72 hours; however, subsamples from the headspace atmosphere were taken every 24 hours. Potential  $CH_4$  oxidation rates at the different concentrations were obtained from the slope of the  $CH_4$  decrease with time ( $r^2 > 0.90$ ; methane oxidation was calculated from the difference between final and initial headspace concentration and volume of the flask; results are expressed per volume unit of wet sediment ( $CH_4$  mL<sup>-1</sup> WW hour<sup>-1</sup>) or per unit dry weight of sediment per one day (mg  $CH_4$  kg<sup>-1</sup> DW day<sup>-1</sup>).

## 2.5. Fluxes of methane across the sediment-water interface

Fluxes of methane across the sediment-water interface were estimated either by direct measurement with benthic chambers or calculated by applying Fick's first law.

### *Benthic fluxes*

The methane fluxes across the sediment-water interface were measured using the method of benthic chambers (e.g. Sansone et al. 1998). Fluxes were measured during the summer months (VII, VIII, IX). The plexiglas chamber (2.6 dm<sup>3</sup>) covered an area 0.0154 m<sup>2</sup>. The chambers ( $n = 7$ ) were installed randomly and gently anchored on the substrate without



disturbing the sediment. Samples to determine of initial concentration of CH<sub>4</sub> were collected from each chamber before the beginning of incubation. Incubation time was 24 hours. Samples of water were stored in 40 ml glass vials closed by cap with PTFE/silicone septum until analysis.

### *Diffusive fluxes*

Fluxes of methane between the sediment and overlying water were calculated from Fick's first law as described by Berner (1980):

$$J = -D_s \times \Phi \times (\Delta C / \Delta x) \quad (1)$$

where  $J$  is the diffusive flux in  $\mu\text{g m}^{-2} \text{s}^{-1}$ ,  $\Phi$  is the porosity of the sediment,  $D_s$  is the bulk sediment diffusion coefficient in  $\text{cm}^2 \text{s}^{-1}$ ,  $\Delta C / \Delta x$  is the methane concentrations gradient in  $\mu\text{g cm}^{-3} \text{cm}^{-1}$ . Bulk sediment diffusion coefficient ( $D_s$ ) is based on diffusion coefficient for methane in the water ( $D_0$ ) and tortuosity ( $\theta$ ) according to the formula:

$$D_s = D_0 \theta^{-2} \quad (2)$$

Tortuosity ( $\theta$ ) is possible calculate from porosity according to equation (Boudreau 1996):

$$\theta^{-2} = 1 - \ln(\Phi^2) \quad (3)$$

Diffusive fluxes of CH<sub>4</sub> were determined at all five study sites along the longitudinal profile of the Sitka stream.

## **2.6. Measurement of emissions**

Gas flux across the air-water interface was determined by the floating chamber method four times during the year period in 2005 – 2006. The open-bottom floating PE chambers (5L domes with an area of 0.03 m<sup>2</sup>) were maintained on the water's surface by a floating body (Styrene) attached to the outside. The chambers ( $n = 4 - 5$ ) were allowed to float on the water's surface for a period of 3 hours. Previous measurements confirmed that time to be quite enough to establish linear dependence of concentration change inside the chambers on time for the gas samples collected every 30 min over a 3 hour period. Due to trees on the banks, the chambers at all study sites were continuously in the shade. On each sampling occasion, ambient air samples were collected for determining the initial background concentrations. Samples of headspace gas were collected through the rubber stopper inserted at the chamber's top, and stored in 100mL PE gas-tight syringes until analysis. Emissions were calculated as the difference between initial background and final concentration in the chamber headspace, and expressed on the 1m<sup>2</sup> area of the surface level per day according to the formula:

$$F = [(c_I - c_R) * V * 24 / t * 1000] / p \quad (4)$$

where  $F$  is a gas flux in  $\text{mg m}^{-2}\text{day}^{-1}$ ;  $c_i$  is a concentration of particular gas in the chamber headspace in  $\mu\text{g L}^{-1}$ ;  $c_R$  is a concentration of particular gas in background air;  $V$  is volume of the chamber in L;  $t$  is time of incubation in hr;  $p$  is an area of chamber expressed in  $\text{m}^2$ . For each chamber, the fluxes were calculated using linear regression based on the concentration change as a function of time, regardless of the value of the coefficient of determination (cf. Duchemin et al. 1999, Silvenoinen et al. 2008).

In order to assess emissions produced from a total stream area, the stream was divided into five stretches according to the channel width, water velocity and substrate composition. For each stretch we have then chosen one representative sampling site (locality I-V) where samples of both stream and interstitial waters and sediments, respectively, were repeatedly taken. Localities were chosen in respect to their character and availability by car and measuring equipments. For calculation of whole-stream gases emissions into the atmosphere, the total stream area was derived from summing of 14 partial stretches. The area of these stretches was calculated from known length and mean channel width (measured by a metal measuring type). Longitudinal distance among the stretches was evaluated by using ArcGIS software and GPS coordinates that have been obtained during the field measurement and from digitalised map of the Sitka stream. The total area of the Sitka stream was estimated to be  $181\,380\text{ m}^2$  or  $0.18\text{ km}^2$ . Stretches have differed in their percentual contribution to this total area and also by their total length (Table 1).

The total annual methane emissions to the atmosphere from the five segments of the Sitka stream,  $E_a$  ( $\text{kg yr}^{-1}$ ) were derived from seasonal average, maximum or minimum emissions measured on every locality and extrapolated to the total area of the particular segment. The total methane emissions produced by the Sitka stream annually were then calculated according to the following formula:

$$E_a = \left( \sum p_i * F_i * 365 \right) / 1000000 \quad (5)$$

where  $E_a$  is average, maximal or minimal assess of emission of methane from the total stream area in kilograms per year;  $p_i$  is an area of stretch (in  $\text{m}^2$ ) representing given locality;  $F_i$  is average, maximal or minimal assess of the methane from a given locality expressed in  $\text{mg m}^{-2}\text{day}^{-1}$ .

## 2.7. Carbon isotopic composition of dissolved methane and carbon dioxide in sediments

Interstitial water samples for carbon isotopic analysis of methane and carbon dioxide were collected in 2010 - 2011 through three courses at study site. Sampling was performed by set of minipiezometers placed in a depth of 20 to 60 cm randomly in a sediment. After sampling, refrigerated samples were transported (within 72 hours) in 250 mL bottles to laboratory at the Department of Plant Physiology, Faculty of Science University of South Bohemia in Ceske Budejovice, which are equipped with mass spectrometry for carbon isotopes measurements. Firstly both water samples, for methane and for carbon dioxide, were extracted to helium headspace. After relaxation time isotopic equilibrium was

achieved and four subsamples of gas were determined by GasBanch (ThermoScientific) and IRMS Delta<sup>plus</sup>XL equipped by TC/EA (ThermoFinnigan) for analysis of  $\delta^{13}\text{CO}_2$ . Afterwards  $\delta^{13}\text{CO}_2$  of water samples were calculated from gaseous  $\delta^{13}\text{CO}_2$  by fractionation factor from a linear equation (Szaran 1997):

$$\varepsilon_{\infty}^{13}\text{C} = - (0.0954 \pm 0.0027) T[^\circ\text{C}] + (10.41 \pm 0.12) \quad (6)$$

Stable isotope analysis of  $^{13}\text{C}/^{12}\text{C}$  in gas samples was performed using preconcentration, kryoseparation of  $\text{CO}_2$  and gas chromatograph combustion of  $\text{CH}_4$  in PreCon (ThermoFinnigan) coupled to isotope ratio mass spectrometer (IRMS, Delta Plus XL, ThermoFinnigan, Brehmen, Germany). After conversion of  $\text{CH}_4$  to  $\text{CO}_2$  in the Finningan standard GC Combustion interface  $\text{CO}_2$  will be tranfered into IRMS. The obtained  $^{13}\text{C}/^{12}\text{C}$  ratios (R) will be referenced to  $^{13}\text{C}/^{12}\text{C}$  of standard V-PDB (Vienna-Pee-Dee Belemnite)(Rs), and expressed as  $\delta^{13}\text{C} = (\text{R}_{\text{sample}}/\text{R}_{\text{standard}} - 1) \times 1000$  in ‰. The standard deviation of  $\delta^{13}\text{C}$  determination in standard samples is lower than 0.1‰ with our instrumentation. From our data, we also calculated an apparent fractionation factor  $\alpha_c$  that is defined by the measured  $\delta\text{CH}_4$  and  $\delta\text{CO}_2$  (Whiticar et al. 1986):

$$\alpha_c = (\delta\text{CO}_2 + 10^3) / (\delta\text{CH}_4 + 10^3) \quad (7)$$

This fractionation factor gives rough idea of magnitude of acetoclastic and hydrogenotrophic methanogenesis.

## 2.8. Abundance of microbial cells and microbial community composition

For measuring of microbial parameters, formaldehyde fixed samples (2 % final conc.) were first mildly sonicated for 30 seconds at the 15 % power (sonotroda MS 73, Sonopuls HD2200, Sonorex, Germany), followed by incubation for 3 hours under mild agitation with 10 mL of detergent mixture (Tween 20 0.5%, vol/vol, tetrasodium pyrophosphate 0.1 M and distilled water) and density centrifugation (Santos Furtado & Casper 2000, Amalfitano & Fazi 2008). For density centrifugaton, the non-ionic medium Nycodenz (1.31 g mL<sup>-1</sup>; Axis- Shield, Oslo, Norway) was used at 4600 G for 60 minutes (Rotofix 32A, Hettich, Germany). After the preparation processes, a 1 mL of Nycodenz was placed underneath 2 ml of treated slurry using a syringe needle (Fazi et al. 2005). 1 ml of supernatant was then taken for subsequent analysis.

## 2.9. Total cell numbers (TCN)

The supernatant was filtered onto membrane filters (0.2 µm GTTP; Millipore Germany), stained for 10 minutes in cold and in the dark with DAPI solution (1 mg/ ml; wt/ vol; Sigma, Germany) and gently rinsed in distilled water and 80 % ethanol. Filters were air-dried and fixed in immersion oil. Stained cells were enumerated on an epifluorescence microscope (Olympus BX 60) equipped with a camera (Olympus DP 12) and image analysis software (NIS Elements; Laboratory Imaging, Prague, Czech Republic). At least 200 cells within at

least 20 microscopic fields were counted in three replicates from each locality. TCN was expressed as bacterial numbers per 1 mL of wet sediments.

## 2.10. Procaryotic community composition

The methanogenic archaea, three selected methanogen families (*Methanobacteriaceae*, *Methanoseaceae* and *Methanosarcinaceae*) and methanotrophic bacteria belonging to groups I and II were detected using FISH (Fluorescence in situ hybridization) with 16S rRNA-targeted oligonucleotide probe labelled with indocarbocyanine dye Cy3. The prokaryotes were hybridized according to the protocol by Pernthaler et al. (2001). Briefly, the supernatants which were used also for TCN were filtered onto polycarbonate membrane filters (0.2 µm GTTP; Millipore), filters were cut into sections and placed on glass slides. For the hybridization mixtures, 2 µL of probe-working solution was added to 16 µL of hybridization buffer in a microfuge tube. Hybridization mix was added to the samples and the slides with filter sections were incubated at 46 °C for 3 hours. After incubation, the sections were transferred into preheated washing buffer (48 °C) and incubated for 15 minutes in a water bath at the same temperature. The filter sections were washed and air-dried. The DAPI staining procedure followed as previously described. Finally, the samples were mounted in a 4:1 mix of Citifluor and Vecta Shield. The methanogens and methanotrophs were counted in three replicates from each locality and the relative proportion of bacteria, archaea, methanogens and methanotrophs to the total number of DAPI stained cells was then calculated.

## 2.11. Nucleic acid extraction and Denaturing gradient gel electrophoresis (DGGE)

Nucleic acids were extracted from 0,3 g of sieved sediment with a Power Soil DNA isolation kit (MoBio, Carlsbad, USA) according to the manufacturer's instructions. 16S rRNA gene fragments (~350 bp) were amplified by PCR using primer pair specific for methanogens. Primer sequences are as follows, 0357 F-GC 5'-CCC TAC GGG GCG CAG CAG-3' (GC clamp at 5'-end CGC CCG CCG CGC GCG GCG GGCGGG GCG GGG GCA CGG GGG G) and 0691 R 5'- GGA TTA CAR GAT TTC AC -3' (Watanabe et al. 2004). PCR amplification was carried out in 50 µL reaction mixture contained within 0.2 mL, thin walled micro-tubes. Amplification was performed in a TC-XP thermal cycler (Bioer Technology, Hangzhou, China). The reaction mixture contained 5 µL of 10 × PCR amplification buffer, 200 µM of each dNTP, 0,8 µM of each primer, 8 µL of template DNA and 5.0 U of FastStart Taq DNA polymerase (Polymerase dNTPack; Roche, Germany). The initial enzyme activation and DNA denaturation were performed for 6 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C and 2 min at 69°C and a final extension at 69°C for 8 min (protocol by Watanabe et al. 2004). PCR products were visualised by electrophoresis in ethidium bromide stained, 1.5% (w/v) agarose gel.

DGGE was performed with an INGENYphorU System (Ingeny, Netherlands). PCR products were loaded onto a 7% (w/v) polyacrylamide gel (acrylamide: bisacrylamide, 37.5:1). The

polyacrylamide gels were made of 0.05% (v/v) TEMED (N,N,N,N-tetramethylethylenediamine), 0.06% (w/v) ammonium persulfate, 7 M (w/v) urea and 40 % (v/v) formamide. Denaturing gradients ranged from 45 to 60%. Electrophoresis was performed in 1×TAE buffer (40 mM Tris, 1 mM acetic acid, 1 mM EDTA, pH 7.45) and run initially at 110V for 10 min at 60°C, afterwards for 16 h at 85 V. After electrophoresis, the gels were stained for 60 min with SYBR Green I nucleic acid gel stain (1:10 000 dilution) (Lonza, Rockland USA) DGGE gel was then photographed under UV transilluminator (Molecular Dynamics). Images were arranged by Image analysis (NIS Elements, Czech Republic). A binary matrix was created from the gel image by scoring of the presence or absence of each band and then the cluster tree was constructed (programme GEL2k; Svein Norland, Dept. Of Biology, University of Bergen).

## 2.12. PCR amplification, cloning and sequencing of methyl coenzyme M reductase (*mcrA*) gene

Fragments of the methanogen DNA (~470 bp) were amplified by PCR using *mcrA* gene specific primers. Primer sequences for *mcrA* gene are as follows, *mcrA* F 5'-GGTGGTGTACGGATTCACACAAGTACTGCATACAGC-3', *mcrA* R 5'-TTCATTGCAGTAGTTATGGAGTAGTT-3'. PCR amplification was carried out in 50 µl reaction mixture contained within 0.2 mL thin walled micro-tubes. Amplification was performed in a TC-XP thermal cycler (Bioer Technology, Hangzhou, China). The reaction mixture contained 5 µL of 10 × PCR amplification buffer, 200 µM of each dNTP, 0.8 µM of each primer, 2 µL of template DNA and 2.5 U of FastStart Taq DNA polymerase (Polymerase dNTPack; Roche, Mannheim, Germany). The initial enzyme activation and DNA denaturation were performed for 6 min at 95°C, followed by 5 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C, and the temperature ramp rate between the annealing and extension segment was set to 0.1°C/s because of the degeneracy of the primers. After this, the ramp rate was set to 1°C/s, and 30 cycles were performed with the following conditions: 30 s at 95°C, 30 s at 55°C, 30s at 72°C and a final extension at 72°C for 8 min. PCR products were visualised by electrophoresis in ethidium bromide stained, 1.5% (w/v) agarose gel.

Purified PCR amplicons (PCR purification kit; Qiagen, Venlo, Netherlands) were ligated into TOPO TA cloning vectors and transformed into chemically competent *Escherichia coli* TOP10F' cells according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). Positive colonies were screened by PCR amplification with the primer set and PCR conditions described above. Plasmids were extracted using UltraClean 6 Minute Plasmid Prep Kit (MoBio, Carlsbad, USA), and nucleotide sequences of cloned genes were determined by sequencing with M13 primers in Macrogen company (Seoul, Korea). Raw sequences obtained after sequencing were BLAST analysed to search for the sequence identity between other methanogen sequences available in the GenBank database. Then these sequences were aligned by using CLUSTAL W in order to remove any similar sequences. The most appropriate substitution model for maximum likelihood analysis was identified by Bayesian Information Criterion implemented in MEGA 5.05 software. The



phylogenetic tree was constructed by the maximum likelihood method (Kimura 2-parameter model). The tree topology was statistically evaluated by 1000 bootstrap replicates (maximum likelihood) and 2000 bootstrap replicates (neighbour joining).

### 3. Results

#### 3.1. Sediment and interstitial water

The physicochemical sediment and interstitial water properties of the investigated sites showed large horizontal and vertical gradients. Sediment grain median size decreased along a longitudinal profile while organic carbon content in a sediment fraction < 1 mm remained unchanged (Table 2). Generally, interstitial water revealed relatively high dissolved oxygen saturation with the exceptions of localities IV and V where concentration of dissolved oxygen sharply decreased with the depth, however, never dropped below ~ 10%. Vice versa, these two localities were characterized by much higher concentrations of ferrous iron and dissolved methane (Table 2) compared to those sites located upstream. Concentration of the ferrous iron reflects anaerobic conditions of the sediment and showed the highest concentration to occur in the deepest sediment layers (40-50cm). Average annual temperatures of interstitial water at localities in downstream part of the Sitka stream were about 2.5 °C higher compared to localities upstream and may probably promote higher methane production occurring here. Precursors of methanogenesis, acetate, propionate and butyrate were found to be present in the interstitial water at all study sites, however, only acetate was measured regularly at higher concentration with maximum concentration reached usually during a summer period.

Variable/ Locality	I	II	III	IV	V
particulate organic C in sediment < 1 mm [%]	0.9	0.9	0.6	1.3	0.7
interstitial dissolved O <sub>2</sub> saturation [%]	80.5	88.1	82.3	38.5	50.9
ferrous iron [mg L <sup>-1</sup> ]	< 1	< 1	1.8	8.1	4.2
acetate [mmol L <sup>-1</sup> ]	0.21	0.34	0.52	1.87	0.29
interstitial CH <sub>4</sub> concentration [μg L <sup>-1</sup> ]	4.9	0.7	8.1	2 480.2	42.8
methanogenic potential [pM CH <sub>4</sub> mL <sup>-1</sup> WW hour <sup>-1</sup> ]	6.6	1.9	2.9	80.7	9.7
methanotrophic activity [nM CH <sub>4</sub> mL <sup>-1</sup> WW hour <sup>-1</sup> ]	0.3	1.3	28.5	30.3	25.1
average daily interstitial water temperature [°C]	8.7	9.4	11.6	11.2	11.4

**Table 2.** Selected physicochemical parameters (annual means) of the hyporheic interstitial water and sediments of studied localities taken from the depth 25-30 cm.

#### 3.2. Methanogenic potential and methanotrophic activity of sediments

Methanogenic potential (MP) was found to be significantly higher in the upper sediment layer compared to that from deeper sediment layer. Generally, average MP varied between 0.74-158.6 pM CH<sub>4</sub> mL<sup>-1</sup> WW hour<sup>-1</sup> with the highest values found at site IV. Average



methanotrophic activity (MA) varied between 0.02– 31.3 nM CH<sub>4</sub> mL<sup>-1</sup> WW hour<sup>-1</sup> and the highest values were found to be at the downstream localities while sediment from sites located upstream showed much lower or even negative activity. Similar to MP, values of MA were significantly higher in sediments from upper layers compared to those from deeper layers (e.g. Figs. 3c, 3d).

**3.3. Methane concentration along the longitudinal profile, vertical and temporal pattern, stable isotopes**

Methane concentrations ranged between 0.18 – 35.47 µg L<sup>-1</sup> in surface water and showed no expected trend of gradual increase from upstream localities to those laying downstream. However, significant enhancement of CH<sub>4</sub> concentration was found on locality IV and V, respectively. Concentrations of dissolved CH<sub>4</sub> in both surface and interstitial waters peaked usually during summer and autumn period (Hlaváčová et al. 2005, Mach et al. in review).

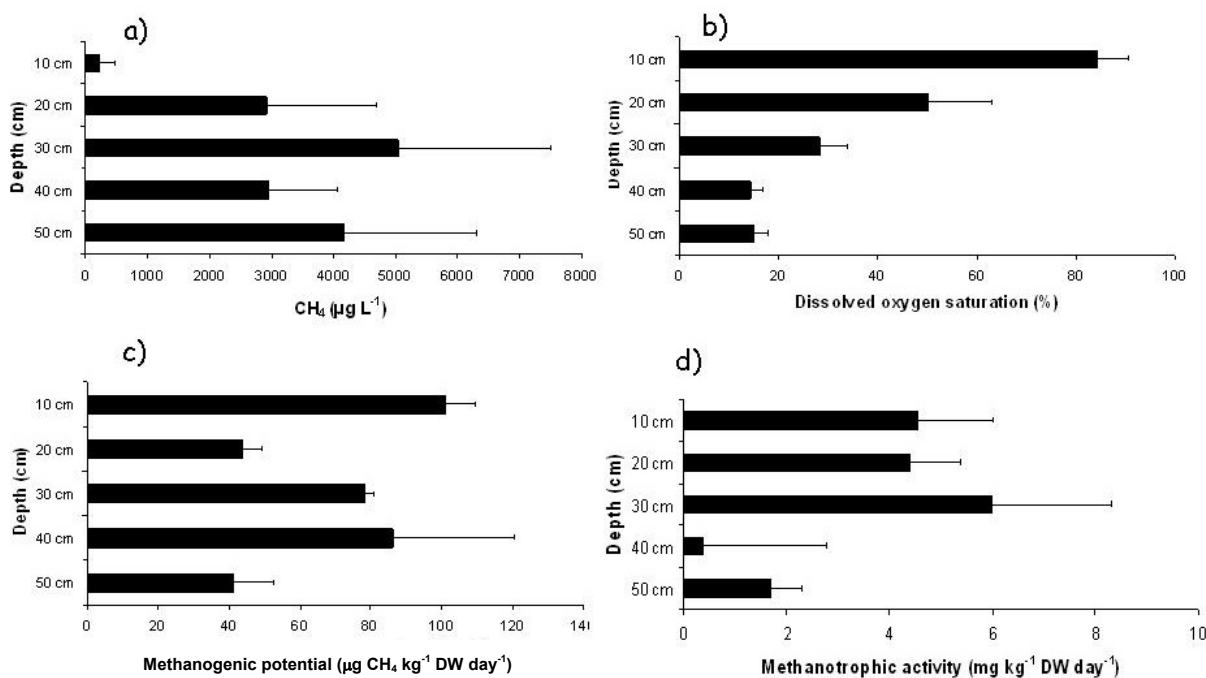
Generally, methane concentrations measured in interstitial water were much higher compared to those from surface stream water and on a long-term basis ranged between 0.19 - 11 698.9 µg L<sup>-1</sup>. Due to low methane concentrations in interstitial water at localities I and II, vertical distribution of its concentrations was studied only at the downstream located sites III-V. Significant increase of the methane with the sediment depth was observed at the localities IV and V, respectively. Namely locality IV proved to be a methane pool, methane concentrations in a depth of 40 cm were found to be one order of magnitude greater than those from the depth of 20 cm (Tab. 3). Recent data from locality IV show much lower methane concentrations in the upper sediment horizons compared to those from deeper layers (Fig. 3a). Considerable lowering of methane concentration in upper sediment horizons is likely caused by oxidizing activity of methanotrophic bacteria (Fig. 3d). while dissolved oxygen concentration sharply decreased with the sediment depth (Fig. 3b).

Locality	Profile (depth)	CH <sub>4</sub> [µg L <sup>-1</sup> ]
III.	Surface water	1.8
	Interstitial water (depth 20cm)	1.44
	Interstitial water (depth 40 cm)	1.52
IV.	Surface water	5.52
	Interstitial water (depth 20 cm)	1 523.9
	Interstitial water (depth 40 cm)	11 390.54
V.	Surface water	4.72
	Interstitial water (depth 20 cm)	6.92
	Interstitial water (depth 40 cm)	24.4

**Table 3.** Average concentrations of methane in the vertical sediment profile at localities III-V compared to those from surface water at the same sites

Usually, both the surface and interstitial water were found to be supersaturated compared to the atmosphere with locality IV displaying saturation ratio  $R$  to be almost 195 000. This high supersaturation greatly promote diffusive fluxes of methane to the atmosphere across air-water interface and is also an important mechanisms for loss of water column  $\text{CH}_4$ .

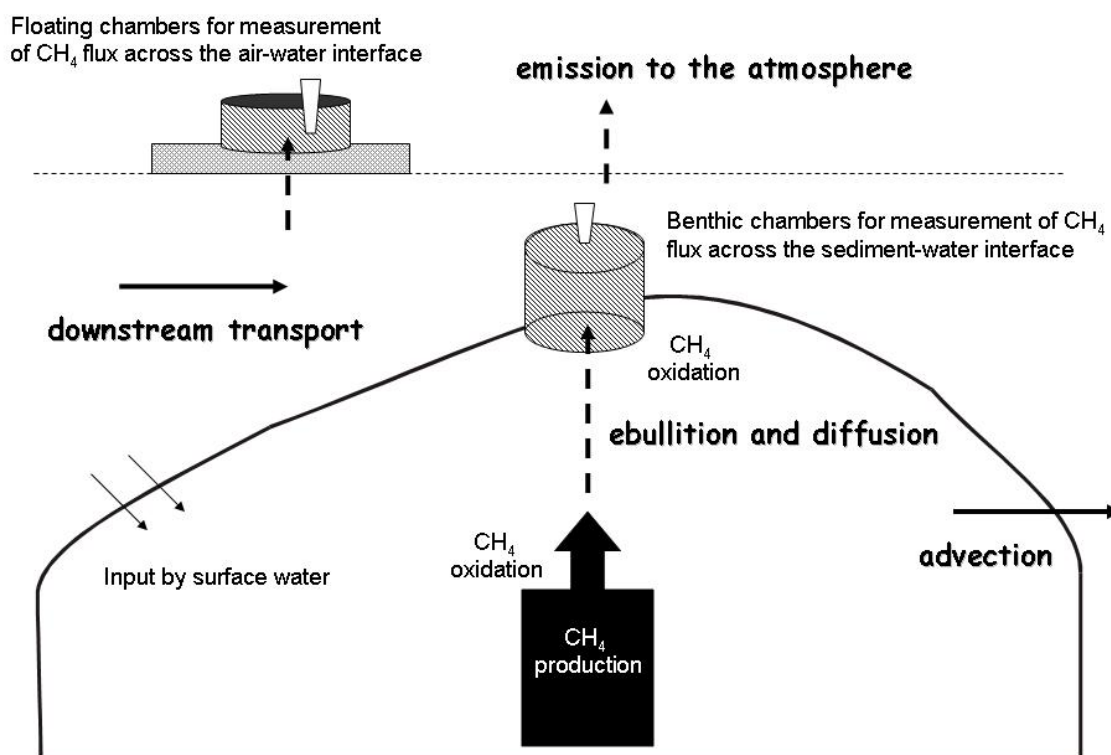
Stable carbon isotope signature of carbon dioxide ( $\delta^{13}\text{C}\text{-CO}_2$ ) measured in the interstitial water ranged from  $-19.8\text{‰}$  to  $-0.8\text{‰}$ , while carbon isotope signature of methane ( $\delta^{13}\text{C}\text{-CH}_4$ ) ranged between  $-72\text{‰}$  to  $-19.8\text{‰}$ . This relatively high variation in the methane isotopic values could be caused due to consequential fractionation effects preferring light carbon isotopes like methane oxidation or fractionation through diffusion and through flow of an interstitial water. Contrary, the narrow range of the  $\delta^{13}\text{C}\text{-CH}_4$  was found in the sediment depth of 40-60 cm where a high methane production has occurred. Here, the  $\delta^{13}\text{C}\text{-CH}_4$  values varied only from  $-67.9\text{‰}$  to  $-72\text{‰}$ . Apparent fractionation factor ( $\alpha_c$ ) varied also greatly from 1,004 to 1,076. Usually values of  $\alpha_c > 1.065$  and  $\alpha_c < 1.055$  are characteristic for environments dominated by hydrogenotrophic and acetoclastic methanogenesis, respectively. Our measurements indicate predominant occurrence of a hydrogenotrophic methanogenesis in the high methanogenic zones where the most amount of methane is produced and  $\delta^{13}\text{C}$  of  $\text{CO}_2$  values were markedly depleted (i.e.  $^{13}\text{C}$  enriched). This could be caused by enhanced carbon dioxide consumption by hydrogenotrophic methanogens, strongly preferring light isotopes. Nevertheless, both acetoclastic and hydrogenotrophic pathways take part in the methanogenesis along the longitudinal profile of the Sitka stream.



**Figure 3.** Vertical distribution of methane concentration in the interstitial water at study site IV, horizontal bars indicate 1 SE

### 3.4. Fluxes of methane across the sediment-water and the air-water interfaces

Methane diffusion rate from deeper sediment layers depends on a methane concentration gradient whilst is affected by oxidation and rate of methanotrophic bacteria consumption. When diffusion fluxes are positive (positive values indicate net  $\text{CH}_4$  production), then surface water is enriched by methane which in turn may be a part of downstream transport or is further emitted to the atmosphere (Fig. 4).



**Figure 4.** Possible fate of the methane within hyporheic zone and two kinds of chambers for measurement of methane fluxes. Providing that some sites along the longitudinal stream profile should be sources of methane for the stream water, we chose locality IV to be suitable for benthic fluxes measurements.

On the contrary, when the fluxes of methane across the sediment-water interface are negative then all methane produced in the sediments is likely oxidized and consumed by methanotrophic bacteria here or transported via subsurface hyporheic flow.

Calculated diffusive fluxes of  $\text{CH}_4$  ranged from 0.03 to 2307.32  $\mu\text{g m}^{-2} \text{ day}^{-1}$  along the longitudinal profile. The lowest average values of diffusive fluxes were observed at study site II ( $0.11 \pm 0.05 \mu\text{g m}^{-2} \text{ day}^{-1}$ ) while the highest average values were those observed at study site IV ( $885.81 \pm 697.54 \mu\text{g m}^{-2} \text{ day}^{-1}$ ). Direct benthic fluxes of  $\text{CH}_4$  using the benthic chambers were measured at study site IV only and ranged from 0.19 to 82.17  $\text{mg m}^{-2} \text{ day}^{-1}$ . We observed clear negative relationships between benthic methane fluxes and the flow discharge. During higher discharges when the stream water is pushed into sediments, methane diffusing from

deeper sediments upward is either transported by advection through sediments downstream or is probably almost completely oxidized by methanotrophic bacteria due to increasing oxygen supply from the surface stream. As a consequence, very low or no benthic fluxes were recorded during the time of high flow discharge. Compared to calculated diffusive fluxes it is clear that fluxes obtained by direct measurement were approximately 15× higher than the fluxes calculated with using Fick's first law. Thus, direct benthic fluxes were used for a calculation of water column CH<sub>4</sub> budget.

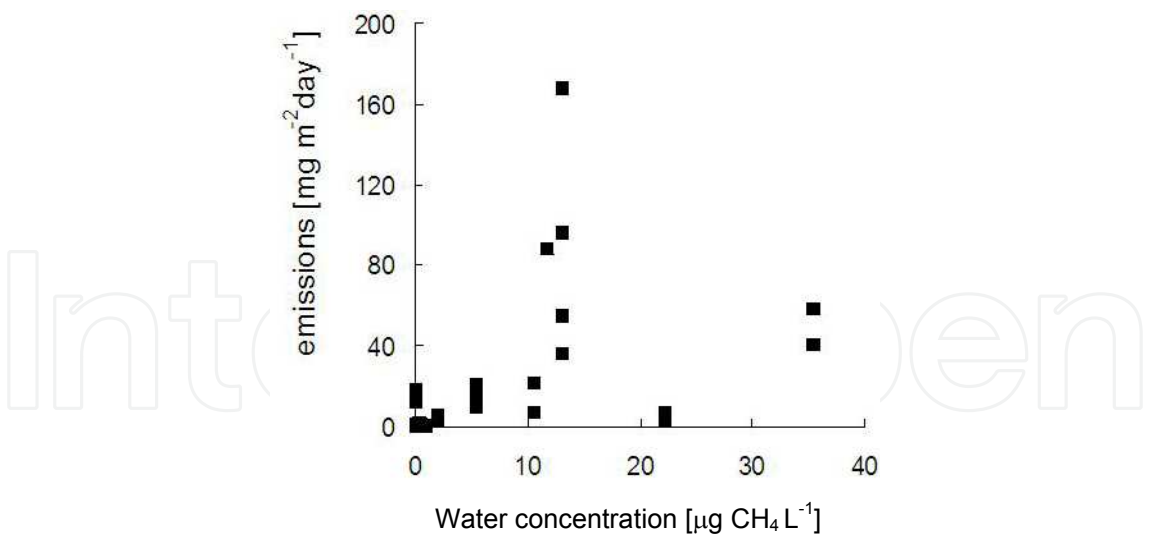
Gaseous fluxes from surface water to the atmosphere were found at all localities except locality I, where emissions were not measured directly but were calculated lately using a known relationships between concentrations of gases in surface water and their emissions to the atmosphere found at downstream laying localities II-V. Methane showed an increase in emissions toward downstream where highest surface water concentrations have also occurred (Table 4). Methane emissions measured at localities II-V ranged from 0 – 167.35 mg m<sup>-2</sup> day<sup>-1</sup> and no gradual increase in downstream end was found in spite of our expectation. However, sharp increase in the amount of methane emitted from the surface water was measured at lowermost localities IV and V (Tab. 4). We found positive, but weak correlation between surface water methane concentrations and measured emissions ( $r_s = 0.45$ ,  $p < 0.05$ )(Fig. 5).

Locality/Gas	CH <sub>4</sub> [mg m <sup>-2</sup> day <sup>-1</sup> ]
Locality I.	2.39
Locality II.	0.25 (0 – 0.6) $n = 9$
locality III.	1.3 (0 – 5.01) $n = 10$
Locality IV.	32.1 (7.3 – 87.9) $n = 8$
Locality V.	36.3 (2.8 – 167.4) $n = 12$

**Table 4.** Average emissions to the atmosphere and their range in parenthesis and from all localities except locality I. Emissions values for the locality I were calculated using a known relationships between concentrations of methane gas in surface water and its emissions to the atmosphere found at downstream laying localities II-V.  $n$  means sample size

### 3.5. Whole-stream emissions $E_a$

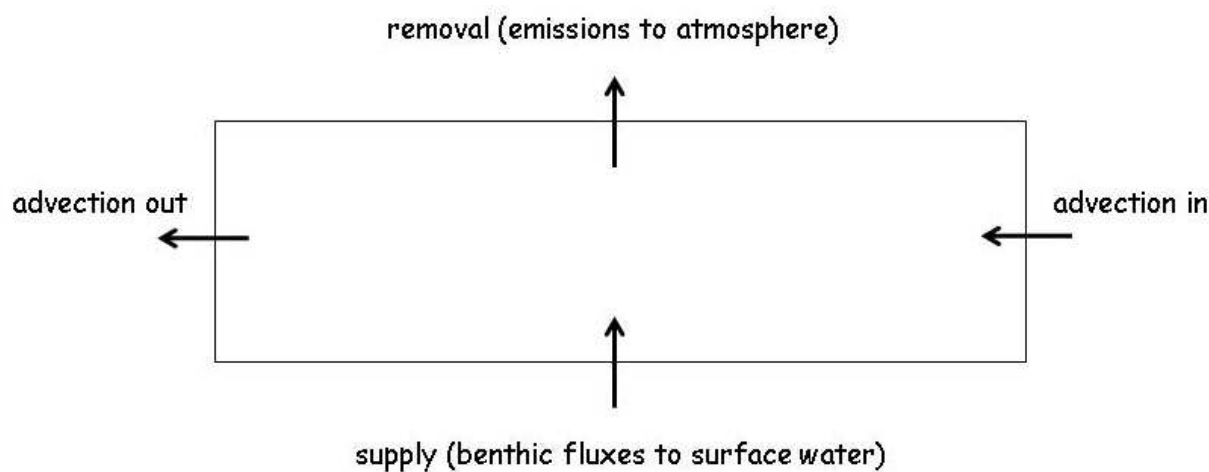
Depending on the time of year we measured the emissions, values of  $E_a$  ranged from 430 to 925 kg year<sup>-1</sup> for methane. Annually, approximately 0.7 tonne of methane was emitted to the atmosphere from the water level of the Sitka stream (total area ca 0.2 km<sup>2</sup>). The majority of annual methane emissions (90 %) occurred in the lower 7 km of the stream (stretch IV and V) that represents only 1/5 of the total stream area. In addition, contribution of methane emissions to the total annual emissions was found to be the highest during spring-summer period (Mach et al. in review).



**Figure 5.** Relationships between atmospheric emissions and surface water concentrations of the methane. Each point represents the mean of five replicate emission measurements and the two replicates of stream water methane concentrations at all

**3.6. Sitka stream water column CH<sub>4</sub> budget for the experimental stretch of a stream**

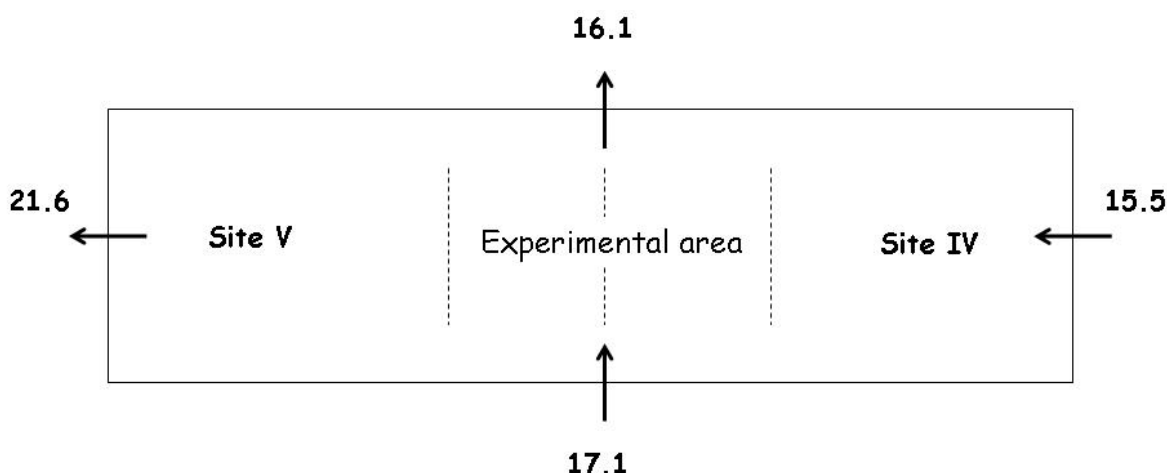
The potentially important source and sinks terms for dissolved methane in the water column of the Sitka stream are shown in Figure 6. Previously calculated rates of inputs (benthic fluxes) and loss of dissolved CH<sub>4</sub> through evasion to the atmosphere can be combined together with advection inputs and losses to yield a CH<sub>4</sub> dynamics (budget) for any particular section of the stream.



**Figure 6.** Simple box model used to calculate a CH<sub>4</sub> budget for the Sitka stream experimental section; advection in + supply = advection out + removal (box adjusted after de Angelis & Scranton 1993)

The CH<sub>4</sub> budget determined for the 2011 sampling period in an experimental stream section is summarized in Figure 7. Benthic fluxes were measured along a stream section 45 m long

with an area being  $\sim 200 \text{ m}^2$ . Positive fluxes of  $\text{CH}_4$  were found to occur at 30.9 % of the study area. Assuming that average benthic flux of methane across the sediment-water interface was  $15.40 \text{ mg m}^{-2} \text{ day}^{-1}$ , the benthic flux of  $3081.39 \text{ mg CH}_4 \text{ day}^{-1}$  should occur from the whole area of  $200 \text{ m}^2$ . Average emission flux of  $\text{CH}_4$  across the water-air interface for all study sites was determined to be  $14.47 \pm 4.73 \text{ mg CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ . This value is slightly lower than the direct benthic flux of  $\text{CH}_4$  and suggests that some portion of methane released from the bottom sediments may contribute to increasing concentration of  $\text{CH}_4$  in the surface water. Average flow of the Sitka stream during time of benthic fluxes measurements was  $0.351 \text{ m}^3 \text{ s}^{-1}$  (i.e.  $351 \text{ L s}^{-1}$ ). Therefore, we may expect that water column was enriched at least by  $187.4 \text{ mg}$  (i.e.  $0.006 \mu\text{g L}^{-1}$ ) of  $\text{CH}_4$  from sediment at  $45 \text{ m}$  long section near study site IV during one day. Next study site V is located some  $4 \text{ km}$  downstream from the site IV. Average  $\text{CH}_4$  concentration difference in the stream water between these study sites was found to be  $3.2 \mu\text{g L}^{-1}$  of  $\text{CH}_4$  indicating that  $\text{CH}_4$  supply exceeds slightly  $\text{CH}_4$  removal. Methane fluxes from the sediment would contribute to this concentration difference only by  $0.6 \mu\text{g L}^{-1}$ , thus, the immediate difference in the  $\text{CH}_4$  budget found between two studied sites IV and V indicates that there must likely be other sources of methane supply to the stream water (Fig. 7). This „missing source“ seems to be relatively small ( $0.9 \text{ mg CH}_4 0.351 \text{ m}^3 \text{ s}^{-1}$ ), however, net accumulation of  $\text{CH}_4$  in the stream water during  $4 \text{ km}$  section of the Sitka stream below study site IV was almost  $78 \text{ g CH}_4$  per one day.



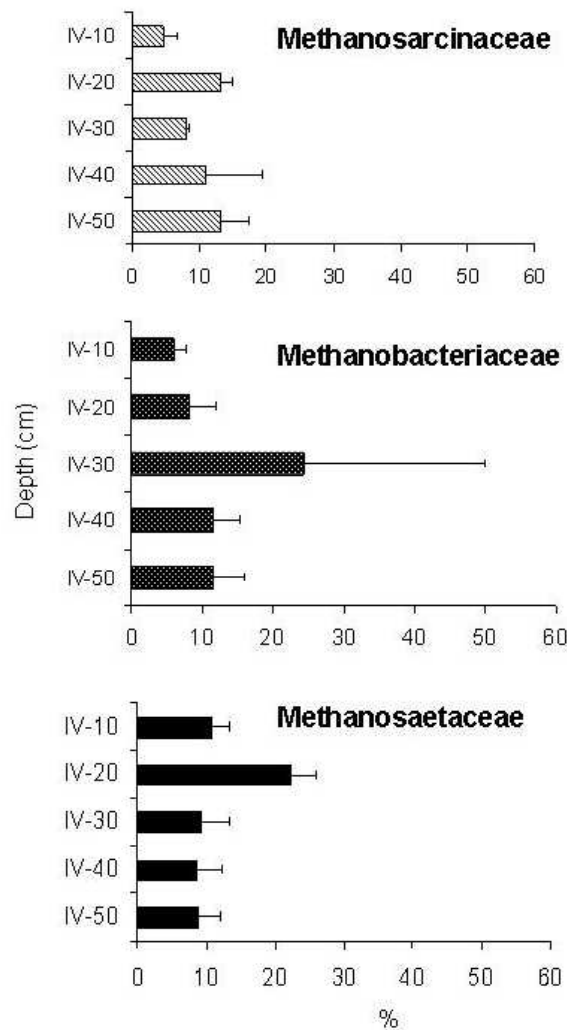
**Figure 7.**  $\text{CH}_4$  budget in  $\text{mol day}^{-1}$  for a section of the Sitka stream between study sites IV and V (length ca  $4 \text{ km}$ ). The arrows correspond to those depicted in Figure 6.

### 3.7. Fluorescence in situ hybridization (FISH)

Both methanogenic archaea and aerobic methanotrophs were found at all localities along the longitudinal stream profile. The proportion of these groups to the DAPI-stained cells was quite consistent and varied only slightly but a higher proportion to the DAPI-stained cells in deeper sediment layer  $25\text{--}50 \text{ cm}$  was observed. On average  $23.4 \%$  of DAPI-stained cells were detected by FISH with a probe for methanogens while type I methanotrophs reached  $\sim 21.4 \%$  and type II methanotrophs  $11.9 \%$ , respectively. All three groups also revealed non-significant higher proportion to the TCN in deeper sediment layer; the abundance of



methanogens and methanotrophs remained almost unchanged with increasing sediment depth. The average abundance of methanogens ( $0,88 \pm 0,28$  and  $1,07 \pm 0,23 \times 10^6$  cells  $\text{mL}^{-1}$  in the upper and deeper layer, respectively) and type II methanotrophs ( $0,44 \pm 0,14 \times 10^6$  cells  $\text{mL}^{-1}$  and  $0,56 \pm 0,1 \times 10^6$  cells  $\text{mL}^{-1}$ ) increased slightly with the sediment depth, while type I methanotrophs revealed average abundance  $0,98 \pm 0,23 \times 10^6$  cells  $\text{mL}^{-1}$  in the deeper layer being lower compared to abundance  $1,07 \pm 0,28 \times 10^6$  cells  $\text{mL}^{-1}$  found in upper sediment layer (Buriánková et al. 2012). Very recently, however, using the FISH method we found that abundance of methanogens belonging to three selected families reached their maximum in the sediment depth of 20-30 cm and had closely reflected vertical distribution of acetate concentrations. Species of family *Methanobacteriaceae* grow only with hydrogen, formate and alcohols (except methanol), *Methanosarcinaceae* can grow with all methanogenic substrates except formate, and members of *Methanosaetaceae* grow exclusively with acetate as energy source. All three families also showed similar proportion to the DAPI stained cells, ranging in average (depth 10-50 cm) from 9.9% (*Methanosarcinaceae*) to 12.3% (*Methanobacteriaceae*) (Fig. 8).



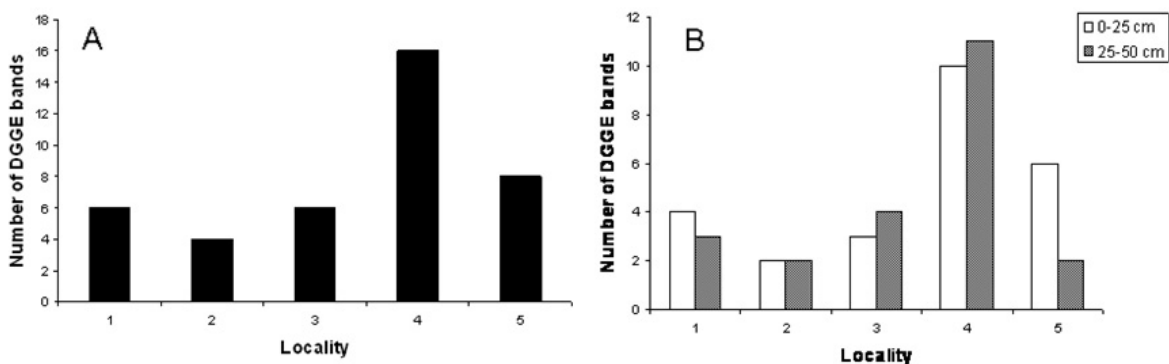
**Figure 8.** The percentage of chosen methanogenic families as compared to the total bacterial cell numbers found in different sediment layers at locality no. IV, horizontal bars indicate 1 SE

### 3.8. Denaturing gradient gel electrophoresis and cloning

Methanogenic communities associated with hyporheic sediments at two different depths (0-25 cm and 25-50 cm) along the longitudinal stream profile were compared based on the DGGE patterns. As shown in Fig. 9, the DGGE patterns varied highly among study localities (Fig. 9A), irrespective of the depth (Fig. 9B). However, presence of the bands in all samples indicates that methanogens may occur up to 50 cm of the sediment depth. The number of DGGE bands of the methanogenic archaeal communities was compared either among localities or among different sediment depths. A total of 22 different bands were observed in the DGGE image ranging from 4 (locality II) to 16 (locality IV) in the samples (Fig. 9A).

The number of DGGE bands also ranged from 2 to 10 for the samples from upper layer (0-25 cm) and from 2 to 11 for the samples from deeper layer (25-50 cm), respectively (Fig. 9B). We found no clear trend in the number of DGGE bands with increasing depth (Fig. 9B). Locality IV appears to be the richest in number of DGGE bands. We suppose that this might be due to most favorable conditions prevailing for the methanogens life as indicated by a relatively low grain median size, lower dissolved oxygen concentration or higher concentration of the ferrous iron compared to other localities (cf. Table 2).

The methanogenic community diversity in hyporheic sediment of Sitka stream was also analysed by PCR amplification, cloning and sequencing of methyl coenzyme M reductase (*mcrA*) gene. A total of 60 *mcrA* gene sequences revealed 26 different *mcrA* gene clones.



**Figure 9.** Number of DGGE bands associated with hyporheic sediments at two different depths along the longitudinal stream profile. A – Total number of all bands detected at each locality; B – number of bands found at different sediment depths

Most of the clones showed low affiliation with known species (< 97% nucleotide identity) and probably represented genes of novel methanogenic archaeal genera/species, but all of them were closely related to uncultured methanogens from environmental samples (> 97% similarity) retrieved from BLAST. The 25 clones were clustered to four groups and were confirmed to be affiliated to *Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales* orders and other unclassified methanogens. The members of all three orders and novel methanogenic cluster were detected to occur in a whole bottom sediment irrespective of a depth, nevertheless, the richness of methanogenic archaea in the sediment was slightly

higher in the upper sediment layer 0-25 cm (15 clones) than in the deeper sediment layer 25-50 cm (11 clones)(Buriánková et al. in review). The clones affiliated with *Methanomicrobiales* predominated in the deeper layer while *Methanosarcinales* clones dominated in the upper sediment layer. This prevalence of *Methanosarcinales* in the upper sediment layer was also confirmed by our FISH analyses as has been mentioned above.

## 4. Discussion

### 4.1. Occurrence of methane in stream water and sediments

In spite of commonly held view of streams as well-oxygenated habitats, we found both surface and interstitial water to be supersaturated with methane compared to the atmosphere at all five localities (Mach et al. in review). Availability of interstitial habitats for bacteria and archaea carrying out anaerobic processes has been confirmed by our previous (Hlaváčová et al. 2005, 2006; Cupalová & Rulík 2007) and contemporary findings. During this study we found relatively well developed populations of methanogenic archaea at all localities and that all localities also showed positive methanogenic potential. Emissions of methane from water ecosystems results from complex microbial activity in the carbon cycle (production and consumption processes), which depends upon a large number of environmental parameters such as availability of carbon and terminal electron acceptors, flow velocity and turbulence, water depth. In our previous paper (Hlaváčová et al. 2006), we suggested that surface water concentrations, and as a consequence methane gas emissions to the atmosphere would result from downstream transport of gases by stream water (advection in/out), and moreover, from autochthonous microbial metabolism within the hyporheic zone. If so, surface water is continually saturated by gases produced by hyporheic metabolism, leading to supersaturation of surface water and induced diffusion of these gases out of river water (volatilizing). Moreover, the run-off and drainage of adjacent soils can also contribute greatly to the degree of greenhouse gas supersaturation (De Angelis & Lilley 1987, Kroeze & Seitzinger 1998, Worral & Lancaster 2005, Wilcock & Sorrell 2008). For example, CH<sub>4</sub> in the estuarine waters may come from microbial production in water, sediment release, riverine input and inputs of methane-rich water from surrounding anoxic environments (Zhang et al. 2008b). For the European estuaries, riverine input contribute much to the estuarine CH<sub>4</sub> due to high CH<sub>4</sub> in the river waters and wetlands also play important roles. However, low CH<sub>4</sub> in the Changjiang Estuary (China) may be resulted from the low CH<sub>4</sub> in the Changjiang water together with the low net microbial production and low input from adjacent salt marshes (Zhang et al. 2008b). Dissolved methane concentrations in a surface water of Sitka stream is consistent with literature data on methane in rivers published by Middelburg et al. (2002) and Zhang et al. (2008b).

### 4.2. Stable carbon isotopes

A knowledge of the stable carbon isotopic ratio of methane  $\delta^{13}\text{C-CH}_4$  in natural systems can be useful in studies of the mechanisms and pathways of CH<sub>4</sub> cycling (Sansone et al. 1997). Values of carbon isotope signature of methane ( $\delta^{13}\text{C-CH}_4$ ) indicate biogenic nature of the

methane, being usually in the range -27 ‰ up to -100 ‰ (Conrad 2004; Michener & Lajtha 2007). Whiticar et al. (1986) demonstrated that methane in freshwater sediments is isotopically distinguished by being relatively enriched in  $^{13}\text{C}$  ( $\delta^{13}\text{C} = -65$  to  $-50\text{‰}$ ) in contrast to marine sediments ( $-110$  to  $-60\text{‰}$ ). Accordingly, the two precursors of methane, namely acetate and  $\text{CO}_2/\text{H}_2$ , yield methane with markedly different  $\delta^{13}\text{C}$  values; methane from acetate is relatively enriched in  $^{13}\text{C}$ . Average minimum in the carbon isotopic composition of  $\text{CH}_4$  ( $-61.4\text{‰}$ ) occurred deeper in sediments (60 cm) while average maximum in  $\delta^{13}\text{C}\text{-CH}_4$  occurred in the lower sediment depth of 30 cm. Enrichment of  $^{13}\text{C}$  in  $\text{CH}_4$  probably reflects aerobic  $\text{CH}_4$  oxidation because oxidation would result in residual  $\text{CH}_4$  with  $\delta^{13}\text{C}\text{-CH}_4$  values less negative than the source  $\text{CH}_4$  (Barker & Fritz 1981; Chanton et al. 2004). However, this effect has been observed only at the study site IV.

### 4.3. Spatial and temporal distributions of emissions

Our working hypothesis suggested that along with the longitudinal profile of a stream, slope and flow conditions also change together with corresponding settling velocity, sediment composition and organic matter content. Thus, according to this prediction, sediment with prevalence of fine-grained particles containing higher amount of organic matter should dominate at the downstream stretches. Moreover, due to prevalence of anoxic environment, production of methane and its emissions was expected to be also higher here compared to that from upstream stretches. Based on our findings, it seems that this presumption is valid for the methane. In addition, we found higher methane concentrations in both the surface and interstitial water at the uppermost locality I compared to lower situated locality II. Similar situation with high methane concentration in the upstream part with subsequent decline further downstream was also reported from USA by Lilley et al. (1996). Dissimilarity of this first stretch is apparent in a comparison with the next, downstream laying stretch (locality II), represented by profile with steep valley and high slope. Generally, there were found very low methane concentrations either in surface or interstitial water and fluxes of emissions to atmosphere were also very low.

Flux rates of gaseous emissions into atmosphere depend on partial pressure of particular gas in the atmosphere and its concentration in a water, water temperature and further on the water depth and flow velocity. Thus, maximum peak of emissions may be expected during summer period and in well torrential stretch of the river. Silvennoinen et al. (2008), for example, found that the most upstream river site, surrounded by forests and drained peatlands, released significant amounts of  $\text{CO}_2$  and  $\text{CH}_4$ . The downstream river sites surrounded by agricultural soils released significant amounts of  $\text{N}_2\text{O}$  whereas the  $\text{CO}_2$  and  $\text{CH}_4$  concentrations were low compared to the upstream site. When consider seasonal distribution of methane emissions, it is clear, in concordance with above mentioned presumption, that majority of methane emissions was released during a warm period of the year (81%). Effect of temperature on methane production was also observed in southeastern USA where the most methane released to the atmosphere during warm months (Pulliam 1993). In addition, close correlation between methane emissions and temperature was reported also from south part of Baltic Sea; the temperature has been found to be a key factor driving methane emissions (Heyer & Berger 2000).

These findings also indicate that we should be very careful in making any generalization in total emissions estimation for any given stream or river. Even though some predictions can be made based on gas concentrations measured in the surface or interstitial water, results may be very different. From this point, noteworthy was locality IV; enormous concentrations of methane found in the deep interstitial water were caused probably by very fine, clayed sediment containing high amount of organic carbon, as well as high DOC concentrations. Supersaturation led also to the enrichment of the surface water with methane - such places may be considered as very important methane sources for surface stream and, consequently source of emissions to the atmosphere.

#### **4.4. Benthic fluxes and potential methane oxidation**

CH<sub>4</sub> can be produced and released into overlying near-bottom water through exchange at sediment-water interface. Methane released from the sediments into the overlying water column can be consumed by methanotrophs. Methanotrophs can oxidize as much as 100 % of methane production (Le Mer & Roger 2001). According to the season, 13-70 % of methane was consumed in a Hudson River water column (de Angelis et Scranton 1993). For the Sitka stream, measurement of benthic fluxes into the overlying surface waters indicates that methane consumption by methanotrophic bacteria is likely a dominant way of a methane loss, nevertheless some methane still supports relatively high average methane concentrations in the surface water and, in turn, high emissions to the atmosphere.

The methane production (measured as methanogenic potential) was found to be 3 orders of magnitude lower than the oxidation (methanotrophic activity), thus, almost all methane should be oxidized and consumed by methanotrophic bacteria and no methane would occur within the sediments. However, situation seems to be quite different suggesting that namely methanotrophic activity measured in a laboratory could be overestimated. Since oxidation of methane requires both available methane and oxygen, methanotrophic activity is expected to be high at sites where both methane and dissolved oxygen are available. Therefore, high values of the MA were usually found in the upper layers of the sediments (Segers 1998) or at interface between oxic and anoxic zones, respectively. Relatively high methanotrophic activity found in deeper sediments of the localities III-V indicates that methane oxidation is not restricted only to the surface sediments as is common in lakes but it also takes place at greater depths. It seems likely that oxic zone occurs in a vertical profile of the sediments and that methane diffusing from the deeper layer into the sedimentary aerobic zone is being oxidized by methanotrophs here. Increased methanotrophic activity at this hyporheic oxic-anoxic interface is probably evident also from higher abundance of type II methanotrophs in the same depth layer. Similar pathway of methane cycling has been observed by Kuivila et al. (1988) in well oxygenated sediments of Lake Washington, however, methane oxidation within the sediments would be rather normal in river sediments compared to lakes. All the above mentioned findings support our previous suggestions that coexistence of various metabolic processes in hyporheic sediments is common due to vertical and horizontal mixing of the interstitial water and occurrence of microbial biofilm (Hlaváčová et al. 2005, 2006).



#### 4.5. Methanogens diversity

The presence of relatively rich assemblage of methanogenic archaea in hyporheic river sediments is rather surprising, however it is in accordance with other studies. The number of total different bands (i.e. estimated diversity of the methanogenes) observed in the DGGE patterns of the methanogenic archaeal communities was comparable with a number of the DGGE bands found in other studies. For example, Ikenaga et al. (2004) in their study of methanogenic archaeal community in rice roots found 15-19 DGGE bands, while Watanabe et al. (2010) showed 27 bands at different positions in the DGGE band pattern obtained from Japanese paddy field soils. Our results from the DGGE analysis are supported by cloning and sequencing of methyl coenzyme M reductase (*mcrA*) gene which also retrieved relatively rich diversity (25 different *mcrA* gene clones) of the methanogenic community in the Sitka stream hyporheic sediments. Similar richness in number of clones was also mentioned in a methanogenic community in Zoige wetland, where 21 different clones were found (Zhang et al. 2008a), while 20 clones were described in the methane cycle of a meromictic lake in France (Biderre-Petit et al. 2011). In addition, soils from Ljubljana marsh (Slovenia) showed 17 clones (Jerman et al. 2009), for example. Both DGGE and *mcrA* gene sequencing results suggest that both hydrogenotrophic and acetoclastic methanogenesis are an integral part of the CH<sub>4</sub> - producing pathway in the hyporheic zone and were represented by appropriate methanogenic populations. Further, these methanogenic archaea form important component of a hyporheic microbial community and may substantially affect CH<sub>4</sub> cycling in the Sitka stream sediments.

#### 5. Conclusion

To our knowledge this study is the first analysis of the composition of active methanogenic/methanotrophic communities in river hyporheic sediments. By use of various molecular methods we have shown that both methanogenic archaea and aerobic methanotrophs can be quantitatively dominant components of hyporheic biofilm community and may affect CH<sub>4</sub> cycling in river sediments. Their distribution within hyporheic sediments, however, only partly reflects potential methane production and consumption rates of the sediments. Rather surprising is the detection of methanotrophs in the deep sediment layer 25-50 cm, indicating that suitable conditions for methane oxidation occur here. In addition, this work constitutes the first estimation of sources, sinks and fluxes of CH<sub>4</sub> in the Sitka stream and in 3rd order stream environment. Fluxes of CH<sub>4</sub> from supersaturated interstitial sediments appear to be a main CH<sub>4</sub> source toward the water column. Compared with CH<sub>4</sub> production rates, the diffusive fluxes are very low due to efficient aerobic oxidation by methanotrophic bacteria, especially during higher flow discharges. Although fluxes to the atmosphere from the Sitka stream seems to be insignificant, they are comparable or higher in comparison with fluxes from other aquatic ecosystems, especially those measured in running waters. Finally, our results suggest that the Sitka Stream is a source of methane into the atmosphere, and loss of carbon via the fluxes of this greenhouse gas out into the ecosystem can participate significantly in river self-purification.



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