PRODUKCE SKLENÍKOVÝCH PLYNŮ (CO_2, CH_4, N_2O) Z TEKOUCÍCH VOD

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ABSTRAKT

Skleníkovým plynům a souvisejícím biogeochemickým koloběhům uhlíku a dusíku je v posledních dvou desetiletích věnována zvýšená pozornost v důsledku globálních změn klimatu. Cílem disertační práce bylo v několika vědeckých studiích kvantifikovat a charakterizovat produkci skleníkových plynů, především metanu, v hyporheických sedimentech malého vodního toku Sitka. Studie, které se zakládají na terénních měřeních, inkubačních experimentech a analýze stabilních izotopů, jsou součástí širšího výzkumu, který se zabývá koloběhem uhlíku v tekoucích vodách. Data z pěti studií obsažených v disertační práci dokládají, že vodní toky uvolňují do atmosféry významné množství metanu, oxidu uhličitého a oxidu dusného. Dále uvedené studie dokládají, že v hyporheických sedimentech dochází k mikrobiálním procesům metanogeneze a metanotrofie a současně k výskytu souvisejících taxonů archeí a bakterií. Závěrečná studie prokazuje, že podíl acetoklastické a hydrogenotrofní produkce metanu v hyporheických sedimentech je v rozsahu typickém pro sladkovodní prostředí. Disertační práce přispěla k vědomostem o anaerobním metabolismu v hyporheických sedimentech a o jejich roli v biogeochemických koloběžích uhlíku a dusíku.

Klíčová slova: skleníkové plyny, metan, oxid uhličitý, oxid dusný, hyporheický sediment, tekoucí vody, metanogeneze

ABSTRACT

In terms of the global climate change, a raised awareness is devoted to greenhouse gas emissions and to the contextual biogeochemical cycles of carbon and nitrogen in the last two decades. The aim of the thesis is to quantify and characterize, in several research papers, greenhouse gas production, especially of methane, in hyporheic sediments of the small water stream Sitka. The research papers based on field measurements, incubation experiments and a stable isotope analysis are a part of a long-term research concerned with the organic carbon cycle in running waters. Data in the five research papers included in the thesis prove that water streams release a significant amount of methane, carbon dioxide and nitrous oxide into the atmosphere. Furthermore the research papers demonstrate that microbial processes of methanogenesis and methanotrophy occur in conjunction with presence of related taxonomic groups of archaea and bacteria. The last research paper approves that partitions of acetoclastic and hydrogenotrophic pathways of methane production in hyporheic sediments are in a range typical for freshwater environments. The thesis contribute to the knowledge about an anaerobic metabolism in hyporheic sediments, and about their role in biogeochemical cycles of carbon and nitrogen.

Key words: greenhouse gases, methane, carbon dioxide, nitrous oxide, hyporheic sediments, running waters, methanogenesis

OBSAH

STRUKTURA DISERTAČNÍ PRÁCE

Disertační práce je založena na níže uvedených publikacích a manuskriptech, které jsou označeny a citovány pod číslicemi I. - V.

I.

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II.

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ÚVOD

Skleníkové plyny

Kromě vodních par jsou třemi nejvýznamnějšími skleníkovými plyny oxid uhličitý, metan a oxid dusný (IPCC 2013). Celkový podíl skleníkových plynů na změnách klimatu ovlivňuje především nárůst jejich atmosférické koncentrace, která od preindustriálního období u všech tří plynů významně stoupla. V roce 2011 byla průměrná globální atmosférická koncentrace oxidu uhličitého 391 ppm, což je nárůst o 41 % oproti preindustriálnímu období, za které je považován rok 1750 (IPCC 2013). V preindustriálním období byla globální atmosférická koncentrace oxidu uhličitého podle měření vzduchu z ledovcových vrtů 278 ppm (Etheridge et al. 1996). Průměrná globální atmosférická koncentrace metanu je 1803 ppb (IPCC 2013), což je výrazný nárůst oproti preindustriálnímu období, kdy byla jeho průměrná globální koncentrace pouze 722 ppb (Eltridge et al. 1998, Dlugokencky et al. 2005), ale předpokládá se, že lidský vliv na globální koloběh metanu začal o tisíce let dříve než na počátku industriálního období (Ruddiman 2003, Ferretti et al. 2005, Ruddiman 2007). V roce 2011 dosáhla průměrná globální atmosférická koncentrace oxidu dusného 324 ppb (IPCC 2013) a od roku 1750, kdy byla 270 ppb, stoupla o 20 % (Prather et al. 2012). Měření izotopického složení oxidu dusného v ledovcových vrtech naznačuje, že vzrůst atmosférické koncentrace tohoto plynu od 50. let 20. století je především v důsledku používání syntetických dusičnanových hnojiv (Rockmann et Levin 2005, Ishijima et al. 2007, Davidson 2009, Syakila et Kroeze 2011).

Schopnost různých skleníkových plynů přispět ke klimatické změně ovlivňuje vedle atmosférických koncentrací také jejich efektivita. Jako metrické nástroje převedení efektu různých skleníkových plynů na společnou stupnici slouží potenciály globálního oteplení (*global warming potentials*, zkráceně GWP). Potenciál globálního oteplení je definován jako v daném čase vypočtené radiační působení určitého skleníkového plynu v poměru ku radiačnímu působení stejné hmotnosti oxidu uhličitého (Houghton et al.

1990). Efekt stejné hmotnostní jednotky metanu i oxidu dusného na oteplení troposféry je výrazně vyšší než u oxidu uhličitého (Myhre et al. 2013).

Produkce biogenních skleníkových plynů

Oxid uhličitý, metan a oxid dusný jsou součástí biogeochemických cyklů uhlíku a dusíku, tudíž kolují mezi rezervoáry, ze kterých jsou odstraňovány ztrátami a doplňovány do nich zdroji. Významným rezervoárem všech tří sloučenin je atmosféra. Ztráty a zdroje skleníkových plynů pro atmosféru se mohou rozdělovat na přirozené a antropogenní. Rovnováha přirozených koloběhů prvků mezi rezervoáry je zpravidla antropogenními vlivy narušována. Příkladem je právě zvyšování koncentrace skleníkových plynů v atmosféře. Důležitou společnou vlastností všech tří plynů je, že mohou vznikat mikrobiálními procesy, které patří mezi jejich významné přirozené i antropogenní zdroje.

K přirozeným zdrojům oxidu uhličitého přibyly v holocénu zdroje antropogenní, kterými jsou spalování fosilních paliv, výroba cementu a změny využití území (např. deforestace). Pouze malý zlomek celkového množství oxidu uhličitého uvolněného ročně do atmosféry je antropogenního původu, ale přesto je tato relativně malá část klíčová v narušení rovnováhy přirozeného koloběhu uhlíku a vzrůstu atmosférických koncentrací oxidu uhličitého.

Podle vzniku lze zdroje metanu do atmosféry rozdělit na tři kategorie: biogenní, termogenní (fosilní) a pyrogenní, které mohou být jak přirozené tak antropogenní. Biogenní emise metanu tvoří asi 65-79 % celkových emisí do atmosféry (Wuebbles et Hayhoe 2002, Mikaloff-Fletcher et al. 2004, Wang et al. 2004, Chen et Prinn 2006). Z hlediska biogenních zdrojů metanu byla zatím věnována pouze malá pozornost řekám a vodním tokům (Jones et al. 1995, Hlaváčová et al. 2005, Sanders et al. 2007, Wilcock et Sorrell 2007, Trimmer 2009, publikace I. a II.).

K nárůstu atmosférické koncentrace oxidu dusného dochází kvůli antropogennímu narušení koloběhu dusíku. Rovnováha je narušována tím, že je zvýšena produkce

reaktivního dusíku (všechny formy dusíku kromě N_2) lidskou činností. Oxid dusný vzniká přirozeně během mikrobiálních procesů nitrifikace a denitrifikace, které jsou zvýšeným obsahem reaktivního dusíku antropogenně zesilovány (Sloss 1992). Nedávné studie potvrzují, že řeky jsou významným zdrojem oxidu dusného (Beaulieu et al. 2011, Rosamond et al. 2012, publikace I.).

Kvůli obavám z globální klimatické změny navrhují mnohé studie možné způsoby a strategie snižování emisí skleníkových plynů z antropogenních zdrojů. Na druhou stranu některé přirozené zdroje těchto plynů jsou opomíjeny (Frankignoulle et Middelburg 2002), přestože je inventarizaci zdrojů skleníkových plynů věnováno značné úsilí. Koncentrace oxidu uhličitého, metanu a oxidu dusného je ve vodních tocích několikanásobně vyšší než rovnovážná s atmosférickou koncentrací (Cole et Caraco 2001, Hlaváčová et al. 2006, Striegl et al. 2012, Yang et al. 2012), což naznačuje, že vodní toky mohou být významným zdrojem těchto plynů. Ačkoliv panuje všeobecná potřeba kvantifikace potenciálních zdrojů skleníkových plynů, nejsou obvykle vodní toky mezi zdroje metanu přímo započítány, jako je to v případě oxidu dusného. Přesto již existuje např. mnoho dokladů, že vodní toky uvolňují také významné množství metanu (Sanders et al. 2007, Wilcock et Sorrell 2008).

Hyporheické sedimenty

Hyporheická zóna je oblast říčního dna vyplněná porézními sedimenty, které jsou saturované říční a podzemní vodou. Hyporheické sedimenty mají zásadní úlohu v procesu samočištění vody a jsou metabolickým centrem vodního toku zodpovědným za zadržování, ukládání a mineralizaci organického materiálu, který je transportován povrchovou vodou (Grim et Fisher 1984). Fyzikální a chemické parametry v hyporheickém sedimentu se vyznačují prostorovou variabilitou.

Díky hyporheickému biofilmu mohou v aerobním prostředí vedle sebe probíhat aerobní a anaerobní procesy, které se odehrávají v jeho různých vrstvách. Protože polysacharidová matrix udržuje propojení mezi jednotlivými mikrokoloniemi různých

bakteriálních a archeálních typů, mohou vznikat místa specifických limitovaných nik. Mikroorganismy požadující specifické substráty, jako jsou např. metanogeny s požadavkem na vodík, jsou schopny růst díky těsnému prostorovému nastavení s ostatními typy bakterií a archeí, které produkují tyto substráty (Korber et al. 1995).

Metanogeneze

Biogenní metan hraje úlohu při rozkladu organických látek v sedimentech a půdách, protože je finálním produktem anaerobní dekompozice organického materiálu. V anaerobních podmínkách je organický materiál nejprve rozložen fermentací na jednoduché látky (oxid uhličitý, kyseliny a alkoholy); poslední krok anaerobního rozkladu pak probíhá, až pokud jsou spotřebovány alternativní anorganické elektronové akceptory (zejména dusičnany, železité ionty a sírany) a je nazýván metanogeneze. Tento mikrobiální proces je umožněn funkční skupinou zvaných metanogeny (metanogenní archea), které fylogeneticky náleží do několika taxonů v doméně *Archea* (Chaban et al. 2006). Metanogeny mohou využívat pouze omezené množství substrátů. Ve sladkovodních ekosystémech se vyskytuje prakticky pouze redukce oxidu uhličitého vodíkem (tzv. hydrogenotrofní metanogeneze) a rozklad acetátu (tzv. acetoklastická metanogeneze) (Davidson et Schimel 1995, Conrad 2007). Ve většině metanogenních prostředí probíhají tyto typy metanogeneze současně a liší se svým podílem na celkové produkci metanu.

Metan produkovaný ve sladkovodním prostředí je často obohacen o těžší izotop uhlíku (^{13}C) v poměru k metanu z mořských sedimentů. To je díky relativně většímu významu acetoklastické metanogeneze ve sladkovodních prostředích oproti mořským sedimentům, kde převládá hydrogentrofní metanogeneze (Blair et al. 1993). Přestože podíl metanogenních reakcí na celkové produkci metanu byl již kvantifikován v mnoha různých prostředích, jako jsou tropická jezera (Conrad et al. 2011), rýžová pole (Sugimoto et Wada 1993, Fey et al. 2003, Penning et Conrad 2007), kořeny rýže (Chin et al. 2004), boreální rašeliniště (Galand et al. 2005), mořské sedimenty (Crill et Martens 1986), mokřady mírného pásu (Hornibrook et al. 1997) či anaerobní reaktory

(Laukenmann et al. 2010), o podílu metanogenních reakcí v říčních ekosystémech máme dosud stále málo informací.

Lepší představu již máme o koloběhu metanu v říčním ekosystému. Metan je produkován v hyporheické zóně, ze které přechází difúzí nebo v podobě bublinek do povrchové vody, odkud je uvolňován dále do atmosféry. Na úbytku metanu v hyporheické zóně se podílí metanotrofie, která je zprostředkována funkční skupinou metanotrofních bakteriií. Tento mikrobiální proces převládá ve více prokysličených částech sedimentu a v některých vodních ekosystémech může zoxidovat až veškerý vyprodukovaný metan (Hanson et Hanson 1996).

CÍLE DISERTAČNÍ PRÁCE

V disertační práci byly sledovány tři hlavní cíle:

Porovnat a kvantifikovat emise a koncentrace skleníkových plynů (metanu, oxidu uhličitého, oxidu dusného) v hloubkovém a podélném profilu malého vodního toku Sitka během jednoho ročního cyklu. (publikace I. a II.).

Pomocí inkubačních experimentů charakterizovat a porovnat mikrobiální procesy spojené s produkcí a oxidací metanu v hloubkovém profilu hyporheického sedimentu a podél říčního kontinua malého vodního toku Sitka (publikace II., III., IV.).

Pomocí analýzy $\delta^{13}C$ a inkubačních experimentů se selektivní inhibicí metanogeneze kvantifikovat substrátovou preferenci metanogenů a charakterizovat mikrobiální procesy v koloběhu uhlíku v hloubkovém profilu hyporheického sedimentu říčky Sitka (publikace V.).

METODIKA

Výzkum probíhal na říčce Sitka, která je z převážné části neregulovaným vodním tokem dlouhým 35 km. Pramení v Nízkém Jeseníku a ústí do říčky Oskavy asi 5 km severně od Olomouce. V podélném profilu vodního toku bylo vytyčeno pět lokalit, které představovaly pravidelná odběrová místa. Horní část toku protéká skrze lesnatou oblast s malou intenzitou antropogenních činností (lokality I-II), zatímco spodní část toku s dobře vyvinutou pobřežní vegetací přirozeně meandruje skrze intenzivně obhospodařovanou zemědělskou krajinu (lokality III-V).

Během výzkumu byly na stanovených lokalitách pravidelně v průběhu roku odebírány vzorky povrchové a intersticiální vody a vzorky hyporheického sedimentu. Intersticiální voda byla odebírána za použití minipiezometrů (Trulleyová et al. 2003) a hyporheické sedimenty byly odebírány pomocí metody freeze-core s tekutým dusíkem jako chladícím mediem (Bretschko et Klemens 1986). Koncentrace oxidu uhličitého, metanu a oxidu dusného v plynných a vodních vzorcích byla měřena plynovou chromatografií.

Měření úniku plynů z vodní hladiny do atmosféry bylo provedeno pomocí metody uzavřených komor (Crill et al. 1988). Vespodu otevřené plovoucí komory byly umístěny na vodní hladinu pomocí plováku a plyny mohly volně emitovat do *headspace* komor. Emise byly spočteny jako rozdíl mezi pozaďovou koncentrací a koncentrací uvnitř komor po uplynutí doby inkubace a vyjádřeny v mg m^{-2} day^{-1}.

Inkubační experimenty pro stanovení potenciální produkce metanu a potenciální oxidace metanu v podélném a hloubkovém profilu hyporheických sedimentů toku Sitka sloužily ke srovnání s početností mikrobiálních populací metanogenů a metanotrofů. Míra potenciální produkce metanu (metanogeneze) je měřena pomocí anaerobních inkubačních experimentů (Segers 1998). Míra potenciální oxidace metanu (metanotrofní aktivita) byla měřena modifikací metody oxidace metanu v půdních vzorcích (Hanson 1998). Potenciální produkce a oxidace metanu byly vypočteny

z rozdílů koncentrací metanu během doby inkubace a byly vyjádřeny v nM CH_4 ml^{-1} mokrého sedimentu za hodinu.

Pro určení substrátové specifity metanogeneze byla použita metoda srovnání izotopového složení uhlíku v produkovaném metanu a jeho prekurzorech během anaerobní inkubace hyporheického sedimentu s a bez přídavku metyl-fluoridu, který specificky inhibuje acetoklastickou metanogenezi. Izotopické složení metanu a oxidu uhličitého bylo provedeno hmotnostním spektrometrem s předřazeným spalováním a plynovým chromatografem (Brand 1996). Izotopická analýza acetátu byla provedena metodou hmotnostní spektrometrie s předřazenou kapalinovou chromatografií (Krummen et al. 2004). Izotopický poměr všech analyzovaných sloučenin byl vyjádřen v hodnotě delta oproti standartě VPDB. Podíl hydrogentrofní metanogeneze byl vypočten podle rovnice (Conrad 2005):

$$f_{mc} = (\delta_{CH4} - \delta_{ma}) / (\delta_{mc} - \delta_{ma})$$

kde f_{mc} je podíl hydrogentrofní metanogeneze na celkové produkci metanu, δ_{CH4}, δ_{ma} a δ_{mc} jsou izotopický signál uhlíku metanu směsného, produkovaného výhradně z acetátu a výhradně z oxidu uhličitého.

SHRNUTÍ VÝSLEDKŮ

V první části práce zaměřené na produkci metanu, oxidu uhličitého a oxidu dusného v hyporheických sedimentech byly naměřeny významné, v porovnání s atmosférou několikanásobně přesycené koncentrace všech sledovaných plynů v intersticiální a povrchové vodě a to v celém podélném profilu toku Sitka. Koncentrace sledovaných plynů v intersticiální vodě několikanásobně převyšovaly koncentrace ve vodě povrchové, což indikuje, že hyporheický sediment je zdrojem těchto plynů pro vodu povrchovou, ze které jsou následně skleníkové plyny uvolněny do atmosféry. Současně byly v celém podélném profilu vodního toku během jedné roční sezóny naměřeny významné atmosférické emise všech tří plynů. Celkové roční emise skleníkových plynů z toku Sitka do atmosféry byly odhadnuty na 637 kg metanu, 210 t oxidu uhličitého a 229 kg oxidu dusného. Pokud převedeme všechny plyny pomocí potenciálů globálního oteplení ve stoletém horizontu na stejné metrické jednotky, přispěje sledovaný vodní tok 300 ekvivalentních tun oxidu uhličitého, z čehož je 7,2 % příspěvek metanu.

V další části práce, která již byla zaměřena pouze na metan, byly naměřeny pozitivní potenciální produkce metanu a potenciální oxidace metanu hyporheického sedimentu z celého podélného profilu sledovaného vodního toku. Naměřené potenciální produkce metanu i potenciální oxidace metanu vykazovaly vyšší hodnoty v dolní části toku než v horní části toku. Při srovnání aktivity obou mikrobiálních procesů mezi svrchní vrstvou (0-25 cm) a spodní vrstvou (25-50 cm) hyporheického sedimentu vykazovala vyšší hodnoty především vrstva svrchní, která celkově obsahovala větší množství mikroorganismů a mikrobiální aktivity. Početnosti metanogenních archeí a metanotrofních bakterií v podélném a vertikálním profilu vodního toku ovšem neodpovídaly relativnímu významu příslušných mikrobiálních procesů.

Poslední část práce zužuje pozornost pouze na mikrobiální procesy v hyporheickém sedimentu na lokalitě IV. Anaerobní inkubační experimenty při detailním hloubkovém rozlišení prokázaly dvě produkční maxima produkce metanu: první v hloubce 0-10 cm a druhá v hloubce 40-50 cm. Produkce metanu se pohybovala ve srovnatelných řádech

jako produkce metanu v jiných sladkovodních ekosystémech, jako jsou jezera nebo rýžoviště a přitom hodnoty $\delta^{13}C$ produkovaného metanu odpovídaly hodnotám pro biogenní metan. Na základě změřených $\delta^{13}C$ smíšeného a výhradně hydrogentrofně produkovaného metanu, $\delta^{13}C$ acetátu a frakcionačních faktorů acetoklastické metanogeneze z literatury byl dopočítán podíl hydrogentrofní metanogeneze, který představuje 40-50 % celkové produkce metanu, což je opět v rozsahu typickém pro sladkovodní prostředí. Výskyt obou typů metanogeneze v hyporheickém sedimentu toku Sitka koresponduje s nálezy z předchozí části výzkumu, kdy byli nalezeni zástupci hydrogentrofních (*Methanobacterium*) i acetoklastických (*Methanosarcina* a *Methanosaeta*) metanogenů. Z nízkých hodnot $\delta^{13}C$ akumulovaného acetátu, který se nahromadil během anaerobní inkubace za přítomnosti metyl-fluoridu, vyplývá, že v hyporheickém sedimentu Sitky probíhá významnou měrou také homoacetogeneze, což je mikrobiální proces produkce acetátu z oxidu uhličitého.

ZÁVĚR

Tato práce přinesla jedny z prvních odhadů kvantifikace celkových ročních atmosferických emisí skleníkových plynů z malého vodního toku. Bylo potvrzeno, že anaerobní mikrobiální procesy hrají významnou roli v koloběhu uhlíku v hyporheických sedimentech a že metan, kterému bylo doposud ve vodních tocích věnováno pouze málo pozornosti, se nezanedbatelnou měrou podílí na klimatickém efektu celkových emisí skleníkových plynů z vodního toku. Také z toho důvodu byl původní záměr, věnovat se v této disertační práci produkci všech tří biogenních skleníkových plynů, posléze zúžen pouze na produkci metanu. Dalším impulsem k této změně byla spoluúčast na výzkumném grantu GAČR „Biogeochemie metanu a detekce metanogenních a metanotrofních bakterií v říčních sedimentech“. Další studie prokázaly, že metanogeneze a metanotrofie jsou významné procesy v sedimentech tekoucích vod, což odpovídá nalezenému bohatému společenstvu metanogenů a metanotrofů. Za použití metody detekce stabilních izotopů a selektivní inhibice metanogeneze během inkubace bylo zjištěno, že na celkové produkci metanu se acetoklastická metanogeneze podílí 50-60 % a hydrogenotrofní metanogeneze 40-50 %. Míra produkce metanu a substrátová preference metanogenů v hyporheickém sedimentu vodního toku se pohybují v řádově podobných hodnotách jaké byly nalezeny v jiných sladkovodních ekosystémech.

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ABSTRAKTY PUBLIKACÍ V DISERTAČNÍ PRÁCI.

I. Seasonal measurement of greenhouse gases concentrations and emissions along the longitudinal profile of small stream

Václav Mach, Lubomír Čáp, Jan Šipoš, Martin Rulík

(*Pol. J. Environ. Stud.* submitted)

In order to find out whether streams might be the considerable source of greenhouse gases to the atmosphere, our investigation sought to determine the total emissions of CH_4, CO_2, and N_2O from the surface water of a small stream. Over a period of a year we used floating chambers to measure gaseous emissions along the longitudinal profile of the Sitka stream (Czech Republic). Additionally, we measured gas concentrations of surface and interstitial waters. We found that interstitial and surface waters were supersaturated by all monitored gases, especially by CH_4 and that the stream is significant emitter of these greenhouse gases. The concentrations and the emission rates of all three gases were higher in the downstream part than in the upstream part of the stream. In the case of CH_4 the majority of the total annual emission (90%) was released from the most downstream section of the stream, representing only 1/5 of the stream's total surface area (0.18 km^2). The total annual emissions of CH_4, CO_2, and N_2O into the atmosphere from the water‘s surface were estimated to be 0.6 t, 210 t, and 0.2 t respectively. After conversion of the greenhouse gas emissions to CO_2 equivalents used by IPCC, CO_2 accounts the most part of total annual emission of greenhouse gases (70.1%), with the second being N_2O (22.7%), and the last CH4 (7.2%), for 100-year time horizon. This work brings worthwhile data of greenhouse gas emissions and concentrations from a small water stream based on seasonal measurement along the longitudinal profile.

II. Methanogenic system of a small lowlandstream Sitka, Czech Republic

Martin Rulík, Adam Bednařík, Václav Mach, Lenka Brablcová, Iva Buriánková, Pavla Badurová, Kristýna Gratzová

(In: Matovic MD et al. *Biomass Now - Cultivation and Utilization,* Chapter 17: 395-426)

Chapter in a book summarized results of long term research focused on organic carbon cycle in running waters and methanogenesis, especially. Methane emissions from environmental sources are presently wide discussed issue due to the role of methane in global warming. The findings from aquatic environments confirmed that amount of microbially produced methane significantly contributes to the total methane concentration in atmosphere. Methane emissions, distribution of microbial activity, methanogenic archaea and methanotrophs were studied in a small lowland stream Sitka in Czech Republic. The methanogens and methanotrophic bacteria were detected using FISH with 16 S rRNA-targeted oligonucleotide probes. The occurence and diversity of methanogenic archaeal community were analyzed by cloning technique and denaturing gradient gel electrophoresis (DGGE).

III. Methanogens and methanotrophs distribution in the hyporheic sediments of a small lowland stream

Iva Buriánková, Lenka Brablcová, Václav Mach, Aneta Hýblová, Pavla Badurová, Jana Cupalová, Lubomír Čáp, Martin Rulík

(*Fundam. Appl. Limnol.* 17: 87-102)

Distribution of microbial activity, methanogenic archaea and type I and II methanotrophs were studied in a small lowland stream Sitka in Czech Republic. The methanogens and methanotrophic bacteria were detected using FISH with 16 S rRNA-targeted oligonucleotide probes. The highest microbial density was obtained in the upper sediment layer 0 – 25 cm; this zone corresponded also to that of the highest metabolic activity, as indicated by the methanogenic potential, methanotrophic activity, INT and FDA profiles. 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 the deeper sediment layer 25 – 50 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 ~ 21.4 % and type II methanotrophs 11.9 %, respectively. The percentage of DAPI-stained cells hybridizing with methanotroph-specific probes was generally higher for type I than type II. Our data show that the methanogenic archaea and aerobic methanotrophs can be numerically dominant components of the hyporheic biofilm community and affect CH_4 cycling in river sediments.

IV. Identification of methanogenic archaea involved in a methane stream cycle by targeting methylcoenzyme M reductase (*mcrA)* gene

Iva Buriánková, Lenka Brablcová, Václav Mach, Petr Dvořák, Martin Rulík

(*PloS One* 8: E80804)

Methanogenic archaea produce methane as a metabolic product under anoxic conditions and they play a crucial role in the global methane cycle. In this study molecular diversity of methanogenic archaea in the hyporheic sediment of the lowland stream Sitka (Olomouc, Czech Republic) was analyzed by PCR amplification, cloning and sequencing analysis of the methyl coenzyme M reductase alpha subunit (*mcrA*) gene. Sequencing analysis of 60 clones revealed 24 different *mcrA* phylotypes from hyporheic sedimentary layers to a depth of 50 cm. Phylotypes were affiliated with *Methanomicrobiales, Methanosarcinales* and *Methanobacteriales* orders. Only one phylotype remains unclassified. The majority of the phylotypes showed higher affiliation with uncultured methanogens than with known methanogenic species. The presence of relatively rich assemblage of methanogenic archaea confirmed that methanogens may be an important component of hyporheic microbial communities and may affect CH_4 cycling in rivers.

V. Methanogenic pathways in sediment of river Sitka

Václav Mach, Martin Bruno Blaser, Peter Claus, Prem Prashant Chaudhary, Martin Rulík

(*Front. Microbiol.* 6:506. doi: 10.3389/fmicb.2015.00506)

Biological methanogenesis is linked to permanent water logged systems, e.g., rice field soils or lake sediments. In these systems the methanogenic community as well as the pathway of methane formation are well-described. By contrast, the methanogenic potential of river sediments is so far not well-investigated. Therefore, we analyzed (a) the methanogenic potential (incubation experiments), (b) the pathway of methane production (stable carbon isotopes and inhibitor studies), and (c) the methanogenic community composition (terminal restriction length polymorphism of *mcrA*) in depth profiles of sediment cores of River Sitka, Czech Republic. We found two depth-related distinct maxima for the methanogenic potentials (a) The pathway of methane production was dominated by hydrogenotrophic methanogenesis (b) The methanogenic community composition was similar in all depth layers (c) The main TRFs were representative for *Methanosarcina, Methanosaeta, Methanobacterium*, and *Methanomicrobium* species. The isotopic signals of acetate indicated a relative high contribution of chemolithotrophic acetogenesis to the acetate pool.

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PRODUKCE SKLENÍKOVÝCH PLYNŮ (CO_2, CH_4, N_2O) Z TEKOUCÍCH VOD

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DISERTAČNÍ PRÁCE

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Prohlášení

Prohlašuji, že jsem diplomovou práci vypracoval samostatně pod vedením doc. RNDr. Martina Rulíka, Ph.D. a jen s použitím citovaných literárních pramenů.

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podpis

ABSTRAKT

Skleníkovým plynům a souvisejícím biogeochemickým koloběhům uhlíku a dusíku je v posledních dvou desetiletích věnována zvýšená pozornost v důsledku globálních změn klimatu. Cílem disertační práce bylo v několika vědeckých studiích kvantifikovat a charakterizovat produkci skleníkových plynů, především metanu, v hyporheických sedimentech malého vodního toku Sitka. Studie, které se zakládají na terénních měřeních, inkubačních experimentech a analýze stabilních izotopů, jsou součástí širšího výzkumu, který se zabývá koloběhem uhlíku v tekoucích vodách. Data z pěti studií obsažených v disertační práci dokládají, že vodní toky uvolňují do atmosféry významné množství metanu, oxidu uhličitého a oxidu dusného. Dále uvedené studie dokládají, že v hyporheických sedimentech dochází k mikrobiálním procesům metanogeneze a metanotrofie a současně k výskytu souvisejících taxonů archeí a bakterií. Závěrečná studie prokazuje, že podíl acetoklastické a hydrogenotrofní produkce metanu v hyporheických sedimentech je v rozsahu typickém pro sladkovodní prostředí. Disertační práce přispěla k vědomostem o anaerobním metabolismu v hyporheických sedimentech a o jejich roli v biogechemických koloběžích uhlíku a dusíku.

Klíčová slova: skleníkové plyny, metan, oxid uhličitý, oxid dusný, hyporheický sediment, tekoucí vody, metanogeneze

ABSTRACT

In terms of the global climate change, a raised awareness is devoted to greenhouse gas emissions and to the contextual biogeochemical cycles of carbon and nitrogen in the last two decades. The aim of the thesis is to quantify and characterize, in several research papers, greenhouse gas production, especially of methane, in hyporheic sediments of the small water stream Sitka. The research papers based on field measurements, incubation experiments and a stable isotope analysis are a part of a long-term research concerned with the organic carbon cycle in running waters. Data in the five research papers included in the thesis prove that water streams release a significant amount of methane, carbon dioxide, and nitrous oxide into the atmosphere. Furthermore the research papers demonstrate that microbial processes of methanogenesis and methanotrophy occur in conjunction with presence of related taxonomic groups of archaea and bacteria. The last research paper approves that partitions of acetoclastic and hydrogenotrophic pathways of methane production in hyporheic sediments are in a range typical for freshwater environments. The thesis contribute to the knowledge about an anaerobic metabolism in hyporheic sediments, and about their role in biogeochemical cycles of carbon and nitrogen.

Key words: greenhouse gases, methane, carbon dioxide, nitrous oxide, hyporheic sediments, running waters, methanogenesis

OBSAH

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OBECNÝ ÚVOD

Skleníkové plyny

Skleníkové plyny jsou plyny, které absorbují a uvolňují záření v rozsahu tepelných infračervených vlnových délek. Tato vlastnost některých plynů je příčinou skleníkového efektu, jehož antropogenní zesilování se zásadní měrou podílí na současné klimatické změně. Kromě vodních par jsou třemi nejvýznamnějšími skleníkovými plyny oxid uhličitý, metan a oxid dusný a růst jejich atmosferických koncentrací je považovaný za klíčový spouštěč globální antropogenní klimatické změny (IPCC 2013).

Přestože je oxid uhličitý pouze stopovým plynem, má jeho obsah v atmosféře zásadní biologický význam pro primární produkci všech ekosystémů (Melillo et al. 1993). Nárůst koncentrací oxidu uhličitého v atmosféře má rozhodující vliv na změnu klimatu a činí z něj jednu z nejsledovanějších komponent atmosféry (IPCC 2013).

Metan je nejhojnější organickou sloučeninou v atmosféře. Jeho emise a atmosférické koncentrace jsou monitorovány z několika důvodů. Především je významným a ve srovnání s oxidem uhličitým relativně účinnějším skleníkovým plynem (Lashof et Ahuja 1990). Zadruhé je nejhojnějším reaktivním stopovým plynem v troposféře a ovlivňuje chemismus troposféry ale také stratosféry. Oxidace metanu hydroxylovými ionty v troposféře vede k tvorbě formaldehydu, oxidu uhelnatého a s dostatkem oxidů dusíku k tvorbě ozónu (Thomposon et al. 1990). Metan ve stratosféře hraje důležitou roli v přeměně reaktivního chloru na méně reaktivní HCl (Burnett et Burnett 1995).

Atmosférické koncentrace a emise oxidu dusného se sledují především z důvodu, že se jedná o relativně efektivní skleníkový plyn. Oxid dusný se významně podílí na úbytku stratosférické vrstvy ozonu (Forster et al. 2007). Ravishankara et al. (2009) tvrdí, že oxid dusný je nejvýznamnější emitovanou sloučeninou podílející se na úbytku ozonu, a předpokládají, že jí po dobu 21. století zůstane.

Celkový podíl skleníkových plynů na změnách klimatu ovlivňuje především nárůst jejich atmosférické koncentrace, která od preindustriálního období u všech tří uvedených skleníkových plynů významně stoupla.

V roce 2011 byla průměrná globální atmosférická koncentrace oxidu uhličitého 391 ppm, což je nárůst o 41 % oproti preindustriálnímu období, za který je považován rok 1750 (IPCC 2013). V preindustriálním období byla globální atmosférická

koncentrace oxidu uhličitého podle měření vzduchu z ledových vrtů 278 ppm (Etheridge et al. 1996). Průměrná roční míra růstu atmosférické koncentrace oxidu uhličitého v období od roku 1960 až 2005 byla 1,4 ppm (Forster et al. 2007). Hlavními charakteristikami průběhu atmosférické koncentrace oxidu uhličitého jsou její dlouhodobý nárůst a sezónní cykly v důsledku fotosyntézy a respirace terestrické biosféry na severní polokouli. Hlavními přispěvateli k dlouhodobému nárůstu atmosferických koncentrací oxidu uhličitého jsou spalování fosilních paliv a změny využití území (Tans 2009).

V roce 2011 byla průměrná globální koncentrace metanu 1803 ppb (IPCC 2013), což je výrazný nárůst oproti preindustriálnímu období, kdy byla jeho průměrná globální koncentrace pouze 722 ppb (Eltridge et al. 1998, Dlugokencky et al. 2005), ale předpokládá se, že lidský vliv na globální koloběh metanu začal o tisíce let dříve než v době označované jako preindustriální (Ruddiman 2003, Ferretti et al. 2005, Ruddiman 2007). Vývoj průměrných globálních koncentrací atmosférického metanu má proměnlivý charakter. Zatímco od začátku osmdesátých let do roku 1998 docházelo ke snižování ročního přírůstku metanu, od roku 1999 do roku 2006 byla průměrná globální atmosférická koncentrace metanu stabilizovaná, ale od roku 2007 do roku 2011 obsah metanu v atmosféře opět roste (Rigby et al. 2008, Dlugokencky et al. 2009). Zatím nebylo dosaženo vědeckého konsensu na příčinách výkyvů míry ročního přírůstku metanu do atmosféry. Dlugokencky et al. (2003) tvrdí, že globální emise jsou konstantní a pokles v roční míře přírůstku byl způsoben vyššími ztrátami hydroxidovými radikály (OH), které obecně způsobují největší ztráty metanu v atmosféře. Ze sledování ^{13}C metanu v atmosféře vyplývá, že by mohlo docházet k poklesu míry ročního přírůstku metanu vlivem poklesu emisí biogenního metanu na severní polokouli (Kai et al. 2011). Další vysvětlení tvrdí, že pokles míry ročního přírůstku byl způsoben snížením emisí z těžby a distribuce zemního plynu, protože docházelo k simultánnímu poklesu atmosférické koncentrace etanu, jehož klíčovým zdrojem jsou emise zemního plynu (Aydin et al. 2011, Simpson et al. 2012). Nejpravděpodobnější příčinou opětovného růstu atmosférické koncentrace metanu byly anomálně vysoké teploty v Arktidě v roce 2007 a nadprůměrné srážky během let 2007 a 2008 (Dlugokencky et al. 2009, Bousquet 2011). Pokud zvážíme nejistoty v příčinách míry ročního přírůstku atmosférické koncentrace metanu a nové potenciálně významné zdroje metanu, jako jsou těžba nekonvenčních fosilních paliv (Howarth et al. 2011, Tollefson 2013) a klatráty metanu v permafrostu (Kvenolden et

al. 1993, Dallimore et Colett 1995) a na dně moří (Buffett et Archer 2004), jsou předpovědi budoucích změn atmosferických koncentrací metanu značně nejisté.

V roce 2011 dosáhla průměrná globální atmosférická koncentrace oxidu dusného 324 ppb (IPCC 2013) a od roku 1750, kdy byla 270 ppb, stoupla o 20 % (Prather et al. 2012). Měření izotopického složení oxidu dusného v ledovcových vrtech naznačuje, že vzrůst atmosférické koncentrace tohoto plynu od 50. let 20. století je především v důsledku používání syntetických dusičnanových hnojiv (Rockmann et Levin 2005, Ishijima et al. 2007, Davidson 2009, Syakila et Kroeze 2011). Od začátku systematického měření oxidu dusného v atmosféře v 70. letech 20. století roste jeho koncentrace v průměru o 0,75 ppb za rok (IPCC 2013). Průměrná atmosférická koncentrace oxidu dusného vykazuje vedle dlouhodobého růstu také sezónní variabilitu. Tato variabilita není způsobena cykly v emisích, ale výměnou vzduchu mezi troposférou a stratosférou, kde je oxid dusný rozkládán fotochemicky (Jiang et al. 2007).

Schopnost různých skleníkových plynů přispět ke klimatické změně ovlivňuje vedle atmosferických koncentrací také jejich efektivita v oteplování troposféry. Jako metrické nástroje převedení efektu různých skleníkových plynů na společnou stupnici slouží potenciály globálního oteplení (*global worming potentials*, zkráceně GWP), které kromě relativní efektivity různých molekul absorbovat infračervené záření započítávají také dobu setrvání plynu v atmosféře a zpětné vazby emisí plynu na chemismus atmosféry. Potenciál globálního oteplení je definován jako v daném čase vypočtené radiační působení určitého skleníkového plynu v poměru ku radiačnímu působení stejné hmotnosti oxidu uhličitého (Houghton et al. 1990). Efekt stejné hmotnostní jednotky metanu i oxidu dusného na oteplení troposféry je výrazně vyšší než u oxidu uhličitého. Pro Pátou hodnotící zprávu Mezivládního panelu pro klimatické změny přehodnotili Myhre et al. (2013) potenciály globálního oteplení metanu. Nově navržené potenciály metanu vzrostly ze 72 na 86 ve dvacetileté perspektivě a z 25 na 34 ve stoleté perspektivě, tudíž je metan považován za ještě významnější skleníkový plyn než dříve. Radiační působení oxidu dusného je relativně ještě silnější - potenciály globálního oteplení pro oxid dusný jsou 268 ve dvacetileté perspektivě a 298 ve stoleté perspektivě (Myhre et al. 2013).

Produkce biogenních skleníkových plynů

Oxid uhličitý, metan a oxid dusný jsou součástí biogeochemických cyklů uhlíku a dusíku, tudíž kolují mezi rezervoáry, ze kterých jsou odstraňovány ztrátami a doplňovány do nich zdroji. Významným rezervoárem všech tří sloučenin je atmosféra. Ztráty a zdroje skleníkových plynů pro atmosféru se mohou rozdělovat na přirozené a antropogenní. Rovnováha přirozených koloběhů prvků mezi rezervoáry je zpravidla antropogenními vlivy narušována. Příkladem je právě zvyšování koncentrace skleníkových plynů v atmosféře. Pro kvantifikaci přirozených a antropogenních zdrojů se využívají metody, které lze rozdělit na *botom-up* a *top-down*. *Top-down* metody většinou sledují změny koncentrace nebo izotopového složení sloučenin prvku v rezervoáru (v čase či prostoru), z čehož se následně vyvozuje příspěvek jednotlivých zdrojů do rezervoáru. *Botom-up* metody přímo měří a inventarizují vydatnost jednotlivých zdrojů přecházejících do rezervoáru. Kombinací obou přístupů lze získat lepší představu o přirozených koloběžích skleníkových plynů a na ně působících antropogenních vlivů. Důležitou společnou vlastností všech tří plynů je, že mohou vznikat mikrobiálními procesy, které patří mezi jejich významné přirozené i antropogenní zdroje.

Oxid uhličitý je z atmosféry odstraňován několika způsoby. Zaprvé je odčerpáván rostlinami fotosyntézou, což ročně představuje 123 ± 8 Pg uhlíku (Beer et al. 2010). Uhlík se tak dostává do biosféry a dále jako odumřelá biomasa do půd. Dále atmosférický oxid uhličitý přechází do povrchové vrstvy oceánu plynovou výměnou, která je poháněna rozdílem parciálního tlaku oxidu uhličitého mezi vzduchem a mořskou vodou. Uhlík je v oceánu transportován třemi mechanismy: rozpouštěním, „biologickou pumpou“ a „karbonátovou pumpou“. Malá část rozpuštěného uhlíku (0,2 Pg C ročně) se pak těmito procesy ukládá v sedimentech na dně moře (Denman et al. 2007). Několika jinými způsoby se oxid uhličitý naopak do atmosféry uvolňuje. Zaprvé je to autotrofní (rostlinná) a heterotrofní (živočišná a mikrobiální) respirace. Mikrobiální respirace zahrnuje rozklad organické hmoty. Dále je uvolňován při disturbancích, jako jsou sporadické požáry v přírodě. Přirozené uvolňování oxidu uhličitého při těchto procesech je dlouhodobě vyrovnané s jeho odčerpáváním při fotosyntéze. Oxid uhličitý se přirozeně uvolňuje také při sopečné činnosti. Tento zdroj je z dlouhodobého hlediska kompenzován sedimentací. V období před průmyslovou revolucí byla atmosférická koncentrace oxidu uhličitého ve

vyrovnaném stavu, jako důkaz malých odchylek atmosférické koncentrace atmosférického oxidu uhličitého je jeho záznam v ledu (Pongratz et al. 2009).

K přirozeným zdrojům oxidu uhličitého přibyly v holocénu zdroje antropogenní, kterými jsou spalování fosilních paliv a výroba cementu, těmi se do atmosféry ročně uvolní 7,8 ± 0,6 Pg uhlíku, a změny využití území (např. deforestace), těmi se ročně vyprodukuje dalších 1,1 ± 0,8 Pg uhlíku (Ciais et al. 2013). Z toho vyplývá, že pouze malý zlomek oxidu uhličitého uvolněného ročně do atmosféry je antropogenního původu, ale přesto je tato relativně malá část klíčová v narušení rovnováhy přirozeného koloběhu uhlíku. Významné množství uhlíku z terestrických ekosystémů (1,7 Pg uhlíku ročně) je transportováno z půd do říčního systému. Část tohoto uhlíku je z řek a jezer uvolněna do atmosféry v podobě oxidu uhličitého, část je uložena ve sladkovodních sedimentech a zbytek (0,9 Pg uhlíku ročně) je dopraven do moře (Tranvik et al. 2009).

Podle vzniku lze zdroje metanu do atmosféry rozdělit na tři kategorie: biogenní, termogenní (fosilní) a pyrogenní. Každá kategorie těchto zdrojů produkuje metan s odlišným izotopovým složením uhlíku (^{13}C) (White et al. 2007) a může být dále rozdělena na antropogenní a přirozené zdroje. Antropogenní zdroje metanu zaujímají zhruba 50-65 % (Ciais et al. 2013). Atmosférický metan je odstraňován především fotochemicky reakcí s OH radikály. Další, menší ztráty metanu z atmosféry probíhají ve stratosféře během reakce s chlorem a kyslíkovými radikály, oxidací v dobře prokysličených půdách a pravděpodobně také reakcí chloru v mořské hraniční vrstvě (Allan et al. 2007).

Mezi termogenní zdroje metanu patří jak přirozené emise fosilního metanu z geologických zdrojů (mořské a terestrické průsaky, geotermální průduchy, bahenní sopky), tak antropogenní emise způsobené úniky z těžby a používání fosilních paliv (zemní plyn, uhlí a ropný průmysl). Pyrogenní zdroje jsou výsledkem neúplného spalování fosilních paliv nebo biomasy (přirozené a antropogenní). Biogenní zdroje zahrnují přírodní emise především z přirozených mokřadů, termitišť a oceánů (Ciais et al. 2013). Předpokládá se, že hlavním přírodním zdrojem metanu jsou přirozené mokřady (Pulliam 1993, Whalen 2005), přesto jsou metanem přesyceny mnohé další povrchové vody. Dobře známými a významnými zdroji biogenního metanu jsou jezera (Bastviken et al. 2004), vodní nádrže (DelSonotro et al. 2010) a ústí velkých řek (Middelburg et al. 2002). Mezi antropogenní zdroje biogenních emisí patří rýžoviště, chov skotu, skládky odpadu, lidmi vytvořená jezera a mokřady.

Obecně je biogenní metan produkován z organického materiálu fermentačním procesem metanogenních mikroorganismů (Conrad 1996). Biogenní emise metanu tvoří při komparaci *top-down* a *botom-up* odhadů asi 65-79 % celkových emisí do atmosféry (Wuebbles et Hayhoe 2002, Mikaloff-Fletcher et al. 2004, Wang et al. 2004, Chen et Prinn 2006). Zatím byla pouze malá pozornost věnována řekám a vodním tokům (Jones et al. 1995, Hlaváčová et al. 2005, Sanders et al. 2007, Wilcock et Sorrell 2007, Trimmer 2009, příloha I. a II.). Ačkoliv jsou většinou koncentrace metanu v tekoucích vodách nižší než ve vodách stojatých, intenzivnější turbulentní proudění způsobuje intenzivnější plynovou výměnu, takže při nižší koncentraci metanu mohou být emise z tekoucích vod srovnatelné s emisemi z vod stojatých.

K nárůstu atmosférické koncentrace oxidu dusného dochází kvůli antropogennímu narušení koloběhu dusíku. V preindustriálním období probíhala tvorba reaktivního dusíku (všechny formy dusíku kromě N_2) z plynného dusíku přirozeně skrze dva procesy: blesky a biologickou fixaci dusíku. Tento vstup reaktivního dusíku do biosféry byl vyrovnáván ztrátami reaktivního dusíku skrze denitrifikaci (Ayres et al. 1994). Tato rovnováha je od začátku průmyslového období narušována tím, že je zvýšena produkce reaktivního dusíku lidskou činností. Existují tři hlavní antropogenní zdroje reaktivního dusíku: 1) Haber-Boschův proces (průmyslová výroba dusíkatých hnojiv); 2) kultivace pícnin a jiných plodin zvyšujících biologickou fixaci dusíku; 3) spalování fosilních paliv, kterým z N_2 vznikají oxidy dusíku (NO_X). Dále také dochází k mobilizaci reaktivního dusíku uloženého v sedimentech (Morford et al. 2011). Množství antropogenně vyprodukovaného reaktivního dusíku převedeného zpět na N_2 denitrifikací je mnohem menší než množství reaktivního dusíku každoročně vyprodukovaného. Celkem je zpět přeměněno asi 30 až 60 % celkové produkce reaktivního dusíku (Galloway et al. 2004, Canfield et al. 2010, Bouwman et al. 2013).

Oxid dusný vzniká přirozeně během mikrobiálních procesů nitrifikace a denitrifikace, které jsou zvýšeným obsahem reaktivního dusíku antropogenně zesilovány (Sloss 1992). Růst emisí oxidu dusného neprobíhá jen na zemědělských půdách, ale také ve vodních ekosystémech, v důsledku splachování z polí, a v ostatních půdách a v oceánech v důsledku atmosférické depozice dusíkatých sloučenin. Kvůli prostorové a časové heterogenitě je velmi obtížné atmosférické emise oxidu dusného odhadnout. V Páté hodnotící zprávě Mezivládního panelu pro

klimatické změny jsou současné celkové přirozené emise oxidu dusného uvolňované do atmosféry odhadnuty na 11,0 Tg N_2O-N ročně a celkové antropogenní emise jsou pak odhadnuty na 6,9 Tg N_2O-N ročně, z nichž na řeky, ústí řek a pobřežní zóny připadá celkem 0,6 Tg N_2O-N ročně (Ciais et al. 2013). Odhady emisí oxidu dusného z vodních ekosystémů jsou ovšem relativně nejisté a zatížené chybami. Emise z řek, ústí řek a kontinentálního šelfu byly předmětem diskusí po řadu let (Seitzinger et Kroeze 1998, De Klein et al. 2007). Nedávné studie potvrzují, že řeky jsou významným zdrojem oxidu dusného, což by mohl být důvod pro další přehodnocení současných odhadů emisí oxidu dusného z vodních ekosystémů (Beaulieu et al. 2011, Rosamond et al. 2012, příloha I.).

Kvůli obavám z globální klimatické změny navrhují mnohé studie možné způsoby a strategie snižování emisí skleníkových plynů z antropogenních zdrojů. Na druhou stranu některé přirozené zdroje těchto plynů jsou opomíjeny (Frankignoulle et Middelburg 2002), přestože je inventarizaci zdrojů skleníkových plynů věnováno značné úsilí. Koncentrace oxidu uhličitého, metanu a oxidu dusného je ve vodních tocích několikanásobně vyšší než rovnovážná s atmosférickou koncentrací (Cole et Caraco 2001, Hlaváčová et al. 2006, Striegl et al. 2012, Yang et al. 2012), což naznačuje, že vodní toky mohou být významným zdrojem těchto plynů. Ačkoliv panuje všeobecná potřeba kvantifikace potenciálních zdrojů skleníkových plynů, nejsou obvykle vodní toky mezi zdroje metanu přímo započítány jako v případě oxidu dusného. Přesto již existuje např. mnoho dokladů, že vodní toky uvolňují také významné množství metanu (Sanders et al. 2007, Wilcock et Sorrell 2008).

Hyporheické sedimenty

Hyporheická zóna je oblast říčního dna vyplněná porézními sedimenty, které jsou saturované říční a podzemní vodou. Hyporheické sedimenty mají zásadní úlohu v procesu samočištění vody a jsou metabolickým centrem vodního toku zodpovědným za zadržování, ukládání a mineralizaci organického materiálu, který je transportován povrchovou vodou (Grim et Fisher 1984). Fyzikální a chemické parametry v hyporheickém sedimentu se vyznačují prostorovou variabilitou.

V prostředí sedimentů jsou téměř všechny bakterie a archea přisedlé na sedimentární částice (Costerton et Lappin-Scott 1995) ve vysoce strukturovaných shromážděních - assemblage. Assemblage se skládají z mikrokolonií jednotlivých druhů bakterií nebo archeí v těsném prostorovém uspořádání s mikrokoloniemi jiných druhů. Jsou

obklopeny komplexní polysacharidovou matrix, která působí jako vysoce selektivní síto, jež limituje průnik plynů, rozpuštěných solí a makromolekul transportovaných směrem k nebo od mikrokolonií (Paerl et Pinckney 1996). Takové prostorové a funkční uspořádání mikroorganismů se nazývá hyporheický biofilm. Díky biofilmu mohou v aerobním prostředí vedle sebe probíhat aerobní a anaerobní procesy, které se odehrávají v jeho různých vrstvách. Kuhl et Jorgensen (1992) ukázali, že se v biofilmu může odehrávat celý cyklus síry a dusíku, takže měření transformace nutrientů ve větším měřítku nemusí odhalit skutečnou diverzitu mikrobiálních procesů odehrávajících se v tomto prostředí. Protože polysacharidová matrix udržuje propojení mezi jednotlivými mikrokoloniemi různých bakteriálních a archeálních typů, mohou vznikat místa specifických limitovaných nik. Mikroorganismy požadující specifické substráty, jako jsou např. metanogeny s požadavkem na vodík, jsou schopny růst díky těsnému prostorovému nastavení s ostatními typy bakterií a archeí, které produkují tyto substráty (Korber et al. 1995).

Metanogeneze

Biogenní metan hraje důležitou úlohu při rozkladu organických látek v sedimentech a půdách, protože je finálním produktem anaerobní dekompozice organického materiálu. V anaerobních podmínkách je organický materiál nejprve rozložen fermentací na jednoduché látky (oxid uhličitý, kyseliny a alkoholy); poslední krok anaerobního rozkladu pak probíhá, až pokud jsou spotřebovány alternativní anorganické elektronové akceptory (zejména dusičnany, železité ionty a sírany) a je nazýván metanogeneze. Tento mikrobiální proces je umožněn funkční skupinou zvaných metanogeny (metanogenní archea), které fylogeneticky náleží do několika taxonů v doméně *Archea* (Chaban et al. 2006). Metanogeny mohou využívat pouze omezené množství substrátů, mezi které patří acetát, oxid uhličitý s vodíkem a metylované sloučeniny (metanol, metylamin, dimetylsulfid). S výjimkou mořských sedimentů a mikrobiálních povlaků slaných pánví se v přirozených ekosystémech vyskytuje prakticky pouze redukce oxidu uhličitého vodíkem (tzv. hydrogenotrofní metanogeneze) a rozklad acetátu (tzv. acetoklastická metanogeneze) (Davidson et Schimel 1995, Conrad 2007). Pro většinu ekosystémů proto postačuje počítat pouze s následujícími reakcemi jako způsoby vzniku metanu (Games et al. 1978, Sugimoto et Wada 1995):

$$CH_3COOH \rightarrow CO_2 + CH_4 \quad (1)$$

$CO_2 + 4\ H_2 \rightarrow 2\ H_2O + CH_4$ (2)

Ve většině metanogenních prostředí probíhají tyto produkční reakce metanogeneze současně a liší se svým podílem na celkové produkci metanu. Stechiometricky připadá z úplné anoxické degradace polysacharidů 67 % na acetoklastickou metanogenezi a zbylých 33 % na hydrogenotrofní metanogenezi (Conrad 1999). Tento stechiometrický poměr ovšem platí, pouze pokud se v sedimentu nevyskytují další konkurenční metabolické reakce, jako jsou např. homoacetogeneze (Schultz et Conrad 1996) nebo syntrofní oxidace acetátu (Nüsslein et al. 2003). Navíc meziprodukty fermentačních procesů mohou být ukládány či transportovány, případně může organická hmota obsahovat kromě polysacharidů také další organické sloučeniny. Proto se v různých prostředích podíl hydrogenotrofní a acetoklastické metanogeneze od teoretického výpočtu rozkladu polysacharidů významně liší (Conrad 2005). Navíc se podíl jednotlivých metanogenních reakcí na celkové produkci metanu může lišit v závislosti na vnějších podmínkách. Conrad et al. (2009) např. zaznamenali posun od smíšené acetoklastické a hydrogentrofní metanogeneze k výlučně hydrogentrofní metanogenezi při zvýšení teploty inkubovaného sedimentu nad 42 °C.

Podíl jednotlivých metanogenních reakcí na celkové produkci metanu je ovlivněn také složením mikrobiálního společenstva metanogenních archeí, protože různé taxony metanogenů utilizují různé substráty. Taxony metanogenních archeí jsou detekovány celou řadou molekulárních metod (Chaudhary et al. 2013, příloha III. a IV.). Mezi hydrogenotrofní metanogeny patří naprostá většina zástupců metanogenů (např. rod *Methanobacterium*). Některé hydrogentrofní metanogeny jsou schopné utilizovat také formiát. Acetát je konzumován zástupci dvou rodů archeí, kam patří *Methanosarcina* a *Methanosaeta*, přičemž první zmíněný rod může konzumovat současně také oxid uhličitý s vodíkem (Miyamoto 1997).

Pro kvantifikaci substrátové specifity metanogeneze bylo používáno několik metod: sledování stabilních izotopů substrátů a produktů, sledování radioaktivních izotopů produktů při inkorporaci značeného substrátu a sledování množství produktu při částečné inhibici metanogeneze (metylfluoridem) nebo sledování množství naakumulovaného substrátu při úplné inhibici metanogeneze (bromo-etan sulfonátem). Další možností je kombinace těchto metod. Zvláště výhodné je sledování stabilních izotopů substrátů a produktů metanogeneze v kombinaci s částečnou inhibicí metanogeneze metylfluoridem (Conrad 2005, Conrad et al. 2011,

příloha V.). Sledování stabilních izotopů uhlíku navíc poskytuje komplexnější pohled na koloběh uhlíkový než přístupy zaměřené pouze na uhlík nebo jeho radioizotopy. Analýza stabilních izotopů metanu navíc nepomáhá rozlišovat pouze různé mikrobiální zdroje metanu, ale celkem snadno lze díky ní rozlišit také termogenní a pyrogenní zdroje metanu. Proto může tato elegantní metoda v kombinaci s *top-down* metodami pomoci s kvantifikací jednotlivých zdrojů metanu v celkové atmosférické bilanci.

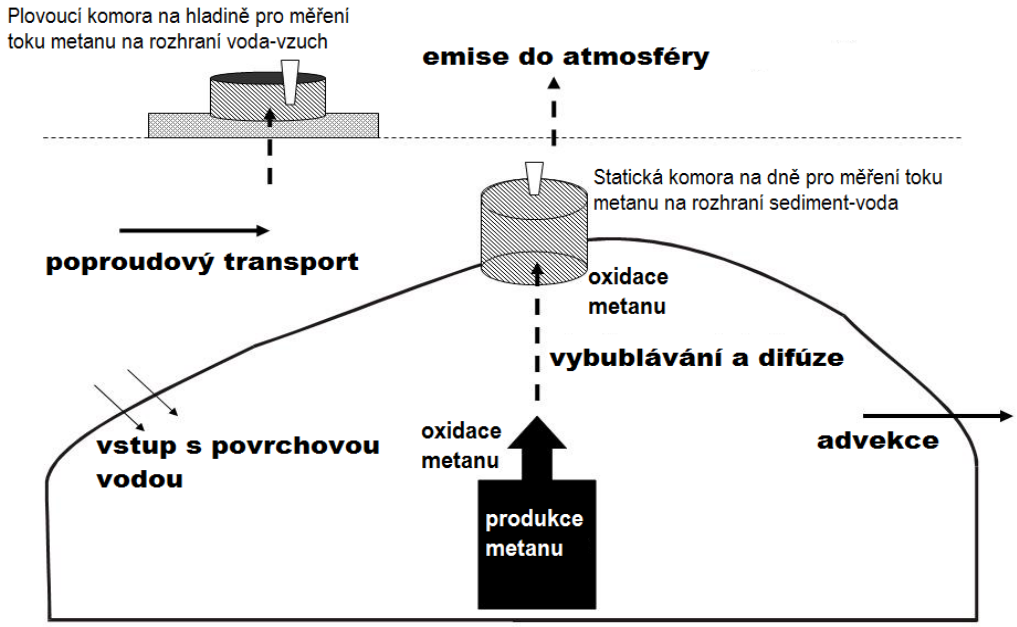
Pro vyjádření izotopového složení uhlíku ve sledovaném vzorku se používá zápis $\delta^{13}C$, který vyjadřuje odchylku v izotopovém složení aktuálního vzorku od obecně uznávané standarty. Vypočítá se podle rovnice (4). Změnu $\delta^{13}C$ během chemické reakce vyjadřují frakcionační faktory reakcí, které se počítají podle rovnice (5). Izotopy uhlíku z metanogenního substrátu (acetátu nebo oxidu uhličitého) frakcionují během obou hlavních metanogenních reakcí jinou měrou. Acetoklastická metanogeneze vykazuje menší kinetický izotopický efekt než hydrogenotrofní produkce metanu, tudíž během acetoklastické metanogeneze dochází k menší diskriminaci těžkých izotopů uhlíku (^{13}C), naopak při hydrogentrofní metanogenezi jsou těžké izotopy uhlíku (^{13}C) diskriminovány více a dochází k většímu upřednostňování lehkých izotopů uhlíku (^{12}C). Z toho plyne, že frakcionační faktor acetoklastické metanogeneze (α = 1.009 až 1.027) je nižší než frakcionační faktor hydrogenotrofní metanogeneze (α = 1.045 až 1.073). Přestože $\delta^{13}C$ produkovaného metanu závisí také na izotopickém složení vstupujících substrátů, je $\delta^{13}C$ metanu produkovaného z acetátu (-27 ‰ až -60 ‰) obvykle vyšší než $\delta^{13}C$ metanu produkovaného z oxidu uhličitého (-60 ‰ až -77 ‰) (Conrad 2005). Z toho vyplývá, že pro přesnou kvantifikaci relativního příspěvku jednotlivých metanogenních reakcí je potřeba znát $\delta^{13}C$ metanu smíšeného z obou reakcí a k tomu buď $\delta^{13}C$ jeho prekurzorů (acetát a oxid uhličitý) a frakcionační faktory obou metanogenních reakcí, nebo $\delta^{13}C$ metanu produkovaného výhradně z jednotlivých produkčních reakcí. Frakcionační faktory obou metanogenních reakcí se ale mohou významně lišit v různých metanogenních ekosystémech a jsou ovlivněny mnoha faktory (např. složení mikrobiálního společenstva, teplota, koncentrace acetátu nebo vodíku), proto většinou nedostačuje pouhé měření izotopového složení prekurzorů metanu (Conrad 2005).

Acetoklastickou metanogenezi lze selektivně inhibovat metyl-fluoridem (Janssen et Frenzel 1997), ale inhibice je úplně selektivní pouze při specifické koncentraci, která

je proměnlivá v závislosti na konkrétním sedimentu. Se vzrůstající koncentrací metyl-fluoridu narůstá inhibice acetoklastické metanogeneze, ale při příliš vysoké koncentraci je ovlivněna také hydrogentrofní metanogeneze (Conrad et Klose 1999). Ovšem pokud se přidá metyl-fluorid v přebytku, není ovlivněno izotopické složení metanu hydrogentrofní metanogeneze, proto lze inhibicí acetoklastické metanogeneze měřit přesně alespoň $\delta^{13}C$ metanu produkovaného výhradně hydrogentrofní metanogenezí. Izotopické složení metanu produkovaného výhradně acetoklastickou metanogenezí lze dopočítat ze změřeného $\delta^{13}C$ acetátu použitím frakcionačních faktorů z literatury. Pak je možné, pokud známe $\delta^{13}C$ směsného metanu, spočíst příspěvek jednotlivých metanogenních reakcí k celkové produkci metanu (Conrad et al. 2011).

Metan produkovaný ve sladkovodním prostředí je často obohacen o těžší izotop uhlíku (^{13}C) v poměru k metanu z mořských sedimentů. To je díky relativně většímu významu acetoklastické metanogeneze ve sladkovodních prostředích oproti mořským sedimentům, kde převládá hydrogentrofní metanogeneze (Blair et al. 1993). Přestože podíl metanogenních reakcí na celkové produkci metanu byl již kvantifikován v mnoha různých prostředích, jako jsou tropická jezera (Conrad et al. 2011), rýžová pole (Sugimoto et Wada 1993, Fey et al. 2003, Penning et Conrad 2007), kořeny rýže (Chin et al. 2004), boreální rašeliniště (Galand et al. 2005), mořské sedimenty (Crill et Martens 1986), mokřady mírného pásu (Hornibrook et al. 1997) či anaerobní reaktory (Laukenmann et al. 2010), o podílu metanogenních reakcí v říčních ekosystémech máme dosud stále málo informací.

Lepší představu již máme o koloběhu metanu v říčním ekosystému. Ten je znázorněn na obrázku 1. Metan je produkován v hyporheické zóně, ze které přechází difúzí nebo v podobě bublinek do povrchové vody, odkud je uvolňován dále do atmosféry. Na úbytku metanu v hyporheické zóně se podílí metanotrofie, která je zprostředkována funkční skupinou metanotrofních bakterií. Tento mikrobiální proces převládá ve více prokysličených částech sedimentu a v některých vodních ekosystémech může zoxidovat až veškerý vyprodukovaný metan (Hanson et Hanson 1996).



Obrázek 1. Předpokládaný koloběh metanu v hyporheické zóně a povrchové vodě. Tok metanu na rozhraní voda-vzduch a sediment-voda je měřen plovoucími nebo statickými komorami.

CÍLE A STRUKTURA DISERTAČNÍ PRÁCE

V disertační práci byly sledovány tři hlavní cíle:

- Porovnat a kvantifikovat emise a koncentrace skleníkových plynů (metanu, oxidu uhličitého, oxidu dusného) v hloubkovém a podélném profilu malého vodního toku Sitka během jednoho ročního cyklu (příloha I. a II.).
- Pomocí inkubačních experimentů charakterizovat a porovnat mikrobiální procesy spojené s produkcí a oxidací metanu v hloubkovém profilu hyporheického sedimentu a podél říčního kontinua malého vodního toku Sitka (příloha II., III., IV.).
- Pomocí analýzy $\delta^{13}C$ a inkubačních experimentů se selektivní inhibicí metanogeneze kvantifikovat substrátovou preferenci metanogenů a charakterizovat mikrobiální procesy v koloběhu uhlíku v hloubkovém profilu hyporheického sedimentu říčky Sitka (příloha V.).

Dizertační práce je tvořena publikacemi a manuskriptem v anglickém jazyce, v nichž jsem hlavním autorem (příloha I. a V.) nebo spoluautorem (příloha II., III., IV.). Stručný popis publikací a podíl autora je uveden níže.

I.

První práce se věnuje produkci biogenních skleníkových plynů (metanu, oxidu uhličitého a oxidu dusného) ve vodním toku Sitka. Studie se zaměřuje na koncentrace skleníkových plynů v intersticiální a povrchové vodě a dále na emise těchto plynů do atmosféry a to na pěti lokalitách v podélném profilu vodního toku. Na základě celoročního měření byly odhadnuty roční atmosférické emise sledovaných plynů, které byly pro zhodnocení jejich klimatického dopadu převedeny na metrické ekvivalenty oxidu uhličitého. Studie prokázala, že metan je významným emitentem z vodních toků nejen z hlediska koloběhu uhlíku ale také kvůli jeho klimatickému dopadu.

Václav Mach se v této práci podílel na terénním měření, odběrech a úpravách vzorků. Samostatně pak vyhodnotil data, interpretoval výsledky, napsal manuskript a finalizoval publikaci.

II.

Druhým příspěvkem k disertační práci je kapitola v knize, která shrnuje výsledky dlouhodobé studie biogeochemie metanu v hyporheickém sedimentu říčky Sitka.

Studie bilancuje výsledky získané pomocí širokého spektra různých metod, které byly použity k popisu metanogeneze a koloběhu uhlíku ve vodním toku. Zabývá se emisemi metanu do atmosféry, substrátovou preferencí metanogenů, metanogenním potenciálem i metanotrofní aktivitou. Dále shrnuje poznatky o distribuci metanogenních archeí, jejich fylogenetickou příslušností a o celkových počtech mikroorganismů v souvislosti s metabolickou aktivitou.

Václav Mach se podílel na terénních měřeních emisí, odběrech vzorků, laboratorních měřeních metodou stabilních izotopů uhlíku, inkubačních experimentech a vyhodnocování a interpretaci příslušných dat. Také se podílel na části manuskriptu a jeho korekci.

III.

Třetí studie se zabývá distribucí a mikrobiální aktivitou metanogenních archeí a metanotrofních bakterií v podélném a hloubkovém profilu hyporheického sedimentu říčky Sitka na pěti vybraných lokalitách. Mikrobiální aktivita metanogenů a metanotrofů byla analyzována laboratorními inkubačními experimenty. K detekci a kvantifikaci metanogenních archeí byla použita metoda FISH s cílovou oblastí v 16S DNA. Obě skupiny mikroorganizmů i jejich aktivity byly detekovány na všech studovaných lokalitách i v obou hloubkách. Vyšší denzita mikrobů byla zjištěna ve svrchní vrstvě sedimentu (0-25 cm), což koresponduje s naměřenými hodnotami metabolické aktivity.

Václav Mach se podílel na odběrech a zpracování vzorků, inkubačních experimentech a vyhodnocení dat metanogenního a metanotrofního potenciálu. Dále se podílel na revizi a korekci manuskriptu.

IV.

Čtvrtá publikace se zaměřuje na odhalení fylogenetické struktury metanogenních archeí ve dvou vrstvách (0-25 cm a 25-50 cm) říčního sedimentu pomocí klonování genu pro expresi metyl-koenzymu M reduktázy (*mcrA*). Tato studie odhalila poměrně bohaté společenstvo metanogenních archeí a přinesla tak nové poznatky o fylogenetické struktuře společenstva metanogenů v hyporheických sedimentech vodních toků.

Václav Mach se podílel na odběrech vzorků a inkubačních experimentech metanogenního potenciálu. Dále se podílel na revizi a korekci manuskriptu.

V.

Pátá studie se zabývá kvantifikací substrátové preference metanogenních bakterií v hloubkovém profilu hyporheického sedimentu na Sitce. Při anaerobní inkubaci sedimentů za přítomnosti a nepřítomnosti metyl-flouridu (inhibitoru acetoklastické metanogeneze) bylo pomocí analýzy $\delta^{13}C$ metanu a jeho prekurzorů zjištěno, že hydrogenotrofní metanogeneze se na celkové produkci metanu podílí 40-50 %. Tato studie přinesla zajímavé poznatky o funkční aktivitě mikrobiálního společenstva při anaerobním rozkladu organického materiálu ve vodním toku včetně pravděpodobného příspěvku homoacetogeneze při produkci acetátu.

Václav Mach se podílel na odběru a převozu vzorků do Německa, dále provedl inkubační experimenty a laboratorní analýzy plynovou chromatografií a hmotnostní spektrometrií, vyhodnotil a interpretoval data. Také napsal první verzi manuskriptu a podílel se na korekci a revizi upraveného manuskriptu.

METODIKA

Studovaná lokalita

Výzkum probíhal na říčce Sitka, která je z převážné části neregulovaným vodním tokem druhého řádu. Sitka je dlouhá 35 km, přičemž pramení v Nízkém Jeseníku ve výšce 650 m. n. m. a ústí do říčky Oskavy asi 5 km severně od Olomouce v nadmořské výšce 213 m. n. m. V podélném profilu vodního toku bylo vytyčeno pět lokalit (I-V, číslováno od pramene směrem k ústí), které představovaly pravidelná odběrová místa. Vybrané lokality reprezentují úseky toku podél měnících se podmínek říčního kontinua. Horní část toku až po město Šternberk protéká skrze lesnatou oblast s malou intenzitou antropogenních činností (lokality I-II), zatímco spodní část toku s dobře vyvinutou pobřežní vegetací přirozeně meandruje skrze intenzivně obhospodařovanou zemědělskou krajinu (lokality III-V). Pro odhad ročních emisí skleníkových plynů byla spočtena celková plocha hladiny z ploch jednotlivých úseků Sitky. Plochy jednotlivých úseků toku byly vyhodnoceny z průměrných šířek úseků za použití softwaru ArcGIS s digitalizovanou mapou toku Sitka a s GPS souřadnicemi získanými v terénu.

Odběr a analýzy vzorků

Během výzkumu byly na stanovených lokalitách pravidelně v průběhu roku odebírány vzorky plynné (viz. oddíl níže), vzorky povrchové a intersticiální vody a vzorky hyporheického sedimentu. Povrchová voda byla odebírána přímo z toku do sterilních vialek se šroubovými uzávěry a septy s PTFE vrstvou. Intersticiální voda byla odebírána za použití minipiezometrů (Trulleyová et al. 2003) rozmístěných náhodně v příslušné hloubce sedimentu. Hyporheické sedimenty byly odebírány pomocí metody freeze-core s tekutým dusíkem jako chladícím mediem (Bretschko et Klemens 1986). Namražené sedimenty byly rozděleny podle hloubkových vrstev a pro další účely byla přes síto vyseparována frakce < 1 mm, protože největší podíl organické hmoty a biomasy mikroorganismů se nachází na nejmenších částicích sedimentu (Leichfried 1988), navíc se tím zvýšila homogenita vzorků. Tato frakce byla dále použita jako výchozí materiál pro inkubace.

Koncentrace oxidu uhličitého, metanu a oxidu dusného v plynných a vodních vzorcích byla měřena plynovou chromatografií. Koncentrace rozpuštěných sledovaných plynů v tekoucí vodě byla stanovena pomocí techniky rovnovážné

umělé atmosféry (Kampbell et al. 1989). Metan byl analyzován na plynovém chromatografu CHROM 5 s plamenovým ionizačním detektorem a s kolonou PORAPAK Q s dusíkem jako nosným plynem. Oxid uhličitý a oxid dusný byly analyzovány na plynovém chromatografu Becker Gas model 419 s elektrony zachytávajícím detektorem a s kolonou POROPAK Q s héliem jako nosným plynem. Saturační poměr (R) byl spočten jako aktuální naměřená koncentrace plynu vydělená rovnovážnou koncentrací plynu při naměřené atmosférické teplotě s využitím dat rozpustnosti pro oxid uhličitý (Weiss 1979), metan (Wiesenburg et Guinasso 1979) a oxid dusný (Weiss et Price 1980).

Měření emisí z vodní hladiny

Tok plynů z vodní hladiny do atmosféry lze stanovit několika metodami. První možností jsou modely difusní plynové výměny, které operují s funkcí proudění vody a s rozdílem mezi aktuální koncentrací plynu ve vodě a vyváženou koncentrací plynu za dané atmosférické koncentrace (DeAngelis et Lilley 1987). Vhodnějším přístupem jsou v této práci použitá přímá měření úniku plynů z vodní hladiny do atmosféry pomocí metody uzavřených komor (Crill et al. 1988), protože jsou tím započítány také možné uniky plynu bublajícího přímo ze sedimentu.

Vespodu otevřené plovoucí komory byly umístěny na vodní hladinu pomocí polystyrénového plováku. Komory byly na hladině inkubovány tři hodiny, po jejichž uplynutí byl dle zkušebních testů nárůst koncentrací v *hedaspace* stále lineární. Emise byly spočteny jako rozdíl mezi pozaďovou koncentrací a koncentrací uvnitř komor po uplynutí doby inkubace dle rovnice:

$$F = [(c_I - c_R) * V * (24 / t) * 1000] / p \quad (3)$$

kde F je tok plynu v mg $m^{-2}day^{-1}$; c_I je koncentrace plynu v komoře po uplynutí inkubace v µg l^{-1}; c_R je koncentrace plynu v okolním vzduchu v µg l^{-1}; V je objem komory v litrech; t je čas inkubace v hodinách; p je plocha hladiny, ze které uniká plyn z hladiny do komory v m^2.

Inkubační experimenty pro stanovení metanogeneze a metanotrofie

Inkubační experimenty pro stanovení potenciální produkce metanu a potenciální oxidace metanu v podélném a hloubkovém profilu hyporheických sedimentů toku Sitky sloužily kesrovnání s aktivitou mikrobiálních populací metanogenů a metanotrofů. Míra potenciální produkce metanu (metanogeneze) je měřena pomocí

anaerobních inkubačních experimentů (Segers 1998). Sediment (100g vlhké hmotnosti) byl umístěn do inkubační láhve (250 ml), kam byl přidán acetát (100 mg C na láhev) a destilovaná voda (cca 180 ml). Headspace (20 ml) v inkubační láhvi byl nahrazen N_2. Míra potenciální oxidace metanu (metanotrofní aktivita) byla měřena modifikací metody oxidace metanu v půdních vzorcích (Hanson 1998). Sediment (100 g vlhké hmotnosti) byl aerobně inkubován v láhvi (250 ml), kam byl přidán metan na 10 % objemu headspace při atmosférickém tlaku. Uzavřené láhve byly u obou experimentů inkubovány při 20 °C po dobu tří dnů, přičemž vzorky z *headspace* byly odebírány každých 24 hodin. Potenciální produkce a oxidace metanu byly vypočteny z rozdílů koncentrací metanu během doby inkubace a byly vyjádřeny v nM CH_4 ml^{-1} mokrého sedimentu za hodinu.

Stabilní izotopy a inhibice metanogeneze metyl-flouridem

Pro určení substrátové specifity metanogeneze byla použita metoda srovnání izotopového složení uhlíku v produkovaném metanu a jeho prekurzorech během anaerobní inkubace hyporheického sedimentu s a bez přídavku metyl-flouridu, který specificky inhibuje acetoklastickou metanogenezi. *Headspace* inkubačních zkumavek bylo nahrazeno dusíkem a v případě inhibice bylo doplněno metyl-flouridem na 3 % objemu. Inkubace probíhala při 25 °C po dobu zhruba sedmi týdnů, kdy byly ze zkumavek opakovaně odebírány vzorky plynu pro zjištění koncentrace a $\delta^{13}C$ metanu a oxidu uhličitého. Po skončení inkubace byl sediment ze zkumavek centrifugován a voda byla přefiltrována skrze 0,2 μm PTFE membránový filtr pro analýzu koncentrací a $\delta^{13}C$ acetátu a dalších mastných kyselin.

Izotopické měření $^{13}C/^{12}C$ v plynných vzorcích bylo provedeno hmotnostním spektrometrem s předřazeným spalováním a plynovým chromatografem (Brand 1996). Plynné sloučeniny byly nejprve rozděleny v plynovém chromatografu Hewlett Packard 6890 s PoraPlot Q kolonou s nosným plynem héliem. Vzorek dále prošel skrze spalovací rozhraní Finnigan Standard GC Combustion Interface III a izotopický poměr $^{13}C/^{12}C$ byl analyzován na hmotnostním spektrometu Finnigan MAT Deltaplus. Izotopická analýza acetátu byla provedena metodou hmotnostní spektrometrie s předřazenou kapalinovou chromatografií (Krummen et al. 2004). Roztok sloučenin byl nejprve separován na vysokotlakém kapalinovém chromatografu Spectra System P1000 vybaveném ionty nepropouštějící kolonou Aminex HPX-87-H. Poměr izotopů uhlíku byl změřen na hmotnostním spektrometru

Finnigan MAT Deltaplus Advantage. Izotopický poměr všech analyzovaných sloučenin byl vyjádřen v hodnotě delta oproti standartě VPDB podle rovnice:

$$\delta^{13}C = 10^3 * (R_{vzorek} / R_{standarta} - 1) \quad (4)$$

kde R je $^{13}C/^{12}C$ vzorku a standarty.

Frakcionační faktory reakcí (A □ B) byly spočteny podle rovnice (Hayes 1993):

$$\alpha_{A, B} = (\delta^{13}C_A + 10^3) / (\delta^{13}C_B + 10^3) \quad (5)$$

Podíl hydrogentrofní metanogeneze je vypočten podle rovnice (Conrad 2005):

$$f_{mc} = (\delta_{CH4} - \delta_{ma}) / (\delta_{mc} - \delta_{ma}) \quad (6)$$

kde f_{mc} je podíl hydrogentrofní metanogeneze na celkové produkci metanu a δ_{ma} je izotopický signál uhlíku metanu produkovaný výhradně z acetátu, který je vypočten z rovnice:

$$\delta_{ma} = (1 / \alpha_{ma}) (\delta_{ac} + 10^3 - \alpha_{ma} * 10^3) \quad (7)$$

kde α_{ma} je frakcionační faktor acetoklastické metanogeneze a δ_{ac} je změřený izotopický signál acetátu. Pro odhad hydrogentrofní metanogeneze byly použity hodnoty α_{ma} z dostupné literatury (Gelwicks et al. 1994, Penning et al. 2006, Goevert et Conrad 2009).

SHRNUTÍ VÝSLEDKŮ

V první části práce zaměřené na produkci metanu, oxidu uhličitého a oxidu dusného v hyporheických sedimentech byly naměřeny významné, v porovnání s atmosférou několikanásobně přesycené koncentrace všech sledovaných plynů v intersticiální a povrchové vodě a to v celém podélném profilu toku Sitka. Koncentrace sledovaných plynů v intersticiální vodě několikanásobně převyšovaly koncentrace ve vodě povrchové, což indikuje, že hyporheický sediment je zdrojem těchto plynů pro vodu povrchovou, ze které jsou následně skleníkové plyny uvolněny do atmosféry. Současně byly v celém podélném profilu vodního toku během jedné roční sezóny naměřeny významné atmosférické emise všech tří plynů. Celkové roční emise skleníkových plynů z toku Sitka do atmosféry byly odhadnuty na 637 kg metanu, 210 t oxidu uhličitého a 229 kg oxidu dusného. Pokud převedeme všechny plyny pomocí potenciálů globálního oteplení ve stoletém horizontu na stejné metrické jednotky, přispěje sledovaný vodní tok 300 ekvivalentních tun oxidu uhličitého, z čehož je 7,2 % příspěvek metanu. Emise metanu z vodního toku představují 0,8 % celkové hmotnosti uhlíku unikajícího jako plyn do atmosféry, proto hraje metan důležitou roli v biogeochemickém koloběhu uhlíku a klimatických dopadech vodních toků.

V další části práce, která již byla zaměřena pouze na metan, byly naměřeny pozitivní potenciální produkce metanu a potenciální oxidace metanu hyporheického sedimentu z celého podélného profilu sledovaného vodního toku. Naměřené potenciální produkce metanu i potenciální oxidace metanu vykazovaly vyšší hodnoty v dolní části toku než v horní části toku. Při srovnání aktivity obou mikrobiálních procesů mezi svrchní vrstvou (0-25 cm) a spodní vrstvou (25-5% cm) hyporheického sedimentu vykazovala vyšší hodnoty především vrstva svrchní, která celkově obsahovala větší množství mikroorganismů a mikrobiální aktivity. Hodnocení potenciální produkce metanu a potenciální oxidace metanu sloužilo také ke srovnání mezi aktivitou mikrobiálních procesů v hyporheickém sedimentu a přítomností odpovídajících funkčních skupin bakterií a archeí detekovaných mikrobiálními metodami. Početnosti metanogenních archeí a metanotrofních bakterií v podélném a vertikálním profilu vodního toku ovšem neodpovídaly relativnímu významu příslušných mikrobiálních procesů.

Poslední část práce zužuje pozornost pouze na mikrobiální procesy v hyporheickém sedimentu na lokalitě IV, kde potenciální produkce metanu vykazovala z celého toku řádově nejvyšší hodnoty. Anaerobní inkubační experimenty při detailním hloubkovém rozlišení prokázaly dvě produkční maxima produkce metanu: první v hloubce 0-10 cm a druhá v hloubce 40-50 cm. Produkce metanu se pohybovala ve srovnatelných řádech jako produkce metanu v jiných sladkovodních ekosystémech, jako jsou jezera nebo rýžoviště a přitom hodnoty $\delta^{13}C$ produkovaného metanu odpovídaly hodnotám pro biogenní metan. Na základě změřených $\delta^{13}C$ smíšeného a výhradně hydrogentrofně produkovaného metanu, $\delta^{13}C$ acetátu a frakcionačních faktorů acetoklastické metanogeneze z literatury byl dopočítán podíl hydrogentrofní metanogeneze, který představuje 40-50 % celkové produkce metanu, což je opět v rozsahu typickém pro sladkovodní prostředí. Malý nárůst koncentrací acetátu během inkubačních experimentů poukázal na téměř kompletní spotřebu acetátu během metanogeneze a tudíž byly pro výpočet použity nižší hodnoty frakcionačního faktoru acetoklastické metanogeneze, což výpočet ještě zpřesnilo. Výskyt obou typů metanogeneze v hyporheickém sedimentu toku Sitka koresponduje s nálezy z předchozí části výzkumu, kdy byli nalezeni zástupci hydrogentrofních (*Methanobacterium*) i acetoklastických (*Methanosarcina* a *Methanosaeta*) metanogenů. Z nízkých hodnot $\delta^{13}C$ akumulovaného acetátu, který se nahromadil během anaerobní inkubace za přítomnosti metyl-fluoridu vyplývá, že v hyporheickém sedimentu Sitky probíhá významnou měrou také homoacetogeneze, což je mikrobiální proces produkce acetátu z oxidu uhličitého.

ZÁVĚR

Tato práce přinesla jedny z prvních odhadů kvantifikace celkových ročních atmosferických emisí skleníkových plynů z malého vodního toku. Bylo potvrzeno, že anaerobní mikrobiální procesy hrají významnou roli v koloběhu uhlíku v hyporheických sedimentech a že metan, kterému bylo doposud ve vodních tocích věnováno pouze málo pozornosti, se nezanedbatelnou měrou podílí na klimatickém efektu celkových emisí skleníkových plynů z vodního toku. Také z toho důvodu byl původní záměr, věnovat se v této disertační práci produkci všech tří biogenních skleníkových plynů, posléze zúžen pouze na produkci metanu. Dalším impulsem k této změně byla spoluúčast na výzkumném grantu GAČR „Biogeochemie metanu a detekce metanogenních a metanotrofních bakterií v říčních sedimentech“. Další studie prokázaly, že metanogeneze a metanotrofie jsou významné procesy v sedimentech tekoucích vod, což odpovídá nalezenému bohatému společenstvu metanogenů a metanotrofů. Za použití metody detekce stabilních izotopů a selektivní inhibice metanogeneze během inkubace bylo zjištěno, že na celkové produkci metanu se acetoklastická metanogeneze podílí 50-60 % a hydrogenotrofní metanogeneze 40-50 %. Míra produkce metanu a substrátová preference metanogenů v hyporheickém sedimentu vodního toku se pohybují v řádově podobných hodnotách, jaké byly nalezeny v jiných sladkovodních ekosystémech.

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I. Seasonal measurement of greenhouse gases concentrations and emissions along the longitudinal profile of small stream

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Seasonal measurement of greenhouse gases concentrations and emissions along the longitudinal profile of small stream

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Abstract:

In order to find out whether streams might be the considerable source of greenhouse gases to the atmosphere, our investigation sought to determine the total emissions of CH4, CO2, and N2O from the surface water of a small stream. Over a period of a year we used floating chambers to measure gaseous emissions along the longitudinal profile of the Sitka stream (Czech Republic). Additionally, we measured gas concentrations of surface and interstitial waters. We found that interstitial and surface waters were supersaturated by all monitored gases, especially by CH4 and that the stream is significant emitter of these greenhouse gases. The concentrations and the emission rates of all three gases were higher in the downstream part than in the upstream part of the stream. In the case of CH4 the majority of the total annual emission (90%) was released from the most downstream section of the stream, representing only 1/5 of the stream's total surface area (0.18 km2). The total annual emissions of CH4, CO2, and N2O into the atmosphere from the water's surface were estimated to be 0.6 t, 210 t, and 0.2 t respectively. After conversion of the greenhouse gas emissions to CO2 equivalents used by IPCC, CO2 accounts the most part of total annual emission of greenhouse gases (70.1%), with the second being N2O (22.7%), and the last CH4 (7.2%), for 100-year time horizon. This work brings worthwhile data of greenhouse gas emissions and concentrations from a small water stream based on seasonal measurement along the longitudinal profile.

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Seasonal measurement of greenhouse gases concentrations and emissions along the longitudinal profile of small stream

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Abstract

In order to find out whether streams might be the considerable source of greenhouse gases to the atmosphere, our investigation sought to determine the total emissions of CH_4, CO_2, and N_2O from the surface water of a small stream. Over a period of a year we used floating chambers to measure gaseous emissions along the longitudinal profile of the Sitka stream (Czech Republic). Additionally, we measured gas concentrations of surface and interstitial waters. We found that interstitial and surface waters were supersaturated by all monitored gases, especially by CH_4 and that the stream is significant emitter of these greenhouse gases. The concentrations and the emission rates of all three gases were higher in the downstream part than in the upstream part of the stream. In the case of CH_4 the majority of the total annual emission (90%) was released from the most downstream section of the stream, representing only 1/5 of the stream's total surface area (0.18 km^2). The total annual emissions of CH_4, CO_2, and N_2O into the atmosphere from the water's surface were estimated to be 0.6 t, 210 t, and 0.2 t respectively. After conversion of the greenhouse gas emissions to CO_2 equivalents used by IPCC, CO_2 accounts the most part of total annual emission of greenhouse gases (70.1%), with the second being N_2O (22.7%), and the last CH_4 (7.2%), for 100-year time horizon. This work brings worthwhile data of greenhouse gas emissions and concentrations from a small water stream based on seasonal measurement along the longitudinal profile.

Key words: methane, greenhouse gases, stream water, carbon dioxide, nitrous oxide

Introduction

In 2011, the global mean concentration of atmospheric CO_2 was 391 ppm [1], and the average rate of increase in the concentration of atmospheric CO_2 over the period spanning from 1960 to 2005 of 1.4 ppm yr^{-1} is therefore considered to be the main driver of climate change [2]. The global mean concentrations of CH_4 and N_2O in 2011 were 1803 ppb and 324 ppb, respectively [1]. The significant ability of CH_4 and N_2O molecules to absorb infrared radiation makes them more efficient greenhouse gases than CO_2, thus resulting in a substantial contribution to the heating effect of the atmosphere in particular, and to climate change in general. In the Fifth Assessment Report of the IPCC, Myhre et al. [3] reconsider the global warming potentials (GWPs) of CH_4 as an instrumental metric for transferring emissions of different gases to a common scale. The recently suggested GWPs of CH_4 rose significantly from 72 to 86 and from 25 to 34 for the 20-year and 100-year time horizon, respectively, which makes CH_4 a more important greenhouse gas. The radiative forcing of a molecule of N_2O is even greater, and the GWPs for N_2O are 268 and 298 respectively for the 20-year and 100-year time horizon.

With respect to concerns about climate change, numerous studies suggest possible ways and strategies to mitigate emissions of greenhouse gases, particularly those originating from anthropogenic sources. On the other hand, however, some natural sources of these gases were omitted owing to the fact that we have only limited information about their role in the production of greenhouse gases [4]. Extensive efforts have been directed towards the identification and quantification of the source strength of greenhouse gas emissions into the atmosphere from a variety of aquatic environments. Wetlands are believed to be a major natural source of CO_2 and CH_4 [5 - 7], however both CO_2 and CH_4 are also supersaturated in many surface waters, with streams and rivers frequently exhibiting gaseous partial pressures many times in excess of the atmospheric equilibrium [8, 9]. The production of N_2O is linked mainly with excesses of nitrogen in agricultural soils and its runoff in drainage water [10]. However, streams similarly show significant supersaturation of N_2O with respect to the atmosphere [11]. Unquantified potential sources of greenhouse gases are of obvious climatic concern, and while streams and rivers are not usually implicated directly, there is a growing body of evidence to suggest that alongside CO_2 and N_2O, rivers and streams can also emit significant quantities of CH_4 [12, 13]. Saarnio et al. [14] estimated CH_4 release from wetlands and watercourses in Europe, but data for rivers and streams are still with high error rate. If we

consider that rivers and streams are likely to cover 0.30-0.56% of the land surface (with a significant partion of third order streams) and make contributions to global processes and greenhouse gas emissions that may be 20-200% greater than those implied by previous estimates [15], explorations of greenhouse gases productions and emissions in river ecosystems are required.

Our long-term study on the Sitka stream documented the production of greenhouse gases in hyporheic sediments [16]. Hlaváčová et al. [17] reported that respiration of oxygen, nitrate, sulphate, and methanogenesis may simultaneously coexist within the hyporheic zone, and that an anaerobic metabolism and methanogenesis appear to be an important pathway in organic carbon cycling in the Sitka stream sediments. Additionally, Cupalová and Rulík [18] found that members of the phylogenetic domain Archaea may commonly occur within well-oxygenated hyporheic sediments. Building on this work, Buriánková et al. [19] found relatively well developed populations of methanogenic Archaea, and significant methanogenic potential in hyporheic sediments of the Sitka stream. The methanogenesis and other degradation processes result in the production of CO_2, CH_4, and N_2O which then supersaturate the interstitial water and eventually emit them into the atmosphere [20]. Based on previous studies, we suppose that stream sediments are a source of gases that are important in global warming. In particular, CH_4 production in hyporheic sediments seems to be novel as published global CH_4 emission estimates from streams are also sparse [21]. Moreover, recent discoveries of metabolic pathways in both the nitrogen and carbon cycle across a spectrum of aquatic ecosystems highlights the need for new directions and a multidisciplinary approach to quantifying the flux of carbon and nitrogen through rivers [22].

Past studies have focused mainly on the downstream section of the Sitka stream, but we were also curious to know if other parts along the stream's profile will also produce greenhouse gases. In contrast to previous studies concerned with greenhouse gas emissions, we extended our exploration to five study sites along longitudinal profile of the Sitka stream. The primary objectives of our study were to measure the concentrations of CH_4, CO_2, and N_2O both in surface and interstitial water, and their emissions at different localities along a stream continuum, and to estimate the total annual emissions of greenhouse gases from surface water to the atmosphere. Our study presents at once total annual emissions of all three biogenic greenhouse gases from a small water stream, and their relative contribution to the total annual emission expressed in standard metric units (CO_2 equivalent emission). This study is

worthwhile mainly because there is paucity of studies estimating greenhouse gas emissions from the whole river ecosystem during a year.

Material and methods

Study site

The sampling sites were located on the Sitka stream, Czech Republic (Fig. 1). The Sitka is an undisturbed, third-order, 35 km long stream originating in the Hrubý Jeseník mountains at 650 meters above sea level. With the exception of short sections, the Sitka stream is unregulated with well-established riparian vegetation. A detailed description of the Sitka stream can be found in Hlaváčová et al. [17]. In order to assess the emissions released from a total stream area, the stream was divided into five stretches according to changing conditions along the river's course (Tab. 1). For each stretch we chose one representative sampling site (localities I-V) where samples of both surface water and air were taken repeatedly. The numbering of each locality corresponds to the numbering of each stretch. The Sitka stream flows in its upper section until it reaches Šternberk town through a forested area with low intensity of anthropogenic effects (localities I-II), while the lower course of the stream is naturally meandering through a more intensively managed agricultural landscape (localities III-V). The areas of the sections of stream were calculated from known lengths and mean channel widths (measured by a metal measuring type at different sites). The longitudinal distances of the sections were worked out using ArcGIS software with digitalized maps of the Sitka stream and GPS coordinates that were obtained during the field measurement. The total area of the Sitka stream was estimated to be 0.18 km^2.

Water sampling

Surface water samples were collected from running water at a depth of about 10 cm below the surface level at all of localities four times from autumn 2005 to autumn 2006 (October 25th, February 28th, May 25th, and July 18th). Three replicates of surface water were collected on each sampling date. All water samples were taken between 9 am and 2 pm. Interstitial water samples were collected using a set of two mini-piezometers randomly placed at a depth of 30 cm in sediments at each study site two times in 2006 (May 25th and July 18th). The initial

100 ml of water from each mini-piezometer was used as a rinse and was discarded. Three replicates of interstitial water from each mini-piezometer were collected from a continuous column of water, by a 100 ml polypropylene syringe connected to a hard PVC tube, and drawn from a mini-piezometer and overflowed and sealed into vials. All water samples were collected into sterile, clear vials (40 ml) with screw-tops, covered by a polypropylene cap with PTFE silicone septa, and stored in ice on the way to the laboratory. On each sampling date, temperatures of interstitial and surface waters were measured for calculation of saturation ratios. 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 sampled water using the solubility data of Wiesenburg and Guinasso [23], Weiss and Price [24], and Weiss [25].

Measurement of emissions

Gaseous fluxes across the air-water interface were determined by the floating chamber method [26, 27] four times from autumn 2005 to summer 2006 (October 25th, February 28th, May 25th, and July 18th). On each sampling date, 4-5 replicated measurements were performed at all the localities between 9 am and 2 pm. The open-bottom floating polyethylene chambers (internal diameter 20 cm, total volume 5.5 L) were held in place on the water surface by a floating body (polystyrene) attached to the outside. The chambers were attached to a metal pole which was anchored to the stream bed in order to hold the chambers in flowing conditions. The chambers were allowed to float on the water surface for a period of 3 hours. Preincubations were performed to assess linearity of gaseous concentrations in headspace of the chambers and establish the incubation time required for reliable flux measurements. Due to trees on the banks, the chambers at all the study sites were continuously in the shade. On each sampling occasion, ambient air samples were collected for determining initial background concentrations. Samples of headspace gas were collected through a rubber stopper inserted at the top of each chamber, and stored in 100 ml polyethylene gas-tight syringes until analysis. Emissions were calculated as the differences between initial background and comparing concentration in the chamber headspace, and expressed on the $1 m^2$ area of the surface level per day according to the formula:

$$F = [(c_I - c_R) * V * 24 / t * 1000] / p \quad (1)$$

where F is a gas flux in mg $m^{-2}day^{-1}$; c_I is a concentration of a particular gas in the chamber headspace in $\mu g\ l^{-1}$; c_R is a concentration of a particular gas in the background air; V is volume of the chamber in L; t is time of incubation in hr; p is an area of the chamber expressed in m^2.

Analysis of gases, calculations and statistics

Concentrations of dissolved gases in the water and air samples were measured using gas chromatography as described in Hlaváčová et al. [20]. Estimations of total annual emissions into the atmosphere from the five segments of the Sitka stream were calculated according to the following formula:

$$E_a = (\sum p_i * F_i * 365) / 1\ 000\ 000 \quad (2)$$

where E_a is average estimation of emission of a particular gas from the total stream area in t yr^{-1} for CO_2 and kg yr^{-1} for CH_4/N_2O; p_i is an area of stretch in m^2 representing the given locality; F_i is the average emission of a particular gas from the given locality, expressed in g $m^{-2}day^{-1}$ for CO_2 and in mg $m^{-2}day^{-1}$ for CH_4/N_2O.

Data analysis was performed using statistical software R (version 2.6.0). Data for regression analyses embody non-parametric distributions, and normality was not achieved by data transformation. Shapiro-Wilk normality test was used ($P < 0.05$). Therefore robust regressions were used and linear models were tested by robust F-test for relations between gaseous concentrations and gaseous emissions and for relations between surface and interstitial water concentrations.

Results

Patterns of gaseous concentrations

Highly supersaturated concentrations of all observed gases were found in both surface and interstitial water at all the localities (Tab. 2) and the saturation ratio R usually reflects those concentrations (Tab. 3).

Measured surface water CH_4 concentrations were between 0.19-35.47 µg l^{-1} and a significant enhancement of CH_4 concentration was found on localities IV and V compared to the upstream localities. Generally, CH_4 concentrations measured in interstitial water were higher as compared to those from surface water and ranged between 0.19-11968.9 µg l^{-1}. The relationship between CH_4 concentrations in the interstitial and surface water was expressed by a positive linear regression ($y = 1.5 + 0.004\ x$, $R^2 = 0.61$, $F = 284.5$, $P < 0.001$). Interstitial concentrations at downstream localities III-V were much higher compared to those at the upstream reach (localities I-II).

Measured surface water CO_2 concentrations varied between 0.64-4.5 mg l^{-1}. Measured interstitial concentrations of CO_2 were higher than those from the surface water and occurred in the range of 0.85-10.82 mg l^{-1}. The relationship between CO_2 concentrations in the interstitial and surface water was expressed by a positive linear relationship ($y = 0.08 + 1.77\ x$, $R^2 = 0.54$, $F = 15.5$, $P < 0.01$). Saturation ratios of CO_2 were found at levels slightly in excess of the atmospheric equilibrium leading to the supersaturation. CO_2 saturation ratios of surface and interstitial water showed only slight variation compared to the CH_4 saturation ratios.

Measured surface water concentrations of N_2O varied between 0-5.8 µg l^{-1}. A remarkable increase in the surface concentration was found on the downstream localities (III-V) compared to the upstream localities (I-II). Measured interstitial N_2O concentrations were found to be higher compared to those from the surface water except locality V and ranged in between 0.1-26.93 µgl^{-1}. No relationship was observed between N_2O concentrations in the interstitial and surface water ($F = 0.5$, $P = 0.48$).

Patterns of emissions

Generally, emissions occurred in the order of CO_2, CH_4, and N_2O where CO_2 emissions were usually three orders of magnitude greater than CH_4 and N_2O. We found differences among greenhouse gas emissions along the longitudinal stream profile. A significant enhancement of emissions for all the three greenhouse gases was found on downstream localities (IV-V) compared to upstream localities (I-III) (Tab. 4). Moreover, we found significant positive relationships between surface water concentration and measured emissions of all the gases (Fig. 2). Measured CH_4 emissions were in the range of 0-167.35 mg m^{-2} day^{-1}. Sharp increase

in the amount of CH_4 emitted from the surface water was measured at lowermost localities (IV-V). We found a positive correlation between the surface water CH_4 concentrations and measured emissions ($y = -3.83 + 1.62\ x$, $R^2 = 0.57$, $F = 664.5$, $P < 0.001$). Emissions of CO_2 varied between 0-17.33 g m^{-2} day^{-1} with a maximum found at the lowermost locality V. CO_2 showed an increase in the amount of emissions in the downstream region (localities IV-V) compared to the upstream part (localities I-III). We found a positive correlation between surface water CO_2 concentrations and measured emissions ($y = -6.06 + 3.66\ x$, $R^2 = 0.77$, $F = 81.3$, $P < 0.001$). N_2O emissions ranged between 0-19.69 mg m^{-2} day^{-1} and were much higher at the downstream localities III-V compared to upstream localities I-II. Measured N_2O emissions positively correlated with the surface water N_2O concentrations ($y = -3.36 + 1.89\ x$, $R^2 = 0.61$, $F = 24.7$, $P < 0.001$). Multiple linear regression with gaseous concentrations and flow velocity as predictor variables and gas emissions as a response variable was not significant for all monitored gases.

On the basis of annual mean emissions of particular gasses and areas of the defined segments approximately 211 tons of greenhouse gases were emitted annually to the atmosphere from the water surface of the Sitka stream with a dominant proportion being CO_2 (Tab. 5). If total annual emissions of each gas is converted to standard metric units (CO_2 equivalent emission) using GWP described in IPCC methodology [3], then CO_2 accounts the most part of total annual emission of greenhouse gases from the Sitka stream, with the second being N_2O, and the last CH_4, for both 20-year and 100-year time horizons. Contribution of CH_4 emissions to the total annual emissions was found to be the highest during the spring and summer periods (81.9 %), while relatively high proportion of CO_2 emissions (65.7 %) was during summer and autumn months. Contribution of N_2O emissions was at its highest during summer (32.3 %) and winter (31.9 %) (Fig. 3).

Discussion

We found both surface and interstitial water to be supersaturated with CH_4, CO_2, and N_2O compared to the atmosphere at all five localities, except for the N_2O concentration in the surface water on locality II. Since CO_2 is produced mainly by decomposition of organic matter, carbonate equilibrium reaction, and respiration; N_2O by nitrification, denitrification, and coupled nitrification-denitrification as a byproduct; supersaturated concentrations of CO_2 and N_2O in interstitial and surface waters are commonly reported [11, 28 - 35]. Dissolved CH_4

concentrations in the surface water of the Sitka stream are in agreement with earlier studies which focused on the measurement of CH_4 concentration in rivers [36, 37]. Some authors suggested that increased concentration of CH_4 in rivers result from lateral diffusion of stream banks and drainage of forest and agricultural soils or from outflow of urbanized areas and sewage treatments [9, 36, 38]. Striegl et al. [8] mentioned that river CH_4 could be derived from multiple sources, including groundwater, surface water runoff, and benthic and water column microbial processing of organic carbon. The exact role of each of these processes is not yet quantified in the overall river carbon budget. Other authors [30, 39] found, as we did, a significant increase of CH_4 in the interstitial water. In spite of a commonly perceived view of streams as well-oxygenated habitats, we assume internal CH_4 production in hyporheic sediments as some other authors have suggested [12, 19, 39, 40]. Moreover, we can exclude the possibility that CH_4 in the Sitka stream sediments is leaked from a natural gas extraction or from a geothermal and volcanic source due to absence of these events in the watershed of the stream. This supposition of biogenic origin of CH_4 is supported by carbon isotope analysis of CH_4 in bubbled gas from hyporheic sediments of the Sitka stream (unpublished data). We also excluded significant contribution of drainage from wetlands and peatlands, as was reported in some cases [41, 42], due to absence of wetland ecosystems in the watershed of the stream. The internal CH_4 production could easily be explained by anaerobic conditions occurring in hyporheic biofilm in micro-scale dimension. Physical and chemical gradients in hyporheic sediments enable a broad spectrum of metabolic pathways to occur within small spatial scales and imply important biological consequences. The seemingly well-oxygenated hyporheic zone contains anoxic and hypoxic pockets associated with irregularities in sediment surfaces, small pore spaces, or local deposits of organic matter. Thus this creates a 'mosaic' structure of various environments, where different microbial populations can live and different microbial processes can occur simultaneously [43]. As the interstitial water in comparison to surface waters were markedly more supersaturated by all monitored gases, we suggest that they are produced in hyporheic sediments of the stream.

Levels of the gaseous concentrations in the surface water do not depend only on gaseous production in hyporheic sediments on a sampling site. However they are affected by some other effects like photosynthesis, methanotrophy, respiration or downstream transport of gases. Moreover, a part of the gases can result from drainage of soils in watershed [42]. Since aquatic macrophytes and evident algal communities were not observed in the stream bed on the localities, therefore we do not expect marked daily variation of CO_2 concentration in

surface water due to photosynthesis; although little fluctuations could be caused by daily temperature fluctuation and microbial biofilms containing primary producers on the streambed.

Downstream localities (III-V) had higher interstitial and surface water concentrations and emissions of all the gases in comparison to upstream localities (I-II). For example, the majority of the total annual CH_4 emission (90%) was released from the two most downstream stretches (IV-V) of the stream, representing only 1/5 of the total stream area. An ideal water stream embody continuum of changing physical conditions downstream with decreasing flow velocity and increasing mass of finer particles and organic matter in hyporheic sediments. [44] Therefore productions of biogenic gases increase downstream together with an amount of organic rich sediments which enable microbial processes. However, in spite of this, the increase of the gaseous concentrations and emissions was not gradual. A real stream does not act as an ideal stream, characterized by gradual continuum of changing physical conditions and increase of organic matter in hyporheic sediments downstream. A real stream constitutes a dynamic water body with heterogeneous changes in physical conditions in longitudinal profile. Torrential sequences interchange with fluviatile sequences and therefore conditions for microbial processes and gas production are changing along the stream. A noteworthy point was that locality IV showed the highest interstitial concentrations for all monitored gases. High concentrations of the gases found in interstitial water (Tab. 2) were caused probably by very fine sediment and substantial amounts of organic matter in this locality. Such place may be considered as a „hot spot" source of the gases for surface water. In turbulent streams gases are repeatedly degassed from reaches containing riffles and replenished in slower moving reaches within pools. High CH_4 concentration in the upstream part, with subsequent decline further downstream, was reported from USA by Lilley et al. [36]. Therefore greenhouse gas concentrations and emissions are more likely related to local conditions than to an accumulation of transported gases from upstream parts of a stream. In the case of the N_2O we suggest that high surface and interstitial concentrations on the downstream localities (III-V), and consequently also emissions, are due to input of nitrate from sewage water outflows from the Šternberk town, located between localities II and III, and due to drainage of agricultural land on downstream part of the watershed. Generally, N_2O concentrations are related to the amount of flooding and leaching of N from a catchment, especially if the catchment contains agricultural areas. The most important factor associated with river N_2O production is nitrate concentration [30, 45].

The range of measured greenhouse gas emissions was in accordance with most other studies. Average values of CH_4 emissions on the Sitka stream are similar to values reported for small streams in Arizona [39] and for rivers in Oregon [36]. The annual mean CO_2 emissions to the atmosphere for the Sitka stream were also similar to those found on estuaries of large European rivers [46]. Wilcock and Sorrell [13] found, as we did, that rivers could emit significant amounts of N_2O. Although the extent of gaseous emissions from the Sitka stream magnify the effect when compared with other streams and rivers, they do not depend only on gaseous concentrations in the surface water, but they are affected by some other effects which can differ on various water bodies.

Generally, gaseous emissions comprise a flux mediated by submerged plants, an ebullition from sediments and a diffusive flux from the water surface [47]. Since we did not record any primary producers in the stream bed, contribution of the plant mediated flux to the overall emissions is the most probably not a case or is negligible at the Sitka stream. However, we observed gas bubbles released from the sediment, specifically on locality IV. Although an ebullition is rarely captured by the floating chamber technique [48] the preference of this method was accurate, because the methods based on calculation models account only diffusive flux. The rate of gaseous diffusion into the atmosphere depends on several factors: partial pressure of a particular gas in the atmosphere and its concentration in water, water temperature, and further on molecular diffusion coefficient of a particular gas, water depth, and flow velocity [38, 49]. Accordingly, a temperature increase which reduces gas solubility could lead to higher emission rates during the summer period. Further, streams with higher flow velocity have higher gas exchange coefficients compared to wetlands or large rivers. Nevertheless, multiple linear regression with gaseous concentrations and flow velocity as predictor variables and gas emissions as a response variable was not significant for all monitored gases, but simple linear regression between gaseous concentrations and emissions was significant for all monitored gases (Fig. 2), as was found on three rivers in China [9]. This contradiction could be caused by highly turbulent flows influencing gas exchange on the air-water interface, which make flow velocity less predictive of gaseous emissions from the stream.

If we consider the seasonal distribution of gas emissions, it is clear, in concordance with the above mentioned relations, that the majority of CH_4 and CO_2 emissions were released during

warmer periods of the year. The effect of temperature on CH_4 and CO_2 production was observed in south-eastern USA, where most of the CH_4 was released into the atmosphere during the warm months [50]. The highest N_2O emissions from the Sitka stream were recorded during summer and winter months and an equivocal seasonal pattern was obtained. But the 31.9% contribution of N_2O emissions during winter to the total annual budget of the stream is not surprising. Nitrate concentrations, the main predictor of N_2O emissions, usually increase during winter and decline during growing seasons because the export of nitrate from the watershed is controlled by plant uptake of inorganic N, which is lower during the non-growing season [51].

Based on our results we suggest that CH_4 and N_2O emission contributes considerably to the total greenhouse gas emission from the stream. We found that CH_4 emissions represent 0.8% of mass of carbon release to the atmosphere from the Sitka stream. Although the total CH_4 mass emitting from the Sitka stream is three order of magnitude lesser than the total CO_2 mass, CH_4 emissions play considerable role in the climate impact of the stream due to its GWP. The total annual CH_4 emissions contribute 7.2% to the total annual emission of all gases, converted to CO_2 equivalents, from the Sitka stream for the 100-year time-horizon, but as much as 16.8% for the 20-year horizon. The total annual N_2O emissions contribute with 22.7% to the total annual emission of all gases, converted to CO_2 equivalents, from the Sitka stream for the 100-year time-horizon, and with 18.8% for the 20-year horizon. These rates indicate that CH_4 and N_2O emissions should not be omitted in greenhouse gas budgets of rivers and streams, especially in a short time scale. Moreover, Campeau and Del Giorgio [52] estimated that greenhouse gas emissions (CO_2 equivalent) from boreal rivers and streams may increase markedly mostly driven by a steep increase in the contribution of CH_4 under plausible scenarios of climate change over the next 50 years. Nevertheless, our results should be interpreted carefully with the up-scaling of emission data for the whole riverine systems, especially because our dataset is spatially and temporally restricted. Moreover, we did not include greenhouse gas emissions from parafluvial zones and the riverine (landscape) level fluxes can be rather different from those gathered from limited local points.

Conclusions

The CH_4, CO_2, and N_2O are produced in hyporheic sediments of the Sitka stream. The longitudinal pattern of greenhouse gas concentrations and emissions along streams is

heterogeneous and reflects a spatial distribution of physicochemical characteristics. Although concentrations and emission rates of the greenhouse gases did not show increasing trends along the stream, sampling sites located downstream indicated higher values of concentrations and emission rates than localities located upstream, and such longitudinally located sampling sites are necessary for an emission estimate. Therefore, we suggest that our approach of the direct measurement on a set of sampling sites along a water stream during a season is proper for an estimation of total annual emissions of greenhouse gases from river ecosystems. The total annual emission of CO_2 to the atmosphere was the highest for all the monitored greenhouse gases from the surface of the Sitka stream and the total annual emissions of CH_4, CO_2, and N_2O were estimated to be 637 kg, 210 t, and 229 kg, respectively. However, counting the global warming potential, the total annual contribution of CH_4 and N_2O to the total annual greenhouse gases emission from Sitka stream is nearly 30% for 100-year horizon. Therefore, CH_4 and N_2O emissions should not be omitted in an evaluation of rivers and water streams to produce greenhouse gases. Our results utilize directly measured gas concentration and emission data to contribute to recent assessments [53 - 56] of the importance of inland waters in regional and global carbon cycles and quantify the importance of CH_4, CO_2, and N_2O emissions in the carbon and nitrogen balance of a small temperate water stream system.

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Tables

Tab. 1 Longitudinal physicochemical patterns of the Sitka stream (means ± standard error).

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
average flow velocity [$m.s^{-1}$]	0.46 ± 0.09	0.19 ± 0.04	0.45 ± 0.08	0.39 ± 0.04	0.20 ± 0.04
stretch surface area [km^2]	0.043	0.06	0.043	0.024	0.012
grain median size [mm]	12.4	12.9	13.2	0.2	5.4
surface dissolved oxygen saturation [%]	101 ± 22.6	110 ± 13.4	104 ± 3.7	114 ± 14.6	103 ± 3.8
hyporheic dissolved oxygen saturation [%]	63 ± 23.6	89 ± 13.8	79 ± 4.4	61 ± 9.2	58 ± 12.7

surface water temperature [°C]	9.1 ± 3.59	9.7 ± 3.56	12 ± 4.39	12.3 ± 4.93	11 ± 5.27
interstitial water temperature [°C]	13.9 ± 1.17	14.8 ± 1.4	17.5 ± 1.72	18.3 ± 2.04	14.2 ± 3.44
surface water DOC [mg/l]	2.47 ± 0.21	0.81 ± 0.19	2.62 ± 0.15	2.69 ± 0.19	3.74 ± 0.39
interstitial water DOC [mg/l]	2.05 ± 0.17	1.31 ± 0.29	2.71 ± 0.32	5.76 ± 0.91	2.62 ± 0.24

Tab. 2 Annual means of gas concentrations in surface and interstitial water at localities of the Sitka stream (mg l^{-1} for CO_2 and µg l^{-1} for CH_4/N_2O) ± standard errors.

Gas	I	II	III	IV	V
surface CH_4	1.25 ± 0.43	0.46 ± 0.2	1.37 ± 0.49	15 ± 6.63	12.12 ± 3.77
surface CO_2	1.76 ± 0.29	1.13 ± 0.18	1.81 ± 0.3	2.01 ± 0.39	3.36 ± 0.45
surface N_2O	1.32 ± 0.81	0.59 ± 0.29	3.14 ± 0.93	1.6 ± 0.54	3.03 ± 0.72
interstitial CH_4	1.34 ± 0.55	0.65 ± 0.26	19.20 ± 16.93	5196.01 ± 2322.28	15.38 ± 7.23
interstitial CO_2	3 ± 0.49	1.46 ± 0.35	2.28 ± 0.38	5.8 ± 2.16	4.34 ± 1.3
interstitial N_2O	4.47 ± 3.02	1.07 ± 0.43	5.62 ± 1.66	13.63 ± 5.59	1.27 ± 0.69

Tab. 3 Annual means of saturation ratio of CH_4, CO_2 and N_2O in surface and interstitial water at all localities ± standard errors.

Locality/Location	CH_4	CO_2	N_2O
I			
surface	25.2 ± 10.22	1.9 ± 0.12	1.9 ± 0.95

	interstitial	29.5 ± 5.14	3.85 ± 0.29	8.6 ± 5.88
	II			
	surface	9.9 ± 5.54	1.3 ± 0.2	0.9 ± 0.4
	interstitial	15.4 ± 8.94	2.1 ± 0.61	2.2 ± 0.16
	III			
	surface	31.5 ± 13.49	2.28 ± 0.51	6.2 ± 2.42
	interstitial	487.9 ± 382.69	3.4 ± 0.98	12.7 ± 5.76
	IV			
	surface	379.5 ± 216.86	2.5 ± 0.7	3 ± 1.14
	interstitial	130 437.4 ± 83 544.46	9.8 ± 4.49	30.4 ± 19.11
	V			
	surface	246.4 ± 81.98	4.1 ± 0.82	5.1 ± 1.81
	interstitial	364.9 ± 190.73	6.15 ± 3.14	4.1 ± 2.98

606
607
608 **Tab. 4** Annual mean gas emissions to the atmosphere (g m^{-2} d^{-1} for CO_2 and mg m^{-2} d^{-1} for
609 CH_4/N_2O) ± standard errors.
610
611

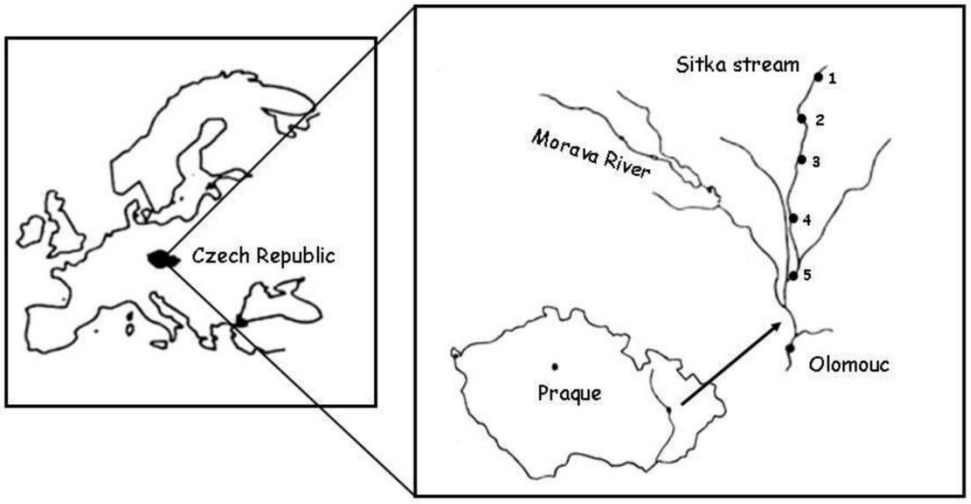
Locality	**CH_4**	**CO_2**	**N_2O**
I	2.39 ± 0.96	3.68 ± 0.71	2.83 ± 2.01

II	0.25 ± 0.08	1.15 ± 0.67	0.8 ± 0.92
III	1.41 ± 0.91	3.28 ± 1.87	6.63 ± 2.48
IV	41.31 ± 20.25	4.15 ± 1.99	3.34 ± 1.69
V	49.33 ± 26.25	9.33 ± 1.69	8.01 ± 3.74

Tab. 5 Estimation of total annual emissions of particular greenhouse gases (t yr^{-1} for CO_2 and kg yr^{-1} for CH_4/N_2O) from the Sitka stream to the atmosphere and relative metric units to 1kg of CO_2 calculated using GWPs for 20-year and 100-year time horizons. The percentage contribution of particular greenhouse gas to relative metric units of the total annual emission is in parenthesis.

	CH_4	**CO_2**	**N_2O**
total annual emission	637.2	210.2	228.6
100-year GWP	21 665 (7.2)	210 200 (70.1)	68 123 (22.7)
20-year GWP	54 799 (16.8)	210 200 (64.4)	61 265 (18.8)

Figure Captions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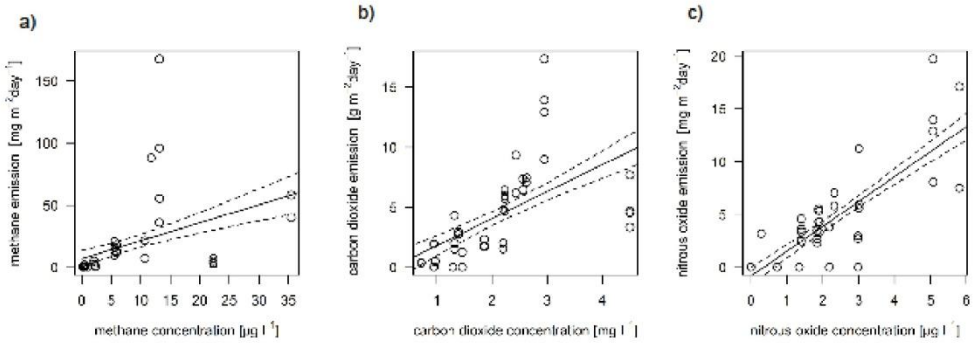
a)
b)
c)
methane emission [mg m⁻²day⁻¹]
methane concentration [µg l⁻¹]
carbon dioxide emission [g m⁻²day⁻¹]
carbon dioxide concentration [mg l⁻¹]
nitrous oxide emission [mg m⁻²day⁻¹]
nitrous oxide concentration [µg l⁻¹]

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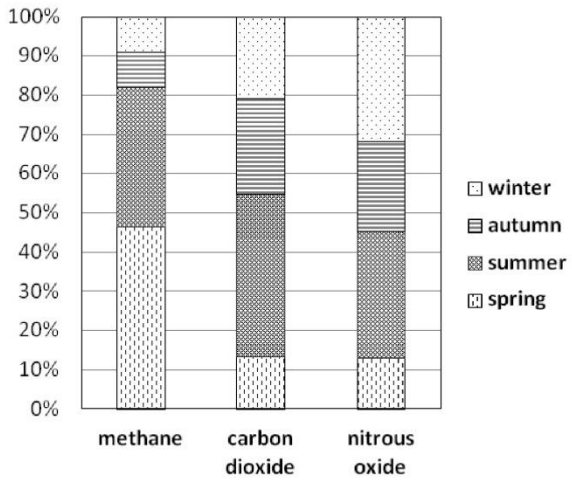

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627 **Fig. 1** A map showing the localities (black circles) on the Sitka stream.

628 **Fig. 2** Relationship between atmospheric emissions and surface water concentrations for CH_4
629 (a), CO_2 (b) and N_2O (c).

630 **Fig. 3** Contribution of gas emissions to the atmosphere from different seasons to the total
631 annual emission of each gas.

Figure 1
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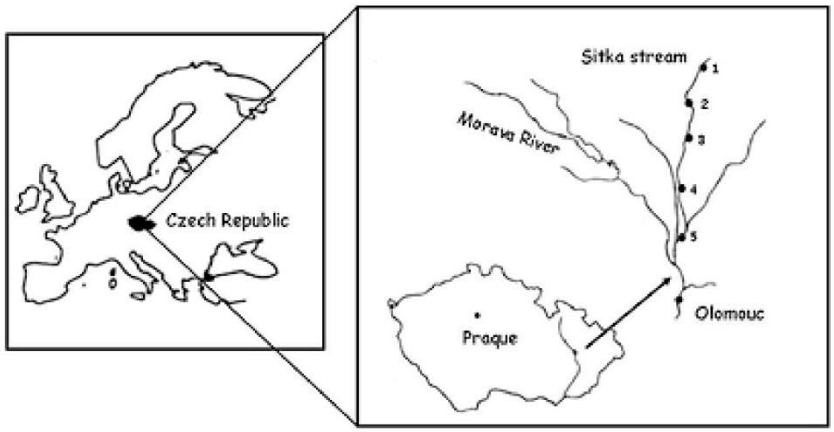

Figure 2
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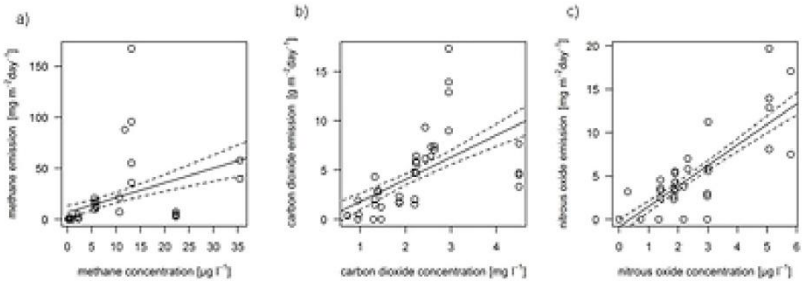

Figure 3

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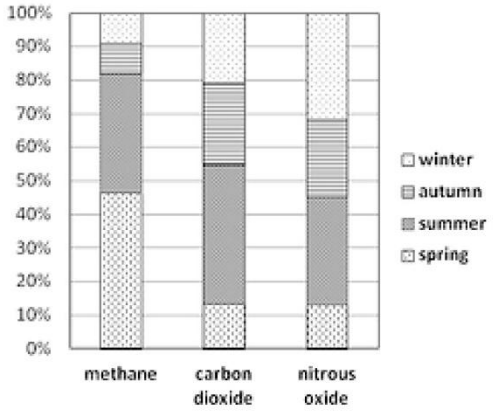

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Figures

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II. Methanogenic system of a small lowlandstream Sitka, Czech Republic

Martin Rulík, Adam Bednařík, Václav Mach, Lenka Brablcová, Iva Buriánková, Pavla Badurová, Kristýna Gratzová

(In: Matovic MD et al. *Biomass Now - Cultivation and Utilization*, Chapter 17: 395-426)

Chapter 17

Methanogenic System of a Small Lowland Stream Sitka, Czech Republic

Martin Rulík, Adam Bednařík, Václav Mach, Lenka Brablcová, Iva Buriánková, Pavlína Badurová and Kristýna Gratzová

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/52718

1. Introduction

Methane (CH_4) 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_4 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_4 during the year 2007 has been recently reported (Rigby et al. 2008), indicating that the sources and sinks of atmospheric CH_4 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 lengh, 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 118.81km^2, 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^3.s^{-1}. 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 confluences 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).

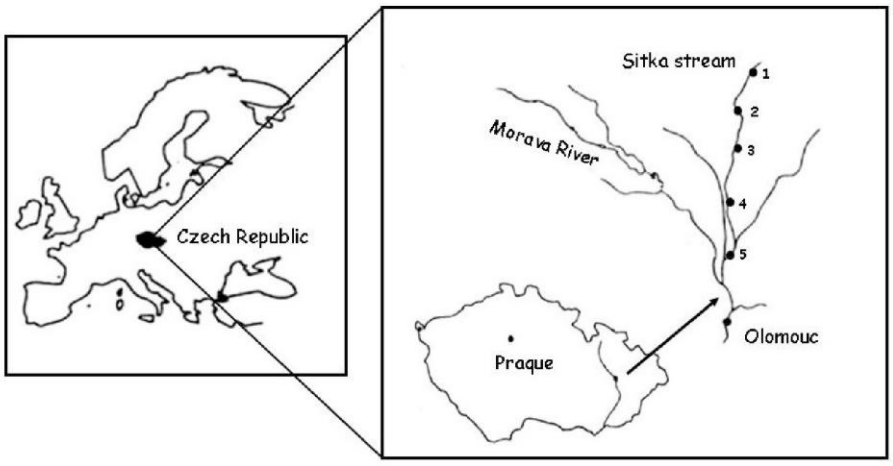

Figure 1. A map showing the location of the Sitka stream. Black circles represents the study sites (1-5)

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).

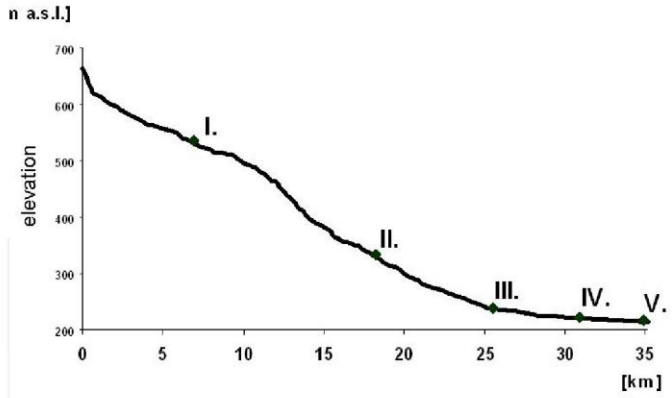

Figure 2. Graphic depiction of the thalweg of the Sitka stream with sampling localities. The main source of pollution is an effluent from Šternberk city sewage water plant, located just in the middle between stretch II and III.

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^{-1}]	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^2]	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_4^{3-} [mg L^{-1}]	0,15	0,24	7,0	2,6	1,8
surface water N - NO_3^- [mg L^{-1}]	0,01	0,21	1,2	0,5	0,18
surface water N - NH_4^+ [mg L^{-1}]	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^{-1}]	107,5	127,5	404,8	394,0	397,7
hyporheic water conductivity [μS.cm^{-1}]	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^{-1}]	2,47	0,81	2,62	2,69	3,74
hyporheic water DOC [mg L^{-1}]	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 (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^{HT} 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 (Fe^{2+}) concentration was measured using absorption spectrophotometry after reaction with 1,10-phenanthroline. Concentrations of organic acids were meausred using capilary electrophoresis equipped with diode array detector HP 3D CE Agilent (Waldbron, Germany). Limits of detection for particular organic acids were set as following: LOD (acetate) = 6,2 μmol L^{-1}; LOD (propionate) = 4,8 μmol L^{-1}; LOD (butyrate) = 2,9 μmol L^{-1}; LOD 32 (valerate) = 1,8 μmol 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 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 2ml of air sub-sample with a gas-tight syringe into a CHROM 5 gas chromatograph, equipped with the flame ionization detector (CH4 detection limit = 1μg 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 ($CH4\ mL^{-1}$ WW $hour^{-1}$) or per unit dry weight of sediment per one day ($\mu g\ CH_4\ kg^{-1}$ DW day^{-1}). 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 ($CH4\ mL^{-1}$ WW $hour^{-1}$) or per unit dry weight of sediment per one day (mg $CH_4\ kg^{-1}$ DW day^{-1}).

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^3) covered an area 0.0154 m^2. 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_4 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):

$$J = -D_S \times \Phi \times \left(\Delta C / \Delta x\right) \quad (1)$$

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

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

Tortuosity (θ) is possible calculate from porosity according to equation (Boudreau 1996):

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

Diffusive fluxes of CH_4 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^2) 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 $1m^2$ area of the surface level per day according to the formula:

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

where F is a gas flux in mg $m^{-2}day^{-1}$; c_I is a concentration of particular gas in the chamber headspace in $\mu g\ L^{-1}$; 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 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 caculated from known lenght 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 m^2 or 0.18 km^2. Stretches have differed in their percentual contribution to this total area and also by their total lenght (Table 1).

The total annual methane emissions to the atmosphere from the five segments of the Sitka stream, E_a (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 annualy 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 m^2) representing given locality; F_i is average, maximal or minimal assess of the methane from a given locality expressed in mg $m^{-2}day^{-1}$.

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^{plus}XL equiped by TC/EA (ThermoFinnigan) for analysis of $\delta^{13}CO_2$. Afterwards $\delta^{13}CO_2$ of water samples were calculated from gaseous $\delta^{13}CO_2$ by fractionation factor from a linear equation (Szaran 1997):

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

Stable isotope analysis of $^{13}C/^{12}C$ in gas samples was performed using preconcentration, kryoseparation of CO_2 and gas chromatograph combustion of CH_4 in PreCon (ThermoFinnigan) coupled to isotope ratio mass spectrometer (IRMS, Delta Plus XL, ThermoFinnigan, Brehmen, Germany). After conversion of CH_4 to CO_2 in the Finningan standard GC Combustion interface CO_2 will be tranfered into IRMS. The obtained $^{13}C/^{12}C$ ratios (R) will be referenced to $^{13}C/^{12}C$ of standard V-PDB (Vienna-Pee-Dee Belemnite)(Rs), and expressed as $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1000$ in ‰. The standard deviation of $\delta^{13}C$ determination in standard samples is lower than 0.1‰ with our instrumentation. From our data, we also calculated an apparent fractionation factor α_C that is defined by the measured δCH_4 and δCO_2 (Whiticar et al. 1986):

$$\alpha_C = \left(\delta CO_2 + 10^3\right) / \left(\delta CH_4 + 10^3\right) \quad (7)$$

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^{-1}; 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, Methanosetaceae* 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-tetramethylenediamine), 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, afterwatds 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 bend 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 x 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 unchaged (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 occuring 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^2 saturation [%]	80.5	88.1	82.3	38.5	50.9
ferrous iron [mg L^{-1}]	< 1	< 1	1.8	8.1	4.2
acetate [mmol L^{-1}]	0.21	0.34	0.52	1.87	0.29
interstitial CH4 concentration [μg L^{-1}]	4.9	0.7	8.1	2 480.2	42.8
methanogenic potential [pM CH_4 mL^{-1} WW $hour^{-1}$]	6.6	1.9	2.9	80.7	9.7
methanotrophic activity [nM CH_4 mL^{-1} WW $hour^{-1}$]	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_4 mL^{-1} WW $hour^{-1}$ with the highest values found at site IV. Average

methanotrophic activity (MA) varied between 0.02– 31.3 nM CH_4 mL^{-1} WW $hour^{-1}$ 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^{-1} in surface water and showed no expected trend of gradual increase from upstream localities to those laying downstream. However, significant enhancement of CH_4 concentration was found on locality IV and V, respectively. Concentrations of dissolved CH_4 inboth 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^{-1}. 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_4 [μg L^{-1}]
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 CH_4.

Stable carbon isotope signature of carbon dioxide ($\delta^{13}C\text{-}CO_2$) measured in the interstitial water ranged from -19.8 ‰ to -0.8 ‰, while carbon isotope signature of methane ($\delta^{13}C\text{-}CH_4$) ranged between -72 ‰ to -19.8 ‰. 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 $\delta^{13}C\text{-}CH_4$ was found in the sediment depth of 40-60 cm where a high methane production has occured. Here, the $\delta^{13}C\text{-}CH_4$ values varied only from -67.9 ‰ to -72 ‰. Apparent fractionation factor (αc) 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 hydrogenothropic and acetoclastic methanogenesis, respectively. Our measurements indicate predominant occurrence of a hydrogenothropic methanogenesis in the high methanogenic zones where the most amount of methane is produced and $\delta^{13}C$ of CO_2 values were markedly depleted (i.e. ^{13}C enriched). This could be caused by enhanced carbon dioxide consumption by hydrogenothrophic methanogens, strongly preferring light isotopes. Nevertheless, both acetoclastic and hydrogenotrophic pathways take part in the methanogenesis along the longitudinal profile of the Sitka stream.

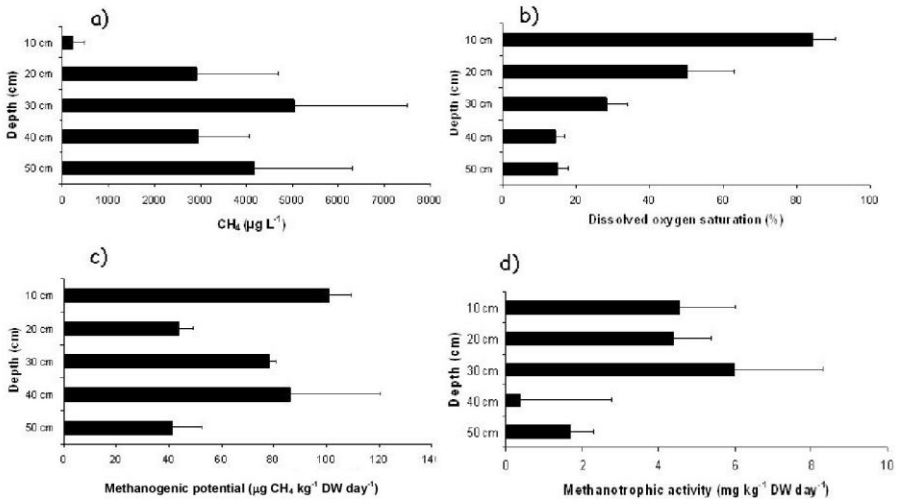

Figure 3. Vertical distribution of methane concentration in the interstitial water at study site IV, horizontal bars indicate 1 SE

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 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).

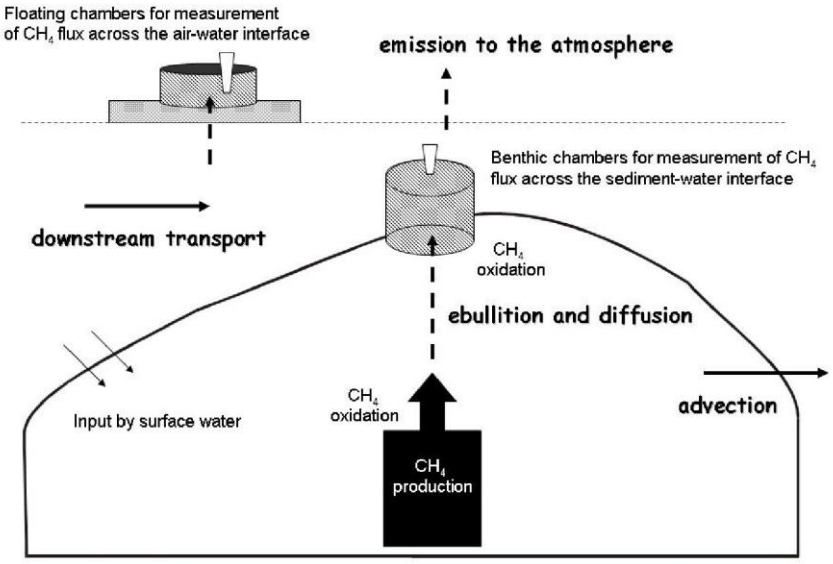

Figure 4. Possible fate of the methane within hyporheic zone and two kinds of chambers for measurement of methane fluxes. Providing that some sites along the longitudinal stream profile should be sources of methane for the stream water, we chose locality IV to be suitable for benthic fluxes measurements.

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 CH_4 ranged from 0.03 to 2307.32 $\mu g\ m^{-2}\ day^{-1}$ along the longitudinal profile. The lowest average values of diffusive fluxes were observed at study site II ($0.11 \pm 0.05\ \mu g\ m^{-2}\ day^{-1}$) while the highest average values were those observed at study site IV ($885.81 \pm 697.54\ \mu g\ m^{-2}\ day^{-1}$). Direct benthic fluxes of CH_4 using the benthic chambers were measured at study site IV only and ranged from 0.19 to 82.17 $mg\ m^{-2}\ day^{-1}$. 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_4 budget.

Gaseous fluxes from surface water to the atmosphere were found at all localities except locality I, where emissions were not mesured 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 occured (Table 4). Methane emissions measured at localities II-V ranged from 0 – 167.35 mg m^{-2} day^{-1} 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_4 [mg $m^{-2}day^{-1}$]
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 $year^{-1}$ 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^2). The majority of annual methane emissions (90 %) occured 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).

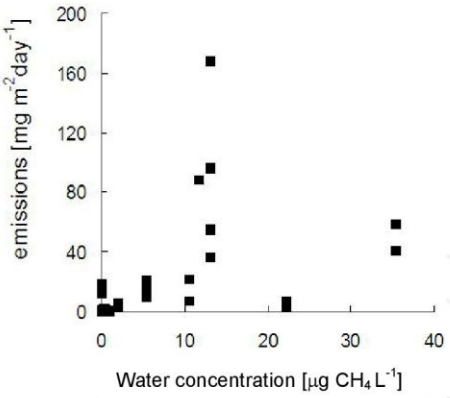

Figure 5. Relationships between atmospheric emissions and surface water concentrations of the methane. Each point represents the mean of five replicate emission measurements and the two replicates of stream water methane concentrations at all

3.6. Sitka stream water column CH_4 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_4 through evasion to the atmosphere can be combined together with advection inputs and losses to yield a CH_4 dynamics (budget) for any particular section of the stream.

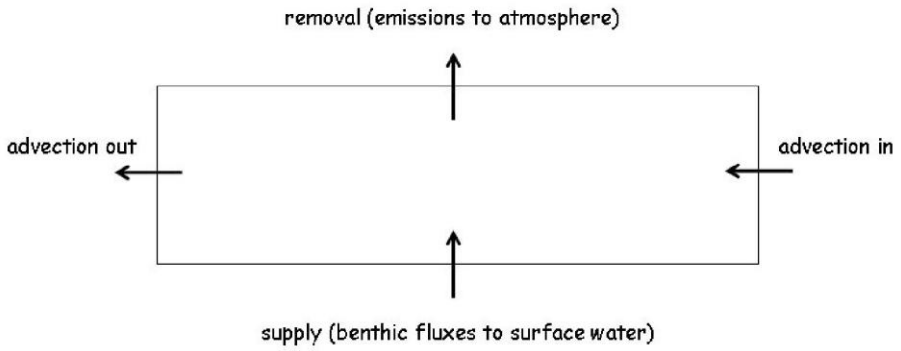

Figure 6. Simple box model used to calculate a CH_4 budget for the Sitke stream experimental section; advection in + supply = advection out + removal (box adjusted after de Angelis & Scranton 1993)

The CH_4 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 ~ 200 m^2. Positive fluxes of 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 mg m^{-2} day^{-1}, the benthic flux of 3081.39 mg CH_4 day^{-1} should occur from the whole area of 200 m^2. Average emission flux of CH_4 across the water-air interface for all study sites was determined to be 14.47 ± 4.73 mg CH_4 m^{-2} day^{-1}. This value is slightly lower than the direct benthic flux of CH_4 and suggests that some portion of methane released from the bottom sediments may contribute to increasing concentration of CH_4 in the surface water. Average flow of the Sitka stream during time of benthic fluxes measurements was 0.351 m^3s^{-1} (i.e. 351 $L^{-1}s^{-1}$). Therefore, we may expect that water column was enriched at least by 187.4 mg (i.e. 0.006 μg L^{-1}) of CH_4 from sediment at 45 m long section near study site IV during one day. Next study site V is located some 4 km downstream from the site IV. Average CH_4 concentration difference in the stream water between these study sites was found to be 3.2 μg L^{-1} of CH_4 indicating that CH_4 supply exceeds slightly CH_4 removal. Methane fluxes from the sediment would contribute to this concentration difference only by 0.6 μg L^{-1}, thus, the immediate difference in the 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 mg CH_4 0.351 $m^{-3}s^{-1}$), however, net accumulation of CH_4 in the stream water during 4 km section of the Sitka stream below study site IV was almost 78 g CH_4 per one day.

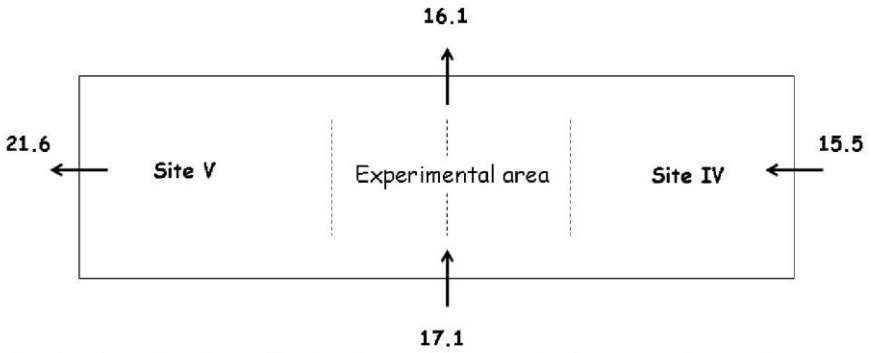

Figure 7. CH_4 budget in mol day^{-1} for a section of the Sitka stream between study sites IV and V (lenght ca 4 km). The arrows correspond to those depicted in Figure 6.

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 25-50 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 ~ 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 ± 0,28 and 1,07 ± 0,23 x 10^6 cells mL^{-1} in the upper and deeper layer, respectively) and type II methanotrophs (0,44 ± 0,14 x 10^6 cells mL^{-1} and 0,56± 0,1 x 10^6 cells mL^{-1}) increased slightly with the sediment depth , while type I methanotrophs revealed average abundance 0,98 ± 0,23 x 10^6 cells mL^{-1} in the deeper layer being lower compared to abundance 1,07± 0,28 x 10^6 cells 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 ecxlusively 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).

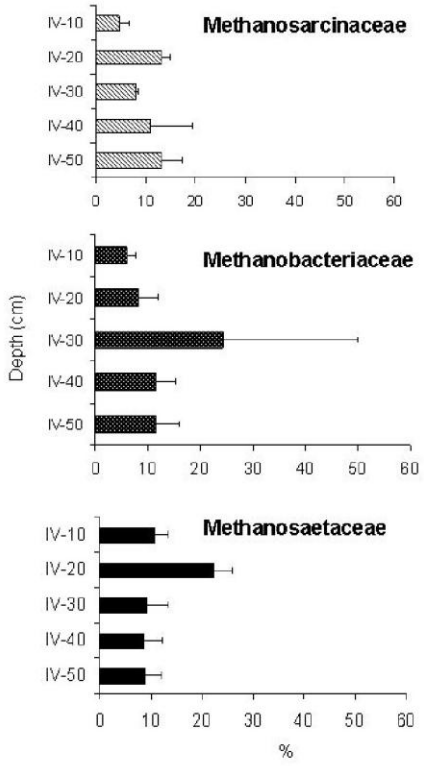

Figure 8. The percentage of chosen methanogenic families as compared to the total bacterial cell numbers found in different sediment layers at locality no. IV, horizontal bars indicate 1 SE

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 localites 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.

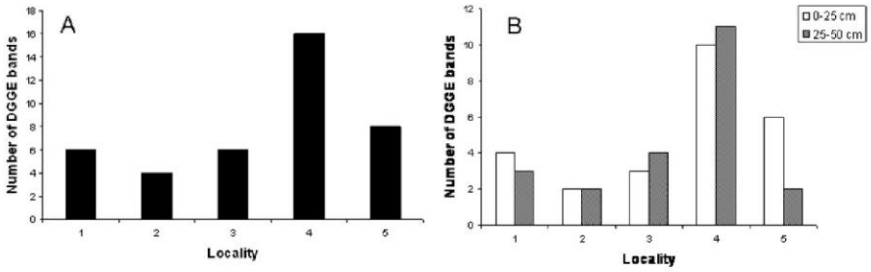

Figure 9. Number of DGGE bands associated with hyporheic sediments at two different depths along the longitudinal stream profile. A – Total number of all bands detected at each locality; B – number of bands found at different sediment depths

Most of the clones showed low affiliation with known species (< 97% nucleotide identity) and probably represented genes of novel methanogenic archeal 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. Occurence 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 (volatizing). 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_4 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_4 due to high CH_4 in the river waters and wetlands also play important roles. However, low CH_4 in the Changjiang Estaury (China) may be resulted from the low CH_4 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 $\delta^{13}C\text{-}CH_4$ in natural systems can be useful in studies of the mechanisms and pathways of CH_4 cycling (Sansone et al. 1997). Values of carbon isotope signature of methane ($\delta^{13}C\text{-}CH_4$) 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}C ($\delta^{13}C$ = -65 to -50‰) in contrast to marine sediments (-110 to -60‰). Accordingly, the two precursors of methane, namely acetate and CO_2/H_2, yield methane with markedly different $\delta^{13}C$ values; methane from acetate is relatively enriched in ^{13}C. Average minimum in the carbon isotopic composition of CH_4 (-61.4 ‰) occurred deeper in sediments (60 cm) while average maximum in $\delta^{13}C$-CH_4 occured in the lower sediment depth of 30 cm. Enrichment of ^{13}C in CH_4 probably reflects aerobic CH_4 oxidation because oxidation would result in residual CH_4 with $\delta^{13}C$-CH_4 values less negative than the source 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 hypotesis 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 containg 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 CO_2 and CH_4. The downstream river sites surrounded by agricultural soils released significant amounts of N_2O whereas the CO_2 and 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 relesed during a warm period of the year (81%). Effect of temperature on methane production was also observed in southeastern USA where the most methane reased 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 carefull 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 a 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 emissons to the atmosphere.

4.4. Benthic fluxes and potential methane oxidation

CH_4 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 methanoges) 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 positiosns 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_4 - 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_4 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_4 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_4 in the Sitka stream and in 3rd order stream environment. Fluxes of CH_4 from supersaturated interstitial sediments appear to be a main CH_4 source toward the water column. Compared with CH_4 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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III. Methanogens and methanotrophs distribution in the hyporheic sediments of a small lowland stream

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Article

Methanogens and methanotrophs distribution in the hyporheic sediments of a small lowland stream

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With 4 figures and 4 tables

Abstract: Distribution of microbial activity, methanogenic archaea and type I and II methanotrophs were studied in a small lowland stream Sitka in Czech Republic. The methanogens and methanotrophic bacteria were detected using FISH with 16 S rRNA-targeted oligonucleotide probes. The highest microbial density was obtained in the upper sediment layer 0–25 cm; this zone corresponded also to that of the highest metabolic activity, as indicated by the methanogenic potential, methanotrophic activity, INT and FDA profiles. 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 the deeper sediment layer 25–50 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 ~21.4 % and type II methanotrophs 11.9 %, respectively. The percentage of DAPI-stained cells hybridizing with methanotroph-specific probes was generally higher for type I than type II. Our data show that the methanogenic archaea and aerobic methanotrophs can be numerically dominant components of the hyporheic biofilm community and affect CH_4 cycling in river sediments.

Key words: methane, hyporheic sediment, methanogenic archaea, methanotrophs, FISH.

Introduction

The hyporheic zone, the 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 reduc-

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ing 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 may be a well-oxygenated habitat even up to a 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). Methane is the second-most abundant gas containing carbon in the atmosphere and has a greenhouse effect some 20 times greater than that of CO_2, resulting in a significant contribution to the radiation forces of the atmosphere and global climate changes (Houghton et al. 2001, Schimel 2004). Methane (CH_4) is an atmospheric trace gas present at concentrations of about 1.8 ppmv representing about 15 % of the anthropogenic greenhouse effect (Forster et al. 2007). The atmospheric CH_4 concentration has increased steadily since the beginning of the industrial revolution (~ 0.7 ppmv) and has stabilized at ~1.8 ppmv from 1999 to 2005 (Forster et al. 2007). An unexpected increase in the atmospheric growth of CH_4 during the year 2007 has been recently reported (Rigby et al. 2008), indicating that the sources and sinks of atmospheric CH_4 are dynamic, 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). However, despite anaerobic metabolism being described in many lake, estuary and wetland sediments, there is still a paucity of information in river ecosystems.

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, for example the aerobic degradation of methyl phosphonates (e.g. Karl et al. 2008) or oxidation of ascorbic acid using iron minerals and hydrogen peroxide (Althoff et al. 2010). Methanogenic archaea, which belong to the kingdom Euryarchaeota, are ubiquitous in anoxic environments. Methanogens 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 et al. 1988) 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). The degradation pathway of polysaccharides, for example, is such that about two-thirds of the produced CH_4 should be derived from acetate and one-third from H_2/CO_2 if steady state conditions exist (Conrad 1999).

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 diver-

sity and activity of methanotrophic communities from the river ecosystems are fragmentary as yet. MOB are grouped within alpha and gamma subdivisions of the Proteobacteria. The α-methanotrophs include the family Methylocystaceae (genera *Methylosinus* and *Methylocystis*), also known as type II methanotrophs, and acidophilic methanotrophs belonging to the family Beijerinkiaceae (*Methylocella* and *Methylocapsa*). The γ-methanotrophs include the family Methylococcaceae, which consists of type I (*Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylosphaera*, *Methylosarcina*, *Methylothermus*) and type X methanotrophs (*Methylococcus* and *Methylocaldum*). 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. Although methanotrophs can oxidize as much as 100 % of methane production (Le Mer & Roger 2001), authors monitoring methanotrophy in riverine ecosystems indicate that CH_4 losses due to microbial consumption are rather less (Zaiss et al. 1982, Lilley et al. 1996).

Estimates of methanogens and methanotrophs abundance in natural samples are based on a number of complementary techniques. Fluorescent in situ hybridization (FISH) with rRNA-targeted oligonucleotide probes has become very useful in studies of microbial ecology, as this method allows target populations to be detected and enumerated directly in their environment without cultivation. A classical FISH method is based on the direct microscopic identification of single cells after hybridization with 16 S rRNA targeted fluorescent dye-labelled oligonucleotide probes. The intensity of probe signal depends on cellular rRNA content, which correlates with the physiological status of the microbial population (Kallistova et al. 2007).

The present results are part of a long-term study of organic carbon dynamics and associated microbial communities in hyporheic sediments in the Sitka Stream, Czech Republic. The main objective of this study was a basic characterization of both methanogenic and methanotrophic populations in different localities along the longitudinal profile of a small lowland stream. We used FISH method for the analysis of microbial community composition and determination of methanogenic and methanotrophic abundance using oligonucleotide probes targeting the 16 S rRNA. In addition, total sediment microbial activity was measured simultaneously with potential methane production and oxidation in order to assess whether a relationship between the activity and distribution of the methanogens and methanotrophs could be found.

Material and methods

Study site

The sampling sites are located on the Sitka stream, Czech Republic (Fig. 1). The Sitka is an undisturbed, third-order, 35 km

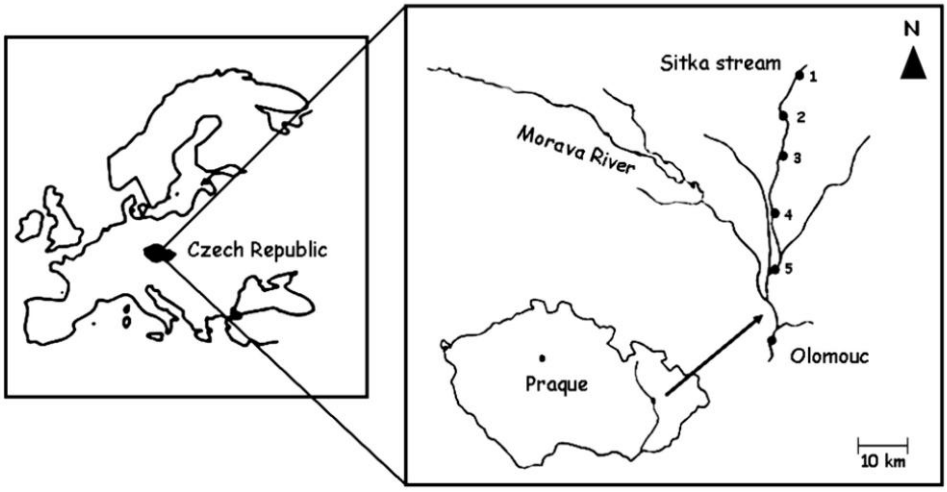

Fig. 1. A map showing the location of the Sitka stream. Black circles represent the study sites (1–5).

long lowland stream originating in the Hrubý Jeseník mountains at 650 m above sea level. The catchment area is 118.81 km^2, 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 $m^3 s^{-1}$. The Sitka stream flows in its upper reach 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 characterized by finer sediment with a median grain size of 2.8 mm. The Sitka stream confluences 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^{2-}) 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).

Sediment sample collecting and sample processing

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

Four 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).

Water samples and analysis of methane

Surface water was collected from the river at a depth of 10 cm below the air-water interface in autumn 2009 at each study site. Interstitial water samples were collected using a set of 5–6 minipiezometers (Trulleyová et al. 2003) randomly placed at depths of about 20–50 cm in the sediments at each study site.

Table 1. Selected longitudinal physicochemical patterns (annual means) of the hyporheic interstitial water taken from the depth 25–30 cm. Saturation ratio R of methane = measured concentration of the gas in the water divided by the concentration in equilibrium with the atmosphere).

Variable/ Locality	I.	II.	III.	IV.	V.
Geographic coordinates	49° 49′ 27.782″ N, 17° 18′ 47.528″ E	49° 45′ 53.953″ N, 17° 19′ 5.141″ E	49° 42′ 46.109″ N, 17° 15′ 36.225″ E	49° 40′ 43.709″ N, 17° 14′ 49.025″ E	49° 38′ 7.977″ N, 17° 14′ 37.068″ E
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
dominant substrate composition	gravel	gravel	gravel	sand-clay	gravel-sand
grain median size [mm]	12.4	12.9	13.2	0.2	5.4
organic carbon in sediment < 1 mm [%]	0.9	0.7	0.6	1.0	1.2
interstitial dissolved oxygen saturation [%]	63.0	89.3	83.5	62.5	59.3
interstitial water DOC [mg L^{-1}]	2.05	1.31	2.71	5.76	2.62
interstitial CH_4 concentration [µg L^{-1}]	1.34	0.65	10.34	7 260.8	18.38
saturation ratio R of interstitial CH_4	29.6	11.3	224.6	146 321	402.1

The initial 50–100 mL of water was used as a rinse and discarded. Then 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. The subsamples were injected into separate 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 at base flow. Interstitial water temperature, dissolved oxygen (mg L^{-1} and percent saturation) and conductivity were measured in the field with a portable Hanna HI 9828pH/ORP/EC/DO meter. Dissolved organic carbon (DOC) was measured by Pt-catalysed high temperature combustion on a TOC FORMACS^{HT} analyser.

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 N_2 and then vigorously shaking the vials for 15 seconds (to release the 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 gas sub-sample with a gas-tight syringe into a CHROM 5 gas chromatograph, equipped with the flame ionization detector (CH_4 detection limit = 1 µg L^{-1}) and with the 1.2 m 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), Weiss & Price (1980), and Weiss (1974).

Methanogenic potential and methanotrophic activity

The rate of methane production (methanogenesis) was measured using the PMP method (Segers 1998). The sediment was sieved and placed into incubation flasks. 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. The substrate (acetate) was chosen according to our previous results obtained for the same study site which showed that acetate is more important than hydrogen for methanogenesis in the hyporheic sediments (Hlaváčová et al. 2005). 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 unit wet weight of sediment per one hour (pM CH_4 mL^{-1} WW $hour^{-1}$).

Rate of potential methane oxidation (methanotrophy) was measured using a modified method of methane oxidation in soil samples from Hanson (1998). The sediment was sieved and placed into incubation flasks. 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 unit wet weight of sediment per one hour (nM CH_4 mL^{-1} WW $hour^{-1}$).

Respiratory – electron transport system (ETS) activity and hydrolytic (esterase) activity

ETS activity was measured using the assay originally proposed by Kenner & Ahmed (1975) and modified by Tóth (1995) and Simčič (2005). Samples of sediment were collected by the freeze-core method and then transported to the laboratory and analysed within 24 hours. The samples (approximately 1 mL of sediment) were gently shaken for 5 minutes (500 rpm; OS Control 10) in 4 mL volume of ice-cold homogenization buffer. After that samples were sonicated (20 seconds, 20 % power; Sonopuls 2200) and centrifuged (5 minutes, 3 000×g; Rotofix 32 A). Then 0.5 mL of supernatant (in triplicate) was incubated in 1.5 mL substrate solution (containing NADH, NADPH) with 0.5 mL reagent solution [2(*p*-iodophenyl)-3-(-*p*-nitrophenyl)-5-phenyl tetrazolium chloride, INT] for 30 minutes at *in situ* temperature. Controls with no supernatant were also incubated. After incubation a stopping solution (formalin conc. : H_3PO_4) was added to all samples (including the controls). The formazan production was determined spectrophotometrically against a calibration curve produced by formazan standards. Results are expressed per unit wet weight of sediment per one hour (µM O_2 $hour^{-1}$ mL^{-1}).

Sediment samples for measurement of esterase activity were also collected by the freeze-core method and then transported to the laboratory and analysed within 24 hours. The samples (approximately 1 mL) were placed into sterile tubes. Secondly 5 mL of phosphate buffer, 4 mL of distilled water and 1 mL of FDA solution were added to all samples. Controls were inactivated with $HgCl_2$. All samples were gently shaken for 5 minutes (500 rpm; OS Control 10) and after that incubated for 30 minutes at in situ temperature. Incubation was stopped with $HgCl_2$, the samples were then sonicated (30 seconds, 40 % power; Sonopuls 2200) and centrifuged (5 minutes, 3 000×g; Rotofix 32 A). Absorbance of the supernatant at 490 nm was measured spectrophotometrically against a fluorescein calibration curve. The assay proposed by Battin (1997), modified by Ntougias et al. (2006), was used for measuring hydrolytic activity. Results are expressed per unit wet weight of sediment per one hour (µM FDA $hour^{-1}$ mL^{-1}).

Abundance of microbial cells and microbial community composition

For measuring 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 (Priemé et al. 1996, Santos Furtado & Casper 2000, Amalfitano & Fazi 2008). For density centrifugaton, the non-ionic medium Nycodenz (1.31 g mL^{-1}; Axis- Shield, Oslo, Norway) was used at 4600G for 60 minutes (Rotofix 32 A, Hettich, Germany). After the preparation processes, 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.

Total cell numbers (TCN)

The supernatant was filtered onto membrane filters (0.2 µm GTTP; Millipore Germany), stained for 10 minutes in cold (4–6 °C) and in the dark with DAPI solution (0.0063g mL^{-1}; wt vol^{-1}; Sigma, Germany) and gently rinsed in distilled water and 80 % ethanol. Filters were air-dried and mounted 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.

Prokaryotes community composition

The methanogenic archaea and methanotrophic bacteria were detected using FISH (Fluorescence in situ hybridization) with 16 S rRNA-targeted oligonucleotide probes labelled with indocarbocyanine dye Cy3 (see Table 2). 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 prokaryotes was then calculated.

Statistics

Data analyses were performed using statistical software R-version 2.6.0 and GLM (General linear models). Analysis of deviance was chosen for the factors cogency. Models with dependent variable (methanogenic potential and methanotrophic activity) were log-transformed (y = log[x+1]) to achieve normality. In the models where the dependent variable was abundance (TCN, FISH), Negative Binomial Model was used. Depending on data distribution Wilcoxon signed rank sum test or paired t-test were used.

Results

General processes

a) Cell counts and in situ hybridization

The total cell number (DAPI-stained cells, TCN) on hyporheic sediments < 1 mm revealed from DAPI staining (TCN) varied between $2.06 \pm 1.7 \times 10^6$ and 12.36 ± 8.7 10^6 cells mL^{-1} wet sediment. DAPI-stained prokaryotes showed significantly higher average cell numbers in the middle part of the stream ($p < 0.001$, n = 120) (Fig. 2a). Generally, cell numbers were higher in the upper 0–25 cm sediment layer ($7.5 \pm 1.9 \times 10^6$ cells mL^{-1}) than at greater sediment depth of 25–50 cm ($4.6 \pm 1.2 \times 10^6$ cells mL^{-1}). The highest number of cells was found in the 0–25 cm layer at the locality IV where the TCN reached up to 18×10^6 cells mL^{-1} wet sediment.

The percentage of cells identified with probe EUB338 which targeted members of the domain Bacteria, accounted for 5.1 to 31.3 % of the DAPI-stained cells. The average portion of EUB-hybridized bacteria in the upper sediment layer (13.8 %) was similar to that in deeper sediments (14.7 %) (Table 3). Probe ARCH915 targeting members of the domain archaea resulted in average cell counts of 10.9 to 14.3 % of the DAPI-stained cells. The portion of arch-hybridized cells slightly increased with the sediment depth, however no significant difference has been found between both sediment layers (Table 3).

Table 2. Sequences and target organisms of used oligonucleotide-probes.

Probe	Target sequence (5'-3')	Target phylogenetic group	Reference
EUB338	GCTGCCTCCCGTAGGAGT	Eubacteria	Amann et al. (1990)
Mγ84	CCACTCGTCAGCGCCCGA	Type I methanotrophs	Eller & Frenzel (2001)
Mγ705	CTGGTGTTCCTTCAGATC	Type I methanotrophs	Eller & Frenzel (2001)
Mα450	ATCCAGGTACCGTCATTATC	Type II methanotrophs	Eller & Frenzel (2001)
ARCH915	GTGCTCCCCCGCCAATTCCT	Archaea	Raskin et al. (1994)
MPB1	CATGCACCWCCTCTCAGC	Methanogen-specific probe	Jupraputtasri et al. (2005)

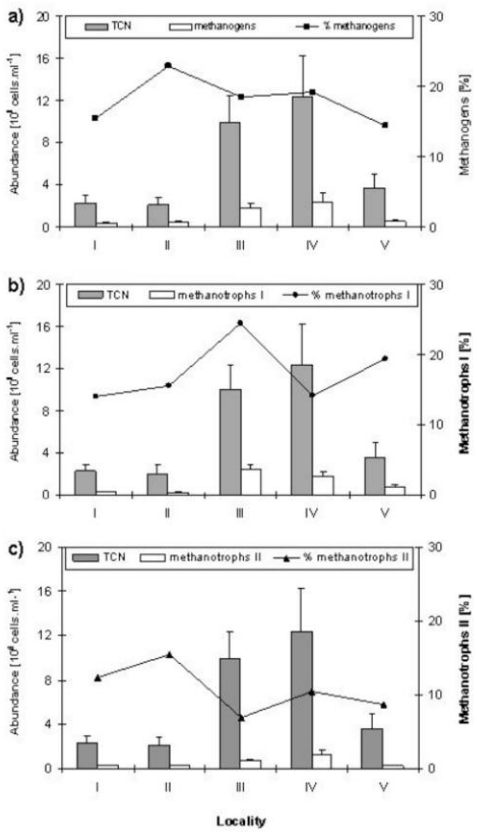

Fig. 2. Total cell numbers (grey bars), methanogens (panel **a**), type I (**b**) and II (**c**) methanotrophs (white bars) at different localities along longitudinal profile of the Sitka stream. The lines indicate the percentage of methanogens or methanotrophs as compared to total bacterial cell numbers. Values are averages for both sediments layers (standard errors are given for bars).

b) ETS and esterase activity

ETS activity measured as INT reduction varied between 0.14–2.84 uM O_2 hour^{-1} mL^{-1} wet weight sediment; however, no differences were found in ETS activity between localities studied. Esterase activity as showed by FDA method was in the range from 0.73 to 16.22 uM FDA hour^{-1} mL^{-1} wet weight sediment and the values were significantly different at various localities ($p < 0.05$, n = 40). Both ETS activity and esterase activity exhibited significantly much higher values for the upper sediment layer at all localities (Table 4). These results supported measurements of MP and MA mentioned above and suggest that the majority of metabolic processes occur in the upper 0–25 cm of the sediments where input of dissolved oxygen from the surface stream is still sufficient.

Methane related processes

a) Methanogens and MOB numbers

Methanogens-targeting probe MPB1 gave a much higher signal for the deeper sediment layer ($p < 0.05$) (Table 3) and percentage of MPB1-hybridized methanogen was in the range of 4.6 to 31 % of the DAPI-stained bacteria. The percentage of DAPI-stained cells hybridizing with methanotroph-specific probes was generally higher for type I than type II, with a pronounced increase in type I towards to the deep sediments (Table 3). Percentage of type I methanotrophs, hybridized with combination of Mγ84 plus Mγ705 probes was in the range 5 to 33.4 % of the DAPI-stained cells and clearly tended to be significantly much higher in deeper sediment layers compared to that from the surface ($p < 0.05$) (Table 3). By contrast, type II methanotrops showed significantly lower percentage of DAPI-stained cells (2.9–18.2 % of the DAPI) and the portion of their cell count was almost the same in both sediment layers (Table 3).

Table 3. Relative numbers of eubacteria, archaea, methanogens and methanotrophs detected by FISH in relation to the total DAPI counts in different sediment depths of the Sitka stream (average ± SE, range in parentheses). EUB338 – a probe that targets all bacteria, ARCH915 – a probe that targets archaea; MPB1 – a probe that is specific for methanogens; Mγ84 plus Mγ705 – a probe combination that is specific for type I methanotrophs; Mα450 – a probe that is specific for type II methanotrophs.

Depth [cm]	EUB338 [%]	ARCH915 [%]	MPB1 [%]	Mγ84 + Mγ705 [%]	Mα450 [%]
0–25	13.8 ± 3.8 (5.1 – 28.5)	10.9 ± 3.5 (3.8 – 25.2)	12.8 ± 3.7 (4.5 – 23.4)	13.7 ± 3.6 (5.3 – 30.9)	8.8 ± 3.7 (2.8 – 17.6)
25–50	14.7 ± 3.9 (5.6 – 31.2)	14.3 ± 2.9 (6.5 – 23.4)	23.4 ± 6.7 (13.6 – 31.0)	21.4 ± 4.8 (14.8 – 33.3)	11.9 ± 2.3 (7.9 – 18.1)

Table 4. Summary of the microbial metabolic activity in the different sediment depths of the Sitka stream (average ± SE).

Depth [cm]	ETS [μM O_2 /mL/hour]	FDA [μM FDA/mL/hour]	MP [pM CH_4/mL/hour]	MA [nM CH_4/mL/hour]
0–25	1.59 ± 0.29	11.4 ± 1.5	38.9 ± 30.1	18.47 ± 6.97
25–50	0.25 ± 0.13	2.4 ± 0.3	1.9 ± 036	15.71 ± 6.48

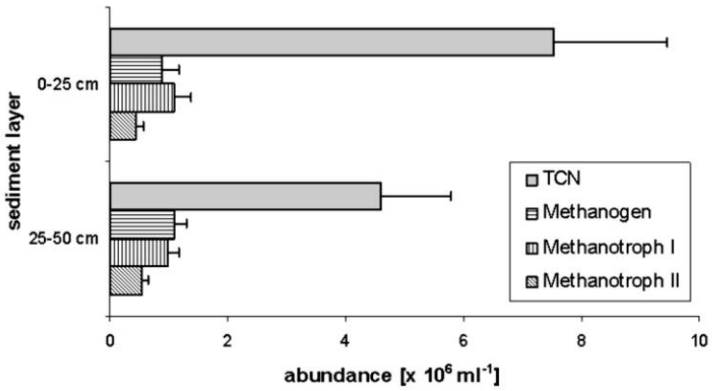

Fig. 3. Average total cell numbers, (DAPI-stained, TCN), methanogens, type I and II methanotrophs found in different sediment layers, horizontal bars indicate 1 SE.

Fig. 2 shows the population of methanogenic and methanotrophic communities along a longitudinal profile of the Sitka stream. Cell numbers of methanogens and type I methanotrophs differed among localities ($p < 0.001$, n = 30) while type II methanotrophs exhibited less significant difference in the cell numbers among localities ($p = 0.026$, n = 30). Generally, both methanogenic archaea and aerobic methanotrophs were found in all samples and at all localities along the longitudinal profile. The proportion of these groups to the TCN was quite consistent and varied only slightly (Fig. 2a–c) along the longitudinal stream profile. The percentage of methanogens to total DAPI counts revealed a maximum (22.9 %) at locality II; despite the fact that this locality showed relatively low TCN as well as number of methanogens. Methanotrophs showed similar trends, however they differ in percentage – type I methanotrophs reached the maximum at locality III (24.8 %) while II type methanotrophs at locality II (15.5 %) (Fig. 2b, c).

All three groups also formed a higher proportion (although non-significant) of the TCN in the deeper sediment layer. Methanogens comprized on average up to 23 % of the total ($p = 0.167$, n = 30), while type I methanotrophs comprized ~22 % ($p = 0.68$, n = 30) and type II methanotrophs comprized 12 % ($p = 0.795$, n = 30) of the total respectively (Table 3). However, the abundance of methanogens and methanotrophs remained almost unchanged with increasing sediment depth, thus their increase in the proportion to the TCN corresponded mostly to the total bacterial cell count decreasing from $7.53 \pm 1.93 \times 10^6$ cells mL^{-1} wet sediment in the upper sediment layer to 4.6×10^6 cells mL^{-1} wet sediment in the deeper sediments (Fig. 3).

The average abundance of methanogens (0.88 ± 0.28 and $1.07 \pm 0.23 \times 10^6$ cells mL^{-1} in the upper and deeper layer, respectively) and type II methanotrophs ($0.44 \pm 0.14 \times 10^6$ cells mL^{-1} and $0.56 \pm 0.1 \times 10^6$ cells mL^{-1}) increased slightly with the sediment depth (Fig. 3), while type I methanotrophs had lower average abundance in the deeper layer ($0.98 \pm 0.23 \times 10^6$ cells mL^{-1}) compared to their abundance $1.07 \pm 0.28 \times 10^6$ cells mL^{-1} in the upper sediment layer (Fig. 3). Increase in the abundance with increasing sediment depth had been expected in the case of methanogens; however, we have no explanation yet for type II methanotrophs.

b) Methanogenic potential (MP) and methanotrophic activity (MA)

Methanogenic potential was significantly higher in the upper sediment layer compared to that from the deeper

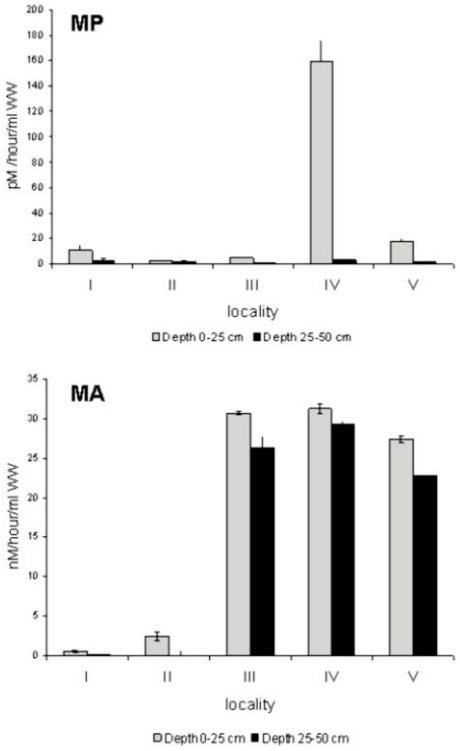

Fig. 4. Methanogenic potential (MP) and methanotrophic activity (MA) along longitudinal profile of the Sitka stream. Vertical bars indicate SE.

sediment layer ($p < 0.001$, n = 30) (Table 4). Generally, average MP varied between 0.74–158.6 pM CH_4 mL^{-1} WW $hour^{-1}$ with the highest values found at site IV located the furthest downstream (Fig. 4a). In the long term, this locality also exhibits both high interstitial methane concentrations and emissions of methane to the atmosphere. Average MA varied between 0.02–31.3 nM CH_4 mL^{-1} WW $hour^{-1}$ and the highest values were found to be at the downstream localities ($p < 0$, n = 30) while sediment from sites located upstream showed much lower or even negative activity (Fig. 4b). Similar to MP, values of MA were significantly higher in sediments from upper layers compared to those from deeper layers ($p < 0.001$, n = 30) (Table 4).

Discussion

During this study we found relatively well developed populations of methanogenic archaea at all localities and that all localities also showed positive methanogenic potential. After DAPI staining, the highest microbial density was obtained in the upper sediment layer 0–25 cm and density decreased with depth. This zone corresponded to that of highest metabolic activity, as indicated by the methanogenic potential, methanotrophic activity, INT and FDA profiles whereas the methane profile increased in the deeper sediment. This observation is in strong concordance with some previous studies which suggested that there may be a spatial uncoupling of bacterial production and organic carbon mineralisation in sediments, with growth occurring predominantly in the near-surface aerobic layer whilst the bulk of mineralisation takes places in deeper, anoxic sediment (Wellsbury et al. 1996).

Artificial electron acceptors such as tetrazolium salts are commonly used as an indicator of electron transport system (ETS) activity. Although it is well established that tetrazolium salts (mostly 5-cyano-2,3-ditolyl tetrazolium chloride, CTC, triphenyl tetrazolium chloride, TTC and INT) can be used to detect specifically the metabolic activity of aerobic bacteria, some results suggest that tetrazolium salts could potentially be used to detect the metabolic activity of bacteria under some anaerobic conditions (Fukui & Takii 1989, Walsh et al. 1995, Smith & McFeters 1997, Bhupathiraju et al. 1999) and a positive response to the INT test has been found even in strictly anaerobic bacteria in activated sludge (Maurines-Carboneill et al. 1998). Hence, measurement of ETS activity in the Sitka stream sediments should not be affected by the prevalence of an anaerobic metabolism in the deeper sediments (Hlaváčová et al. 2005, 2006) and, moreover, ETS activity can be considered as a measure of the total microbial respiratory potential in the sediments (Simčič & Mori 2007). Though one would expect that freezing and thawing of sediment samples may affect cell numbers and ETS activity we never observed any serious changes. Similarly, activities of extracellular α-glucosidase, β-glucosidase and β-xylosidase that were repeteadly measured in frozen and thawed hyporheic sediments and compared to those from the samples taken by handy shovel showed also no significant difference (Rulík & Spáčil 2004). Hence, number of cells and microbial activities should not be affected by freezing and thawing. On the other hand, results from some experiments revealed that the tetrazolium salt INT is toxic to some groundwater bacteria at concen-

trations normally used for ETS assays, and may thus alter the composition of the microbial community under study. The results also suggest that some viable and actively growing bacteria do not reduce tetrazolium salts and tetrazolium salt assays are likely to dramatically underestimate total ETS activity in groundwater (Hatzinger et al. 2003). Fluorescein diacetate (FDA) is hydrolysed by non-specific esterases and can be considered as a useful indicator of cell activity (Battin 1997). In our study, we have used this method to measure esterase activity of the sediment biofilm. The distribution pattern of both ETS and esterase activity showed the highest values in the upper sediment layer and also reflect to some extent the methanogenic and methanotrophic activities mentioned above. This observation is in accordance with the results of ETS measurements by Vosjan (1982) in sediments of the Wadden Sea and also with our previous study focusing on enzyme activity (Rulík & Spáčil 2004). Surface sediments exhibited significantly higher enzyme activities compared to deeper layers and we had suggested that higher activity in surface sediments could be attributed to the occurrence of biofilm internal DOC cycling mediated by epilithic algae. Under light conditions, autotrophic algae in the biofilm are a possible source of labile compounds that may be used by biofilm bacteria living in close proximity (Rulík & Spáčil 2004).

The method we have used for characterisation of ETS activity did not allow us to determine the number of metabolically active cells. However, if the abundance of metabolically active cells correlates with total cell numbers (Haglund et al. 2002), then we can suppose that sediment microbial community of the upper 0–25 cm layer exhibits a larger proportion of active cells compared to the deeper sediment layer (see Fig. 3).

Methanogenic processes

Measurement of potential methane production could be affected by a different methodological approach, because there is no standardized approach. The MP range is quite broad and may differ up to three orders of magnitude (10^{-2} to 10^{1} μmol m^{-3} s^{-1}); however, depending on temperature or availability of electron acceptors for methanogens the values of MP may reach up to 10^{3} μmol m^{-3} s^{-1} (Segers 1998). Our data from the Sitka stream showed much greater differences with maximum value being 230 times higher compared to the lowest one. Anaerobic incubation of sediment from various depths (0–8 cm) of chalk streams revealed maximum MP in the depth of 6 cm (16.5 CH_4 nmol g^{-1} wet sed h^{-1}) with MP decrease with increasing sediment depth (Sanders et al. 2007). This finding corresponds to our own research that methane production in the upper 0–25 cm sediment layer was higher compared to the lower sediment depth.

Methane production was detected in all samples; generally, upper sediment layer 0–25 cm showed much higher potential methane production compared to lower 25–50 cm layer. We have no exact explanation for these findings. In the case of locality IV, where considerable methane production has been found in the upper sediment, we suppose that predominance of clay sediment in deeper layers could be a reason explaining the failure of methanogenesis at this locality. Another suggestion could be better substrate provision in the upper sediment layer (Galand et al. 2002, Kobabe et al. 2004). These authors studied methanogens and their activity in peat bogs or fens and found that most of the degradation of organic matter occurs in the surface layers. The low potential methane production in the deeper layers of the peat can then be explained by the lack of suitable substrate or the presence of less favourable substrate for the methanogens at those depths (Valentine et al. 1994). In their study, Chan et al. (2005) observed that the vertical decrease of methane production rates correlated better with the similar decrease of bacterial rather than archaeal numbers. This assumption is based on the fact that very first steps of organic matter degradation were found to be rate-limiting for methane production and that the decrease in bacterial numbers reflects a similar decrease in methane production (Chan et al. 2005). Indeed, our results show that the total number of microbial cells decreased with depth. However, decrease in total numbers of microorganisms is the rule for stratified lake sediments (Zepp-Falz et al. 1999, Haglund et al. 2003, Koizumi et al. 2003) as fresh substrates are only supplied from above (Schulz & Conrad 1995). Distribution of organic matter in running waters is dependent on many factors such as stream velocity or river bed topography (Rulík 1994). We have found no clear difference in the organic carbon content between different sediment layers, thus the hypothesis of the better substrate provision might be refused. However, much higher microbial cell numbers and both ETS and esterase activity in the upper sediment layer compared to deeper layer (cf. Table 4) would indicate that the 'hot spot' of microbial metabolic activity indeed occurs in the surface sediment layer and can support methanogenic populations living here.

The distribution pattern of all methanogens did not reflect the sediment methanogenesis activity as

it did in the study of Casper et al. (2003). Generally, the number of methanogens increased slightly with increasing depth; however methanogenic potential in deeper sediments remained very low, as has been showed earlier. One reason for such a difference could be adding only acetate as a methanogenic substrate for MP measurements. Our current results of stable carbon isotopic analysis of interstitial methane indicate that hydrogenotrophic methanogenesis predominates in the sediment zones where the most amount of methane is produced (unpublished data). 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 Methanomicrobiaceae grow only with hydrogen, formate and alcohols (except methanol), Methanosarcinaceae can grow with all methanogenic substrates except formate, and members of Methanosetaceae grow exclusively with acetate as energy source. All three families also showed similar proportion to the DAPI stained cells, ranging from 9.9 % (Methanosarcienaceae) to 12.3 % (Methanobacteriaceae). Thus, these results indicate that low methanogenic activity within the deeper sediments are unlikely to be caused by the presence of predominantly hydrogenotrophic methanogens which did not grow in the MP assay when amended by acetate.

Generally, the proportion of prokaryotic cells were low in the samples. The factors limiting detection of microbial cells by FISH might be the abundance of ribosomes per cell, accessibility of the rRNA and cell wall permeability (e.g. Cotrell & Kirchmann 2000, Bouvier & del Giorgio 2003). In addition, methanogens seem more abundant than total archaea in hyporheic sediments of the Sitka stream. One reason could be a lack of specificity of the ARCH915 domain probe, which has been shown to hybridize non-specifically to some members of the Bacteria (see e.g. Battin et al. 2001, Simon et al. 2000, Lehours et al. 2005). The observation of the number of methanogens is similar to that made by Kotsyurbenko et al. (2004) in an acidic West-Siberian peat bog who also found a slight increase with depth. The proportion of methanogens (15–28 % of total microbial cell numbers) in our study is similar to those found by Casper et al. (2003) and Kobabe et al. (2004), but rather higher than mentioned by other authors (Zepp-Falz et al. 1999, Koizumi et al. 2003, Chan et al. 2005).

One might wonder how methanogenic archaea can occur in the river surface sediments fully saturated with dissolved oxygen. One possibility could be the existence of hyporheic microbial biofilm attached to the sediment particles where separated layers allow coexistence and growth of various microorganisms (Koutný & Rulík 2007). Although we tried to explain the discordance between the number of methanogens and potential methanogenic production in the vertical profile of the sediment, there is still a question to be answered: why analysis of interstial water usually showed higher methane concentrations in deeper sediments compared to the upper sediment layers (unpublished data). A possible explanation might be that methane concentrations in the upper sediments are continually lowered by oxidation and consumption by aerobic methanotrophic bacteria. Nevertheless, we should be very careful when comparing sediment methanogenic activity, interstitial water methane concentration and distribution of methanogenic Archaea in the hyporheic sediments. Firstly, river bed sediments are already much more heterogeneous compared to the lake sediments. We must take into account that our data on number of bacterial/archaeal cells are based on numbers obtained from sediment particles smaller than 1 mm; therefore, distribution pattern of microbial cells reflects to some extent the distribution of those particles, which may vary from 2 up to more than 60 % of the Sitka stream bulk sediment (unpublished data). Secondly, vertical distribution of organic matter, and particularly dissolved oxygen in the river sediments, is quite different from those in lakes and may change substantially step by step due to unpredictable movement of the interstitial water. Finally, we should also consider that we measured methanogenesis on substrate amended with acetate as has been mentioned above.

Although only a small basis of experimental methods for measurement of potential oxidation of methane are reported, MA may vary in three orders of magnitude (Segers 1998). In Lake Müggelsee, MA depending on the location varied from 0.68 to 10.10 mmol m^{-2} day^{-1} and correlated also with the measured MP (Rolletschek 1997) while Sanders et al. (2007) found values to be 0.145 µmol CH_4 g^{-1} wet sediment h^{-1} in a chalk stream. 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 the interface between oxic and anoxic zones. Relatively high methanotrophic activity found in deeper sediments of the localities III–V (Fig. 4b) indicates that

methane oxidation is not restricted only to the surface sediments as is common in lakes but also takes place at greater depths. It seems likely that an 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 (Fig. 3). A similar pathway of methane cycling has been observed by Kuivila et al. (1988) in well oxygenated sediments of Lake Washington. Nevertheless, all the above mentioned findings support our previous measurements 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).

Rather surprisingly we found a relatively high number of methanotroph cells compared to the total number of cells determined by DAPI counting. In our sediments methanotrophs accounted for 10.6 to 25.7 % of the total cell numbers while papers focused on peat bog or rice soils mention much lower values (0.8 % – 4.9 %) (Dedysh et al. 2003a, b, Eller & Frenzel 2001). An exception is Kallistova et al. (2007) who found that methanotrophs accounted for about 50 % of the total bacterial population. The number of aerobic methanotrophic bacteria in the sediments of three coastal thermal springs of Lake Baikal varied between 10^3–10^4 cells mL^{-1}, with the highest number of methanotrophs (10^8 cells mL^{-1}) found in the Sukhaya spring. These values for methanotrophs were much higher than their numbers in the deepwater sediments of Lake Baikal: 10^2–10^3 cells mL^{-1} (Zelenkina et al. 2009). Total methanotrophs in Lake Washington were estimated to be 3.6×10^8–7.4×10^8 cells g^{-1} dry weight sediment (Costello et al. 2002). The total number of type I methanotrophs g^{-1} dry weight sediment was found to be 3.4×10^8–6.7×10^8, while the total number of type II methanotrophs was found to be 2.3×10^7–6.8×10^7 cells g^{-1} dry weight sediment (Costello et al. 2002).

In soils type II methanotrophs are found more frequently than type I methanotrophs. Type II methanotrophs were dominant in boreal peatland soil, type II methanotrophs were also found to be the dominant methanotrophs in peat bogs, whereas type I methanotrophs seem to prevail in aquatic environments, such as lake water and lake sediments (Henckel et al. 1999). Our data correspond to this premise. Type I methanotrophs were found in higher density and percentage to DAPI stained cells compared to type II methanotrophs. Similar results were also published by Costello et al. (2002) in Lake Washington sediments and Rahalkar et al. (2009) in Lake Constance sediments. Moreover, methanotrophs of type I also dominated over type II in the studies by Kallistova et al. (2007), Urmann et al. (2009) and Wang et al. (2008).

Several hypotheses have been raised for the ecological differences between Type I and Type II methanotrophs. For example, it has been hypothesized that Type I methanotrophs prefer relatively low CH_4 and high O_2 concentrations, while Type II methanotrophs prefer relatively high CH_4 and low O_2 concentrations (Amaral & Knowles 1995). A test of this hypothesis using Italian rice field soil showed that Type I in contrast to Type II methanotrophs indeed prefer relatively low CH_4 concentrations, but show no preference for high versus low O_2 concentrations (Henckel et al. 2000, Conrad 2007). In the Sitka stream, type I and type II methanotrophs were found at all localities in nearly the same abundance, thus, it is not clear whether any preference for methane concentration or dissolved oxygen exists between both types of methanotrophs. However, higher abundance of the type II methanotrophs (compared to those from upper layer found in the deeper sediment layer 25–50 cm in depth where also high methanotrophic activity has been detected) may suggest that suitable conditions for type II methanotrophs would occur here. However, since methanotrophs are able to survive periods of CH_4 or O_2 deficiency (Roslev & King 1994, Schnell & King 1995), this suggestion is only speculation.

Conclusions

To our knowledge this study is the first analysis of the composition of active methanogenic/methanotrophic communities in river hyporheic sediments. By use of FISH we have shown that both methanogenic archaea and aerobic methanotrophs occur commonly within river sediments, however their distribution only partly reflects potential methane production and consumption rates measured simultaneously. Rather surprising is the detection of type I and II methanotrophs in the deep sediment layer 25–50 cm, indicating that suitable conditions for methane oxidation occur here. Since we have only used oligonucleotide probes to specifically detect all methanogens and type I and II methanotrophs, we cannot show whether a function-structure relationship exists along longitudinal and vertical profile of the Sitka stream sediments. Thus, our future investigations will focus more on FISH population

analysis with family and genus specific probes and phylogenetic analyses conducted by DGGE and sequencing.

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IV. Identification of methanogenic archaea involved in a methane stream cycle by targeting methylcoenzyme M reductase (*mcrA)* gene

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Identification of Methanogenic archaea in the Hyporheic Sediment of Sitka Stream

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Abstract

Methanogenic archaea produce methane as a metabolic product under anoxic conditions and they play a crucial role in the global methane cycle. In this study molecular diversity of methanogenic archaea in the hyporheic sediment of the lowland stream Sitka (Olomouc, Czech Republic) was analyzed by PCR amplification, cloning and sequencing analysis of the methyl coenzyme M reductase alpha subunit (*mcrA*) gene. Sequencing analysis of 60 clones revealed 24 different *mcrA* phylotypes from hyporheic sedimentary layers to a depth of 50 cm. Phylotypes were affiliated with *Methanomicrobiales, Methanosarcinales* and *Methanobacteriales* orders. Only one phylotype remains unclassified. The majority of the phylotypes showed higher affiliation with uncultured methanogens than with known methanogenic species. The presence of relatively rich assemblage of methanogenic archaea confirmed that methanogens may be an important component of hyporheic microbial communities and may affect CH_4 cycling in rivers.

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Introduction

The decomposition of organic matter in aquatic sediments is an important process in global and local carbon budgets, as it ultimately recycles complex organic compounds from terrestrial and aquatic environments into carbon dioxide and methane. The latter is a major component in the carbon cycle of anaerobic aquatic systems. Since a relatively large amount of methane production has been observed in river sediments [1–4], we hypothesised that river sediments may act as a considerable source of methane gas emission into the environment [5].

Methane (CH_4) is produced mostly by methanogenic archaea [6,7] as a final byproduct of anaerobic respiration and fermentation but there is also aerobic formation of methane by the aerobic degradation of methyl phosphonates [8] or by oxidation of ascorbic acid using iron compounds and hydrogen peroxide [9]. Methanogenic archaea belonging to the *Euryarchaeota* phylum are divided into seven orders: *Methanopyrales, Methanococcales, Methanobacteriales, Methanomicrobiales*, *Methanosarcinales* and the recently recognized groups *Methanocellales* and *Methanoplasmatales* [10,11]. Methanogenic archaea are ubiquitous in anoxic environments and require a redox potential of less than -300 mV for their growth [12]. They can be found in moderate habitats such as rice paddies [13], soils [14], lake sediments [15], in extreme conditions such as hydrothermal vents [16], permafrost soils [17,18] and also in the gastrointestinal tract of animals [19]. Freshwater sediments, including wetlands, rice paddies and lakes, are thought to contribute 40 to 50% of the annual atmospheric methane flux [20].

Rates of methane production and consumption in sediments are controlled by the relative availability of substrates for methanogenesis. The most important immediate precursors of methanogenesis are acetate and H_2/CO_2. The acetate is converted into CH_4 and CO_2 by acetoclastic methanogens while hydrogenotrophic methanogens convert CO_2 and H_2 or formate to CH_4 [21]. Acetate is consumed by a limited number of strains such as *Methanosarcina* spp. and *Methanosaeta* spp., the latter are incapable of using hydrogen. A large quantity of the acetate is produced in natural ecosystems and acetoclastic methanogens are responsible for 30–70% of methane production from freshwater sediments [22]. Hydrogenotrophic methanogens of the genus *Methanobacterium*, are important in maintaining low levels of atmospheric H_2 [23]. According to Conrad et al. [24], the degradation pathway of polysaccharides in methanogenic sediments (lakes, bogs, paddy fields, marine etc) is such that

about two-thirds of the produced CH_4 is theoretically derived from acetate and one third from H_2/CO_2 if steady state conditions exist. The universal distribution of the hydrogenotrophic pathway suggests that hydrogenotrophic methanogenesis may be the ancestral form of biological methane production and that hydrogenotrophic methanogenesis appears only once in evolution [25]. Some studies showed that temperature conditions can be helpful for defining the structure and function of the methanogenic microbial community. Noll et al. [26] observed functional changes in rice fields soil from a mixture of acetoclastic and hydrogenotrophic methanogenesis to exclusively hydrogenotrophic methanogenesis over a temperature range of 42–46°C. Another study indicated that at 30°C, the methanogenic community in soil consists mainly of *Methanosarcinaceae*, whereas at 15°C, the diversity of methanogenic archaea is greater and includes for example members of the *Methanosaetaceae* family [27]. However, despite the fact that anaerobic metabolism is described in many lakes, estuaries and wetland sediments, there is a paucity of information on the methanogen diversity in river ecosystems.

Methanogenic archaea express the enzyme methyl-coenzyme M reductase which catalyzes the terminal step in biogenic methane production [28,29]. This enzyme complex is present in methanogens and methane oxidizers, making it a suitable tool for specific detection of methanogens. Methyl-coenzyme M reductase (*mcr*) constitutes about 5-12% of methanogen cellular protein and has been resolved into three components - A, C, and a small cofactor B. Component C is thought to be the site for methyl reduction and it is composed of three subunits; α, β and γ which are coded for by *mcrA, mcrB*, and *mcrG* genes respectively [30]. The genomes of all methanogenic archaea encode at least one copy of the *mcrA* operon [28]. The gene coding for *mcr* has been the target for many molecular ecological studies of methanogens [30-32]. The *mcr* operon exists in two forms, *mcrA* and *mrtA* gene coding. The *mcrA* gene is thought to be present in all methanogens, while the *mrtA* gene has only been demonstrated in members of the orders *Methanobacteriales* and *Methanococcales* [33].

The aim of this study was to identify and investigate the distribution of methanogens in two sediment depths (0-25 cm and 25-50 cm sediment layer) using the functional gene marker (the α-subunit of the methyl-coenzyme M reductase – *mcrA gene*). We used PCR, cloning and sequencing analysis for the determination of the methanogenic phylogenetic composition. In addition, analysis of dissolved methane, total cell numbers, abundance of methanogens and potential methane production were also measured at the chosen locality simultaneously. The results are part of a long-term study of organic carbon dynamics and associated microbial communities in hyporheic sediments of the small lowland Sitka stream in Olomouc, a city in the Czech Republic. Earlier measurements of relatively high methane production confirmed the suitability of the field site for the study of methane cycling [1,34].

Materials and Methods

Ethics statement

No specific permits were required for the described field studies. No permission was required for any locations or any activity. The locations are not privately owned or protected in any way. No activity during field study involved any endangered or protected species.

Study site

The sampling sites are located on the Sitka stream, Olomouc, Czech Republic. Five study localities are placed along the Sitka stream and they were studied as a part of long term research on methanogenesis. Localities I and II are situated in an upper forested area. Localities III-V are situated in agricultural landscape. This study was on the locality IV, in particular. The Sitka is an undisturbed, third-order stream, 35 km long lowland stream originating in the Hrubý Jeseník mountains 650 m above sea level. The catchment area is 118.81 km^2, the 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^3s^{-1}. The Sitka stream flows in its upper reach 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 is several kilometres away from the confluence and is characterised by finer sediment with a median grain size of 2.8 mm. More detailed characteristics of the geology, gravel bar, longitudinal physicochemical (e.g. temperature, pH, redox, conductivity, O_2, CH_4, NO_3^-, SO_4^{2-}) patterns in the sediments and a schematic view of the site with sampling point positions have been published [34,35]. 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 [1,5,34].

Collection and processing of sediment sample

Based on the conclusions of the previous research carried on the Sitka stream in 2009 [36], locality no. IV was chosen from five localities for sampling sediment and interstitial water. The values of selected physico-chemical parameters of five localities, used in this study (Table 1), were the annual mean values of the reading collected during the year 2009 to 2011. Locality no. IV shows long- term extreme values in most cases (organic carbon in sediment, dissolved ferrous iron, acetate and methanogenic potential) (Table 1) and was chosen for more detailed study of the dynamics of methane and vertical distribution of methanogens. All samples were collected and parameters measured at 4th Oct 2010 (Table 2). The mean physico-chemical parameters and SDs were calculated for values of upper sediment layers (0-25cm) and deeper sediment layers (25-50 cm). Hyporheic sediments were collected with a

Table 1. Selected physico-chemical parameters (annual means 2009-2010) of the hyporheic interstitial water and sediment along the longitudinal stream profile (average ± SD).

Variable/ Locality	I	II	III	IV	V
Organic carbon in sediment < 1 mm [%]	0.98 ± 0.12	0.91 ± 0.02	0.57 ± 0.31	1.31 ± 0.63	0.74 ± 0.30
Dissolved oxygen saturation [%]	80.48 ± 6.65	88.01 ± 2.91	82.38 ± 5.66	38.45 ± 29.37	50.91 ± 24.66
Ferrous iron Fe^{2+} [mg L^{-1}]	< 1	< 1	1.78 ± 0.15	8.08 ± 5.76	4.23 ± 4.01
Acetate [mmol L^{-1}]	0.21 ± 0.13	0.34 ± 0.18	0.52 ± 0.17	1.87 ± 0.55	0.29 ± 0.17
Dissolved methane concentration [µg L^{-1}]	4.94 ± 3.45	0.71 ± 0.15	8.06 ± 1.65	2 480.19 ± 1145.10	42.83 ± 32.11
Methanogenic potential [pmol CH_4 g^{-1} DW h^{-1}]	1.73 ± 1.70	0.45 ± 0.02	0.53 ± 0.50	18.45 ± 25.16	1.71 ± 2.00
Interstitial water temperature [°C]	8.70 ± 0.85	9.44 ± 0.37	11.60 ± 1.27	11.20 ± 0.14	11.40 ± 3.53

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Table 2. Vertical gradient of physico-chemical parameters and variables of the hyporheic interstitial water and sediment at the locality no. IV (average ± SD).

Variable/ Depth	0-25 cm	25-50 cm
Dissolved oxygen saturation [%]	59.37 ± 21.83	17.54 ± 6.02
Ferrous iron Fe^{2+} [mg L^{-1}]	3.70 ± 3.98	12.26 ± 1.49
Acetate [mmol L^{-1}]	1.72 ± 0.73	2.02 ± 0.12
Dissolved methane concentration [µg L^{-1}]	2262.65 ± 2053.41	3856.01 ± 898.02
Methanogenic potential [pmol CH_4 g^{-1} DW h^{-1}]	191.5 ± 75.14	173,87 ± 61.84
Total cell number [10^6 cells g^{-1}DW]	4.28 ± 6.53	6.18 ± 6.13
Methanogens abundance [10^6 cells g^{-1}DW]	0.65 ± 1.21	1.01 ± 1.16

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freeze-core using liquid N_2 as a coolant [37]. Altogether, three cores were gathered and taken for subsequent analyses. After sampling two layers, the surface 0-25 cm and 25-50 cm depth were immediately separated and stored at low temperature during transport to the laboratory. Immediately following thawing the wet sediment of each layer was sieved and only particles < 1 mm were considered for the following molecular analyses since most of the biofilm is associated with this fraction [38]. Four randomly selected subsamples (1 mL) from each core were used for the extraction of microbial cells and subsequently used for the estimation of bacterial and archaeal numbers. Four other subsamples were used for DNA extraction.

Sediment organic matter content was determined by oven-drying at 105°C to constant weight and subsequent combustion at 550°C for 5 h to obtain ash-free dry weight (AFDW). Organic matter values were then converted to carbon equivalents assuming 45% carbon content of organic matter [39].

Collection of water samples and methane analysis

Interstitial water samples were collected using a set of 5–6 minipiezometers [40] randomly placed into hyporheic sediments on locality IV at specified depths (Table 2, for more details see File S1). The initial 50–100 mL of water was used as a rinse and discarded. Two subsamples of interstitial water from each minipiezometer were then collected from a continuous column of water with a 100 mL polypropylene syringe connected to a stiff PVC tube. The subsamples were injected into separate sterile, clear vials (40 mL) with screw-tops, covered by a polypropylene cap with PTFE silicone septa (for analysis of dissolved gases) and stored before returning to the laboratory. All samples were taken in the morning and all measurements were done at base flow. Interstitial water temperature, dissolved oxygen (percent saturation) were measured in the field with a portable Hanna HI 9828 pH/ORP/EC/DO multimeter (Fischer Scientific, USA). Dissolved ferrous iron (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 (Waldbron, Germany). Limits of detection (LOD) for particular organic acids were set as follows: LOD (acetate) = 6.2 µmol L^{-1}; LOD (propionate) = 4.8 µmol L^{-1}; LOD (butyrate) = 2.9 µmol L^{-1}; LOD (valerate) = 1.8 µmol L^{-1}.

Concentrations of dissolved methane in the interstitial water were measured directly using a headspace equilibration technique. Dissolved methane was extracted from the water by replacing 10 mL of water with N_2 and then the vials were vigorously shaken for 15 s (to release the gas from the water to facilitate equilibration between the water and gas phases). All samples were equilibrated with air at room temperature. Methane was analysed from the headspace of the vials by injecting 2 mL of gas subsample with a gas-tight syringe into a CHROM 5 gas chromatograph, equipped with the flame ionization detector (CH_4 detection limit = 1µg L^{-1}) and with the 1.2 m PORAPAK Q column (I.D. 3 mm, Sigma-Aldrich, Germany), with nitrogen as a carrier gas. Gas concentration in water was calculated using Henry's law.

Analysis of methanogenic potential

The rate of methane production (methanogenesis) was measured using the potential methane production method [41]. The sediment was sieved and placed in incubation flasks. C-amended solutions (flushed for 5 min with N_2) with acetate Ca $(CH_3COO)_2$ (100 mg C in the incubation flask) were used for examination of the methanogenic potential. The substrate, acetate, was chosen as our earlier results for the same study site showed that acetate is more important than hydrogen for methanogenesis in hyporheic sediments [1]. 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 samples (methanogenesis was inhibited by addition of 1.0 mmol chloroform) from each depth were stored at 20°C in the dark and the incubation time was 72 h; however, subsamples from the headspace atmosphere were taken every 24 h. Gas production was calculated from the difference between initial and final headspace concentration and volume of flask; results are expressed per unit dry weight of sediment per one hour (pmol CH_4 g^{-1} DW h^{-1}).

Abundance of microbial cells and methanogens

For measuring microbial parameters, paraformaldehyde fixed samples of sediment were sonicated followed by incubation with detergent mixture (Tween 20, 0.5% v/v) and density centrifugation (non-ionic medium Nycodenz, 1.31 g mL^{-1}) was used.

The supernatant was filtered onto membrane filter. The abundance of methanogenic archaea was identified by fluorescence *in situ* hybridization (FISH) with 16S rRNA-targeted methanogen-specific oligonucleotide probe MPB1 5'-CAT GCA CCW CCT CTC AGC -'3 [42] labelled with indocarbocyanine dye Cy3. The MPB1 probe was designed to target position 978–996 (*E.coli*). The prokaryotes were hybridized according to the protocol [43]. Staining with DAPI solution (6.3 mg mL^{-1}; w/v) for total cell number (TCN) estimation was then used (more detailed protocol is described in [41]). Stained cells were enumerated on an epifluorescence microscope (Olympus BX 60, 1000 × magnification, Olympus corporation, Japan) 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 sediment layer. Total cell numbers (TCN) were expressed as cell numbers per 1 g of dry sediments.

Nucleic acid extraction and PCR amplification

Nucleic acids were extracted from 0.5 g of sieved sediment with a Power Soil DNA isolation kit (MoBio, Carlsbad, USA) according to the manufacturer's instructions. Fragments of the methanogenic DNA (~ 470 bp) were amplified by PCR using *mcrA* gene specific primers. Primer sequences for *mcrA* gene are as follows,

mcrA F 5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3',

mcrA R 5'-TTCATTGCRTAGTTWGGRTAGTT-3'

[29]. PCR amplification was carried out in a 50 µL reaction mixture 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 µmol of each dNTP, 0.8 µmol 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 30 s at 95°C, 30 s at 55°C and 30 s 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. The ramp rate was then set to 1°C/s, and 30 cycles were performed under the following conditions: 30 s at 95°C, 30 s at 55°C, 30 s at 72°C and a final extension at 72°C for 8 min – according to the protocol [44]. PCR products were visualised by horizontal gel electrophoresis in ethidium bromide stained, 1.5% (w/v) agarose gel.

Cloning, sequencing and phylogenetic analysis

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 as 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 sequence data were then analyzed by BLAST software to search for the similarity with other methanogen sequences available in the GenBank database. The sequences were then aligned by using CLUSTAL W [45] software in order to remove any chimeric sequences. The most appropriate substitution model for maximum likelihood analysis was identified by Bayesian Information Criterion implemented in MEGA 5.05 software [46]. The phylogenetic tree was constructed by the maximum likelihood method (Kimura 2-parameter model, gamma variation across sites). The tree topology was statistically evaluated by 1000 bootstrap replicates (maximum likelihood) and 1000 bootstrap replicates [47]. The cut-off value for determination of identical sequences was 97%.

GenBank accession numbers for methanogenic *mcrA* gene sequences retrieved from Sitka are as follows:

KC952027-KC952036, KC952039, KC952041-KC952042, KC952043-KC952048, KC952050-KC952052 and KF156778-KF156780.

Statistics

The analyses were performed using statistical software R version 2.6.0. Studied parameters were analyzed by a Wilcoxon signed rank sum test. All tests were considered significant at probability level $p < 0.05$.

Results and Discussion

Environmental and microbial parameters of the river hyporheic sediment

Generally, interstitial water revealed relatively high dissolved oxygen saturation with the exceptions of localities V and IV where the concentration of dissolved oxygen sharply decreased with depth. However, it never dropped below ~ 10% of the oxygen saturation (data not shown). Vice versa, these two localities were also characterized by much higher concentrations of dissolved ferrous iron and dissolved methane than sites located upstream (Table 1). Concentration of ferrous iron reflected the anaerobic conditions of the interstitial

environment and showed the highest concentration in the deepest sediment layer (25-50cm) ($p=0.02$; $n=5$) at locality IV (Table 2). In aquatic environments, iron can be found in two forms. In oxic environments iron occurs in the form of ferric iron (Fe^{3+}) and in anoxic enviroment ferrous iron (Fe^{2+}) dominates. Ferric iron is reduced to ferrous iron in anoxic conditions by bacteria which utilize this reduction process for their growth. This reduction can be carried out using one of several reductans such as hydrogen, pyruvate, lactate, acetate etc. [48].

Localities IV and V are situated in the lowland part of the stream which naturally meanders through an intensively managed agricultural landscape with increasing trophic level in the environment. The river bed sediment in lowland parts is characterised by fine sediment with organic matter accumulation. These budgets of organic matter allow local anoxic conditions. Locality IV, in particular, showed high concentration of organic carbon, ferrous iron and acetate in the sediment. These parameters facilitate the presence of methanogens in sediment and this is supported by the high concentration of dissolved methane in intestitial water and the methanogenic potential measurements (Table 1).

The average annual temperature of interstitial water at localities in the downstream parts of the Sitka stream was about 2.5°C higher than in localities upstream and this may result in higher methane production in the region.

The precursor of methanogenesis, acetate was found in the interstitial water at all study sites and measured regularly at higher concentration with maximum concentration usually during the summer period (Table 2). However, the concentration of other precursors such as propionate, valerate and butyrate were also measured but the values were under detection limits (data not shown).

At locality no. IV the mean methane concentration in the interstitial water ranged between 2262.65 - 3856.01 $\mu g\ L^{-1}$ at 0-25 and 25-50 cm depths, respectively (Table 2). However the differences were not significant ($p=0.11$; $n=5$). Generally, the methanogenic potential (MP) varied around 180 pmol $CH_4\ g^{-1}$ DW h^{-1} (0,21 nmol CH_4.g.WW.h^{-1}, respectively) at the study site. The methanogenic potential was found to be similar in both sediment layers ($p=0.82$; $n=5$) (Table 2). These results show decreased readings compared to our previous study [34], in which a considerable amount of methane production was found in the upper sediment layer. To date, there is no standardised approach to measuring potential methane production and hence different results could be due to different methodologies. The MP range is quite broad and may differ by up to three orders of magnitude (10^{-2} to 10^{1} $\mu mol\ m^{-3}\ s^{-1}$,, 10^{3}-10^{6} $nmol\ m^{-3}\ h^{-1}$ respectively); however, depending on temperature or availability of electrons, it can reach up to 10^{8} $nmol\ m^{-3}\ h^{-1}$. Increasing temperature apparently raises the ability to produce methane [41]. Study of anaerobic incubation of sediment acceptors for methanogens of chalk streams revealed maximum MP at a depth of 6 cm (16.5 CH_4 $nmol\ g^{-1}$ wet sed. h^{-1}) with MP decrease with increasing sediment depth. However these authors only investigated a depth 0-8 cm under the river bed [2].

Although there was found a lot of variability in interstitial methane concentration and methanogenic potential, the values of both the parametres suggest that the studied locality (no. IV) produces a lot of methane and hence would be suitable for analysis of methanogen diversity in the region. Fluctuation in the data even during measurements conducted at the same day should not be suprising when considering the very dynamic system of hyporheic zone. Concentrations of methane may differ by up to several orders of magnitude both in horizontal and vertical profile of the sediments [49].

Total prokaryote cell numbers showed a significantly higher value in the deeper sediment layer (6.18×10^{6} cells g^{-1}DW) than in the upper sediment layer (4.28×10^{6} cells g^{-1}DW) ($p=0.02$; $n=15$). The abundance of methanogenic archaea identified with probe MPB1 was higher in deeper sediment layer, however the values were not significantly different ($p=0.06$; $n=15$) (Table 2). The values of the total cell numbers obtained from river sediment were relatively low compared to that of other sediments which varied from 10^{8} - 10^{10} per g^{-1}DW [50,51]. This could be explained by the use of density centrifugation and sonication which can potentially damage prokaryote communities and influence total cell numbers and diversity.

However, additional purification of cells by a combination of sonication and detergent treatment, followed by density gradient centrifugation is often recommended for soil, sediment or biofilm samples [52]. Even if the total cell numbers are slightly underestimated, sonication and density centrifugation techniques are still powerful enough to enable comparison between equally treated samples [53]. Further, checking of direct microscopic cell counts after sonication showed that the efficiency of the sonication was 85–90% (as shown in our previous experiments). Direct counting of bacteria in sediment is limited due to masking of bacteria by sediment particles. Density centrifugation results in the separation of bacteria from sediment particles and improves the purity of cell suspensions [54].

Identification of methanogens based on mcrA genes

The methanogenic community in the hyporheic sediment of the Sitka stream was also analyzed by PCR amplification, cloning, and sequencing of the methyl coenzyme M reductase (*mcrA*) gene. A total of 60 *mcrA* gene sequences revealed 24 different phylotypes. These phylotypes were clustered into four groups and they confirmed affiliation to *Methanosarcinales* (10 phylotypes), *Methanomicrobiales* (10 phylotypes, one phylotype was found in both sediment layer, respectively) and *Methanobacteriales* orders (3 phylotypes) and uncultutured group (1 phylotype) of methanogens. Six members of the *Methanosarcinales* order were affiliated to the *Methanosarcinaceae* family and the nearest identical uncultured sequences were obtained from fen soil in Germany [55], Tibetian Zoige wetland sediment [56] and rice roots grown in Holland [57]. Four phylotypes were related to the acetoclastic methanogen *Methanosaeta concilii* and showed the highest similarity with sequences retrieved in a meromictic lake sediment in France [58] and flooding soil in Holland [59]. Within the *Methanomicrobiales* order, ten different phylotypes were detected and clustered along with the uncultured

sequences, which were obtained from humic bog lake [60], acidic peatland [61], peat soil from Finland [62], Tibetian wetland soil [56] and rice roots in Holland [57]. Three phylotypes were related to the *Methanobacteriales* order and of these three phylotypes, one was closely affiliated to *Methanobacterium* sp. which was isolated from a Western Siberian peat bog [63] and the second one was clustered with clone originating in flooding soil [59] and rice root samples in Holland [57]. One single clone remains unclassified and was clustered with a sequence, which came from a biogas plant reactor in India [64]. It however showed 76% identity with *Methanobacterium* sp. Most of the clones assigned in this study showed low affiliation with known methanogenic species and were closely related to uncultured methanogens obtained from other similar environments (Figure 1). Some environmental studies also confirmed that some clones may constitute an unclassified methanogenic cluster [56–58].

Members of all three orders were detected in a whole bottom sediment irrespective of depth. The number of clones affiliated with *Methanomicrobiales* predominated in the deeper layer while numbers of *Methanosarcinales* clones in general were higher in the upper sediment layer. However, higher number of *Methanosarcinaceae*-like clones were found in the upper layer and *Methanosaetaceae*-like clones prevalenced in the deeper layer of sediment (Table 3).

The coverage (C) of each clone library, a measure of captured diversity, was calculated as: C=1-(n/N), where *n* is the number of different phylotypes from a clone library that were encountered only once and *N* is the total number of sequenced clones in the library [65]. The coverage of each library was 76,5% for upper sediment layer (0-25 cm) and 76,9% for deeper sediment layer (25-50 cm).

Of the total number of 24 methanogenic phylotypes identified from hyporheic sediment, 13 phylotypes (34 clones, 57% from total number of clones) were found in the upper sediment layer (0-25 cm). One phylotype obtained from the upper layer (1 clone, 3%) was affiliated to the *Methanobacteriales*. Phylotypes related to the *Methanosarcinales* order including 5 phylotypes (20 clones, 59%) *Methanosarcinaceae*-like and one phylotype (1 clone, 3%) *Methanosaetaceae*-like member. Larger number of *Methanosarcinales*-like archaea in the upper sediment layer was also confirmed by FISH analyses (unpublished data). Five phylotypes (11 clones, 32%) retrieved from the upper layer were related to the *Methanomicrobiales*. A single unclassified methanogen clone (3%) was retrieved from the upper sediment layer.

Twelve phylotypes (26 clones, 43% from total number of clones) were detected in the deeper sediment layer (25-50 cm). Six phylotypes (14 clones, 54%) were related to the *Methanomicrobiales*. Four phylotypes (7 clones, 27%) were affiliated to the *Methanosarcinales* order, *Methanosarcinaceae*-like member including one phylotype (1 clone, 4%) and *Methanosaetaceae*-like member consists of three phylotypes (6 clones, 23%). Two phylotypes (5 clones, 19%) of these were associated with the genus *Methanobacterium*,

Our results indicate the presence of both hydrogenotrophic and acetoclastic methanogens in river sediment. These observations are supported by the stable carbon isotope signature of methane ($\delta^{13}CH_4$) which shows that both acetoclastic and hydrogenotrophic pathways take part in methanogenesis along the vertical profile of the Sitka stream [66]. The latest results show that the acetoclastic pathway predominates over the hydrogenotrophic pathway in a whole bottom sediment irrespective of depth and contributes to the methanogenesis in the Sitka stream with approximately 70 - 80% (unpublished data).

Methanomicrobiales group of methanogens only grow in the presence of hydrogen, formate and alcohols with the exception of methanol. The *Methanosarcinaceae* can utilize all methanogenic substrates except for formate but the *Methanosaetaceae* grow exclusively using acetate as an energy source [67] whereas *Methanobacteriales* grow by CO_2 reduction. In the Sitka stream sediment, the number of phylotypes related to *Methanomicrobiales* and *Methanosarcinales* was equivalent. However the number of *Methanosarcinales* clones (n=28) was higher over *Methanomicrobiales* clones (n=25). As suggested in an earlier study [68], members of these two orders may be efficient syntrophic partners in the complete degradation of organic biomass in freshwater sediments. Only one study mentions methanogens in river sediment. This research investigated microbial populations in the extremely metal-contaminated Coeur d'Alene River sediments but the authors found just three methanogen phylotypes related to the *Methanosarcinales* order [69]. Most of the earlier reports on methanogens diversity were came from ruminants [70–72].

Moreover, DGGE analyses based on 16S rDNA of the methanogen community of the Sitka stream hyporheic sediments also retrieved a resembling number of taxonomic units at locality no. IV and this supports the results of this study (unpublished data).

The application of PCR-based technologies for the investigation of naturally occurring methanogen populations has several advantages [72]. These methods are effective for detecting novel sequences, indicating unculturable new species and providing more complete description of the methanogen community structure. However, molecular methods introduce their own bias, such as the favoured lysis of one cell type over another, leading to the recovery of unrepresentative DNA fractions or skewed PCR amplification, where certain bands are favoured over others [33]. Another form of PCR bias is template reannealing which may occur during PCR when a high concentration of a product has accumulated and similar products and templates reanneal to each other, inhibiting primer binding and further amplification of a product [73]. It has also been suggested that the variability in copy numbers and intraspecies and interspecies heterogeneity of functional genes may represent a source of biases in microbial ecological studies [74].

Conclusion

To the best of our knowledge, this study is the first analysis of the methanogenic community composition in river hyporheic sediments with respect to the process of methanogenesis. The presence of methanogenic archaea was detected using *mcrA*

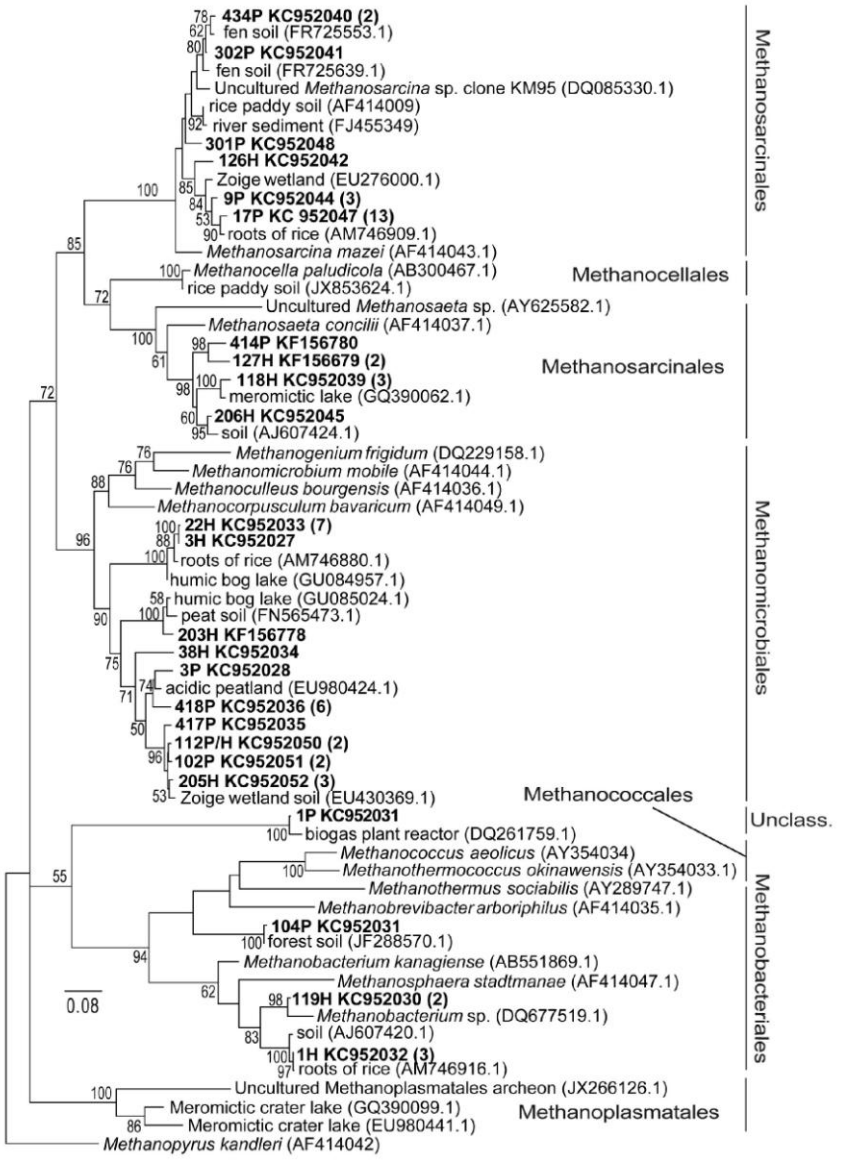

Figure 1. Phylogenetic tree of *mcrA* gene clone (phylotype) sequences retrieved from hyporheic river sediment. The clones come from upper sediment layer (0-25 cm depth) are described as „P", clones come from deeper sediment layer (25-50 cm depth) are described as „H". The numbers in parenthesis indicate the number of clones. The phylogenetic tree is rooted with *Methanopyrus kandleri*.

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Table 3. Number of clones and phylotypes and their phylogenetic affiliation to each library.

Variable/ Depth				
Phylogenetic affiliation	Methanomicrobiales	Methanosarcinales (Methanosarcinaceae/ Methanosaetaceae)	Methanobacteriales	unclassified
No. of clones				
[depth 0-25 cm]	11	21 (20/1)	1	1
[depth 25-50 cm]	14	7 (1/6)	5	0
No. of phylotypes				
[depth 0-25 cm]	5	6 (5/1)	1	1
[depth 25-50 cm]	6	4 (1/3)	2	0
Clones occurrence [%]				
[depth 0-25 cm]	32	59/3	3	3
[depth 25-50 cm]	54	4/23	19	0

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gene marker and FISH (MPB-1 oligonucletide probe) to a 50 cm river sediment depth. The data from the *mcrA* gene sequencing, retrieved a relatively large number of methanogenic phylotypes. These results support our previous measurements and suggest that methanogens contributes significantly to the hyporheic microbial community and may affect CH_4 cycling in the Sitka stream sediments. The results also indicate the presence of both hydrogenotrophic and acetoclastic metabolic pathways in the Sitka river sediment. We hope these findings will be helpful for further research on the ecological function of methanogens in the carbon cycle in river hyporheic sediments.

Supporting Information

File S1. Original data of physico-chemical parameters and variables of the hyporheic interstitial water and sediment used for Table 2 calculation.

(XLS)

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Author Contributions

Conceived and designed the experiments: IB LB MR. Performed the experiments: IB LB VM. Analyzed the data: IB LB VM PD PPC. Contributed reagents/materials/analysis tools: IB LB VM PD. Wrote the manuscript: IB MR.

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V. Methane production potentials, pathways, and communities of methanogens in vertical sediment profiles of river Sitka

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Methane production potentials, pathways, and communities of methanogens in vertical sediment profiles of river Sitka

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Biological methanogenesis is linked to permanent water logged systems, e.g., rice field soils or lake sediments. In these systems the methanogenic community as well as the pathway of methane formation are well-described. By contrast, the methanogenic potential of river sediments is so far not well-investigated. Therefore, we analyzed (a) the methanogenic potential (incubation experiments), (b) the pathway of methane production (stable carbon isotopes and inhibitor studies), and (c) the methanogenic community composition (terminal restriction length polymorphism of *mcrA*) in depth profiles of sediment cores of River Sitka, Czech Republic. We found two depth-related distinct maxima for the methanogenic potentials (a) The pathway of methane production was dominated by hydrogenotrophic methanogenesis (b) The methanogenic community composition was similar in all depth layers (c) The main TRFs were representative for *Methanosarcina*, *Methanosaeta*, *Methanobacterium*, and *Methanomicrobium* species. The isotopic signals of acetate indicated a relative high contribution of chemolithotrophic acetogenesis to the acetate pool.

Keywords: methane production potential, river sediment, stable carbon isotope, isotope fractionation, depth profile, methyl fluoride, mcrA, T-RFLP

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Introduction

Biogenic methane production is carried out by highly specialized, oxygen sensitive methanogenic archaea. Usually methanogenesis is therefore restricted to water-logged systems like freshwater sediments, rice field soils or gut systems (Ciais et al., 2014). Rivers as turbulent systems usually have well-aerated water bodies. Hence they are not considered to be an important source of atmospheric methane (Conrad, 2009; Ciais et al., 2014). Even when the methane emission of different fresh water systems (lakes, wetlands etc.,) is compared, the emission rates of rivers are usually low (**Table 1**).

Methane emission from fresh water systems is usually estimated using the CH_4 released from open water bodies to the atmosphere. These kind of measurements are showing high spatial fluctuations of methane concentrations (Berger and Heyer, 1989; Lilley et al., 1996; Moura et al., 2008; Wang et al., 2009; Gar'Kusha et al., 2010; Striegl et al., 2012; Musenze et al., 2014) as well as seasonal dynamics (Sanders et al., 2007; Gar'Kusha et al., 2010; Musenze et al., 2014).

However, methane measurements of river water body may not give a conclusive picture of the methanogenic potential of river ecosystems, since the well-aerated water bodies render optimal conditions for methanotrophic bacteria possibly scavenging a large portion of the methane

TABLE 1 | Methane emissions from wetlands.

River	Methane emission rate (mg CH_4 m^{-2} h^{-1})	References
River (Nome Creek)[a]	3.5	Crawford et al., 2013
River (Sitka)	0.3–1.6	Hlavacova et al., 2006
Rivers[b]	0.01–6.67	Bastviken et al., 2011
Rivers	10.5	Ortiz-Llorente and Alvarez-Cobelas, 2012
Lakes	18.1	Ortiz-Llorente and Alvarez-Cobelas, 2012
Wetlands	13.6	Ortiz-Llorente and Alvarez-Cobelas, 2012
Estuaries	3.3	Ortiz-Llorente and Alvarez-Cobelas, 2012

[a]*Given as 58.2 nmol CH_4 m^{-2} s^{-1}* [b]*Calculated from Supplementary Material.*

produced in the anoxic parts of the river sediment. Indeed the methane concentrations in the sediment are usually two orders of magnitude higher than in the surface water as can be seen for our study site river Sitka (Hlavacova et al., 2005; Rulik et al., 2013) and several other river sediments (Zaiss, 1981; De Angelis and Scranton, 1993; Trimmer et al., 2009; Gar'Kusha et al., 2010).

In contrast to these *in situ* measurements, which to some extent may be influenced by aerobic methanotrophic activities, the methanogenic production potential of river sediments can be obtained with incubation experiments under strict anoxic conditions in the laboratory. Such experiments have so far only been conducted for mixed top sediments (Jones et al., 1995; Avery and Martens, 1999). In river Sitka preliminary methane production potentials have been estimated with short time incubations under substrate additions (ca 8 μM acetate) (Rulik et al., 2013). Since earlier reports in sediment profiles show vertically dispersed methane concentrations (De Angelis and Scranton, 1993; Schindler and Krabbenhoft, 1998; Gar'Kusha et al., 2010; Chen and Yin, 2013) we decided to test the methanogenic potential of different depth layers of two sediment cores to define whether these differences are due to different methanogenic potentials.

In addition we aimed to differentiate the underlying pathway of methane production. In the well-studied systems (e.g., rice paddies and lake sediments) methane emission can be linked to two dominating processes: acetoclastic (Equation 1) and hydrogenotrophic (Equation 2) methanogenesis:

$$CH_3COOH \rightarrow CO_2 + CH_4 \quad (1)$$

$$CO_2 + 4H_2 \rightarrow 2H_2O + CH_4 \quad (2)$$

To distinguish the two dominant methanogenic pathways the natural abundance of stable carbon isotopes can be used if the $\delta^{13}C$ of methane and of its precursors and the methanogenic fractionation factors are known (Conrad, 2005). The acetoclastic methanogenesis expresses a smaller kinetic isotopic effect (KIE = 1.009−1.027) (Gelwicks et al., 1994; Penning et al., 2006; Goevert and Conrad, 2009) than the hydrogenotrophic methane formation (KIE = 1.045−1.073) (Valentine et al., 2004). The inhibition of acetoclastic methanogenesis by methyl fluoride (CH_3F) allows quantifying the contribution of both pathways (Janssen and Frenzel, 1997; Conrad et al., 2011).

While the acetoclastic pathway is dominating in e.g., rice paddy soils [up to 67% of methane release (Conrad, 1999)] freshwater sediments and gut environments are dominated by hydrogen driven methanogenesis (Conrad, 1999). The hydrogenotrophic contribution to methane relase for White Oak River sediments was reported to be 37–39% (Avery and Martens, 1999).

As a third aspect we were interested in quantifying the methanogenic community in river sediment profiles and contrast these findings to well-described ecosystems: Lake sediments are dominated by *Methanomicrobiales* and *Methanosaetaceae*. They show gradual vertical changes in methanogenic potential, pathway usage and community composition (Chan et al., 2005). Investigations of mudflat sediments of Yangtze River estuary, China showed a dominance of *Methanomicrobiales* and *Methanosarcinales* (Zeleke et al., 2013). In freshwater systems *Methanomicrobiales* have been shown to increase in relative abundance with depth while *Methanosarcinales*/*Methanosaetaceae* decrease (Chan et al., 2005; Zeleke et al., 2013). Oxygenated upland soils contain a less developed methanogenic community than permanently water-logged systems and are dominated by *Methanocellales* and *Methanosarcinales* (Angel et al., 2012). Rice field soils are generally characterized by the most complex methanogenic community (Chin et al., 1999; Lueders et al., 2001; Ramakrishnan et al., 2001), which has been attributed to the seasonal change of oxic and anoxic conditions. We speculated that the methanogenic community of river sediments will be similar to lake sediments.

In this study, we investigated the methanogenic potential, pathway usage and community structure in river sediment depth profiles. We had three main objectives: (1) we wanted to investigate how the potential methane production rates differ over a vertical profile of two sediment cores in order to validate the potential methane emission rates of river sediment compared to other water logged systems. (2) We wanted to characterize the underlying pathway usage of methane production using the natural stable carbon isotope signals. (3) We were interested in comparing the methanogenic community of river sediments to community profiles of other well-characterized soil systems.

In general we hypothesized that river sediments will share some common features with other freshwater sediments but may also have distinct characteristics due to the water movement and the higher oxygen load of the overlaying water.

Methods

Sampling Site

The sampling site is situated ca 10 km north of the city Olomouc in an agricultural field area. Stream width ranges between 4 and 6 m during a year. Bottom sediments are composed of clay, sand and gravel having a median grain size of 0.2 mm. More physicochemical parameters (e.g., grain median size, organic carbon content, dissolved O_2, DOC, interstitial, CH_4 concentration) in the sediments have already been reported (Buriankova et al., 2012) as locality IV.

Sediment Sampling

Two sediments cores (60 cm deep) were collected using the freeze core method (Bretschko and Klemens, 1986) at morning in April 2012. Sediment cores were split up in layers of 10 cm, sieved with distilled deionized water to 1 mm grain size and stored at 4°C under river water in closed plastic jars.

Incubation Experiments

For determining the methanogenic potential of sediment and carbon isotopic composition of methane and carbon dioxide, the samples were incubated in triplicates under wet anoxic conditions: 5 g of wet sediment samples were supplemented with 2 ml of distilled water and placed in 27-ml pressure tubes, closed with butyl rubber stoppers and incubated under N_2 at 25°C; if needed 3% (v/v of the headspace) methyl fluoride (CH_3F) was added to specifically inhibit acetoclastic methanogenesis (Janssen and Frenzel, 1997; Conrad and Klose, 1999). Gas subsamples (0.1–0.4 ml) were taken repeatedly from the headspace using a gas-tight syringe (VICI) and analyzed for concentration and $\delta^{13}C$ of CH_4 and CO_2. Methane production potentials were calculated as slope of the methane concentration over time using at least three data points during the linear phase of methane release. The production potentials are given in nmol CH_4 per gram dry weight (DW) per day. The water content of fresh samples was approximately 24.6% ± 4.

At the end of the incubation, the vials were sacrificed, sediments were centrifuged and the supernatants were filtered through 0.2 − μm polytetrafluoroethylene (PTFE) membrane filters and stored at −20°C for later analysis of concentration and $\delta^{13}C$ of acetate (and other fatty acids).

In-Situ Gas Measurements

At morning time in October 2012, sampling of gas ebullition from river sediments was carried out at the same stream stretch from where sediment cores were collected. Ebullition samples were taken in water depths varying from 30 to 80 cm according to spatial changes in the water level. To collect the samples we modified the method described by Martens et al. (1992). The gas was collected in an inverted funnel (20 cm diameter) and transferred into a 6 ml gas tight syringe. The gas samples (2 ml) were then transferred into 12-ml glass vials containing N_2 previously sealed with butyl rubber stopper. Nine samples were sent for carbon isotopic analysis of methane and carbon dioxide to the Max-Planck Institute for terrestrial Microbiology, Marburg (Germany).

Chemical and Isotopic Analyses

CH_4 was analyzed by gas chromatography (GC) using a flame ionization detector (Shimadzu, Kyoto, Japan). CO_2 was analyzed after conversion to CH_4 with a methanizer (Ni-catalyst at 350°C, Chrompack, Middelburg, Netherlands). Isotope measurements of $^{13}C/^{12}C$ in gas samples were performed on a gas chromatograph combustion isotope ratio mass spectrometer (GC-C–IRMS) system (Thermo Fisher Scientific, Bremen, Germany). The principle operation was described by Brand (1996). The gaseous compounds were first separated in a Hewlett Packard 6890 GC using a Pora Plot Q column (27.5 m length, 0.32 mm internal diameter, and 10 μm film thickness; Chromopack Frankfurt, Germany) at 30°C and He (99.996% purity; 2.6 ml/min) as carrier gas. The sample was run through the Finnigan Standard GC Combustion Interface III and the isotope ratio of $^{13}C/^{12}C$ was analyzed in the IRMS (Finnigan MAT Deltaplus). The reference gas was CO_2 (99.998% purity) (Air Liquide, Düsseldorf, Germany), calibrated with the working standard methylstearate (Merck). The latter was intercalibrated at the Max Planck Institute for Biogeochemistry, Jena, Germany (courtesy of W. A. Brand) against the NBS-22 and USGS-24 standards and reported in the delta notation vs. Vienna Pee Dee Belemnite.

$$\delta^{13}C = 10^3(R_{sample}/R_{standard} - 1) \quad (3)$$

with $R = {}^{13}C/{}^{12}C$ of sample and standard, respectively.

Isotopic analysis and quantification of acetate were performed on a high pressure liquid chromatography (HPLC) system (Spectra System P1000, Thermo Fisher Scientific, San Jose, CA, USA; Mistral, Spark, Emmen, the Netherlands) equipped with an ion-exclusion column (Aminex HPX-87-H, BioRad, München, Germany) and coupled to Finnigan LC IsoLink (Thermo Fisher Scientific, Bremen, Germany) as described (Krummen et al., 2004). Isotope ratios were detected on an IRMS (Finnigan MAT Deltaplus Advantage).

The $\delta^{13}C$ in the organic matter was analyzed at the University of Göttigen (Germany) using an elemental analyzer (Fisons EA 1108) coupled to a mass spectrometer. The C, N, and H content of the sediments were quantified on a CHNS-element analyzer by the Analytical Chemical Laboratory of the University of Marburg.

Calculations

The carbon isotopic signature was given in the delta notation relative to the Vienna Pee Dee Belemnite (V-PDB) standard. The fractionation factor α for a reaction A → B are defined after (Hayes, 1993):

$$\alpha_{A,B} = (\delta^{13}C_A + 10^3)/(\delta^{13}C_B + 10^3) \quad (4)$$

Isotopic calculations of fractionation factors and estimation of the approximate partition of hydrogenotrophic methanogenesis of the total methanogenesis were calculated according to Conrad (2005):

The apparent fractionation factor (α_{app}) for conversion of CO_2 to CH_4 is given by:

$$\alpha_{app} = (\delta CO_2 + 10^3)/(\delta CH_4 + 10^3) \quad (5)$$

where δCO_2 and δCH_4 are directly measured isotopic signatures of the carbon in CO_2 and CH_4, respectively.

Fractionation factor for hydrogenotrophic methanogenesis (α_{mc}) is given by:

$$\alpha_{mc} = (\delta CO_2 + 10^3)/(\delta_{mc} + 10^3) \quad (6)$$

where δ_{mc} is carbon isotopic signature of methane solely produced from carbon dioxide (directly measured from assays

inhibited by methyl fluoride). Partition of hydrogenotrophic methanogenesis is calculated by the following mass balance Equation (7):

$$f_{mc} = (\delta_{CH4} - \delta_{ma})/(\delta_{mc} - \delta_{ma}) \quad (7)$$

where f_{mc} is the partition of hydrogenotrophic methanogenesis and δ_{ma} is carbon isotopic signature of methane solely produced from acetate. It is calculated from the following equation:

$$\delta_{ma} = (1/\alpha_{ma})(\delta_{ac} + 10^3 - \alpha_{ma}{}^*10^3) \quad (8)$$

where α_{ma} is fractionation factors for acetoclastic methanogenesis and δ_{ac} is the measured isotopic signal of acetate. Several published α_{ma} have been used to estimate the contribution of hydrogenotrophic methanogenesis e.g., (Gelwicks et al., 1994; Penning et al., 2006; Goevert and Conrad, 2009).

Molecular Analyses

DNA was extracted from the fresh sediment before the start of the incubation and at the end of the incubations (with and without methyl-fluoride) using the PowerSoil DNA Isolation Kit (MO BIO, USA), according to the manufacturer's instructions. The extracted DNA was used to characterize the *mcrA* gene by T-RFLP (Terminal-restriction length polymorphism) according to Chin et al. (Liu et al., 1997; Chin et al., 1999) using the primers *mcrA* f (TAY GAY CAR ATH TGG YT) and *mcrA* r (ACR TTC ATN GCR TAR TT) published by Springer et al. (1995) with a FAM (6-carboxyfluorescein)-label at the forward primer. The *mcrA* gene amplicons were digested with Sau96I (Fermentas), and the products were size-separated in an ABI 3130 DNA sequencer (Applied Biosystems, Darmstadt, Germany). For downstream analysis only fragments between 80 and 520 bp have been considered to avoid analysis of false signals originated from primer residuals, primer dimmers, and undigested PCR product. The normalization and standardization of the T-RFLP profiles was done according the method from Dunbar et al. (2001). The relative abundance was calculated using the ratio between the height of the fluorescence signal and the total height of all signals in one sample. To assign the resulting fragments we used a clone library which was constructed in our lab in a framework to characterize the methanogenic community at different locations and depth of River Sitka (Figure S9). The dominant peaks well-reflect published literature values of other water logged systems (Lueders et al., 2001; Ramakrishnan et al., 2001; Chin et al., 2004; Kemnitz et al., 2004; Conrad et al., 2008).

Results

All samples of core I and almost all samples of core II (except the 20–30 and 30–40 cm depth and the 10–20 cm depth under inhibited conditions) released methane and all samples released carbon dioxide under the chosen incubation conditions (**Figure 1**, Figures S1, S2). Both cores showed the same vertical pattern of methane emission rates (**Figure 1**, Figure S1): The highest average methane production rates (up to 34 ± 11 nmol CH_4 g^{-1} DW day^{-1}) were found in the top 10 cm and in the 40–50 cm depth layer. The 10–40 cm depth layers as well as the 50–60 cm depth layer proved low methane production rates (below 9 ± 9 nmol CH_4 g^{-1} DW day^{-1}) for the first core and negligible if any methane production for the second core. Roughly threefold more methane was released under uninhibited conditions in the top 10 cm; the 40–50 cm peak was doubled in the absence of methyl fluoride. In the presence of methyl fluoride methane production rates followed the same pattern, again showing the highest values in the 40–50 cm depth layer of both cores.

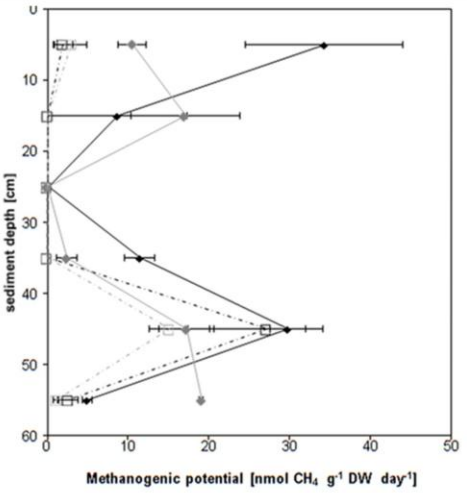

FIGURE 1 | Vertical profile (60 cm depth sampled in 10 cm slices) of the methanogenic potential of two sediment cores. Core I uninhibited control N_2♦, core I methyl fluoride (N_2 + 3% CH_3F)◆, core II uninhibited control N_2□, core II methyl fluoride (N_2 + 3% CH_3F)□. The methanogenic potential (in nmol per g dry weight (DW) per day) has been calculated using the slope of the methane concentration over the last 10–11 day of the incubation (compare Figure S1). The values of the individual layers (e.g., 0–10 cm) are given as average (e.g., 5 cm) The rates are given ± standard deviation ($n = 3 - 5$).

The concentrations of free carbon dioxide in the headspace increased in all sediment layers under all tested treatments in both cores (Figure S2). During the methanogenic lag phase carbon dioxide concentrations of both cores increased faster and later on the increase was slowing down up to the end of incubation. The upper 10 cm of both cores showed the highest concentrations. Generally methyl fluoride amendment did not systematically affect the carbon dioxide concentrations.

In both cores the $\delta^{13}C$ of methane for uninhibited controls was in the range of −98.6 to −48.2‰ and for inhibited incubations in the range of −116.3 to −74.5‰ (**Figure 2**, Figure S3). The $\delta^{13}C$ of methane was not affected by the sampling depth. The *in-situ* $\delta^{13}C$ of methane (−59.0 ± 1.2‰, $n = 9$) was very close to the methane measured in the maximum methanogenic depth layers for uninhibited control assays (−59 to −62‰).

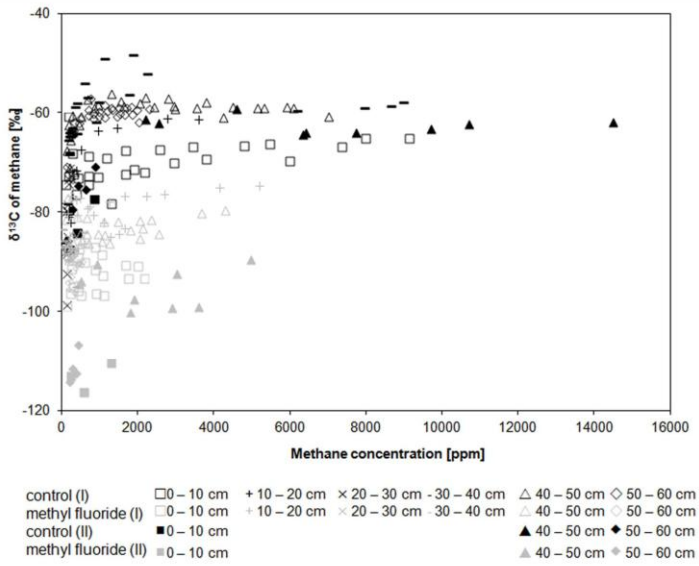

FIGURE 2 | Course of the $\delta^{13}C$ of CH_4 along CH_4 concentrations in both inhibited and uninhibited control assays for core (I) open symbols and core (II) filled symbols. Uninhibited control incubations (N_2) in black, methyl fluoride inhibited samples (N_2 + 3% CH_3F) in gray. Details are likewise plotted in Figures S1, S3.

The $\delta^{13}C$ of carbon dioxide was irrespective of the treatment during the incubations in the range of −18.8 to −36.0‰ for all depth layers and both cores (Figure S4). The initially light CO_2 (−18.8 to −25.3‰) usually became heavier during the incubation; only the samples showing high methane production rates had lighter CO_2 in the end (Figure S4). The *in-situ* $\delta^{13}C$ carbon dioxide was slightly heavier (−16.3 ± 1.2‰, $n = 9$).

We calculated the apparent fractionation (α_{app}) for uninhibited control and inhibited samples using Equations (5) and (6), respectively, (**Figure 3**). While the apparent fractionation of core I for the uninhibited samples was on average 1.046 ± 0.009 ($n = 54$) ranging from 1.039 (50–60 cm) to 1.062 (20–30 cm), the inhibited samples were approximately 20‰ more depleted in ^{13}C: 1.065 ± 0.006 ($n = 43$) ranging from 1.057 (10–20 cm) to 1.073 (0–10 cm). Only three depth layers (0–10, 40–50, and 50–60 cm) of core II could be fully evaluated using prolonged incubation times (30–80 days). The apparent fractionation of the uninhibited samples ranged from 1.039 to 1.065. The inhibited samples again were approximately 29‰ more depleted in ^{13}C and ranged from 1.069 to 1.088. It is worth noting that the two depth layers with the highest methane production potentials showed distinct apparent fractionations: in the top layer the average apparent fractionation was 1.073 in the inhibited and 1.050 in the uninhibited samples; in the 40–50 cm depth layer the apparent fractionation was 1.062 and 1.040, respectively. The average apparent fractionation factor for the *in situ* samples was 1.045 ± 0.002 ($n = 9$).

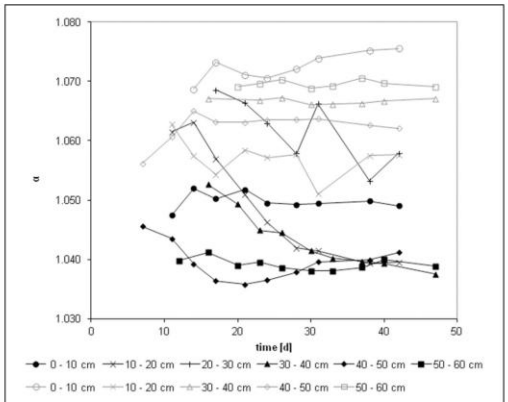

FIGURE 3 | Course of fractionation factors during incubations: apparent fractionation factor(α_{app}) for the core I. Uninhibited incubations in black, methyl fluoride inhibited samples in gray.

Carbon contents in incubated sediments are listed in **Table 2**. They showed no vertical pattern but differed in the two sediment cores. The average carbon isotope values of organic matter was −26.3‰ (± 0.1‰, $n = 12$). The acetate concentrations at the end of the incubations stayed at a relatively low level (<0.02 mM

TABLE 2 | Depth profiles of two sediment cores.

Depth [cm]	C_{soil} [%]	$\delta^{13}C_{soil}$ [‰]	Uninhibited		methylfluoride	
			acetate [mM]	$\delta^{13}C_{acetate}$ [‰]	acetate [mM]	$\delta^{13}C_{acetate}$ [‰]
CORE I.						
0–10	0.6	−26.2	n.d.	n.d.	1.53 ± 0.6	−42.0 ± 1.2
10–20	2.3	−25.9	n.d.	n.d.	0.47 ± 0.17	−47.8 ± 7.9
20–30	0.9	−25.8	n.d.	n.d.	n.d.	n.d.
30–40	2.9	−26.8	0.02 ± 0.00	−27.4 ± 1.4	0.58 ± 0.16	−34.9 ± 1.9
40–50	2.3	−26.8	0.03 ± 0.00	−27.8 ± 0.2	0.79 ± 0.07	−34.3 ± 1.0
50–60	1	−26.3	0.02 ± 0.00	−29.6 ± 0.4	0.08 ± 0.06	−31.0 ± 1.2
CORE II.						
0–10	0.5	−26.4	n.d.	n.d.	0.31 ± 0.41	−50.7 ± 1.0
10–20	0.7	−26.3	n.d.	n.d.	n.d.	n.d.
20–30	0.9	−26.3	n.d.	n.d.	n.d.	n.d.
30–40	0.7	−26	n.d.	n.d.	n.d.	n.d.
40–50	6.8	−26.7	0.02 ± 0.00	−27.5 ± 1.0	1.16 ± 0.27	−40.7 ± 4.9
50–60	2.4	−26.2	n.d.	n.d.	0.02 ± 0.01	−38.6 ± 1.3

Soil carbon content and delta ^{13}C values of the original sediments are given together with the acetate concentrations and isotopic signals of uninhibited and inhibited incubation (CH_3F) experiments at the end of the incubation. n.d. not detected. Values are given ± standard deviation n = 3.

uninhibited; up to 1.5 mM under CH_3F) again showing a peak in the top 10 cm and for the 40–50 cm depth layer. The $\delta^{13}C$ values of acetate were in the range of −50.7 to −31‰ and −30.8 to −27.5‰ for inhibited and uninhibited incubation assays respectively, (**Table 2**). For all sediment samples, the $\delta^{13}C$ of produced acetate was lower than the $\delta^{13}C$ of organic matter. Other parameters (e.g., H and N content) are listed in Table S1.

The contribution of hydrogenotrophic methanogenesis (f_{mc}) was calculated by Equation (7) incorporating measured $\delta^{13}C$ of methane (δ_{CH4}), methane produced purely from hydrogenotrophic methanogenesis (δ_{mc}) and an estimate for the methane produced from acetate (δ_{ma}) based on measured ^{13}C acetate and fractionation factors of acetoclastic methanogenesis presented in literature. The time courses of f_{mc} in the core (I) calculated with $\alpha_{ma} = 1.009$ (Goevert and Conrad, 2009) is shown in **Figure 4**. In the beginning almost all methane was produced from hydrogen; later the contribution of hydrogenotrophic methanogenesis dropped to about 40%. In core II only three depth layers could be evaluated during the second half of the incubation period. These samples showed a contribution of 26–45% of hydrogenotrophic methanogenesis to the released methane.

The molecular analysis of the methanogenic marker-gene (*mcrA*) revealed a significant different methanogenic community for the top layer in contrast to deeper layers (**Figure 5**). The community profile (T-RFLP of *mcrA*) resolves in up to 11 fragments (Figure S8). The microbial community was not affected by the incubation under N_2 or $N_2 + CH_3F$. In all depth layers and under all incubation conditions *Methanosarcinacea* were the dominant group (22–52%) followed by *Methanobacteriacea* (24–56%); *Methanomicrobiales* were only detectable in the two active layers (up to 12%). *Methanosaetacea* were almost absent in the top layer (below 3%) and reached a higher relative abundance in deeper layers (10–25%). The samples of core II have not been analyzed by T-RFLP. However, a core sampled in 2014 at the same location did confirm the overall pattern of the T-RFLP but showed a more gradual change of the community over the depth profile.

Discussion

Methane Production Potentials in River Sediments

Estimations of the methane production potentials of river sediments have so far only been made for mixed top sediments: e.g., White Oak River sediment incubations at 25°C had methane production potentials of approximately 250 nmol gDW^{-1} d^{-1} (originally given as 8 μM hr^{-1}) (Avery and Martens, 1999). Incubations of fresh top sediment layers or river Sitka sampled in spring 2014 and incubated under similar conditions as reported in this study resulted in more than tenfold larger methane production potentials of 469 nmol gDW^{-1} d^{-1} (Bednarik unpublished) compared to 34 nmol CH_4 gDW^{-1} d^{-1} reported for the top 10 cm in this study. While top sediments of the Elbe River had maximum potential methane production rates of 552 nmol gDW^{-1} d^{-1} (Matoušů in preparation). This would suggest that the methane production potential of river sediments reaches methane production potentials up to 552 nmol gDW^{-1} d^{-1}. For comparison lake sediments have a methane production potential of e.g., 9–3380 nmol gDW^{-1} d^{-1} (Conrad et al., 2010, 2011) while rice field soils show methane production rates of 3360–7920 nmol gDW^{-1} d^{-1} (Conrad et al., 2009a, 2012).

In order to better understand the methanogenic potential of river sediments we incubated several depth layers of two sediment cores under anoxic conditions in the laboratory. While published data of *in situ* measurement of methane concentrations in river sediments pointed to diverse vertical profiles, reaching

several hundred μM (**Table 3**) it is as well-possible that the *in situ* concentrations are independent from the underlying methane production potential.

Indeed we find two distinctive peaks of methane production in the present study (up to 34 nmol gDW^{-1} d^{-1}) which correlated with higher CO_2 production in these layers and acetate accumulation in the inhibited samples. These peaks are present in both cores which have been separately analyzed. The earlier reported preliminary methane production potentials for River Sitka under substrate addition (ca. 8 μM acetate) were much lower (below 6 nmol gDW^{-1} d^{-1}) and only based on two time points and a very short incubation time (72 h) (Rulik et al., 2013). Since we could show that roughly 40% of methane is produced hydrogenotrophically, these short time incubations under substrate addition may not reflect the natural conditions. However, already these incubations showed two distinct peaks for the top sediment and 40–50 cm depth. In this respect it is worth to note that the lag phase of our incubation experiments lasted for about 15–35 days (Figure S1), which is most probable due to the presence of other electron acceptors which have to be depleted before methanogenesis starts.

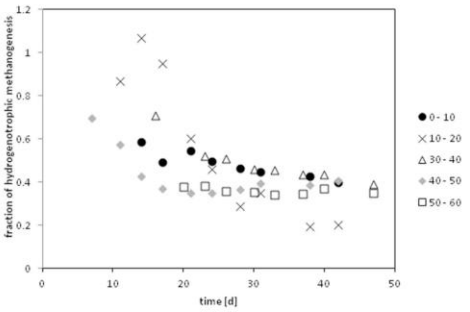

FIGURE 4 | Relative contribution of hydrogenotrophic methanogenensis to the released methane of the depth profile of core I. Calculated assuming a fractionation factor of $\alpha_{app} = 1.009$ for acetoclastically produced methane. (Compare Figures S5, S6). The 20–30 cm depth did not release methane under inhibited conditions; hence the contribution of hydrogenotrophic methanogens could not be calculated for that sample.

The methane production potential of the top layer is paralleled by high oxygen saturation (>80%) (Rulik et al., 2013), low *in situ* methane concentrations (**Table 3**) and high activities of methanotrophic bacteria [Figure S7 and (Rulik et al., 2013)]. The second peak goes along with lower oxygen saturation (17.5%) (Rulik et al., 2013), intermediate *in situ* methane concentration, and reduced methanogenic activity. However, it is presently not clear why the intermediate zone (10–30cm) shows almost no methanogenic potential.

Methanogenic Pathways in River Sediments

Our result shows that carbon isotopic values of methane measured both *in situ* (−59‰) and in different incubations of depth layers (−68 to −59‰) were in the broad range of $\delta^{13}C$ of methane measured in other studies in rivers e.g., *in situ* measurements from the Amazonian rivers ranged from −75

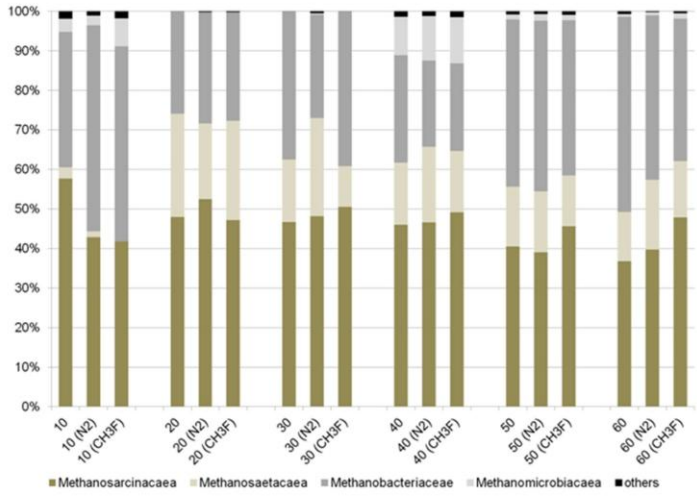

FIGURE 5 | Relative abundance of important methanogenic groups as determined by T-RFLP for core I (different depth layers up to 60 cm depth). Results are given for samples before the incubation as well as at the end of incubations under N_2 (control incubations) or N_2 + 2% CH_3F (inhibition of acetoclastic methanogenesis). Details on T-RFLP results are given in Figure S8.

TABLE 3 | Methane concentration in depth profiles of river sediments.

River	Sample description	Depth (cm)	CH_4 concentration (μM)	References
Hudson	Station 118 Aug. 23, 1991	50	0.105	De Angelis and Scranton, 1993
		1000	0.99	De Angelis and Scranton, 1993
		2000	0.108	De Angelis and Scranton, 1993
Severnaya Dvina River	Station 20	0-5	0.5	Gar'Kusha et al., 2010
(White Sea)		5–10	3	Gar'Kusha et al., 2010
Allequash Creek	Lower site	20	430	Schindler and Krabbenhoft, 1998
(Wisconsin, USA)		60	410	Schindler and Krabbenhoft, 1998
		180	400	Schindler and Krabbenhoft, 1998
	Upper site	top	2	Schindler and Krabbenhoft, 1998
Jiulong River Estuarine		90	6	Chen and Yin, 2013
		100–140	2–3	Chen and Yin, 2013
		150	6	Chen and Yin, 2013
Sitka	Location IV	10	20	Rulik et al., 2013
		20	175	Rulik et al., 2013
		30	300	Rulik et al., 2013
		40	175	Rulik et al., 2013
		50	260	Rulik et al., 2013

to −53‰ (Moura et al., 2008) but slightly heavier than methane collected from interstitial water at 40–50 cm depth in Sitka (−72 to −68‰) (Rulik et al., 2013).

Assuming complete inhibition of acetoclastic methanogenesis in the presence of methyl fluoride (CH_3F) the isotopic signal of the methane can be completely attributed to hydrogenotrophically produced CH_4 (δ_{mc}). The range for the apparent fractionation reported in our study (α_{app} = 1.04 to 1.06) have quite commonly been observed in e.g., rice field soils (Sugimoto and Wada, 1993; Chidthaisong et al., 2002; Penning and Conrad, 2007; Conrad et al., 2009b).

The fractionation factor (α_{ma}) during conversion of total acetate to methane in *Methanosarcina acetivorans* and *M. barkeri* ranges from α_{ma} of 1.012–1.027 (Gelwicks et al., 1994; Conrad, 2009; Goevert and Conrad, 2009), whereas isotope fractionation in *Methanosaeta* spp. is weaker, i.e., α_{ma} of 1.007–1.009 (Valentine et al., 2004; Penning et al., 2006). From an earlier study, it was found that both acetoclastic genera *Methanosarcina* spp. and *Methanosaeta* spp. occur in Sitka sediments (Buriankova et al., 2013). Therefore, we calculated the contribution of hydrogenotrophic methanogenesis with all published fractionation factors ranging up to α_{ma} = 1.027 (Figure S5). However, α_{ma} = 1.009 is maybe most reasonable because fractionation factors of acetoclastic methanogenesis under environmental settings are approximately 5–10‰ less negative than in pure culture, which is probably due to limitation by acetate (Penning and Conrad, 2007; Conrad, 2009; Goevert and Conrad, 2009). We used the isotopic value of the total acetate for our calculations, which may be incorrect since we find a high contribution of acetogenesis to the acetate pool (compare Discussion below). If we use the isotopic signal of the soil organic carbon as a proxy for the acetate values we get almost the same results (Figure S6 and accompanying discussion). When carbohydrates are methanogenically degraded f_{mc} is expected to be 33%, which is commonly observed in e.g., rice field soils (Conrad, 1999). Other environments like e.g., lake sediments can have much larger contributions of hydrogenotrophically produced methane (Conrad, 1999). Estimates of f_{mc} for White Oak River sediments were reported to be 37–39% (Avery and Martens, 1999) which is in good agreement with our own results (40%). Comparing the different layers of our depth profile it was found that the upper maximum (0–10 cm) produce slightly more hydrogenotrophic methane 42–51% than the 40–50 cm layer 36–46%.

Isotope Fractionation during Acetate Production

While the major sink of acetate in methanogenic environments is methane two dominant mechanisms are known to replenish the acetate pool: Acetate is produced either by fermentation of organic matter or by reduction of CO_2 with H_2 via the acetyl-CoA pathway (acetogenesis) (Drake et al., 2006). Hence the *in-situ* $\delta^{13}C$ value of acetate is influenced by all three reactions (Heuer et al., 2010; Conrad et al., 2014). Acetoclastic methanogenesis has a moderate fractionation around α = 1.01 (see Discussion above), fermentation has only a very weak preference for either carbon isotope [α < 1.009 (Blair et al., 1985; Penning and

Conrad, 2006)], a stronger preference for light carbon has been determined for the acetyl-CoA pathway [$\alpha = 1.06$ (Gelwicks et al., 1989; Blaser et al., 2013)]. In principle syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis is an alternative route to deplete acetate (Zinder and Koch, 1984; Conrad and Klose, 2011; Rui et al., 2011; Dolfing, 2014).

As a result of all three reactions the acetate signatures in environmental samples are usually in the range of the soil organic carbon (± 10‰) e.g., (Conrad et al., 2011, 2014). The presence of methyl fluoride blocks the only acetate depleting reaction (in our experimental set up) and hence results in an accumulation of acetate. In most studies this acetate however does no significantly differ from the acetate signature of the uninhibited control incubations under N_2 e.g., (Heuer et al., 2010; Conrad et al., 2011). In our sample the acetate signatures of the uninhibited samples are similar to the ^{13}C values of soil organic carbon, while inhibited samples are always depleted in ^{13}C relative to the soil organic carbon (−5 to −24‰; compare **Table 1**). This may point to a relative high contribution of the strong fractionating acetyl-CoA pathway to the acetate signature under these conditions.

If we assume complete inhibition of acetoclastic methanogenesis in these samples and no fractionation during fermentation, the contribution of the acetyl-CoA pathway can be calculated to be 8–41% (Table S2). If a stronger fractionation during fermentation ($\alpha = 1.01$) is assumed the contribution is between 0 and 29%. In comparison we calculated a lower contribution of acetogenic bacteria for data published by Conrad et al. (2011) on anoxic lake sediments: 0–19% (no fractionation scenario) or 0–3% ($\alpha = 1.01$).

Under methyl fluoride inhibition the acetyl-CoA pathway competes with hydrogenotrophic methanogenesis for the substrates hydrogen and carbon dioxide which are either reduced to acetate or to methane. Since methanogenesis is energetically more favorable than acetogenesis (131 vs. 95 kJ mol^{-1}) it outcompetes acetogenesis in many environments (Kotsyurbenko et al., 2001). Acetogenesis can become dominating under elevated hydrogen partial pressures: e.g., Heuer et al. reported strongly depleted acetate ($\delta_{acetate} = -48.8$‰ for lake sediments incubated under elevated hydrogen partial pressure (Heuer et al., 2010). Likewise low temperatures favor the prevalence of acetogens over hydrogenotrophic methanogens (Kotsyurbenko et al., 2001). Oxygen is a third factor in favor of acetogenic bacteria which are better adopted to aerated environments than methanogens (Kuesel and Drake, 1995).

Our data suggest that acetogenic bacteria contribute up to 40% of the produced acetate in river sediments (under CH_3F inhibition) and that they can effectively compete with hydrogenotrophic methanogens. Therefore, acetogens may play an important yet not well-characterized role in river sediment ecology.

Methanogenic Community Profile

The methanogenic community based on T-RFLP of *mcrA* has so far primarily been described for rice field soils (Lueders et al., 2001; Ramakrishnan et al., 2001; Chin et al., 2004; Kemnitz et al., 2004; Conrad et al., 2008). Most of the fragments we found in the clone library of river systems were identical with previously published T-RF's. The only exception was the 473 bp fragment, which is distinct from the 470 bp fragment of *Methanobacteria* (Lueders et al., 2001; Chin et al., 2004) and could be assigned to the order of *Methanomicobiales* using cloning and sequencing (Figure S9). This fragment was only present in the two layers showing high methanogenic potentials. The absence of *Methanosaetacea* in the top layer is plausible since they are commonly found in permanent anoxic systems like fresh water sediments (Banning et al., 2005; Chan et al., 2005; Youngblut et al., 2014) but only dominate in rice paddies when acetate is scarce (Lueders et al., 2001; Ramakrishnan et al., 2001; Chin et al., 2004; Kemnitz et al., 2004; Conrad et al., 2008). This has been attributed to a reduced stress tolerance of these strains e.g., lower oxygen tolerance (Erkel et al., 2006; Yuan et al., 2011). Molecular data based on the *mcrA* gene suggest that the methanogenic community is stable over the depth (ca. 10^7 *mcrA* copies g^{-1} DW, Chaudhary et al., in preparation). Likewise the pathway usage (compare Discussion above) is only mildly affected by the sediment depth. It is therefore most plausible that the differences in the methane production potential are caused by the activity of different methanogenic archaea and may as well be influenced by substrate availability. Indeed the 40–50 cm depth peak has the highest organic carbon content in core II (compare **Table 2**).

Our study revealed no difference in the T-RFLP profiles before and after incubations suggesting that the methanogenic community was rather stable over the approximately 2 month incubation period. Similar results have been found for rice field soil incubations (Yuan et al., 2011; Ma et al., 2012) and river sediment (Beckmann and Manefield, 2014). It can therefore be assumed that the differences in the methanogenic potential are regulated on the RNA or activity level of *mcrA* rather than caused by growth of the methanogenic archaea. This would also explain why the second methanogenic peak in the potential measurements (40–50 cm) could not be anticipated by the molecular data alone. The presence of methyl fluoride did not impact the T-RFLP profiles. This is in agreement with Daebeler et al. which showed that the presence of methyl-fluoride impacts the methanogenic activity rather than changing the community composition of methanogenic archaea (Daebeler et al., 2013).

Conclusions

Our experiments show that methane is produced in anoxic incubations of river sediment cores. Methane production is vertically organized showing two distinct maxima in the top layers and in 40–50 cm depth. The magnitude of the calculated methane production rates in rivers covers a broad range but is on average lower than the reported potential of other water logged systems (lakes, rice paddies). Likewise, the pathway usage (contribution of hydrogenotrophic methanogenesis) is comparable to previously studied fresh water systems. Under methyl fluoride inhibition the ^{13}C value of acetate is unusually light pointing to a high contribution of acetogenic bacteria. The methanogenic community composition was different in the top

sediment while the lower segments share similar methanogenic fingerprints.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00506/abstract

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VI. PUBLIKAČNÍ ČINNOST

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VII. ŽIVOTOPIS