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Ageing of organic matter in incubated freshwater sediments; inferences from C and H isotope ratios of methane

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The freshwater sediments were incubated under anaerobic conditions for 570 and 879 days to investigate the potential variations in methanogenic pathways due to increasing sediment age and recalcitrance of organic matter. The methanogenic pathways did not shift from acetate fermentation toward CO_2 reduction, as indicated by the observed variations of the isotopic composition of methane in natural conditions. It appeared, however, that the observed decrease of methane concentration (from 86 to 39%) and continuous increase in $\delta^{13}C(CH_4)$ (from –69.7 to –59.0‰) and $\delta D(CH_4)$ values (from –381 to –320‰) resulted mainly from exhaustion of at least one methanogenic substrate in the incubated sediments. To better understand processes controlling the variations of $\delta^{13}C(CH_4)$ and $\delta D(CH_4)$ values relative to ageing of organic matter, the method of principal component analysis (PCA) was used. This method offers good comparison of the relationships between variables when a larger number of parameters control a given process in the same time period. In this study, the PCA indicated three distinctive factors that controlled decomposition of organic matter during the incubation. Factor 1 explained 33% of observed variations among the variables and had positive (0.93–0.92) loadings for electric conductivity and DIC concentration and negative loading for $\delta^{13}C(CH_4)$ values (–0.72). Factor 2 accounted for 28% and had high positive loading for $\delta D(CH_4)$ value (0.86) and high negative loading for methane concentration (–0.81). Factor 3 accounted for 19% and exhibited high positive loadings for temperature (0.90) and $\delta^{13}C(DIC)$ value (0.69). Factors 1 and 2 were directly linked to the methanogenesis and indicated that bigger accumulation of bio-products in sediments is likely important for variations of $\delta^{13}C$ and δD of methane. This study shows that method of principal component analysis might be a useful tool while studying biogeochemical carbon cycle during early digenesis of freshwater sediments.

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INTRODUCTION

Methane is one of the end products of microbial decomposition of organic matter. It is produced in marine and freshwater anaerobic sediments mainly due to disproportionation of acetate (acetoclastic methanogenesis) and CO_2 reduction by H_2 (hydrogenotrophic methanogenesis) (e.g., Schoell, 1980; Whiticar *et al*., 1986). The carbon isotope analyses are commonly used to distinguish methanogenic pathways and to determine sources of methane. The carbon isotope mass balance of methane is often used in atmospheric studies (e.g., Wahlen *et al*., 1989; Quay *et al*., 1991). To obtain a correct isotope mass balance of methane, the representative $\delta^{13}C(CH_4)$ values of each contributing source have to be known. Such calculations, however, are difficult because carbon isotope composition of methane undergoes spatial and temporal variations (e.g., Martens *et al*., 1986; Whiticar *et al*., 1986; Jędrysek, 1995; Blair, 1998; Hornibrook *et al*., 2000). The mechanism of these variations is still enigmatic, but four main factors are generally considered to be responsible for these variations:

1. Changes of methanogenic pathways;
2. Availability of substrates (e.g., Miyajima *et al*., 1997; Hornibrook *et al*., 1997, 2000; Waldron *et al*., 1998; Jędrysek, 1999; Lojen *et al*., 1999);
3. Temperature variations (Games *et al*., 1978; Krzycki *et al*., 1987; Gelwick *et al*., 1994);
4. Distribution of labile organic carbon in depth profiles of sediments or water reservoirs (e.g., Hornibrook *et al*., 1997, 2000; Jędrysek, 1997*a*, *b*, 2005).

The importance of each factor may vary and change significantly in particular environments.

It is believed that changes of methanogenic pathways are mainly controlled by the availability of substrates. Methanogens are *Archaea* strains and stay at the end of food chain in anaerobic environments. They are strongly dependent on other microbial products of decomposition of organic matter, mainly delivered by fermentative, acetogenic and homoacetogenic bacteria. It is well evidenced that acetoclastic methanogenesis predominates in freshwater sediments (Takai, 1970; Winfrey and Zeikus, 1979*a*, *b*; Lovely and Klug, 1982; Phelps and Zeikus, 1984) that are distinctly enriched in labile organic substances. The hydrogenotrophic methanogenesis is more abundant in marine sediments (e.g., Whiticar *et al*., 1986) that are depleted in labile organic carbon due to elevated concentrations of sulfate and activation of sulfate microbial reducers. These microorganisms may easy outcompete methanogens for labile substrates (e.g., Lovley and Klug, 1983).

Carbon isotope fractionation during the methanogenesis is, to large extent, controlled by temperature. Games *et al*. (1978), Krzycki *et al*. (1987) and Gelwick *et al*. (1994) demonstrate that increasing temperature lowers carbon isotope fractionation between substrates and methane, thus causing the increase of $\delta^{13}C(CH_4)$ value. The isotope fractionation factor may vary from 1.021 to 1.079, and is distinctly smaller for acetoclastic methanogenesis (Sugimoto and Wada, 1993; Gelwick *et al*., 1994) than hydrogenotrophic methanogenesis (Games *et al*., 1978; Krzycki *et al*., 1987). The temperature also affects other microorganisms in the anaerobic chain of decay that provide methanogens with substrates. Typically, at lower temperatures, the supply of substrates for methanogens, that are products of metabolism of secondary fermenters, is limited. For example, the hydrogenotrophic methanogens may be dependent on H_2 availability, which is the other important substrate for this type of methanogenesis. Schütz *et al*. (1990) and Chin and Conrad (1995) showed that H_2 production in anaerobic sediments was strictly controlled by temperature and its higher production rates took place at higher temperatures. For that reason it is believed that hydrogenotrophic methanogens may be more restricted by H_2 supplies than temperature. It should be pointed out, however, that the influence of temperature on methanogenic pathways was demonstrated for natural samples from only one site (Swenson, 1984). This author noticed that different optimum temperature for particular methanogens involved changes in methanogenic pathways, hence the change in $\delta^{13}C(CH_4)$ value, as the methanogens utilizing acetate are more active at lower temperature (the optimum is at 20°C) than hydrogenotrophic methanogens (the optimum is at 28°C). Consequently, it may be expected that, in some cases, $\delta^{13}C(CH_4)$ values would be higher at lower temperatures, when acetoclastic methanogenesis is dominant, and lower at higher temperatures when hydrogenotrophic methanogenesis is dominant.

The most enigmatic is the understanding of the spatial redistribution of methanogenic pathways in depth profiles of sediments and water column. A decreasing trend of $\delta^{13}C(CH_4)$ values with depth of sediments (e.g., Jędrysek 1997*b*, 1998; Hornibrook, 1997, 2000) lead to a conclusion that acetoclastic methanogenesis changes toward CO_2 reduction with increasing depth. It is in a good agreement with a distribution of labile organic carbon, which decreases downward in sediment profiles and a bigger carbon isotope fractionation resulting from the increasing hydrogenotrophic methanogenesis. It does not correlate, however, with increasing methane concentration downward of sediment depth and optimum temperatures for particular methanogens in which the methanogesis is the most efficient (e.g., Waldron *et al*., 1998). The most problematic in this case is that the deeper layers of sediments store the produced gases from several seasons or years. Therefore, the $\delta^{13}C(CH_4)$ values represent a sum of different processes of organic matter decomposition and may be controlled by several factors which variations depend on time of sediment deposition and recalcitrance of organic matter as well as the activity and character of microbial community. The observed similar decrease in $\delta^{13}C(CH_4)$ values with decrease of water column in lakes was also explained by changes of methanogenic pathways from acetoclastic to hydrogenotrophic methanogenesis with increasing depth of the water column (Jędrysek, 1997*b*, 1999, 2005).

In this paper, we investigate the changes of methanogenic pathways using carbon and hydrogen isotope composition of methane relative to the availability of methanogenic substrates and time of decomposition of organic matter in freshwater sediments as well as the concentration of DIC (dissolved inorganic carbon) in water column. Miyajima *et al*. (1997) has evidenced that increased recalcitrance of soil organic matter results in greater production of CH_4 through the hydrogenotrophic methanogenesis in tropical wetlands. The same conclusion was drawn by Hornibrook *et al*. (2000) for freshwater wetlands. They suggested that lower rates of acetoclastic methanogenesis with increasing depth was a result of decrease of labile organic carbon with increasing recalcitrance of biodegraded organic matter. On the other hand, ageing of sediment *via* incubations that could simulate depth difference in labile organic matter is very difficult to achieve. Under laboratory conditions, the incubation of sediments may proceed for few years what typically could correspond to the time of accumulation of few millimetres of sediment material. For example, in wetlands soils, stable isotope variations over one or two metres depths of peat typically represent one or two thousand years of accumulation and decomposition of organic matter. However, the most advanced methanogenic decomposition of organic matter takes palace in the most fresh organic sediment. The laboratory experiments proceed under controlled conditions (limited factors controlling isotope ratios in methane) with small amounts of sediments. Therefore, it can be expected that decrease in labile organic carbon will be more noticeable under laboratory condition. To verify the possible influence of sediment ageing on variations in methanogenic pathways, we incubated freshwater sediments over relatively long time interval (570 and 879 days) with assumption that the time of incubation is sufficient to observe the variations in methanogenic pathways as a result of sediment ageing and changes in substrate availability for methanogens. Generally, in our study the time of incubation was considerably longer compared to previous experiments on methanogenesis (<100 days). It was our expectation that increasing time of incubation should result in the change in methanogenic pathways from acetoclastic to hydrogenotrophic methanogenesis reflected in carbon and hydrogen isotope composition of methane.

METHODS

INCUBATED SEDIMENTS

The sediments for incubation experiment were collected from the abandoned basaltic quarry in Nowa Cerekiew (SW Poland). The average thickness of sediments at the bottom of the lake is 25 cm. They are mostly composed of the products of basalt weathering and authigenic organic matter (Jędrysek, 1999). The sediment was collected from a water depth of 8 m in the central part of the lake by dragging. The sediment was homogenized and deposited into three plastic barrels of 65 cm height and 50 cm in diameter (volume of 110 l). The thickness of sediments deposited in the incubators was 10 cm. The incubators were filled with double-distilled water. The height of water column was 25 cm (incubator B3) and 60 cm (incubator B4). In aquatic environment, the water column isolates the sediments from the oxidative conditions dominant at the surface. In the laboratory, it was not possible to simulate the 8 m high of water column that covered the sediments before they were collected for the experiment. Therefore, we simulated the smaller difference in the height of the water column (60 and 25 cm) to check whether the height of water column importantly influences the rates of methane production and carbon and hydrogen isotope composition of methane, as it has been observed in natural conditions (Jędrysek *et al*., 1997, 1999). It was suspected that the water column may considerably affect a variation in temperature and the O_2 content.

Along the course of incubation, the incubators were sealed by a plastic twist cover with a rubber gasket for the most of the duration of the experiment, with exception of opening for several minutes for sampling. The incubation proceeded 879 days. From day 247 to 369 and from day 610 to 729 incubators were open and exposed to light (halogen light with 150 W intensity). The light is a major factor controlling the process of photosyntesis which enhance the production of planktonic matter. Plankton is a dominant source of labile organic carbon to anaerobic sediments. Simulation of this process in B3 and B4 allowed to investigate weather the fresh organic matter may involve greater production of methane due to acetoclastic methanogenesis.

As a control incubator we used incubator B2. Because B2 was also used as a control incubator for another experiment to simulate anthropogenic sulfate impact on freshwater sediments (Szynkiewicz *et al*., 2008), this incubator was not available for the current experiment at its early stages. Incubation in B2 started on the 312th day of incubation in B3 and B4 and proceeded for next 570 days. In the control incubator (B2), the thickness of sediment and water column was 10 and 60 cm, respectively. In contrast to B3 and B4, incubator B2 was not exposed to light.

Incubators used in this study were much larger than those in other incubation experiments that used smaller volumes of sediments (e.g., Dannenberg *et al*., 1997; Miyajima *et al*., 1997). During experiments of this type, it is crucial to maintain anaerobic conditions, but in the presence of small amounts of sediment and very efficient methanogenesis, the exhaustion of substrates for methanogens proceeds very fast. Consequently, it is not possible to precisely estimate the influence of natural sediment ageing and potential variations in methanogenic pathways. Moreover, the incubation based on small amounts of sediments creates conditions which significantly differ from the natural environments. For example, in such small-volume experiments, the headspace of incubators contains high concentrations of helium or nitrogen as these gases are used to remove the oxygen from the incubators or help in sampling procedures. Therefore, the incubation proceed under higher partial pressure (caused by gases artificially introduced to the incubators) than it is in the natural environments. Because of this, our experiments may raise some concerns, especially with respect to the volume of the incubators and potential oxygen access to the sediments. Nevertheless, we believe that our experimental setup better simulate the natural conditions where water column serves not only as a barrier but also as a carrier to exchange gases between air and sediment. During the incubation experiment, the stratification in the water column was always observed and during each sampling the incubated sediments showed O_2 concentration close to 0 mg/l. It evidences that anaerobic condition was in general maintained inside the incubated sediments.

SAMPLING PROCEDURE AND MEASUREMENTS

The water for analysis of DIC concentrations and carbon isotope measurements was collected from sediment/water interface by slow release *via* a stop-cock mounted across the wall of the incubator. Afterwards, bubble methane was sampled to glass bottles filled with double-distilled water after agitation of sediments (Jędrysek, 1995). During the incubation, only bubble methane concentrated in pore gases inside the sediments was analysed. At the end of each sampling, the sediment was agitated and mixed with water. It was done to release small amounts of residual pore gases which were still left in the sediment and to homogenize the concentration and $\delta^{13}C$ value of DIC in water column and sediment. The sampling took place in 30 day intervals. During mixing of water with the sediment after sampling, it was important to remove all methane cumulated in the sediment during 30 days incubation (the time span between subsequent sampling). Previous observations in natural conditions show that procedure of mixing can be important even when sampling is carried out in several hours interval (Jędrysek, 1995). We did not observe any significant influence in increase of O_2 concentration in the incubated sediments after mixing. The direct measurements of O_2 concentration carried out a few seconds after mixing showed 0 mg/l both in the entire water column (surface water and sediment/water interface) and in the sediment deposited after the mixing. Based on these observations, we assumed that the procedure applied had negligible effect on the amount of oxygen in the incubated sediments.

During incubation, temperature, electric conductivity and O_2 content measurements were carried out using electrodes *Senix 41-*, *TetraCon 325-3* and oxygen probe *CellOx 325*, respectively. The electrodes were connected with *Multi 340i* meter (WTW, Germany). The precision of temperature measurement was ±0.1°C, electric conductivity ±1 μS/cm, O_2 content ±0.5%. The main targets were concentrations and $\delta^{13}C$ values of DIC in the sediment/water interface, and methane concentration and its $\delta^{13}C$ and δD in pore gases in the sediment.

The pH of water column and sediments showed small variation around 6.5. Therefore, we accepted that DIC in the water was mainly represented by aqueous CO_2 and HCO_3^- ions, because at pH=6.5 and temperature 20°C, the activity of these forms of DIC are similarly high, and CO_3^{2-} concentration is negligible (Fetter, 1994). The analysis of DIC concentration were done only with respect to HCO_3^- concentration by HCl titration of water sample in the presence of methyl orange and expressed in mg of HCO_3^- per litre. The precision was better than <3 mg/L. Before titration, the water was filtered using cotton wool to minimize the possible effect of dissolution of small carbonate particles by HCl used to titration. The $\delta^{13}C$ value of DIC was determined by precipitating of DIC species to barium carbonate by increasing the pH of water sample up to 10–11 and addition of 10% solution of $BaCl_2$ (Bishop, 1990; Szynkiewicz *et al*., 2006). Dried $BaCO_3$ was decomposed under vacuum in the reaction with H_3PO_4 to CO_2 (McCrea, 1950). We collected bubble methane to glass bottles after agitation of sediment and this sampling procedure caused release of some methane to the atmosphere. Therefore, this sampling procedure did not allow for calculation of the maximum amount of methane produced during 30 days incubation interval. The concentration of methane was determined by mean of gas chromatography with a thermal conductivity detector ("*Elwro chromatograf 504*"). The concentration of methane was expressed in % values. The analytical error was ±1%.

The pore gasses taken from the incubated sediments contained some amounts of CO_2. For that reason, before carbon and hydrogen determination, methane was separated from CO_2 using the molecular sieves. Afterwards methane was passed twice through a copper oxide furnace (850–900°C) where it was combusted to H_2O and CO_2, which were then cryogenically separated (e.g., Jędrysek, 1999). At the end, H_2O was reduced on zinc to H_2 at 480°C (Demeny, 1995). The carbon isotopic composition of DIC and methane was analysed on the mass spectrometer and presented as $\delta^{13}C$ value relative to PDB standard with analytical error average ±0.5 and 0.4‰, respectively. The hydrogen isotopic composition of methane was analysed on *Finnigan Mat DeltaS* mass spectrometer and expressed as δD value relative to V-SMOW standard and the analytical error average ±2‰.

PRINCIPAL COMPONENT ANALYSIS

The incubation using large volumes of sediments was influenced by many factors such as duration of experiment, temperature and biological activity at the same time. Therefore, to interpret the observed variations among geochemical data relative to the long-term incubation of sediments under different physical condition, we used the method of principal component analysis (PCA). This method allowed limiting the number of variables and describing the relationships among variables (Manly, 1998). Factor analysis requires normal distribution of all variables (Drever, 1997; Manly, 1998), and this condition was confirmed by Shapiro-Wilk test of normality for all variables used in transformation. Normal distribution for DIC concentration in the sediment/water interface was well attained by log transformation (Shapiro *et al*., 1968).

PCA transformation was done for standardized parameters: temperature, methane concentration, $\delta^{13}C(CH_4)$ and $\delta D(CH_4)$ values and for the sediment/water interface standardized parameters: electric conductivity, DIC concentration and $\delta^{13}C(DIC)$ value. Because some of the samples did not have a complete set of physicochemical and isotopic measurements, a main correlation matrix was created for 39 data by parawise deletion of missing data. The PCA was done simultaneously using above listed variables for all incubators.

During standardized processing, all values of selected variables were replaced by standardized values (0 is a mean value, –1 and 1 are standard deviations). Transformation factors were estimated based on PCA to create the matrix of loadings. For better clarity, the factor scores were calculated based on additional correlation matrix created for 68 data for which missing data were replaced by mean values. The additional matrix showed an identical distribution of factor loadings and scores like the main correlation matrix. The values calculated from factor loadings vary from –1 to 1. Based on Child (1970), it was arbitrary accepted that values from –0.6 to 0.6 are "significant" and were included to interpretation. If loadings for a particular factor have positive numbers, the variables are positively correlated. If they have negative numbers, the variables are negatively correlated (Drever, 1997). In order to achieve a more accurate interpretation of the results, the "varimax" rotation was used, and the percentage quota of each factor was calculated (Johnson, 1978). In this matrix, the number of factors was determined based on the statistical scree test (Cattell, 1966). The calculated factors were identified with the particular process that created the observed variability of physical and chemical/isotopic compositions for all incubators. Changes with time for particular factor scores were also calculated.

The method of parametric Spearman's rank correlation was used for correlation analyses. The significance level (p) of 0.05 was usually considered. All numbers calculated for the significance level are presented in graphs (Figs. 1–3).

RESULTS

METHANE IN SEDIMENTS

In the course of incubation, the permanent decreases of CH_4 concentrations in sediments, from 86 to 39% in B2 (R = –0.83) and from 76 to 32% in B3 (R = –0.76), were observed (Fig. 1). In B4, the decrease of CH_4 concentration showed weak correlation (R = –0.032) with time and widely varied from 89 to 47% (Fig. 1). In all incubators, the increase of $\delta^{13}C(CH_4)$ values was noted, from –68.9 to –61.4‰ (R = 0.72) in B2, from –67.0 to –60.6‰ (R = 0.62) in B3, and from –69.7 to –59.0‰ (R = 0.74) in B4 (Fig. 2). This was accompanied by continuous increase of $\delta D(CH_4)$ values, from –381 to –343‰ (R = 0.70) in B2, from –379 to –320‰ (R = 0.75) in B3, from –392 to –345‰ (R = 0.51) in B4 (Fig. 3).

The concentration of DIC in the sediment/water interface varied along the incubation time from 101 to 217 mg/l in B2, from 70 to 107 mg/l in B3 and from 67 to 104 mg/l in B4 (Table 1). The value of $\delta^{13}C(DIC)$ widely varied from –6.3 to

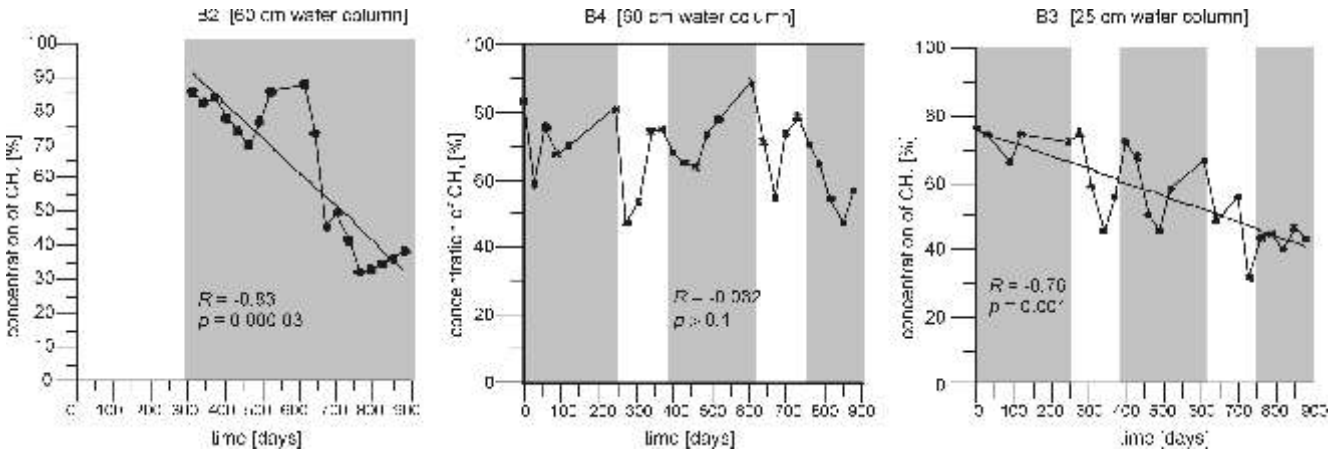

Fig. 1. Variations of methane concentration in sediment along the entire incubation

Grey colour — no exposure to light

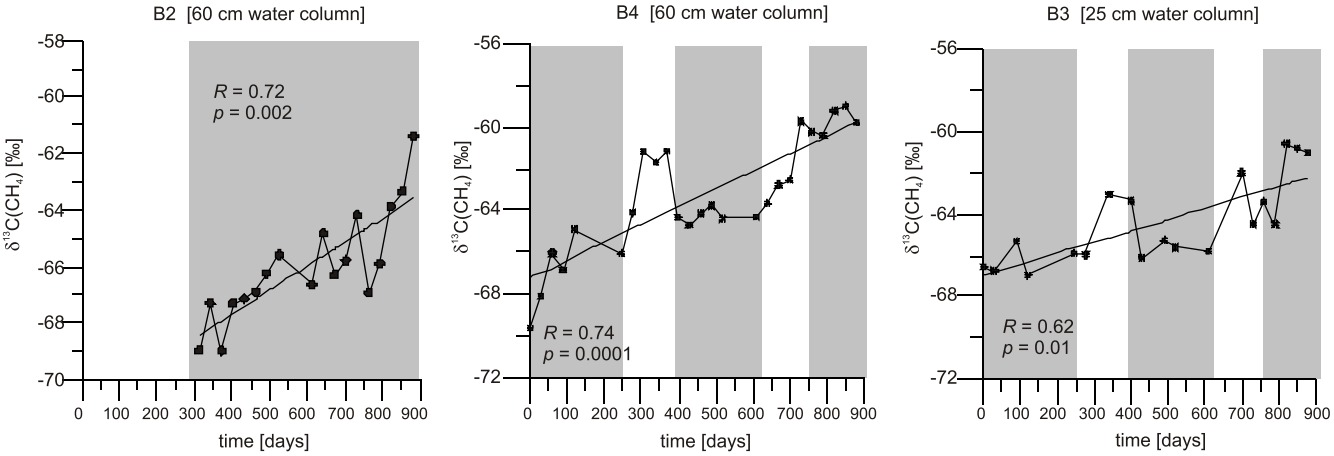

Fig. 2. Variations of $\delta^{13}C(CH_4)$ values in sediments along the entire incubation

Grey colour — no exposure to light

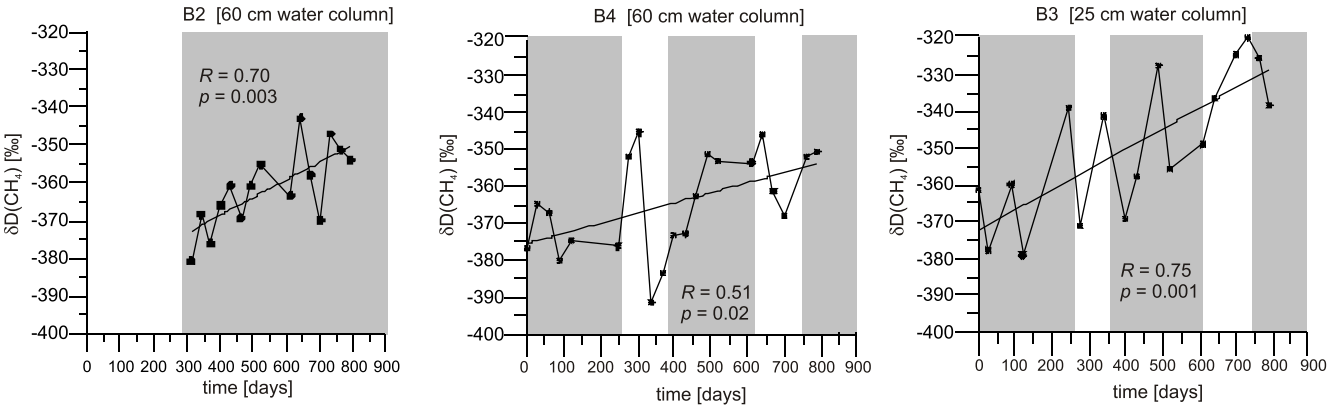

Fig. 3. Variations of $\delta D(CH_4)$ values in sediments along the entire incubation

Grey colour — no exposure to light

Table 1

Variation of temperature, EC (electric conductivity), concentration and $\delta^{13}C$ of dissolved inorganic carbon (DIC), concentration and $\delta^{13}C$–δD of methane during the incubation of B1, B2, B3 and B4

Time [day]	Temperature [°C]	EC [µS/cm]	DIC		Methane		
			Concentration [mg/l]	$\delta^{13}C$ [‰]	Concentration [%]	$\delta^{13}C$ [‰]	δD [‰]
INCUBATOR B2 [60 cm water column, control incubator]							
312	18.3	164	119	–7.9	86	–69.0	–381
342	17.8	203	127	–7.3	82	–67.3	–368
372	17.4	230	134	–6.6	84	–69.0	–376
402	16.5	263	195	n.a.	78	–67.3	–366
432	16.5	259	198	–6.3	74	–67.1	–361
463	18.7	258	207	–6.6	70	–66.9	–369
492	18.5	247	217	–7.7	77	–66.2	–361
523	21.0	409	211	–7.7	86	–65.6	–355
612	19.8	310	156	–7.3	88	–66.6	–363
642	19.6	257	113	–8.4	73	–64.8	–343
672	18.9	245	134	–7.9	46	–66.3	–358
702	18.9	247	104	–7.4	50	–65.8	–370
732	18.4	241	134	–11.6	42	–64.2	–347
762	18.2	235	125	–10.7	32	–66.9	–351
792	18.0	194	101	–11.1	33	–65.9	–354
822	18.6	211	107	–11.9	35	–63.9	n.a.
852	19.6	225	113	–12.0	36	–63.3	n.a.
882	19.6	232	125	–11.4	39	–61.4	n.a.
INCUBATOR B3 [25 cm water column, light radiation]							
1	17.1	371	n.a.	n.a.	76	–66.6	–361
29	17.2	343	n.a.	n.a.	75	–66.8	–378
60	17.4	288	n.a.	–6.9	n.a.	n.a.	n.a.
89	18.3	212	n.a.	–8.4	66	–65.3	–360
120	18.9	182	n.a.	–9.8	75	–67.0	–379
246	18.9	223	n.a.	n.a.	72	–65.9	–339
276	23.5	261	n.a.	n.a.	75	–66.0	–371
306	21.5	202	n.a.	–2.7	59	n.a.	n.a.
339	19.5	150	82	–3.9	46	–63.1	–341
369	20.5	191	107	0.6	56	n.a.	n.a.
399	16.2	125	92	–10.6	72	–63.3	–369
429	16.1	158	92	–10.4	68	–66.1	–358
459	18.1	145	88	–10.7	50	n.a.	n.a.
489	18.6	139	92	–12.2	46	–65.3	–328
519	19.9	149	82	–12.0	58	–65.6	–356
609	19.5	150	88	–11.9	67	–65.8	–349
639	21.5	156	70	–0.3	49	n.a.	–336
669	22.1	171	76	–1.5	n.a.	n.a.	n.a.
699	23.1	147	73	–4.7	56	–62.0	–325
729	20.7	139	70	–8.8	32	–64.5	–320
759	17.8	145	85	–13.5	44	–63.4	–325
789	17.4	151	88	–13.6	45	–64.5	–338
819	18.2	141	95	–12.9	40	–60.6	n.a.
849	18.9	136	95	–12.2	46	–60.8	n.a.
879	19.3	130	88	–11.3	43	–61.0	n.a.

Tab. 1 cont.

Time [day]	Temperature [°C]	EC [µS/cm]	DIC		Methane		
			Concentration [mg/l]	$\delta^{13}C$ [‰]	Concentration [%]	$\delta^{13}C$ [‰]	δD [‰]
INCUBATOR B4 [60 cm water column, light radiation]							
1	16.8	165	n.a.	n.a.	83	–69.7	–377
29	16.9	228	n.a.	–7.1	59	–68.1	–365
60	17.1	226	n.a.	–9.4	76	–66.0	–367
89	18.0	232	n.a.	–9.0	68	–66.9	–380
120	18.6	182	n.a.	n.a.	70	–64.9	–375
246	18.7	206	n.a.	–10.9	81	–66.1	–376
276	21.9	152	n.a.	n.a.	47	–64.1	–352
306	19.7	242	n.a.	n.a.	53	–61.2	–345
339	18.0	151	101	–0.2	74	–61.7	–391
369	19.1	137	101	1.2	75	–61.1	–384
399	15.8	129	101	–8.4	68	–64.3	–373
429	15.8	172	104	–9.2	65	–64.7	–373
459	17.5	175	85	–9.4	64	–64.1	–363
489	18.4	168	98	–10.7	73	–63.8	–351
519	19.6	209	104	–9.8	78	–64.4	–353
609	19.5	141	98	–10.7	89	–64.3	–354
639	19.7	165	95	–3.3	71	–63.7	–346
669	20.4	154	85	–1.0	55	–62.8	–361
699	20.8	129	70	–3.8	73	–62.5	–368
729	19.5	133	70	–4.6	78	–59.7	n.a.
759	17.2	115	73	–8.7	71	–60.2	–352
789	16.9	128	79	–9.9	65	–60.4	–351
819	17.7	121	67	–10.4	54	–59.2	n.a.
849	18.6	149	92	–11.3	47	–59.0	n.a.
879	19.1	150	92	–11.5	57	–59.8	n.a.

Grey colour indicates periods under light radiation, n.a. — not analysed

–12.0‰ in B2, from –0.3 to –13.6‰ in B3 and from –0.2 to –11.5‰ in B4 (Table 1). Generally, at the end of incubation the lowest values of $\delta^{13}C(DIC)$ were observed in all incubators, however, a considerably higher values of $\delta^{13}C(DIC)$ were noted along the cycles of exposition of B3 and B4 to the light (Table 1).

The sediment temperatures of B2 varied from 16.5 to 21.0°C and were slightly lower compared to B3 and B4 which were exposed for radiation, from 16.2 to 23.5°C and from 15.8 to 21.9°C, respectively (Table 1). The electric conductivity varied in similar range, from 164 to 409 µS/cm in B2, from 130 to 371 µS/cm in B3, and from 141 to 242 µS/cm in B4 (Table 1).

PRINCIPAL COMPONENT ANALYSIS

The PCA explained 80% of the observed variables in B2, B3 and B4 (Table 2). The remaining 20% constitutes random noise, impossible to interpret (Drever, 1997; Manly, 1998). Based on this method, three factors that accounted for 80% of the total variance in all incubators were identified (Table 2). Factor 1 accounts for nearly 33% of the total variance and had high positive loadings for electric conductivity (0.93) and DIC concentration (0.92) in the sediment water interface and negative loading values for $\delta^{13}C(CH_4)$ in sediment (–0.72). Factor 2 accounts for 28% of the total variance and had high positive loading for $\delta D(CH_4)$ value (0.86) and high negative loadings for methane concentration (–0.81) in the sediment. Factor 3 accounts for 19% of the data's total variance and exhibited high positive loadings for temperature (0.90) and $\delta^{13}C(DIC)$ value in the sediment water interface (0.69).

The variation of factor scores shows that the influence of each factor changed relative to the duration of incubation and was slightly different for B2 and B3 compared to B4 (Fig. 4). The values of factor scores are lower or higher than 0. These values relate to the intensity of chemical processes that each factor represents. Extreme negative values (< –1) reflect a period of time not affected by the specific process that each factor represents, in contrast to positive values (> +1) which indicate period of time under strong influence of the process.

In control incubator B2, the intensity of factor 1 and 3 increased until the 520 day of incubation; afterward they showed the continuous decrease until the end of incubation (Fig. 4). In contrast, the intensity of factor 2 showed steady decrease relative

Table 2

Factor's quota and factor loadings for the Varimax Rotated 3 Factor Model

Variables	Factor 1	Factor 2	Factor 3
Conductivity sediment/water interface [μS/cm]	**0.93**	–0.03	0.10
DIC concentration sediment/water interface [mg/l]	**0.92**	–0.17	–0.12
$\delta^{13}C$(DIC) sediment/water interface [‰]	–0.04	–0.59	**0.69**
Temperature sediment [°C]	–0.06	0.27	**0.90**
CH_4 concentration sediment [%]	0.24	**–0.81**	0.02
$\delta^{13}C(CH_4)$ sediment [‰]	**–0.72**	0.29	0.12
δD (CH_4) sediment [‰]	–0.17	**0.86**	0.20
Factor quota [%]	**33**	**28**	**19**

to increasing time of incubation. In B3, the intensity of factor 1 and 2 decreased relative to time, and factor 3 showed the highest intensity while incubator was exposed to light (Fig. 4). In B4, all three factors showed wide variety in the intensity during entire incubation. During the second exposition to light, however, the intensity of each factor decreased with time of incubation.

DISCUSSION

METHANOGENESIS AND SEDIMENT AGEING

The acetate is a fermentative product of easy degradable organic compounds and represents a fraction of labile organic carbon in sediments. It is also well documented that acetate is the main substrate for methanogenesis in freshwater sediments (Takai, 1970; Winfrey and Zeikus, 1979*a*, *b*; Lovely and Klug, 1982; Phelps and Zeikus, 1984; Whiticar, 1999). Methane originated from acetoclastic methanogenesis is characterized by higher values of $\delta^{13}C$ (from –40 to –30‰) as compared to methane formed due to hydrogenotrophic methanogenesis (from –110 to –60‰) (e.g., Whiticar *et al*., 1986; Conrad, 2005). The CO_2 reduction may contribute important amounts of methane from freshwater sediments (Zaiss, 1981). Recent studies (e.g., Nüsslein *et al*., 2001, 2003) report that isotopic patterns of methane in freshwater sediments may be affected by syntrophic acetate oxidation coupled with CO_2 reduction methanogenesis rather than by direct acetoclastic methanogenesis. Typical $\delta^{13}C(CH_4)$ values for acetoclastic methanogenesis are significantly higher than reported for freshwater sediments (Sugimoto and Wada, 1993). For that reason, syntrophic methanogenesis by acetate fermentation better explains the observed lower values of $\delta^{13}C(CH_4)$ in lake sediments (from –65 to –50‰), as CO_2 reduction causes larger carbon isotope fractionation (resulting in lower $\delta^{13}C(CH_4)$ values).

Changes in methanogenic pathways from acetate fermentation to CO_2 reduction are usually described as a negative trend in $\delta^{13}C(CH_4)$–$\delta D(CH_4)$ system (e.g., Burke, 1993; Hornibrook *et al*., 2000). It is a result of different carbon and hydrogen isotope fractionation during methanogenesis. We assumed that this trend should also appear relative to ageing of sediment. However, along the entire incubation, we observed a weak positive trend in $\delta^{13}C(CH_4)$–$\delta D(CH_4)$ system (Fig. 5). The positive trend may reflect one of the two processes: 1) methane oxidation, or 2) enrichment of methanogenic substrates in heavier carbon and hydrogen isotopes along the incubation.

From other experiments, we noticed that anaerobic and/or aerobic oxidation of methane was negligible because of lack of adequate bacterial community inside the sediments to enhance this process (Szynkiewicz *et al*., 2008). Therefore, we link the observed changes in methane isotopic composition with exhaustion of one methanogenic substrate. It can be expected that ageing of sediment decreases the concentration of substrates in the incubated sediments and this is followed by general decrease of methane concentration in all incubators with time. The observed range of $\delta^{13}C(CH_4)$ and $\delta D(CH_4)$ values, however, overlaps with both methanogenic pathways reported for freshwater sediments. Therefore, it is not possible to directly indicate the type of methanogenesis. Generally, microbial processes involve the preferential uptake of light isotopes, thus, the remaining substrates should show isotopic enrichment under closed condition with time due to kinetic isotope effect. Indeed, the observed increase of $\delta^{13}C(CH_4)$ and $\delta D(CH_4)$ values may suggest that one of the methanogenic substrates, enriched by heavier isotopes, could have caused this migration towards higher values (Figs. 2 and 3) This could be acetate or another methylated compound which has been found to be important for methanogenesis in freshwater sediments.

BLOOMING IN THE WATER COLUMN

It is believed that plankton is very important primary substrate for acetate formed in freshwater sediments. The planktonic matter is produced in freshwater environments by photosynthesis in water column. After its death, the planktonic particles are deposited at the sediments surface and are decomposed by microbial communities to the final product which is acetate. To simulate this process and, consequently, observe the variations in carbon isotopic composition of methane, the incubators B3 and B4 were exposed to the light radiation in two cycles, from 247 to 369 and from 610 to 729 days of incubation. In anaerobic sediments, the acetate is enriched in ^{13}C (e.g., Sugimoto and Wada, 1993) and we expected that, among other consequences, decomposition of the fresh plankton in the incubated sediments may result in increase of $\delta^{13}C(CH_4)$ values during the exposition to the light. This was observed on diurnal basis in natural systems, and explained mainly by variations in CO_2/acetate pathways and phothosynthesis/respiration processes (e.g., Jędrysek, 1995, 1999). We noticed a significantly higher $\delta^{13}C(CH_4)$ values only in the first cycle of exposure in B4 (Fig. 2) inferring possible shift toward acetoclastic methanogenesis. During second exposure to light blooming was less intensive and there was no essential shift toward higher $\delta^{13}C(CH_4)$ values in B4 as it was observed in the first exposure. Most likely, the amount of produced plankton was too small to change the $\delta^{13}C$ value of substrates for methanogenesis in the incubated sediments, thus, the increase of $\delta^{13}C(CH_4)$

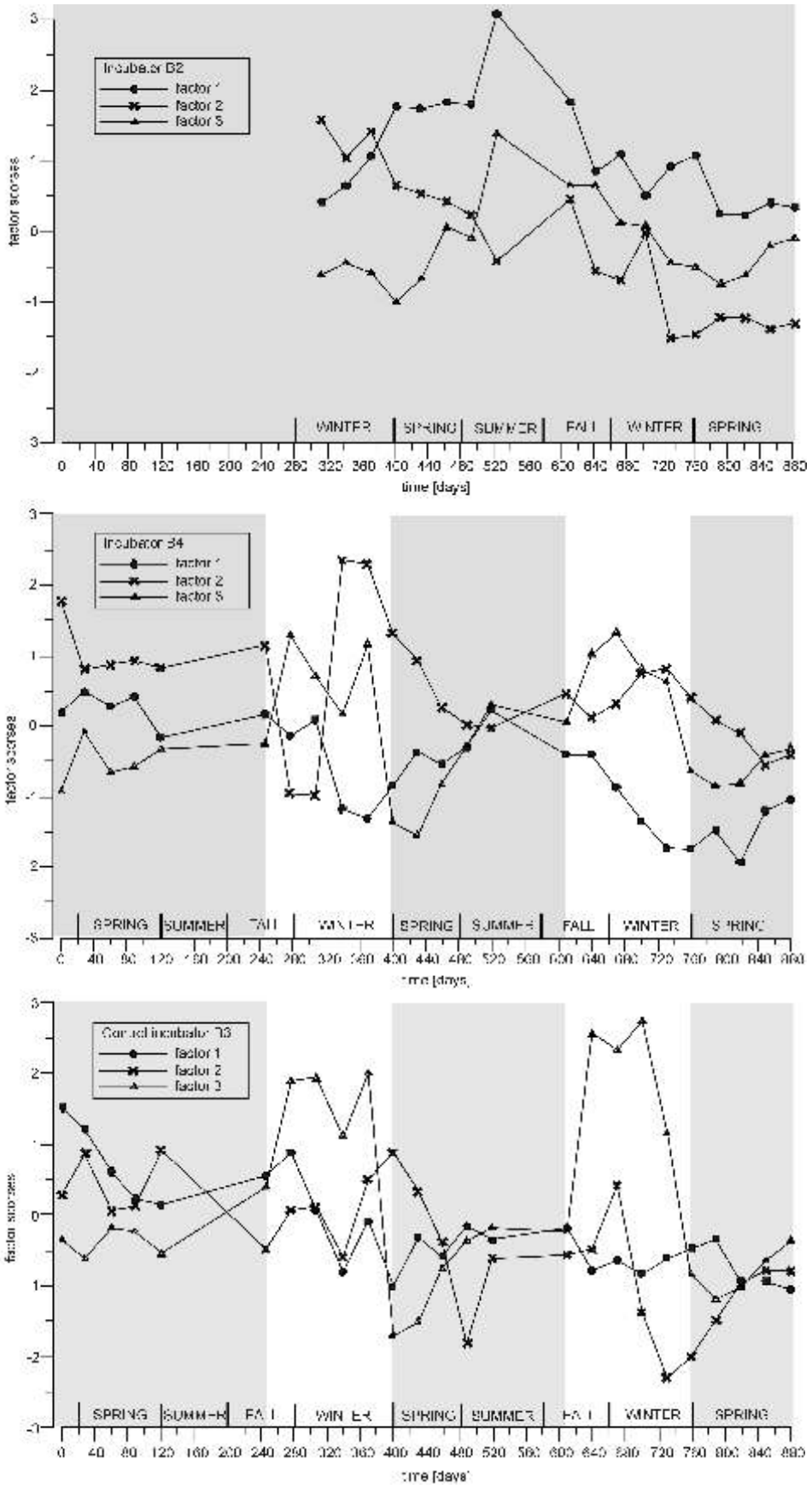

Fig. 4. Variations of factor scores in the incubated sediments relative to increasing duration of incubation

Grey colour indicate time without exposure to light radiation

value apparently resulted from kinetic isotopic fractionation due to exhaustion of methanogenic substrate.

Alongside plankton blooming, the exposure to light radiation concurrently increased the temperature of water column for about 1–3°C, both in B3 and B4 (Szynkiewicz, 2003), and in turn, increased the temperature of sediments (Table 1). The influence of temperature on isotope composition of methane has been considered by many authors with respect to carbon isotopes. It was suggested that higher temperatures involve smaller carbon isotope fractionation (Games *et al*., 1978; Krzycki *et al*., 1987; Sugimoto and Wada, 1993; Gelwick *et al*., 1994). On the other hand, in natural conditions, it was observed that temperature is an important, although not the dominant factor controlling $\delta^{13}C$ value in freshwater methane (e.g., Jędrysek, 1997, 1998, 1999). In general, the increase of temperature leads to increase of $\delta^{13}C(CH_4)$ value. The reported α values in the substrate-methane system vary from 1.021 to 1.079 and they are distinctively smaller for acetate fermentation (Sugimoto and Wada, 1993; Gelwick *et al*., 1994) than for CO_2 reduction (Games *et al*., 1978; Krzycki *et al*., 1987). B3 and B4 did not show an apparent relationship with temperature, however, it may be assumed that temperature could have resulted in increase of $\delta^{13}C(CH_4)$ value during light radiation.

PRINCIPAL COMPONENT ANALYSIS

As it was shown above, based on isotopic composition of methane alone it is difficult to find a direct evidence of changes from acetoclastic to hydrogenotrophic methanogenesis due to ageing of sediments. Simulation of different physical conditions (e.g., exposure to light, difference in water table) on incubated sediments involved additional changes in the same time that could have considerably affected the methanogenesis. Because simple correlation between analysed variables did not show significant and clear correlation, we used PCA analysis that offers better comparison of the relationships among variables when a larger number of parameters control a given process in the same time period and over long periods of time.

PCA suggests that decomposition of organic matter were controlled by at least three factors during entire incubation in all incubators (Table 2). Two factors, 1 and 2, may be linked to the methanogenesis because of their relation to $\delta^{13}C(CH_4)$, $\delta D(CH_4)$ and methane concentration that are directly affected by this process. Factor 1 explains 33% of observed variations among the variables studied and had positive (0.93–0.92) loadings for electric conductivity and DIC concentration in the sediment water interface and negative loading for $\delta^{13}C(CH_4)$ value (–0.72). As only double-destilled water was used during incubation and the chemical weathering of basaltoids, being the mineral components of incubated sediments, is relatively slow, the electric conductivity was controlled mainly by changes in DIC concentration (Fig. 6). The highest value of electric conductivity was always observed within the incubated sediments (Szynkiewicz, 2003) inferring that decomposition of organic matter instead of dissolution of atmospheric CO_2 was a main source of DIC species in the water column. Generally, the increase in DIC concentration in the sediment/water interface was accompanied by decrease of $\delta^{13}C(CH_4)$ in the incubated

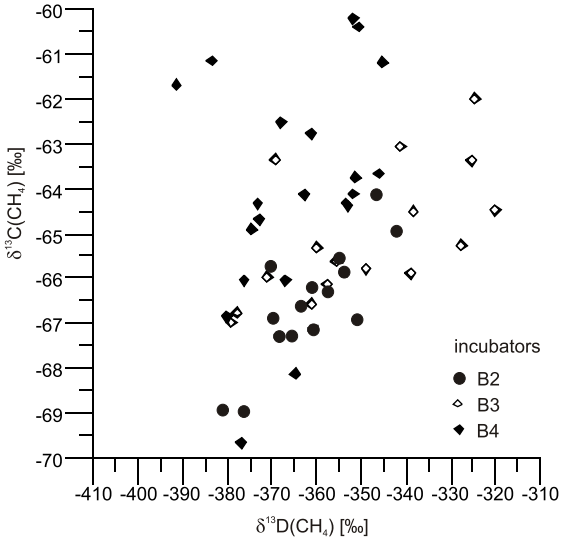

Fig. 5. Comparison of $\delta^{13}C(CH_4)$ and $\delta D(CH_4)$ values between incubators B2, B3 and B4

sediments, implying its significance on carbon isotopic composition of methane. Many recent studies emphasize the role of synthrophic acetate oxidation coupled to CO_2 reduction methanogenesis instead of direct acetoclastic methanogenesis (e.g., Nüsslein *et al*., 2001, 2003). Translating this to our experimental conditions, this suggests that in all incubators, the size of carbon pool significantly affected in the $\delta^{13}C(CH_4)$ value. It should be pointed out that sampling procedures involved significant dilution of DIC species within the water column and sediment, and the continuous removal of gases (e.g., CH_4, CO_2) formed by decomposition of organic matter. In natural condition, biologically produced DIC species, including gaseous CO_2, might be stored under elevated content in sediments for several years; this was not achieved during incubation. The low values of $\delta^{13}C(CH_4)$ are referred to conditions when DIC concentration was the highest (Table 2). As CO_2 reduction leads to formation of methane with lower $\delta^{13}C$ values, our results suggest that during the incubation this methanogenic pathway was partially limited because of constant dilution by mixing the water with sediment and general exhaustion of organic substrates. This suggests that more abundant CO_2 reduction with increasing depth of sediments (e.g., Jędrysek 1997*a*, *b*; Hornibrook *et al*., 2000) may result mainly from bigger accumulation of bio-products coming from decomposition of organic matter. As CO_2 represents the last product of biodegradation, its steady accumulation might favour the CO_2 reduction deep in sediments instead of acetoclastic methanogenesis.

Factor 2 explains 28% of the observed variations among variables in the incubated sediments and had positive (0.86) loading for $\delta D(CH_4)$ opposite to negative (–0.81) loading for methane concentration. This infers that lower values of $\delta D(CH_4)$ are followed by increasing methane concentration in sediments. Variations in $\delta D(CH_4)$ value were mainly considered with respect to methanogenic pathways. Generally, CO_2 reduction shows higher values of $\delta D(CH_4)$ compared to acetoclastic methanogenesis that shows more negative values of

$\delta D(CH_4)$ (e.g., Shoell, 1980; Woltemate *et al*., 1984; Whiticar *et al*., 1986). At the beginning of incubation, both DIC and methane concentration was the highest, and $\delta^{13}C(CH_4)$ value showed the lowest values inferring a bigger contribution of methane from CO_2 reduction. In spite of this, the $\delta D(CH_4)$ showed the lowest values at the beginning. This implies that observed changes do not relate directly to changes in methanogenic pathways. In contrast, Burke (1993) showed that partial pressure of H_2 may affect $\delta D(CH_4)$ value that decreases while H_2 pressure increase. As the rates of organic decomposition were generally higher at the beginning of incubation, the production of H_2 in sediments was probably also more efficient and could have affected the value of $\delta D(CH_4)$. On the other hand, the general exhaustion of methanogenic substrates might have influenced on $\delta D(CH_4)$ value, as well, by kinetic isotopic fractionation of methanogenic substrate. For that reason, according to obtained results it is difficult to indicate which process was more important, because both the decrease of H_2 content and exhaustion of methanogenic substrates would involve the decrease of $\delta D(CH_4)$ value followed by decrease in methane concentration in sediments.

Factor 3 explains 18% of the observed variations among variables and showed the positive (0.90–0.69) factor loadings for temperature of sediments and $\delta^{13}C(DIC)$ value in sediment/water interface. We linked this factor with seasonal changes because temperature was controlled by seasonal changes in experimental room and varied from 16°C in winter to 21°C during summer. Additionally, in B3 and B4 temperatures were controlled by exposure to light radiation. Carbon isotope exchange between DIC species is relatively fast and it is in large extent controlled by temperature (e.g., Clark and Fritz, 1997). The results obtained due to PCA analysis implies that isotopic equilibrium was achieved in the sediment/water interface.

In control incubator B2, the score of factor 3 showed similar changes to score of factor 1 (Fig. 4). The highest intensity of those factor scores was observed for summer period when temperatures were the highest in the experimental room. Numerous studies show that increasing temperature activates microbial processes (e.g., Westermann, 1993), thus, the highest intensity of factor 1 during the summer and its positive loading for DIC concentration may be related to more intensive biodegradation of organic compounds in the incubated sediments. Factor 2 is linked directly with methanogenesis by its relation to methane concentration (Table 2) and showed the continuous decrease with time inferring the exhaustion of primary methanogenic substrates. In contrast, in B3 and B4 the increase of temperature by exposition to light involved the relatively wide variation in the intensity of factors 1 and 2 (Fig. 4). In our opinion, it may relate to higher oxygenation of water column due to photosynthesis activated by light radiation in B3 and B4. While the mean content of O_2 in the sediment/water interface in B2 was 0.19 mg/l, in B3 and B4 during exposition to light it increased up to 4.90 and 6.48 mg/l, respectively (Szynkiewicz, 2003). In the next period, that appeared directly after light radiation, the O_2 content in sediment/water interface was importantly higher, mean 1.63 mg/l in B3 and 1.89 mg/l in B4, compared to control incubator B2. This implies that the exchange of gasses between sediments and water column would have involved different intensity in factors 1 and 2 defining in our experiment the methanogenesis and other anaerobic processes of organic matter degradation.

A wide and independent variety in the score intensity of factors 1, 2 and 3 for different incubators suggest that physical condition may strongly affect the way of organic matter decomposition in sediments but on the other hand it had a minor effect on carbon and hydrogen isotopic composition of methane that seems to relate mainly to the primary isotopic composition of methanogenic substrates and their availability in the incubated sediments; this is confirmed by similar constant increase of $\delta^{13}C(CH_4)$ and $\delta D(CH_4)$ values with time (Figs. 2 and 3).

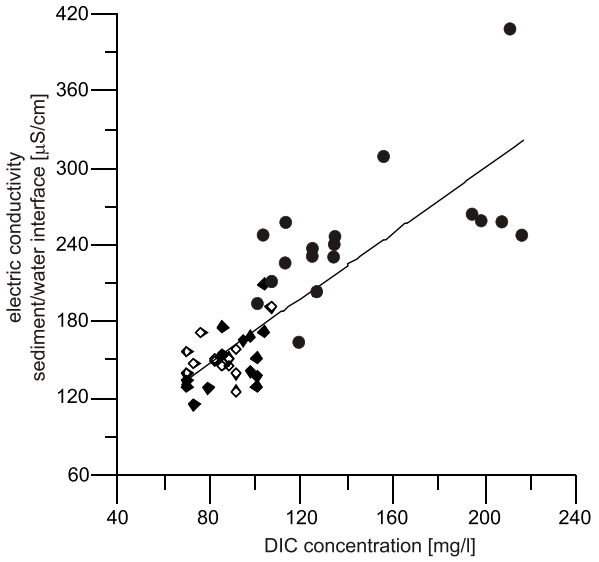

Fig. 6. Comparison of electric conductivity and DIC concentration between incubators B2, B3 and B4

For explanations see Figure 5

CONCLUSIONS

The sediment ageing and increasing recalcitrance of organic matter in anaerobic sediments did not result in negative trend in $\delta^{13}C(CH_4)$–$\delta D(CH_4)$ system as it was expected for a long-term incubation. Therefore, based on isotopic composition of methane, it was not possible to establish which methanogenic pathway was dominant during incubation of freshwater sediments. Most likely, the increase of $\delta^{13}C(CH_4)$ and $\delta D(CH_4)$ values with time, followed by general decrease of methane concentration, resulted mainly from the kinetic isotope fractionation of methanogenic substrate(s) in the incubated sediment.

The principal component analysis indicated three distinctive factors/processes influencing decomposition of organic matter during the long-term incubation of sediments. First of all, it appeared that $\delta^{13}C(CH_4)$ value was importantly controlled by the availability of DIC. Generally, the negative $\delta^{13}C(CH_4)$ values were typical while the DIC concentration was higher in sediment/water interface indicating that methanogenesis based on CO_2 reduction may be considerably limited by DIC availability

in sediments and/or water column. As in natural conditions, the freshwater sediments are characterized by bigger accumulation of bio-products, the steady accumulation of CO_2 may likely favour the CO_2 reduction deep in sediments instead of acetoclastic methanogenesis. Secondly, the $\delta D(CH_4)$ values were negatively correlated with the concentrations of methane and might have resulted from both the decrease of H_2 content and exhaustion of methanogenic substrate in the incubated sediments. Lastly, the seasonal changes of temperatures considerably controlled $\delta^{13}C$(DIC) values only, and likely corresponded to the carbon isotope exchange that took place between particular DIC species when the equilibrium was achieved.

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