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Isotopic Evidence for Anaerobic Oxidation of Methane in the Freshwater Sediments of Reservoirs: The Impact of Selected Environmental Factors

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Abstract: This paper presents the results of research conducted in 2018–2019 on the anaerobic oxidation of methane (AOM) in reservoir sediments. Located in SE Poland, Maziarnia, Nielisz and Rzeszów Reservoirs were selected for the purposes of the research. Rates of AOM were determined via 50-day incubation of sediment from the 0–5, 5–10 and 10–15 cm layers, to which a $^{13}CH_4$ isotope tracer was added. The sediments had been collected from a single station at each reservoir in places that had earlier reported high levels of emission of CH_4 to the atmosphere. Results demonstrate ongoing AOM processes in the kinds of freshwater ecosystem represented by reservoirs, further implying the existence of an important sink for CH_4. More specifically, however, AOM rates were found to differ among both the reservoirs, and the layers of sediment, studied. Preliminary analysis of selected environmental factors capable of affecting AOM failed to suggest the availability of electron acceptors (NO_3^-, SO_4^{2-}, Fe^{3+}) as key controlling factors. Important factors also proved to be sediment pH, the quality of organic matter (especially the content of organic electron acceptors), the salinity of pore water, and—primarily—the presence of the microorganisms actually responsible for AOM. The results here are important, given the low level of knowledge of AOM process in reservoirs. They therefore help supply key information on the functioning of these ecosystems and the role in global climate change they play.

Keywords: methane; anaerobic oxidation of methane; reservoir; sediment; greenhouse gases

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1. Introduction

Methane (CH_4) is one of the main greenhouse gases, and furthermore, it is one whose concentration in the atmosphere is increasing steadily, with the result that average air temperatures also go up [1]. On that basis, researchers around the world seek to identify the sources of CH_4 in the atmosphere and to quantify level of emission. Among other sources, freshwater ecosystems such as lakes and reservoirs have been shown to be of significance globally, where CH_4 is concerned [2–7]. Since reservoirs as anthropogenic, methane-emitting bodies of water are continuing to increase in number worldwide, their emissions of CH_4 have proved to be of special interest. A specific feature of the functioning of these ecosystems is the production of organic matter during primary production in situ as well as its continuous transport from the catchment.

Under anaerobic conditions, the organic matter accumulated in sediments decomposes, with CH_4 being among the end products. Spatial variation in emissions of this gas from reservoirs has been shown to be significant, with zones located near tributaries considered hot spots for CH_4 [8–15]. However, it should be stressed at the outset that amounts of CH_4 emitted to the atmosphere are not equivalent to what is produced in sediments, as part of that gas is oxidised there, or on the way through the water column. That leaves it all the more important for the processes contributing to a reduction in CH_4 emissions from reservoirs to be well understood.

One of the least recognised sinks for CH_4 involves the anaerobic oxidation of methane (AOM) in sediments—a process in which microorganisms of the *Archaea* group participate, with the role of electron-acceptor played by such substances other than oxygen such as the ions NO_3^-, SO_4^{2-}, Fe^{3+} and Mn^{4+} [16–20]. Where sulphates are present, it is specific groups of *Archaea* (i.e., ANME-1, ANME-2a,b,c and ANME-3) that oxidise methane, while in the presence of nitrate (V), the role is played by ANME2-d, and in the presence of iron (III), it is played by microorganisms associated with the ANME-2d group [21–24]. In order to identify these microorganisms in sediments, characteristic lipid biomarkers (e.g., *archaeol*, *crocetane*, *PMI*, *pristane*, *phytane* and *squalane*) are most often used [25–28].

However, as the AOM process was until recently thought to be confined to environments with saline waters, the importance of the process in inland waters was anyway neglected. Therefore, there is a great need for research in this area. The few scientific reports about AOM in freshwater sediments mainly concern lakes and wetlands [29–33]. Relevant data on reservoirs prove to be entirely lacking.

The main aim of the presented work was to determine the rates of the anaerobic oxidation of methane (AOM) in freshwater sediments of reservoirs and to determine the biotic and abiotic factors potentially controlling this one. The obtained results of the research are very important and of a cognitive nature. Recognition of the AOM process can contribute to changing existing views about the cycle of CH_4 in freshwater ecosystems and provides significant information on functioning of reservoirs and their role in global climate change.

2. Materials and Methods

2.1. Study Area

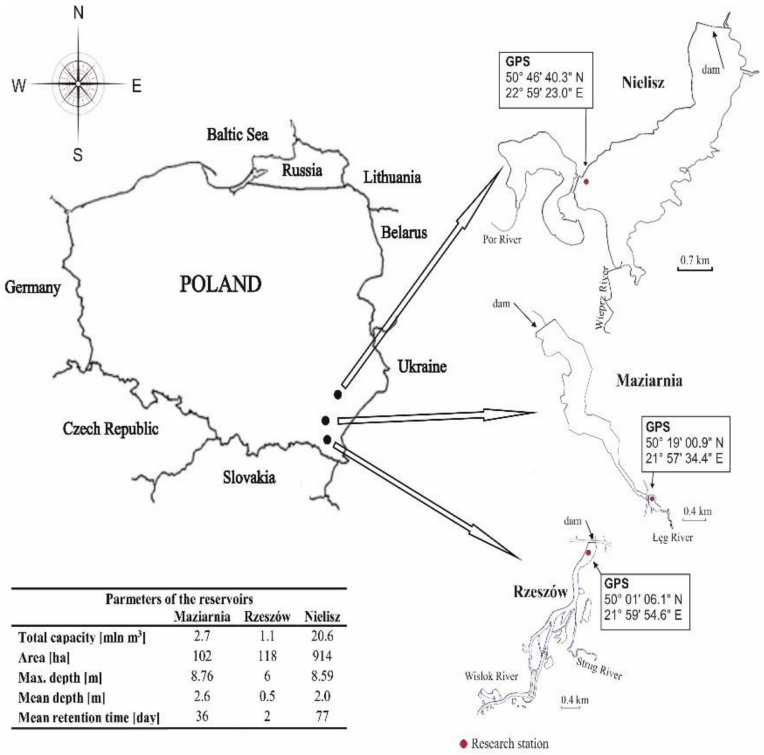
The objects of research were the sediments present in selected reservoirs of SE Poland, located in Podkarpackie Province (Rzeszów and Maziarnia Reservoirs) or Lubelskie Province (Nielisz Reservoir).

Rzeszow Reservoir was created in 1974. The main aim of the construction of the reservoir was the proper operation of the water intake for the city of Rzeszow and recreational-sports use. The surroundings of the reservoir are wasteland, cultivated fields and few scattered buildings. The catchment area of the reservoir, apart from a few industrial centres, is mainly agricultural in nature. Over the years, as a result of sediment accumulation and intensive deposition of debris carried by tributaries, the reservoir has become significantly siltation and shallow. Reclamation activities are currently underway at the reservoir.

Established in 1988, the Maziarnia Reservoir serves many functions, including: water supply, irrigation, fish farm, recreation and retention. The latter task is significantly limited due to insufficient capacity. The catchment of the reservoir is predominantly arable, with mixed crop and meadows zones accounting for about 70% of the area. A dominant role is played by individual-scale farming in highly fragmented holdings of land.

Nielisz Reservoir, commissioned in 2008, is surrounded by a belt of coniferous forests and consists of two parts: the main reservoir and the preliminary reservoir. The basic tasks of the reservoir include: flood protection, limiting water table fluctuations during the bird breeding season, energy generation (there is a 362 kW hydroelectric power plant on the dam), and use in recreation, leisure and amateur fishing.

The objects selected for the research differed, e.g., in size, nature of catchment and degree of degradation. Selected parameters of the studied reservoirs and their catchments are as shown in Figure 1.



Parmeters of the reservoirs	Maziarnia	Rzeszów	Nielisz
Total capacity [mln m^3]	2.7	1.1	20.6
Area [ha]	102	118	914
Max. depth [m]	8.76	6	8.59
Mean depth [m]	2.6	0.5	2.0
Mean retention time [day]	36	2	77

Figure 1. Location of studied reservoirs and sampling station.

2.2. Methods

2.2.1. Sediment Sampling and Preparation

Sediments from Rzeszów Reservoir were collected in April 2018, while those from Maziarnia and Nielisz Reservoirs were collected in April 2019. In each of the reservoirs, sediments were collected from a single station located in the river zone (Maziarnia and Nielisz) or near the dam (Rzeszów) (Figure 1). The height of the water column over the sediment at the sampling stations was approx. 1 m. These research stations were selected by reference to the conclusions of previous studies showing that emissions of CH_4 to the atmosphere were higher at these stations than others, amounting to 29.13 mmol/m^2·d on average (Rzeszów), as well as 372.43 (Maziarnia) and 209.55 mmol/m^2·d (Nielisz) [34]. The earlier study also confirmed a distinct nature to the pathways leading to the generation of CH_4 in the different production pathways was found between reservoirs. While acetate fermentation prevailed at the Nielisz site, CO_2 reduction prevailed at Maziarnia, while the roles played by the two pathways were similar in Rzeszów Reservoir [35,36].

The work described here thus proceeded on the assumption that the various biotic and abiotic parameters prevailing in the sediments at the stations selected for study would illustrate various courses to the AOM process representing the focus of the work.

Five intact sediment cores were collected from each reservoir, into plexiglass pipes, using a sampler constructed specially [37]. These were transported to the laboratory along with overlying water. Sediment cores were then expelled from pipes using a piston in such a way as to ensure division into three 5 cm layers, i.e., 0–5, 5–10 and 10–15 cm. Pore water was centrifuged from each layer of the first core (with an MPW-352 laboratory centrifuge) prior to determinations being made of concentrations of: organic carbon (TOC_W), inorganic carbon (IC), as well as ions of nitrate (III) (NO_2^-), nitrate (V) (NO_3^-), ammonium (NH_4^+), sulphate (SO_4^{2-}), chloride (Cl), sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}). Pore-water from the second core, set aside for determinations of the concentrations of iron ions (Fe^{2+} and Fe^{3+}) was extruded directly into gas-tight vials using a modified squeezer [38] to ensure that contact with air was avoided. Different sediment layers of the third core were dried at 60 °C, and milled, before being tested in regard to: pH,

organic matter (OM), total organic carbon (TOC_S) and humic substances (HS). The fourth sediment core, divided into layers, was first stored at −20 °C prior to the determination of contents of selected lipid biomarkers of microorganisms participating in AOM. The fifth core was deployed in studying the AOM process, with all analytical procedures performed in a helium atmosphere (glove bag).

2.2.2. Sediment Analysis

Sediment pH was determined potentiometrically (MultiLine P4, WTW, Reutlingen, Germany) in suspension with 1 N KCl. The content of organic carbon (TOC_S) was determined using a CN elemental analyser (Flash EA 1112, ThermoQuest, USA). Quality control relied on standard samples of known composition of carbon and nitrogen (acetanilide—C_8H_9NO). Prior to the determination of TOC content, samples of dried and milled sediment were placed in a desiccator with concentrated HCl vapours [39] for 72 h to ensure the removal of carbonates. Prior to analysis, sediment samples were again dried to constant mass at 60 °C. The content of humic substances in sediments was assessed according to the modified Griffith–Schnitzer method [40]. Granulometric analysis of sediments was performed in line with the PN-EN ISO 165 17892-4: 2017-01 standard. Areometric analysis (sedimentation method based on the Stokes law) was used to determine the content of boundary fractions for particles with a diameter of 0.063 to 0.002 mm and less than 0.002 mm. Sieve analysis was used for fractions with a particle diameter of 2–0.063 mm. Sediments were in turn classified in accordance with the PN-EN ISO 14688-2: 2018-05 standard.

Lipids (*pristane*, *crocetane*, *squalane* and *archaeol*) were extracted from 10 g portions of wet sediments by sonication in a sequence of solvent mixtures of increasing (1:2, 2:1, 1:0) dichloromethane: methanol (DCM:MeOH) ratios. Extracts were pooled and partitioned against a pre-extracted 0.05 M KCl-solution. The organic phase was collected and the solvent evaporated, yielding a total lipid extract (TLE). An aliquot of such TLE was then saponified with 6% KOH in methanol (80 °C, 3 h). Once cooled, this mixture was diluted with 0.05 M KCl-solution with neutral lipids then released from the basic mixture through a process of extraction with n-hexane. The neutral lipids were separated by solid-phase extraction (SPE) into three compound classes of (1) hydrocarbons (n-hexane:dichloromethane, 95:5, *v*:*v*) and (2) alcohols (dichloromethane:acetone, 9:1). Prior to analysis, alcohols were silylated with 50 µL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 100 µL pyridine for 1 h at 70 °C. In the case of alcohols and hydrocarbons, the analysis in question was performed using Thermo Electron Finnigan GC-MS (USA) equipped with a 60 m ZB-5 ms capillary column (0.32 mm i.d., 0.25 µm film thickness). Helium was supplied as the carrier gas at a flow rate of 1.2 mL/min. The GC temperature programme used was as follows: initial temperature 60 °C (1 min isothermal—alcohols; 2 min isothermal—hydrocarbons), from 60 to 150 °C (at 10 °C/min—alcohols; at 15 °C/min—hydrocarbons) and from 150 to 320 °C (at 5 °C/min—alcohols and hydrocarbons), 320 °C (25 min isothermal—alcohols and 2 min isothermal—hydrocarbons) [41–44].

2.2.3. Pore-Water Analysis

Ion concentrations for: nitrate (III) (NO_2^-), nitrate (V) (NO_3^-), ammonium (NH_4^+), sulphate (SO_4^{2}), chloride (Cl^-), sodium (Na^+), potassium (K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) were analysed in pore-water samples using a DIONEX ICS-5000 ion chromatograph. Concentrations of total organic carbon (TOC_w) and inorganic carbon (IC) were determined using a Shimadzu TOC-V CPN analyser, concentrations of Fe^{2+} and Fe^{3+} ions via the spectrophotometric method with ferrozine [45].

2.2.4. $^{13}CH_4$ Incubation Experiment

To determine rates of AOM, 7 g portions of wet sediment from the different (0–5 cm, 5–10 cm and 10–15 cm) layers were placed into the incubation vessels with 14 mL of deionised water deoxygenated by nitrogen (99.99% Air Products, USA). All operations

were carried out in a glove box (830-ABB Compact Glove Box Plas-Labs, USA), with the commencement of work preceded by 7–8 changes of the air in the glove box, which was replaced with helium (99.9999% Air Products). The prepared samples were preincubated to remove oxygen residues reflecting biochemical processes. This was completed by placing those samples in a thermostat for 10 days (at 15 °C, in the dark). Thereafter, 1 cm^3 of the $^{13}CH_4$ isotope tracer (99%, Sigma-Aldrich 490229–1L-EU, USA) was introduced into the headspace, prior to 40 days of incubation of the sediments (in the case of Rzeszów Reservoir), or 50 days (in the cases of Maziarnia and Nielisz Reservoirs). To provide evidence that the CO_2 produced by the AOM process is derived from the isotopic tracer, sediment incubation without $^{13}CH_4$ was carried out simultaneously. Incubation temperature was 15 °C, as this corresponded with the in situ temperature of the sediments collected from the first reservoir (April 2018, Rzeszów). The concentration of CO_2 in the headspace was determined on day 0, and then, successively on days 3, 10, 20, 30, 40 and 50, concentrations of both CO_2 and δ^{13}C-CO_2 were determined. The CO_2 concentrations at time zero were subtracted from those measured in subsequent days of incubation.

To determine the CO_2 concentration in the headspace, 1 cm^3 of the gas was taken in a gas-tight syringe (1001SL Hamilton, USA), with the difference made up each time using 1 cm^3 of helium (99.9999% Air Products). CO_2 concentrations were measured using a Shimadzu GC-2010 Plus gas chromatograph equipped with a Barrier Discharge Ionization Detector (BID) and a Shin Carbon ST column (2 m, 1.00 mm ID, Mesh 100/120). Samples were injected manually into the GC apparatus with the detector working at 250 °C. Temperatures were in turn 150 °C in the case of the injection port, and 60 °C in the column (the latter maintained for 13 min). The carrier gas was helium (99.9999% Air Products), supplied at a flow rate of 50 mL/min.

The carbon isotopic composition of CO_2 was determined using an isotope ratio mass spectrometer on-line with a gas chromatograph (GC-CIII-IRMS DELTAPlus Finnigan, Thermo Electron Corporation, USA) by injecting 0.1 cm^3 of the headspace. Results have been presented in the form of δ^{13}C expressed in ‰ relative to the PDB (Pee Dee Belemnite) standard.

Values obtained for CO_2 concentrations (ppm) and δ^{13}C-CO_2 (‰) were used to calculate the anaerobic oxidation of methane (AOM).

2.2.5. Calculations

The AOM rate (nmol/g·h) and amount of CH_4 oxidised (nmol/g) were calculated using Equations (1) and (2) [29,31]. The values δ^{13}C-CO_2 were converted to ^{13}C AP% in line with Equation (3):

$$Rate\ of\ AOM = \frac{\frac{^{13}CAT\%}{100} \cdot [CO_2] \cdot \frac{V_g}{V_m} \cdot 1000}{m \cdot t} \quad (1)$$

$$Amount\ of\ CH_4\ oxidised = Rate\ of\ AOM \cdot t \quad (2)$$

$$\begin{aligned} {}^{13}C\ AP &= \frac{\delta^{13}C\ PDB+1000}{\delta^{13}C\ PDB+1000+\frac{1000}{R_{std}}} \cdot 100 = \frac{\left[^{13}CO_2\right]}{\left[^{13}CO_2\right]+\left[^{12}CO_2\right]} \cdot 100 \\ &= \frac{\left[^{13}CO_2\right]}{[CO_2]} \cdot 100 \end{aligned} \quad (3)$$

where $^{13}C\ AP$—^{13}C atom percent (%), CO_2—carbon dioxide concentration at time (ppm), V_g—volume of headspace in vial (0.096156 dm^3), V_m—molar volume of ideal gas at 25 °C (24.45 dm^3/mol), m—dry sediment weight (g), t—incubation time (h), R_{std}—absolute ratio constant in the standard (0.0111803).

3. Results

3.1. Sediment Characteristics

The sediments studied were found to differ in their granulometric composition (Table 1). The silty fraction dominated in all sediment layers from Maziarnia Reservoir

(44.09–50%). In the 0–5 and 5–10 cm layers, the smallest share was accounted for by the sand fraction (17.49% and 25.18%), while in the deepest layer, it was the clay fraction (22%). The analysed sediments were loam down to a depth of 10 cm, though with clay loam present in the 10–15 cm layer. The analysis of the granulometric composition of the Nielisz Reservoir sediments confirmed these as clay-loam sediments throughout the studied depths. In the 5–10 and 10–15 cm layers, it was the silty fraction that predominated, with the respective shares being 45.98 and 56.05%, and with clear increases occurring at successively greater depths. The sand fraction took the highest share in the 0–5 cm layer, (51.27%). It was also in these sediments that the smallest clay-fraction share was noted (in the range 15.44–22.64%). In the sediments from Rzeszów Reservoir, as in those from the Maziarnia site, the silty fraction dominated, though with a much larger share (73.15–77.35%). The sand fraction proved to be the smallest, accounting for between 3.50 and 5.23% of the total (Table 1). The surface layer thus consisted of silty loam sediments, while the deeper layers were classified as silty clay.

Table 1. Granulometric composition of the studied sediments.

Reservoir	Depth (cm)	Fraction (%)			Type
		Fsa 2–0.063 mm	Fsi 0.063–0.002 mm	Fcl < 0.002 mm	PN-EN ISO 14688–2: 2018–05
Maziarnia	0–5	17.49	50.00	32.51	loam (Cl)
	5–10	25.18	44.09	30.72	loam (Cl)
	10–15	32.18	45.82	22.00	clay loam (sasiCl)
Nielisz	0–5	51.27	33.28	15.44	clay loam (sasiCl)
	5–10	34.03	45.98	19.98	clay loam (sasiCl)
	10–15	21.31	56.05	22.64	clay loam (sasiCl)
Rzeszów	0–5	3.50	77.35	19.15	silty loam (clSi)
	5–10	5.23	73.15	21.61	silty clay (siCl)
	10–15	4.01	73.76	22.22	silty clay (siCl)

Notes: Fsa—sand fraction, Fsi—silty fraction, Fcl—clay fraction.

The pH values for the analysed sediments ranged from 6.39 to 8.03, with greatest acidity characterising those from Maziarnia, and highest alkalinity those from the Nielisz site (Table 2). While it was the deeper-lying sediments from Maziarnia Reservoir that had lower pHs, the trend for Rzeszów Reservoir was the reverse. No such trends could be noted for Nielisz Reservoir.

Table 2. Selected parameters characterising sediments in the reservoirs studied.

Reservoir	Depth (cm)	pH	OM (%)	TOC_S (%)	HS (mg/g)	*Pristane* (µg/g)	*Crocetane* (µg/g)	*Squalane* (µg/g)	*Archaeol* (µg/g)
Maziarnia	0–5	7.13	4.44	1.38	14.20	0.082	0.16	0.20	1.00
	5–10	6.39	4.71	1.79	18.20	0.046	0.07	0.16	0.65
	10–15	6.44	3.38	1.29	13.40	0.042	0.07	0.49	0.47
Nielisz	0–5	8.01	13.16	5.22	48.00	0.117	0.13	0.22	3.14
	5–10	7.99	13.18	4.94	49.60	0.101	0.13	0.19	3.48
	10–15	8.03	12.47	4.97	50.50	0.075	0.10	0.17	3.34
Rzeszów	0–5	7.51	9.65	3.79	34.90	0.106	0.14	0.24	1.42
	5–10	7.58	8.18	3.84	34.70	0.072	0.09	0.27	2.44
	10–15	7.71	5.99	2.66	22.90	0.057	0.07	0.68	2.06

Contents of OM and TOC_S showed strong correlations with each other ($r = 0.9831$, $p < 0.001$), with TOC_S being 31–47% of OM. The sediments from Nielisz Reservoir displayed highest contents of OM and TOC_S, in the respective ranges 12.47–13.16% and 4.94–5.22%. Lowest contents of OM and TOC_S were recorded in the sediments of Maziarnia Reservoir, and were respectively in the 3.39–4.71% and 1.29–1.79% ranges (Table 2).

HS, as natural organic compounds found commonly in the aquatic environment, were found to correlate closely with OM ($r = 0.9899$, $p < 0.001$) and TOC_S ($r = 0.9909$, $p < 0.001$). It was in the Nielisz sediments that HS contents were highest (in the 48.00–50.50 mg/g range), while lowest values were those in the 14.2–18.20 mg/g range reported from Maziarnia Reservoir (Table 2).

Where the analysed lipid biomarkers were concerned, sediments showed highest contents of *archaeol* (in the 0.47–3.48 µg/g range), as well as lowest contents of *crocetane* (0.07–0.14 µg/g) (Table 2). Typically, it was deeper layers of sediment that were found to contain smaller amounts of the lipid biomarkers. However, there was also a general observation that sediments from Nielisz Reservoir stood out in terms of their greatest level of enrichment in *archaeol*.

3.2. Pore-Water Characteristics

Table 3 presents concentrations of NO_2^-, NO_3^-, NH_4^+, SO_4^{2-}, Cl^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, Fe^{2+}, Fe^{3+} ions, as well as IC and TOC_W, in the pore water of sediments in the reservoirs studied.

Table 3. Results obtained for the chemical parameters of pore water in the reservoirs studied.

Reservoir	Depth cm	NO_2^- mg/dm^3	NO_3^- mg/dm^3	NH_4^+ mg/dm^3	SO_4^{2-} mg/dm^3	Cl^- mg/dm^3	Na^+ mg/dm^3	K^+ mg/dm^3	Mg^{2+} mg/dm^3	Ca^{2+} mg/dm^3	TOC_w mg/dm^3	IC mg/dm^3	Fe^{2+} µmol/dm^3	Fe^{3+} µmol/dm^3
Maziarnia	0–5	0.032	0.040	5.53	14.49	17.30	16.29	5.10	4.98	50.72	20.06	64.08	69.70	896.33
	5–10	0.028	0.071	6.64	10.61	17.88	15.77	5.70	6.92	63.39	36.39	85.71	172.45	736.61
	10–15	0.024	0.037	9.43	6.30	16.93	13.36	4.19	9.88	78.81	44.34	101.66	321.61	401.51
Nielisz	0–5	0.010	0.024	16.58	11.34	6.37	6.21	5.24	12.37	119.34	52.74	147.12	110.80	845.55
	5–10	0.015	0.029	12.70	9.70	4.81	5.59	2.63	9.73	80.62	28.54	114.30	162.17	778.95
	10–15	0.018	0.036	8.07	8.40	3.20	4.99	1.35	9.00	64.79	20.64	101.50	259.78	741.85
Rzeszów	0–5	0.015	0.037	4.75	0.87	19.81	17.12	6.85	18.92	97.06	19.88	149.14	68.41	279.75
	5–10	0.018	0.052	9.61	2.56	16.70	15.77	7.46	21.79	118.59	31.44	141.44	75.93	177.15
	10–15	0.015	0.020	8.04	2.37	16.12	16.43	8.64	22.67	124.42	36.56	131.80	100.52	39.45

Analysis of concentrations of nitrogen compounds revealed that these were slightly higher in Maziarnia Reservoir than the other sites, where NO_2^- and NO_3^- were concerned. Respective values ranged from 0.024 mg/dm^3 in the deepest layer to 0.032 mg/dm^3 in the surface layer, as well as from 0.037–0.071 mg/dm^3 in the 5–10 cm layer. The lowest recoded concentrations of NO_2^- and NO_3^- characterised Nielisz Reservoir, even as this was the reservoir distinguished from the others in having the highest observed concentrations of NH_4^+, which also decreased with sediment depth—from 16.58 to 8.07 mg/dm^3.

The highest concentrations of SO_4^{2-} were the 6.30–14.49 mg/dm^3 recorded in Maziarnia Reservoir, while figures were slightly lower at the Nielisz site (8.40–11.34 mg/dm^3), as well as much lower in Rzeszów Reservoir (0.87–2.56 mg/dm^3). In the first two reservoirs, the concentration of SO_4^{2-} was lower at greater depth.

Concentrations of Cl^-, Na^+ and K^+ ions were found to be present at similar levels in the Maziarnia and Rzeszów Reservoirs, respectively, varying across the ranges 16.12–19.81 mg/dm^3, 13.36–17.12 mg/dm^3 and 4.19–8.64 mg/dm^3. Only much lower values were reported from Nielisz Reservoir, in the ranges 3.20–6.37 mg/dm^3, 4.99–6.21 and 1.35–5.24 mg/dm^3, respectively.

The highest concentrations of $Mg^{2+}$and Ca^{2+} ions characterised Rzeszów Reservoir (18.92–22.67 and 97.06–124.42 mg/dm^3), with figures slightly lower in Nielisz Reservoir (at 9.00–12.37 and 64.79–119.34 mg/dm^3), as well as being much lower in Maziarnia Reservoir (4.98–9.88 and 50.72–78.81 mg/dm^3). Concentrations of these ions showed statistically significant positive correlations with each other ($r = 0.8704$, $p < 0.01$) as well as with

concentrations of IC ($r = 0.8136$, $r = 0.8953$, $p < 0.01$). Moreover, the concentration of Mg^{2+} correlated significantly with that of SO_4^{2-} ($r = -0.8839$, $p < 0.01$).

All reservoirs had pore-water concentrations of Fe^{3+} that decreased with depth. The highest values were found for Nielisz Reservoir (741.85–845.55 μmol/dm^3), although figures for Maziarnia (401.51–896.33 μmol/dm^3) were similar, while those for Rzeszów Reservoir were far lower (39.45–279.75 μmol/dm^3). Trends were the opposite for Fe^{2+} concentrations, which were greater at greater depth. However, no significant differences between the reservoirs were observed. It was also possible to note a statistically significant correlation between the concentrations of Fe^{3+} and SO_4^{2-} ($r = 0.9349$, $p < 0.001$).

In the case of TOC_W concentrations, it proved impossible to note significant differences between the reservoirs, with no clear relationships of any kind found. In Maziarnia and Rzeszów Reservoirs, TOC_W concentrations were greater further down into the sediment, differing across respective ranges of 20.06–44.34 and 19.88–36.56 mg/dm^3. In Maziarnia Reservoir, a high of 52.74 mg/dm^3 was noted for pore water of the surface sediment layer even as a low figure of 20.64 mg/dm^3 characterised the 5–10 cm layer.

3.3. Rate of AOM

The addition of the $^{13}CH_4$ isotope tracer to the incubations resulted in an increase in CO_2 concentrations and in values for ^{13}C AP in the headspace (Figure 2)—a circumstance taken to confirm the presence of the AOM process in sediments investigated. In comparison, in incubations without the isotopic tracer, ^{13}C AP values varied slightly over time from a value of about 1.05 to a maximum of 1.46. However, the reservoirs differed in the intensity of the process (Figure 3a,c,e). In almost all cases, it was the Nielisz sediments that had the highest CH_4 oxidation rates (in the 0.20–0.81 nmol/g·h range), with similar rates in the 0–5 cm and 10–15 cm layers, as well as slightly lower ones in the 5–10 cm layer. In the other two reservoirs, AOM rates were lower than at the Nielisz site. In the surface 0–5 cm layer of sediments from Rzeszów Reservoir, AOM rates were about twice as high as in Maziarnia Reservoir, with ranges of 0.35–0.53 and 0.12–0.28 nmol/g·h, respectively. In the deeper (5–10 and 10–15 cm) layers, there was no clearly higher AOM rate in one reservoir or another. In addition, it was typical for the maximum AOM rate to be noted 3 or 10 days into the incubation process.

The amounts of CH_4 oxidised during the incubation are as shown in Figure 3b,d,f. In Nielisz Reservoir (where AOM was most intensive), the maximum amount of CH_4 oxidised characterised day 30 in the 0–5 cm and 5–10 cm layers, or day 40 in the 10–15 cm layer. In Maziarnia Reservoir, a maximum on day 40 was only recorded for the surface sediment layer, with deeper layers reporting their maximum as early as on day 20. In Rzeszów Reservoir, the amount of CH_4 oxidised continued to increase through to day 40 in all sediment layers, it not therefore being known if maxima had been reached or if further increases might have been noted.

With a view to factors controlling the studied process in reservoir sediments being determined, further analysis considered relationships between the AOM rates obtained and certain parameters characterising both pore water and sediments. Pearson correlation coefficients (r) for rates of AOM on days 3, 20 and 40 are presented in Tables 4 and 5.

Table 4. Pearson correlations (r) between AOM rate and pore-water parameters.

	NO_2^-	NO_3^-	NH_4^+	SO_4^{2-}	Cl^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	TOC_w	IC	Fe^{2+}	Fe^{3+}
AOM rate (3 day)	−0.3293	−0.0577	−0.0922	−0.0694	−0.3295	−0.3015	−0.2884	0.0333	−0.0914	−0.6362	0.2740	−0.3431	0.2296
AOM rate (20 day)	−0.5329	−0.2925	0.5989	0.2206	−0.7804 *	−0.8589 **	−0.6647	−0.2689	0.0055	0.2515	0.2625	0.4285	0.4657
AOM rate (40 day)	−0.8646 **	−0.5910	0.3810	−0.2938	−0.6325	−0.5763	−0.1690	0.3968	0.4691	−0.0911	0.6676 *	−0.1430	−0.0673

Notes: * $p < 0.05$, ** $p < 0.01$.

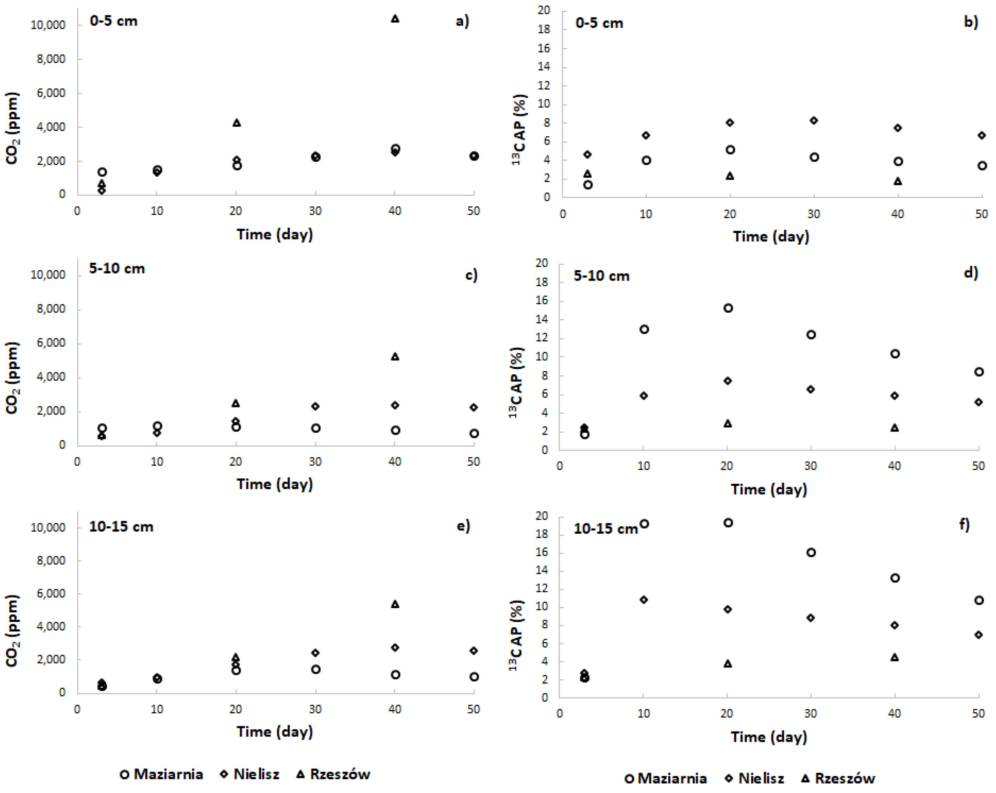


Figure 2. Changes in CO_2 concentrations – panels (**a,c,e**) and values of ^{13}C AP – panels (**b,d,f**) over the time of incubation of sediments.

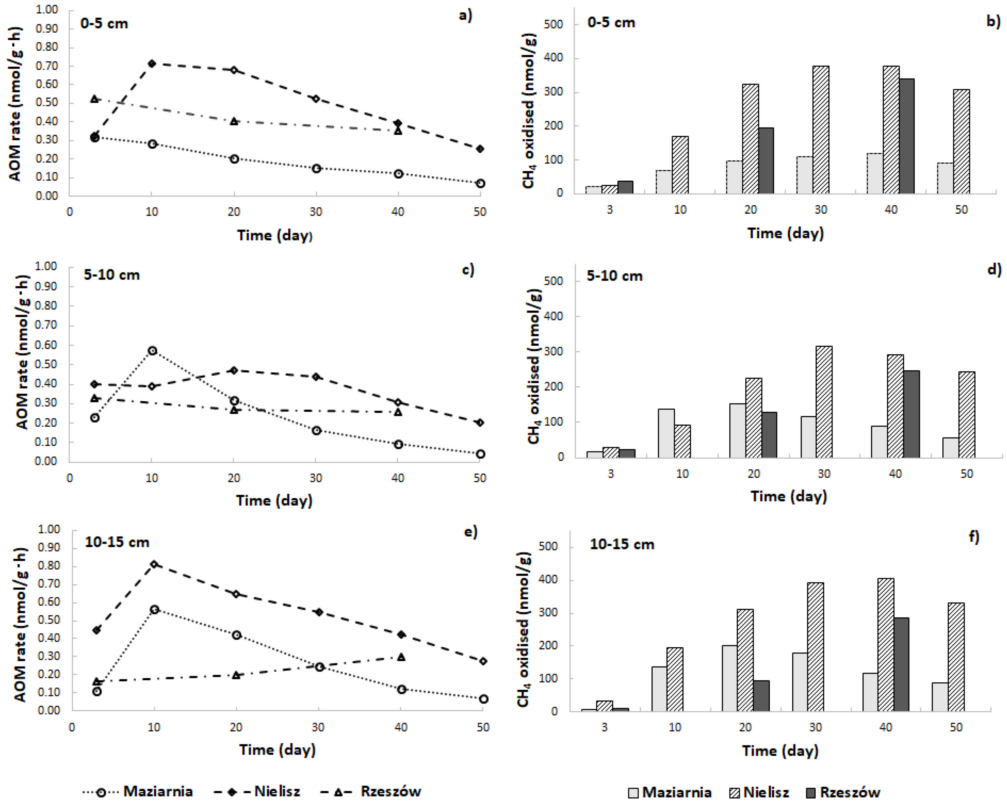


Figure 3. Changes in AOM rates – panels (**a,c,e**) and amounts of CH_4 oxidised – panels (**b,d,f**) over the time of incubation of sediments.

Table 5. Pearson correlations (*r*) between AOM rate and sediment parameters.

	pH	OM	TOC_S	HS	*Pristane*	*Crocetane*	*Squalane*	*Archaeol*
AOM rate (3 day)	0.5889	0.7057 *	0.6676 *	0.6840 *	0.7495 *	0.6514	−0.6621	0.4943
AOM rate (20 day)	0.4402	0.7513 *	0.7077 *	0.7452 *	0.4804	0.1649	−0.4038	0.5778
AOM rate (40 day)	0.9026 ***	0.8691 **	0.8998 ***	0.8712 **	0.6574	0.2801	−0.0890	0.8244 **

Notes: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

The experiments run with $^{13}CH_4$ tracer evidenced that a process of the anaerobic oxidation of methane that takes place in reservoir sediments and may therefore represent an important sink for CH_4 (even) in the ecosystems of inland waters. Rates for AOM obtained for the sediments in reservoirs located in SE Poland are of 0.05 to 0.81 nmol/g·h (Figure 3a,c,e). While these are different values from those reported in other aquatic ecosystems, they do fall within the overall reported range. Thus, AOM rates for the sediments of certain wetlands and swamps in the USA ranged from 0.00047 to 0.00254 nmol/g·h [29], while—in the more familiar circumstances of marine environments, obtained AOM values have included 5 nmol/g·h reported for sediments in the North Sea, 15.63 nmol/g·h in Japan's Nankai Trough, and 22.92 nmol/g·h in the Hydrate Ridge (USA) [46]. However, the much higher rate noted for Eckernforde Bay (Germany) was of 11,920 nmol/g·h [47,48].

In the course of the studies, it was possible to observe the formation of $^{13}CO_2$, with the AOM process proving most intense 3 or 10 days into the process of incubation, after which a decrease in the rate of oxidation of CH_4 could be observed. Decreasing AOM rates during incubation have also been reported by other researchers [31,49,50]. A decrease in ^{13}C AP values was primarily after day 20 (Figure 2b,d,f), with the CO_2 concentration usually still increasing to day 40 (Figure 2a,c,e). Maxima for amounts of CH_4 oxidised characterised days 20, 30 or 40, depending on the reservoir and sediment layer involved, with a decrease then ensuing (Figure 3b,d,f). This certainly reflected a gradual depletion of electron acceptors and an increase in the endogenous activity of microorganisms responsible for methanogenesis going hand in hand with a decline in concentrations of $^{13}CO_2$ [50,51].

In regard to the vertical distribution of AOM rates in sediments, it was only those in Rzeszów Reservoir that showed a markedly reduced intensity of the process at greater depths. Similar trends have been noted by other researchers [52–54], which was attributed to a greater availability of electron acceptors close to the sediment–water interface [53,55]. Given that Maziarnia and Nielisz Reservoirs failed to report any such clear decrease in AOM in deeper sediment layers, it needs to be assumed that the availability of electron acceptors is not the only controlling factor but also certain properties of both sediments and their pore waters.

Indeed, the process of AOM in the bottom sediments of aquatic ecosystems can be seen to depend on many factors, with their impact proving very hard to determine given the complexity of processes. Temperature, which was not addressed in this study at all, is believed to be a key factor, with the literature often invoking its positive impact [50,52,56–58]. Some papers confirm the sensitivity of the AOM process to temperature change and imply a peak level ~25 °C [59].

A priori consideration of influences on AOM suggested the kind of positive correlation with concentrations of electron acceptors in pore water that the experiments in fact failed to detect (Table 4). While concentrations of NO_3^- were low in all the sediments we investigated, and while no significant differences between reservoirs in this respect were observed, concentrations of SO_4^{2-} and Fe^{3+} were already higher and clearly differentiated. Thus, while these ions were present in rather low concentrations in the Rzeszów sediments, in those of the Maziarnia and Nielisz Reservoirs, they were higher, with SO_4^{2-} present at

above 60 μmol/dm^3—i.e., the threshold value at which reduction in sulphates may surpass methanogenesis [60]. Despite concentrations of SO_4^{2-} and Fe^{3+} in the Nielisz and Maziarnia Reservoirs being similar, there were significant differences in measured rates of AOM. This perforce confirmed the hypothesis advanced above that rates of AOM are controlled not solely by the availability of electron acceptors but also by other environmental factors.

One such factor may have been sediment pH, given the positive relationship with AOM rate we were able to observe (Table 5). While the microorganisms involved in AOM do have adapted to environments across a wide range of pH, optimal conditions for them are nevertheless associated with just pH 7.7–7.9. In turn, in extremely acidic conditions (pH < 2) or those featuring extremely alkaline pHs (∼12), the oxidation of CH_4 is—unsurprisingly—inhibited, inter alia because the growth of the *Archaea* involved is arrested [61,62]. While the sediments under study displayed far more moderate pHs, the observed range of 6.39–8.03 mostly denoted circumstances outside the aforementioned optima (Table 2). Furthermore, differences in pH may affect not only the microorganisms responsible for AOM but also other circumstances relevant to the process. For example, lower pH values favour the solubility of iron (III) hydroxide and increase the bioavailability of Fe^{3+}, thereby accelerating AOM [62].

Another factor of undoubted influence on AOM relates to amounts and types of organic matter. We obtained statistically significant positive correlations between OM and TOC_S on the one hand and rate of AOM rate on the other (Table 5). It is presumed that sediments richer in OM will stimulate AOM, e.g., by organic electron acceptors such as humic substances which are used by microorganisms in their anaerobic respiration [23]. Such a hypothesis may gain confirmation through our statistically significant positive relationships found between rate of AOM and HS content (Table 5) as well as the observation that the highest AOM rate coincided with Nielisz Reservoir sediments richest in HS (Table 2). Moreover, strong statistically significant correlations were found between contents of OM and TOC_S ($r = 0.9898$, $p < 0.001$) and OM and HS ($r = 0.9908$, $p < 0.001$). Positive correlations between rates of AOM and contents of OM and HS have also been observed in other studies [49,63]. Thus, the labile part of OM present in freshwater sediments can promote methanogenesis [64], while a slowly decomposing humic part can prevent CH_4 emission by acting as the final electron acceptor driving AOM [20].

The highest level of statistical significance characterised positive correlations between AOM rate and the content of the *pristane* and *archaeol* lipid biomarkers characteristic for *Archaea* (Table 5). *Archaeol* and *pristane* are both ether lipids, whose presence denotes *Archaea* of the ANME-1 group. These are common in sediments given a higher degree of adaptability in environments that can be regarded as unfavourable (in terms of temperature or pH) than is the case for ANME-2 and ANME-3 [65,66]. As *Archaea* from this group are responsible for an AOM process combining with sulphate reduction (AOM-SR), the correlations obtained may point to this mechanism of CH_4 oxidation being the main one ongoing in the sediments under study. This hypothesis would be confirmed by positive correlations between AOM rate and the concentration of SO_4^{2-} in pore water—of the kind not in fact observable in our work (Table 4). However, measured AOM rates might not solely reflect AOM-SR, given the way that other studies point to complex relationships between metals and sulphates or humic substances in sediments, and hence to the co-occurrence of CH_4 oxidation via a variety of mechanisms [20,55,57,62,67–69].

Analysis of obtained results also showed statistically significant negative correlations between the concentrations of Cl^-, Na+, K+ in the pore water and the AOM rates, suggesting that salinity may impact upon the AOM process in a negative way (Table 4). An inverse relationship between concentrations of Cl^- and rates of AOM has in fact been confirmed by other studies [70–72]. High concentrations of Cl^- have been linked with reduced biodiversity of both bacteria and *Archaea* [73].

5. Conclusions

The results presented show how the process of AOM can represent a sink for CH_4 in freshwater sediments. Rates of AOM were found to differ both from one studied reservoir to another and with different layers of sediment. It was observed that the AOM process was seen to have achieved peak intensity after 3 or 10 days into incubation, which is followed by a slowdown, most likely as available electron acceptors become depleted. After day 20 of incubation, the coexisting process of methanogenesis could gain in significance.

Preliminary analysis of the environmental factors controlling AOM pointed not only to the availability of electron acceptors but also to parameters characterising both sediments and their pore water. Higher pH values of sediments could be associated with intensified AOM, presumably as conditions favourable to the development of microorganisms were present or because other processes responsible for the availability of electron acceptors were being supported. We further suggest that an increase in the salinity of pore waters may limit the AOM process, which is possibly due to a negative effect on the growth of microorganisms. Our results also suggest that the intensity of AOM in sediments is affected to a significant degree by the availability, not only of NO_3^-, SO_4^{2-} and Fe^{3+}, but also of other organic electron acceptors such as humic substances. However, the key controlling factor appears to be the availability of the microorganisms oxidising CH_4 anaerobically.

Our analysis further suggests an overall AOM process in sediments entailing very complex mechanisms in need for further elucidation. Matters of particular importance in this regard will be the recognition of the role played by potential electron acceptors as well as species identification and the singling-out of those *Archaea* responsible for the anaerobic oxidation of methane in reservoir sediments (phylogenetic analysis of 16S rRNA).

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