



The natural chlorine cycle – Formation of the carcinogenic and greenhouse gas compound chloroform in drinking water reservoirs



Sándor T. Forczek ^{a,*}, Milan Pavlík ^a, Josef Holík ^a, Luděk Rederer ^b, Martin Ferenčík ^{b, c}

^a Isotope Laboratory, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220, Prague, Czech Republic

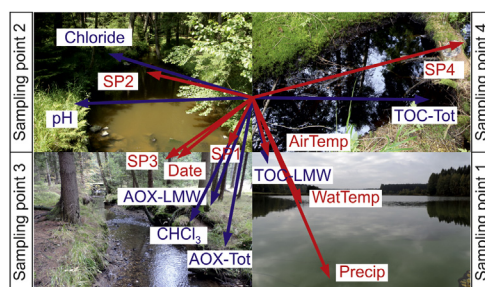
^b Povodí Labe, State Enterprise, Váta Nejedlého 951, CZ-50003, Hradec Králové, Czech Republic

^c Institute of Environmental and Chemical Engineering, Faculty of Chemical Technology, University of Pardubice, Studentská 573, CZ-53210, Pardubice, Czech Republic

HIGHLIGHTS

- CHCl_3 is formed naturally in the chlorine cycle, beginning and ending with chloride.
- Soil organic carbon is chlorinated to large adsorbable organohalogen molecules.
- CHCl_3 is emitted during biotransformation of adsorbable organohalogenes.
- The process is influenced by physicochemical factors, mainly by pH & redox potential.
- Ratios of non-chlorinated and chlorinated substrates to CHCl_3 influence its formation.

GRAPHICAL ABSTRACT



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ABSTRACT

Chlorine cycle in natural ecosystems involves formation of low and high molecular weight organic compounds of living organisms, soil organic matter and atmospherically deposited chloride. Chloroform (CHCl_3) and adsorbable organohalogenes (AOX) are part of the chlorine cycle. We attempted to characterize the dynamical changes in the levels of total organic carbon (TOC), AOX, chlorine and CHCl_3 in a drinking water reservoir and in its tributaries, mainly at its spring, and attempt to relate the presence of AOX and CHCl_3 with meteorological, chemical or biological factors.

Water temperature and pH influence the formation and accumulation of CHCl_3 and affect the conditions for biological processes, which are demonstrated by the correlation between CHCl_3 and $\Sigma\text{AOX}/\text{Cl}^-$ ratio, and also by $\text{CHCl}_3/\Sigma\text{AOX}$, $\text{CHCl}_3/\text{AOX}_{\text{LMW}}$, $\text{CHCl}_3/\Sigma\text{TOC}$, $\text{CHCl}_3/\text{TOC}_{\text{LMW}}$ and $\text{CHCl}_3/\text{Cl}^-$ ratios in different microecosystems (e.g. old spruce forest, stagnant acidic water, humid and warm conditions with high biological activity). These processes start with the biotransformation of AOX from TOC, continue via degradation of AOX to smaller molecules and further chlorination, and finish with the formation of small chlorinated molecules, and their subsequent volatilization and mineralization. The determined concentrations of chloroform result from a dynamic equilibrium between its formation and degradation in the water; in the Hamry water reservoir, this results in a total amount of 0.1–0.7 kg chloroform and 5.2–15.4 t chloride. The formation of chloroform is affected by Cl^- concentration, by

* Corresponding author.

E-mail address: alex067@biomed.cas.cz (S.T. Forczek).

concentrations and ratios of biogenic substrates (TOC and AOX), and by the ratios of the substrates and the product (feedback control by chloroform itself).

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1. Introduction

Until the 1980's, halogenated compounds present in nature were considered to be exclusively industrial products. The AOX method (Müller, 2003) was introduced to detect possibly harmful halogenated organic compounds (aka organohalogenes) in natural environment; it was originally developed to quantify anthropogenic pollution of surface waters. The majority of substances determined by AOX contain chlorine bound to organic molecules and are called chlorinated organic compounds or organochlorines (Cl_{org}). Subsequent investigations have clearly shown that organohalogenes, which are also measured as AOX (Müller, 2003), are derived from natural ecosystems (Silk et al., 1997).

Abiotic and biotic reactions in soil cause chlorination of organic compounds by inorganic chlorine. High molecular weight Cl_{org} (HMW- Cl_{org}), such as chlorohumus, chlorinated humic- and fulvic acids (Myneni, 2002; Leri et al., 2006) and naturally occurring low-molecular weight Cl_{org} (LMW- Cl_{org} , 0–500 Da) such as aromatic Cl_{org} (chlorophenol analogs (Ph- Cl_{org})) and aliphatic Cl_{org} (A- Cl_{org}) called also alkyl Cl_{org} (Hoekstra et al., 1999a; Keppler et al., 2000). Some A- Cl_{org} belong to highly volatile organochlorines (HV- Cl_{org}), which cannot be determined by the AOX method. At the same time LMW- Cl_{org} are formed from HMW- Cl_{org} by degradation (mineralization).

A considerable attention has been given to conversions of LMW- Cl_{org} in soil explaining the formation of Cl_{PhA} (Hoekstra et al., 1999a), chloroacetic acids (Hoekstra et al., 1999a; Matucha et al., 2007a; Matucha et al., 2007b), HV- Cl_{org} , e.g. chloroform - $CHCl_3$ (Hoekstra et al., 1999a), and further A- Cl_{org} or HV- Cl_{org} (Forczek et al., 2015). Through volatilization and mineralization from soil (abiotic redox reactions), water and organisms (fungi, bacteria and plants), chlorine gets back into the atmosphere (A- Cl_{org} or HV- Cl_{org}), where its biogeochemical cycle continues (Harper, 1985; Keppler et al., 2000; Winterton, 2000; Öberg, 2002); (Öberg, 2003; Hamilton et al., 2003; Forczek et al., 2015).

Among other Cl_{org} that were found to be carcinogenic in animal tests, chloroform is one of the most strictly controlled and regulated compounds in drinking water. Limits of chloroform in drinking water vary among different countries of Europe, USA and the Czech Republic (30–200, 70 and 50 $\mu\text{g L}^{-1}$). Anthropogenic sources of chloroform and other Cl_{org} cannot be distinguished from natural sources. Chloroform represents the most abundant halocarbon in the atmosphere (Cappelletti et al., 2012; Harper, 2000) but the anthropogenic flux of chloroform into the environment is much too low to account for observed background concentrations. Budget calculations indicate that the terrestrial ecosystem may also be an important source of volatile halocarbons or Cl_{org} , thereby contributing to the global halogen or more precisely, to the global chlorine cycle (Winterton, 2000; Öberg, 2002).

The biggest share of AOX, represented by various types of Cl_{org} formed in the forest soil and determined as AOX, usually falls between 200 and 1000 mg Cl kg^{-1} soil and its amount is proportional to the total carbon content of the soil and often exceeds its free inorganic chloride (Cl^-) content (Öberg et al., 2005). The dissolved Cl_{org} in waters from the forest ecosystem can end up in reservoirs of drinking water, where these substances then contribute to increased concentration of $CHCl_3$, HV- Cl_{org} and AOX. The AOX

indicator reports on potential deterioration of water quality. It is important to understand the biogeochemical cycle of chlorine and to separate natural processes from anthropogenic influences in forest ecosystems, in order to identify the threats stemming from human activities. High AOX levels in drinking water can pose health risk, which raises concerns about the health of its consumers. A water source that can be subject to these risks can be found in the catchment area of the Hamry water reservoir (Czech Republic).

The aim of our study was to characterize the levels of chlorine, AOX and $CHCl_3$ in the reservoir and in its tributaries, mainly at its spring, in a spruce forest stand and find out whether it is possible to relate the presence of AOX and $CHCl_3$ with meteorological, chemical or biological factors. The anticipated findings can show a possible correlation between increased levels of Cl^- , TOC, AOX and $CHCl_3$ with environmental factors in different microecosystems in the catchment of the Hamry water reservoir. According to our previous laboratory findings the chemistry of the water, including the content of carbon- and chlorine-containing compounds with different molecular weights and pH can influence the formation of organochlorines, but has never been confirmed in natural circumstances. Studying the changes in the above factors along a small river feeding the reservoir offers the possibility to gain insight into the chlorine cycle directly in nature.

2. Materials and methods

2.1. Sampling strategy and site description

The catchment of the river Chrudimka was monitored on four characteristic plots (Sup. Table 1, Sup. Fig. 1), including the Hamry water reservoir, where drinking water is prepared, for meteorological parameters (maximum daily air temperature, and precipitation), physico-chemical properties of the water (temperature, pH) and changes of the concentration of chloride (Cl^-) and adsorbable organically bound halogens (AOX), total organic carbon (TOC) and chloroform content ($CHCl_3$). The area is covered by Norway spruce forest community (vegetation type *Piceion abietis* alliance), and peatland forests (vegetation type *Vaccinio uliginosi-Pinetum sylvestris* alliance).

2.2. Physico-chemical parameters of water

pH: A portable pH meter (EcoScan pH 6, Eutech Instruments, 0.01 pH resolution) was used to determine air and water temperature and pH on site.

Cl^- : The concentration of chloride ion was determined in the laboratory by chloride ion-selective electrode (CyberScan Ion 510, Eutech Instruments).

Chloroform was determined according to the method of Cho et al. (2003) by gas chromatography (Varian CP-3800 with an autosampler CombiPal) with electron capture detection (GC-ECD) on a capillary column: VF-1ms (15 m, 0.25 mm ID, D_f 0.25 μm), using solid phase microextraction (SPME) technique. GC column program was the following: 40 $^{\circ}\text{C}$ (2 min), 25 $^{\circ}\text{C min}^{-1}$ to 130 $^{\circ}\text{C}$ (4 min), 25 $^{\circ}\text{C min}^{-1}$ to 290 $^{\circ}\text{C}$ (4 min). Injector temperature: 230 $^{\circ}\text{C}$, detector temperature: 290 $^{\circ}\text{C}$. The volatile compounds were sampled by SPME fiber from the headspace over a 10 ml sample in a

20 ml vial. The SPME fiber was coated with 100 μm layer of polydimethylsiloxane (PDMS) as a sorbent polymer (Supelco), SPME sample extraction was done with agitation (40 $^{\circ}\text{C}$, 15 min). Trihalomethanes Calibration Mix (Supelco) was used for calibration of CHCl_3 . 10 parallel samples were measured for each sampling, blank samples were inserted on a regular basis to control background purity.

AOX and TOC determinations The determination of AOX and their LMW fractions in water samples was carried out according to the EN ISO 9562:2004 guidelines of technical standards for water quality and analysis (European Standard, 2004). In brief, AOX were determined as follows: 50 mg of activated carbon and 5 ml of an acidic nitrate solution (0.02 M HNO_3 , 0.2 M NaNO_3) and 0.1 ml of concentrated HNO_3 (yielding a $\text{pH} < 2$) was added to a 100 ml homogenized water sample in a 250 ml Erlenmeyer flask. The flask was shaken for 1 h, and then the sample was filtered through a polycarbonate membrane filter with a pore size of 0.45 μm on which the activated carbon was retained. Reminders of inorganic halide on the filter were removed by washing with acidic nitrate solution (0.001 M HNO_3 , 0.01 M NaNO_3 , a total volume 25 ml). The filter with the activated carbon was transferred to a Analytik Jena Multi X 2500 AOX analyzer (Jena, Germany) in which the carbon is burned at 950 $^{\circ}\text{C}$ in a stream of oxygen. The halide formed during combustion is determined by microcoulometric titration with silver ions.

The determination of TOC and their LMW fractions in water samples was performed according to the EN 1484:1997 guidelines of technical standards for water quality and analysis (European Standard, 1997). In brief, TOC is calculated by subtracting the result of the low temperature measurement (B) from the high temperature measurements (A) using the TOC Analyzer Formacs HT/TN CA16 (Skalar Analytical B.V., Breda, The Netherlands). First measurement (A) is carried out by injecting water by means of automated septumless rotary port into the high-temperature reactor at 950 $^{\circ}\text{C}$ with cobalt as an oxidant catalyst. All organic and inorganic carbon (Total Carbon, TC) is quantitatively oxidized to the gaseous CO_2 , which is transported by air flow (purity 5.0, SIAD) into the infrared detector and measured at 4.2 μm . A second injection of water sample is made into the low temperature liquid reactor containing 2% (v) phosphoric acid at room temperature. All inorganic carbon (IC) is oxidized to gaseous CO_2 and measured by IR detector. TOC value is calculated from the equation: $\text{TOC} = \text{TC} - \text{IC}$.

2.3. Sample preparation, fractionation

Isolation of low molecular weight (LMW) fractions: Ultrafiltration under inert conditions (nitrogen atmosphere) was used for the separation of LMW compounds (0–500 Da) (Millipore membrane filter YC 500), according to the method of Lin et al. (1999). The ultrafiltration was carried out in a Millipore 8400 stirred ultrafiltration cell type with RC800 minireservoir. The ultrafiltration membranes were washed prior to with use boiling water double-distilled from quartz apparatus. The AOX and TOC concentrations were measured in LMW fractions.

2.4. Statistical procedures

Correlations between meteorological and chemical data (Sup. Table 2) were calculated by polynomial of the third degree using Microsoft Excel. The data were further statistically analyzed using CANOCO software version 4.5. In case of CHCl_3 analysis the outlier values were rejected by Dixon's Q test. We used redundancy analysis (RDA) to characterize the relationship between environmental measures (sampling date and location, water- and air temperature, precipitation) and the presence of chemical species present (chloride, CHCl_3 , pH , ΣTOC , TOC_{LMW} , ΣAOX , AOX_{LMW}). RDA is a direct

gradient analysis technique which combines multiple regressions with principal component analysis and constrains the extracted pattern of species to a linear combination of the measured environmental variables, and it is a proven, robust method for describing species-environment relationships (Palmer, 1993). All the default options were selected in the RDA (i.e., no special weighting of species or environmental factors, no samples were excluded from the analysis). Each canonical axis is a linear combination of all explanatory variables.

3. Results

We studied a natural ecosystem composed of animals, plants and microorganisms, which is not affected by anthropogenic influences, yet chlorinated compounds (AOX, chloroform) were found there. The presence of these chlorinated compounds is allocated to anthropogenic but also to natural sources. Regarding the accumulation of chloroform (CHCl_3) in the studied ecosystem, metabolism by microorganisms and the pH are the two most significant influencing factors. As seen from our measurements, the largest CHCl_3 content and also the largest fluctuation is at the SP3 sampling point (Chart 2). In this context, it is interesting that the second largest CHCl_3 content and also the highest average content of chloroform are found at locality SP4 (Chart 2). The area of SP4 together with other similar tributaries supplies primarily Krejcarský creek in SP3, and thus significantly affects the accumulation of CHCl_3 in this location (Chart 2).

The locality of SP4 has a relatively stagnant water rich in microbial activity, degrading and transforming plant residues also illustrated in Fig. 1. Locations with higher elevations (such as SP4) form the spring area of the creek at SP3. Draining canals in the forest are overgrown with peat moss, the water is dark due to decaying plant residues and the presence of a high content of organic matter (determined as ΣTOC parameter) washed off from peatlands at SP4 (Chart 3). The degradation of TOC in the presence of chloride ions results in the formation of chlorinated humic acids and chlorinated fulvic acids. These acids are the source of compounds subsequently determined as ΣAOX (Chart 4) and their low molecular weight degradation products, determined as AOX_{LMW} . The activity of microorganisms that decompose organic aquatic plant residues is strongly dependent on water temperature of their environment. This influence is confirmed by the correlation between water temperature and the content ratio of $\text{CHCl}_3/\text{AOX}_{\text{LMW}}$ in



Fig. 1. Locality SP4, spring area.



Fig. 2. Locality SP3, stream area.



Fig. 3. Locality SP2, river area.



Fig. 4. Locality SP1, Hamry water reservoir.

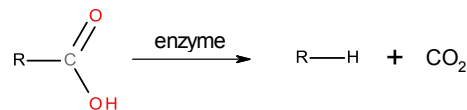


Fig. 5. Scheme of decarboxylation of organic acids.

microorganisms, particularly at SP4, significantly influences the ratios of $\text{CHCl}_3/\text{Cl}^-$ (correlation $R^2 = 0.828$), $\text{CHCl}_3/\Sigma\text{TOC}$ (correlation $R^2 = 0.918$), $\text{CHCl}_3/\Sigma\text{AOX}$ (correlation $R^2 = 0.639$) and $\text{AOX}_{\text{LMW}}/\text{TOC}_{\text{LMW}}$ (correlation $R^2 = 0.521$). The influence of water temperature on the ratios of the above substances shows an interconnection between degradation and biodegradation processes leading from TOC through AOX to CHCl_3 . That is why in flowing water with higher current (locations SP3, SP2) and lower current (SP1) these correlations are decreasing, cf. the $\text{CHCl}_3/\text{Cl}^-$ ratio (correlation $R^2 = 0.779$ at SP3, 0.617 at SP2 and 0.615 at SP1, resp.). Due to various types of precipitation, the discontinuous drainage of the spring area results in dynamic changes that determine the high fluctuation of the measured parameters at SP4, where the lowest pH and highest values of AOX and TOC can be found. It is therefore possible to divide the localities into two groups. The first group contains SP1-3 while the second contains only SP4 and differs considerably from the previous sites, which can be also seen on the canonical analysis (Chart 6).

The second most important factor affecting the formation and accumulation of CHCl_3 is water pH. The pH is strongly acidic at SP4, while at SP3 it has the second highest value, and can be characterized in most measurements as neutral (mean pH 7.71, with a range of 5.70–7.72; Chart 1). The soil water at SP4 is normally strongly acidic due to formation of humic substances of decaying organic matter. Soil biota under anaerobic conditions is forming organic acidic compounds, which is also reflected in TOC values (Chart 3). The acidic precipitation also uniformly contributes to acidification of soils at SP1-SP4, and deposits in the studied area $0.388 \text{ g} \cdot \text{Cl}^-/\text{m}^2/\text{yr}$ in a long-term average; we do not expect any significant difference in the amount of deposition between the sampling points which are at a distance of approximately 2 km from each other, with similar elevation and exposure. The pH increase at SP3 is primarily due its lower elevation, as the connecting stream flows through a mixed forest which is related with increased soil erosion (Fig. 2). The calculated correlations (Table 2) show that the accumulation of CHCl_3 is influenced by the pH and by free chlorine, which is necessary for the formation of the analyzed AOX fractions and CHCl_3 (Charts 7–10).

Although ΣTOC is converted to TOC_{LMW} , ΣTOC to ΣAOX , TOC_{LMW} to AOX_{LMW} , ΣAOX to AOX_{LMW} , and AOX_{LMW} is mainly converted to CHCl_3 , their influence on the accumulation of CHCl_3 was not observed simultaneously in all localities. This can only be explained through the many different and independently running abiotic and biotic processes, involving different organisms which affect the accumulation of CHCl_3 in the studied locations and in the given ecosystem. It is apparent from the correlations in Table 2 that pH strongly influences the amount of determined CHCl_3 in all localities during the studied period (25th March – 1st September). The pH, which affects free chlorine, $\Sigma\text{AOX}/\text{Cl}^-$, and $\Sigma\text{TOC}/\text{Cl}^-$ in locations SP3 and SP2, also strongly affects the accumulation of CHCl_3 determined in the water (as indicated by the correlations in Table 2). In most locations the CHCl_3 content strongly influences the ratio of low molecular weight TOC to free chlorine ($\text{TOC}_{\text{LMW}}/\text{Cl}^-$), as is apparent from the correlation coefficients in Table 2. Relations between various forms of chlorinated and non-chlorinated compounds (e.g. $\text{AOX}_{\text{LMW}}/\Sigma\text{TOC}$, correlation $R^2 = 0.666$) demonstrate the importance of microbial processes on the accumulation of CHCl_3 , which are the most remarkable at SP4.

locations SP3 and SP4 (correlation $R^2 = 0.816$ and 0.884, resp.). The temperature of stagnant water, which determines the activity of

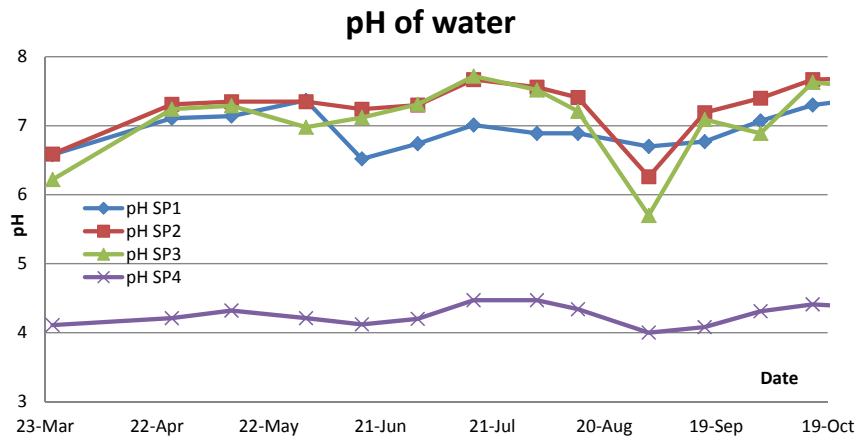


Chart 1. pH of water.

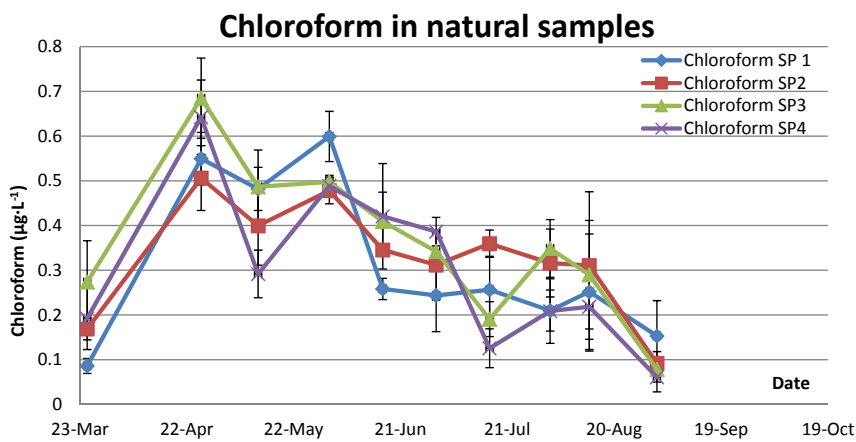


Chart 2. Chloroform in natural samples.

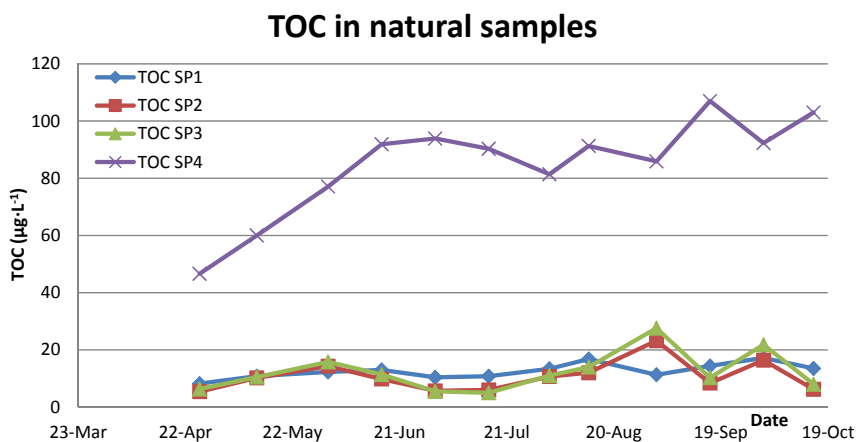


Chart 3. TOC in natural samples.

The $\text{CHCl}_3/\Sigma\text{AOX}$, $\text{CHCl}_3/\text{AOX}_{\text{LMW}}$, $\text{CHCl}_3/\Sigma\text{TOC}$, $\text{CHCl}_3/\text{TOC}_{\text{LMW}}$ and $\text{CHCl}_3/\text{Cl}^-$ ratios therefore depend on the accumulation of CHCl_3 , as follows from the calculated correlations in Table 2.

While the significance of microbial biotransformation for the transformation of CHCl_3 from AOX at locations SP3 and SP4 is evident, an increasing effect of pH is obvious at locations SP1 and SP2 (Chart 1), which is apparent from the very high correlation

coefficients in Table 2. Location SP2 also shows a maximum average pH (7.3) and the highest minimum pH (6.52). Consequently, the increased chlorine content (Chart 5) affects the content of CHCl_3 in SP2 and SP3. The highest average values of chlorine can be found in these very areas, while the actual content of chlorine (Cl^-) reaches extreme values (Chart 5). The relationships between CHCl_3 , Cl^- , and SP1 and SP2 locations are also apparent from the complex graph

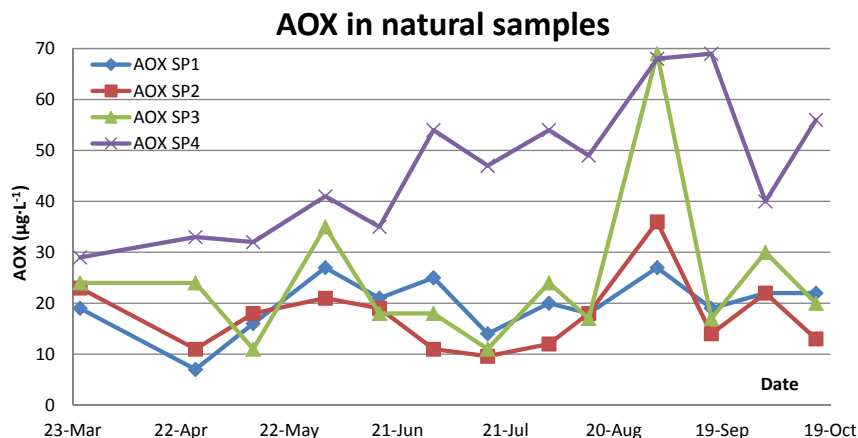


Chart 4. AOX in natural samples.

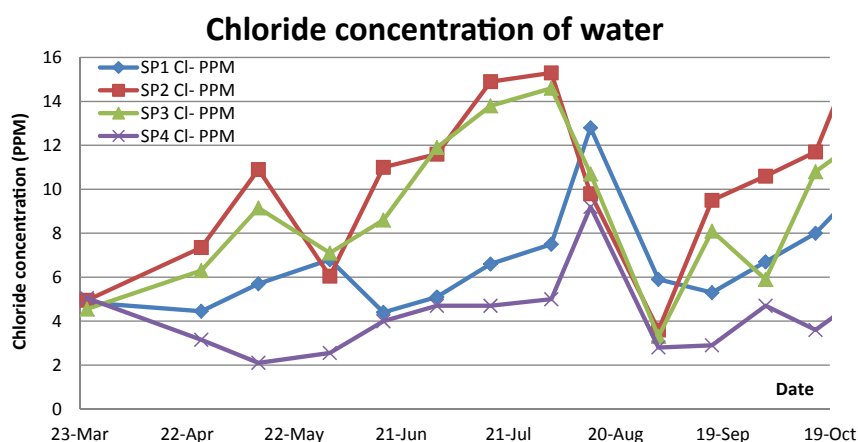


Chart 5. Chloride concentration of water.

showing the canonical analysis of our results (Chart 6).

Redundancy analysis generally showed relationships between environmental factors and formation of complex substances (AOX, TOC, CHCl_3 ; Chart 6). Canonical analysis shows a correlation between precipitation, air temperature and water temperature. The highest correlation can be found between air temperature and concentrations of ΣAOX , AOX_{LMW} , TOC_{LMW} and CHCl_3 . Furthermore,

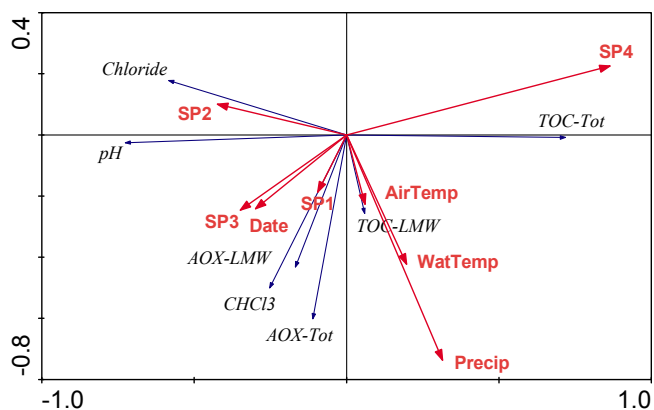


Chart 6. Canonical analysis of data, parameters: localities SP1–SP4, sampling date, air and water temperature, precipitation, pH, total TOC, TOC_{LMW} , total AOX, AOX_{LMW} , CHCl_3 , chloride.

Chart 6 indicates a strong negative correlation between ΣTOC and pH and a correlation between the sampling locations and the sampling date. Furthermore, correlation has been observed between AOX forms and CHCl_3 ; these factors are also close to TOC_{LMW} .

It is apparent from the analyses of chlorophenol analogues that various derivatives of chlorobenzenes, dichlorobenzenes, and trichlorobenzenes were determined below the limits of detection, i.e. $<0.1 \text{ mg L}^{-1}$. These compounds are the source of chloroacetic and dichloroacetic acids, which have been determined below the values of detection limit, $<0.7 \text{ mg L}^{-1}$, at all locations. Similarly, trichloroacetic acid level has been determined below the values of detection limit, i.e. $<0.2 \text{ mg L}^{-1}$. All chloroacetic acids including the significant chloroacetic acid arise naturally from AOX. Chloroform is then formed by decarboxylation of trichloroacetic acid or by oxidation of AOX. The lowest minimum concentrations of CHCl_3 ($0.061 \mu\text{g L}^{-1}$) were found at SP4, where the main process is biotransformation from trichloroacetic acid or from AOX. The rate of enzymatic biotransformation from substrate to product in living organisms is affected not only by the nature of the substrate but often by the product as well through a negative feedback on the enzyme activity. This is supported by the results in Table 2 that show significant correlations between the ratios of $\text{CHCl}_3/\Sigma\text{AOX}$, $\text{CHCl}_3/\text{AOX}_{\text{LMW}}$, $\text{CHCl}_3/\text{TOC}_{\text{LMW}}$, and $\text{CHCl}_3/\text{Cl}^-$ at all locations.

The highest minimum value of CHCl_3 has been determined at SP2 ($0.092 \mu\text{g L}^{-1}$, Table 1), where abiotic transformation of AOX to CHCl_3 or decarboxylation of trichloroacetic acid predominates due to the higher pH of the aqueous matrix. The highest maximum

Table 1Summary of changes in the studied parameters during the typical growing season at the SP1–SP4 localities. Δ - difference, \emptyset – average.

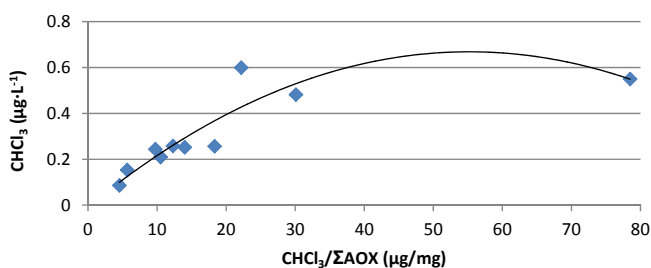
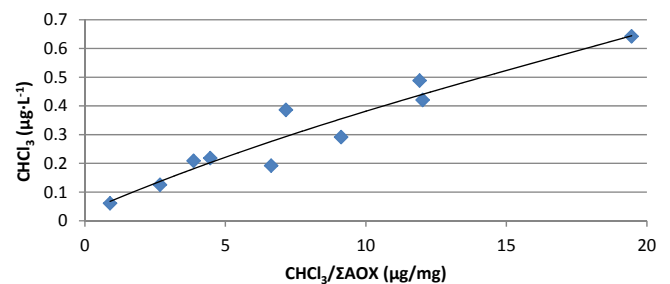
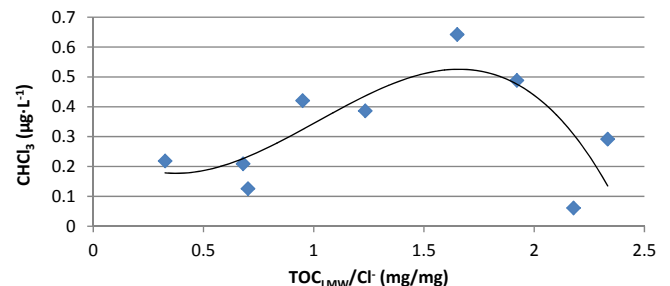
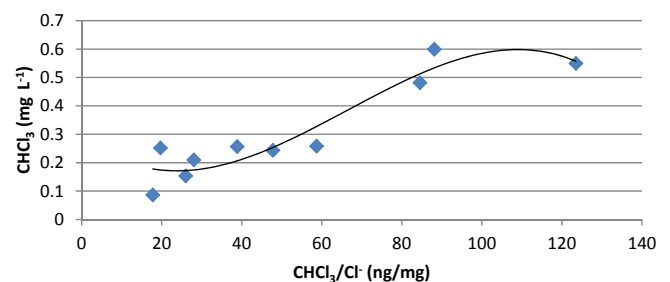
Year	2010	Place	SP1	SP2	SP3	SP4	SP1–4
Place	Units	Date	25.3.–15.11.	25.3.–15.11.	25.3.–15.11.	25.3.–15.11.	25.3.–15.11.
pH	–	Min-Max	6.52–7.42	6.26–7.69	5.70–7.72	4.00–4.47	4.00/7.72
		Δ/\emptyset	0.90/6.99	1.43/7.3	2.02/7.1	0.47/4.3	3.72/6.4
Chlorid	ppm	Min-Max	4.40–12.80	3.60–18.50	3.30–14.60	2.10–9.20	2.10–18.50
		Δ/\emptyset	8.40/7.05	14.90/10.76	11.30/9.37	7.10/4.54	16.40/7.93
Σ AOX	$\mu\text{g L}^{-1}$	Min-Max	7.0–27.0	9.6–36.0	11.0–69.0	29.0–69.0	7.0–69.0
		Δ/\emptyset	20.0/19.77	26.4/17.51	58.0/24.46	40.0/46.69	62.0/27.11
AOX _{LMW}	$\mu\text{g L}^{-1}$	Min-Max	1.1–16.0	1.0–12.0	2.2–39.0	1.7–28.0	1.0–39.0
		Δ/\emptyset	14.9/6.59	11.0/5.46	36.8/14.87	26.3/16.44	38.0/10.84
Chloroform	$\mu\text{g L}^{-1}$	Min-Max	0.086–0.599	0.092–0.506	0.078–0.685	0.061–0.642	0.061–0.685
		Δ/\emptyset	0.513/0.309	0.414/0.329	0.607/0.360	0.581/0.304	0.624/0.325
Σ TOC	Mg L^{-1}	Min-Max	8.2–17.2	5.4–23.2	5.0–27.6	46.6–141.0	5.0–141.0
		Δ/\emptyset	9.0/12.51	17.8/10.32	22.6/12.01	94.4/89.36	136/31.05
TOC _{LMW}	Mg L^{-1}	Min-Max	0.68–5.00	0.52–4.20	0.50–6.00	2.60–7.20	0.50–7.20
		Δ/\emptyset	4.32/1.78	3.68/1.52	5.50/1.57	4.60/4.42	6.70/2.32

Table 2

Correlation coefficients showing the effects of changes in physical and chemical parameters characterizing the water matrix at the SP1–SP4 localities during the year (weather course, life cycle and the number of organisms).

Locality	SP1	SP2	SP3	SP4
Parameters	CHCl ₃	CHCl ₃	CHCl ₃	CHCl ₃
pH	0.861	0.730	0.631	0.660
Cl [–]	0.021	0.728	0.798	0.256
Σ AOX	0.377	0.524	0.438	0.413
AOX _{LMW}	0.439	0.095	0.507	0.427
Σ TOC	0.237	0.765	0.402	0.433
TOC _{LMW}	0.324	0.700	0.077	0.648
Σ AOX/Cl [–]	0.099	0.654	0.670	0.353
AOX _{LMW} / Σ TOC	0.102	0.496	0.373	0.666
CHCl ₃ / Σ AOX	0.821	0.834	0.623	0.917
CHCl ₃ /AOX _{LMW}	0.523	0.737	0.508	0.739
CHCl ₃ / Σ TOC	0.959	0.840	0.740	0.947
CHCl ₃ /TOC _{LMW}	0.838	0.740	0.717	0.867
TOC _{LMW} /Cl [–]	0.225	0.842	0.757	0.547
CHCl ₃ /Cl [–]	0.893	0.500	0.714	0.920

value of CHCl₃ (0.360 $\mu\text{g L}^{-1}$, Table 1) has been determined at SP3, where biotransformation occurs simultaneously with abiotic transformation by decarboxylation due to higher pH values. The average concentrations of CHCl₃ declined gradually from 0.360 $\mu\text{g L}^{-1}$ (SP3) over 0.329 $\mu\text{g L}^{-1}$ (SP2) to 0.309 $\mu\text{g L}^{-1}$ (SP1; where abiotic transformation predominates) and to the lowest level of 0.304 $\mu\text{g L}^{-1}$ at SP4 (Table 1). This decrease is inversely proportional to the increasing water flow rate, increasing water volume, oxygen content and oxidation/reduction potential (ORP) in the watercourse from localities SP1 through SP2 (mean 10.9 mg L^{-1} O₂, 150.8 mV ORP) to SP3 (11.0 mg L^{-1} O₂, ORP 131.4 mV). Site-specific conditions prevail at SP4; biotransformation predominates in the lake environment, where specific control of CHCl₃ formation occurs

**Chart 7.** The effect of CHCl₃/ Σ AOX ratio on CHCl₃ during growing season at SP1. Third order polynomial trendline, reliability $R^2 = 0.8211$.**Chart 8.** The effect of CHCl₃/ Σ AOX ratio on CHCl₃ during growing season at SP4. Third order polynomial trendline, reliability $R^2 = 0.9172$.**Chart 9.** The effect of TOC_{LMW}/Cl[–] ratio on CHCl₃ during growing season at SP4. Third order polynomial trendline, reliability $R^2 = 0.5473$.**Chart 10.** The effect of CHCl₃/Cl[–] ratio on CHCl₃ during growing season at SP1. Third order polynomial trendline, reliability $R^2 = 0.8929$.

(Table 2). Due to decomposition of organic matter (mineralization with a mean 13.0 mg L^{-1} O₂, 190.7 mV at SP4) the highest average values of Σ TOC and TOC_{LMW} and of the compounds formed from

them (ΣAOX and AOX_{LMW}) were determined at this locality. The mean concentration of these compounds gradually decreases in the areas of SP3 and SP2, and the lowest average values were determined at SP2 (Table 1). Locality SP1 can also be characterized as a relatively stagnant water of the Hamry reservoir (SP1: $9.7 \text{ mg L}^{-1} \text{ O}_2$ and ORP 171.7 mV), which is fed with water from location SP2 and additionally from other tributaries (Vortovský creek, Valčice creek). Another factor influencing the contents of ΣTOC , TOC_{LMW} , ΣAOX , and AOX_{LMW} , including CHCl_3 in locality SP1 is the maximum mean temperature of the water, which increases the evaporation of CHCl_3 from the water matrix.

Unlike several individual components of AOX, such as analogs of chlorinated phenols, chlorinated aliphatic hydrocarbons and chlorinated acetic acids, CHCl_3 is a compound which is relatively stable to biodegradation. CHCl_3 is accumulated from the minimum mean value of 0.061 mg L^{-1} (SP4) to the maximum mean value of 0.685 mg L^{-1} (SP3) in the analyzed water matrices. Precursors of CHCl_3 are reactive compounds, and their transformation or degradation leads to the final, less reactive CHCl_3 , which therefore accumulates in the analyzed stream water samples that are coming from the studied ecosystem.

4. Discussion

Despite the use of modern analytical techniques (Cincinelli et al., 2012), it is hard to differentiate anthropogenic chlorinated compounds, e.g. antibiotics (Hanekamp and Bast, 2015) primarily produced by biotechnological processes from substances naturally occurring in the ecosystem. At the same time, anthropogenic chlorinated compounds (e.g. pentachlorophenol, still used as a fungicide) and natural AOX (e.g. drosophilin (Anchel, 1952)) may have common degradation products. Therefore we chose for our study the Žďárské vrchy Protected Landscape Area (Lusk, 1993), which also serves as a first-degree hygienic protection zone of drinking water sources (Figs. 1–4). It represents an extensive – ecological type of forestry and farming intended to protect soil and soil microflora. Only combined fertilizers of low solubility can be used to a limited extent in the vicinity of the localities.

Naturally occurring chlorinated compounds (AOX) are formed from TOC mainly by the metabolism of microorganisms – algae, fungi, bacteria and lichens and less often by higher organisms – plants, freshwater organisms, insects and higher animals, even by mammals (Öberg, 2002). Therefore we continuously monitored the concentration of ΣTOC (Table 1, Chart 3), along with pH (Chart 1), TOC_{LMW} , chlorides (Chart 5), ΣAOX (Chart 4), AOX_{LMW} , besides meteorological data, e.g. water and air temperature (Chart 6; Sup. Table 2). The structural diversity of AOX (from CHCl_3 to isocomplestatin) is reflected in their large numbers (Winterton, 2000; Gribble, 2003, 2015). Natural and anthropogenic AOX are both continually degraded in the ecosystems (Leys et al., 2013); as a result, a dynamic equilibrium takes place between emerging and degraded AOX (Chart 4). Our analyses did not show the occurrence of any anthropogenic chlorinated compounds, including trichloroacetic acid (TCAA). At the same time, TCAA formation from natural sources has not been demonstrated by some authors (Hoekstra et al., 1999b) but its natural occurrence in ecosystems was later detected by an exact ^{36}Cl radiotracer method (Matucha et al., 2007a). This fits well with the fact that on the one hand the use of TCAA as herbicide has been prohibited in the whole country for more than 30 years while, on the other hand, its persistence in soil (when applied at 2 kg ha^{-1} dosage) is 21–90 days (Tomlin, 1994). The only representative of organic chlorinated compounds found here was CHCl_3 (Charts 2 and 6).

The formation of CHCl_3 requires high AOX concentration, which is apparent both from our results (Table 1; Chart 6), and from a

series of works listed in Sup. Table 4. These works show that chloroform is formed in ecosystems from natural sources, i.e. from AOX. This formation is mainly influenced by the activities of various organisms and their enzymes. As seen from Sup. Table 4, this reaction is therefore affected not only by the environmental conditions, but also by the ratio of substrates to products. However, one should note that a hormesis effect can arise at low concentrations of toxic compounds, in this case chloroform (Calabrese and Baldwin, 2003), which is described by “hormesis curves” which are functions of second or third degree polynomials. It is therefore also apparent from our results (Tables 2–4) that the production of chloroform is affected by the ratio of products and substrates (CHCl_3/AOX – Charts 7 and 8; $\text{TOC}_{\text{LMW}}/\text{Cl}^-$ – Chart 9; $\text{CHCl}_3/\text{Cl}^-$ – Chart 10).

The microecosystem of SP4 locality is characterized by the presence of catabolic and mineralization processes in decaying biomass caused by saprophytic microorganisms, and consequently by the lowest water pH values of all localities (Chart 1). Biomass is continually replenished by plants that contribute to this diverse microecosystem. According to Keppler et al. (2000), redox potential (ORP) of the environment plays a significant role in the formation of AOX from TOC. AOX, alkyl halides and CHCl_3 are formed by oxidation of TOC and humic substances. This explains why the average values of ORP, ΣTOC , TOC_{LMW} , ΣAOX and AOX_{LMW} at SP4 are the highest among all locations (Table 2). Due to their continuous replenishment, their levels are not decreased by the runoff water from the spring region. At the same time, it is also logical that the lowest chloride concentrations can be found at SP4 because Cl^- in the chlorine cycle is replenished mainly from precipitations and is then leached from the locality. In contrast, the seemingly illogical lowest average content of CHCl_3 (Table 2) at SP4, which contradicts the findings of Keppler et al. (2000), is due to saprophytic microorganisms actively interfering with HV-Clorg-forming processes by their enzymatic apparatus. These enzymes, including peroxidases (Sup. Table 4), permanently reduce CHCl_3 at SP4.

According to Hoekstra et al. (1999a), due to the very low water pH in this area TCAA should be formed at the expense of CHCl_3 . The measured absence of TCAA and the determined content of CHCl_3 at SP4 can thus be attributed to the metabolic action of organisms present in this microecosystem. Compared with the stagnant water at SP4, the running water at SP3 and SP2 locations has a significantly higher pH; this leads to the absence of TCAA and formation of CHCl_3 whose levels in those locations are the highest (Table 2). The metabolic pathway of TCAA formation from AOX has been described primarily in soil (Matucha et al., 2007b) or is due to low pH (Hoekstra et al., 1999a); this was confirmed by ^{36}Cl radiotracer method (Matucha et al., 2007a). Nevertheless, a very close relationship between pH and CHCl_3 determined in water was found in all the locations, as demonstrated by the correlation coefficients (Table 4). This shows that another factor, either abiotic (e.g. oxidation-reduction potential) or biotic, must play a role in the production of CHCl_3 apart from the abiotic factor of pH. Oxidation-reduction potential is an important factor in the formation and

Table 3

Correlations showing the effect of ratios of chemical compounds on the accumulation of Cl^- in the aqueous matrix at the SP1–SP4 localities during the year (course of weather, life cycle and the number of organisms).

Locality	SP1	SP2	SP3	SP4
Parameters	Cl^-	Cl^-	Cl^-	Cl^-
$\Sigma\text{AOX}/\text{Cl}^-$	0.356	0.847	0.784	0.684
$\text{AOX}_{\text{LMW}}/\text{Cl}^-$	0.704	0.861	0.785	0.748
$\text{CHCl}_3/\Sigma\text{TOC}$	0.312	0.536	0.533	0.508
$\Sigma\text{TOC}/\text{Cl}^-$	0.673	0.687	0.756	0.645

Table 4

Correlations showing the effect of physico-chemical parameters and ratios of chemical compounds on the accumulation of Σ AOX in the aqueous matrix at the SP1–SP4 localities during the year (course of weather, life cycle and the number of organisms).

Locality	SP1	SP2	SP3	SP4
Parameters	Σ AOX	Σ AOX	Σ AOX	Σ AOX
pH	0.559	0.756	0.806	0.422
Σ TOC	0.555	0.929	0.861	0.451
TOC _{LMW}	0.171	0.793	0.258	0.598
Σ AOX/Cl [−]	0.573	0.925	0.946	0.552
CHCl ₃ / Σ AOX	0.554	0.868	0.898	0.651
CHCl ₃ /AOX _{LMW}	0.542	0.737	0.932	0.736
CHCl ₃ / Σ TOC	0.546	0.843	0.814	0.646

degradation of organic compounds by oxidation in soil; at the same time halocarbons can be formed by their halogenation (Keppler et al., 2000). Although Hoekstra et al. (1999a) generally reject CHCl₃ formation by decarboxylation of TCAA, several works have described thermal decarboxylation of TCAA in soil (Matucha et al., 2006; Dickey et al., 2005). Subsequent work described that CHCl₃ arises not only from thermal decarboxylation of TCAA, but also from other chlorinated compounds present in the soil organic matter (Albers et al., 2010). Temperatures suitable for thermal decarboxylation mentioned by Matucha et al. (2006) are dramatically higher than those monitored in the studied locations, including SP4. Therefore, though the effect of temperature definitely plays a role in TCAA degradation, thermal decarboxylation is not so significant in the ecosystems of Central Europe. This corresponds well with the work of Breider and Albers (2015), which is in accordance with our findings that thermal decarboxylation is not a major process in nature.

During chlorination of drinking water the high concentrations of chlorine used cause that trihalomethanes (THMs), chloroacetic acids (CAAs) and other disinfection byproducts (DBPs) are formed from organic matter (Reckhow et al., 1990) by non-enzymatic processes, which are probably typical for flowing water, as demonstrated by the correlations of Cl[−]/CHCl₃ at SP2 and SP3 (Table 2). Regarding the active chlorine in the reaction, abiotic formation may be similar to natural conditions, where HOCl is formed by enzymatic processes, which are probably typical for standing waters, as demonstrated by the correlation of Cl[−]/CHCl₃ (Table 2; Hoekstra et al., 1999b; Albers et al., 2010) or CHCl₃/Cl[−] (Table 2; Breider and Albers, 2015) at SP1 and SP4, and is presumably converted to Cl₂. Anabolic (Hoekstra et al., 1999b; Albers et al., 2010) and catabolic processes (Breider and Albers, 2015) are also typical for enzymatic processes. Resorcinol- and phenol-like aromatic structures and aliphatic compounds have also been found to form DBP and are well known precursors of THMs (Dickenson et al., 2008). Another possible parallel mechanism of CHCl₃ formation during water chlorination is decomposition of polar aromatic DBPs (Zhai and Zhang, 2011). Decomposition of trihaloacetic acids, also producing haloforms at relatively high temperatures (Pan et al., 2014) in controlled environment, can simulate processes happening under natural conditions. However, these processes may be distorted by lower temperatures and concentrations of reactants (Table 1.) and by the destruction of unstable intermediate products by subsequent reactions with soil components.

Breider and Albers (2015) pointed out the poorly understood biotic degradation of AOX, including decarboxylation of TCAA by enzymes present in soil matrix and especially in living organisms (Sup. Table 4). The enzymatic decarboxylation of TCAA, which leads to the biosynthesis of CHCl₃, has been indicated by e.g., Matucha et al. (2006). Enzymatic decarboxylation (Fig. 5) releases CO₂ into

the ecosystem after decarboxylation of acids by fungal enzymes (Anke, 1997). This CO₂ was determined after decarboxylation of TCAA as well (Tomlin, 1994). On the other hand, Breider and Hunkeler (2011) described the biosynthesis of CHCl₃ from AOX as a result of enzymatic processes in living cells such as those catalyzed by chloroperoxidases (Sup. Table 4). Koppen et al. (1988) and Christensen et al. (1988) described similar enzymatic processes in the liver which lead from trichloroethylene via TCA to the formation of CHCl₃. Biological processes at the studied locations have a major influence on the formation of CHCl₃ inside living cells, from which biosynthesized CHCl₃ is released into the environment. Our results indicate that abiotic factors such as temperature do not play a significant role (Matucha et al., 2006; Dickey et al., 2005; Breider and Albers, 2015). Temperature is important only indirectly, as a requirement for growth, development and reproduction of bacteria, fungi (Sup. Table 4), and other microorganisms constituting the ecosystem of the Žďárské vrchy Protected Landscape Area.

As TCAA was not determined in the studied ecosystem, it can be concluded that CHCl₃ is formed directly by biodegradation of AOX and not via TCAA. This is in accordance with the described degradation and biodegradation of AOX to volatile chlorinated aliphatic hydrocarbons, including CHCl₃ (Sup. Table 4). Unfortunately, the results show (Sup. Table 4) that only very little is known about the biotic processes, involvement of enzymes and their regulation, which affect the formation of CHCl₃. Sup. Table 4 also shows that degradation of AOX to CHCl₃ can take place via TCAA decarboxylation. The hypothesis of decarboxylation of TCAA forming CHCl₃ has been rejected in many works; however, it has been conclusively proven by ³⁶Cl radiotracer method (Matucha et al., 2007a).

The subsequent degradation of chlorinated aliphatic hydrocarbons to Cl[−] and to volatile hydrocarbons (Sup. Table 3) or their biodegradation rate depends for example on the formation of their products, e.g. propane, which is one of the mineralization products of TOC (Frasconi et al., 2008). That is why significant correlations were found showing the effect of compound ratios on the accumulation of CHCl₃ (Table 2) and also the effect of ratios of selected compounds on the Cl[−] concentration (Table 3). Relatively lower correlations indicate that the source of Cl[−] in the analyzed water matrix comes not only from the dissociated chlorides eluted from soil, but also from chlorine dissolved from the atmosphere. In this ecosystem the concentration of chlorine in the atmosphere (Kolářová and Ostatnická, 2015) and in the water is very low (Table 1). Tables 2–4 therefore integrate two basic steps of the natural chlorine cycle, which are the incorporation of chlorine into organic compounds by formation of AOX (Table 4) and their subsequent biodegradation through CHCl₃ (Table 1) to Cl[−] (Table 3).

Biological processes leading to the accumulation of CHCl₃ and its subsequent slower biodegradation are influenced by both abiotic and biotic factors of the environment, in which the living organisms accumulate natural AOX, or biodegrade AOX to CHCl₃. These processes are significantly affected by antagonistic and symbiotic relationships between organisms. These relationships between organisms apply not only in a water matrix, but also in the soil and particularly in the rhizosphere of plants. Similarly to abiotic factors, which affect the development and growth of organisms, abiotic factors determine the activity of intracellular enzymes (Sup. Table 4), which are involved in the metabolism of AOX and in the subsequent release of CHCl₃ into the ecosystem. Besides physico-chemical factors, metabolic rate is affected by substrate concentration. However, the enzyme activity involved in intracellular catabolic and anabolic processes is typically regulated by various metabolites, by pH, by feedback control of products, or by the substrate/product ratio (Sup. Table 4). Our results show (Tables 2 and 3) that the formation of CHCl₃ and Cl[−] in the studied ecosystem is natural and has a biogenic character. Its

biodegradation affects various organisms (Sup. Table 4). In 1.2 million m³ water, the Hamry water reservoir stores a total of 0.1–0.72 kg CHCl₃ (0.086–0.599 μg L⁻¹), which dynamically changes throughout the year. This chloroform is a potential source of atmospheric pollution and a difficulty during drinking water treatment. The current year average total amount of precipitation at the Hamry dam was 887 mm, and the yearly amount of 2.00–13.98 kg chloroform is transported from the catchment area. Since the catchment area is 54.0 km², where a total amount of 6950 kg chloride is deposited, approximately the same amount is transported by the river Chrudimka as chloride, and 0.11% is transported as chloroform.

5. Conclusions

The conditions of CHCl₃ formation (quantity and temporal distribution changes in accumulation) are primarily related to the activity of biological processes that are determined by the environment. The results and subsequent discussion show that the chlorine cycle in natural ecosystems is not only complicated, but also poorly understood.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2016.05.017>.

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