Oecologia Montana 2020,
29, 1-8

Impact of biodegradable lubricant oils on soil element concentrations

M. MICHALÍK, Z. KOMPIŠOVÁ BALLOVÁ and M. KOMPIŠ

Institute of High Mountain Biology, Žilina University, Tatranská Javorina 7, SK-059 56, Slovak Republic; e-mail: ballova1@uniza.sk

Abstract. This study deals with the biodegradation of vegetable oils commonly used in the forestry industry. Sampling was performed in the National nature reserve Slnečné skaly in Rajecká basin, Slovakia. Eighty soil samples contaminated with biodegradable lubricant oil and eighty control samples were collected. The samples were collected from ten pairs of plots (oil and control) once a week for eight calendar weeks following chain saw use. Biodegradable vegetable oil had significant effects on the concentration of several elements. Amounts of K, Ti, Mn, Cr, Fe, Rb, Sr, Zr and Ba significantly decreased in some of the oil plots when compared to control plots. However, this effect was found to be related to the unique environmental conditions of these plots. Element concentrations in soil samples were observed to be reduced approximately three weeks following the commencement of the experiment, corresponding to the estimated time of degradation of our used oil. The biodegradable oil had a negative effect on Ca binding in the soil and this study found that oil tends to have an effect on the binding of Ca in the soil after 5 weeks. The oil samples had higher levels of Pb and S than the control samples, whereas the contents of S and Pb were closely related. Sulphur likely decreased soil pH and increased availability and mobility of Pb. Additionally, the biodegradable lubricant oil had a slight non-significant negative impact on amounts of Cu found in the soil.

Key words: environmental pollution, ecotoxicity, oils, biodegradability, soils, heavy metals

Introduction

Forestry machinery used on forest roads and in forest areas presents an ecological threat due to pollution caused by the leakage of lubricant oil and other petroleum products (Stanovský *et al.* 2013). Recently, effort has been put into developing environmentally friendly lubricants, which are biodegradable or of biological origin. From an environmental point of view, however, the utilization of recycled synthetic or mineral oils has a negative effect on the soil and water in these ecosystems (Stanovský *et al.* 2013; Nowak *et al.* 2019). Oils produced from crude oil (mineral oils) cause serious contamination of soils, groundwater, and can accumulate in plant tissues (Nowak *et al.* 2019). Forestry workers avoid bio-oils because their lubricating characteristics are not as good as those of conventional lubricants are much more expensive. Bio-oil does not provide better lubrication for chainsaws and in some cases, it performs worse than used synthetic motor oil (Skoupý 2004). However, vegetable oil-based lubricants biodegrade much more quickly compared to mineral-oil based or synthetic lubricants (Erhan and Asadauskas 2000; Haigh 1995).

The biodegradability means the tendency of a lubricant to be ingested and metabolized by microorganisms. Complete biodegradability indicates that the lubricant has essentially returned to nature (Bartz 1998). Biodegradability varies depending on the type of oil, and the presence of toxic compounds affecting microorganisms plays a part in this process. Toxic compounds in mineral oils could include metals, degradation products like acrolein, peroxides or polymers. Sometimes vegetable oil contains aldehydic compounds, as a consequence of thermal degradation (Cecutti and Agius 2008). Vegetable or natural oils are triglycerides of natural fatty acids, palmitic acid, stearic acid, oleic acid, vegetable oils, linolenic acid, etc. One of the most important natural oils is rape seed oil. Due to its high content of unsaturated fatty acids, natural oils tend to have low oxidation stability (Bartz 1998). According to Lauhanen *et al.* (2000), the degradability of rape seed oil is faster than that of mineral oils in the field. Biodegradability of lubricants varies; from 21 to 24 days after use for bio-oils, 35 to 40 days for mineral oils (Cecutti and Agius 2008), and 30 days for synthetic oils (Haigh 1995). The bio vegetable oil that was used in this study also achieved a high degree of biodegradability - within 3 weeks (BIPOL L1 2017). The decomposition time of oils depends on many factors, including temperature, humidity, quantity and type of bacteria, quantity of oxygen, and other biological, ecological and physical factors (Stanovský *et al.* 2013). Additionally, temperature can have a strong impact on biodegradation efficiency (Ribicic *et al.* 2018; Skoupý *et al.* 2010). Ribicic *et al.* (2018) found that different oil types may influence degradation rates and microbial community structure, especially at low temperatures.

The oil used for lubrication enters the forest environment directly and completely (Skoupý 2004). Bio-lubricants of vegetable origin do not migrate as deep as the mineral oils, but none of these compounds migrate further than 60 cm in depth in the soil within a certain period of time (Cecutti and Agius 2008). A chainsaw requires 0.05 litres of lubricating oil to produce $1 m^3$ of wood, while a harvester needs less than half that amount of lubricating oil (i.e. only 0.02 litter) (Nowak *et al.* 2019). For example, in the state forest enterprise of Slovakia alone, 1.8 million litres of oil were spilt into the soil from chain saw operation alone (Stanovský *et al.* 2013). Oil pollution causes serious damage to soils, due to the physicochemical processes leading to a change in the distribution of organic matter. Consequently, the correct functioning of the ecosystem may be disturbed (Abosede 2013).

Soil type and permeability have an impact on how oils affect the environment (Vähäoja *et al.* 2005). The properties of forest soils vary depending on the parent material, i.e. igneous or sedimentary rocks and Neogene-Quaternary sediments (Leitgeb *et al.* 2019). Forest soils are generally characterized by deeply rooted trees, wide varieties of soil-dwelling organisms, and recycling of organic matter and nutrients, including wood (Boyle 2005). The soils in forested areas are primarily affected by harmful and trace elements that result from atmospheric deposition (Baize and Oort 2014). These atmospheric pollutants have an effect on soil chemical properties, which modify solubility, mobility and availability of soil elements to plants (Tyler and Olsson 2002). The toxicity of heavy metals and their availability to forest habitats increase due to soil acidification caused by sulphur deposition (Temminghoff *et al.* 1997). Physical site manipulations in forest harvesting such as mechanical removal of competing vegetation and forest floor organic matter can alter soil physical properties such as porosity, infiltration capacity, and susceptibility to erosion. Crucial parameters that can positively influence the retention capacity of forest soils include the humus concentration, pH and saturation levels (Utermann *et al.* 2019).

Biodegradation of mineral and synthetic base oils is well documented and therefore we focused on the study of biodegradability impacts on vegetable oils, as they are commonly used in forestry. The aim of this study was to determine the effects of biodegradable lubricant oil on changes in the element composition of forest soils.

Material and Methods

Study area and sample collection

The site where sampling was performed is located in the National nature reserve (NNR) Slnečné skaly in Rajecká basin, Slovakia. The area of the NNR Slnečné Skaly is morphologically formed from dolomite. Field research took place from between 3 February and 24 March 2017. The altitude of the field was 550 meters above sea level. The sampling site was approximately one hectare in size, and forested with non-native black pine (*Pinus nigra*) and common spruce (*Picea abies*). In the field harvesting occurred in a ratio of 70 % common spruce to 30 % black pine. Trees were sawed by standard technique (Slovak technical standard STN 48-00-50), using the principle of directional sawing with two cuts. After cutting the trees, a soil sampling methodology was developed. Ten stumps of spruce with a minimum distance of ten meters from one another were selected. The oil was preserved mainly on the soil surface under the point of cut. Soil samples were taken once per week around each stump. The soil samples had a base of limestone bedrock. We placed the obtained soil samples in 10 x 20 cm PVC bags. Samples were taken in a semi-circular shape around the stumps for eight weeks, totalling 80 samples from ten stumps. In order to establish the time distribution of the decomposition of biodegradable oil we used, control samples were necessary. These were obtained at a distance of ten meters from the upper edge of the stumps up the slope. A total of 80 control samples were collected over eight weeks. All together 160 soil samples were collected. To harvest trees we used standard forestry tools, including OLEO-MAC 947 handsaw and the HUSQVARNA 365 XP. These chainsaws are commonly used in forestry for different purposes. Both are powered by BA 95 fuel mixed 1 : 50 with STIHL HP self-mixing oil. Bipol biodegradable vegetable-based lubricating oil was used in both chainsaws.

Laboratory and statistical analyses

Soil samples were dried at 70° C for 12 h in a Memmert IF 160 laboratory Plus dryer (Memmert, Germany). All samples were ground and homogenized into a fine dust in the Retsch Cryomill. The samples were analysed by X-ray fluorescence, using the hand-held XRF Spectrometer DELTA CLASSIC (Innov-X Systems, Inc., Woburn, MA, USA). We used multiple-beam measurement, in which every measurement consisted of 3 beams for 80 seconds, repeated three times, and then averaged. The results were given in ppm (part per million) units. The minimum value of a particular element represented the current detection limit of the spectrometer for the measured material. We used an additional calibration matrix to correctly measure for the specific needs of plant material analysis using certified plant standards INCT-PVTL-6 (ICHTI, Poland) and BCR-19, as well as NIST 1575a for the soils. The DELTA Spectrometer is designed for accurate investigation of heavy metals, transition metals, as well as rare earth elements. This instrument has a high analytical accuracy and precision of measurements (Innov-X Systems 2010). In cases of rare earth elements XRF measurements correspond with AAS results, but the spectrometer measures several light elements (mainly P) in wider limits. The XRF spectrometer can be used to determine variability and interrelationships of elements in samples by using multivariate statistical analyses. In our study, the effect of elements was evaluated by principal component analysis (PCA) which is widely used in ecotoxicological studies. It is a variable reduction technique that maximizes the amount of variance accounted for in the observed variables by a smaller group of variables called components or factors.

We repeated measurements with certified standards, to demonstrate repeatability. The standard deviation (SD) was stable and minimal for all repeated measurements for measured elements. Control measurements of standard reference materials were consistent with the certified values within the uncertainty limit of <10 % (RSD) for relative standard deviation. The detection limits change for each sample and for each element during current measurements, therefore it is not possible to state the detection limits in the methodology. The minimum detection limit of the device is 1 ppm.

Results

According to the results of PCA, out of the 16 principal components only the first 4 components account for meaningful amounts of variance in the investigated plots (Table 1).

The highest variance of factor 1 reflects differences in soil composition among different plots (Table 2). Factor 1 (K, Ti, Cr, Mn, Fe, Rb, Sr, Zr, Ba) has a major effect on the soil and concentrations of these elements simultaneously increase or decrease. In plots 7 to 10, the elements that comprise factor 1 increase (Fig. 1a). Factor 1 shows different effects of lubricant oil on various types of soils. However, in Fig. 1a, there is a difference between oil and control plots, with significant higher concentrations of the investigated elements (K, Ti, Cr, Mn, Fe, Rb, Sr, Zr, Ba) found in control plots. The date of sample collection does not have a significant effect on the soil element composition (Table 3), although the elements were slightly depleted from the oil plots approximately three weeks into the experiment (Fig. 1b).

Factor 2 clearly shows that more calcium appeared in the control plots (Fig. 2a). The oil has an overall negative effect on calcium binding in the soil. The effect of oil on calcium concentration is not significant because the oil tends to act on the binding of calcium in the soil after approximately 5 weeks (Fig. 2b).

According to factor 3, there are clearly differences in the accumulation of S and Pb in soils from plots 7 – 10 (Fig. 3a). The concentrations of S and Pb tended to increase in oil plots (Fig. 3a), however this effect mainly occurred in the second half of the experiment (Fig. 3b).

In factor 4, the presence of oil presumably suppressed the amount of copper in the soil in plots 6 through 9. More copper remains in the control samples (Fig. 4a). However, the effect of lubricant oil on the binding of copper in the soil could not be established between different types or dates of samples and plots (Fig. 4a, b). There is evidently a large variance between individual plots.

Discussion

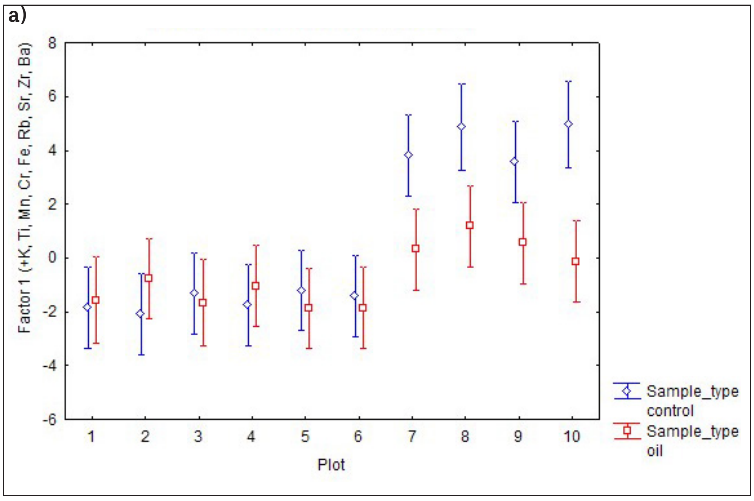
We found that oil may have a different effect on different types of soil or different plots. This phenomenon is mainly evident in factor 1 (Table 2). Amounts of K, Ti, Mn, Cr, Fe, Rb, Sr, Zr and Ba significantly decreased in oil plots 7 to 10 (Fig. 1a). Decomposition of plant material highly influences the concentration of nutrients in forest soils (Aerts and Chapin 1999). The amount of litter and the thickness of organic soil horizon have significant impact on the concentration of nutrients, such as K (Pompeani *et al.* 2018). The decline in bioavailable K in the oil soil suggests its removal by mineralization (Qualls 2000) and subsequent utilization by plants. Mineralization increases the bioavailability of the nutrients in decomposing organic compounds for plants. The process of oil biodegradation can, in some cases, lead to complete mineralization of organic matter into carbon dioxide, water, inorganic compounds, and cell protein, or the breakdown of complex organic contaminants into other simpler organic compounds by microorganisms (Das and Chandran 2011). Decreasing soil nutrients, minerals and trace elements may be also caused by forest harvesting alone. This removes elements from the system in larger quantities at once (Federer *et al.* 1989). We found that time plays a significant role in the effect of biodegradable lubricant oil on soil element elimination. The depletion of elements from the soil starts approximately three weeks after the beginning of the experiment (Fig. 1b) and this time period corresponds to the estimated time of degradation of oil that was used in this study.

Soil Ca content has a fundamental effect on tree growth or indirect effects on soil pH. The content of Ca in soil is essential for multiple ecosystem processes, including soil organic matter decomposition and availability of nutrients such as N and P (Page and Mitchell 2008). Surface geology, soil depth, landscape position, hydrology, and slope characteristics are considered crucial internal landscape drivers of forest Ca cycling and export (McLaughlin 2014). We found that biodegradable oil has a negative effect on the binding of Ca in the soil. Thus, forest harvesting practices that utilize lubricant oil (biodegradable or unbiodegradable) may have an impact on forest Ca cycling (Zetterberg *et al.* 2013). A soil's capacity to neutralize acids is required for forest growth, as it establishes conditions for soil food-web functions and nutrient cycling (Park *et al.* 2008). However, forestry practices such as harvesting and regeneration may further intensify soil acid cation and anion production and leaching of calcium from the soil (Likens *et al.* 1998). The impact of lubricant oils can be intensified by clear-cutting of forests, which leads to radical changes in the underlying soil, humus layer, and soil chemistry (Berthelsen and Steinnes, 1995). Long-term acidification of forest soils can lead to changes in the Ca dynamic, which may subsequently affect nutrient limitation (Leys *et al.* 2016). Calcium is an important element for neutralizing soil acidity in forests (Dijkstra 2003). The effects of lubricant oil differed between plots, however, increased Ca depletion in plots 7 - 10 may be a result of variations in Ca cycling between various tree species, which affects nutrient balances in the soil (Dijkstra 2003). The common spruce was predominant on the plots that were investigated throughout this study, but black pine was also observed within the sample area.

We found that soils reflect a high variance in element compositions. Surface soil systems are unstable with respect to heavy metal accumulation and large concentration variations are frequently

Element	Factor 1	Factor 2	Factor 3	Factor 4
S	-0.16	-0.10	**0.74**	0.24
Cl	-0.61	0.10	-0.22	-0.02
K	**0.94**	-0.02	-0.00	-0.10
Ca	0.35	**-0.81**	0.25	-0.18
Ti	**0.98**	0.06	-0.03	-0.05
Cr	**0.82**	0.39	-0.11	0.04
Mn	**0.97**	-0.08	0.03	-0.04
Fe	**0.93**	0.29	0.10	0.08
Cu	-0.10	0.34	0.43	**-0.82**
Zn	-0.68	0.40	0.24	-0.01
Rb	**0.95**	0.23	-0.06	0.07
Sr	**0.81**	-0.22	0.10	-0.04
Zr	**0.97**	0.11	-0.11	-0.02
Mo	-0.70	0.54	-0.06	0.02
Ba	**0.95**	0.24	-0.03	-0.02
Pb	0.28	0.25	**0.64**	0.34
Variance (%)	57.8	10.7	8.5	5.6

Table 1. Component weights of the principal component analysis at the first four most important factors. Factors indicate the process of element manifestation in the examined soils.



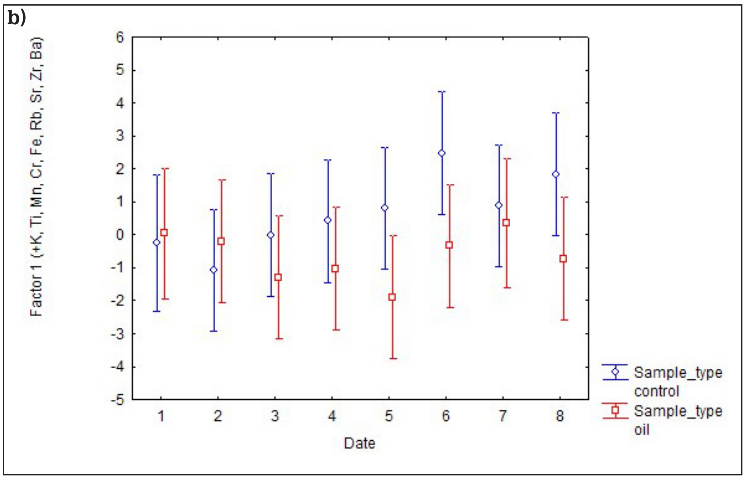


Fig. 1. Oil significantly influenced concentrations of many elements in the soil. Amounts of K, Ti, Mn, Cr, Fe, Rb, Sr, Zr and Ba significantly decreased at the oil plots from 7 to 10 against to control plots (**a**), and the elements were reduced from the soil approximately three weeks after beginning of the experiment (**b**). Two-way ANOVA of principal component scores is presented in Tables 2 and 3.

Factor	Plot	Sample type	Plot/ Sample type interaction
Factor 1	$F(9, 136) = 13.97$, $p = 0.000$***	$F(9, 136) = 17.55$, $p = 0.000$***	$F(9, 136) = 4.08$, $p = 0.000$***
Factor 2	$F(9, 136) = 5.85$, $p = 0.000$***	$F(9, 136) = 4.04$, $p = 0.046$*	$F(9,136) = 0.94$, $p = 0.494$
Factor 3	$F(9, 136) = 0.54$, $p = 0.846$	$F(9, 136) = 5.06$, $p = 0.026$*	$F(9, 136) = 1.11$, $p = 0.362$
Factor 4	$F(9, 136) = 0.70$, $p = 0.078$	$F(9, 136) = 0.12$, $p = 0.727$	$F(9, 136) = 1.21$, $p = 0.295$

Table 2. Differences in the effects of element concentrations in soil samples between plots and sample type. Two way ANOVA of principal component scores. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

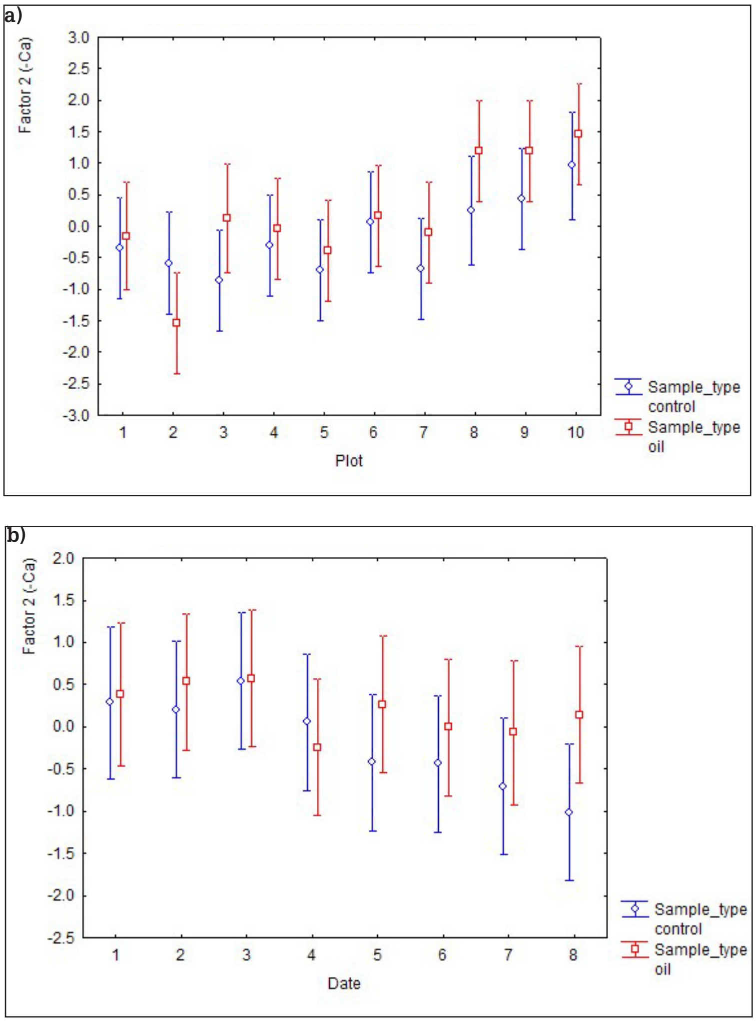


Fig. 2. Oil significantly removes calcium at plot 7 to 10 (**a**). Although the calcium tended to decrease after five weeks from the beginning of the experiment, the difference in this phenomenon was not significant between oily and control samples (**b**). Two-way ANOVA of principal component scores is presented in Tables 2 and 3.

found within small areas (Berthelsen and Steinnes 1995). Heavy metals have an affinity for accumulation in surface organic layers and consequently affect the biological activity in forest soils (Hernandez *et al.* 2003). Stefanowicz *et al.* (2009) found that the functional diversity of bacterial communities in soil decreased with increasing metal concentrations in forest ecosystems. We found that the oil samples had higher levels of lead and sulphur than the control samples. Sulphur significantly decreases soil pH and increases the availability and mobility of heavy metals (Cui *et al.* 2004). One of the most important soil parameters affecting metal content in bioavailable forms is pH (Takáč *et al.* 2009). Low pH and a high level of organic matter in soil result in high mobility of heavy metals (Muhammad *et al.* 2011). An increase in Pb content in the soils is due to both the influence of humus dynamics and the close correlation

Factor	Date	Sample type	Date / Sample_type interaction
Factor 1	F (7, 140) =0.98, p =0.445	F (7, 140) =7.06, p =0.009***	F (7, 140) =1.07, p =0.383
Factor 2	F (7, 140) =1.59, p =0.142	F (7, 140) =3.39, p =0.068	F (7, 140) =0.60, p =0.754
Factor 3	F (7, 140) =7.30, p =0.000***	F (7, 140) =7.05, p =0.009***	F (7, 140) =2.41, p =0.023**
Factor 4	F (7, 140) =1.71, p =0.112	F (7, 140) =0.13, p =0.714	F (7, 140) =0.90, p =0.508

Table 3. Differences in the effects of element concentrations in soil samples between dates and sample type. Two way ANOVA of principal component scores. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

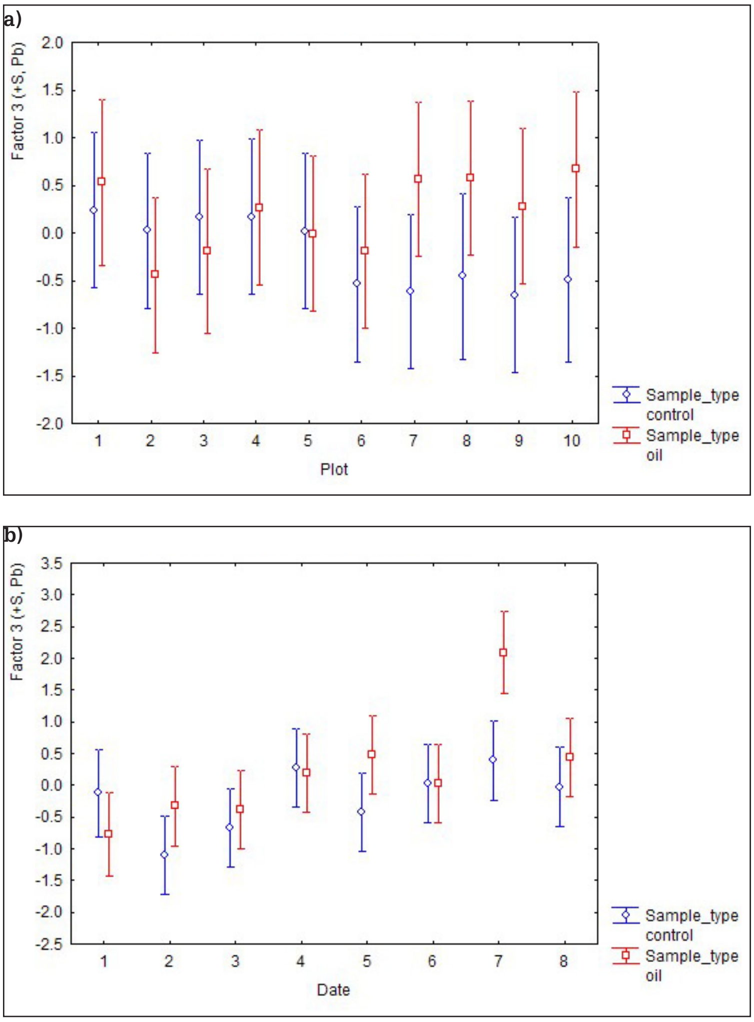


Fig. 3. Acidification by sulphur and synergic pollution by lead are more depended on the plot than on the sample type (**a);** Table 1) and mainly occurred in the second half of the experiment (**b**); Table 3.).

between measurements of heavy metals and organic matter (Utermann *et al.* 2019). The behaviour of Pb in organic surface soils is determined by soil chemical factors (Berthelsen and Steinnes, 1995). Generally, Pb availability increases as soil pH decreases (Siebielec *et al.* 2006). Soil contaminated by sulphur is characterized by an increased bioavailability of Pb in roots and plants in the forest. We found that sulphur and lead in forest soils are closely related. Similar findings were reported by Holah *et al.* (2010), demonstrating that increasing concentrations of sulphur caused a considerable increase in lead content in plants.

We found that biodegradable lubricant oils have an insignificant negative impact on the amount of copper in the soil (Fig. 4a, 4b). In most cases, more copper was present in control samples. Cu is strongly associated with biomass in organic surface soils (Berthelsen and Steinnes 1995).

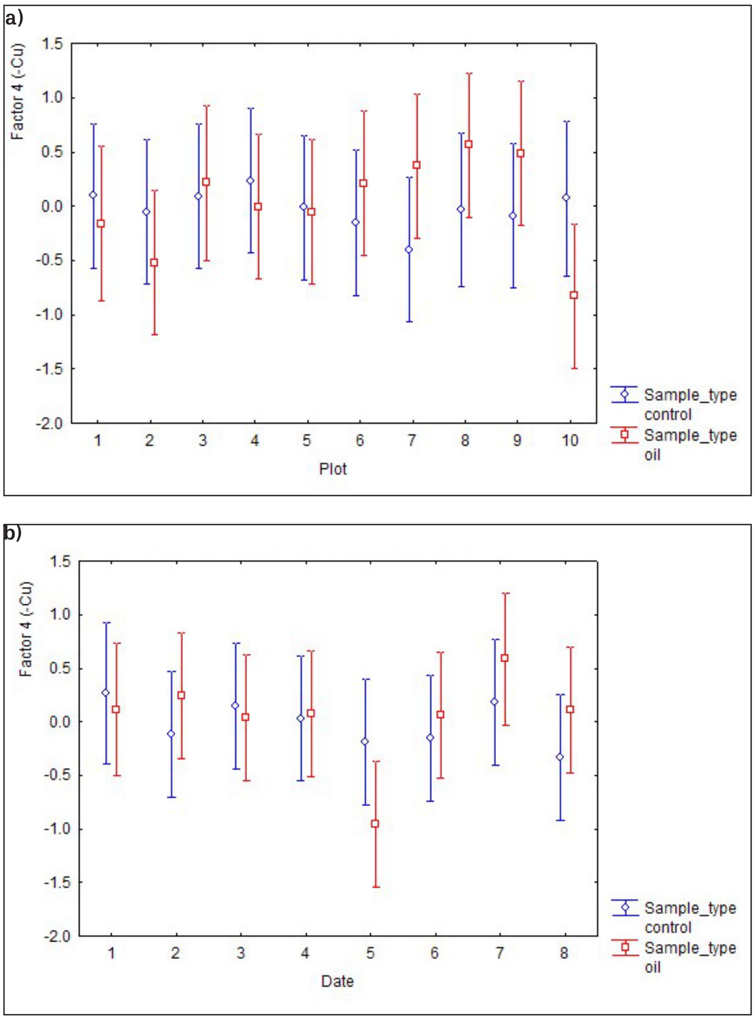


Fig. 4. Amount of copper did not significantly differ in dependence on type of samples, plots (**a**) or experiment chronology (**b**) (Tables 2 and 3).

According to Tyler and Olsson (2002), most of the Cu occurs as a soluble complex in organic matter. This finding may be related to the increase in organic litter present in plots with standing trees, consistent with those that used for the collection of control samples.

We can conclude, therefore, that biodegradable lubricant oils can influence the composition and concentration of elements in soils, regardless of this product's rapid degradation and very low eco-toxicity (Stanovský *et al.* 2013). There is an assumption that the biodegradation of bio-oils affects mineralization processes, pH, nutrient availability, and the mobility and bioavailability of heavy metals such as Pb. This study is the first step in analysing the effect of vegetable oils on soil processes, but these issues deserve further exploration, as there is no doubt that biodegradable lubricant oils are significantly less harmful than synthetic or mineral oils.

Acknowledgments

We would like to thank Ondrej Mahút and Radovan Kovalčík for their help during fieldwork. This study was supported by project ITMS (Grant No. 26110230078).

References

Abosede, E.E. 2013: Effect of crude oil pollution on some soil physical properties. *J. Agric. Vet. Sci.*, **6**: 14-17.

Aerts, R. and Chapin III, F.S. 1999: The mineral nutrition of wild plants revisited: A re-valuation of processes and patterns. *Adv. Ecol. Res.*, **30**: 1-67.

Bartz, J.W. 1998: Lubricants and the environment. *Tribol. Int.*, **31**: 35-47.

BIPOL_L1. 2017: Chain lubricating oil. RIWALL. Product specification. Online: https://www.mountfield.sk/olej-na-mazanie-retazi-bipol-1-l-1phm1008?gclid=EAIaIQobChMIzqfBoMyG7QIVk_hRCh2Z8ARAEAAYASAAEg-K6cvD_BwE. (retrieved 25.4.2020).

Baize D., van Oort F. 2014: Potentially Harmful Elements in Forest Soils. In: *PHEs, Environment and Human Health* (eds. C. Bini and J. Bech), pp. 1.51-198 Springer, Dordrecht.

Berthelsen, B.O. and Steinnes, E. 1995: Accumulation patterns of heavy metals in soil profiles as affected by forest clear-cutting. *Geoderma*, **66**: 1-14.

Boyle, J.R. 2005: Forest soils. In: *Encyclopedia of Soils in the Environment* (ed. D. Hillel), pp. 73-79. Academic Press, Oxford, UK, St Louis, MO.

Cecutti, Ch. and Agius, D. 2008: Ecotoxicity and biodegradability in soil and aqueous media of lubricants used in forestry applications. *Bioresour. Technol.*, **99**: 8492-8496.

Cui, Y., Dong, Y., Li, H. and Wang, Q. 2004: Effect of elemental sulfur on solubility of soil heavy metals and their uptake by maize. *Environ. Int.*, **30**: 323-328.

Das, N. and Chandran, P. 2011: Microbial degradation of petroleum hydrocarbon contaminants: an overview. *Biotechnol. Res. Int.*, **11**: 1-13.

Dijkstra, F.A. 2003: Calcium mineralization in the forest floor and surface soil beneath different tree species in the northeastern US. *For. Ecol. Manag.*, **175**: 185-194.

Erhan, S.Z. and Asadauskas, S. 2000: Lubricant basestocks from vegetable oils. *Ind. Crops. Prod.*, **11**: 277-282.

Federer, C.A., Hornbeck, J.W., Tritton, L.M., Martin, C.W., Pierce, R.S. and Smith, C.T. 1989: Long-term depletion of calcium and other nutrients in eastern US forests. *Environ. Manage.*, **13**: 593-601.

Haigh, S.D. 1995: Fate and effects of synthetic lubricants in soil: biodegradation and effect on crops in field studies. *Sci. Total. Environ.*, **168**: 71-83.

Hernandez, L., Probst, A., Probst, J.L. and Ulrich, E. 2003: Heavy metal distribution in some French forest soils: evidence for atmospheric contamination. *Sci. Total. Environ.*, **312**: 195-219.

Holah, S., Kamel, M.M., Taalab, A.S., Siam, H.S., El-Rahman and Eman, A. 2010: Effect of elemental sulfur and peanut compost on the uptake of Ni and Pb in bazil and peppermint plants grown in polluted soil. *Int. J. Acad. Res.*, **2**: 211-219.

Innov-X Systems. 2010: User Manual. Delta™ Family: Handheld XRF Analyzers.

Lauhanen, R., Kolppanen, R., Takalo, S., Kuokkanen, T., Kola, H. and Valimaki, I. 2000: Effects of biodegradable oils on forest machines and forest environment. In: *Proceedings of the Scientific Conference on Forest and Wood Technology vs. Environment*, pp. 203-207. Brno.

Leitgeb, E., Ghosh, S., Dobbs, M., Englisch, M. and Michel, K. 2019: Distribution of nutrients and trace elements in forest soils of Singapore. *Chemosphere*, **222**: 62-70.

Leys, B.A., Likens, G.E., Johnson, C.E., Craine, J.M., Lacroix, B., and McLauchlan, K.K. 2016: Natural and anthropogenic drivers of calcium depletion in a northern forest during the last millennium. *PNAS*, **113**: 6934-6938.

Likens, G.E., Driscoll, C.T., Buso, D.C., Siccama, T.G., Johnson, C.E., Lovett, G.M., Fahey, T.J., Reiners, W.A., Ryan, D.F., Martin, C.W. and Bailey, S.W. 1998: The biogeochemistry of calcium at Hubbard Brook. *Biogeochemistry*, **41**: 89–173.

McLaughlin, J.W. 2014: Forest soil calcium dynamics and water quality: implications for forest management planning. *Soil. Sci. Soc. Am. J.*, **78**: 1003-1020.

Muhammad, S., Shah, M.T. and Khan, S. 2011: Heavy metal concentrations in soil and wild plants growing around Pb–Zn sulfide terrain in the Kohistan region, northern Pakistan. *Microchem. J.*, **99**: 67-75.

Nowak, P., Kucharska, K. and Kaminski, M. 2019: Ecological and health effects of lubricant oils emitted into the environment. *Int. J. Environ. Res. Public Health*, **16**: 3002.

Page, B.D. and Mitchell, M.J. 2008: Influences of a calcium gradient on soil inorganic nitrogen in the Adirondack Mountain, New York. *Ecol. Appl.* **18**: 1604-1614.

Park, B.B., Yanai, R.D., Fahey, T.J., Bailey, S.W., Siccama, T.G., Shanley, J.B., and Cleavitt, N.L. 2008: Fine root dynamics and forest production across a calcium gradient in northern hardwood and conifer ecosystems. *Ecosystems*, **11**: 325-341.

Pompeani, D.P., McLauchlan, K.K., Chileen, B.V., Wolf, K.D., and Higuera, P.E. 2018: Variation of key elements in soils and plant tissues in subalpine forests of the northern Rocky Mountains, USA. *Biogeosci. Discuss.*, 1-19.

Ribicic, D., McFarlin, K.M., Netzer, R., Brakstad, O.G., Winkler, A., Holst, M.H. and Størseth, T.R. 2018: Oil type and temperature dependent biodegradation dynamics - Combining chemical and microbial community data through multivariate analysis. *BMC Microbiol.*, **18**: 1-15.

Qualls, R.G. 2000: Comparison of the behavior of soluble organic and inorganic nutrients in forest soils. *For. Ecol. Manag.*, **138**: 29-50.

Siebielec, G., Stuczyński, T. and Korzeniowska-Pucułek, R. 2006: Metal bioavailability in long-term contaminated Tarnowskie Gory soils. *Pol. J. Environ. Stud.*, **15**: 121-129.

Skoupý, A. 2004: Biologically degradable oils at working with power saws. *J. For. Sci.*, **50**: 542-547.

Skoupý, A., Klvac, R. and Hosseini, S. 2010: Changes in the external speed characteristics of chainsaw engines with the use of mineral and vegetable oils. *Croat. J. For. Eng.*, **31**: 149-155.

Stanovský, M., Schürger, J., Jankovský, M., Messingerová, V., Hnilica, R. and Kučera, M. 2013: The Effect of lubricating oil on temperature of chainsaw cutting system. *Croat. J. For. Eng.*, **34**: 83-90.

Stefanowicz, A.M., Niklińska, M. and Laskowski, R. 2009: Pollution-induced tolerance of soil bacterial communities in meadow and forest ecosystems polluted with heavy metals. *Eur. J. Soil. Biol.*, **45**: 363-369.

Takáč, P., Szabová, T., Kozáková, Ľ. and Benková, M. 2009: Heavy metals and their bioavailability from soils in the long-term polluted Central Spiš region of SR. *Plant Soil Environ.*, **55**: 167-172.

Temminghoff, E.J., Van der Zee S.E. and de Haan F.A. 1997: Copper mobility in a copper contaminated sandy soil as affected by pH and solid and dissolved organic matter. *Environ. Sci. Technol.*, **31**: 1109-1115.

Tyler, G. and Olsson, T. 2002: Conditions related to solubility of rare and minor elements in forest soils. *J. Plant. Nutr. Soil Sci.*, **165**: 594-601.

Vähäoja, P., Roppola, K., Välimäki, I. and Kuokkanen, T. 2005: Studies of biodegradability of certain oils in forest soil as determined by the respirometric BOD OxiTop method. *Int. J. Environ. Anal. Chem.*, **85**: 1065-1073.

Utermann, J., Aydın, C.T., Bischoff, N., Böttcher, J., Eickenscheidt, N., Gehrmann, J., König, N., Scheler, B., Stange, F. and Wellbrock, N. 2019: Heavy metal stocks and concentrations in forest soils. In: *Status and Dynamics of Forests in Germany* (eds. N. Wellbrock and A. Bölte), pp. 199-229. Springer, Cham, Switzerland.

Zetterberg, T., Olsson, F.A., Löfgren, S., von Brömssen, C., and Brantberg, P.O. 2013: The effect of harvest intensity on long-term calcium dynamics in soil and soil solution at three coniferous sites in Sweden. *For. Ecol. Manag.*, **302**: 280-294.

Received 11 June 2020; accepted 28 July 2020.

Oecologia Montana 2020,
29, 9-16

Bacteria in the water from the snow from the area Ružomberok

J. GUZYOVÁ, Z. HREHOVÁ and D. MIHÁLIK

Institute of High Mountain Biology, Žilina University, Tatranská Javorina 7, SK-059 56, Slovak Republic; e-mail: ihmb@uniza.sk

Abstract. The aim of our study was to investigate the possible environmental pollution by the Mondi SCP pulp mill in Ružomberok. Bacterial communities were examined by T-RFLP analysis of the 16S rRNA gene. This gene was subjected to restriction digestion by using the restriction enzyme MspI. Terminal restriction fragments (T-RFs) from 60-924 bp were evaluated. An experimental size of fragments was compared to in silico sizes T-RF from the database of 16S rDNA sequences by using the MiCA program. Amplification and sequencing of 16 rDNA from DNA isolated from bacteria in the snow samples indicate the presence of bacteria. It was established three categories of bacteria. The first representated the dominant phyla of 5 to 25 % (*Proteobacteria* 19.5 %, *Firmicutes* 18.2 %, *Actinobacteria* 13.4 %, *Bacteroidetes* 11.1 %, and *Chloroflexi* 10.1 %). The second group has a relatively low percentage of bacterial phyla 1 to 5 % (*Planctomycetes, Acidobacteria, Tenericutes, Planctomycetes, Acidobacteria*, *Tenericutes*, *Spirochaetes*, *Cyanobacteria*, *Deinococcus-Thermus*, *Gemmatimonadetes*, *Fusobacteria*, *Nitrospirae*). In the last one, the percentage of bacterial phyla ranged from 0 to 1% (*Defferibacteres*, *Chlorobi*, *Verrucomicrobia*, *Synergistes*, *Fibrobacteres*, *Thermotogae*, *Caldiserica*, *Thermodesulfobacteria*).

Key words: snow-bacteria, mg DNA, pulp mill, Mondi SCP Ružomberok, T-RFLP

Introduction

We chose to study bacteria in snow melt surrounding the Mondi SCP mill near Ružomberok to show what type of bacteria occur in this environment. It is known that paper and pulp processing results in the production of many highly toxic chlorinated organic compounds. Of prime concern are chlorinated phenols, guaiacols, catechols, furans, dioxins, aliphatic hydrocarbons and highly mutagenic agents. In addition, two natural wood resin acids, neoabietic and 7-oxodehydroabietic acids, exhibit mutagenic activity (Murray 1992). Recent research on bacteria in the snow and their influence show that an abundance of bacteria are closely related to dust (Chuvochina *et al.* 2011; Yamaguchi *et al.* 2012). Bacterial concentration positively correlates with the distribution of dirty layers (Zhang *et al.* 2008). Other reports suggest that microorganisms impact composition, abundance of nutrients, and the dynamics and it seems bacteria have a role in governing redox conditions and also play a part in Fe, S, N, and P cycling (Hodson *et al.* 2008). It is likely that they are also responsible for the metabolism and transformation of environmental contaminants such as mercury (Barkay and Poulain 2007).

Our aim was to collect snow samples around Mondi SCP in Ružomberok and isolate the DNA of bacteria in samples of melted snow. For monitoring we chose to use Terminal restriction fragment length polymorphism analysis (T-RFLP), which is a cultivation independent method of microorganism identification (Marsh 1999).

Psychrophilic bacteria covered an extensive range of phylogenetic diversity (Bowman *et al.* 1997). They represente important functional groups such as methanotrophic organisms (Bowman *et al.* 1997), sulphate-reducing (Knoblauch *et al.* 1999), and methanogenic organisms (Franzmann *et al.* 1997).

Terminal restriction fragment length polymorphism

T-RFLP analysis is a technique used to study complex microbial communities based on variation in the 16S rRNA gene (Osborn *et al.* 2000). In this way in is possible to determine if new microbes are present in a particular environment. The length of the bacterial 16S rRNA gene is approximately 1540 base pairs. This is why it can be quickly and inexpensively copied and sequenced (www.microbeworld.org 2015).

Total DNA is first extracted from the microbial community and the16S rRNA gene is isolated from samples using fluorescently-labelled forward and reverse primers. Next, the PCR product is purified and subjected to restriction enzyme digestion with enzymes that have 4 base pair recognition sites. This step generates fluorescently labelled terminal restriction fragments. The digested products are then separated and detected on an appropriate electrophoresis platform. For a given sample the terminal fragments will contain a fluorescent label at the 5'- end and will therefore be detected.

The output will be a series of peaks (fragments) of various sizes and heights that represents the profile of that sample (Osborn *et al.* 2000).

Material and Methods

Study area and sample collecting

Collection of samples was conducted during snowy periods in January-February 2015 the Mondi SCP mill in Ružomberok (N: 49˚ 4´ 50.18", E: 19˚ 19´ 35.44").

The sample collection period was chosen based on predicted snowfall (during the winter 2014/2015). Samples were collected from two localities. The first sampling location was in Lisková which is located to the east and 2 km away from Mondi SCP Ružomberok (N: 49˚ 5´ 27.93"; E: 19˚ 21´ 0.45V). The second control location was in Hrboltová which is located to the west and 6,5 km away from Mondi SCP Ružomberok (N: 49˚ 6´ 1.14"; E: 19˚ 14´ 40.58").

The first sample of snow was collected on January 8. Samples were collected from both sampling sites once per day. Due diligence during sampling processes was important to ensure minimal contamination. Snow was melted slowly to 5˚ C (up to 48 h) to avoid stress to the bacteria during melting. Depending on the snow texture, melted snow resulted in 500 - 850 ml melt water, which could be compared to previous observations (Ferrari *et al.* 2004). All of the samples were collected between 7:30 am and 6:30 pm, but most of them between 12:00 - 4:00 pm. During sampling we recorded the time of sampling, temperature and weather. A total of 61 samples were collected (Table 1).

T-RFLP analysis

DNA isolation

Melted snow was filtered by a vacuum filtration system (KNF Neuberger, Inc., USA). Until further processing in laboratory, the filters were stored at a temperature of 4˚ C, and filled with 50 ml of water to avoid drying out the filters. Following the collection of all the samples, DNA was isolated using the PowerWater® DNA Isolation Kit. DNA was isolated according to the manual.

Following DNA isolation, the concentration and purity of DNA was measured by the NanoPhotometer P330 (Implen, Germany) device and isolated DNA was stored at -20˚ C.

Agarose gel electrophoresis

DNA extracts were verified by gel electrophoresis, using 1 % Agarose gel (1 g of Agarose and 100 ml 1xTBE). They were observed under UV light with the addition of ethidium bromide (50 µg for 100 ml of gel).

PCR

16S rRNA genes were amplified by PCR (polymerase chain reaction). This step was carried out using universal primers for bacteria 8F (Edwards *et. al.* 1989) and 926R (Muyzer *et al.* 1995). The forward primer was fluorescently labelled at the 5'- end with Cyanine5 (Cy5'). For PCR were prepared with master mix in 50 µl volumes. The PCR master mix contained reagents with the following final concentrations: 1x PCR buffer, 1.5 mM $MgCl_2$, 200 mM of dNTPs, 0.1 µM of each primer, 2.5 U Fire Pol DNA Polymerase.

Date	Age of snow in days	Number of samples	Lisková		
			Time	T [˚ C]	Actual weather
3.12.2014	0	I	14:00	-1	overcast
8.1.2015	4	III	13:15	-7	overcast
10.1.2015	6	1	14:20	4	overcast
22.1.2015	18	3	16:00	4	overcast
25.1.2015	1	5	15:10	-3	snowing
26.1.2015	2	7	13:45	1	snowing
27.1.2015	-	-	-	-	-
28.1.2015	4	11	14:00	1	overcast
29.1.2015	0	13	13:15	1	snowing
30.1.2015	0	15	12:45	4	overcast
31.1.2015	0	17	14:00	-3	overcast
1.2.2015	1	19	15:34	-2	overcast
2.2.2015	2	21	13:30	1	overcast
3.2.2015	3	23	13:30	0	overcast
4.2.2015	0	25	12:10	2	snowing
5.2.2015	1	27	10:15	-1	sunny
6.2.2015	2	29	12:30	2	sunny
7.2.2015	3	31	14:00	-2	sunny

continued...

8.2.2015	0	33	11:20	-1	snowing
9.2.2015	0	35	12:30	1	snowing
10.2.2015	1	37	18:30	2	overcast
11.2.2015	2	39	15:00	4	overcast
12.2.2015	3	41	13:30	7	overcast
13.2.2015	4	43	16:00	4	sunny
15.2.2015	6	45	15.30	3	sunny
17.2.2015	8	47	13:30	2	sunny
18.2.2015	9	49	13:30	1	sunny
19.2.2015	10	51	13:30	2	sunny
20.2.2015	11	53	13:30	6	sunny
23.2.2015	14	55	15:30	7	overcast
24.2.2015	15	57	9:00	-1	overcast
6.3.2015	3	59	9:30	4	overcast

			Hrboltová		
			Time	**T [° C]**	**Actual weather**
3.12.2014	0	II	14:30	-1	overcast
8.1.2015	4	IV		0	overcast
10.1.2015	6	2	15:00	0	overcast
22.1.2015	18	-	-	-	-
25.1.2015	1	6	14:20	-3	snowing
26.1.2015	2	8	17:00	0	snowing
27.1.2015	-	10	13:05	0	snowing
28.1.2015	4	12	12:15	-1	overcast
29.1.2015	0	14	12:15	-6	snowing
30.1.2015	0	16	14:15	2	overcast
31.1.2015	0	18	14:30	-3	overcast
1.2.2015	1	20	14:40	-2	overcast
2.2.2015	2	22	12:00	0	snowing
3.2.2015	3	24	11:30	0	overcast
4.2.2015	0	26	10:30	1	overcast
5.2.2015	1	28	8:00	-4	sunny
6.2.2015	2	30	14:40	-4	sunny
7.2.2015	3	32	14:40	-2	sunny
8.2.2015	0	34	12:00	-1	snowing
9.2.2015	0	36	13:30	-1	snowing
10.2.2015	1	38	16:30	0	overcast
11.2.2015	2	40	14:00	2	overcast
12.2.2015	3	42	14:00	3	overcast
13.2.2015	4	44	13:15	4	sunny
15.2.2015	6	46	16:10	2	sunny
17.2.2015	8	48	14:00	3	sunny
18.2.2015	9	50	14:00	0	sunny
19.2.2015	10	52	14:00	2	sunny
20.2.2015	11	54	14:00	4	sunny
23.2.2015	14	56	14:15	8	overcast
24.2.2015	15	58	7:30	2	overcast

Table 1. Database of samples with characterization of time of sampling, temperature and weather.

The PCR reactions were run over in the thermocycler (Standart PCR, Bio-Rad C1000 TOUCH) by using PCR program (Table 2).

Used primers: **8F** 5' Cy5-GAGTTTGATCCTGGCTCAG 3' and **926R** 5' CCGTCAATTCCTTTRAGTTT 3'.

PCR conditions were optimized, and the first PCR test was performed on 10 samples. The chosen samples were exposed to PCR amplification.

PCR reactions were verified by electrophoresis in 1 % agarose gel (1 g of Agarose and 100 ml 1 × TBE). It was visualised under UV light and addition of ethidium bromide (50 µg for 100 ml of gel).

Temperature profile:			
Initial denaturation	95° C	3 min.	
Denaturation	94° C	30 sec.	
Annealing	56° C	45 sec.	35 cycles
Extension	72° C	2 min.	
Final Extension	72° C	3 min.	

Table 2. PCR program for amplification of 16S rRNA gene.

Ethanol precipitation

PCR products were precipitated according to Ondreičková *et al.* (2014). A volume of 1/10 volume 3M sodium acetate (pH 5.2) was added to the PCR products. Followed by three times the total volume of chilled 96 % ethanol. The mixture was thoroughly mixed and incubated for 20 minutes at -80° C, then centrifuged at 13 500 rpm for 20 minutes at 4° C. The supernatant was carefully poured down after centrifugation, as the pellet must not be removed. 70 % ethanol (250 µl) was added, and then the sample was centrifuged at maximum speed for 5 minutes at 4° C. Following this, supernatant was again carefully poured down. The pellet was left to dry at room temperature and then dissolved in sterile water.

Restriction digest

The purified PCR products were subjected to restriction by the following procedure:

A reaction mixture was prepared of 10 µl mg DNA, 2 µl of 10x buffer for a given restriction enzyme, and 20 units appropriate restriction endonucleases. Distilled water was added to the reaction mixture to a volume of 20 µl and allowed to incubate for 3 hours at 37° C. The restriction enzyme MspI (New England Biolabs, USA) was used. The digested PCR products were precipitated by ethanol. It was added 1/10 volume of 3M sodium acetate (pH 5.2) to the PCR products. Then it was added 3 multiple of the volume of chilled 96 % ethanol. The mixture thoroughly mixed and incubated for 20 minutes at -80° C. Centrifuged at 13 500 rpm, 20 min at 4° C. Supernatant was carefully poured down after centrifugation, because pellet has to be not removed. It was added 70 % ethanol (250 µl). Centrifuged at maximum speed for 5 min, 4° C. Subsequently carefully poured down the supernatant. Pellet was left to dry at room temperature and then the pellet resolved in the sterile water.

Automated separation of T-RFs

The purified terminal restriction fragments were separated by capillary electrophoresis by an automated sequencer (GenomeLab GeXP, Beckman-Coulter, U.K.). The separation of the fragments is carried out in polyacrylamide gel during denaturing conditions. Composition of the mixture to fragment analysis was: SLS (38.5 µl, AB Sciex, Canada), standard MapMarker 1000 labelled with D1 (0.5 µl Bioventures, Inc., USA) and 1 µl of DNA.

Evaluation of T-RFs profile and statistical analyses

Terminal restriction fragments (T-RFs) were defined as those between 60 and 924 bp. Using by GenomeLab GeXP software. We compared these fragments with in silico sizes T-RF from the 16S rDNA sequence database using the MiCA 3 program (Microbial Community Analysis III; Shyu *et al.* 2007).

To compare bacterial communities in the samples, T-RFLP profiles were evaluated based on the presence of T-RF. Size T-RF were expressed as present (1) or absent (0) in the samples. Due to this constellation of binary system PCA (Principal Component Analysis) by the Statistica 8 program was used.

Our spreadsheet also includes the age of snow, defined by three categories. The first category was snow 0 to 1 days old, followed by the second category; snow 2 to 4 days old. The third category was snow older than 5 days.

The month in which samples were collected was also recorded. Months 1 includes the first half of winter and Months 2 the second half. Three categories of weather were recorded: overcast, sunny, or snowing. The last set of data were temperatures, either below ° C or above 0° C. These data were analysed by One-way ANOVA and factorial ANOVA.

Results

We collected 61 samples. DNA concentration level varied from 4 to 64 ng/µl. The ratio of absorbance at 260 nm and 280 nm is used to assess DNA purity. If the ratio is considerably lower than 1.8, it may indicate the presence of protein, phenol or other contaminants. The purity of PCR products after precipitation ranged from 1.4 - 2.3.

The second measured of purity was (A260/230). Expected values of A260/230 are usually in the range of 2.0 - 2.2. If it is considerably lower, it may also indicate the presence of contaminants. The purity of PCR products after precipitation was ranged 0.24 – 4.93.

To define the genetic diversity of the bacterial community, we chose the 16S rRNA gene. This gene isolated in the DNA by universal primers 8F and 926R (Fig. 1). The first primer was fluorescently labelled at the 5'end with Cyanine -5 (Cy-5').

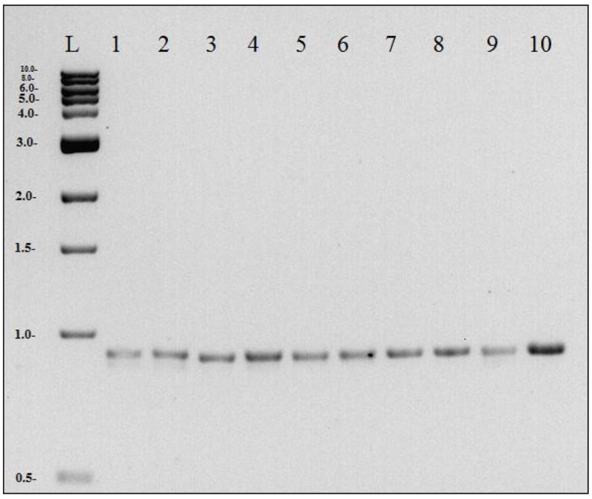


Fig. 1. PCR products - 16S rRNA gene of 10 chosen samples. L - 1kb DNA ladder. 1-10 - number of samples putted to lane in gel.

Conclusion T-RFLP analysis

Using T-RFLP analysis, fragments of digestion were compared with the in silico T-RF sizes. We determined relative abundance phyla. We created three groups According to the percentage of bacterial phyla. The first group includes a representation of the dominant phylum of 5 to 25 %. The second group had a relatively low percentage of bacterial phylum (1-5 %). In the last group, the phylum of the minority representation was up to 1 % (Table 3).

Phylum	%	Percentage in Lisková (1)	Percentage in Hrboltová (2)
Dominant phyla 5-27 %			
Proteobacteria	19.5	19.7	19.3
Firmicutes	18.2	18.2	18.1
Actinobacteria	13.4	13.1	13.6
Bacteroidetes	11.1	11.7	10.6
Chloroflexi	10.1	10.1	10.1
Percentage of phyla 1-5%			
Planctomycetes	4.7	5.1	4.4
Acidobacteria	4.5	4.6	4.4
Tenericutes	3.9	3.7	4.1
Spirochaetes	2.4	2.4	2.4
Cyanobacteria	2.4	2.2	2.5
Deinococcus-Thermus	1.8	1.5	2.1
Gemmatimonadetes	1.6	1.6	1.6
Fusobacteria	1.2	1.0	1.4
Nitrospirae	1.0	0.9	1.0
Percentage of phyla to 0-1%			
Defferibacteres	0.9	1.0	0.9
Chlorobi	0.8	0.8	0.8
Verrucomicrobia	0.8	0.6	1.0
Synergistes	0.7	0.6	0.7
Fibrobacteres	0.5	0.6	0.5
Thermotogae	0.2	0.2	0.2
Caldiserica	0.2	0.3	0.1
Thermodesulfobacteria	0.1	0.0	0.2

Table 3. Relative abundance phyla compared by the sizes of T-RFs obtained by digestion with the enzyme MspI with the in silico T-RF sizes from the database of the 16S rDNA sequences.

T-RFs were detected in the samples in various amounts. T-RFs were detected in 61 samples from 2 localities. The most T-RFs were detected in sample number 12 (with 182 fragments), and the least in sample number 28 (with 6 fragments). The average value of fragments of all samples collected was 59. To compare bacterial communities in the samples T-RFLP profiles were evaluated using the presence of T-RF. Due to this constellation of binary system PCA (Principal Component Analysis) with a statistical program was used.

The complex of data used in the statistical program included records of the age of the snow, its temperature, month collected, and locality. We determined a correlation between these and the presence of T-RF. We focused on the 3 most significant of 7 tested principal components (factors).

Under the principal component Factor 3, correlation between localities and months was found. It shows that during the first months of winter, composition of bacterial diversity differs from the localities, though it is not statistically significant (Fig. 2)

A correlation between localities and samples shows that there are not significant differences between localities of bacterial diversity in the individual samples (Fig. 3). We compared fragments from samples 18, 42, and 44 (representing the Hrboltová locality) as well as fragments of samples 17, 23, and 25 (represent the Lisková locality) with the remaining samples.

Discussion

Amplification and sequencing of 16 rDNA from DNA isolated from bacteria in our snow samples indicate the presence of bacteria. We organized these into three categories. The first includes the representation of the dominant phyla of 5 to 25 %. The second group has a relatively low percentage of bacterial phyla (1 to 5 %). In the last one, the percentage of bacterial phyla ranged from 0 to 1 %. We devoted the most attention to bacterial phyla in the first category. This catecory includes *Proteobacteria* (19.5 %), *Firmicutes* (18.2 %), *Actinobacteria* (13.4 %), *Bacteroidetes* (11.1 %), and *Chloroflexi* (10.1 %). First we searched what type of bacteria are typical in cold environments, including psychrophilic microorganisms, which are restricted to permanently cold habitats. Several studies include psychrophilic

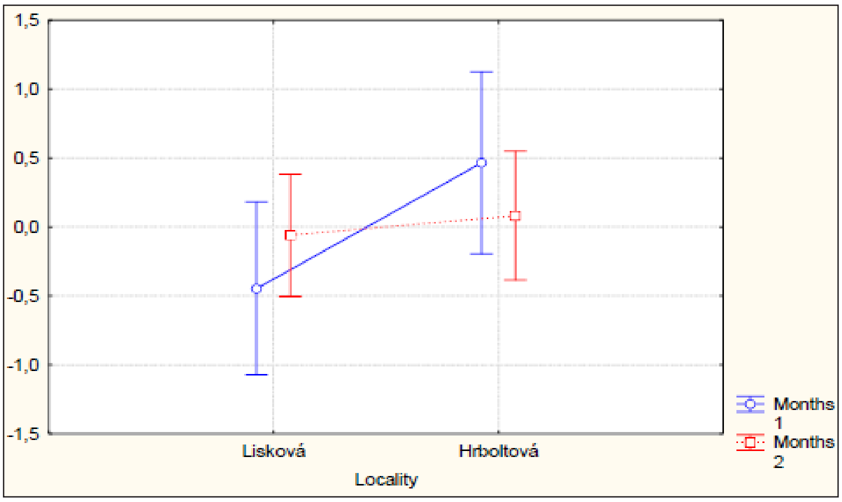


Fig. 2. The effects of increase or decrease bacterial diversity correlation between localities and months. (One – way ANOVA F (1,53)= 1.9220, p = 0.1714).

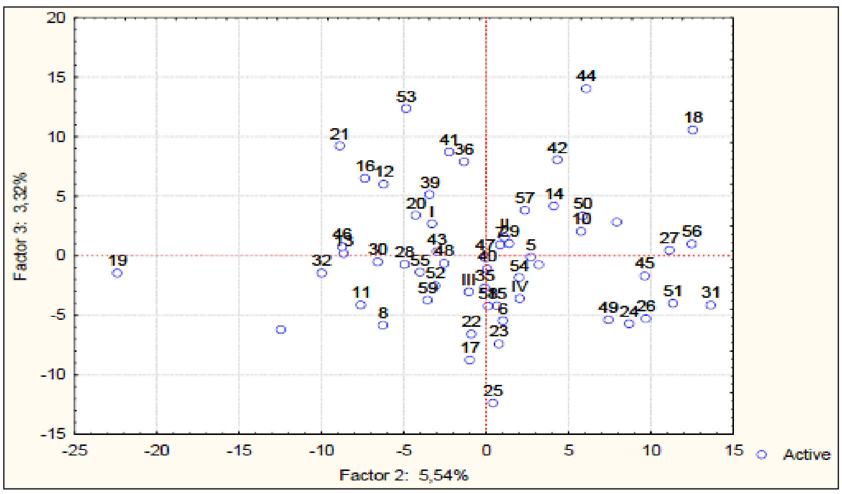


Fig. 3. The effects of increase or decrease bacterial diversity correlation between samples and Factor 3.

bacteria of phyla such as: *Proteobacteria, Bacteroidetes*, and *Firmicutes* (Gounot 1986). We compared studies researching bacteria found in snow. Bacterial diversity in snow varied among different glaciers. For example, members of *Bacteroidetes*, α, β, γ – *Proteobacteria, Actinobacteria*, and *Firmicutes* were dominant in studies of glaciers by Liu *et al.* (2009). In another study, they induct the most dominant group of α, β, γ – *Proteobacteria, Actinobacteria, Firmicutes, Verrucomicrobia, Cyanobacteria*, and *Flavobacterium* (Larose *et al.* 2010). This is similar to other research. In the study by Liu *et al.* (2009), *Proteobacteria, Bacteroidetes*, and *Cyanobacteria* dominated, though *Actinobacteria* and *Firmicutes* were also abundant. In the deepest snow layer, large percentages of *Firmicutes* and *Fusobacteria* can be found. In freshwater, *Bacteroidetes, Actinobacteria* and *Verrucomicrobia* were the most abundant phyla while and relatively few *Proteobacteria* or *Cyanobacteria* were present (Møller *et al.* 2011). We can see that phyla in the studies are sonsistent with our dominant phyla with the exception of *Chloroflexi*. This difference was the topic of our discussion.

The production of paper is the main source of halogen materials, which are used in whitening silks of cellulosic. In this procedure we can see the reaction of chlorine with organic material resulting in chlorinated organic materials, mainly polychlorinated dibenzodioxins and polychlorinated biphenyls (PCB) (Raclavská *et al.* 2008).

The available information indicates that the ring structures of dibenzo-p-dioxin, dibenzofuran (DF), and related compounds are degraded by aerobic bacteria containing aromatic hydrocarbon dioxygenases having broad substrate specificity. In addition to this type of biodegradation, another process of dioxin biotransformation has been recognized; reductive de-chlorination by anaerobic microorganisms (Yoshida *et al.* 2005). This trait, halorespiration - the use halogenated organics as energy sources - is found in some species belonging to three major phyla: *Proteobacteria, Chloroflexi* and *Firmicutes*. Whereas bacteria belonging to the phylum *Proteobacteria* (i.e. *Anaeromyxobacter, Desulfomonile, Desulfuromonas, Desulfovibrio* and *Geobacter*), and the phylum *Firmicutes* (i.e. *Desulfitobacterium*) are facultatively dehalorespiring bacteria. Strictly dehalorespiring microorganisms are limited to only two bacterial genera: *Dehalobacter* (*Firmicutes*) and *Dehalococcoides* (*Chloroflexi*) (Hiraishi 2008). As mentioned by Yoshida *et al.* (2005) aerobic bacteria of the abovementioned phyla are likely responsible for the oxidative degradation of dechlorinated products in the microcosm. Members of the "*Dehalococcoides*" group were reported to be putative de-chlorinators in anaerobic enrichment cultures capable of dechlorinating. The detection of *Dehalococcoides* and its relatives in polluted environments and dechlorinating enrichment cultures indicates that members of the *Dehalococcoides* group are widely distributed in nature and play major roles in the transformation of environmental organohalide pollutants (Yoshida *et al.* 2005). According to Japanese scientists, the detection of *Dehalococcoides* would provide more direct evidence for the involvement of the organism in de-chlorination, and for the presence of this group of bacteria worldwide in organohalide-polluted environments.

We endeavoured to explain the rising representation of steam *Chloroflexi* as well as *Proteobacteria* in the localities we sampled. In these localities we can assume contamination is caused by organic chlorinated materials. The steam *Chloroflexi* was the 5th most occurring steam, and its occurrence is generally atypical in snowy conditions. When taking into consideration the genre *Dehalococcoides* and its characteristic occurrence in organohalide-polluted environments we must consider the potential contamination caused by the nearby pulp and paper mill. Indeed 32 of 61 samples were contaminated. The organic halogens which were present in our samples could be a result of contamination by proximity to this facility. They may serve as culture medium for the increase of steam *Chloroflexi*, as well as more notably, *Dehalococcoides*. It is assumed that naturally occurring chlorophenols are candidates for the original substrates for the *Dehalococcoides* (Hiraishi 2008). A connection might also be made between *Dehalococcoides* and the *Deltaproteobacteria* found in the T-RFs profiles of samples 135, 512, 513, called facultatively dehalorespiring bacteria.

The usage of polychlorinated biphenyls is also interesting; as they are no longer used in the machines used for production of paper in the paper mill SCP Ružomberok.

The SCP paper mill in Ružomberok purchased a product called Delotherm DH which contains PCB. We are not aware of the quantity of this product but the usage of PCB is well know in the production of copy paper, which did not contain the carbon. (Murín *et al.* 2003).

PCBs are materials that inflict long lasting destruction, their concentration rises when the temperatures are low, and they can be carried over long distances (Kimáková 2009). They may also play a role in the increasing of the steam *Chloroflexi*. Bacteria like those from the group *Dehalococcoides* are able to consume PCB, so it's presence may increase their prevalence as the chlorine in PCB molecules acts as a metabolic fuel. Scientists from Rensselaer Polytechnic Institute lead by Donn Bedard proved that these bacteria are abundant in locations where there is desensitization caused by chloric disincorporation as well as polychlorinated biphenyls (DeMarco 2007). For this reason we began to investigate the influence of paper mill on the presence of *Dehaloccoides*, and the phylum of *Chloroflexi*, in our samples.

The correlation between localities and samples, which can be seen in Fig. 3, represents a diverse percentage of phyla in the remaining samples; particularly in fragments of samples number 18, 42, and 44 (represent locality Hrboltová), and fragments of samples number 17, 23, and 25 (represent locality Lisková). In the case of sample 44, differences could be a result of the prevalence of phylum *Chloroflexi* (12.4 %), since the remaining samples show percentages of phylum *Chloroflexi* between 8-10 %. Differences shown by sample 25 include most notably a decrease in the most abudant phylum; *Proteobacteria* (14.8 %), while the percentage of phylum *Proteobacteria* in redundant samples is around 20 %.

More attention should be paid toward the chemical cycle of halogenated compounds in which anaerobic dehalogenating microorganisms may play important ecological roles. Thsee organism could be the key to developing methods that help detoxify commercial PCB compounds.

Acknowledgments

This research was supported by the project ITMS (Grant No. 26110230078).

References

Barkay, T. and Poulain, A.J. 2007: Mercury (micro) biogeochemistry in polar environments. *Microbiol. Ecol.*, **59**: 232-241

Bowman, J.P., McCammon, S.A, Brown, M.V, Nichols, D. S. and McMeekin, T.A. 1997: Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl. Environ. Microbiol.*, **63**: 3068-3078.

DeMarco, G. 2007: Bacterium could treat PCBs without the need for dredging. Online: https://news.rpi.edu/luwakkey/1969 (retrieved: 10.5.2016).

Chuvochina, M.S., Marie, D., Chevaillier, S., Petit, J.R., Normand, P., Alekhina, I.A. and Bulat, S.A. 2011: community variability of bacteria in alpine snow (Mont Blanc) containing Saharan dust deposition and their snow colonisation potential. *Microbes Eviron.*, **26**: 237-247.

Edwards, U., Rogall, T., Blöcker, H., Emde, M. and Böttger, E.C. 1989: Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic acids res.*, **17**: 7843-7853.

Ferrari, C.P., Dommergue, A., Boutron, C.F., Skov, H., Goodsite, M. and Jensen, B. 2004: Nighttime production of elemental gaseous mercury in interstitial air of snow at Station Nord, Greenland. *Atmos. Environ.*, **38**: 2727-2735.

Franzmann, P.D., Liu, Y., Balkwill, D.L., Aldrich, H.C., De Macario, E.C. and Boone, D.R. 1997: Methanogenium frigidum sp. nov., a psychrophilic, H2-using methanogen from Ace Lake, Antarctica. *Int. J. Syst. Evol. Microbiol*, **47**: 1068-1072.

Gounot, A.M. 1986: Psychrophilic and psychrotrophic microorganisms. *Experientia*, **42**: 1192-1197.

Hiraishi, A. 2008: Biodiversity of dehalorespiring bacteria with special emphasis on polychlorinated biphenyl/dioxin dechlorinators. *Microbes Environ.*, **23**: 1-12.

Hodson, A., Anesio, A.M., Tranter, M., Fountain, A., Osborn, M., Priscu, J., Laybournparry, J and Sattler, B. 2008: Glacial ecosystems. *Ecological monographs*, **78**: 41-67.

Kimáková, T. 2009: Zaťaženie životného prostredia polychlórovanými bifenylmi na východe Slovenska. In: *Škola a zdraví pro 21. století: aktuální otázky výchovy ke zdraví* (ed. Evžen Řehulka), pp. 315-320. Masarykova univerzita, Brno.

Knoblauch, C., Sahm, K., and Jørgensen, B. B. (1999). Psychrophilic sulfate-reducing bacteria isolated from permanently cold Arctic marine sediments: description of *Desulfofrigus oceanense* gen. nov., sp. nov., *Desulfofrigus fragile* sp. nov., *Desulfofaba gelida* gen. nov., sp. nov., *Desulfotalea psychrophila* gen. nov., sp. nov. and *Desulfotalea arctica* sp. nov. *Int. J. Syst. Evol. Microbiol*, **49**: 1631-1643.

Larose, C., Berger, S., Ferrari, C., Navarro, E., Dommergue, A., Schneider, D., and Vogel, T.M. 2010: Microbial sequences retrieved from environmental samples from seasonal Arctic snow and meltwater from Svalbard, Norway. *Extremophiles*, **14**: 205-212.

Liu, Y., Yao, T., Jiao, N., Kang, S., Xu, B., Zeng, Y.H. Liu, X., Huang, S.J. and Xu, B. 2009: Bacterial diversity in the snow over Tibetan Plateau Glaciers. *Extremophiles*, **13**: 411-423.

Marsh, T.L. 1999: Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.*, **2**: 323-327.

Møller, A.K., Barkay, T., Al-Soud, W.A., Sørensen, S.J., Skov, H. and Kroer, N. 2011: Diversity and characterization of mercury-resistant bacteria in snow, freshwater and sea-ice brine from the High Arctic. *FEMS Microbiol. Ecol*,, **75**: 390-401.

Murín, M., Pilváňová, A., Kissová, M. and Lenková, K. 2003: Inventarizácia PCB. In: *Inventarizácia perzistentných organických látok v Slovenskej Republike* (ed. R. Chriašteľ, K. Magulová, M.Murín and J. Gavora), pp. 10-36. MŽP SR – SHMÚ, Bratislava.

Murray, W. 1992: Pulp and Paper: The reduction of toxic efluents. Library of Parliament Research Branch, Ottawa.

Muyzer, G., Teske, A., Wirsen, C.O. and Jannasch, H.W. 1995: Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.*, **164**: 165-172.

Ondreičková, K., Babulicová, M., Mihálik, D., Gubišová, M. and Gubiš, J. 2014: Screening of bacterial populations in crop rotations with different proportion of cereals. *Agriculture (Poľnohospodárstvo)*, **60**: 31-38.

Osborn, A.M., Moore, E.R. and Timmis, K.N. 2000: An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ. Microbiol.*, **2**: 39-50.

Raclavská, H., Kuchařová, J. and Plachá, D. 2008: Halogenované organické látky (AOX). Podklady k provádění Protokolu o PRTR – Přehled metod a identifikace látek sledovaných podle Protokolu o registrech úniků a přenosů znečišťujících látek v únicích do půd.MŽP, Praha.

Shyu, C., Soule, T., Bent, S.J., Foster, J.A., and Forney, L. J. 2007: MiCA: a web-based tool for the analysis of microbial communities based on terminal-restriction fragment length polymorphisms of 16S and 18S rRNA genes. *Microbial. Ecol.*, **53**, 562-570.

Yamaguchi, N., Ichijo, T., Sakotani, A., Baba, T., and Nasu, M. 2012: Global dispersion of bacterial cells on Asian dust. *Sci. Rep.*, **2**: 525-530.

Yoshida, N., Takahashi, N., and Hiraishi, A. 2005: Phylogenetic characterization of a polychlorinated-dioxin-dechlorinating microbial community by use of microcosm studies. *Appl. Environ. Microbiol.*, **71**: 4325-4334.

www.microbeworld.org 2015. Online: http://www.microbeworld.org/careers/tools-of-the-trade/genetic-tools-and-techniques/16s-rrna (retrieved: 8.5.2015).

Zhang, S., Hou, S., Wu, Y., and Qin, D. 2008: Bacteria in Himalayan glacial ice and its relationship to dust. *Biogeosciences*, **5**: 1741-1750.

Received 15 April 2020; accepted 9 June 2020.

Oecologia Montana 2020, **29,** 17-22

Haemosporidian infection in passerine birds from high elevation in the Tian Shan, Kyrgyzstan

M. HAAS and J. KISKOVÁ

Institute of High Mountain Biology, Žilina University, Tatranská Javorina 7, SK-059 56, Slovak Republic; e-mail: martina.haas@uniza.sk

Abstract. The study of blood parasites in alpine environments allows us to look into host-parasite relationships under very specific conditions. The objective of our study was to detect the presence of blood parasites in Alpine accentors (*Prunella collaris*) living in the Central Tian Shan region, Kyrgyzstan. However, an examination of the specimens of the genus *Prunella* confirmed the absence of blood parasites in all tested samples (species: *P. atrogularis, P. collaris rubida, P. fulvescens*). In addition to the target species, other songbirds were also examined to determine the presence of haemosporidian parasites in the region. A total of 52 birds of 21 species of 6 families were examined. In the samples, the presence of parasites of the genus *Haemoproteus* (8 positives individuals) and *Leucocytozoon* (4 positives individuals) was detected in the host species: *Emberiza buchanani, Luscinia megarhynchos, Motacilla cinerea, Phoenicurus caeruleocephala, Serinus pusillus*.

Key words: blood parasites, *Haemoproteus, Leucocytozoon*, *Prunella* sp., high altitude

Introduction

Mountain regions are characterized by specific and demanding conditions and they vary geographically based on altitude, water availability, and seasonality (Grabherr *et al.* 2010; Körner *et al.* 2011), all of which determine the biotic composition of alpine communities (Winkler *et al.* 2019). The patterns of climate and landscape are major determinants of the distribution of biodiversity (Pearson and Dawson 2003; Foley *et al.* 2005), the principles of which are also true for parasites (Pérez-Rodríguez *et al.* 2013). Understanding the patterns and factors that determine and shape parasite community composition leads to our knowledge of host-parasite relationship and dynamics. The environmental conditions generally influenced by climate change, like temperature, humidity, availability of vectors, constitute a good model to examine variation in host-parasite interactions. Of the abiotic variables, temperature is one of the most important environmental factors that affects the structure, distribution and diversity of species along elevational gradients (Oommen and Shanker 2005). In this sense, generally, parasite abundance declines with elevation (Badyaev 1997; Álvarez-Ruiz *et al.* 2018).

Parasites from order Haemosporida, whose species (mainly *Haemoproteus*, *Plasmodium*, and *Leucocytozoon*) infect birds, have a broad range of vertebrate host and vectors (blood-sucking insects - Diptera) worldwide (Valkiunas 2005). Increased parasite infection can have negative effects on the host such as a diminished immune response (Merino *et al.* 2000; Tomás *et al.* 2007), reduced reproductive success and growth of birds (Hamilton and Zuk 1982; Marzal *et al.* 2005; Valkiunas *et al.* 2006), as well as a reduction in fitness (Schmid-Hempel 2011; Asghar *et al.* 2015). The epidemiology of avian malaria is regulated by many dynamic spatio-temporal factors, but particularly by ecological (season, habitat quality, elevation), demographical (host and vector density, age host), and environmental factors (temperature, precipitation) (see e.g. Ishtiaq and Barve 2018; Wood *et al.* 2007; Cosgrove *et al.* 2008; Paaijmans *et al.* 2009; Lachish *et al.* 2011; van Rooyen *et al.* 2013; Liao *et al.* 2017). Many factors affect the prevalence of malaria parasites, but the keys to this prevalence seem to include the birds' habitat, precipitation and ambient temperature (which decreases at higher elevations). The prevalence of parasitemia fall as elevation increases, because vectors decline in numbers and become more seasonal at higher altitudes (Atkinson 2005). Monitoring haemosporidian parasites on the altitudinal gradient indicates the correlation of parasites species with altitude. At lower altitude a higher occurrence of *Plasmodium* and *Haemoproteus* can be observed (van Riper *et al.* 1986; Harrigan *et al.* 2014; Zamora-Vilchis *et al.* 2012), while several studies have confirmed the common occurrence of *Leucocytozoon* parasites at higher altitudes (Haas *et al.* 2012; Imura *et al.* 2012; van Rooyen *et al.* 2013; Lotta *et al.* 2015).

To understand host-vector-parasite interactions, and the infection dynamics of haematozoa, it is important to investigate the prevalence of haematozoa among bird communities in specific environmental conditions. Despite a significant number of studies in the last two decades (Marzal 2012), there are few studies about haemoparasites of avifauna located over 2000 m a.s.l. worldwide, (Gonzalez *et al.* 2015) as well as a lack of publications from the Central Asia region.

The objective of the study was to estimate the prevalence of haemosporidian infection in passerine birds at high elevations. Our previous results from the West Carpathians (High Tatras Mountains), in the species *Prunella collaris* confirm zero prevalence (Haas and Kisková 2010). We sought to discover if the prevalence of blood parasites in the genus *Prunella* is similar in another alpine region. We also wanted to collect data on infection with Haemoproteids in other passerine birds, living in the same biotope. The birds were trapped during a research expedition in Kyrgyzstan in August-September 2008.

The Tian Shan is the largest mountain system located in Central Asia and is also the largest isolated east-west stretching mountain range. This region is a distinctly continental climate with cold, snowy winters contrasting with hot, dry summers. The climatic conditions are further modified by the mountainous terrain which creates microclimates and pronounced vertical zonality in the climate and ecology (IUCN 2016). Several species of genus *Prunella* are naturally found in this area, especially: *P. collaris rubida*, *P. fluvescens, P. atrogularis* (Hatchwell 2005).

Material and Methods

A total of 52 birds of 21 species and 6 families were captured and examined for the presence of haematozoan parasites using the PCR method of blood analysis. Microscopic examination of blood smears was only perfomed in genus *Prunella*.

Study area

Birds were captured at five sites using ornithological mist nets or ornithological clap traps.

Site 1: Karaburra pass N: 42° 12'11.62" E: 71° 36'41.13"

This site is located in West Tian-Shan in the Chatkal range at an altitude of 3000 m a.s.l. The terrain was rocky, with grazed alpine meadows and extensive screes sparsely covered with juniper. There was a small stream in the vicinity. Birds were captured in mist nets between August 26-28th 2008.

Site 2: Too-Ashu pass N: 42° 20'39.65" E: 73° 49'46.42"

This site is located in the Northern Tian-Shan range close to a major North-South traffic route. The area is covered with alpine meadows and heavily grazed. It is located in the vicinity of a small stream. Altitude is 3050 m a.s.l. Birds were captured with mist nets between August 23-24th 2008.

Site 3: Too-Ashu valley N: 42° 20'34.36" E: 73° 50'12.66"

This site is located approximately 300 m above Site 2 (3350 m a.s.l.) in rocky terrain with sparse vegetation in the vicinity of a small stream. Birds were captured between August 21-22nd 2008.

Site 4: Ak-Sai valley N: 42° 32' 4.76" E: 74° 31'45.03"

This site is located in the Northern Tian-Shan range approximately 30 km north of the Kyrgyz capital Bishkek in Ala Archa National Park. Birds were captured with mistnets and fall-traps on two occasions between September 10-12 2008 in the vicinity of a climbing camping site at an altitude of 3370 m a.s.l. The site is located in the alpine zone with rocky terrain and sparse grass vegetation.

Site 5: Altyn-Arashan valley N: 42° 23'20.95" E: 78° 35'37.15"

This site is located at an altitude of 2500 m a.s.l. in the Central Tian-Shan range of the Altyn-Arashan valley in the Karakol province. The location was a grassland used for cattle grazing, covered with scattered shrubs and low trees. Birds were captured with mist nets on September 6, 2008.

Field procedure and laboratory analysis

After trapping was complete, standard morphometric measurements were taken for each individual. Adult birds were sexed. The blood was taken by puncture from the vena brachialis. Bleeding was stopped using pressure with a paper swab and thus a blood sample for DNA analysis was obtained. The blood smears were only made from birds of genus *Prunella*. These were further processed by staining according to Pappenheim (Doubek *et al.* 2003). The smears were examined microscopically under 1000× magnification for the presence of blood parasites.

Genomic DNA was extracted from dry blood spots on a paper swab (after blood collection) using the QIAamp DNA Mini Kit (Qiagen, Germany). PCR amplification was carried out according to previous studies (Bensch *et al.* 2000; Hellgren *et al.* 2004; Waldenström *et al.* 2004). Based on the sequence homology between aligned sequences of the blood parasites, initial primers were HaemNFI (5'-CATATATTAAGAGAAITATGGAG-3') and HaemNR3 (5'-ATAGAAAGATAAGAAATACCATTC-3') used to amplify parasite mitochondrial DNA (gene of the cytochrome b, 617 bp large fragment) from both genera of *Haemoproteus*, and *Leucocytozoon*. For the second PCR the following primers were used: for *Haemoproteus* spp. HaemF (5'-ATGGTGCTTTCGATATATGCATG-3') and HaemR2 (5'-GCATTATCTGGATGTGATAATGGT-3') to amplify a 480 bp large fragment, for Leucocytozoon spp. HaemFL (5'-ATGGTGTTTTAGATACTTACATT-3') and HaemR2L (5'-CATTATCTGGATGAGATAATG-3') to amplify a 478 bp large fragment. The first PCR (using HaemNFI–HaemNR3 primers) was performed in using 20 µl reaction volumes, which included 50 ng of total genomic DNA, 1x reaction buffer (15mM Tris-HCl, (pH 8.2 at 25° C) 30mM KCl, 5mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.02 % BSA), 200 µM of each dNTP, 0.5 µM of each primer and 0.5 U DynaZyme DNA polymerase (Finnzymes OY). The PCR amplification protocols were as follows: initial denaturation 94° C for 10 min, then 20 cycles of 94° C for 30 s, 50° C for 30 s and 72° C for 1 min, finally 72° C 10 min.

The product of the first PCR was taken (2 µl) as the template for the second PCR, 2 µl for *Leucocytozoon* spp. (HaemFL-HaemR3L) and 2 µl for *Haemoproteus* spp. (HaemF-HaemR2). These PCR's were performed separately in 20 µl volumes with the same proportions of reagents as in the initial PCR reactions. The thermal profile of the PCR was identical to the initial PCR but performed for 35 cycles. The PCR products were shown on a 2 % agarose gel stained with ethidium bromide.

Results

In the investigated birds the predominant parasite was *Haemoproteus* with 8 infected birds (15.7 %). The *Haemoproteus* was found in three birds species: *Emberiza buchanani, Luscinia megarhynchos* and *Serinus pusillus*. *Leucocytozoon* was detected in four birds (7.8 %) in the following species: *Motacilla cinerea*, *Phoenicurus caeruleocephala* and *Serinus pusillus*. *Serinus pusillus* was infected by both parasites. The investigetion of blood samples (including blood smears) confirmed our previous assumption of the absence of haemosporidian parasites in Prunellidae living in the alpine zone (Table 1).

Discussion

Parasitism is a strong selective force in nature and significantly affects host fitness, thereby affecting its ability to survive and reproduce. The host-parasite relationship thus depends on ecology, behaviour and life history of both host and parasite (Vicente *et al.* 2007; Woodhams *et al.* 2008). More than 200 malarian parasites of the genera *Plasmodium*, *Haemoproteus* and *Leucocytozoon* have been morphologically described among the 4000 bird species investigated worldwide (Valkiunas 2005). The prevalence of blood parasites is highly dependent on diagnostic method, season, and region (John 1997). Presently, the polymerase chain reaction (PCR) is widely used to identify haematozoa parasites in bird blood (e.g. Križanauskiené *et al.* 2010; Bell *et al.* 2015; Ishtiaq *et al.* 2017; Chaisi *et al.* 2019). PCR methods are more sensitive than microscopy, but in the case of chronic infections because numbers of parasites are low, microscopy is still considered the „gold standard" for malaria diagnosis (Atkinson 2005). We chose the PCR method for easier handling and transport of samples. Our resultes based on PCR, confirm the appearance of parasites from genera *Haemoproteus* and *Leucocytozoon* in the study sites, all at an elevation of more than 2500 m a.s.l. We assume this is a pilot study on blood parasites in this region.

The family Fringillidae was classified as highly susceptible to infection by blood parasites (Atkinson and Van Riper 1991). Yakunin and Zhazyltaev (1977) report positive findings in the species

Birds species	**n**	**Sex** **Male/Female/ Juvenile**	***Haemoproteus*** **Male/Female/ Juvenile**	***Leucocytozoon*** **Male/Female/ Juvenile**
Anthus trivialis (*richardi*)	1	0/1/0		
Calliope pectoralis	1	0/0/1		
Cardualis caniceps	1	0/1/0		
Carpodacus sp.	1	0/1/0		
Cinclus cinclus	1	0/1/0		
Emberiza buchanani	2	2/0/0	1/0/0	
Luscinia megarhynchos	1	1/0/0	1/0/0	
Luscinia sp.	1	1/0/0		
Luscinia svecica	1	0/1/0		
Motacilla cinerea	1	1/0/0		1/0/0
Motacilla personata	1	0/0/1		
Phoenicurus caeruleocephala	4	1/3/0		1/0/0
Phoenicurus erythrogaster	1	1/0/0		
Phoenicurus ochruros	1	1/0/0		
Phylloscopus inornatus	1	NI		
Phylloscopus trochiloides	1	1/0/0		
Prunella atrogularis	1	1/0/0		
Prunella collaris rubida	10	6/3/1		
Prunella fulvescens	5	2/3/1		
Rhodospiza obsoleta	1	0/1/0		
Saxicola torquata	2	1/0/1		
Serinus pusillus	12	6/4/2	3/2/1	1/0/1
Total	**51**	**25/19/7**	**5/2/1**	**3/0/1**

Table 1. Occurrence of haematozoa in investigated passeriform birds from Tian-Shan (NI - unidentified sex).

Emberiza buchanani and *Motacilla cinerea* with same genera of parasites as the wild birds of Kazakhstan. However they did not record a positive finding in *Phoenicurus caeruleocephala* (Yakunin and Zhazyltaev 1977). The absense of Haemosporida in *Serinus pusillus* is documented by Nourani *et al.* (2018) from Iran.

There is a higher prevalence of infection by genus *Haemoproteus*, which is in contrast to studies that show a higher prevalence of genus *Leucocytozoon* at higher altitudes (Haas *et al.* 2012; Imura *et al.* 2012; van Rooyen *et al.* 2013; Lotta *et al.* 2015). This conclusion may be due to the fact that *Haemoproteus* had a high prevalence in *Serinus pusillus*, who was also infected with *Leucocytozoon*. A possible explanation for the presence of blood parasites is the ecology of the host species, which is an altitudinal migrant. Elevational migrants may hmave more parasites as they are exposed to high prevalence areas at low elevations with optimal climatic conditions for parasite transmission (Waldenström *et al.* 2002). They have also adapted physiologically to fluctuations in environmental hypoxia during elevation migraqtion and through changes in physiological parameters associated with blood oxygen-carrying capacity such as haemoglobin concentration and haematocrit (Ishtiaq and Barve 2018). Bird distribution in the high mountains is very variable. Some species strictly inhabit narrow altitudes during a specific period of the year e.g. during breeding, while others live at cold high elevations year-round (Price *et al.* 2011; Dixit *et al.* 2016). Elevational migrants, in the wintering grounds, encounter a diverse fauna of parasites as compared to sedentary species and may act as reservoirs for blood parasites.

The main aim of testing birds for the presence of blood parasites in this region was to detect haemosporidian infection in *Prunella collaris*. Our previous findings confirmed the absence of blood parasites in the species *P. collaris* from the High Tatras, Slovakia (Haas and Kisková 2010) as well as in individuals *P. collaris* from Rila Mountain, Bulgaria (2010; unpublished data). In Tian Shan, (Kyrgyzstan) we did not detect infection through the PCR method or microscopic examination in ten caught individuals. The infection was also not detected in other species of *Prunella* - *P. atrogularis* and *P. fulvescens*. The only species in this family where haemosporidian has been documented to date is *P. modularis*, which occurs at lower altitudes (e.g. Merino *et al.* 1997; Palinauskas *et al.* 2005; Hauptmanová *et al.* 2006; Haas *et al.* 2012). For the resident strongly high elevation adapted bird species, the key factor in the development of infection is the ambient temperature on which the haematophagous arthropod vectors depend (Dunn *et al.* 2013; Ishtiaq and Barve 2018). Higher elevation, low temperatures, fewer water reservoirs and windy conditions can help to reduce both the development of avian haematozoa and the abundance of parasite vectors, and hence parasite prevalence (Zamora-Vilchis *et al.* 2012). An alternative explanation for the absence of blood parasites could be either a low host density or insufficient time for the co-evolution of the host, vectors and parasites (Bennett *et al.* 1992; Rytkönen *et al.* 1996; Valera *et al.* 2003).

Variation in ambient temperature can increase or decrease host condition and parasite virulence can lead to a critical influence on the host-parasite interactions, even over relatively small temperature ranges (Thomas and Blanford 2003). In the context of climatic changes, it is presumably that with increasing temperatures of environments, parasites adapt to the biotic and abiotic conditions of the highlands (Gonzalez *et al.* 2015) which can effect vector distribution, leading to the emergence of infectious disease in the new hosts (Imura *et al.* 2012). Knowledge of how different environmental factors affect host-parasites as well as how different environmental factors affect host-parasite interactions will allow us to predict future parasite impacts and their potential effects on biodiversity.

Acknowledgments

We would like to thank to prof. Marián Janiga for bird trapping and their determination, to Dr. Martin Lukáň for help during preparation this article and to Peter Macuľa, Ivan Hanula, Martina Júnová, Elena Markuseková, Veronika Justová, and Filip Fiath, for help in the feldwork. This research was supported by the project ITMS (Grant No. 26210120016).

References

Álvarez-Ruiz, L., Megía-Palma, R., Reguera, S., Ruiz, S., Zamora-Camacho, F. J., Figuerola, J. and Moreno-Rueda, G. 2018: Opposed elevational variation in prevalence and intensity of endoparasites and their vectors in a lizard. *Curr. Zool.*, **64**: 197-204.

Asghar, M., Hasselquist, D., Hansson, D., Zehtindjiev, P., Westerdahl, H. and Bensch, S. 2015: Hidden costs of infection: chronic malaria accelerates telomere degradation and senescence in wild birds. *Science*, **347**: 436-448.

Atkinson, C.T. 2005: Ecology and diagnosis of introduced avian malaria in Hawaiian forest birds. USGS FS 2005-3151. Geological Survey (U.S.) Online: https://pubs.usgs.gov/fs/2005/3151/report.pdf (retrieved: 20.3.2020).

Atkinson, C.T. and van Riper III., C. 1991: 2. Pathogenicity and epizootiology of avian haematozoa: *Plasmodium, Leucocytozoon*, and *Haemoproteus*. *Bird-parasite interactions: ecology, evolution, and behaviour*, **2**: 19.

Badyaev, A.V. 1997: Altitudinal variation in sexual dimorphism: a new pattern and alternative hypotheses. *Behav. Ecol.*, **8**: 675-690.

Bell, J.A., Weckstein, J.D., Fecchio, A. and Tkach, V.V. 2015: A new real-time PCR protocol for detection of avian haemosporidians. *Parasites & vectors*, **8**: 383.

Bennett, G.F., Earlé, R.A. and Peirce, M.A. 1992: The Leucocytozoidae of South African birds: Passeriformes. *Onderstepoort J. Vet. Res*, **59**: 235.

Chaisi, M.E., Osinubi, S.T., Dalton, D.L. and Suleman, E. 2019: Occurrence and diversity of avian haemosporidia in Afrotropical landbirds. *Int. J. Parasitol. Parasites. Wildl.*, **8**: 36-44.

Cosgrove, C.L., Wood, M.J. and Sheldon, B.C. 2008: Seasonal variation in *Plasmodium* prevalence in a population of blue tits *Cyanistes caeruleus*. *J. Anim. Ecol.*, **77**: 540-548.

Hatchwell, B.J. 2005: Family Prunellidae (Accentors) In: *Handbook of the birds of the world. Vol. 10, Cuckoos-shrikes to Trushes* (eds. J.D. del Hoyo, A. Elliott, J. Sargatal, and N. Arlott), pp.496-513. Lynx Editions, Barcelona.

Dixit, S., Joshi, V. and Barve, S. 2016: Bird diversity of the Amrutganga Valley, Kedarnath, Uttarakhand, India with an emphasis on the elevational distribution of species. *Check List*, **12**: 1874.

Doubek, J., Bouda, J., Doubek, M., Fürll, M., Knotková, Z., Pejřilová, S., Scheer, P., Svobodová, Z. and Vodička, R. 2003: Veterinární hematologie. Noviko, Brno.

Dunn, J. C., Goodman, S. J., Benton, T. G. and Hamer, K. C. 2013: Avian blood parasite infection during the non-breeding season: an overlooked issue in declining populations? *BMC ecology*, **13**: 30.

Foley, J.A., DeFries, R., Asner, G.P., Barford, C., Bonan, G., Carpenter, S.R., Chapin, F.S., Coe, M.T., Daily, G.C., Gibbs, H.K., Helkowski, J.H., Holloway, T., Howard, E.A., Kucharik, C.J., Monfreda, C., Patz, J.A., Prentice, I.C., Ramankutty, N. and Snyder, P.K. 2005: Global consequences of land use. *Science*, **309**: 570-574.

González, A.D., Lotta, I.A., García, L.F., Moncada, L.I. and Matta, N.E. 2015: Avian haemosporidians from Neotropical highlands: evidence from morphological and molecular data. *Parasitol. Int.*, **64**: 48-59.

Grabherr, G., Gottfried, M. and Pauli, H. 2010: Climate change impacts in alpine environments. *Geogr. Compass*, **4**: 1133-1153.

Haas, M. and Kisková, J. 2010: Absence of blood parasites in the Alpine Accentor *Prunella collaris*. *Oecologia Montana*, **19**: 30-34.

Haas, M., Lukáň, M., Kisková, J. and Hrehová, Z. 2012: Occurrence of blood parasites and intensity of infection in *Prunella modularis* in the montane and subalpine zone in the Slovak Carpathians. *Acta Parasitologica*, **57**: 221-227.

Hamilton, W.D. and Zuk, M. 1982: Heritable True and Bright Birds: A role for Parasites? *Science*, **218**: 384- 386.

Harrigan, R.J., Sedano, R., Chasar, A.C., Chaaves, J.A., Nguyen, J.T., Whitaker, A. and Smith, T.B. 2014: New host and lineage diversity of avian haemosporidia in the northern Andes. *Evol. Appl.*, **7**: 799e811

Hauptmanová, K., Benedikt, V. and Literák, I. 2006: Blood parasites in passerine birds in Slovakian East Carpathians. *Acta Protozool.*, **45**: 105-109.

Hellgren, O., Waldenström, J. and Bensch, S. 2004: A new PCR assay for simultaneous studies of *Leucocytozoon, Plasmodium*, and *Haemoproteus* from avian blood, J. *Parasitol.*, **90**: 797-802.

Imura, T., Suzuki, Y., Ejiri, H., Sato, Y., Ishida, K., Sumiyama, D., Murata, K. and Yukawa, M. 2012: Prevalence of avian haematozoa in wild birds in a high-altitude forest in Japan. *Vet. Parasitol.*, **183**: 244-248.

Ishtiaq, F. and Barve, S. 2018: Do avian blood parasites influence hypoxia physiology in a high elevation environment? *BMC ecology*, **18**: 15.

Ishtiaq, F., Rao, M., Huang, X., and Bensch, S. 2017: Estimating prevalence of avian haemosporidians in natural populations: a comparative study on screening protocols. *Parasites & vectors*, **10**:127.

IUCN 2016: Western Tien-Shan. Kazakhstan, Kyrgzyzstan Uzbekistan. ID 1490. IUCN Evaluation Report, May 2016.

John, J.L. 1997: The Hamilton-Zuk theory and initial test: an examination of some parasitological criticism. *Int. J. Pathol.*, **27**: 1269-1288.

Körner, C., Paulsen, J. and Spehn, E.M. 2011: A definition of mountains and their bioclimatic belts for global comparisons of biodiversity data. *Alp. Bot.*, **121**: 73.

Križanauskiené, A., Perez-Tris, J., Palinauskas, V., Hellgren, O., Bensch, S. and Valkiunas, G. 2010: Molecular phylogenetic and morphological analysis of haemosporidian parasites (Haemosporida) in a naturally infected European songbird, the blackcap *Sylvia atricapilla*, with description of *Haemoproteus pallidulus* sp. nov. *Parasitology*, **137**: 217-227.

Lachish, S., Knowles, S.C., Alves, R., Wood, M.J. and Sheldon, B.C. 2011: Infection dynamics of endemic malaria in a wild bird population: parasite species-dependent drivers of spatial and temporal variation in transmission rates. *J. Anim. Ecol.*, **80**: 1207-1216.

Liao, W., Atkinson, C.T., LaPointe, D.A. and Samuel, M.D. 2017: Mitigating future avian malaria threats to Hawaiian forest birds from climate change. *PLoS ONE*, **12**: e0168880.

Lotta, I.A., Gonzalez, A.D., Pacheco, M.A., Escalante, A.A., Valkiunas, G., Moncada, L.I. and Matta, N.E. 2015: *Leucocytozoon pterotenuis* sp. nov. (Haemosporida, Leucocytozoidae): description of the morphologically unique species from the Grallariidae birds, with remarks on the distribution of *Leucocytozoon* parasites in the neotropics. *Parasitol. Res.*, **114**: 1031e1044.

Marzal, A. 2012: Recent advances in studies on avian malaria parasites. Malaria Parasites. In: Malria parasites (ed. Omolade O. Okwa), pp. 135-157. IntechOpen. Online: https://www.intechopen.com/books/malaria-parasites/recent-advances-in-studies-on-avian-malaria-parasites (retrieved: 18.3.2020).

Marzal, A., de Lope, F., Navarro, C. and Müller, A.P. 2005: Malarial parasites decrease reproductive success: an experimental study in a passerine bird. *Oecologia*, **142**: 541-545.

Merino, S., Moreno, J., Sanz, J.J. and Arriero, E. 2000: Are avian blood parasites pathogenic in the wild? A medication experiment in blue tits (*Parus caeruleus*). *Proc. R. Sci. Lond. B*, **267**: 2507-2510.

Merino, S., Potti, J. and Fargallo, J.A. 1997: Blood parasites of passerine birds from Central Spain. *J. Wildl. Dis.*, **33**: 638-641.

Nourani, L., Aliabadian, M., Mirshamsi, O. and Djadid, N.D. 2018: Molecular detection and genetic diversity of avian haemosporidian parasites in Iran. *PloS one*, **13**: e0206638.

Oommen, M.A. and Shanker, K. 2005: Elevational species richness patterns emerge from multiple local mechanisms in Himalayan woody plants. *Ecology*, **86**: 3039-3047.

Paaijmans, K.P., Read, A.F. and Thomas, M.B. 2009: Understanding the link between malaria risk and climate. *P. Natl. Acad.Sc. USA*, **106**: 13844-13849.

Palinauskas, V., Markovets, M.Y., Kosarev, V.V., Efremov, V.D., Sokolov, L.V. and Valkiunas G. 2005: Occurrence of avian haematozoa in Ekaterinburg and Irkutsk districts of Russia. *Ekologija*, **4**: 8-12.

Pearson, R.G. and Dawson, T.P. 2003: Predicting the impacts of climate change on the distribution of species: are bioclimate envelope models useful? *Glob. Ecol. Biogeogr.*, *12*: 361-371.

Pérez-Rodríguez, A., Fernández-González, S., de la Hera, I. and Pérez-Tris, J. 2013: Finding the appropriate variables to model the distribution of vector-borne parasites with different environmental preferences: climate is not enough. *Glob. chang. biol.*, **19**: 3245-3253.

Price, T.D., D. Mohan, D.T. Tietze, D.M. Hooper, C.D.L. Orme and P.C. Rasmussen. 2011: Determinants of northerly range limits along the Himalayan bird diversity gradient. *Am. Nat.*, **178**: 97-108.

Rytkönen, S., Ilomäki, K., Orell, M. and Welling, P. 1996: Absence of blood parasites in Willow Tits Parus montanus in northern Finland. *J. Avian Biol.*, **27**: 173-174.

Schmid-Hempel, P. 2011: Evolutionary Parasitology: The Integrated Study of Infections, Immunology, Ecology, and Genetics. Oxford University Press, New York.

Thomas, M.B. and Blanford, S. 2003: Thermal biology in insect-parasite interactions. *Tree*, **18**: 344-350.

Tomás, G., Merino, S., Moreno, J., Morales, J., Martínez-de la Puente, J. 2007: Impact of blood parasites on immunoglobulin level and parental effort: a medication field experiment on a wild passerine. *Funct. Ecol.*, **21**: 125-133.

Valera, F., Carrillo, C.M., Barbosa, A. and Moreno, E. 2003: Low prevalence of haematozoa in Trumpeter finches *Bucanetes githagineus* from south-eastern Spain: additional support for a restricted distribution of blood parasites in arid lands. *J. Arid. Envir.*, **55**: 209-213.

Valkiunas, G. 2005: Avian Malaria Parasites and Other Haemosporidia. CRC Press, Boca Raton, Florida.

Valkiunas, G., Zickus, T., Shapoval, A,P. and Iezhova, T.A. 2006: Effect of *Haemoproteus belopolskyi* (Haemosporida: Haemoproteidae) on body mass of the blackcap Sylvia atricapilla. *J. Parasitol.*, **92**: 1123-1125.

van Riper III., C., van Riper, S.G., Goff, M.L. and Laird, M., 1986: The epizootiology and ecological significance of malaria in Hawaiian land birds. *Ecol. Monogr.*, **56**: 327e344.

van Rooyen, J., Lalubin, F., Glaizot, O. and Christe, P. 2013: Altitudinal variation in haemosporidian parasite distribution in great tit populations. *Parasites & Vectors*, **6**: 139.

Vicente, J., Höfle, U., Fernández-De-Mera, I.G. and Gortazar, C. 2007: The importance of parasite life history and host density in predicting the impact of infections in red deer. *Oecologia*, **152**: 655-664.

Waldenström, J., Bensch, S., Hasselquist, D. and Östman, Ö. 2004: A new nested polymerase chain reaction method very efficient in detecting Plasmodium and *Haemoproteus* infections from avian blood. *J. Parasitol.*, **90**: 191-194.

Waldenström, J., Bensch, S., Kiboi, S,, Hasselquist, D. and Ottosson, U. 2002: Crossspecies infection of blood parasites between resident and migratory songbirds in Africa. *Mol. Ecol.,* **11**: 1545-1554.

Winkler, D.E., Kaitlin, C., Lubetkin, K.C., Carrell, A.A., Jabis, M.D., Yang, Y. and Kueppers, L.M. 2019: Chapter 12: Responses of alpine plant communities to climate warming. In: *Ecosystem consequences of soil warming. Microbes, vegetation, fauna, and soil biogeochemistry* (ed: J.E. Mohan), pp. 297-346. Academic Press, Elsevier, London.

Wood, M.J., Cosgrove, C.L., Wilkin, T.A., Knowles, S.C., Day, K.P. and Sheldon, B.C. 2007: Within-population variation in prevalence and lineage distribution of avian malaria in blue tits, *Cyanistes caeruleus*. *Mol. Ecol.*, **16**: 3263-3273.

Woodhams, D.C., Alford, R.A., Briggs, C.J., Johnson, M. and Rollins-Smith, L.A. 2008: Life-history trade-offs influence disease in changing climates: strategies of an amphibian pathogen. *Ecology*, **89**: 1627-1639.

Yakunin, M.P. and Zhazyltaev, T.A. 1977: The blood parasite fauna of wild and domestic birds from Kazakstan. *Trudy instituta zoologie Akademie Nauk Kazakhstan SSR*, **37**: 124-148. (in Russian)

Zamora-Vilchis, I., Williams, S.E. and Johnson, C.N. 2012: Environmental temperature affects prevalence of blood parasites of birds on an elevation gradient: implications for disease in a warming climate. *PloS one*, **7**: e39208.

Received 29 April 2020; accepted 15 June 2020.

Oecologia Montana 2020,
29, 23-27

Effects of flooding on the physical and chemical water composition of the alpine lake Kolové pleso (High Tatra, West Carpathians)

K. HRIVNÁKOVÁ, M. JANIGA and A. POGÁNYOVÁ

Institute of High Mountain Biology, Žilina University, Tatranská Javorina 7, SK-059 56, Slovak Republic; e-mail: poganyova@gmail.com

Abstract. This study is a part of the physical and chemical limnology multiannual research of lake Kolové pleso to understand the response of the high mountain lake ecosystem to seasonal, climatic and anthropogenic impacts. Due to the extensive rainfall in July 2018, during the experiment, we had the opportunity to observe the effect of flooding on the lake. Flooding had a significant impact on the organic composition of the lake (COD) as well as on some chemical elements (S, K, Rb, Mo, Cd). The values of COD and measured elements sharply decreased after the flood disturbance. On the contrary, the pH level increased and the water in the lake became less acidic. We observed a negative correlation between pH and COD. We can concluded, that the flood disturbance had a major effect on the organic matter content in the water. The flood washed out deposits sediments. This plays a role in the saturation of water of organic and inorganic compounds and thus affects the overall chemical and physical state of the water in the lake and also will influence the ecosystem for the following months.

Key words: High Tatras, alpine lake, extensive rainfall, flood impact, organic composition, acidity (pH)

Introduction

Standing water is characterized by the absence of flow predetermining some of its physicochemical properties, which are significantly different from flowing water. Standing water includes lakes, and this study focuses on a glacial lake in the alpine level of High Tatra Mountains. Glacial lakes represent 90% of all lakes in Slovakia (Bitušík *et al.* 2006) and are mostly oligotrophic standing waters with specific characteristics (Psenner 1989; Drever and Zobrist 1992; Beracko *et al.* 2014). These ecosystems have been identified as key sites for studying global environmental change (Pienitz *et al.* 1997a). Despite their geographical isolation, without direct anthropogenic influences, due to their specific characteristics, alpine lakes are excellent indicators of seasonal changes, as well as increasingly frequent (IPCC 2001) climate change (Wathne *et al.* 1995). Current climate models show an increase in the frequency of irregular phenomena such as extreme rainfall, but also widespread drought, which disturbs water level fluctuations in lakes (IPCC 2001). These current trends have raised the question of how water level fluctuations and the floods impact on lakes (Wantzen *et al.* 2008). Because of fluctuating water levels, floods are notable biochemical events for lake ecosystems (McClain *et al.* 2003). Most lakes are heterotrophic and dependent on organic inputs from their basins and subsoil (Sobek *et al.* 2007). Floods have the effect of rapidly mobilizing and accumulating organic carbon and nutrients (Nogueira *et al.* 2002; Wantzen *et al.* 2008), which can lead to "wash out" and also cause leaching of heavy metals into the environment from lake bottom sediments; posing a potential risk to water quality (Chrastný *et al.* 2006). Alpine lakes are likely to experience dramatic future physical, chemical and biological changes (Antoniades *et al.* 2003). Therefore, understanding the impact of past, present, and likely future anthropogenic influences and climate change depends on understanding the basic state of lakes (Hamilton *et al.* 2001; Michelutti *et al.* 2002).

The aim of this study is, describe the impact of the flood on the monitored alpine lake. The data will serve as a reference for future programs for monitoring global environmental changes (Pienitz *et al.* 1997a) and the impact of flooding on the lakes.

Material and Methods

Field experiments

The Tatra Mountains are the highest mountain range of alpine character in Slovakia. Lake Kolové pleso (GPS position data: N 49° 13′ 13″; E 20° 11′ 28″) is situated in Kolová dolina valley at an altitude of 1565 m a s.l. It has an area of 18 280 m^2, a perimeter of 735 m, a water volume of 10 846 m^3, a length of 225 m, a width of 123 m, a maximum depth of 1.2 m, and an average depth 0.59 m (Marček 1996).

Samples were taken monthly between August 2017 and December 2018. During sampling in the summer (18th of July 2018), the Tatra Mountains were hit by floods. To measure physical parameters of the water samples including water temperature, salinity, pH, U, conductivity, TDS (total dissolved solids) (mg/l), soluble oxygen / (oxygen level) and saturation of the water, we used a portable multimeter WTW 3430 (GEOTECH, Weilheim, Germany) in the field. Along with the Multi 3430 we used compatible probes: IDS pH electrode Sen TixR 940-3, conductivity electrode TetraCon 925-3 and an optic oxygen electrode FDO 925-3. For the subsequent analysis, other samples were placed into plastic containers containing 0.7 l. Water samples were taken at 0.5 meters from the left shore of the lake. Before the sampling, all containers were properly labeled and disinfected. We were careful about the proper transportation of the samples and their preservation, trying to keep intervals between sampling and analysis as short as possible.

Laboratory analyses and statistics

For the detection of the required components of the samples, we've used the following methods. Chemical oxygen demand (COD) - conventional potassium permanganate method, based on the oxidation of organic substances with 20 ml potassium permanganate (K_2MnO_4). It is defined as the amount of oxygen which, under specified conditions, is consumed for the oxidation of organic substances in water by a strong oxidizing agent (Mn - Manganese) (Diviš 2008). The x-ray method determines the values of some chemical elements (trace elements). We used a handle ED-XRF spectrometer DELTA (Innov-X Technologies, Canada) and analyzed the water sample in a plastic vial (minimum 15 mm of sample depth in a vial). We used multiple-beam measurement, in which every measurement consisted of 3 beams for 80 seconds, repeated three times, and then averaged. The results were given in ppm (parts per million) units. Standards used for basic calibration of device were in a clean homogenous SiO_2 matrix without interfering elements. We used also an additional calibration matrix for the correct measuring of surface water samples, which correspond to internationally accepted standards for measurement of elements in surface waters SPS-SW2 (Spectrapure Standards, Norway). Measurements were realized following the working manual DELTA handheld XRF Analyser, Canadian edition, 2015. Detections limits (X-ray) differ for different elements based on and fulfill the criteria described in the manual to Delta XRF.

For statistical analysis (Pearson´s correlation matrix, one way ANOVA and graphs) we used STATISTICA 8 software.

Results

In July of 2018, the High Tatra experienced an extensive flood and in the lake Kolové pleso the significant decrease in concentrations of sulfur (S), molybdenum (Mo), potassium (K) and rubidium (Rb) values were observed (Table 1).

The second impact of the flood was observed in the organic composition of the lake (COD). The level of COD after the summer of 2017 and during the winter months of 2017 and beginning of the 2018 was naturally slightly decreasing. The level of COD starts to increase in the spring months. This trend was interrupted by flood. The organic components were immediately washed out on the day of the flood, and the other element's values fell on the level of detection limits (Table 1).

After flooding acidity significantly decreased (Fig. 1). We can observe a negative correlation relationship between COD and pH level. The data confirm that flooding plays an important role in the life of healthy mountain lake ecosystems, it highly influences the concentrations of elements for a long period and improves the acidity of the water. In the present, flood effects also affect the accumulation of anthropogenic contaminants and as in the case of natural elements, they may cause their elution and further distribution in the watercourse to lowlands.

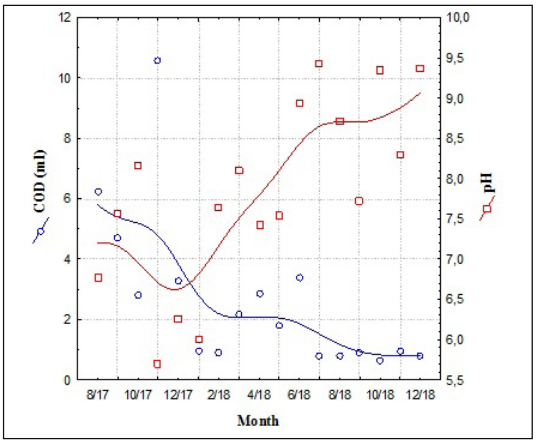


Fig. 1. Change in concentration of organic components (COD) and acidity (pH) values due to extensive summer flood in July 2018. The values were measured from August 2017 to December 2018.

Discussion

Oxygen concentration in Kolové pleso was around 10 mg/l and the average saturation was 98 %, which is characteristic for chemically pure waters (Diviš 2008) such as Tatra Mountain lakes (Bitušík 2013). Based on these parameters, particularly pH values, we can classify the Kolové pleso among oligotrophic lakes (Beracko *et al.* 2014). PH values during the analyzed annual cycle ranged from 5.7-9.4, which are also typical for this type of mountain lakes (Douglas and Smol 1994; Antoniades *et al.* 2000; Hamilton *et al.* 2001; Lim *et al.* 2001; Michelutti *et al.* 2002; Kopáček *et al.* 2006). The acidity of water in the lake seasonally fluctuated, the phenomenon may be caused by natural processes as well as industry located in the wider area of the Tatra Mountain region (Camarero *et al.* 2009; Van Drooge *et al.* 2011).

COD concentrations in the alpine environment range from 0.6 to 10 ml (Pienitz *et al.* 1997b). Low values and limited range are related to an almost complete absence of vegetation and poor drainage in the area (Antoniades *et al.* 2003). The effects of decreased acidity and increased washing of organic components from the lake water is known from several studies (Keddy and Fraser 2000; Nogueira *et al.* 2002; Coops *et al.* 2003). The behavior of the lake and its COD levels has shown that floods mobilize organic

Month	COD (ml)	S (ppm)	K (ppm)	Rb (ppm)	Mo (ppm)	Cd (ppm)
August 2017	6.25	76 ± 64	187 ± 12	1.6 ± 0.6	1.3 ± 0.5	ND (DL = 6)
September 2017	4.70	ND (DL = 62)	196 ± 12	1.6 ± 1.1	1.1 ± 1	ND (DL = 6)
October 2017	2.79	59 ± 48	210 ± 10	1.2 ± 0.8	1.1 ± 0.8	ND (DL = 4)
November 2017	10.59	96 ± 67	252 ± 15	1.5 ± 0.5	1.1 ± 1	ND (DL = 6)
December 2017	3.27	93 ± 34	193 ± 13	1.5 ± 0.6	ND (DL = 1)	ND (DL = 6)
January 2018	0.93	ND (DL = 56)	203 ± 11	1 ± 1	1.3 ± 0.9	ND (DL = 5)
February 2018	0.92	ND (DL = 57)	215 ± 12	1.2 ± 0.5	1.3 ± 0.4	ND (DL = 5)
March 2018	2.18	ND (DL = 63)	187 ± 13	ND (DL = 1.1)	ND (DL = 1)	ND (DL = 6)
April 2018	2.85	196 ± 145	134 ± 9	ND (DL = 0.4)	ND (DL = 0.1)	ND (DL = 6)
May 2018	1.80	ND (DL = 138)	152 ± 9	1.3 ± 0.2	ND (DL = 0.1)	ND (DL = 6)
June 2018	3.41	187 + 144	133 ± 8	1 ± 0.4	ND (DL = 0.1)	ND (DL = 6)
18th of July 2018	**0.82**	**ND (DL = 137)**	**137 ± 8**	**ND (DL = 0.4)**	**ND (DL = 0.1)**	**ND (DL = 6)**
August 2018	0.80	16 ± 12	1 ± 0.1	ND (DL = 0)	ND (DL = 0)	ND (DL = 0)
September 2018	0.91	ND (DL = 11)	1 ± 0.1	ND (DL = 0)	ND (DL = 0)	ND (DL = 0)
October 2018	0.66	ND (DL = 12)	1.1 ± 0.1	ND (DL = 0)	ND (DL = 0)	ND (DL = 0)
November 2018	0.98	ND (DL = 12)	1.1 ± 0.1	ND (DL = 0)	ND (DL = 0)	ND (DL = 0)
December 2018	0.77	13 ± 6	1 ± 0.1	ND (DL = 0)	ND (DL = 0)	ND (DL = 0)

Table 1. Average concentrations of chemical elements and organic components in the typical West Carpathian alpine lake. Extensive unusual flooding occurred in July 2018. COD – chemical oxygen demand values, ± standard error shown in 2-sigma - 95 % confidence, ND – not detected, DL – detection limit showed in ppm. Delta ED-XRF device in case of not detected values shows the actual detection limit for the measuring element in the sample.

nutrients in the lake (Mooij *et al.* 2005; Wantzen *et al.* 2008). The process is influenced by a release of organic sediment deposition which usually participates in water saturation by dissolving organic substances. Due to the flood, these organic substances were washed out and stored in the aquatic-terrestrial transition zone (Grossart and Simon 1998). We expect that this effect also caused the observed decrease in monitored elements values. This trend has been observed mainly in the level of potassium, but we also found the decreasing trend in sulfur, rubidium, molybdenum, and cadmium. Decrease of heavy metals concentrations in surface waters after flooding has been confirmed by Chrastný *et al.* (2006) whose study of the impact of floods on heavy metals revealed that floods may in particular cause the release of cadmium into the environment. Potassium concentrations in alpine waters are strongly governed by the chemical weathering of silicates (rock-forming minerals) such as biotite, K-feldspar, and clay minerals. It reflects qualitatively the relative abundance of silicates in the catchment and works as a nutrient for aquatic plants (Zobrist 2010). In work Wu *et al.* (2013) authors show the specific ecosystem relations between decreasing or increasing of this element and vegetation richness and aboveground biomass and also wider relations of this element with concentrations of main macronutrients - phosphorus and nitrogen in soil and water. Although our study only shows measurements of selected elements (K, S, Rb, Mo, Cd), we can expect that also other unmeasured macronutrient elements (P, N) are washed on during the flood, and limitate biomass development in following months.

One of the most important observations in our monitoring is founding the increase of pH level after the flood. The elements which caused the acidity of water were washed out. In surface waters, the pH is most influenced by sedimentation (McNeely *et al.* 1979; Michelluti *et al.* 2002) and precipitation (Judová *et al.* 2015). Lowes pH values were measured in winter months. This corresponds to the potential pollution effect from fossil fuel burning in winter months, which could influence the mountain ecosystem with acid precipitation. Sulfur compounds from precipitation also play a role in the process of transformation and subsequent deposition of inorganic sulfur in sediments (Evans and Monteith 2001; Liu *et al.* 2017). This deposition of sulfur was washed out by the flood with massive sediment removal. With this process also Acid Neutralising Capacity (ANC) increased proportionally to reductions in SO_x, as mentioned by Monteith *et al.* (2014) in the long-term study of surface waters.

Finally, we confirm a negative correlation between concentrations of organic components and levels of pH. This negative correlation was measured in many alpine lakes (Donahue *et al.* 1998; Evans and Monteith 2001; Kopáček *et al.* 2003; Kopáček *et al.* 2006). Harriman and Taylor (1999) point out in their work that, regardless of the cause, the rising values of organic composition will have a significant impact on the lake's acidity. In our case, flood and precipitation reduced the amount of organic matter and therefore affected the acidity of lake Kolové pleso.

Conclusions

Our research noted a less frequent phenomenon of natural floods and their impact on the aquatic ecosystem of alpine lake waters in high mountains.

The phenomenon of flooding is specific because it does not condition an annual but several-year cycle of water biota. In this long-term cycle, the ecosystem has to adapt after major disturbance and in the long run, many organisms are forced to overcome physiological, morphological and genetic changes to survive. Due to this, we consider this study as exceptional.

Acknowledgments

This research was funded by ITMS project Grant No. 26210120016.

References

Antoniades, D., Douglas, M.S.V. and Smol, J.P. 2000: Limnology and autecology of freshwater diatoms from Alert, northern Ellesmere Island, Nunavut. *Proceedings of the 6th National Student Conference on Northern Studies*, 1-11.

Antoniades, D., Douglas, S.V.M. and Smol, J.P. 2003: The physical and chemical limnology of 24 ponds and one lake from Isachsen, Ellef Ringnes Island. *Canadian High Arctic Internat. Rev. Hydrobiol.*, **88**: 519-538.

Beracko, P., Bulánková, E. and Stloukalová, V. 2014: Freshwater ecosystems. Comenius University publisher, Bratislava.

Bitušík, P. 2013: A recent limnological survey of the Tatra Mountain lakes. *Environment*, **47**: 134-139.

Bitušík, P., Svitok, M., Kološta, P. and Hubkova, M. 2006: Classification of the Tatra Mountain lakes (Slovakia) using chironomids (*Diptera, Chironomidae*). *Biologia (Bratislava)*, **61**: 191-201.

Camarero, L., Botev, I., Muri, G., Psenner, R. and Rose, N. 2009: Trace elements in alpine and arctic lake sediments as a record of diffuse atmospheric contamination across Europe. *Freshwater Biol.*, **54**: 2518-2532.

Coops, H., Beklioglu, M. and Crisman, T.L. 2003: The role of water-level fluctuations in shallow lake ecosystems - workshop conclusions. *Hydrobiologia*, **506**: 23-27.

Diviš, M. 2008: Water monitoring. SPŠ, Karviná.

Donahue, W.F., Schindler, D.W., Page, S.J. and Stainton, M.P. 1998: Acid-induced changes in DOC quality in an experimental whole-lake manipulation. *Environ. Sci. Technol.*, **32**: 2954-2960.

Douglas, M.S.V. and Smol, J.P. 1994: Limnology of high arctic ponds (Cape Herschel, Ellesmere Island, N. W. T.). *Arch. Hydrobiol.*, **131**: 401-434.

Drever, J.I. and Zobrist, J. 1992: Chemical weathering of silicate rocks as a function of elevation in the southern Swiss Alps. *Geochim. Cosmochim. Ac.*, **56**: 3209-3216.

Evans, C.D. and Monteith, D.T. 2001: Chemical trends at lakes and streams in the UK Acid Waters Monitoring Network, 1988–2000: Evidence for recent recovery at a national scale. *Hydrol. Earth Syst. Sci.*, **5**: 351-366.

Grossart, H.P. and Simon, M. 1998: Bacterial colonization and microbial decomposition of limnetic organic aggregates (lake snow). *Aquat. Microb. Ecol.*, **15**: 127-140.

Hamilton, P.B., Gajewski, K., Atkinson, D.E. and Lean, D.R.S. 2001: Physical and chemical limnology of 204 lakes from the Canadian Arctic Archipelago. *Hydrobiologia*, **457**: 133-148.

Harriman, R. and Taylor, E.M. 1999: Acid Neutralising Capacity (ACN) and Alkalinity (ALK): Concepts and Measurement. Report SR (99) 06F, Pitlochry: Freshwater Fisheries Laboratory.

Chrastný, V., Komarek, M., Tlustoš, P., and Švehla, J. 2006: Effects of flooding on lead and cadmium speciation in sediments from a drinking water reservoir. *Environmental monitoring and assessment*, **118**: 113-123.

IPCC 2001: Climate Change 2001: Synthesis Report. Intergovernmental Panel on Climate Change (IPCC), Geneva, Switzerland.

Judová, J., Šalgovičová, D., Pavlovičová, D., Kosová, I. 2015: Environmental monitoring. Institute of High Mountain Biology, University of Žilina, Tatranská Javorina, Slovakia.

Keddy, P. and Fraser, L.H. 2000: Four general principles for the management and conservation of wetlands in large lakes: the role of water levels, nutrients, competitive hierarchies and centrifugal organization. *Lakes and Reservoirs: Research and Management*, **5**: 177-185.

Kopáček, J., Hejzlar, J., Kaňa, J., Porcal, P. and Klementová, Š. 2003: Photochemical, chemical, and biological transformations of dissolved organic carbon and its impact on alkalinity production in acidified lakes. *Limnol. Oceanogr.*, **48**: 106-117.

Kopáček. J., Stuchlík, E. and Hardekopf, D. 2006: Chemical composition of the Tatra Mountain lakes: Recovery from acidification. *Biologia*, **61**: 21-33.

Lim, D.S.S., Douglas, M.S.V., Smol, J.P. and Lean, D.R.S. 2001: Physical and chemical limnological characteristics of 38 lakes and ponds on Bathurst Island, Nunavut, Canadian High Arctic. *Internat. Rev. Hydrobiol.*, **86**: 1-22.

Liu, J., Jiang, T., Huang, R., Wang, D., Zhang, J., Qian, S. and Chen, H. 2017: A simulation study of inorganic sulfur cycling in the water level fluctuation zone of the Three Gorges Reservoir, China and the implications for mercury methylation. *Chemosphere*, **166**: 31-40.

Marček, A. 1996: Tarns of Tatras. *Vysoké Tatry*, **6**: 18.

McClain, M.E., Boyer, E.W., Dent, C.L., Gergel, S.E., Grimm, N.B., Groffman, P., Hart, S.C., Harvey, J., Johnston, C., Mayorga, E., McDowell, W.H. and Pinay, G. 2003: Biogeochemical hot spots and hot moments at the interface of terrestrial and aquatic ecosystems. *Ecosystems*, **6**: 301-312.

McNeely, R.N., Neimanis, V.P. and Dwyer, L. 1979: Water quality sourcebook: a guide to water quality parameters. Environment Canada, Inland Waters Directorate, Water Quality Branch, Ottawa, Ontario, Canada.

Michelutti, N., Douglas, M.S.V., Lean, D.R.S. and Smol, J.P. 2002: Physical and chemical limnology of 34 ultra-oligotrophic lakes and ponds near Wynniatt Bay, Victoria Island, Arctic Canada. *Hydrobiologia*, **48**: 1-13.

Monteith, D.T., Evans, C.D., Henrys, P.A., Simpson, G.L. and Malcolm, I.A. 2014: Trends in the hydrochemistry of acid-sensitive surface waters in the UK 1988–2008. *Ecol Indic*, **37**: 287-303.

Mooij, W.M., De Senerpont Domis, L.N., Nolet B.A., Bodelier, P.L.E., Boers, P.C.M., Pires, L.M.D., Gons, H.J., Ibelings, B.W., Noordhuis, R., Portielje, R., Wolfstein, K. and Lammens, E.H.R.R. 2005: The impact of climate change on lakes in the Netherlands a review. *Aquat. Ecol.*, **39**: 381.

Nogueira, F., Couto, E.G. and Bernardi, C.J. 2002: Geostatistics as a tool to improve sampling and statistical analysis in wetlands: A case study on dynamics of the organic matter distribution in the Pantanal of Mato Grosso, Brazil. *Braz. J. Biol.*, **62**: 861–870.

Pienitz, R., Smol, J.P. and Lean, D.R.S. 1997a:. Physical and chemical limnology of 59 lakes located between the southern Yukon and the Tuktoyaktuk Peninsula, Northwest Territories (Canada). *Can J Fish Aquat Sci*, **54**: 330-346.

Pienitz, R., Smol, J.P. and Lean, D.R.S. 1997b: Physical and chemical limnology of 24 lakes located between Yellowknife and Contwoyto Lake, Northwest Territories (Canada). *Can. J. Fish Aquat. Sci.*, **54**: 347-358.

Psenner, R. 1989: Chemistry of high mountain lakes in siliceous catchments of the Central Eastern Alps. *Aquat. Sci.*, **51**: 108-128.

Sobek, S., Tranvik, L.J., Prairie, Y.T., Kortelainen, P. and Cole, J.J. 2007: Patterns and regulation of dissolved organic carbon: an analysis of 7,500 widely distributed lakes. *Limnol. Oceanogr.*, **52**: 1208-1219.

Van Drooge, B.L., López, J., Fernández, P., Grimalt, J.O. and Stuchlík, E. 2011: Polycyclic aromatic hydrocarbons in lake sediments from the High Tatras. *Environ. Pollut.*, **159**: 1234-1240.

Wantzen, K.M., Wolfgang, J.J. and Rothhaupt, K.O. 2008: An extension of the floodpulse concept (FPC) for lakes. *Hydrobiologia*, **613**: 151-170.

Wathne, B.M., Patrick, S.T., Monteith, D.T. and Barth, H. 1995: AL: PE, acidification of mountain lakes; paleolimnology and ecology. Ecosystems Research Report 9, European Commission, D-G XII, Luxembourg.

Wu, G.L., Ren, G.H., Wang, D., Shi, Z.H. and Warrington, D. 2013: Above and below ground response to soil water change in an alpine wetland ecosystem on the Qinghai-Tibetan Plateau, China. *J. Hydrol.*, **476**: 120-127.

Zobrist, J. 2010: Water chemistry of Swiss alpine rivers. In: *Alpine waters. The handbook of environmental chemistry, Vol 6* (ed. U. Bundi), pp: 96-117. Heidelberg: Springer, Berlin.

Received 4 July 2020; accepted 21 July 2020.

Oecologia Montana 2020,
29, 28-38

The biology of the Alpine accentor *Prunella collaris*. IV. Maintenance activities and their clusters from late autumn to early spring. 20 year observation

M. JANIGA

Institute of High Mountain Biology, Žilina University, Tatranská Javorina 7, SK-059 56, Slovak Republic; e-mail: janiga@uniza.sk

Abstract. The maintenance activities and seasonal rhythms in behaviour and behavioural clusters of Alpine accentor (*Prunella collaris*) were studied between 1984 to 2003 at Malinô Brdo, Great Fatra NP, a ski resort in the West Carpathians, Slovakia. The following episodes of behaviour were recorded: feeding, standing (sitting alert), sitting (drowsily), preening, flights, calls and songs.

Notes on behaviour were simply written or dictated onto tape. One minute of an activity of one bird constituded one field sample. In total, 21 282 samples of feeding, 1699 of preening, 18 411 of seating, 2417 of standing, 3775 of songs or calls, and 1630 of flights (longer than 100 metres) were collected. Daily activity of accentors ranged frorn 8 hours per day in late December to approximately 11 hours per day in April. During November, daily feeding schedules were bipolar, with peaks in early morning and at two o´ clock p.m. Rest periods rapidly increased during December. Resting was accompanied by the disappearence of vocalisation and a rapid reduction in preening. In January, the accentors exhibited a series of activities comparable to the structure of behaviour in November. The increased length of daylight in February likely induced hormonal activity in birds, and the amount of time devoted to singing rapidly increased. Subsongs often alternated with preening. In March, throughhout a day, birds displayed a mixture of many different kinds of activities including singing, preening, short flights, and standing alert. In April, accentors exhibited a series of activities that corresponded to pre – mating behaviour. Generally, birds were more stressed during feeding on cloudy than sunny days. The association between flocking and resting without additional activity, such as preening, supports the notion of a link between flocking and an efficient use of energy during winter.

Key words: Alpine accentor, behaviour, maintenance activities, winter ecology

Introduction

In general, maintenance mechanisms in animals are designed to enable them to behave in the most profitable manner, without an undue waste in energy. At the same time, animals must contend with a number of potential hazards and dangers. Many species follow specific routines, based on their internal clock. The time of day and season can be important stimuli for appetite, rest or preening.

Seasonal rhythms in maintenance activities of Alpine accentors wintering in a ski resort in the Western Carpathians (Slovakia) are the subject of this study. Alpine accentors vary in their wintering strategies. Mainly older males are resident, engaging in only local movements (Martín-Vivaldi *et al.* 1995; Heer 1996; Heer and Fraenkl 1999). Migratory birds usually include a large proportion of females and yearlings (Nakamura *et al.* 1996; Nakamura and Nishiumi 2000; Henry 2011). Extreme changes in climatic factors, such as temperature, light intensity, winds, and snow cover, affect accentors directly. However, indirect climate effects are also a factor, including fluctuations in food availability, numbers of predators, etc. Seasonal and circadian rhythms enable accentors to anticipate changes in environmental conditions (Janiga and Romanová 1997). Such mechanisms facilitate the accurate timing of specific types of behaviour or events, especially under winter conditions. Many events tend to be grouped in time. For example, a hungry accentor performs a series of maintenance activities while searching for food, such as standing, waiting or calling. These activities tend to form a group or a cluster of activities that occur for a given period of time and then cease, to be replaced by a different group; perhaps sitting, sleeping, and preening (Janiga and Romanová 1996). Clusters of activities may be effectively differentiated by multivariate statistics when a suitable amount of quantitative data exists. In this study, the general types of behaviour of wintering Alpine accentors are described. The variation in the structure of clusters was analysed in relation to different variables such as month or flock size. Of particular interest are seasonal variations in maintenance activities.

Material and Methods

Data collection in field

The field work was conducted between 1984 and

Year	84/85	85/86	86/87	87/88	88/89	89/90	90/91	91/92	92/93	93/94	94/95
No. of birds	10	9	8	7	4	7	4	4	2	1	2
No. of visits	15	18	24	13	25	10	9	19	5	5	4
Year	95/96	96/97	97/98	98/99	99/00	00/01	01/02	02/03	03/04	04/05	
No. of birds	2	3	5	3	3	4	4	1	0	0	
No. of visits	12	9	11	7	9	10	5	3	5	5	

Table 1. Maximum number of birds seen in one day during the winter period at Malinô Brdo.

2005 in the Great Fatra Mountains National Park, at the Malinô Brdo ski resort (Table 1). Generally, birds were monitored from the beginning of November to the end of April. Details on maximum number of birds seen in respective years and the number of visits per year are presented in Table 1. In the majority of years, the presence of birds was dependent on heavy snow and deep snow cover in the mountains, but in some years, they also stayed in the resort on sunny days when snow cover disappeared in many patches. Thus, the presence of a bird in the wintering area was often individually motivated, and often the occurrence of accentors was dependent on the type of food offered by tourists, hotel managers or skiers. The survey area was restricted to the ski resort, approximately 1 km long and 2 kms wide, where accentors fooraged near hotels, pensions or restaurants. Daily visits lasted a minimum of five hours, but many visits lasted from dusk till dawn. 27 birds were captured with a cheese/millet-baited fallingtrap. Each bird was individually marked with colourings and measured. The majority of birds were individually distinguished and resighted over the next years. Binoculars of various powers were of assistance for observation. Notes on behaviour were simply written or dictated onto tape. During simultaneous observation of multiple birds, individuals were videorecorded, and the recordings were later analysed in the laboratory.

Description and classification of behaviour

Detailed classification of behaviours was described in studies by Janiga and Romanová (1996, 1997). The following episodes were recorded: feeding, standing (sitting alert), sitting (drowsily), preening, flights, calls and songs. The notion of general maintenance activities usually encompasses a wide variety of behaviour patterns (McFarland 1987).

Feeding (Fig. 1)

This category encompasses appetitive behaviour, and includes activities involved in searching, obtaining, handling, and ingesting food. This category also included “hunting” (for example, the birds hunted flies in sunny March and April days near the windows and roof of the hotel). “Hunting” also involved the activity of “capturing”. The exploratory phase of feeding was often associated with “foraging”. “Drinking” was also included in this category, although feeding was not always a direct stimulus for drinking. In winter, birds tended to eat and drink in synchrony by consuming snow.

Standing (Fig. 2)

The term was given to a somewhat heterogeneous group of behavioural patterns which all had something to do with active immobility. The category included: *Watching* the members of the family. The birds looked for the members of a wintering group, and possibly benefited from watching their activities. *Standing* on one leg – whereby the birds preserved body heat. *Standing* alert during feeding. Birds had to be alert to the possibility of being surprised by predators, particularly domestic cats. Standing before feeding when accentors had access to abundant and predictable sources of food (bird feeder). Generally, during the early morning, following arrival at the ski resort, birds did not immediately seek food sources, but stood, very often without any movement in selected sites beneath the roof of the hotel. *Standing - intention* movements - a bird standing on the ground was about to take off in fllght, or to feed.

Fig. 1. Feeding (Photo: Marián Janiga).

Fig. 2. Standing (Photo: Marián Janiga).

Preening (Fig. 3)

These activities all had something to do with body care, whether it was routine maintenance, as in the preening of birds or rernoval of parasites. The category consists of pethaps twenty different activities, the most of them were active means by which the bird defended its integuments against attack from biological factors, specifically ectoparasites. "Preening" included postbathing preening, body shaking, wing whirring, oiling, and scratching. The behaviours of "sitting + preening" or "standing + preening" were always considered equivalent to simply, "preening". Displacement preening was also included in this category.

Fig. 3. Preening (Photo: Marián Janiga).

Resting (sitting drowsily; Fig. 4)

Accentors often rest during a day. Resting is most commonly associated with sessions lasting many minutes, during which a bird sits and remains immobile, sometimes moving only its head. During the daylight, the category was often associated with active (not quiet) sleep.

Fig. 4. Resting or sitting (Photo: Marián Janiga).

Flights

Usually short flights from one site to a second site. Flights can be used by birds in a number of behavioural contexts, and may be misleading to researchers. All types of flight (McFarland 1987) were included in the category of flight.

Calls and songs

Category consists from all manners of sounds, for example calls of adults during depart. Calls and songs. This category consists of all manner of sounds. Examples include the calls of adults during departure from the locality or long continual subsongs during sunny days in early spring. The "sitting + song" or "calls + flight" were considered equivalent.

Classification of activity budgets - months, decades and hours

I recorded the behaviour of accentor(s) once per minute. This was the shortest interval in which I was still able to record the behaviour in the field. The description represents all categories which occurred in each designated minute. Categories were designed in such a way that the members of one class did not also occur in another class. For example, accentors somtimes were observed "sitting" and "preening" during a given minute. In this case, I separately scored the two categories in this minute. One minute of an activity per one bird was a field sample. If ten birds were seen in a minute, then ten samples were collected. So, the variable "feeding" in a hour means the number of samples of feeding per hour. The activity of birds was recorded between November 1st and April 30th. The following number of samples was collected per month (Table 2).

Hour	Nov.	Dec.	Jan.	Febr.	March	April
6.00					222	439
7.00	235	5	109	1311	1553	287
8.00	669	314	1589	2222	1206	239
9.00	134	544	907	1276	1432	101
10.00	93	299	567	976	798	211
11.00	134	467	488	1280	1008	235
12.00	231	380	435	694	1533	338
13.00	214	715	562	671	919	404
14.00	495	392	127	1858	477	300
15.00	776	641	1350	1732	1676	267
16.00	494	849	1134	1450	1592	758
17.00	6	4	63	741	953	291
18.00				6	125	239
19.00						12

Table 2. Amounts of samples (one minute per a bird equals one sample) used for the computation of relative hourly activity budgets. Eastern European Time.

In total, 21 282 samples of feeding, 1699 of preening, 18 411 of seating, 2417 of standing, 3775 of songs or calls, and 1630 of flights (longer than 100 metres) were collected. For each month, a model of a day was separately constructed.

Classification of activity clusters and statistics

Ratio scale

Hourly sums of the minutes of observations of four bird activities - sitting (resting), standing (siting alert and watching), feeding and preening composed their ratio. For example: 10 : 2 : 25 : 3 means 10 minutes of sitting, 2 of standing, 25 of feeding, and 3 of preening in a hour (e.g. between 10:00 and 11:00). As mentioned above, one minute of observation of one bird equaled one sample, so if more birds were watched in a minute, more samples in a minute were obtained. All arithmetical operations dealing with activity clusters were carried out in these hourly ratio scales. The ratio scale has all characteristics of an interval scale, the unit of measurement is equal in all four classes of activities, and scaling is independent of the observer 's viewpoint. The internal structure of ratios was examined in relation to weather conditions, flock size observed in the field, and calendary months.

Principal component analysis is a multivariate technique that may be used for summarizing data sets combining large numbers of variables The importance of principal components can be judged from the amount of variance associated with them, and from the signs of their weight (coordinates) elements (Jolicoeur 1963; Lawley and Maxwell 1971) The designed ratios were the fundamental inputs to the data matrix for PCA. Principal components (factors) were computed from correlation matrix of variables (Table 3), i.e. relative amounts of minutes spent for each category in a ratio. Analysis of variance was used to test for effects of the different factors on principal component coordinates of cases (Sommers 1986). The calculations were carried out using the statistitical package STATISTICA 12.

Results

Daily routine

The daily routine of wintering accentors differs in different months and also in different hours of a month. On the basis of direct observations in the field, Alpine accentors arrived to the ski resort approximately fifteen minutes after dawn and departed from fifteen to twenty minutes before dusk. The daily activity of accentors ranged from 8 hours per day in late December to approximately 11 hours per day in April (Table 4). During November, the scheme of daily feeding in birds was bipolar with peaks in early morning and around two o´ clock p.m. (Fig. 5a). Birds mainly rested from 10 to 11 o´ clock with increased amounts of time devoted to postbreeding preening (Fig. 5b).

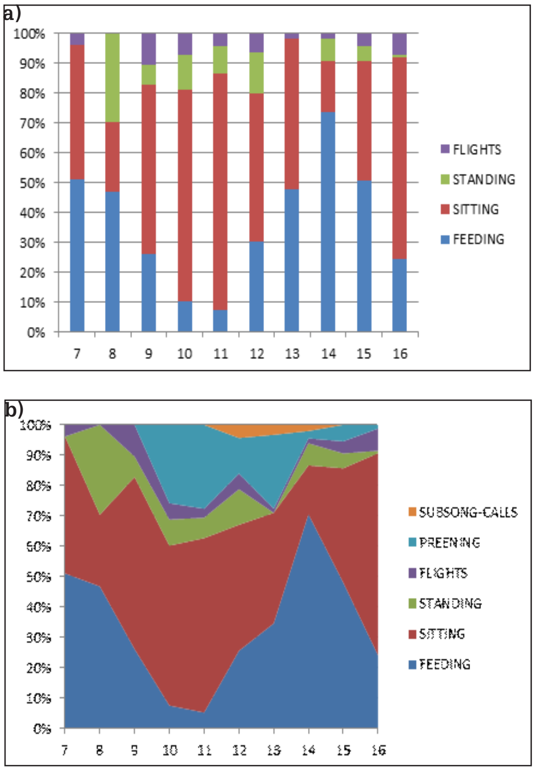


Fig. 5. Hourly activity budgets of wintering Alpine accentors in November. **a)** Structure of four most important maintenance activities. **b)** Maintenance activities accompanied with preening and vocalisation.

Amounts of minutes devoted to resting (sitting, sitting drowsily) rapidly increased in December (Fig. 6a). Birds more or less foraged for the duration of the day. Resting in December was accompanied with the disappearence of vocalisation and a rapid reduction of preening in comparison to the behavioural scheme from November. Mobility of birds in the area during a day was also reduced (Fig. 6b).

In January, accentors exhibited a series of activities comparable to the structure of behaviour in

Variable	PC1	PC2	PC3	PC4
Sitting	-0.855532	**0.267957**	-0.149336	**0.417088**
Standig	-0.626596	**-0.449398**	**0.636626**	0.011238
Feeding	-0.619934	**-0.577808**	**-0.509397**	-0.149446
Preening	-0.759451	**0.540585**	0.058789	**-0.357136**
Variance in %	52	22	17	8

Table 3. Factor coordinates of included variables into principal component analysis. Structure of fundamental behavioural groups in Alpine accentors in winter. Numbers, which denote the main behavioural schemes are in bold.

Date	Arrival (EET)	Departure (EET)	Month/Activity length	Dawn	Dusk
Nov. 7th	16:10				
Nov. 20th	17:07				
Nov. 20th	17:10				17:25
Nov. 22nd	16:54				17:15
Nov. 23rd	7:43				
Nov. 23rd	7:48				
			End of November app. 9 hrs. 15 min		
Dec. 8th	7:58				
Dec. 9th	8:05				
Dec. 10th		17:01			
Dec. 17th	8:12				
Dec. 21th	8:05				
Dec. 22nd	8:05			7:55	
Dec. 26th		16:46			
Dec. 28th		15:40			
Dec. 29th	8:29			8:10	
			End of December app. 8 hrs. 15 min		
Jan. 1st		16:44			
Jan. 2nd	8:2			8:00	
Jan. 4th		17:16			17:30
Jan. 7th		16:38			
Jan. 8th	8:12	17:20			
Jan. 8th		16:50			
Jan. 15th		16:55			
Jan. 15th		17:15			
Jan. 23rd		17:00			
Jan. 24th	8:05				
Jan. 25th	8:10				
Jan. 27th		17:46			
Jan. 29th	7:55				
Jan. 29th	8:05				
Jan. 30th		17:07			
			End of January app. 9 hrs.		
Febr. 1st	8:00				
Febr. 2nd	7:50				
Febr. 5th		16:40			
Febr. 6th		16:59			
Febr. 7th	7:59				
Febr. 8th	8:10				
Febr. 10th	7:40				
Febr. 11th	7:28			7:28	
Febr. 11th	7:42				
Febr. 12th		16:37			
Febr. 15th		17:23			

continued...

Febr. 18th		18:00		
Febr. 19th	7:20			7:00
Febr. 19th		18:01		
Febr. 20th	7:10			
Febr. 21 th	7:55			
Febr. 22nd		16:57		
Febr. 23rd		16:10		
Febr. 24th		17:10		
Febr. 27th		17:44		
			End of February 9 hrs. 30 min	
March 1st		17:30		
March 1st		18:16		
March 1st	7:25			
March 2nd		18:06		
March 3rd	7:15			7:00
March 3rd		18:18		
March 4th	7:00			
March 6th	7:30			6:50
March 7th		17:50		
March 26th	7:21	18:24		6:20
			End of March app. 10 hrs. 30 min	
April 3rd		18:48		
April 4th		18:13		
April 15th		18:35		
April 15th		19:05		
April 15th		19:15		
			April – app. 11 hrs. and more	

Table 4. Seasonal variation in the length of daily activity of Alpine accentors in the wintering area, Malinô Brdo, NP Great Fatra mountains, Slovakia. EET – East European Time.

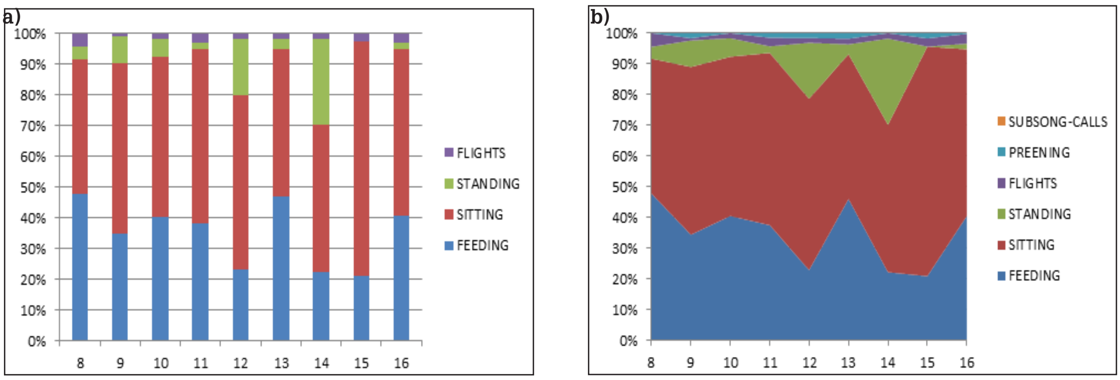


Fig. 6. Hourly activity budgets of wintering Alpine accentors in December. **a)** Structure of four most important maintenance activities. **b)** Maintenance activities accompanied with preening and vocalisation.

November. In deep winter they usually started to feed immediately after arrival. In the mornings, they spent approximately 80 per cent of their time feeding (Fig. 7a). After 10 o´ clok birds usually started to rest (sitting) and the second smaller peak of feeding occurred one hour before departure (Fig. 7b).

In February, accentors foraged in the early morning as well as in the afternoon, from one to two hours before departure (Fig. 8a). The length of daylight during February is probably sufficient to activate hormonal activity in birds, and the amount of time devoted to singing rapidly increased. Singing often alternated with preening (Fig. 8b).

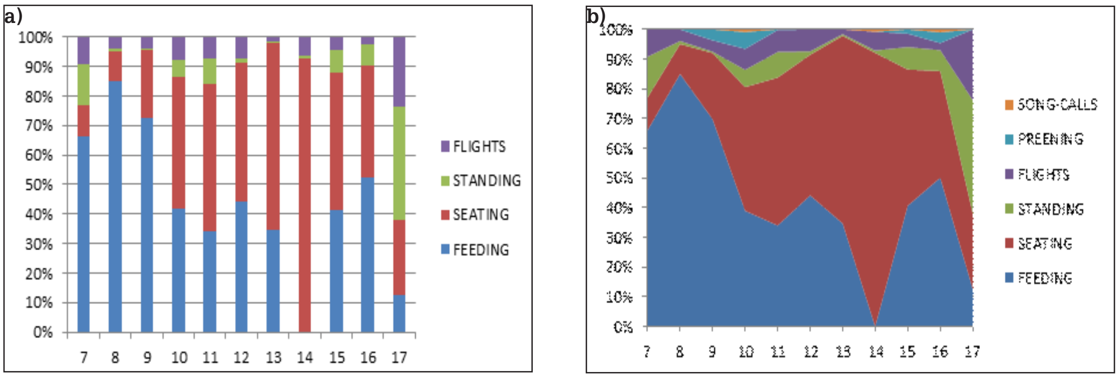


Fig. 7. Hourly activity budgets of wintering Alpine accentors in January. **a)** Structure of four most important maintenance activities. **b)** Maintenance activities accompanied with preening and vocalisation.

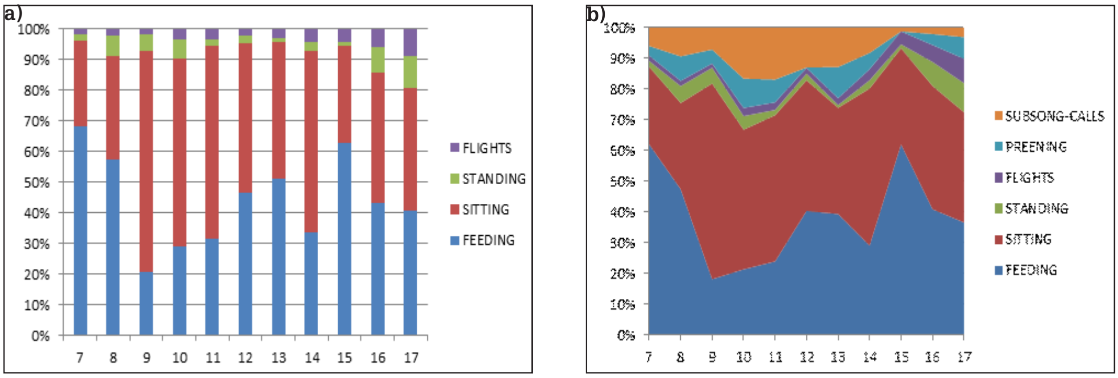


Fig. 8. Hourly activity budgets of wintering Alpine accentors in February. **a)** Structure of four most important maintenance activities. **b)** Maintenance activities accompanied with preening and vocalisation.

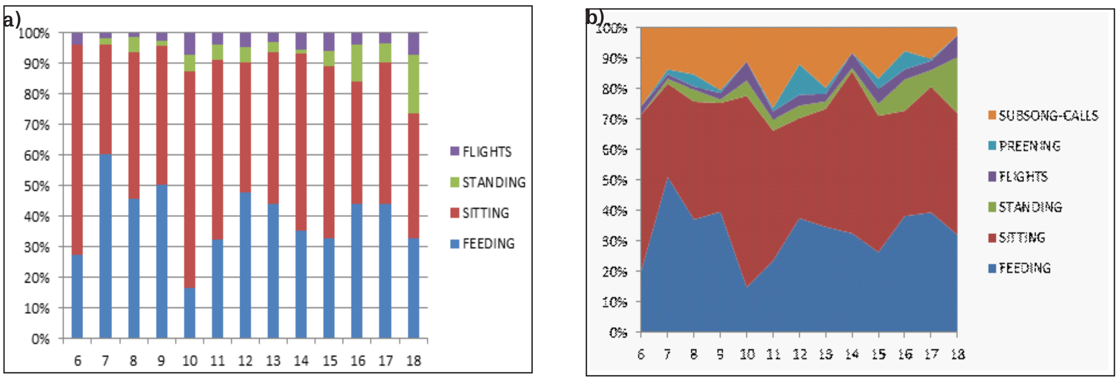


Fig. 9. Hourly activity budgets of wintering Alpine accentors in March. **a)** Structure of four most important maintenance activities. **b)** Maintenance activities accompanied with preening and vocalisation.

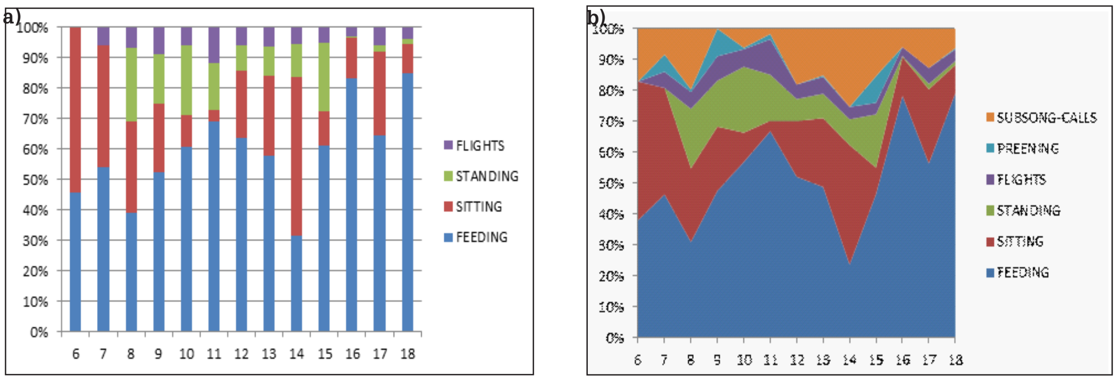


Fig. 10. Hourly activity budgets of wintering Alpine accentors in April. **a)** Structure of four most important maintenance activities. **b)** Maintenance activities accompanied with preening and vocalisation.

In March, the structure of daily behaviour rapidly changed in comparison to February. Birds foraged throughout the day but foraging decreased to 30 - 40 % of hourly behavioural budgets (Fig. 9a). Throughout a day birds displayed a mixture of many different kinds of activities including singing (Fig. 9b).

During April, the number of minutes devoted to feeding rapidly declined in the early morning and significantly increased in the late afternoon (Fig. 10a). Birds often started to forage in open grassy areas. During a day, accentors exhibited a series of activities that corresponded to pre – mating behaviour. The amount of time devoted to short distance flights, standing alert and singing increased (Fig. 10b), especially mid-day during sunny days. The rhythm of intensive feeding usually began in the late evening, from one to two hours prior to daily departure.

Seasonal routine

In late autumn, birds foraged during the early morning, immediately after arrival. In November, Alpine accentors could find food in rocky parts of the resort and wind-blown patches where the soil remained free of snow. In this late post-breeding period, before real winter, their diet consisted of vegetable matter. However, following the first heavy snows, which could occur as early as November, the moutain hotel and restaurants attracted several accentors. In addition to a feeder, the birds found scraps on the terraces of restaurants. When they found good foraging sites with predictable diet, their behaviour strategy changed. From the beginning to the end of November, the daily time budgeted for feeding activity decreased from 70 to 40 per cent and the time devoted to seating (resting) rapidly increased (Fig. 11). Having a predictable site for feeding, accentors effectively saved energy, particularly in December. The daily budget for feeding decreased to 30 percent, and more than 50 % of daily time was devoted to resting (Fig. 12a). Vocalisation, preening or watching in alert (standing) was reduced to the minimum value (Fig. 12b). In birds, the gonads are mainly photo-stimulated and short periods of daylight in December likely contributes to the inactivity of accentors. Thus, December is probably the only month of the year when birds survive winter with minimal energy expenditure. The increased amount of daylight in January and

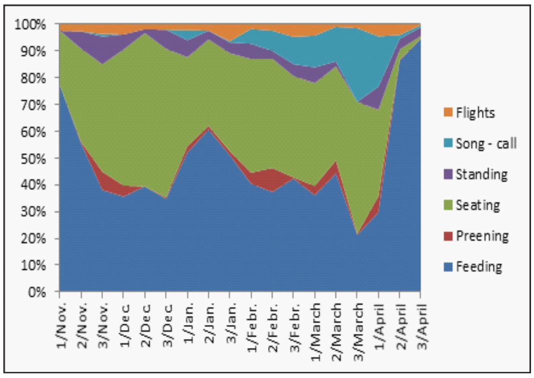


Fig. 11. Relative seasonal variation in the main activities of Alpine accentors in winter. Each month is divided into decades. For number of samples see Table 2.

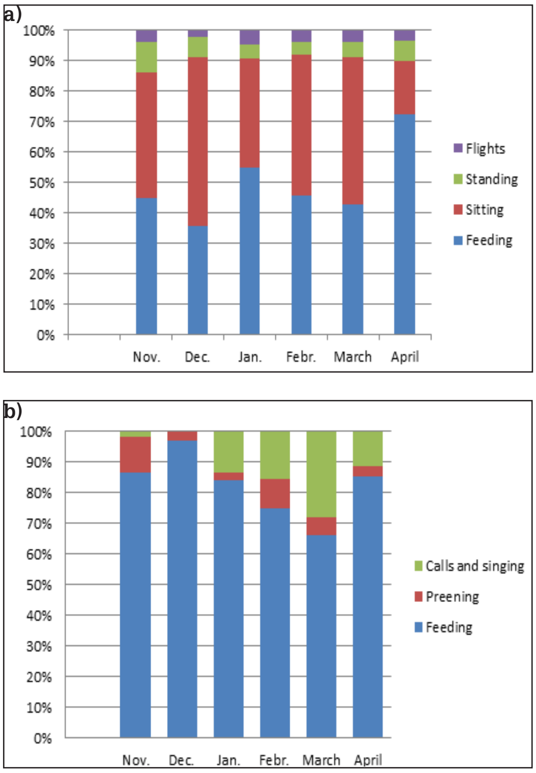


Fig. 12. a) Relative montly variation in main maintenance activities of wintering Alpine acctentors in the Great Fatra NP, West Carpathians. **b)** Feeding activity of accentors in winter related to preening and vocalisation.For number of samples see Table 2.

Factor	Exploratory versus resting activity clusters - PC2 (feed+stand versus sit+preen)	Alarm behaviour during feeding - PC3 (feed or stand and watch)	Sit and preen or sit and rest – PC4
Weather	F (4,118)=1.65, P=0.16, NS	**F (4,118)=4.1, P=0.004****	F (4,118)=1.9, P=0.1, NS
Month	**F (5,131)=2.78,P=0.02***	F (5,131)=0.9, P=0.48, NS	F (5,131)=1.5, P=0.2, NS
Flock size	F (3,133)=2.18, P=0.09, NS	F (3,133)=0.86, P=0.46, NS	**F (3,133)=2.7, P=0.048***

Table 5. Effects of weather, months (season) and number of birds in a flock on the behavioural schemes of Alpine accentors in winter. One - way ANOVAs (F) and their significance levels (P) refer to comparisons of pricipal component coordinates of cases (hourly observations of bird behaviour in field). The significant differences in some groups are shown in bold and in Figs. 13, 14, and 15.

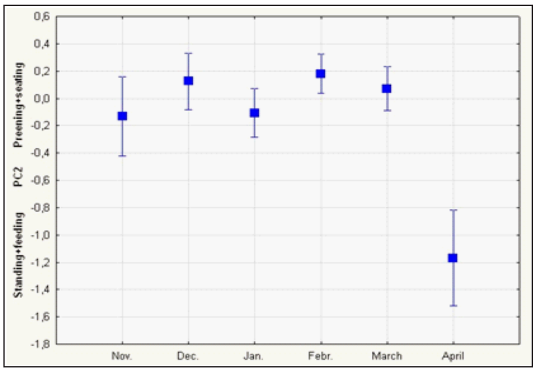


Fig. 13. Mean values of principal coordinates of cases on PC2 Vertical bars denote +/- standard errors. From November to March, Alpine accentors devoted more time to preening and sitting (resting) and less to feeding and standing alert than in April. In April, the exploratory activies (see Table 5) proportionally increased to resting activities.

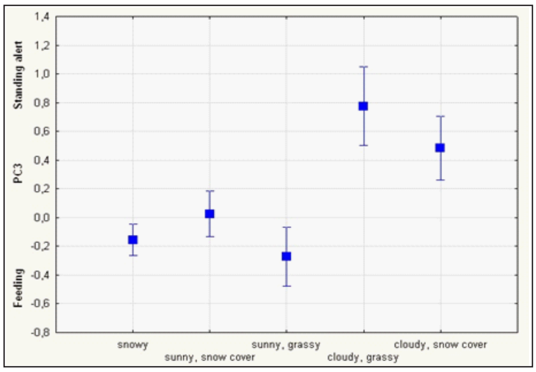


Fig. 14. Mean values of principal coordinates of cases on PC3 Vertical bars denote +/- standard errors. In cloudy weather, the accentors devoted proportionally more time to watching surroundings during feeding than in the sunny winter days or during snowfall.

February activate hormonal cycles. Alpine accentors often warbled subsong when resting under the roof of mountain hotel. Subsong activity increased toward March (Figs. 11, 12 b.) Subsongs were most intense on sunny mid-days. Several birds could be seen singing at a distance of one meter under the same roof. In early spring, during April, accentors changed their scheme of behaviour. Birds increased the time spent foraging, and feed mainly on open patches of grassy areas exposed by snow melt. Starting in March, accentors were seen catching dipterous near the windows and under the roof of hotels and collecting arthropods on the snow. Birds evidently started to look for animal proteins which enable quick body restoration following winter. By the end of April birds spent more time feeding than in winter months (Figs. 11, 12a).

Activity clusters

As mentioned in the Introduction, many activties of animals tend to be grouped by time and by basic maintenance activity. For example, feeding, may be a member of different behavioural clusters. A hungry accentor performs a series of activities while searching for food and eating it, such as standing or calling. These activities form a group which occurs for a given period of time and concrete space and then ceases, to be replaced by a different group; perhaps feeding, sitting and preening. For the analysis of behavioural clusters principal component analysis was used. The first principal component (Table 3) is an indication of researcher activity, and the correlations on PC 1 are of the same sign. The variation of data in PC1 refers to the ability of the observer to find and watch the birds. This was a reason why this component was not analysed in more detail. The next principal components (PC2, PC3, PC4, Table 3) are statistically independent of the first and decribe real clusters of accentor behaviour.

Exploratory versus resting activity clusters (PC2, Table 5, Fig. 13). This type may be called „resting versus exploratory“ because sitting is positively associated with preening, and negatively with feeding and standing alert (Table 3). This activity does not relate to different weather conditions, nor to the size of the wintering flock. However, in this cluster, the exploratory activities – feeding and standing alert – significantly increased in April in comparison to winter months. In winter, birds spent proportionally more time resting and preening than feeding or standing (Table 5, Fig. 13).

Watching and alarm responses during feeding (PC3, Table 5, Fig. 14). If an accentor senses a sign of danger, it stands alert, watching surroundings or members of the group. In PC3, this activity was negatively correlated with feeding. Birds were more stressed during feeding on cloudy than sunny days (Fig. 14). On cloudy days they spent more time standing alert than during snowfall. Cloudy days with fog probably mean less visibility for birds.

Type of a rest (PC4, Table 5, Fig. 15). In winter, birds can simply sit and rest or sit and preen. More birds in a flock, less preening and more simple resting. Individual birds spent proportionally more time preening when sitting (Table 5, Fig. 15). Visual responses are commonly found in birds that live in enlarged groups. In winter, accentors show more variation int he amount of flocking from place to place. Flocking usually correlates with the pres-

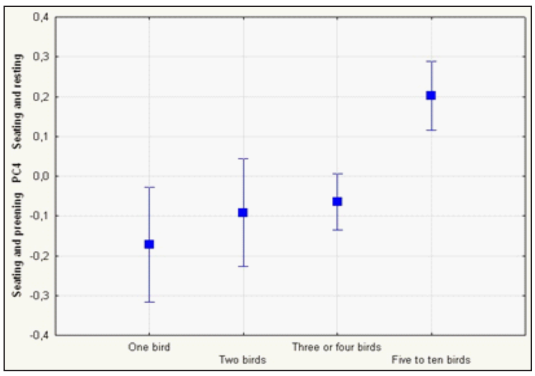


Fig. 15. Mean values of principal coordinates of cases on PC4 (for significancy see Table 5). Vertical bars denote +/- standard errors. Larger flocks tend to spend more time for simple resting and preen less during resting than smaller flocks or single individuals. Singles and small flocks preened more during sitting and resting.

ence or absence of environmental factors. In wintering accentors, the association between flocking and resting without additional activities like preening supports the notion of a link between flocking and the efficiemt use of energy in winter.

Discussion

During wintertime, Alpine accentors forage within their summer territories, in surrounding mountain regions, roofs of mountain hotels and restaurants or fly down to lower-located areas (e.g. Praz 1976; Schmidt 1985; Cramp 1988; Martí *et al.* 1989; Martín-Vivaldi *et al.* 1995; Heer 1996; Heer and Fraenkl 1999; Henry 2011; Lukac *et al.* 2016). Ringed animals from the Tatra mountains show us that some birds extend their range considerably; as far as Hungary, for example. The wintering ecology of these migrants is poorly known. Wintering Alpine accentors at Malinô Brdo were probably first recorded in the 1970s (Hudec 1983). Hotel Malina and cable car were built during the 1960s. Accentors have been contributing to knowledge about this wintering ground for more than 30 years. Rapid change occurred after the political break in 1989. The ski resort was drastically enlarged and the hotel reconstructed in a way that was no longer suitable for perching of birds, and the amount of people visiting the site increased exponentially. These changes were accompanied by acoustic and artificial light smog. Behaviour in the last of the specimens was observed in 2003, when the birds largely disappeared from the site. These winter aggregations of birds were studied in detail between winter 1984/85 and winter 2002/2003 (Table 1).

In Europe, November brings great changes to time budgets of Alpine accentors. In the Slovakian Tatra mountains post-breeding migration starts at the beginning of November (cf. Maruyama *et al.* 1972), and migrants reach their wintering ground by mid-November. Once settled at their wintering site some birds may be sedentary, while some individuals may exploit feeding sites a few kilometres away. Some birds, usually dominant males, commute between their breeding territories on sunny days and feeding sites in the surrounding villages during snowfall or heavy freezing periods. Most birds descend below the snowline or seek snow-free patches during winter. Continuous snow cover means a fundamental change in maintenance activity time budgets in November, and the amount of time spent foraging and feeding decreases, while the amount of time spent resting and saving energy increases.

Diet, and the interaction between day-length and circadian rhythm in birds may be critical factors for change in bird behaviour in November. Photoperiodic regulation of migration is also achieved by the appropriate phasing of endogenous circadian and circaannual rhythms of endocrine function and of tissue sensitivity (Follett 1973). Thus, in accentors, the mediation of migration in November and the definite reduction of reproductive behaviour in December and January must be considered in terms of circadian and circannual periodicities whose phase relationships are an adaptive function of the environmental photoperiod. Distribution and an increased amount of light pulses (Lofts and Lam 1973) during daytime are probably the most important in stimulating photoinduced subsongs of accentors in February. At this time, the daily activity of accentors lasts approximately 9 hours and 30 minutes and daylight lasts approximately 10 hours. The amount of subsong increases in March and the beginning of April (Figs. 11 and 12b). Subsong activity may thus last for three months, increases toward spring, and is most intense on sunny days. At feeding sites, subsong does not cause overt agression and several males may be seen singing close to one another. Males switch from subsong to full song approximately one month before the beginning of the breeding season, usually at the end of April (Heer 1994) and in the beginning of May. The onset of developmet of adult protuberances occurs in the late May (Nakamura 1990; Nakamura and Matsuzaki 1995) when the period of daily activity lasts approximately 14.5 hours (Janiga and Romanová 1997).

In mid- winter (December), reduced time budget for feeding and increased time spent resting probably reflect the shortest period of daylight as well as an increased energy demand for maintenance due to low and freezing temperatures. In late autumn (November), and in February and March, accentors devoted more time to preening. Many passerine birds show an increase in infestation by ectoparasites during winter months (for example, Hamstra and Badayev 2009). The most favourable period for feather eating lice is winter. At this time, the number of lice increases (Janiga and Kubašková 2000), and the size of adult lice is largest, possibly due to a decrease in preening-mediated selection in December. In October and November, Alpine accentors compose feeding aggregations that can include hundreds of birds. Their body mass can increase from 40 to more than 50 grams (Janiga pers. obs.), and they have new plumage. The few individuals of lice that survive moult may trigger an increase that leads to larger infestations of lice in winter. Wintering birds in December spend most of the short daylight period feeding and resting without preening. Less frequent preening, reduced daylight in December, and relatively new plumage provides the lice with a very favourable environment. Mid - winter is an excellent period for the growth and development of Ischnoceran lice (Janiga 2018). The ratio of male to female lice does not differ except during spring, but during most seasons lice in females is more prevalent (Janiga and Mičková 2004). We can hypothesize that preening played the most iportant role in this louse reduction during early spring. Moreover, from February through March, the fat stores of birds are quickly reduced (Heer 1998), reflecting changes in their behaviour and physiology. The lice found on the bodies of birds were smaller in the spring than in winter but nymphs were found, indicating an occurrence of a new parasite generation (Janiga 2018)

In addition to the above-mentioned factors, a range of additional effects can influence the correct interpretation of the structure of Alpine accentor behaviour in winter. They include exo- and endogenous variables such as local weather or aggregation (flock) size. The winter habitat feature of the accentors is a predictably unfavourable climate. During the winter season, mean daily temperatures may be very low. In late autumn and early spring, the amount of precipitation may be high. In this study it was confirmed that cloudy days with fog could cause devation from normal behaviors for several days, as experienced ornithologists know that birds see very poorly in the fog. In cloudy and foggy days, accentors devoted more time to standing alert and less to feeding. Behaviour of birds may also depend on the size of their flock. Autumn and winter flocking in accentors is associated with diet. At this time, the species eats seeds, and granivorous birds are more likely to show flocking behaviour compared to birds that eat mainly animal matter, including insects. In passerine birds, in addition to synchrony of movements, other behaviural patterns such as feeding or sleeping show a strong tendency to be synchronous within a flock. For example, when one or two individuals start sleeping or resting, the others rapidly follow suit. Flock synchrony is based on visual signals or on contact calls (McFarland 1987). In wintering accentors, the association between flocking and resting (without preening) supports the notion of a link between flocking and energy preservation in winter.

Acknowledgements

I gratefully acknowledge the indispensable assistance of Mrs. Jana Repetná in database creation. I thank Amanda Clarahan for assistance in article editing. And I also thank to many friends who helped me in the field work.

References

Cramp, S. (ed.) 1988: Handbook of the Birds of Europe the Middle East and North Africa. The Birds of the Western Palearctic. Volume V. Tyrant Flycatchers to Thrushes. Oxford University Press, Oxford, New York.

Follett, B.K. 1973: Circadian rhythms and photoperiodic time measurement in birds. *J. Reprod. Fert. Suppl.*, **19**: 5-18.

Hamstra, T.L. and Badayev, A.V. 2009: Comprehensive investigation of ectoparasite community and abundance across life history stages of avian host. *J. Zool.*, **278**: 91-99.

Heer, L. 1994: Zur sozialen Organisation und Brutbiologie der Alpenbraunelle MSc. Thesis, Institute of Zoology, University of Bern, Bern.

Heer, L. 1996: Cooperative breeding by Alpine accentor *Prunella collaris*: polygynandry, territoriality and multiple parternity. *J. Ornithol.*, **137**: 35-51.

Heer, L. 1998: The polygandrous mating system of the Alpine accentor *Prunella collaris*, individual reproductive tactics, breeding adaptations on high mountain conditions and winter ecology. PhD Thesis, Institute of Zoology, University of Bern, Bern.

Heer, L. and Fraenkl, A.C. 1999: Zur Verbreitung, sozialen Organisation, Raum- und Habitatnutzung der Alpenbraunelle *Prunella collaris* in Winter. *Orn. Beob.*, **96**: 25-36.

Henry, P.-Y. 2011: Differential migration in the polygynandrous Alpine accentor *Prunella collaris. Bird Study,* **58**:160-170.

Hudec, K. (ed.) 1983: Fauna ČSSR, Ptáci Aves. Díl III/1. Academia, Praha.

Janiga, M. 2018: Adaptive placticity in insect parasites – *Philopterus* lice and their accentor passerine hosts. *Pol. J. Ecol.*, **66**: 395-406.

Janiga, M. and Kubašková, Ľ. 2000: The biology of the Alpine accentor *Prunella collaris* III. The coevolution of Alpine accentors and lice (Phthiraptera). *Oecologia Montana*, **9**: 24-28.

Janiga, M. and Mičková, A. 2004: The biology of the Alpine accentor *Prunella collaris* V. The sex ratio and transmission of lice *Philopterus emiliae. Oecologia Montana*, **13**: 17-22.

Janiga, M. and Romanová, E. 1996: The biology of the Alpine accentor *Prunella collaris.* I. Behaviour: Principal component aualyses of orgauization of activity clusters. *Oecologia Montana*, **5**: 71-78.

Janiga, M. and Romanová, E. 1997: The biology of the Alpine accentor *Prunella collaris.* II. Behaviour: Rhythmic aspects of maintenance activities. *Oecologia Montana*, **6**: 45-48.

Jolicoeur, P. 1963: Note: the multivariate generalization of the allometry equation. *Biometrics*, **19**: 497-499.

Lawley, D.N and Maxwell, A.E. 1971: Factor analysis as a statistical method Butterworths, London.

Lofts, B. and Lam, W.L. 1973: Circadian regulation of gonadotropin secretion. *J. Reprod. Fert. Suppl.*, **19**: 19-34.

Lukač, G., Vujčič-Karlo, S., Milovac, M. and Adžič, I. 2016: Breeding and winter distribution of Alpine accentor (*Prunella collaris*) on the Eastern coast of Adriatic sea. *Larus*, **51**: 7-16.

Martí, R., Gómez-Manzaneque, A. and Perales, J.A. 1989: Diferencias segun edad y sexo en los movimientos dispersivos de una población de acentor alpino (*Prunella collaris*) en Espana central. *Ardeola*, **36**: 224-226.

Martín-Vivaldi, M., Marín, J.M. and Villar, M. 1995: Selección de habitat, tamano de bando y movimientos locales del acentor alpíno (*Prunella collaris*) en Sierra Nevada (SE de Espana). *Ardeola*, **42**: 11-20.

Maruyama, N., Kawano, M., Atsumi, H, Ueki, K. and Nezu, W. 1972: The social organization and the distribution of the Alpine accetttor, *Prunella collaris*, at the Kubiki and Togakushi mountain range. *Tori*, **27**: 325-338.

McFarland, D. 1987: The Oxford companiou to animal behaviour. Oxford University Press, New York.

Nakamura, M. 1990: Cloacal protuberance and copulatory behavior of the Alpine accentor (*Prunella collaris*). The *Auk*, **107**: 284-295.

Nakamura, M. and Matsuzaki, Y. 1995: Sex detetmination based on cloacal plotubetances in the Japanese Accentor *Prunella rubida. J. Yamashina Insf. Ornithol.*, **27**: 78-88.

Nakamura, M., Matsuzaki, Y. and Ootaka, H. 1996. Social unit of the Alpine accentor *Prunella collaris* in the nonbreeding season. *Jpn. J. Ornithol.*, **45**: 71-82.

Nakamura, M. and Nishiumi, I. 2000. Large variation in the sex ratio of winter fl ocks of the Alpine accentors *Prunella collaris. Jpn. J. Ornithol.*, **49**: 145-150.

Praz, J.-C. 1976. Notes sur l'Accenteur alpin *Prunella collaris* dans le Val d'Hérens (Valais). *Nos Oiseaux,* **33**: 257-264.

Schmidt, E. 1985. IX. A Havasi Szurkebegy (*Prunella collaris*) Magyarországon. *Aquila*, **92**: 105-111.

Sommers, K.M. 1986: Multvariate allometry and removal of size wrth principal component analysis. *Systenatic Zoology*, **35**: 359-368.

Received 4 May 2020; accepted 21 July 2020.