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Original Research

Effects of Soil Moisture and Nickel Contamination on Microbial Respiration Rates in Heavy Metal-Polluted Soils

Justyna Morawska-Płoskonka*, Maria Niklińska

Institute of Environmental Sciences, Jagiellonian University,
Gronostajowa 7, 30-387 Kraków, Poland

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Abstract

Soil microorganisms may be both sensitive and resilient to various disturbances. The effects of a single stressor on soil microorganisms have been well studied, but only limited research has been carried out to test the effects of simultaneous action of diverse stressors.

Soil samples were collected from a long-term polluted zinc and lead site and an unpolluted site. Modeling studies assumed spiking soils with five different concentrations of nickel (400, 800, 1.600, 3.200, and 6.400 mg $Ni \cdot kg^{-1}$ dry weight soil) and their incubation under different humidity conditions (10%, 75%, and 120% of water holding capacity). We wanted to test if additional environmental disturbances have a different effect on microorganisms from polluted and unpolluted soils.

The study showed that after 30 and 120 days of incubation, increasing Ni pollution inhibited microbial respiration rate (R), both in unpolluted and long-term metal polluted soils, irrespective of soil moisture. After 30 days of the experiment, microbial communities in both soils demonstrated a similar response to the additional toxicant. However, after 120 days of exposure to Ni, microbial communities from the unpolluted soil showed much higher inhibition of R than microbes from the polluted soils ($p<0.001$). The results might suggest that Ni co-tolerance mechanisms occurred in long-term metal polluted microbial communities.

Keywords: heavy metals, resistance, soil humidity, microbial respiration rate

Introduction

Microorganisms form a vital part of the soil food chain. Their basal respiration is one of the basic parameters of ecological stress, examining the effectiveness of degradation of organic carbon sources.

Respiration rate reflects the level of general microbial activity. Moreover, soil respiration can be used to estimate soil microbial biomass and gives some insight into nutrient cycling in the soil and decomposition rate. Therefore, respiration measurements are the easiest and fastest method of determining the quality of a microbial population subjected to a majority of environmental disturbances, whether as a result of changes in natural factors or pollution.

Soil respiration is directly dependent on the availability of easily degradable carbon compounds and nutrients, as an energy source for microbes [1-3]. Microbial activity also is commonly reported to be directly related to temperature [4-6] and strongly dependent on soil moisture [7, 8] or pH. Conant et al. [5] showed that respiration rates of microorganisms from soils incubated under different soil moisture levels was generally directly related to temperature, but responses were ameliorated with decreases in soil moisture. Weyman-Kaczmarkowa [9] also showed that at high mois-

*e-mail: j.morawska@uj.edu.pl

ture levels (100% WHC) microbial respiratory rate can be raised over twice as high as in dry soils (35% WHC). Similarly, changes in soil pH adversely affect microbial communities' activity. Microbial parameters like respiration, microbial biomass, phospholipid fatty acids composition, or net nitrification also are often reported to be linearly correlated with pH changes, thus strongly disturbed by acidification [10-12]. Furthermore, it is well known that soil acidity, as well as the amount of soluble organic matter, are important factors affecting metal mobility in soils [13, 14], and thus their absorption by organisms.

A wide range of metals plays diverse functions in microorganisms, including the stabilization of cell walls and proteins or osmotic regulation. Their effects on microbial activity and diversity considerably depend on concentration as well as chemical form. For example, some elements, including transition metals such as V, Mo, W, Mn, Fe, Co, Ni, Cu, and Zn, act as co-factors in microbial enzymes. However, at high concentrations even these metals can reduce soil microbial activity by inhibiting, i.e., extracellular enzyme activity [15, 16]. There are many studies confirming that microbial activity in soils highly polluted with metals such as Cu, Zn, Pb, or Cd is significantly inhibited in comparison to communities from a clean environment [17-19]. Similarly, Ni, despite being one of the essential nutrients, in concentrations exceeding the natural level (< 10 mg·kg^{-1}) might be one of the most dangerous metals in the environment [20, 21]. A deposition of 300 mg Ni·kg^{-1} dwt soil can cause a 60% decrease in the number of oligotrophic bacteria and inhibits nitrification processes by up to 20%, in comparison to clean soils [22]. Antil et al. [23] have reported that microbial biomass decreased with increasing concentrations of Ni in soil contaminated with industrial wastewater.

Therefore, it is interesting that microbial communities from polluted soils often reveal resistance mechanisms to additional pollution (stress factor) [24-26]. Many studies show that exposure to one type of stress (e.g. heavy metal pollution) may cause development of community tolerance toward this particular stress, but may also cause development of co-tolerance to another type of stress or disturbance (e.g. other metal, antibiotics). Results showed by Bérard et al. [26] demonstrate that microorganisms after a 21-day combined heat-drought disturbance were affected more than microbes pre-exposed to a 73-day severe drought. Tobor-Kaplon et al. [25] also showed that microbes from soils polluted with heavy metals and from unpolluted soils reveal no difference in resistance and resilience to additional chemical stress (lead pollution), but natural factor disturbances caused different stabilities in polluted and unpolluted soils.

The aim of the present study was to compare the respiratory response of microbial communities from unpolluted and long-term metal polluted soils (Zn, Cd, Pb) to additional chemical stress (Ni treatment) under different moisture levels (changes of soil moisture). We also wanted to check if microbial organisms from investigated soils developed resistance mechanisms to additional stressors.

Methods and Materials

Site Description and Soil Sampling

The soil samples were collected from the topsoil (depth 0-10 cm) of podzolic forest soils near a former Zn and Pb smelter, in the vicinity of Olkusz (southern Poland). Soils were taken from two remote sites located on a pollution transect: a polluted site located next to the Zn smelter (OLK P), and an unpolluted control site located 40 km away from the smelter (OLK C). The polluted site was heavily contaminated, mostly with Zn and Pb. Based on some studies done at the same sites by Stefanowicz et al. [27], we assumed that both studied soils (OLK P, OLK C) differed markedly in total heavy metals concentrations (6.151 mg Zn·kg^{-1} dwt soil, 2.206 mg Pb·kg^{-1} dwt soil and 109 mg Zn·kg^{-1} dwt soil and 157 mg Pb·kg^{-1} dwt soil) for polluted and unpolluted soil. However, the concentration of nickel was low and quite similar (18.6 and 5.18 mg Ni·kg^{-1} dwt soil). Both experimental plots are covered with Scots pine forest (*Pinus sylvestris*) with some birches (*Betula pendula*) admixture. At each site, a 100 m^2 square plot was set out, and three random samples were taken and directly sieved in situ (mesh size, 10 mm). Next, in the laboratory, soil samples were sieved through a 2 mm grid and thoroughly mixed on the day of collection. Soil was stored at 4ºC for 2 weeks before the experiment, in breathable cotton bags. At the beginning of the experiment, soils were transferred to plastic jars (100 ml), 15 g dwt of soil per jar.

Experimental Design

In the laboratory, soils from polluted and unpolluted sites were spiked with inorganic forms of nickel solution ($NiCl_2 \times 6H_2O$) in five different concentrations corresponding to 400, 800, 1.600, 3.200, and 6.400 mg Ni·kg^{-1} dwt soil. For both soils, control treatment was set up as well (0 mg Ni·kg^{-1} dwt soil). All soil samples (clean and contaminated with different doses of Ni) were incubated at constant temperature (22ºC) and under different levels of moisture (10%, 75%, and 120% WHC), which were controlled twice a week. Tap water was adjusted. Incubation jars were sealed by perforated lid, to limit excessive desiccation. Five replicates for each treatment were prepared, thus a total of 90 samples were used in the laboratory experiment (5 replicates × 3 levels of WHC × 6 Ni concentrations).

To test the effect of additional chemical stress on microbes from polluted and unpolluted soils in time, microbial respiration rate were performed after 30 (named experiment A) and 120 (named experiment B) days of incubation with nickel at constant temperature and varying soil moisture.

Physicochemical Analyses

Dry weight of soil was measured after 15 h of drying at 105ºC. Organic matter content in soils was determined as loss on ignition at 550ºC. Water holding capacity was assessed by standard gravimetric method; measurements

Table 1. Mean value of soil parameters.

Site	Ni* (mg/kg dwt soil)	Pb* (mg/kg dwt soil)	Zn* (mg/kg dwt soil)	pH_{H_2O}	%OM
OLK C-unpolluted	5.18	157	109	5.29	12.3
OLK P-polluted	18.6	2,206	6,151	5.41	28.0

*Stefanowicz et al. [27]

were made in 10 replicates for polluted and unpolluted soils. Soil pH was measured in H_2O at a 1:10 (w/v) ratio [28] with a digital pH-meter (CP-401 Elmetron, Poland).

For determination of exchangeable Ni forms (Ni^{2+}), 2g of air-dried soil was shaken (800 RPM) in 20 ml 0.01M $CaCl_2$ for 1h [29]. Concentrations of Ni were measured by graphite furnace atomic absorption spectrometry (AAnalyst 800, PerkinElmer, Boston, MA, USA).

Microbial Analyses

The microbial basal respiration rate (R) was measured as the amount of CO_2 (mmoles $CO_2 \cdot g^{-1}$ OM 24 h^{-1}) evolved from soil samples. Soil samples (20 g dwt soil) were adjusted to 10%, 75%, and 120% of their maximum water-holding capacity and incubated in gas-tight jars at 22ºC. The jars contained small vessels with 5 ml 0.2M NaOH. The evolved CO_2 was absorbed in NaOH solution. After opening the jars, 2 ml $BaCl_2$ was added to the NaOH and the excess hydroxide was titrated with 0.1M HCl, in the presence of phenolphthalein as dye indicator [30].

Statistical Analyses

Before statistical analyses, the skewed data (Ni concentrations) was respectively log-transformed to fulfil the normality criterion. Analysis of variance (one-way ANOVA) was performed to test differences in microbial respiration rate (R) and differences in concentration of exchangeable Ni in the investigated soils. Fisher least significant differences (LSD) test was run if significant differences were found ($p<0.05$). Correlation analysis was used to determine the relation of Ni exchangeable forms and pH to applied $NiCl_2$ dose. Pearson's correlation coefficient (r) was used to define the relationship between variables, significant differences were determined as $p<0.05$. To test the effect of interaction between soil sites (categorical factor), Ni and moisture (quantitative factors) on microbial respiration rate (R), the general linear model (GLM) was used. Non-significant interactions and variables were removed consecutively from the model as long there were any interactions/variables with $p<0.05$. The percentage of variance explained by the model was reported as r^2 value, standard error of means was determined as SE. To illustrate changes in the respiration rate in time (after 30 and 120 days of experiment) and pH dependence from applied $NiCl_2$ in OLK C and OLK P soil, comparison of regression lines was performed. For determination of nickel toxicity the EC_{50} values were estimated using the function:

$$y=100/(1+(\log Ni/EC_{50})\text{^}b)$$

...where: 100 – maximum effect (100% decrease of respiration rate), EC_{50} – concentration in which 50% effect is expected (50% decrease of respiration rate), and b – slope of regression curves. All analyses were performed with the Statgraphics Plus 5.0 software.

Results

Physico-Chemical Properties of Soils

The forest soils in the studied polluted (OLK P) and unpolluted (OLK C) areas differed significantly in organic matter content (OM) and heavy metals concentrations. The polluted sites (OLK P) had much higher organic matter content in comparison to the unpolluted sites (OLK C). Soil pH measured in both soils was slightly acidic.

The total concentrations of Pb and Zn were measured in OLK P and OLK C soils by Stefanowicz et al. [27]. Detailed data on total metal concentrations and other chemical characteristics is given in Table 1.

Correlation analysis showed that input of different Ni doses to investigated soils caused a significant pH decrease in polluted and unpolluted soils. The pH values measured at the end of the experiment were strongly negatively correlated with the applied Ni doses, especially in unpolluted soil ($p < 0.0001$, $r = -0.89$), whereas in polluted soil the correlation was weaker ($p < 0.0001$, $r = -0.56$). In OLK C soil, the pH decreased from 5.29 to 3.8, whereas in OLK P the decrease ranged from 5.41 to 4.82.

Exchangable Ni Forms

Different moisture levels did not significantly affect the Ni concentration in soils.

In both soils, the concentrations of the available form of nickel were strongly correlated with the applied doses of $NiCl_2$ ($p < 0.0001$, $r = 0.59$ for OLK C soil and $r = 0.52$ for OLK P soil). One-Way ANOVA performed to detect differences in concentrations of $CaCl_2$-extracted Ni showed that significantly higher amounts of extracted Ni were reported in clean soils ($p < 0.0041$; 95% LSD) in comparison to polluted soils (Table 2).

Respiration Rate

Analysis of variance (ANOVA) showed that irrespective of treatment (soil moisture, Ni pollution), the microbial

Table 2. $CaCl_2$-soluble nickel concentrations (mg·kg^{-1} dwt) in nickel-treated soils measured after 120 days of experiment.

Site	0 $NiCl_2$	400 $NiCl_2$	800 $NiCl_2$	1600 $NiCl_2$	3200 $NiCl_2$	6400 $NiCl_2$
OLK C-unpolluted	0.5	128.7	324.4	766.4	1,912	5,561
OLK P-polluted	0.8	35.3	99.4	243.0	758.2	3,224

Table 3. EC_{50} value of respiration rate for clean soil (OLK C) and polluted soil (OLK P) after 30 and 120 days of soil incubation at different moisture levels.

Storage	Soil	Moisture	EC_{50}	EC_{50}	Asymptotic 95.0% Confidence Interval	
					Lower	Upper
			Log Ni	Ni (mg·kg^{-1} dwt soil)	for mean	
30d	OLK C	10	3.47	2,951	3.34	3.60
30d	OLK C	75	3.26	1,820	2.82	3.69
30d	OLK C	120	4.40	25,119	3.40	5.40
30d	OLK P	10	3.86	7,244	3.56	4.17
30d	OLK P	75	3.19	1,549	3.07	3.31
30d	OLK P	120	3.75	5,623	3.47	4.03
120d	OLK C	10	3.57	3,715	3.43	3.71
120d	OLK C	75	2.36	229.1*	2.08	2.64
120d	OLK C	120	4.16	14,454	3.20	5.11
120d	OLK P	10	3.67	4,677	3.48	3.86
120d	OLK P	75	3.09	1,230*	3.00	3.18
120d	OLK P	120	3.38	2,399	3.24	3.52

*confidence intervals do not overlap

activity measured as respiration rate (R), regardless of time of measurement, was always higher in unpolluted (OLK C) than in polluted (OLK P) soils ($p<0.0001$). The ANOVA also showed that the highest value of respiration rate was found in optimal moisture conditions (75% maximum WHC) in both soils, irrespective of nickel contamination level ($p<0.0001$). Lower activity was observed in soils at 120% WHC, the lowest at 10% WHC, in both polluted (OLK P) and unpolluted sites (OLK C).

GLM analysis performed to test interaction effects of all investigated factors (soil type as categorical factor; moisture and Ni as quantitative factors) on respiration (R), after 30 (A) and 120 days (B) of the experiment, showed that all variables used in the models were highly significant ($p<0.0001$ for A and B).

The model explained 52.19% (A) and 53.36% (B) of variance. The R was the most dependent on soil type and Ni pollution in both models ($p<0.0001$), as well as on soil moisture ($p<0.0001$ and $p<0.0004$ for A and B, respectively). Microbes from OLK P and OLK C, shortly after Ni spiking (A), reacted to Ni pollution in similar ways ($p<0.05$, Fig. 1), but R activity after 120 days of experiment (B) differed significantly between soils ($p<0.001$, Fig. 2). Soil type or moisture conditions didn't affect Ni toxicity in soils, thus interactions were removed from the models.

Comparison of regression lines performed to test the effects of additional stress on microbial activity in time also showed that after 30 days of the experiment Ni contamination affected microorganism activity (R), likewise in both soils ($p<0.0618$ for the slopes; Fig. 3 A). In turn, after 120 days the respiration rate in unpolluted soil decreased significantly, while microbes from soils already affected by heavy metals revealed slight R inhibition ($p<0.0001$ for the slopes, Fig. 3 B). OLK C soil had higher respiration rates in both models ($p<0.0001$ for intercept, Figs. 3 A and B).

EC_{50} value of the respiration rate calculated for soils incubated in optimal conditions clearly showed that microorganisms in OLK C soil after 120 days of incubation were much more sensitive to nickel treatment than soils originating from an area already polluted by heavy metals. The effect of Ni pollution was especially noticeable in optimal moisture conditions (75% WHC, Table 3). The smallest dose of Ni (400 mg·kg^{-1} dwt soil) caused a rapid decrease in the respiration rate in unpolluted soil (OLK C), while a corresponding dose applied to contaminated soil (OLK P) did not cause a similar effect (asymptotic 95%

confidence intervals do not overlap). In suboptimal moisture conditions (10 and 120% WHC), the difference in respiration inhibition between the soils was not reported, R strongly declined in both clean and polluted soils.

Discussion

Complex interactions between heavy metal pollution and changing environmental factors, such as soil moisture, temperature, organic matter content, or pH make it difficult to distinguish which factor has the greatest impact on the microbial parameters and what level of metal contamination is still safe for the soil ecosystem. The problem is especially important in ecological risk assessment. Having complex toxicological data is critical to the development of mathematical models and simulations for better understanding the distribution and toxicity of pollutant mixtures to organisms and, furthermore, for efficient decision making [31].

Modeling laboratory experiment showed that additional chemical and water stress had a strong impact on microbial activity measured as respiration, but it depends on the level of metal pollution in soils.

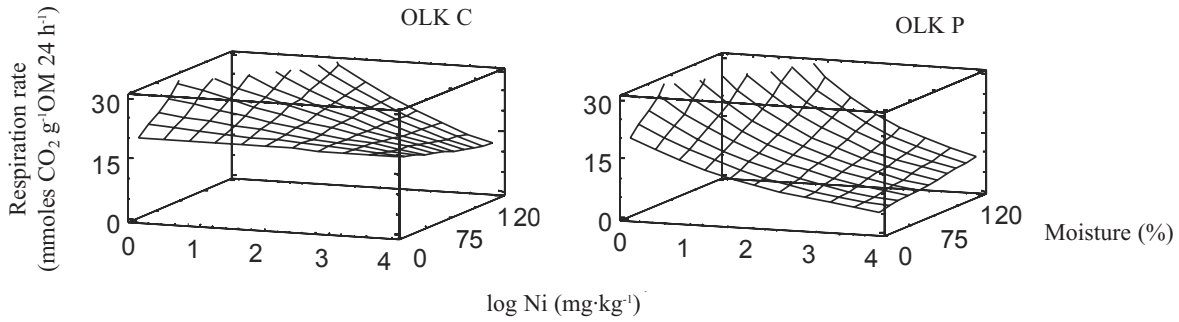

Fig. 1. Relationship between respiration rate (R) and soil moisture and concentration of Ni (log) after 30 days of experiment in OLK C (r^2 = 61.1, SE = 5.7) and OLK P soils (r^2 = 64.4, SE = 2.7) at optimal moisture level (75% WHC).

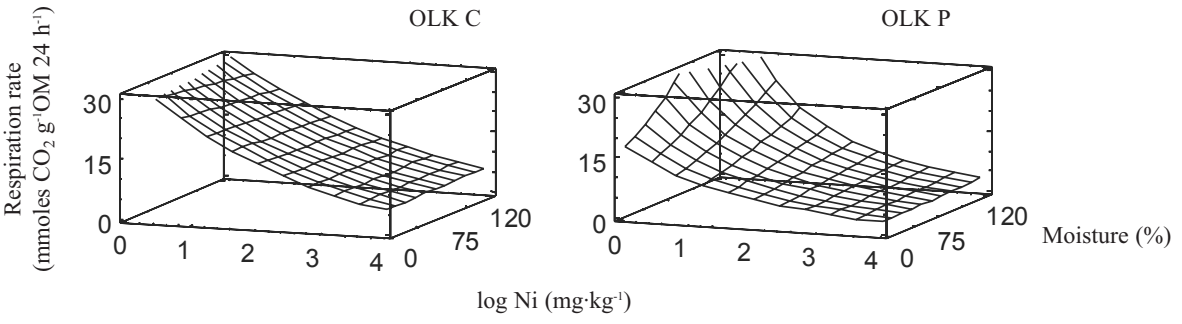

Fig. 2. Relationship between respiration rate (R) and soil moisture and concentration of Ni (log) after 120 days of experiment in OLK C (r^2 = 59.1, SE = 2.6) and OLK P soils (r^2 = 67.2, SE = 1.5) at optimal moisture level (75% WHC).

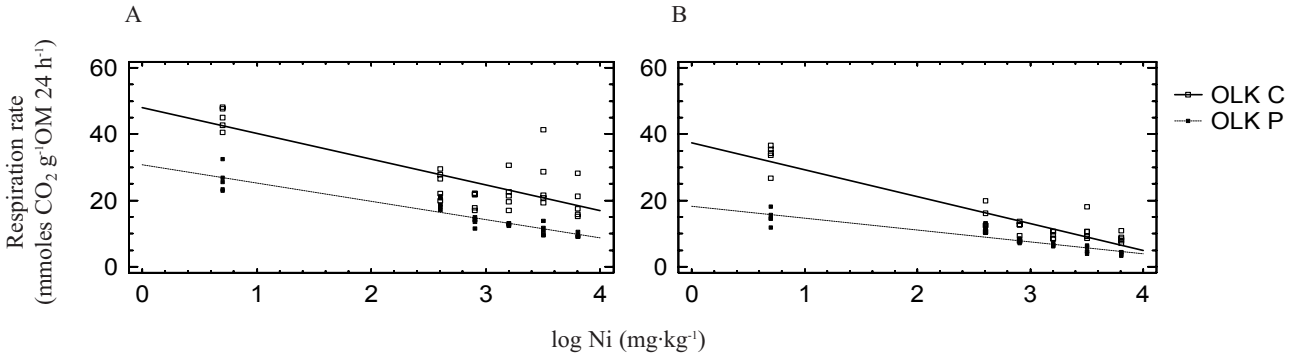

Fig. 3. Comparison of regression lines for nickel effects on the respiration rate of OLK C and OLK P soils after 30 days (A) and 120 days (B) of incubation. Solid line for OLK C (A: y = -7.762x + 48.0079, $p < 0.0618$ for slopes, $p < 0.0001$ for intercept, r^2 = 28.1; B: y = -8.128x + 37.4055, r^2 = 39.4) and dashed line for OLK P (A: y = -5.515x + 30.7909, r^2 = 39.2; B: y = -3.558 + 18.2497; $p < 0.0001$ for intercept and slopes, r^2 = 47.5).

The study showed that additional chemical stress (Ni) inhibited microbial activity in both investigated soils, but the effect was significantly modified by natural factors, such as shifts of soil moisture, pH changes, soil organic matter content – and also most importantly – time of interaction.

Inhibition of the respiration rate due to Ni pollution was especially reported in optimal moisture conditions, while a similar effect was not reported in unfavorable moisture conditions (10, 120% WHC), where microbial activity was strongly inhibited in both studied soils.

An explanation for this phenomenon might be the fact that irrigation that exceeds the soil water holding capacity (120% WHC) could disturb air diffusion in soil pores, and thus access to oxygen. On the other hand, strong desiccation stress (10% WHC) could limit substrate bioavailability, which resulted in a dormant state or death of microorganisms [5]. Similar relationships between soil moisture and microbial activity were observed by Barros et al. [32]. The authors demonstrated, with calorimetric methods, that microorganisms had higher heat emissions and growth rate in optimal moisture conditions in comparison to drought conditions.

It is worth to notice that not only changes the level of moisture, but also pH decrease itself had an adverse effect on measured microbial respiration rate (R). Soil acidification is commonly reported as being unfavorable for microbial parameters, such as total microbial activities, bacterial growth rates, abundance and microbial diversity [10-12]. Moreover, any pH decrease influences heavy metal mobility by increasing the bioavailability of metals and, therefore, enhancing their toxicity [33, 34]. However, some authors report that higher heavy metals pore-water concentrations (Zn, Cd) due to acidification of polluted soils could be not accompanied by increased toxic effects on microorganisms [35, 36]. The decreased metal toxicity observed at low pH might be caused by competition of H^+ with metal cations at the uptake systems of microorganisms [21].

In our experiment, pH significantly decreased due to Ni salt addition to polluted (OLK P) and unpolluted (OLK C) soils, which could affect the microbial activity.

However, it is interesting to see the effect of time of exposition to additional stress as adverse influence on microbial activity. Just after Ni application to soils, microbes from both soils revealed a decrease in R to a similar extent, in order to $NiCl_2$ concentration. However, with prolonged time of exposition to additional toxicant, microbes from clean soil demonstrated higher sensitivity to unfavorable conditions in comparison to communities already affected by metals.

The respiratory inhibition in OLK C soil, after 120 days of experiment, might be explained by fact that Ni applied to soil was in varying degree absorbed by soil particles, and thus availability of Ni was changed. Unpolluted OLK C soil was poor in organic matter, had lower pH, thus microbes could be affected by higher concentration of bioavailable metal ions. Polluted soil (OLK P) had twice as high amount of organic compounds as the unpolluted soil (OLK C) and it is possible that more metal ions were bound with soil particles; the pH in OLK P soil was also higher. The results of Van Beelen [35] show that due to pH increase in soil, zinc and cadmium reveal more sorption to soil particles. Our results for concentration of exchangeable forms of Ni in soils seem to confirm this regularity (Table 2). Soil concentration of available Ni in OLK P is lower than in OLK C. Similarly, in other study of Van Beelen et al. [37], it was reported that high percentage of organic matter decreased the bioavailability of Cu.

During experimental time (120 days) metal added to the soils could be less available because of soil aging processes. Recent studies show that over time, heavy metals in contact with soil particles are less exchangeable, thus less available to soil organisms [38-41]. Oorts et al. [39] confirmed in their studies that Ni solubility declines with aging and the effect is noticeably pronounced, compared to Cu and Zn. Similarly, Co availability to organisms in time is reported to be drastically lower in comparison to Ni or Cu. The aforementioned mechanism may be a result of several processes, such as metal incorporation into mineral structures or chemical complexations with soil solids, diffusion into pore spaces within minerals, precipitation or mineral surface oxidation [41].

Thus, higher sensitivity of microbes from clean soils, after longer exposure to stress, might be caused by higher amount of free ions in soil solution, but it is as well possible that organisms had limited access to an easily degradable carbon source, which declines with time [42-44]. Microbial communities from unpolluted soil could be especially exposed to the above mentioned stress due to lower, basic content of OM in soil. Niklińska et al. [42] reported that the slope of the relation between the respiration rate and metal dose was less steep after 125 day storage than 75 days, what confirms the hypothesis about declined access to easily degradable organic particles.

The above-mentioned factors had an undeniably strong impact on the studied soils. But it has to be taken into account that, despite favorable OM content and lower concentration of Ni ionic forms in polluted soils, microorganisms in OLK P were strongly affected by very high pollution from Zn, exceeding 6.000 $mg \cdot kg^{-1}$ dwt soil, and Pb, exceeding 2.000 $mg \cdot kg^{-1}$ dwt soil, which must have had an adverse effect on the whole microbial community.

Therefore, results of the experiment showing higher stability of R in OLK P soil, after longer exposure to additional chemical stress, suggest that co-tolerance mechanisms to Ni, as additional stress, could occur. It is frequently reported that selective pressures from a metal-polluted environment may lead to the development of resistance response to heavy metals [16, 24, 33, 38, 39, 43-46]. To test this hypothesis, more sophisticated analyses are needed.

Changes in environmental conditions, such as shifts of soil pH or soil moisture may remobilize fixed pools of metals and increase their toxicity to soil environment. Therefore, wide measurements of microbial parameters are critical in order to understand the environmental stressors' impact on soil, as there is still a lack of sufficient toxicological data on the effects of interaction between metal pollution and natural factors for reliable decision making in risk assessment.

Conclusions

The obtained results proved that freshly spiked soils, even with the smallest dose of Ni treatment, inhibited microbial respiration rate both in unpolluted and long-term Zn/Pb-polluted soil, especially in unfavorable natural conditions (pH, moisture or OM content).

But microbes from polluted and unpolluted soils react differently to additional stressors, regardless of whether they are natural disturbances (water level) or chemical pollutants (Ni).

It is worth noting that, regardless of the level of laboratory exposure to Ni microbes from the clean soils are more sensitive (less active) than microorganisms from soils polluted with metals long-term. That effect could be enhanced by other natural factors like pH and lower access to easily degradable OM. Therefore, it must also be taken into account that in unexpected environmental conditions additional natural or anthropogenic abiotic factors may drastically affect toxicity of metals, thus soil microbial processes. Consequently, because the interaction of different factors may have an unpredictable effect, all toxicity tests must be adjusted to assessment of the effect of interactions between numerous factors on microbial communities, because the same dose of pollutant may influence the soil microbial community differently due to natural factor changes.

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