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Relationships
Between Microbial and Chemical Properties
in Mine Soils Reclaimed for Forestry**

1. Introduction

One of the most important goals of post-mining barrens reclamation is to create stabile and productive ecosystems with effective nutrient cycling. In all terrestrial ecosystems soil microorganisms are of crucial importance for element cycling [3]. Therefore, soil microbial properties are often used in the assessment of reclamation success [7, 9]. The most commonly used microbial properties include microbial biomass, basal respiration and activities of some soil enzymes – in particular dehydrogenase, phosphatase and urease [7]. In recent years measurements of soil microbial diversity have become increasingly popular in the assessment of reclamation success [9]. One of the most commonly used methods for microbial diversity assessment is Biolog® – an assay based on the measurement of a number of sole carbon substrates by microbes extracted from a soil [10].

One of the most important limitations in the use of microbial properties in reclamation success assessment is a short time over which the soil samples may be stored prior to the analysis. Since the microbial methods are often time consuming and expensive, usually only a limited number of analyses can be carried out. In a comprehensive soil analysis usually several microbial properties should be included [9]. Therefore, the chosen properties should maximize information on the soil microbial communities.

The objectives of this work were to assess the relationships between different microbial properties of mine soils reclaimed for forestry and to assess dependence of the microbial properties on chemical and physico-chemical soil properties. The chosen microbial properties are commonly used in reclamation success assessment [7–9].

2. Material and Methods

2.1. Soil SAMPLING

Samples (n = 90) of the uppermost 5 cm of mineral soils were taken from sites covered by pure Scots pine (*Pinus sylvestris*), birch (*Betula pendula*), larch (*Larix decidua*) and common alder (*Alnus glutinosa*) forest stands, and from mixed pine--alder and birch-alder stands located in the areas reclaimed by sand quarry Szczakowa and lignite mine Bełchatów (external dump of the mine). All the sampled soils were light textured (sands and loamy sands). Each sample consisted of five sub-samples (area of each subsample = 0.16 m^2) taken at the corners and in the middle of 3 m × 3 m square.

The samples were sieved (2 mm mesh) and divided into two parts. One part was air-dried and used for physical, physico-chemical and chemical analyses, and the other was stored at 4°C and used for microbial analyses. Prior to microbial analyses the samples were adjusted to 50% of the maximum water holding capacity (WHC) and pre-incubated at 22°C for 6 days.

2.2. Chemical and Physico-Chemical Analyses

The pH of the samples was measured in 1M KCl solution (soil : liquid ratio 1:2.5, w:v) with a digital pH-meter (CP-401, ELMETRON). Content of C and N was determined by dry combustion with a CN analyzer (Vario Max, Elementar Analysensysteme GmbH).

2.3. Microbiological Analyses

To measure basal respiration and microbial biomass (C_{mic}), samples (50 g d.w.), unamended for basal respiration measurements and amended with 100 mg glucose monohydrate for C_{mic} measurements were incubated at 22°C in gas-tight jars. The incubation time was 24 hours for determination of basal respiration and 4 hours for C_{mic}. The jars contained small beakers with 5 ml 0.2M NaOH to trap the evolved CO_2. After the jars were opened, 2 ml of 1M $BaCl_2$ was added to the NaOH and the excess of sodium hydroxide was titrated with 0.1M HCl in the presence of phenolphthalein as indicator. Microbial biomass was calculated from the substrate induced respiration rate according to the equation given by Anderson and Domsch [2]: C_{mic} [mg g^{-1}] = 40.04y + 0.37, where y is ml CO_2 h^{-1} · g^{-1}.

Dehydrogenase (DHG) activity was determined according to von Mersi [19]. The soil samples (1 g d.w.) were mixed with 1.5 ml Tris buffer (pH 7) and 2 ml 0.5% INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenyl tetrazolium chloride) solution, and incubated at 40°C for 2 h. The reduced iodonitrotetrazolium formazan

(INTF) was extracted with 10 ml dimethyloformamid/ethanol (1:1) and measured photometrically at 464 nm. DHG activity was expressed as µg INTF $g^{-1} \cdot h^{-1}$.

Acid phosphatase (AcPHP) activity was measured as described by Margesin [13]. The soil samples (1 g d.w.) were mixed with 1 ml disodium p-nitrophenyl phosphate solution (115 mM) and 4 ml buffer solution (pH 6.5) and incubated at 37°C for 1 hour. The p-nitrophenol released by phosphatase activity was extracted and colored with NaOH and determined photometrically at 400 nm. AcPHP activity was expressed as µg p-NP $g^{-1} \cdot h^{-1}$.

Urease (URE) activity was determined as described by Kandeler [11]. The soil samples (5 g d.w.) were mixed with 2.5 ml urea (720 mM) and 20 ml borate buffer (pH 10) and incubated at 37°C for 4 hours. The released ammonium was extracted with acidified potassium chloride solution, coloured in the modified Berthelot reaction and measured photometrically at 690 nm. URE activity was expressed as µg N $g^{-1} \cdot h^{-1}$.

The physiological profiles of the microbial communities were analyzed using Biolog® Ecoplates containing 31 different C substrates [10]. Samples (10 g d.w.) were shaken for 60 min in 20 ml of a 10 mM Bis-Tris solution (pH 7) and allowed to settle for 30 min. Then the extracts containing microbes were decanted and diluted with Bis-Tris solution to obtain 0.5 µg C_{mic} in 1 ml solution. Then the solutions were inoculated on microplates (100 µl per well) and incubated at 22°C. Substrate utilization was monitored by measuring light absorbance at 590 nm. The first measurement was made immediately after inoculation, and the subsequent ones at 12 h intervals for 6 days. The readings for individual substrates were corrected for background absorbance by subtracting the absorbance of the control (water) well. The corrected absorbance values were used to calculate the area under the absorbance curve (AUC). The calculated AUC values were standardized by dividing them by the average area under the curve (AAUC) [10]. The standardized AUC values were used for characterization of community-level physiological profiles and statistical analyses. Average area under curve (AAUC) was used to express overall microbial activity on the plates.

2.4. Statistical Analysis

All analyses were performed in triplicate and the mean values were used in further calculations. The data presented in the text are mean values of eight samples per site.

The functional diversity of microbial communities was calculated using the Shannon index: $H' = \sum_{i=1,...,n} p_i (\ln p_i)$, where n is the number of wells and p_i is the use of the ith substrate (AUC value) as a proportion of the sum of the use of all substrates on a plate.

PCA performed on correlation matrix was used to analyze physiological profiles of microbial communities (Statgraphics Plus 5.1 software, Statistical Graphics Corporation).

Relationships between the analyzed properties were studied using Spearman rank correlations. The Spearman correlation does not require normal distribution of the analyzed properties and does not assume linear relationship between the properties. Significance level for the relationships was calculated with Bonferroni correction and set up on $p = 0.0005$. The strength of correlations was assessed according to values of Spearman correlation coefficients (r) using the following criteria: $|r| = 0.90–1.00$ – very strong correlation, $|r| = 0.70–0.89$ – strong correlation, $|r| = 0.50–0.69$ – moderate correlation, $|r| = 0.30–0.49$ – weak correlation, $|r| < 0.29$ – very weak correlation.

3.   Results and Discussion

The C content in the analyzed samples varied between 3.3 mg·g^{-1} and 60.3 mg·g^{-1} and N content between 0.11 mg·g^{-1} and 3.81 mg·g^{-1} (Tab. 1). The C/N ratio in most of the samples was narrow and pH values low. Low contents of C and N in the reclaimed mine soils resulted probably from low contents of these elements in substrates from which the mine soils developed. The substrates did not contain organic matter and the increase of C and N in mine soils – in particular those light textured – is very slow [5].

Table 1. Microbial and chemical properties of the studied soils

Property	Units	Percentile						
		min	10%	25%	50% median	75%	90%	max
C_{mic}	µg·g^{-1}	18	40	100	191	299	450	757
RESP	µM CO$_2$ g^{-1}·24 h^{-1}	0.31	0.60	1.01	6.36	17.48	28.92	36.99
DHG	µg INTF g^{-1}·h^{-1}	7.3	13.7	17.5	28.6	52.3	76.2	135.0
AcPHP	µg p-NP g^{-1}·h^{-1}	21	28	40	135	241	389	761
URE	µg N g^{-1}·h^{-1}	1.0	1.8	3.4	6.2	10.2	18.3	47.0
AAUC		64.4	75.0	83.5	96.1	112.0	122.7	149.7
H'		2.90	3.00	3.07	3.19	3.24	3.27	3.33
PC1		–5.76	–3.61	–2.92	–0.17	2.65	4.02	8.28
PC2		–5.37	–2.81	–1.37	–0.13	1.82	2.80	4.60
C	mg·g^{-1}	3.3	4.4	6.0	10.5	21.3	30.9	60.3
N	mg·g^{-1}	0.11	0.18	0.20	0.54	1.20	1.80	3.81
C/N		14.8	15.5	18.0	20.9	26.4	32.4	39.7
pH$_{KCl}$		3.4	3.6	3.8	4.1	5.6	6.6	7.4

Most of the samples exhibited low values of C_{mic}, RESP and dehydrogenase, acid phosphatase and urease activities (Tab. 1). The C_{mic} strongly correlated with C and N contents ($r = 0.87$ and 0.84, respectively) but only moderately with RESP ($r = 0.66$) (Tab. 2). The RESP was positively related to C and N contents but the dependence was weaker than in the case of the C_{mic}. In turn, stronger was the correlation between RESP and pH ($r = 0.74$). The enzyme activities, C_{mic} and RESP correlated with C/N ratio, however in all cases the relationships were negative ($r = -0.51$ to -0.79). The obtained results indicate that the growth of microbes in the studied soil is limited by low contents of C and N. However, negative correlation with C/N ratio suggests that N content limits the microbial growth to a larger extent than C content. Positive relationship between microbial biomass and activity and organic matter content has been commonly reported in natural forest soils [3].

Low values of C_{mic}, RESP DHG, AcPHP and URE in the studied soils resulted also from their light texture. Heavier textured soils may contain larger microbial biomass as they provide better protection from faunal predation and are characterized by less fluctuation of water availability [6, 14]. Another reason for low C_{mic} and RESP in the studied soils might have been low pH of the soils. Bauhus and Khanna [3] reported pH to be one of the most important factors affecting microbial biomass in the forest soils.

Activities of all analyzed enzymes were strongly or very strongly correlated with each other and C_{mic} ($r = 0.81–0.91$). For DHG the strong correlation with C_{mic} was expected as dehydrogenases are intracellular enzymes that exist only in living cells [15] and C_{mic} was determined using substrate induced respiration (SIR) the method that measures mainly the active part of soil microbial community. Urease and acid phosphatase are hydrolases involved in cycling of N and P, respectively. Urease plays an important role in the release of NH_4^+ from non-urea [4], whereas acid phosphatase catalyses the hydrolysis of organic P esters to inorganic P [18]. In natural soils high activities of these enzymes may indicate N and P deficiency [17]. However, exact interpretation of the results may be hindered since enzyme activities depend also on the microbial biomass and soil C content [1]. Strong and very strong correlations of AcPHP and URE with C_{mic} and the strong positive correlation between URE and N content ($r = 0.83$) indicate, that in the studied mine soils activity of acid phosphatase and urease depended on the C_{mic} and organic matter content and did not inform of the availability of P and N for soil microbes.

PCA analysis reduced the number of variables describing physiological profiles of soil microbial communities to two principal components (PC1 and PC2), which explained 30.2% and 14.9% of variance in the Biolog® data, respectively. The PC1 values varied from -5.76 to 8.28 and the PC2 values from -5.37 to 4.60 (Tab. 1).

Table 2. Spearman rank correlation coefficients (r) between microbiological and chemical properties of mine soils reclaimed for forestry

	RESP	DHG	AcPHP	URE	AAUC	H'	PC1	PC2	C	N	C/N	pH_{KCl}
C_{mic}	0.66	0.89	0.87	0.86	n.s.	0.53	0.40	-0.42	0.87	0.84	-0.62	0.70
RESP		0.69	0.66	0.67	0.53	0.72	0.66	-0.69	0.65	0.67	-0.51	0.74
DHG			0.91	0.81	0.38	0.54	0.44	-0.41	0.90	0.90	-0.78	0.66
AcPHP				0.82	n.s.	0.53	0.41	-0.38	0.93	0.93	-0.79	0.59
URE					0.37	0.51	0.44	n.s.	0.84	0.83	-0.64	0.63
AAUC						0.84	0.98	n.s.	n.s.	n.s.	n.s.	0.58
H'							0.88	n.s.	0.47	0.48	-0.41	0.73
PC1								n.s.	0.37	0.39	-0.36	0.65
PC2									-0.38	-0.38	n.s.	-0.44
C										0.98	n.s.	0.61
N											-0.86	0.56
C/N												n.s.

Presented values are significant at $p < 0.0005$; n.s. – statistically not significant dependence, C_{mic} – microbial biomass, RESP – basal respiration, DHG – dehydrogenase activity, AcPHP – acid phosphatase activity, URE – urease activity, AAUC – mean activity on the Biolog® microplates, H' – Shannon index, PC1 and PC2 – principal components based on Biolog® data.

Despite the same inoculum density used in the Biolog® analysis the microbial activity on the plates (AAUC) differed from 64.4 to 149.7 and the Shannon index from 2.90 to 3.33. Most of the Biolog® derived parameters correlated with C_{mic}, RESP and soil enzyme activities but usually the relationships were weak (Tab. 2). This means that Biolog® assay carries additional information on the soil microbial communities. The AAUC values did not correlate with C_{mic} indicating effectiveness of the applied inoculum standardization.

Despite the standardization, AAUC correlated positively with RESP ($r = 0.53$) indicating that microbial communities that are active in soils exhibit also high activity on the plates. Biolog® assay has been criticized as it does not regards the whole soil microbial community but only its part that is able to survive under the experimental conditions on the plates [10].

Positive correlation between AAUC and RESP suggest that the results of Biolog® analysis may be regarded as representative for the entire soil microbial community.

Soil pH had the strongest effect on the Biolog® derived parameters (Tab. 2). Importance of soil pH for physiological profiles has been previously shown for organic layers in the Olkusz region [16]. Shannon index H' correlated strongly with RESP ($r = 0.72$) and depended on C_{mic} ($r = 0.53$). Positive correlation between H' and C_{mic} indicates that larger microbial communities were also more functionally diverse. Similar dependence was observed by Yan et al. [20] in tropical soils and Lynch et al. [12] reported that to a certain threshold an increase of microbial biomass results in increased functional diversity of soil microbial communities.

4. Conclusions

1) Most of microbial properties were correlated with each other. Biolog® derived parameters weakly correlated with C_{mic} and enzyme activities what indicates that they bear complementary information on soil microbial communities.

2) Activities of acid phosphatase and urease depended mainly on microbial biomass, and indirectly on C and N contents and did not bear information on the availability of P and N. Dehydrogenase activity and C_{mic} were highly correlated with each other indicating that these two analyses reveal the same information on soil microbial communities.

3) Positive correlation between Shannon index based on Biolog® data and microbial biomass indicates that an increase of biomass in the mine soil increases also physiological abilities of soil microbial communities.

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