Development of microbial properties in a chronosequence of sandy mine soils

Marcin Chodak a,*, Marcin Pietrzykowski b, Maria Niklińska c

a Department of Open-strip Mining, AGH University of Science and Technology, al. Mickiewicza 30, 30-059 Kraków, Poland
b Department of Forest Ecology, Agricultural University, al. 29 Listopada, 31-425 Kraków, Poland
c Institute of Environmental Sciences, Jagiellonian University, ul. Gronostajowa 7, 30-387 Kraków, Poland

1. Introduction

Areas degraded by open-cast mining are often reclaimed for forestry. The ultimate goal of this kind of reclamation is to restore a stable (i.e., able to withstand a disturbance) and productive forest ecosystem. This goal can be achieved only if soil functionality is restored. Soil microbial communities are crucial to the functioning of soils. This is because soil microbes are responsible for establishing biogeochemical cycles and for energy transfer, and are involved in forming soil structure (Diaz-Raviná et al., 1993; Bauhus and Khanna, 1999; Preston et al., 2001). Several gross microbial properties such as the amount of microbial biomass, soil respiration rate and metabolic quotient (Graham and Haynes, 2004; Frouz and

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* Corresponding author. Tel.: +48 12 617 2197; fax: +48 12 617 3546.
E-mail address: chodak@agh.edu.pl (M. Chodak).

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doi:10.1016/j.apsoil.2008.11.009
Novakova, 2005; Šourková et al., 2005) have been used to assess soil development in reclaimed post-mining lands. For numerous chronosequences of reclaimed mine soils a gradual increase of organic C and microbial biomass has been reported (Ruzek et al., 2001; Graham and Haynes, 2004; Šourková et al., 2005). Increasing contents of Corg and microbial biomass may result in increased functional diversity of soil microbial communities (Yan et al., 2000) and thus increased functionality and stability of soil ecosystems (Degens et al., 2001; Lynch et al., 2004).

In recent years, different analytical methods of assessing soil microbial diversity have been proposed as measures of ecosystem restoration (Mummey et al., 2002a,b; Graham and Haynes, 2004; Machulla et al., 2005). Measurement of the functional diversity is considered to provide information relevant to the functioning of soils (Nannipieri et al., 2003; Graham and Haynes, 2004). One of the methods that can be applied to measure functional diversity of microbial communities is the Biolog® test. The Biolog® test, a method of analyzing the metabolic abilities of microbial communities, uses a number of sole carbon substrates. The carbon substrate utilization pattern (referred to as the community-level physiological profile – CLPP) can potentially provide information on the functional abilities of the microbial community. CLPPs have been used to assess the influence of plants on soil microbes (Grayston et al., 1998), the effects of acid precipitation and pollutants on soil microbial communities (Fritze et al., 2000; Pennanen, 2001) and the effects of forest management techniques on soil microbes (Pietikäinen et al., 2000). However, the Biolog® assay has several limitations. The method is sensitive to inoculum density (Insam, 1997) and selects for culturable microorganisms capable of growing under experimental conditions (Garland and Mills, 1991). Thus, Biolog® CLPPs reflect the functional abilities not of the entire microbial community but of limited subset of microbial genera (Ros et al., 2008). Furthermore, the method favors fast-growing bacteria and structural changes may occur during the incubation (Smalla et al., 1998). Despite these drawbacks, Biolog® test could be a valuable tool for evaluating mine soil rehabilitation processes as it is a rapid and convenient analytical technique.

During afforestation of post-mining barrens seedlings of selected tree species are planted in appropriately prepared and fertilized spoil material, and the growing plants transform the raw parent material into soil. Sometimes vegetation from primary succession on the abandoned mine land is used in reclamation (Jochimsen, 1996), since the succession of plants may lead to spontaneous soil formation. Frouz and Novakova (2005) reported that gross microbial properties (FDA activity, respiration, microbial biomass) in mine soils spontaneously developing on brown coal mining heaps were similar to or higher than the levels in reclaimed mine soils. However, little is known about the functional diversity and metabolic abilities of microbial communities in spontaneously developing mine soils.

The objective of this study was to compare the temporal development of microbial biomass, soil respiration, potential N mineralization rate and the physiological profiles of microbial communities in mine soils reclaimed for forestry and in mine soils developing under vegetation from natural succession. Microbial properties measured in the natural forest soils were used as a reference.

2. Materials and methods

2.1. Study site

The study was carried out in Upper Silesia, Poland (19°26’E; 50°16’N) on the grounds of the Szczakowa open-cast sand quarry and its surroundings. The climate is temperate, with mean annual precipitation of ca. 700 mm and mean annual temperature of 8 °C. Soils of the study area (mainly Podzols) developed from sands. The sand deposits are fluvioglacial Quaternary sediments of a pre-Quaternary morphological depression. The dominating tree species in the forests surrounding the sand quarry is Scots pine (Pinus sylvestris), which constitutes 72.9% of the forest stands, followed by common birch (Betula pendula) constituting 16.2% of the stands (data from the Chrzanów Forest District administration).

The Szczakowa open-cast quarry has been extracting sand since 1954. Mining created an open cast 5–25 m deep, covering over 2700 ha. Since the late 1950s it has been reclaimed and reforested. The standard reclamation procedure included forming and leveling off the surface and adding an organic amendment (approx. 300 m³ ha⁻¹). The added amendment was a mixture of forest floor (O horizon) and mineral horizons (horizons A₀, E and partly B) with average organic C content of 0.3–1.0%, collected from forest soils in areas to be mined (Strzyszczcz, 2004). Then the reclaimed sites were limed (1.5 Mg dolomite ha⁻¹), and lupine (Lupinus latius L.) was cultivated for 2 years. The lupine cultivations were fertilized with NPK (140 kg N ha⁻¹, 300 kg P₂O₅ ha⁻¹, 180 kg K₂O ha⁻¹). After 2 years, the lupine biomass was ploughed into the soil as green manure and the sites were afforested with 1-year-old seedlings of Scots pine (P. sylvestris), common birch (Betula pendula) and some other deciduous trees. Over the last 25 years, certain modifications were introduced to the reclamation methods; these included cessation of liming, decreasing the quantity of organic amendment and NPK mineral fertilizers (information from the Szczakowa Sand Quarry). Changes in reclamation procedures caused the reclaimed sites sampled in our study were to be treated differently. The oldest site was reclaimed according to the standard procedure as described above. The intermediate site was not limed but received the same amount of organic amendment and mineral fertilizers as the oldest one. At the youngest reclaimed site no liming was applied, NPK fertilizers were applied at lower rates (50 kg N ha⁻¹, 140 kg P₂O₅ ha⁻¹, 120 kg K₂O ha⁻¹) and the organic amendment (amount decreased to approx. 30 m³ ha⁻¹) was not spread over the entire reclaimed area but applied directly under the seedlings during planting.

In the 1970s and 1980s, some parts of the open cast were abandoned, only to be mined again some 10–20 years later. They were eventually abandoned due to falling demand for sand. Vegetation appeared there spontaneously, and initial soil formation began by natural primary succession. At the abandoned sites, herbaceous communities dominated by gray hair-grass (Corynephorus canescens L.) were the first to establish. They were followed by groups of trees with over 50%
domination of Scots pine (P. sylvestris L.) and common birch (Betula pendula Roth.), with occasional trembling poplar (Populus tremula L.) (Pietrzykowski, 2005).

2.2. Soil sampling

Samples of mineral soil (0–5 cm) were taken in October 2006 at ten sites (20 m × 50 m): a degraded site prior to reclamation (MS), a site after 2 years of lupine cultivation (LUP), reclaimed post-mining sites afforested with Scots pine (6, 20 and 28 years old – R6, R20 and R28, respectively), post-mining sites with spontaneously developing pine forest stands (6, 20 and 27 years old – S6, S20 and S27, respectively), and two natural pine forest stands as reference soils (forest stands under 30 and 108 years old – F30 and F100, respectively; Table 1). At each site, eight mixed samples consisting of five subsamples (area of each subsample = 0.16 m²) were taken. 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 8°C for 14 days. Maximum water holding capacity (WHC) was determined gravimetrically according to Schlichting and Blume (1966).

2.3. Physical, physico-chemical and chemical analyses

The pH of the samples was measured in deionized water and 1 M KCl solution at a 1:10 soil:liquid ratio (w:v) using a digital pH-meter (CP-401, ELMETRON). The content of organic C (Corg) and total N (Nt) was determined by dry combustion using a CN analyzer (Vario Max, Elementar Analysensysteme GmbH). Available P (Pav) was measured according to the Egner-Riehm method (Lityński et al., 1976). Briefly, the soil samples (2 g) were shaken with 40 ml of 0.04 M calcium lactate at pH 3.7. Then, the suspensions were filtered through a fine-pore filter. The P concentration in the filtrates was determined colorimetrically with a continuous flow auto-analyzer (FIA-compact, MLE, Dresden, Germany). N mineralization was calculated as the difference in mineral N concentrations between the extracts obtained before and after incubation of the samples and based on daily mineralization rates.

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Soil texture was determined using the hydrometric method of Sheldrick and Wang (1993) in composite samples made by mixing eight samples from each sampling site.

2.4. Microbial analyses

To measure basal respiration (RESP) and microbial biomass (Cmic), samples (50 g d.w.) unamended for RESP measurements and amended with 100 mg glucose monohydrate for SIR measurements were incubated at 22°C in gas-tight jars. The incubation time was 24 h for determination of RESP and 4 h for Cmic. The jars contained small beakers with 5 ml 0.2 M NaOH to trap the evolved CO2. After opening of the jars, 2 ml of BaCl2 were added to the NaOH and the excess of hydroxide was titrated with 0.1 M HCl in the presence of phenolphthalein as an indicator. Cmic was calculated from the substrate-induced respiration rate according to the equation of Anderson and Domsch (1978): Cmic [mg g⁻¹] = 40.04y + 0.37, where y is given in ml CO2 h⁻¹ g⁻¹.

For determination of the potential N mineralization rate (Minₙ), samples (50 g) were incubated aerobically at 22°C for 42 days at 50% of their maximum WHC. Subsamples were extracted with 2 M KCl directly before and after incubation. Mineral N (NO3-N + NH4-N) in the extracts was measured colorimetrically with a continuous flow auto-analyzer (FIA-compact, MLE, Dresden, Germany). N mineralization was calculated as the difference in mineral N concentrations between the extracts obtained before and after incubation of the samples and based on daily mineralization rates.

The physiological profiles of the microbial communities were analyzed using Biolog® Ecoplates (Insam, 1997). 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 subsamples of the extracts (2 ml) were immediately frozen in liquid nitrogen and stored at −70°C until analysis. In order to ensure similar inoculum density (Insam and Goberna, 2004), prior to analysis the thawed extracts were diluted with Bis-Tris solution to obtain 0.5 µg Cmic 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

<table>
<thead>
<tr>
<th>Site</th>
<th>Age</th>
<th>Dominant tree species</th>
<th>Accompanying tree species</th>
<th>Canopy closure</th>
<th>Herb and shrub undergrowth</th>
</tr>
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<tr>
<td>MS</td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Poor herbal floor, Corynephora canescens L.</td>
</tr>
<tr>
<td>LUP</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Poor herbal floor, C. mic</td>
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were standardized by dividing them by the average area under the curve (AAUC) (Garland, 1997; Insam and Goberna, 2004). 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. Substrate richness determined as a total number of substrates utilized was used to roughly assess the metabolic abilities of soil microbial communities from the studied soils. In order to compare metabolic preferences of the microbial communities the substrates were grouped into four guilds – carbohydrates (CH), carboxylic acids (CA), amino acids (AA) and others (polymers, amines and amides, aromatic compounds and phosphorylated compounds; Sharma et al., 1998). For each guild the AUC values of the substrates were summarized and expressed as a percentage of total AUC value of the plate.

2.5. 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 \( i \)th substrate (AUC value) as a proportion of the sum of the use of all substrates on a plate.

Physiological profiles of microbial communities (CLPP) were used to compare metabolic abilities of soil microbial communities from the studied soils. The CLPPs from different sites were compared by cluster analysis. Clustering was performed on correlation matrix using Ward’s method based on City Block distances (Manly, 1989).

The relationship between Biolog-derived parameters (AAUC and \( H \)) and C\(_{\text{mic}}\) was tested using either linear or logarithmic regression models.

Between-site differences in microbial and chemical properties were tested by one-way ANOVA. The least significant difference (LSD) test for multiple comparisons was run if significant differences were found (\( p < 0.05 \)). Right-skewed data (C\(_{\text{mic}}\), RESP, C\(_{\text{mic-to-Corg}}\) ratio, Min_N, C\(_{\text{org}}\), N\(_{\text{t}}\), P\(_{\text{avl}}\)) were either log- or square root-transformed, and left-skewed data (Shannon index) were power-transformed (5th power) prior to ANOVA in order to approach normality.

All statistical analyses employed Statgraphics Plus version 5.1 (Manugistics Inc.).

3. Results

3.1. Physical and chemical characteristics of the soils

All analyzed soils had sandy texture (Table 2), with low silt and clay content (<7%). Only soils S20 and S27 contained more silt particles (10% and 9%, respectively). S27 also had the highest share of clay particles (4%) among the analyzed soils. Soil MS had the lowest content of C\(_{\text{org}}\) (0.18 mg g\(^{-1}\)) and \( N_{\text{t}} \) (0.004 mg g\(^{-1}\)). Soil LUP had higher C\(_{\text{org}}\) and \( N_{\text{t}} \) content than soil MS, but the difference was statistically significant only for C\(_{\text{org}}\).

In the afforested mine soils (reclaimed and successional), C\(_{\text{org}}\) Table 2 – Soil texture (USDA classification) and chemical properties of the soils. For the chemical properties, mean values (\( n = 8 \)) and standard errors (in parentheses) are presented. Values followed by the same letter within a column are statistically not different (Fisher’s LSD test, \( p < 0.05 \)).
and N content increased with increasing soil age, and reached the maximum values in soil R28 ($C_{org} = 5.01$ mg g$^{-1}$) and soil S27 ($N = 0.148$ mg g$^{-1}$). The reclaimed soils tended to have higher $C_{org}$ content than successional soils at the same age, but the differences were statistically insignificant. $C_{org}$ and N contents in the natural forest soils (F30 and F100) were significantly higher than in the mine soils (Table 1).

The $P_{avl}$ content was the highest in soils LUP (17.2 mg kg$^{-1}$) and F30 (16.8 mg kg$^{-1}$), and the lowest in F100 (4.5 mg kg$^{-1}$) and MS (5.3 mg kg$^{-1}$). In the younger successional mine soils (S6 and S20) $P_{avl}$ content was low and increased significantly only in S27. Among the reclaimed mine soils, $P_{avl}$ content was the highest in R6; in R20 and R28 it was lower and did not differ significantly between the two.

The pH values (in water and in KCl) were the highest in soil MS (7.10 and 6.46, respectively; Table 1). The pH values were significantly lower in soil LUP ($pH_{H_2O} = 6.72$; $pH_{KCl} = 6.13$), and the lowest in the afforested mine soils.

3.2 Microbial properties of the soils

The lowest $C_{mic}$, RESP, and Min_N were measured in soil MS (Fig. 1). All the gross microbial properties tended to be higher in LUP than in MS, but the differences were not statistically significant. In the successional mine soils, $C_{mic}$, RESP, and Min_N increased gradually with soil age, but the differences were significant only for RESP. Among the reclaimed mine soils, $C_{mic}$, RESP, and Min_N were the highest in soil R6. In R20 and R28 the values were significantly lower and did not differ between each other. In the natural forest soils, $C_{mic}$, RESP, and Min_N were relatively low and similar to the values for afforested mine soils.

All the gross microbial properties were higher in soil R6 than in S6 (Fig. 1). None differed between soils R20 and S20; whereas all microbial properties were higher in soil S27 than in R28, the corresponding reclaimed mine soil.

The $C_{mic}$-to-$C_{org}$ ratio was the highest in soils MS and LUP, and decreased rapidly in the afforested mine soils (Fig. 1). The decrease was more pronounced in reclaimed than in successional mine soils.

Activity on the Biolog plates (AAUC) was the highest in both natural forest soils, followed by soil LUP (Fig. 2). In the afforested mine soils the AAUC values increased gradually with age, reaching maximum values in soils S27 and R28. The lowest AAUC was measured for soil MS. There was a weak ($r^2 = 0.08$) but significant ($p < 0.05$) linear relationship between AAUC and $C_{mic}$ values (Fig. 3).

The relative use of CH varied between 20.3% and 28.8%, of CA between 15.1% and 23.5%, of AA between 22.0% and 33.7% and of other compounds between 26.1% and 34.1% (Table 3). There were no significant differences in the use of CA, CH, and the other compounds between the analyzed soils. Significant differences were found only for AA. This guild was preferably used in soils S20 and R20 compared to all remaining soils with the exception for soils R28 and S6.

The highest substrate richness was determined for soil LUP and the two natural forest soils. In the mine soils the richness was significantly lower (Fig. 2). In the successional mine soils the number of substrates utilized increased gradually with soil age. No such trend was recorded for the reclaimed soils; the lowest richness was determined in soil R20. Among the analyzed soils the lowest number of substrates was degraded in soil MS.

The highest $H'$ value, indicative of the highest functional diversity, was calculated for soil LUP, followed by the two natural forest soils (Fig. 2). The $H'$ values were lower for the afforested mine soils and did not differ between the same age successional and reclaimed mine soils. The lowest Shannon
The diversity index was calculated for soil MS. The relationship between \( H^0 \) and \( C_{\text{mic}} \) was weak \((r^2 = 0.11)\) but significant \((p < 0.05)\). The logarithmic model was the best fitting one (Fig. 4).

Cluster analysis of the Biolog\(^\text{®}\) data yielded five distinct clusters – a forest soil cluster containing 14 of 16 analyzed forest soil samples, and four clusters containing the mine soil samples (Fig. 5). Two of the mine soil clusters were relatively uniform and contained the samples of soils MS and LUP, respectively. The other two mine soil clusters contained the samples of afforested mine soils. The first included the samples similar to soil MS; here were mainly the samples of soil S20 \((n = 7)\) and S6 \((n = 5)\). The second contained the samples more similar to soil LUP and to the forest soils; here were the oldest mine soil samples \((S27: n = 7; R28: n = 6)\) and the samples of soil R6 \((n = 6)\).

### 4. Discussion

#### 4.1. Physical and chemical properties

Soil texture is known to affect carbon accumulation and soil microbial properties (Van Cleve and Powers, 1995; Buscot, 2005). In our study, all analyzed soils represented the same textural class - the sands (USDA classification). The differences in
Fig. 5 – Cluster analysis based on community-level physiological profiles (CLPPs) assessed with Biolog® plates. Clustering was done using Ward’s method; distances between samples were measured using the City Block method.

Texture were relatively small and involved only a few soils. This enabled us to assess the effects of reclamation measures in relation to spontaneous soil development under relatively similar conditions.

In the mine soils, $C_{\text{org}}$ content increased from pure sand (MS) to the oldest soils (S27, R28). The soils of reclaimed sites tended to have a higher $C_{\text{org}}$ content than the same age succession soils, but in all cases the differences were not statistically significant. The slightly higher $C_{\text{org}}$ content of the reclaimed mine soils probably was the result of the applied reclamation measures (lupine cultivation and organic amendment). In another comparative study of soil development under succession and after reclamation in the same area, Pietrzykowski and Krzaklewski (2007) found significantly higher C accumulation in reclaimed soils. However, those authors examined entire soil profiles (Oi–Oe–A–AC), whereas our study analyzed only the uppermost 5 cm of mineral soil. When only $A_1$ horizon was considered, there was no significant difference between 20 and 25 years old reclaimed mine soils and the same age succession soils (Pietrzykowski and Krzaklewski, 2007).

The mine soils contained much lower $C_{\text{org}}$ than the natural forest soils. In a study of organic matter status in reclaimed sand dunes in South Africa, Graham and Haynes (2004) reported that 25 years after reclamation the $C_{\text{org}}$ content of the topsoil of reclaimed dunes afforested with sweet thorn (Acacia karoo) was similar to that of the soils of natural sweet thorn (Acacia karoo) forest. Higher $C_{\text{org}}$ accumulation reported by Graham and Haynes (2004) was probably caused by different tree species used for reclamation. Sweet thorn is a leguminous tree and legumes are known to increase soil C content (Rothe et al., 2002).

Total N content in most of the mine soils was very low. Two years of lupine cultivation did not significantly increase soil $N_\text{t}$, but note that we used sieved samples; most of the N could have been in plant residues larger than 2 mm and thus eliminated from the analysis. The highest $N_\text{t}$ content was in the oldest mine soils (S27 and R28). Even there, however, $N_\text{t}$ was distinctly lower than in the natural forest soils. Similarly, Šourković et al. (2005) reported higher $N_\text{t}$ in the uppermost 5 cm of mineral soil in natural alder forest than in reclaimed mine soils afforested with alder species.

The increase of $C_{\text{org}}$ content in the mine soils was accompanied by decreasing soil pH. Among the analyzed soils the highest pH values (both in water and in KCl) were for soil MS. Lupine cultivation significantly decreased soil pH; lupine is a leguminous plant, and the growth of actively N$_2$-fixing legumes causes soil acidification (Haynes, 1983). The afforested mine soils had a further significant decrease in pH, possibly related to the build-up of humic substances over time in the mine soils. Humic substances contain acidic carboxylic and phenolic groups, which may contribute to soil pH decline.

In addition, the dominating tree species, P. sylvestris, is known to cause soil acidification (Arnold, 1992; Emmer and Sevink, 1994). A decrease in pH with soil development is a common phenomenon and has been observed at other reclaimed sites (Graham and Haynes, 2004; Šourković et al., 2005) as well as during primary succession on sand dunes (Emmer and Sevink, 1994).

The effect of reclamation measures was evident in the case of $P_{\text{avl}}$ content. Among the mine soils it was the highest in soil LUP, followed by soil R6. High $P_{\text{avl}}$ content in the youngest reclaimed mine soils probably derived from fertilization applied during reclamation measures. In the chronosequence of successional mine soils, $P_{\text{avl}}$ content gradually increased with soil age. The content of $P_{\text{avl}}$ varied over a wide range in the forest soils; for this reason, $P_{\text{avl}}$ content in the mine soils fell within the range of the natural forest soils of the area.

4.2. Microbial properties

The values for gross microbial properties ($C_{\text{mic}}$, RESP and $\text{Min}_N$) were low in all analyzed soils. The lowest values were measured in soil MS, where the lack of organic substrate was an obvious reason for low microbial activity. Two years of lupine cultivation increased the amount of soil microbial biomass and its activity, but the increase was not significant. In the successional mine soils, $C_{\text{mic}}$, RESP and $\text{Min}_N$ increased with soil age, indicating gradual soil development. In the reclaimed mine soils the time trend for gross microbial properties was different. The values of $C_{\text{mic}}$, RESP and $\text{Min}_N$ were the highest for soil R6, and significantly lower for soils R20 and R28. Such a pattern of development indicates a strong effect of reclamation measures on soil microbial properties.

We presume that planting P. sylvestris seedlings densely spaced (1 m × 1 m) had a pronounced effect on the development of soil microbial communities. Owing to this initial
effect, soil R6 was microbiologically much more active than the same age successional mine soil (S6), but the effect of reclamation measures on soil microbes was relatively short-lived; there was no difference in $C_{\text{mic}}$ and RESP between the 20-year-old reclaimed mine soil and successional mine soil, and $\text{Min}_N$ was even higher in the latter. After nearly 30 years the microbial community of the successional mine soil (S27) had significantly higher biomass and was more active than the community of the reclaimed mine soil (R28). Those lower values for the microbial community of soil R28 might have resulted from differences in texture. The parent material of R28 was nearly pure sand, without any clay particles and with only 2% silt, whereas soil S27 had 4% clay and 9% silt. Soils with higher clay content can maintain larger microbial communities, probably because they provide better protection from faunal predation and are characterized by less fluctuation of water availability (Franzluebbers et al., 1996; Müller and Höper, 2004).

The gross microbial properties measured in the mine soils were in the range measured in the reference natural forest soils. This indicates relatively rapid quantitative restoration of soil microbes in the top layer of mine soils. Other studies have yielded similar results. In reclaimed sand dunes in South Africa, for example, microbial biomass and basal respiration reached the levels characteristic of reference natural forest soil as early as 20 years after reclamation (Graham and Haynes, 2004). Frouz and Novakova (2005) studied microbial properties in a chronosequence of post-mining plots covered by spontaneously developed vegetation on spoil dumps in Czech Republic. They reported respiration rates and bacteria counts in the mine soils comparable to or even higher than the values from local semi-natural oak and alder forests.

It is considered that up to a certain threshold an increase of microbial biomass induces also an increase of functional diversity (Lynch et al., 2004). Regression analysis (Fig. 4) indicated that $C_{\text{mic}}$ was positively related to functional diversity. However, the relationship was significant only because of the soil MS samples (Fig. 4). After excluding these samples (data not shown) the relationship was not significant ($r^2 = 0.03$, $p = 0.12$). Yan et al. (2000) reported a broken stick relationship between the functional diversity measured with Biolog$^\text{\textregistered}$ test and soil microbial biomass. They used data given by Sharma et al. (1997) and found that the functional diversity increased with increasing $C_{\text{mic}}$ up to a threshold of 105.6 $\mu$g g$^{-1}$ and remained constant above this value. The results presented in Fig. 4 suggest that in the studied sandy soils the threshold value was much lower and approximated 35 $\mu$g g$^{-1}$.

The $C_{\text{mic}}$-to-$C_{\text{org}}$ ratio in the mine soils decreased rapidly with soil age, but did not attain the values of the reference forest soils. A similar pattern of decline in the proportion of $C_{\text{org}}$ present as $C_{\text{mic}}$ was observed in reclaimed soils after lignite mining returned to deciduous forest (Insam and Domsch, 1988), in sand dunes reclaimed to pine forest (Graham and Haynes, 2004) and during secondary succession on abandoned arable fields (Šantročíková, 1992). A diminishing $C_{\text{mic}}$-to-$C_{\text{org}}$ ratio indicates a rapid decrease in the availability of soil C due to progressive accumulation of recalcitrant humic material in soil (Graham and Haynes, 2004).

Analysis of substrate preferences in the Biolog$^\text{\textregistered}$ plates did not reveal differences between the analyzed soils. Similar substrate preferences in the afforested soils were probably because in all of these soils the main source of organic C available for microbes was pine litter. The youngest mine soils (MS and LUP) tended to have a higher use of carbohydrates and a lower use of amino acids. However, the differences were not statistically significant, probably due to very high variability in the use of CH and AA in MS soils (Table 3).

More detailed analysis of CLPPs (using individual substrates) revealed that the natural forest soils differed considerably from the mine soils. Nearly all natural soil samples were grouped in a separate cluster (Fig. 5). The microorganisms of the natural forest soils were significantly more active on the Biolog$^\text{\textregistered}$ plates, and more diverse as demonstrated by higher $H'$ values, than those of the mine soils (except for soil LUP). They were also able to degrade more C substrates on the plates. This indicates that restoration of the metabolic abilities of microbial communities is slower than restoration of gross microbial properties. Our results are in agreement with the findings of Graham and Haynes (2004), who reported that the catabolic response profiles of 25-year-old reclaimed mine soils differed from those of native forest soils. Similarly, Mummey et al. (2002a) applied FAME analysis to assess ecosystem recovery after surface mine reclamation in south-eastern Wyoming and reported that nearly 20 years after reclamation there was still a difference in FAME profiles between the microbial communities of the reclaimed and native soils.

Reclamation measures affected the development of the metabolic abilities of the mine soil microbial communities. The soil MS samples were grouped in a separate sub-cluster. The microbial communities of this extremely poor soil were inactive on the Biolog$^\text{\textregistered}$ plates, able to degrade only few C substrates and characterized by low functional diversity. Two years of lupine cultivation created a unique soil microbial community able to metabolize numerous C substrates and characterized by high functional diversity ($H'$ value even higher than for natural forest soils). The soil LUP samples differed distinctly from the remaining mine soil samples and were grouped in a separate sub-cluster. The CLPPs of LUP samples were more similar to the profiles of the natural forest soils than to the profiles of soil MS.

In the successional mine soils the metabolic abilities of microbial communities developed gradually. The substrate richness increased with the soil age. The CLPPs of the youngest successional soils (S6 and S20) resembled the physiological profiles of soil MS more than those of forest soils and only for the soil S27 samples the opposite was the case. On the contrary, most of the reclaimed mine soils (including the youngest ones) were clustered closer to the forest soils than to soil MS. This indicates that reclamation measures not only enhanced gross microbial properties but also promoted development of the metabolic abilities typical for the forest soils. In the longer term, however, the development of metabolic abilities led to similar results in the successional and reclaimed mine soils as most of the samples of soils S27 and R28 were grouped in the same cluster.

Regression analysis indicated a significant relationship between AAUC values and $C_{\text{mic}}$ (Fig. 3). Since clustering apparently depended on AAUC (the samples with low and high AAUC were clustered separately) it is possible that clustering resulted partly from differences in $C_{\text{mic}}$. However, the effect of
differences in $C_{mm}$ on AAUC values was weak (only 8% of the variability in AAUC values was explained by differences in $C_{mm}$) and statistically significant only due to the presence of the soil MS samples.

The effect of inoculum density is a serious issue in Biolog© assay as the differences in CLPPs may not result from metabolic abilities of the microbial communities but from different cell numbers (or microbial biomass) in the wells (Preston-Mafham et al., 2002). In our study we standardized inoculum density according to microbial biomass of the samples (Insam and Goberna, 2004) and standardized the results (AUC values) by dividing them by the AAUC value (Garland, 1997). Therefore, we suppose that the differences in AAUC and consequently in the clustering resulted mainly from differences in intrinsic properties of the studied microbial communities. However, the significant relationship between $C_{mm}$ and AAUC indicated that the applied standardization methods were not sufficient enough to entirely remove the effect of soil $C_{mm}$ on the CLPPs. A different pattern of the substrate use in soil MS might have resulted both from different metabolic abilities of microbial communities but also from lower inoculum densities of the soil MS samples.

Biolog© Ecoplates measure the activity of only the selected groups of bacteria capable of growing in the plates. Indeed, Ros et al. (2008) reported that different CLPP patterns can be generated on the basis of only 3-4 genera. Therefore, the differences demonstrated in our study refer to only that small component of the microbial communities. To evaluate a larger representation of soil microorganisms, additional tests with molecular techniques (e.g., PLFA or molecular techniques) could be used (Winding et al., 2005).

Despite the shortcomings of the Biolog© assay, application of this method did provide additional information on the microbial communities of the mine soils. The information on functional diversity so obtained should prove useful for assessing the results of mine soil rehabilitation practices.

Acknowledgement

The study was financed by the Polish Ministry of Science and Higher Education, grant no. 2PO4G02230.

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