Algae and cyanobacteria colonizing toxic soils on coal-mining dumps

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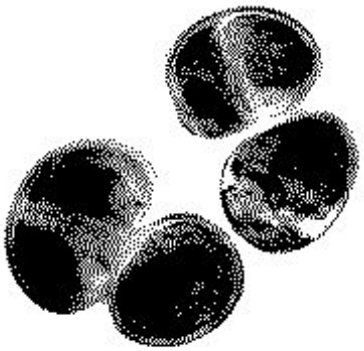

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Annotation

Species composition of soil algal and cyanobacterial communities was investigated in thirteen sites of different toxicity of spoil material on dumps in the Sokolov mining area (Czech Republic). The adaptation ability of various algal and cyanobacterial species to live in toxic environment and the effect of different amendments (wooden coal, organic matter, dolomitic limestone) of toxic soils were tested both in laboratory and field experiments. According to results, species composition corresponded to environmental characteristic (pH, conductivity, substrate type). Some green unicellular algae grew successfully in extracts from the most toxic substrate and seemed to be well adapted to low pH conditions. Results indicate that increase of pH is a basic precondition for the establishment of more diverse and abundant algal flora in highly acidic sites.

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Kristýna Mohlová

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Contents

1. Preface

Soil restoration is the basic precondition of establishing new quality ecosystem in post-mining landscapes. Unlike many other industrial areas, the toxicity of post-mining spoil material is not caused by pollution coming from external sources, but it is predetermined by chemical and physical properties of spoil material. Once the material is uncovered, massive weathering occurs and leads to formation of extreme conditions for living such as low pH, high salinity or high availability of some metals (Jenner&Janssenmommen, 1993; Bradshaw, 1997) (Sharmasarkar&Vance, 1997; Sample&Suter, 2002). The substrate toxicity makes hence the reclamation of post-mining sites difficult.

Sokolov mining district is the large area affected by former mining activity in the northwestern part of Bohemia. Although the area has been recultivated by various techniques, there are still sites, mainly extremely toxic requiring recovery.

Soil algae and cyanobacteria are photoautotrophic microorganisms occurring in various natural habitats as well as in disturbed environments all over the world (Shtina, 1974; Shtina *et al.*, 1985). They participate significantly in many edaphic interactions and are involved in numerous food webs (Zenova *et al.*, 1995). As primary producers they play an important role in colonization of barren soils (Metting, 1981; Maxwell, 1991) and thus in acceleration of plant succession.

Since some algal and cyanobacterial species are able to cope very well with severe conditions of spoil material (Shubert&Starks, 1980) they are potential source of alternative ways for reclamation on post-mining sites.

The goals of this study were 1) to compare algal communities developing in spoil materials of different toxicity 2) to test the survival and possible growth of the most important colonizers isolated from studied dumps in extremely toxic substrates 3) to test the effect of various amendments added to toxic substrates on algal community development.

2. Introduction

2.1. Mining activity

Strip mining is the most common method of coal mining. This type of mining uses some of the largest machines on earth, including bucket-wheel excavators which can move as much as 12,000 cubic meters of earth per hour. Therefore the impact on the topography, vegetation, and water resources is not negligible. During the mining activity, the spoil material is excavated and deposited in adjacent surroundings of mines to form the large area of dumps.
The spoil material comes from 0 - 200 metres and may vary in chemical and physical properties. It is often sterile and characterised by extreme values of pH, high salinity, low organic carbon content, lack of essential trace elements and sensitivity to erosion (Jenner&Janssenmommen, 1993; Piha *et al.*, 1995; Bradshaw, 1997; Sharmasarkar&Vance, 1997; Sample&Suter, 2002) All these features make the spoil material toxic and hardly recoverable.

2.2. Soil developement

Based on pedological, sedimentological, mineralogical and hydrochemical measurements, it is obvious that the rate of soil development is mainly governed by inherited factors of parental waste materials (Neel *et al.*, 2003). Soil profiles are influenced mainly by parent material, climate, topography, organisms and time.
The productivity and stability of soil as a medium for plant growth depends greatly on the balance between living and non-living components. Energy from the sun and nutrients essential for growth are stored in vegetation and recycled through decomposition by micro and macroorganisms in soil. The soil organic matter formed during this process serves as a continuous nutrient supply and a factor stabilizing the soil physical environment. In natural systems, the action of soil microbes and fauna are major determinants of efficient nutrient cycling and plant growth. Therefore biological decomposition of plant residue is the largest source of nutrients.

2.3. Sokolov mining district

Sokolov mining district is situated in the northwest part of the Czech Republic and covers ca 100 km^2. The entire mining area is operated by Sokolovská uhelná spol. s.r.o. Pursuant to the state law mining companies are obligated to restore post-mining landscapes to the original appearance. The recovery occurs either by industrial restoration techniques or by using principles of spontaneous succession nowadays highly accepted by specialists (Prach *et al.*, 2001). The Sokolov post-mining area has been partly afforested, mostly by deciduous trees, some parts of the area have been left to spontaneous succession. The Institute of Soil Biology of Biology Centre AS CR and ENKI o.p.s. with important support of Sokolovská uhelná spol. s.r.o. participate significantly in work related to Sokolov mining area (Frouz *et al.*, 2001; Nováková, 2001; Pižl, 2001; Sklenička *et al.*, 2004; Šourková *et al.*, 2005). Due to the complex approach, which is the combination of biological, social and economical points of view, the Sokolov post-mining landscape is going to develop successfully. A few studies there were carried out to judge the impact of used reclamation methods (Nováková, 2001; Šourková *et al.*, 2005) whilst other described the spontaneous successional processes on the dumps (Frouz&Nováková, 2005; Frouz *et al.*, 2007).

2.4. Algal and cyanobacterial colonization

Algae and cyanobacteria play crucial role as pioneer photosynthetic colonizers of barren soils (Lund, 1962; Metting, 1981), similar to those formed on dumps. Provided there is enough moisture, soil surface is soon become colonised by a microfloral community of algae, including cyanobacteria in association with bacteria and fungi. Such biological crusts prevent soil from erosion (Rushforth&Brotherson, 1982). Due to the ability to consolidate soil particles (Bailey *et al.*, 1973), soil algae ameliorate structure and fertility of soils (Booth, 1941). Thus they encourage the development of higher vegetation. In addition diazotrophic cyanobacteria enrich newly created soils with nitrogen (Shields&Durrell, 1964).

Although soil is the most studied terrestrial algal habitat (Hoffmann, 1989), only a few reports have been published on microfloral community structure in relation to toxic soils. Shubert and Starks studied colonization together with soil-algal relationships on a reclaimed surface mined area in western North Dakota (Shubert&Starks, 1979; 1980). They stressed the importance of algae in soil genesis and subsequent succession. They stated that the restoration of the appropriate soil microflora and soil fertility is the key factor in the land reclamation process.

The same authors determined the impact of various amendments of spoil material on algal community structure (Shubert&Starks, 1979). Moreover they compared the soil microflora developing on sites with and without topsoil cover (Starks&Shubert, 1982). Maxwell (1991) was interested in floristic changes in soil algae and cyanobacteria in metal contaminated land in Canada. He studied treated and untreated post-mining sites in terms of time. Kabirov monitored the development of algal communities on dumps in Kan-Achinsk in relation to dump age and vegetation cover (Kabirov, 1997). Douglas *et al.* (1998) investigated the composition, diversity and distribution of algae in Malaysian mining areas contaminated with potentially toxic trace heavy metals. Megharaj (2000) focused on changes in algal species composition as a response to the soil contamination by petroleum hydrocarbons. The growth of soil algae in cryptogamic crusts on top soil and processed oil shale in Uintah Basin of Utah was reported by Ashley and Rushforth (1984). Phototrophic biofilms of restored fields in the rhenish lignite mining area were examined by Jahnke and Priefer (2002). Lukešová and Komárek (1987) studied spontaneous succession of algae and cyanobacteria in coal mining region in Most in the Czech Republic. Lukešová compared algal communities in soils between two mining areas in Sokolov (Czech Republic) and Cottbus (Germany) (Lukešová, 2001).

2.5. Biological tests

Since the spoil material is sterile after the excavation, it is very suitable for studying the process of colonization and succession. Similarly it provides favourable environment for application of biological tests. Biological tests applied in post-mining areas serve as a good clue for understanding the problems of their toxicity.

Only a few papers concerning biological toxicity tests of post-mining sites have been published. Galli *et al.* (1994) focused on hydrological tests in post-mining sites. The impact of heavy minerals mined along south african coast on vegetation was described by De Villiers *et al.* (1999). Accumulation of heavy metals in plants growing on overburden soils was documented with potential use for phytoremediation (Deo, 2004). The bioaccumulation of metals originating from mining spill was studied in fish, clams and oysters in the Guadalquivir estuary (Riba *et al.*, 2005). The response of the freshwater bivalve *Pyganodon grandis* to increased metal exposure in the mining area in northwestern Quebec was studied by Wang *et al.* (1999). Frouz *et al.* (2005) tested toxicity of spoil substrate after coal mining using a laboratory reproduction test with *Enchytraceus crypticus* (Oligochaeta). The

abnormalities among foraminifera were used as biomarkers for evaluating trends in the biological impact resulting of submarine tailings disposal (Elberling *et al.*, 2003). A bioassay measuring the mycorrhizal population levels in five soils disturbed by mining activities was carried out in southeastern Spain (Diaz&Honrubia, 1993). The toxicity of the substances in the soils were tested using several representative soil organisms such as plants, nematodes, microorganisms and soil algae (Debus&Hund, 1997). A test with the soil alga *Chlorococcum infusionum* has been developed to assess the ecotoxic potential of soil contaminants (Hammel *et al.*, 1998). Microalgal bioassays were used to assess the impact of copper from copper mining on a coastal area in Northern Chile. Stauber *et al.* (2005) studied growth inhibition of *Nitzschia closterium* and enzyme inhibition in *Dunaliella tertiolecta.* Antunes *et al*. (2007) evaluated water column and sediment toxicity from an abandoned uranium mine using a battery of bioassays. Megharaj *et al.* (2000) carried out a screening experiment for total petroleum hydrocarbons and their toxicity to soil algal populations. He also published a study investigating the effects of DDT and its metabolites on enzymatic activities and algal populations in soil (Megharaj *et al.*, 1999). Bérard *et al.* (2004) evaluated the toxicity of herbicides on the soil-based photosynthetic activity of soil algae and detected changes in algal communities by diatom identification and counting.

A series of tests in this study was conducted in order to outline reasons for the substrate toxicity and to attempt to find alternative techniques for reclamation of such devastated site using soil photoautotrophic microflora.

3. Materials and methods

3.1. Locality

The study was conducted in Sokolov mining district in the northwestern part of the Czech Republic, (50°09' - 50°15' N, 12°32' - 12°45' E). The altitude of the area ranges from 450 to 550 m a. s. l. The annual air temperature varies between 7.0 - 8.1°C, mean precipitation is 600 - 650 mm per year.

The investigated plots were situated in dumps of different age (from ten to forty years old). Most of them had no or only rare vegetation. The older plots (VA2, VA4) were covered by a sparse forest formation of *Betula pendula* Roth, the undergrowth consisted mainly of juvenile *Quercus robur* L., *Avenella flexuosa* (L.) Drejer and *Festuca ovina* L. with occasional

occurrence of *Lathyrus pratensis* L., *Vicia villosa* Roth, *Hieracium cf. sabaudum* L., *Leucanthemum vulgare* Lamk., *Trifolium repens* L., *Calamagrostis epigejos* (L.) Roth and *Arrhenatherum elatius* (L.) J. Presl et C. Presl. *Phragmites australis* (Cav.) Steud. accompanied by *Tussilago farfara* L. dominated in TC11.

Studied spoil material included alkaline tertiary clay cypris serie (TC - 5 substrates, from those one acidified by jarosite - TC9, and one with high content of salts - TC11), neutral or acidic volcanic ashes (tufits, VA - 4 substrates), and highly acidic coal rich clays (CC - 4 substrates) (Tab.1).

Table 1. Physical and chemical properties of investigated sites with substrate type (CC - coal clays, VA - volcanic ashes, TC - tertiary cypric clays) and soil properties. Toxicity was defined according to **Frouz *et al.* (2005)** (1 - toxic, 0 - non-toxic site). The values of pH, conductivity (EC) and soil moisture are stated as average values with standard deviations.

site	substrate	abbrev.	age [years]	tox	pH	EC [µS*cm-1]	soil moisture [%]	org.C content [%]	veg.cover [%]
1	CC	**CC1**	30	1	3.05 ± 0.27	282 ± 253	75.4 ± 6.9	11.8	0
2	VA	**VA2**	30	1	3.58 ± 0.29	89 ± 57	66.2 ± 5.8	12.4	85
3	CC	**CC3**	40	1	3.45 ± 0.11	131 ± 98	72.4 ± 2.7	13.4	0.4
4	VA	**VA4**	40	0	3.91 ± 0.11	106 ± 88	71.7 ± 2.6	11.4	80
5	VA	**VA5**	10	0	3.43 ± 0.44	189 ± 76	82.5 ± 8.4	5.4	0.3
6	TC	**TC6**	20	0	8.25 ± 0.04	222 ± 48	81.6 ± 11.1	5.6	45
7	TC	**TC7**	21	0	8.33 ± 0.03	219 ± 43	82.7 ± 10.1	5.3	0.2
8	TC	**TC8**	15	0	8.04 ± 0.24	767 ± 744	79.8 ± 12.6	5.7	5
9	TC	**TC9**	15	1	2.95 ± 0.41	2341 ± 1249	65.8 ± 3.1	6.7	0
10	CC	**CC10**	15	1	2.37 ± 0.05	1230 ± 306	84.5 ± 8.6	6.4	3
11	TC	**TC11**	15	0	8.33 ± 0.06	1855 ± 1606	70.1 ± 5.9	6.2	95
12	CC	**CC12**	20	1	2.47 ± 0.27	1680 ± 1046	91.2 ± 0.3	6.2	0
13	VA	**VA13**	10	0	7.36 ± 0.28	114 ± 75	84.0 ± 0.0	8.0	0

Table 2. Twelve studied sites with aditional information: contents of sodium (Na), potassium (K), aluminium (Al), iron (Fe) and polyphenols. Data obtained in **Frouz *et al.* (2005).**

site	Na [mg/kg]	K [mg/kg]	Al [mg/kg]	Fe [mg/kg]	polyfenoly [mg/kg]
CC1	0.102	0.016	1188.4	116.3	12.7
VA2	0.101	0.048	998.7	135.5	19.1
CC3	0.076	0.016	766.8	221.7	18.6
VA4	0.017	0.095	258.6	5.2	4.6
VA5	0.104	0.027	1098.0	152.3	5.8
TC6	0.082	0.150	0.5	1.1	0.8
TC7	0.364	0.177	60.4	14.3	1.7
TC8	0.165	0.187	2.3	1.5	0.4
TC9	0.132	0.005	30.9	15.7	2.0
CC10	0.145	0.012	3062.0	892.4	23.7
TC11	1.748	0.182	10.0	2.0	2.9
CC12	0.040	0.018	321.6	26.9	4.7

3.2. Sampling

Sampling was performed three times - in fall 2005 and in spring 2006, 2007. Soil samples were collected from thirteen plots of different toxicity of spoil material. Toxicity as well as detailed soil properties were described in previous study (Frouz *et al.*, 2005). The vegetation cover and slope of plots were characterized. Five subsamples (5x5cm) of unvegetated substrate were taken aseptically with a shovel from 1cm upper layer at each site and mixed together. The material was transported to the laboratory in plastic bags, then sieved and analysed.

3.3. Analyses

pH, conductivity and content of organic carbon were evaluated for each soil sample. pH was measured potentiometrically using glass and reference calomel eletrode as active pH in water extract (1:2.5, water : dried soil). Water suspension (1:5, dried soil : distilled water) was shaked for 30 minutes and subsequently used for determining electric conductivity. Conductivity was measured with conductive electrode in conductometer. The content of organic carbon was expressed by incineration of soil samples in 500°C for 6 hours (Howard&Howard, 1990). Information of sodium, potassium, iron and polyphenols contents concerning twelve investigated plots (CC1 - CC12) were received from Frouz *et al.* (2005) (Tab.2).

3.4. Species cultivation, determination and quantification

Species composition of soil algae and cyanobacteria was determined using dilution plate method followed by isolation, cultivation and life cycle studies if necessary (Lukešová, 2001). The colonies grown on BBM medium (Bischoff&Bold, 1963) solidified with 1,7% agar in Petri dishes were isolated using laboratory loop, then cultivated on slant agar in test tubes. The strains were maintained in aerobic conditions, under light - dark (16:8) regime of light intensity of 30 μmol photons.m^{-2}.s^{-1} and in constant temperature about 22°C. Dominants of visible algal crusts were identified directly using light microscope Olympus BX 51. Photographs and measurements of species were taken by digital camera DP 50 equipped with analySIS software version 3.2. Nomenclature was used according to Ettl & Gärtner (Ettl&Gärtner, 1995)

Abundance of algae was assessed as direct cell counts in soil suspension using epifluorescence microscope Olympus BX 51 (Lukešová, 2001).

3.5. Experiments

15 species of soil algae and cyanobacteria previously isolated from studied plots *(Botrydiopsis intercedens, Bracteacoccus minor, Coenochloris sp., Chlamydomonas macrostellata, Chlorella saccharophila, Diplosphaera chodatii, Elliptochloris subsphaerica, Heterococcus sp., Klebsormidium flaccidum, Myrmecia bisecta, Nostoc sp., Phormidium autumnale, Pleurastrum sarcinoideum, Pseudococcomyxa simplex, Xanthonema debile)* were used for series of tests (Fig.2).

3.5.1. Pilot test

For the pilot test, a group of species was divided into four mixtures (Tab.3). Mixtures were prepared from fresh algal biomass scrapped off agar plate and diluted in BBM, each component was added in equal density. Five grams of dried sieved substrate (CC12 - supposedly the most toxic one, Tab.1; Tab.2) were put into small Petri dish, moistened with 4ml BBM and inoculated with relevant mixture. The presence of viable cells in soil suspensions was checked under epifluorescence microscope after two weeks. The soil suspensions were prepared as follows: 5g of tested soil were mixed with 45ml of sterile water. Subsequently 20 µl of soil suspension was put under 22x22mm cover slip and cells with autofluorescence were revised under bright light.

Table 3. Mixtures (mix 1 - 4) with particular species composition.

mix 1	*Diplosphaera chodati*	*Pseudococcomyxa simplex*	*Bracteacoccus minor*	*Xanthonema debile*
mix 2	*Klebsormidium flaccidum*	*Elliptochloris subsphaerica*	*Heterococcus sp.*	*Chlamydomonas macrostellata*
mix 3	*Chlorella saccharophila*	*Pleurastrum sarcinoideum*	*Nostoc sp.*	*Botrydiopsis intercedens*
mix 4	*Coenochloris sp.*	*Phormidium autumnale*	*Myrmecia bisecta*	

The same species were tested for survival in extremely toxic water extract prepared from the substrate CC12 (substrate:water - 1:5) and in its dilutions in BBM. Dense algal/cyanobacterial suspensions prepared by scrapping the biomass from the surface of agar plate and mixed with 1.8ml BBM were inoculated to particular extract. The 100% toxic extract and 50% diluted extract were tested in 96-well micro-plates, 20 µl of dense algal suspension was added to 200

µl of extract in eight replications for each species. Parallelly 90% concentrated toxic extract was tested in test tubes. Living cells were checked microscopically after one week of exposure. The growth was evaluated using semiquantitative scale: growth (2) , survival (1), death (0).

3.5.2. Growth and survival tests

Furthermore, 8 species - *Nostoc sp.* (Cyanophyceae), *Bracteacoccus minor, Chlorella sp.1, Chlorella saccharophila, Pseudococcomyxa simplex* (Chlorophyceae), *Botrydiopsis intercedens, Heterococcus sp., Xanthonema debile* (Xanthophyceae) were investigated for their growing and survival ability in 13 water extracts (1:5) prepared from substrates of different toxicity (corresponding to 13 plots investigated for algal and cyanobacterial communities, Tab.1). 20µl of dense algal suspension, prepared by the procedure described above, was added to 200µl of particular extract in 96-well micro-plates in six replications for each species and each substrate. The optical density (OD) of inoculated cultures maintained in light - dark regime in 22°C was measured at the beginning of the experiment and after two weeks of exposure. OD was evaluated spectrophotometrically at 663nm wavelength against OD of corresponding plain extract using Multidetection Microplate Reader Synergy 2 made by (BIOTEK). Data were processed in Gen5 TM Sofware. The viability of organisms was verified microscopically.

3.5.3. Amelioration tests

19 selected species - *Nostoc sp., Phormidium autumnale* (Cyanobacteria), *Klebsormidium flaccidum, Stichococcus bacillaris* (Charophyceae), *Actinochloris sp., Chlamydomonas macrostellata, Chlorococcum sp.* (Chlamydophyceae), *Bracteacoccus minor, Chlorella saccharophila, Coenochloris sp., Diplosphaera chodatii, Elliptochloris subsphaerica, Myrmecia bisecta, Pleurastum sacrinoideum, Pseudococccomyxa simplex* (Chlorophyceae), *Botrydiopsis intercedens, Chlorellidium tetrabotrys, Heterococcus sp., Xanthonema debile* (Xanthophyceae) were tested in laboratory experiment. 20µl of dense algal suspension was inoculated to 200µl of 8 different extracts in six replications for each species. Experiment was designed for 96-well micro-plates. Water extracts (1:5) were prepared from extremely toxic substrate (CC12) and from the same toxic substrate enriched with various amendments: dolomitic limestone - LM (40g. kg^{-1} $CaCO_3$/toxic substrate), cow manure - M (40g. kg^{-1}

organic matter/toxic substrate) and wooden coal - WC (5g. kg^{-1} wooden coal/toxic substrate) and their combinations. Extract of plain toxic substrate was considered to be a control in the test.

Table 4. Treatments with pH values of water extracts.
Extracts made from: S - substrate CC2, M - cow manure, LM - dolomitic limestone, WC - wooden coal.

treatment	pH
S	2.51
S+M	4.18
S+WC	2.50
S+LM	7.84
S+M+LM	7.78
S+WC+LM	7.97
S+M+WC	4.18
S+M+WC+LM	7.58

The optical density of inoculated cultures was measured both at the beginning of the experiment and after two weeks of exposure. OD was evaluated spectrophotometrically at 663nm wavelength against OD of relevant treatment using Multidetection Microplate Reader Synergy 2 made by (BIOTEK). Data were processed in Gen5 TM Sofware. The results were verified microscopically.

Additionally, data from a field experiment established by Jan Frouz from the Institute of Soil Biology AS CR and provided by Alena Lukešová were used in this study. Four types of amendments - limestone powder (40g. ha^{-1}) and the same dose of limestone powder in combinations with freshly mined cyprises from a nearby pit (40g. ha^{-1}), commercially available bark (40g. ha^{-1}) and cow manure (40g. ha^{-1}) were added to toxic soil on experimental site CC12 (Tab.1). Each treatment was applied on plots of $1m^2$ in three replications. The application of limestone raised pH value to about 5.5. The richness of algal microflora was investigated two years after application.

3.6. Statistical analyses

Data were analysed using the CANOCO for Windows programme version 4.5 (Ter Braak&Šmilauer, 1998)

Unimodal indirect canonical correspondence analysis (CCA) was used to assess the effects of environmental factors explaining the variance of algal/cyanobacterial communities. Forward selection was applied for choosing the main variables. Monte Carlo permutation test was used

to test the significance of thirteen environmental factors (three types of substrate, pH, conductivity, content of organic carbon, age, slope, vegetation cover and toxicity, contents of potassium, sodium, aluminium, iron and polyphenols). CCA was also used to show the effect of age in terms of species diversity.

Redundancy analyses (RDA) were performed to show the preference to substrate type sinificantly correlated with pH. The RDA was based on non-standardized data. RDA analysis was also computed to test the influence of different additions to soil on algal/cyanobacterial growth. Standardized as well as non-standardized data were used for analysis.

4. Results

4.1. Physical and chemical properties

Big differences of studied properties were found among the investigated plots (Tab.1). They included extremely acid as well as alkaline substrates (pH ranged from 2.37 to 8.33). Maximal conductivity reached up to 2341 $\mu S/cm^{-1}$ in TC9. Soils contained from 5.3 to 13.4% of organic carbon. High soil moisture corresponded to early spring and late autumn sampling occasions.

4.2. Algal and cyanobacterial community structure in post-mining soils

In total 71 species of algae and cyanobacteria was found in investigated soils in the Sokolov mining area (10 Cyanophyceae, 8 Bacillariophyceae, 1 Euglenophyceae, 1 Eustigmatophyceae, 7 Charophyceae, 6 Chlamydophyceae, 30 Chlorophyceae, 6 Xanthophyceae, 2 Zygnemaphyceae) (see species list). Only few species of Chlorophyceae in negligible amounts were isolated from extremely acid coal rich clays. Chlorophyceae prevailed also in acidic volcanic ashes (VA) and less acid coal rich clays (CC). In terms of species diversity Chlorophyceae were followed by Chlamydophyceae. Zygnemaphyceae occured exclusively in acid volcanic ashes, in contrast to the only species of Eustigmatophyceae being unique to the most alkaline plot. Both Cyanophyceae and Xanthophyceae prefered alkaline tertiary cypric clays (TC). Observed cyanobacterial species belonged mainly to Oscillatoriales. Representatives of Noctocales were less present.

Bacillariophyceae were the most abundant and diverse in alkaline substrates with higher conductivity (Fig.1, 2).

Although the total number of isolated algal and cyanobacterial species was the highest in TC (62) and decreased in VA (36) and CC (22), both maximal and average values of total abundance were the highest in VA (1.9 x 10^6, and 9.3 x 10^5, respectively) (Fig.3).

Hardly determinable but dominating *Chlorella*-like species were isolated particularly from highly acidic sites (CC, VA and TC9).

Visible algal crusts were found on acidic sites (CC1, VA2, CC3, VA4, VA5). The crusts were dominated mostly by filamentous green alga *Klebsormidium crenulatum,* in some cases alternated by two other very similar species. *Klebsormidium crenulatum* was often accompanied by different *Klebsormidium* species (e.g. *Klebsormidium mucosum).* Surprisingly *Klebsormidium* crusts were also massively developed on slightly alkaline substrate of VA13.

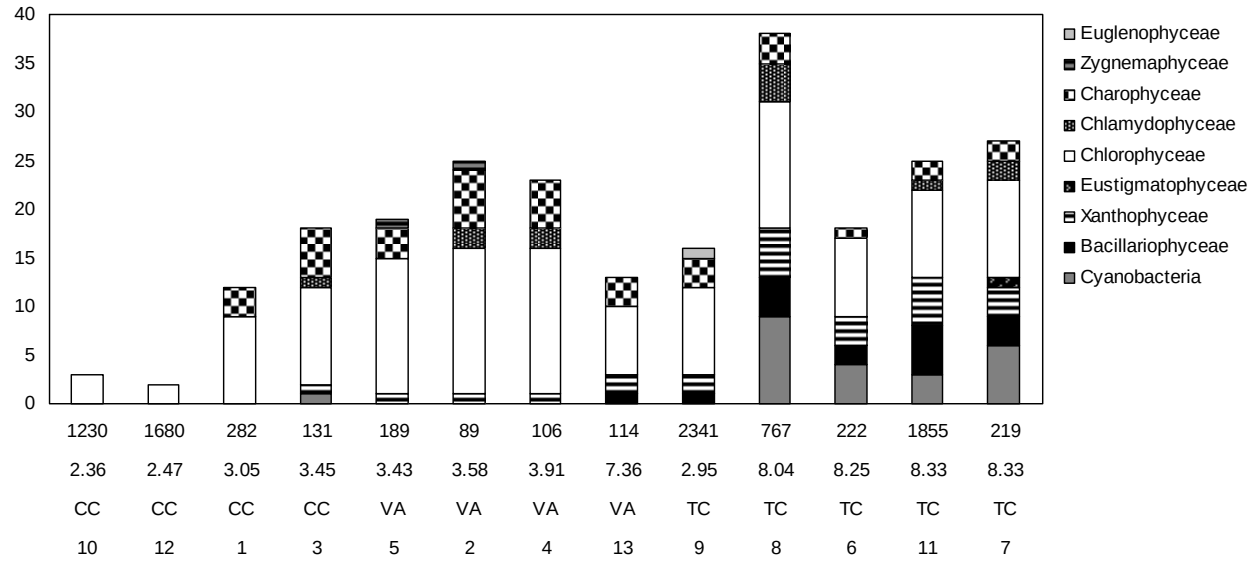

Figure 1. Structure of algal and cyanobacterial communities on 13 investigated plots (1 - 13). Values of conductivity and pH are added as well as substrate type (CC - coal clays, VA - volcanic ashes, TC - tertiary cypric clays).

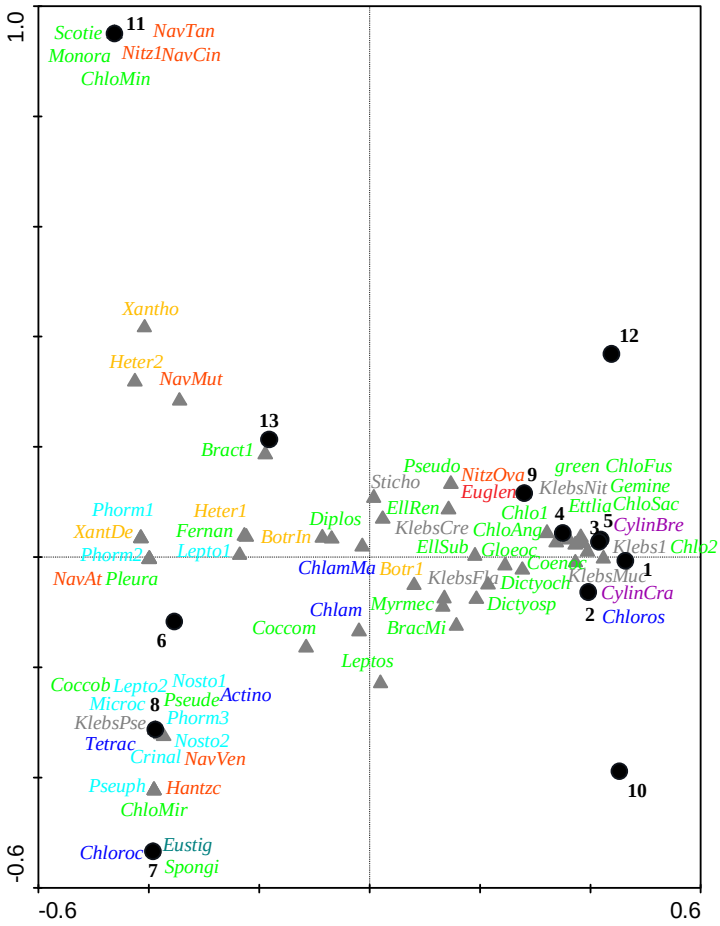

Figure 2. CCA analysis showing the occurence of algal and cyanobacterial species (in italics) in 13 investigated plots (1-13).

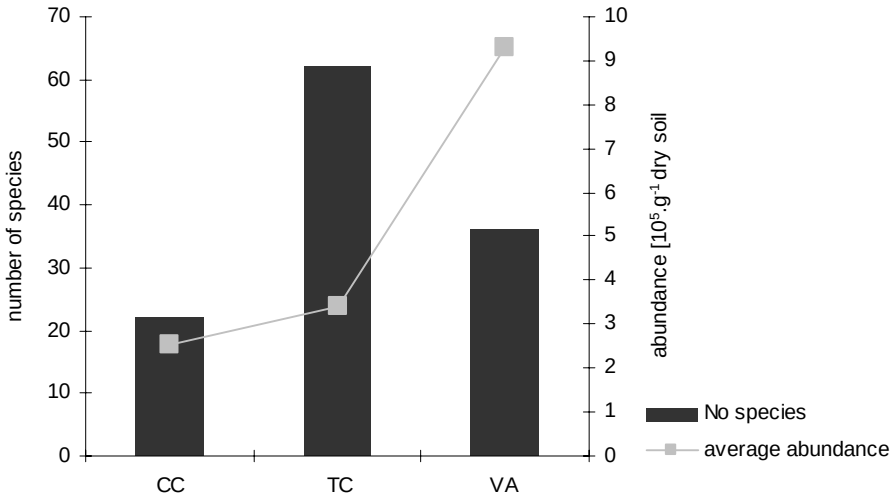

Figure 3. The comparison of total average abundance of living cells and species number dependent on substrate type (CC - coal rich clays, TC - tertiary cypric clays, VA -volcanic ashes).

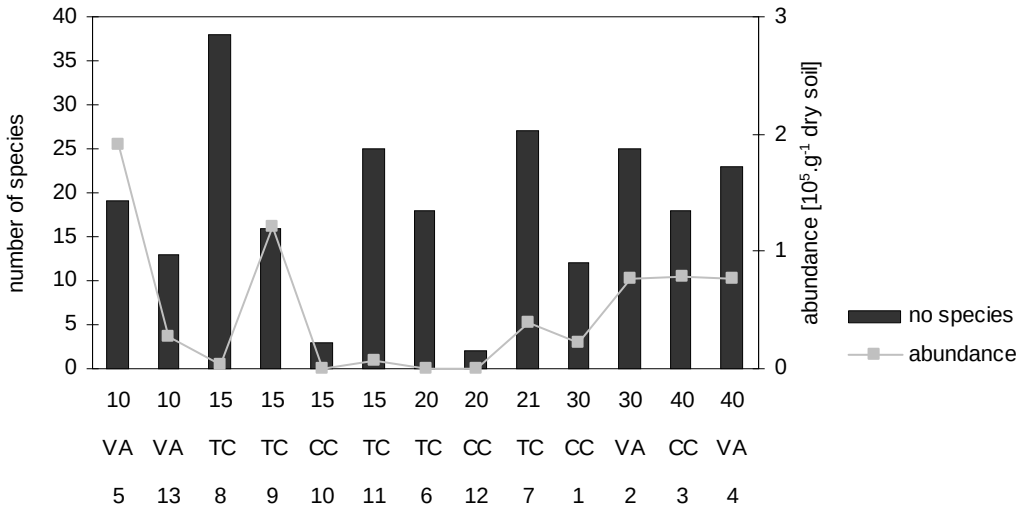

Figure 4. The comparison of total average abundance of living cells and number of species present in thirteen investigated plots, The numbers below X-axis express the age of particular plot in years. The sites are described by substrate type (CC - coal rich clays, TC - tertiary cypric clays, VA -volcanic ashes) and site number (1-13).

Thirteen environmental factors such as substrate type, age of dumps, toxicity, pH, conductivity, organic carbon content, and contents of sodium, potassium, aluminium, iron, and polyphenol of twelve investigated plots were analysed. According to canonical correspondence analysis (CCA), the significance of pH (p-value = 0.0020; F-ratio = 3.07), slope (p-value = 0.0260; F-ratio = 1.53) and conductivity (p-value = 0.0260; F-ratio = 1.59) was proved. Values of pH, slope and conductivity were assessed as the main environmental factors explaining 46.6% of variability in aquired data (Fig.5). Substrate type, toxicity as well as potassium content were highly correlated with pH gradient. The effect of sodium content in soil was marginally significant (p-value = 0.0520; F-ratio = 1.65).

The analysis confirmed the presence of Cyanophyceae, Xanthophyceae and Bacilllariophyceae in sites with higher pH, unlike Chlorophyceae, Charophyceae, Euglenophyceae and Zygnemaphyceae occuring in more acidic soils. Considerably, Cyanophyceae prefered steeper slopes. Moreover, Bacillariophyceae developed best in sites with higher conductivity levels.

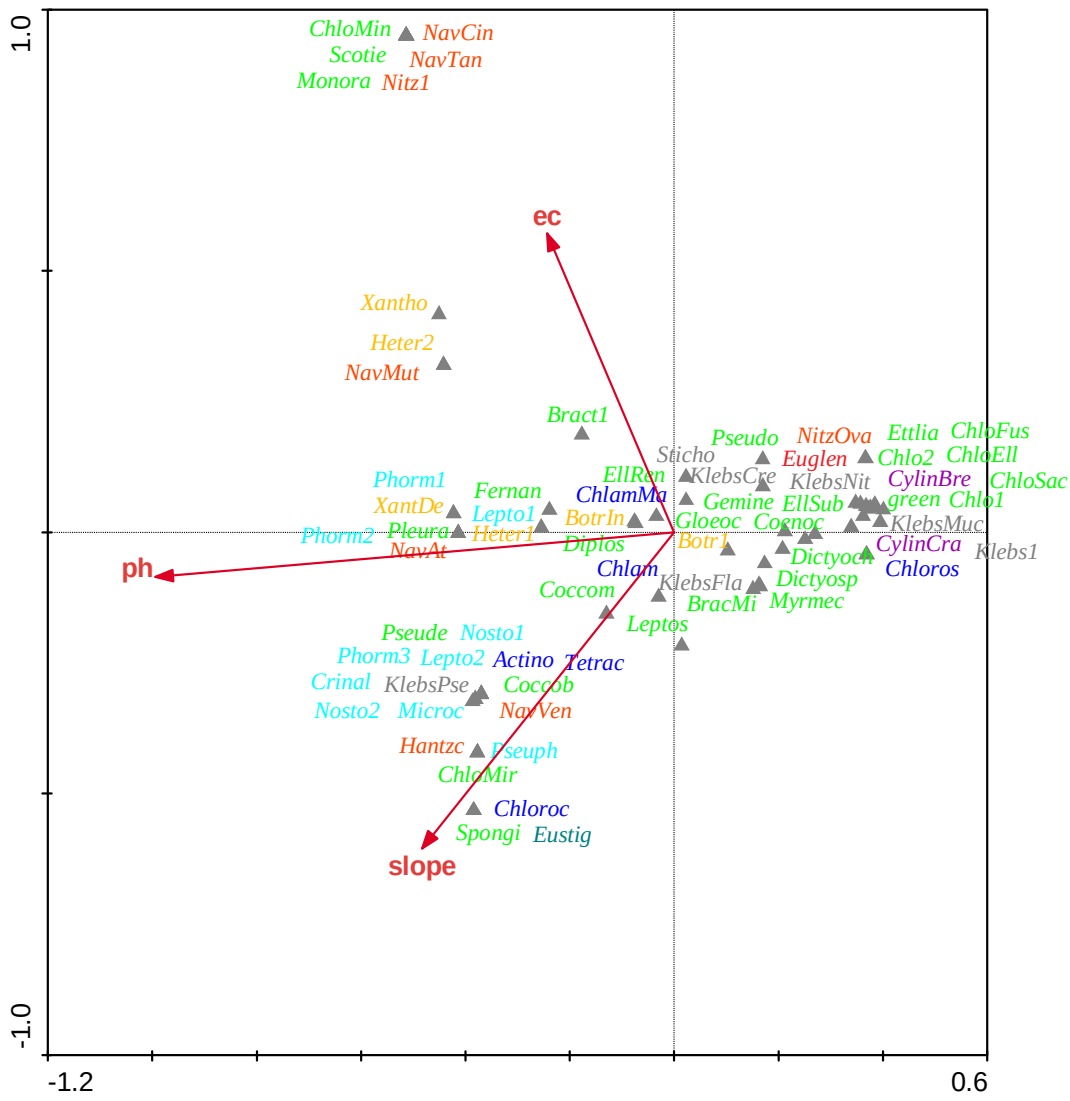

Figure 5. Canonical correspondence analysis (CCA). The first axis explains 23.6% of variability. All factors together explain 46.6% of total variability. The environmental factors showing gradients are labeled by arrows, species are written in italics.

In terms of time changes in algal community structure were observed. Communities of algae and cyanobacteria had a tendency to decrease the species diversity with increasing age (Fig.6). No dependence of total cell abundance on age was proved (p-value = 0.7835; F-ratio = 0.0793). Linear dependence of pH on age was determined. *Chlorella fusca* dominated in soils of older plots reaching pH value ca 4. Younger successional stages were characterized by either more acidic or more alkaline conditions. Particularly earlier stages formed on coal rich clays created extreme environment for living. Coccal green algae accompanied by diatoms were found to be first colonizers of those acid soils of dumps. On the other hand younger alkaline sites were initially dominated by cyanobacteria encouraged by Bacillariophyceae and Chlorophyceae (Fig.6).

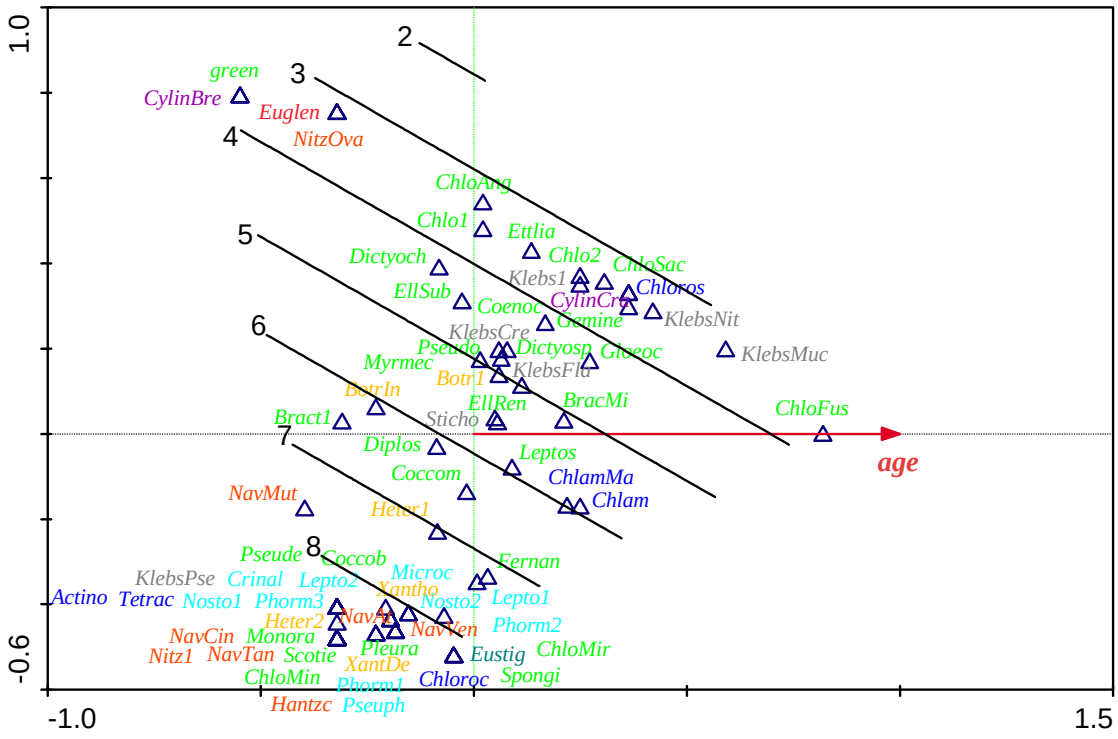

Figure 6. Canonical correspondence analysis (CCA) showing algal/cyanobacterial colonization trends with pH/age dependence. Lines with numbers mean pH values, arrow indicates age gradient, species written in italics are marked with triangles.

4.3. Experiments

4.3.1. Pilot test

It was found that mainly representatives of green algae survived and grew in solid toxic substrate CC12 (Fig.7). Microscopic revision revealed viable cells of *Pseudococcomyxa simplex* and *Elliptochloris subsphaerica* being the most resistant, rarely *Chlamydomonas macrostellata* occured. High number of living cells of *Chlorella saccharophila* was counted. *Botrydiopsis intercedens* and *Myrmecia bisecta* were also occasionally found. Total abundance of living cells in particular mixtures ranged from 1.8×10^3 to 1.8×10^6.

Subsequent test confirmed that only green algae endured conditions of toxic extracts, cells of other species were bleached out after the exposure. *Chlorella saccharophila* and *Chlamydomonas macrostellata* prosper in all concentrations, even in 100% toxic extract. On the contrary *Bracteacoccus minor* was able to grow just in the mildest tested concentration.

Additionally it was noticed that some species (e.g. *Pseudococcomyxa simplex, Elliptochloris subsphaerica*) withstood worse toxic conditions in case of being tested in test tubes. However, nine of total fifteen analysed species did not survive in toxic extracts at all (Fig.7).

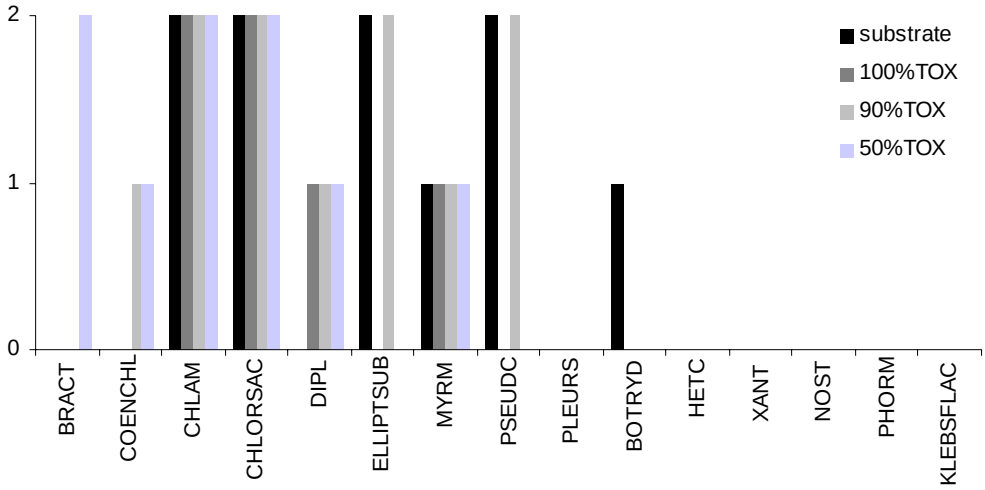

Figure 7. Species survival and growth in toxic substrate (CC12), toxic water extract and its dilutions in BBM (100%TOX, 90%TOX, 50%TOX). DIPL - *Diplosphaera chodatii*, XANT - *Xanthonema debile*, BRACT - *Bracteacoccus minor*, PSEUDOC - *Pseudococcomyxa simplex*, CHLAM - *Chlamydomonas macrostellata*, HETC - *Hetrococcus sp*., ELLIPTSUB - *Elliptochloris subsphaerica*, KLEBSFLAC - *Klebsormidium flaccidum*, NOST - *Nostoc sp*., BOTRYD - *Botrydiopsis intercedens*, PLEURS - *Pleurastrum sarcinoideum*, CHLORSAC - *Chlorella saccharophila*, COENCHL - *Coenochloris sp*., PHORM - *Phormidium autumnale*, MYRM - *Myrmecia bisecta.*

4.3.2. Growth and survival tests

All tested species prefered tertiary cypric clay substrate (p-value = 0.001; F-ratio = 14.42) to coal clays or volcanic ashes. Cypric substrate represented 73.1 % of total variability (Fig.8). pH highly correlated with substrate type was considered to be the most important factor affecting the quality algal growth. The executed RDA analysis revealed (Fig.9) that pH explained 70.4 % of total variability (p-value < 0.001; F-ratio = 26.19). Measured pH values of tertiary cypric clays were slightly alkaline, ranged from 7.72 to 8.42 (except for TC9) in contrast to pH of acid coal clays (pH = 2.29 - 3.56) and volcanic ashes (pH = 2.82 - 7.64). However, it was observed that some species e.g. *Pseudococcomyxa simplex* were able to grow even in very acidic coal rich clays (Fig.10), but the test did not prove any significance (p-value = 0.212; F-ratio = 1.31).

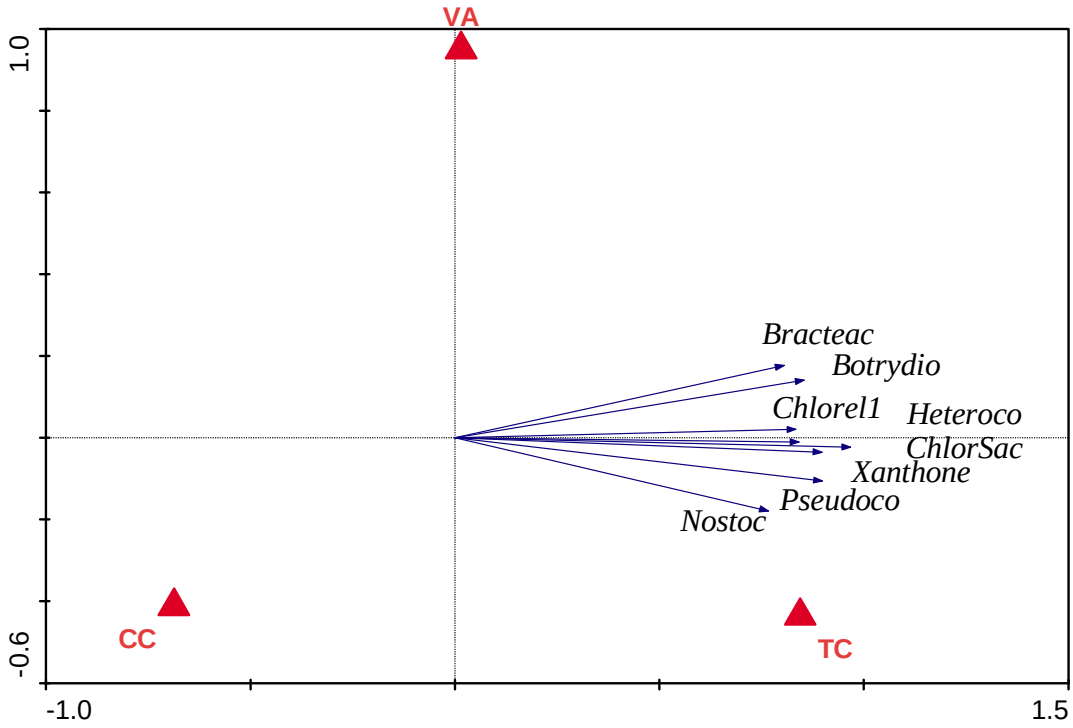

Figure 8. Redundancy analysis (RDA) based on non-standardized data. The arrows mean tested species of algae and cyanobacteria, substrates (CC - coal clays, TC - tertiary cypric clays, VA - volcanic ashes) are represented by centroids. The first axis explains 73.1 % of total variability.

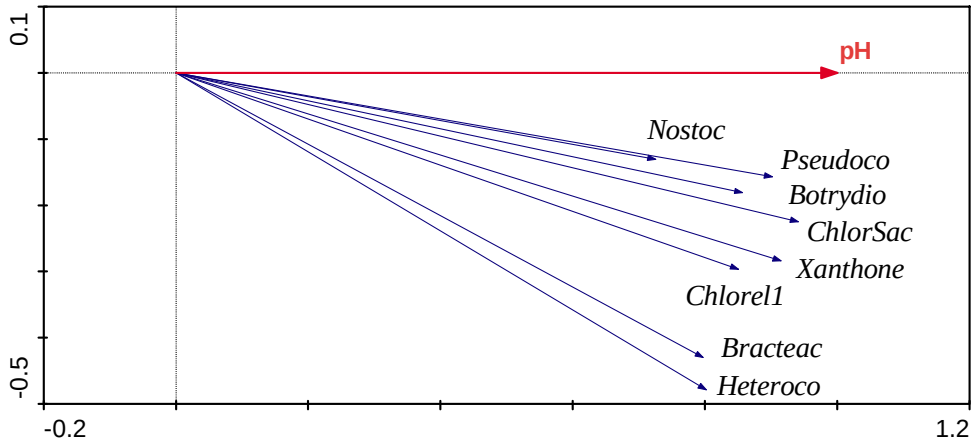

Figure 9. Redundancy analysis (RDA) with non-standardized data. The first axis represents 70.4% variability.

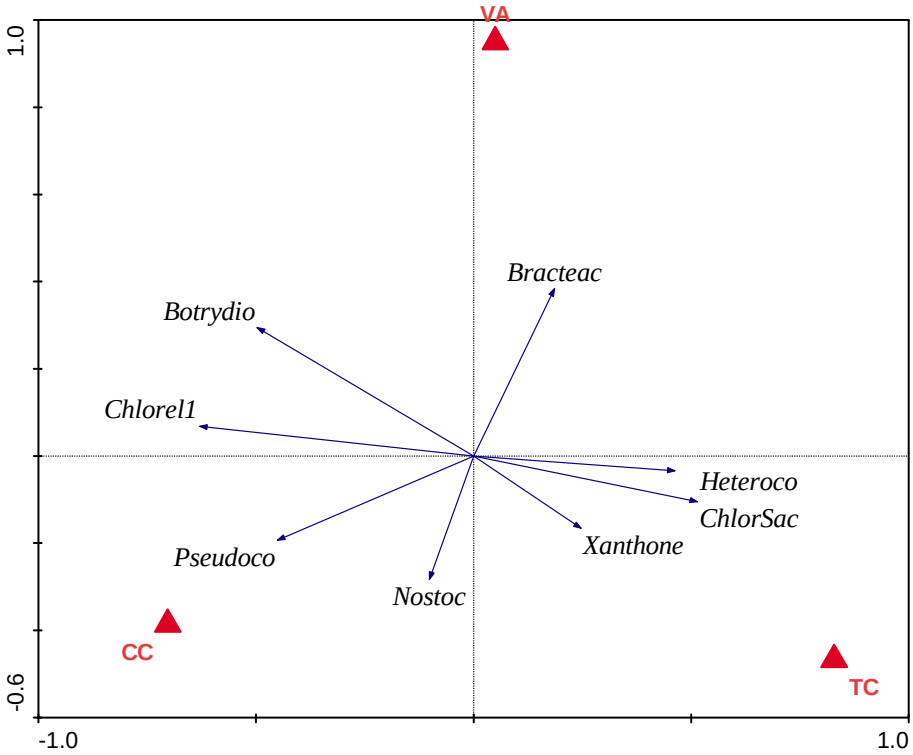

Figure 10. Redundancy analysis (RDA) based on standardized data. The first axis explains only 15.7 % variability.

4.3.3. Amelioration tests

According to redundancy analysis (RDA) all tested species tended to grow well in amendmets with dolomitic limestone rather than in other treatments (p-value = 0.0380; F-ratio = 8.58). The first axis explained 68.2% of variability in data (Fig.11). The addition of limestone led to increase of pH (reaching up to 7.97), therefore even the species sensitive to low pH were able to develop.

However, the significant differences among species behaviour in various treatments were observed (p-value = 0.0260; F-ratio = 4.290). RDA analysis based on standardized data showed that mainly Cyanobacteria and Xanthophyceae prefered alkaline conditions in contrast to green algae growing succesfully in all treatments (Fig.12).

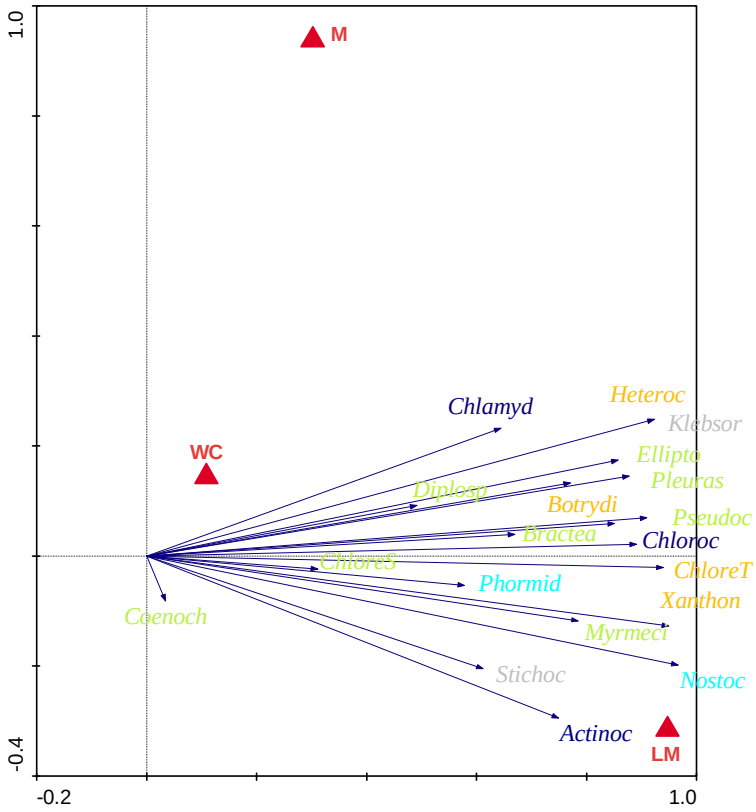

Figure 11. Redundancy analysis (RDA) based on non-standardized data showing the preference to limestone treatment. Treatments are represented by centroids (LM - dolomitic limestone, WC - wooden coal, M - manure additions), arrows stand for tested species. The first axis explains 68.2% of variability.

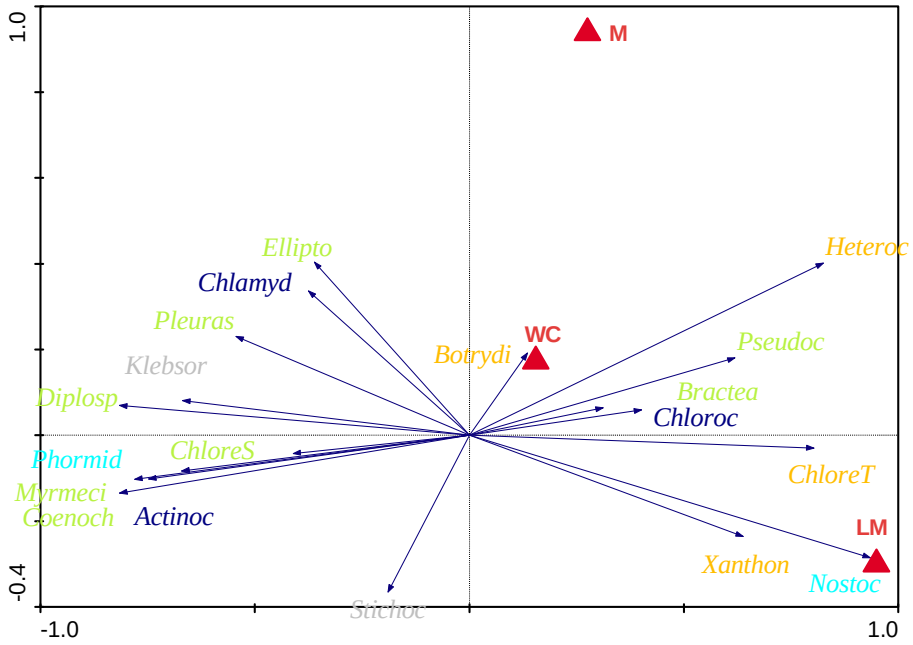

Figure 12. Redundancy analysis (RDA) based on standardized data showing the differences among species growth trends in dependence on treatment. Treatments are represented by centroids (LM - dolomitic limestone, WC - wooden coal, M - manure additions), arrows stand for tested species. The first axis explains 57.1% of variability.

Field experiment confirmed the importance of higher pH as a predictor for establishing more abundant and diverse soil microflora on industrially devastated sites. Besides it has been shown that cypric clay enrichment as well as bark and manure additions had extra positive effects on algal and cyanobacterial developement. The soil ameliorated with limestone powder in combination with cypric clay substrate was proved as the best growing medium in terms of species diversity (Fig.13).

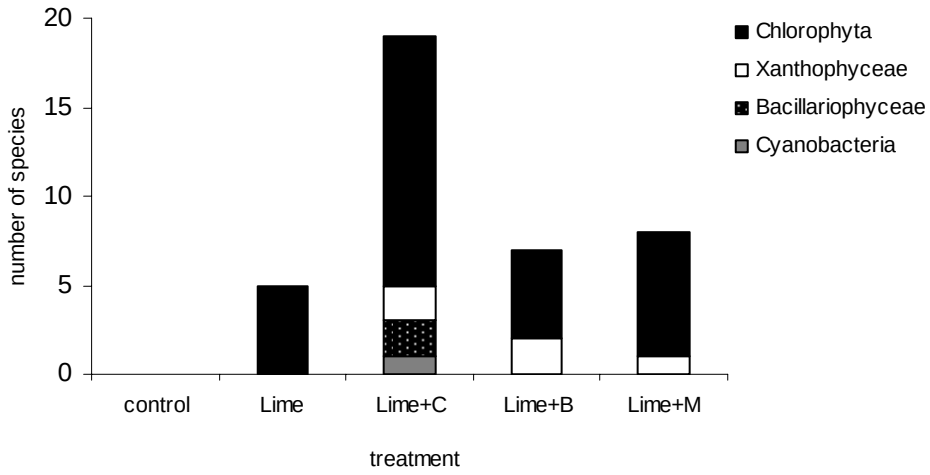

Figure 13. Stucture of algal/cyanobacterial communities growing in soils with various treatments (Lime - limestone powder, C - tertiary cypric clays, B - bark, M - manure). Plain toxic substrate was considered as a control.

5. Discussion

5.1. Algal and cyanobacterial community structure in post-mining soils

The algal and cyanobacterial species diversity found in soils of Sokolov post-mining area is higher than in disturbed soils elsewhere. Shubert&Starks (1980) identified 11 species in freshly mined soils from Arizona, New Mexico and North Dakota. The same authors isolated 41 species from orphaned spoils, 31 species from non-topsoiled and 16 species from top-soiled sites in coal mining area in Western North Dakota (Shubert&Starks, 1979; 1982) Compared with reports from Sokolov district (Lukešová, 2001) the species richness is similar. This can be caused by both the specific properties of investigated soils near Sokolov and new approaches to identification and classification of algal and cyanobacterial species.

Species of algae in this survey were identified and classified according to Ettl & Gärtner (Ettl&Gärtner, 1995). Since the book was published, many new findings in taxonomy and

phylogeny of green algae have been discovered. Some species were transferred to different or new classes or divisions using molecular biology. For example, many species previously belonging to Chlorophyceae were reclassified to Trebouxiophyceaea (Friedl, 1997; Rindi *et al.*, 2007). Charales have been recently separated from Chlorophyta to form an independent group of Streptophyta (Turmel *et al.*, 2007). Diatom taxonomy has also undergone the changes in nomenclature. *Navicula* genus have been splitted up, and selected species were transferred to newly established genera, e.g. renamed *Luticola, Diadesmis* (Denys&DeSmet, 1996). The nomenclature followed in this study was chosen for easy comparison with other literature data and in order to unify the terminology.

The structure of algal communities in investigated soils corresponds with former studies. The occurence of Chlorophyceae in highly acid substrates and their dominance in acid and neutral soils has already been decribed (Hoffmann, 1989; Maxwell, 1991). This can be explained by the tolerance of Chlorophyceae to wide range of pH. Zygnemaphyceae are usually bound to acid environments such as peat bogs, thus their occurrence in acid coal clays is not surprising. According to ecological demands, Cyanophyceae occupy soil habitats with pH higher than 4.4 (Brock, 1973). In accordance with this statement they were found only in alkaline or neutral substrates studied during this work.

As being mentioned in several studies (Hodkinson *et al.*, 2003; Chan *et al.*, 2003; Fermani *et al.*, 2007) Cyanophyceae are the pioneer colonizers of barren soils, with exception for acid soils, particularly due to their ability to fix nitrogen and consolidate soil particules, thereby stabilize the soil surface. Findings of mainly representatives of *Oscillatoriales* and *Nostocales* confirm entirely this fact.

Also Xanthophyceae, representing another important component of soil microflora, occur mostly in alkaline environments (Lukešová&Hoffmann, 1996). The presence of Bacillariophyceae in soils with higher conductivity coincides well with cited literature (Metting, 1981; Hoffmann, 1989).

It has been shown that crusts and biofilms are developed mainly in extreme environments (Jahnke&Priefer, 2002) where they play essential role due to nitrogen fixation, binding soil particles, preventing soil erosion, etc. Although the investigated dumps were in some cases covered by vegetation, *Klebsormidium* crusts occured exclusively on unvegetated places. *Klebsormidium* crusts seem to be a common phenomenon on barren acidic soils on dumps (Durrell&Shields, 1961; Lukešová, 2001). Well developed crusts were observed, however, also on volcanic ashes (VA13) reaching slightly alkaline pH. The coarse substrate texture

seems to support colonization by *Klebsormidium* and its key role in crust formation (Pluis, 1994).

The majority of identified genera can be characterised as typical terrestrial microflora (e.g. *Chlorella, Chlorococcum, Stichococcus, Botrydiopsis, Heterococcus, Xanthonema*). Isolated species of pennate diatoms are commonly found in soils, species such as *Hantzschia amphioxys, Navicula mutica, Navicula ventricosa, Navicula atomus* (see species list) are typical soil and aerophytic species (Starks *et al.*, 1981; Hoffmann, 1989). Many interesting *Chlorella* and *Chlorella*-like species were isolated from highly acidic substrates. The reliable identification to the species level is problematic using only morphology. Therefore the further investigations with the use of molecular methods are necessary and planned for the future. Similar situation was noticed with *Klebsormidium* species dominating visible crusts. Also those should be solved by molecular approach.

In terms of abundance, algal and cyanobacterial species in Sokolov post-mining soils were detected in equal or slightly lower amounts than others observed in disturbed soils (Lukešová, 2001). Different methods of algal quantification used by other algologists, e.g. biomass expression as *chlorophyll a* (Shubert&Starks, 1979) make the comparison difficult. The highest numbers of living cells in volcanic ashes are caused by maximal development of Chlorophyceae. Coccal green algae are able to multiply quickly, thus they can reach high cell counts. In spite of the fact that *Klebsormidium* crusts can produce huge biomass visible by naked eye, quantification based only on counting their relatively big cells (cell abundance) can underestimate biomass and real importance of this species.

Following the conducted analysis, pH, conductvity and slope of the site are the main environmental factors affecting species diversity. In agreement with ecological demands of induvidual species or entire groups, the effect of pH as well as conductivity is not surprising.

Slope also plays a significant role in composition of algal and cyanobacterial community. I suppose that the major occurrence of Cyanobacteria in steep sites is determined by their ability to form so-called mats (Booth, 1941). Being firmly attached to the substrate, these mats resist well the potential soil erosion frequently occuring on steeper slopes. This makes cyanobacteria more successful, compared to other species, in initial successional stages in such non-acidic sites.

The influence of sodium content on the structure of algal communities can differ according to micro-algal group. As me and Shubert&Starks (1979) observed, the sodium had the inhibition effect on algal growth concerning particularly green algae. However, the same authors described the opposite trend for Cyanobacteria (Shubert&Starks, 1979)

Although the type of substrate seems to be significant in determining the algal composition, it is highly correlated with pH, thus it loses its importance. In contrast to alkaline tertiary cypric clays possessing rich species diversity with presence of Cyanobacteria and Xanthophyceae, acid coal clays as well as volcanic ashes are characteristic for their acidophilous microflora (namely Chlorophyceae; *Chlamydomonas macrostellata, Chlorella saccharophila*).

The substrate toxicity defined on the base of former study using enchytraeids as testing organisms showed the positive correlation with pH (Frouz *et al.*, 2005). In spite of various soil properties being toxic to model animal, algae displayed the resistance to most of them except for pH.

5.2. Succession of algal and cyanobacterial communities

As being decribed in several studies (Shubert&Starks, 1979; 1982; Lukešová&Komárek, 1987) the structure of algal communities is changing over the time. The decrease in species diversity in later successional stages has been noticed. According to the literature the same trends were observed in case of higher vegetation as well as in animal communities (Dunger *et al.*, 2001). It is known that during the succession the properties of soils are stabilized and thus the community reaches the equlibrium in number of species.

Despite the literature describing trends towards the increase of the total abundance over the time concerning animals (VanAarde *et al.*, 1996), plants (Skousen *et al.*, 1990; Sanger, 1995) and algae (Shubert&Starks, 1979), no dependence of algal biomass on age was recorded in this study. The algal abundance can be affected by toxicity (low pH) and local conditions in studied sites rather than by their age.

The high abundance especially in ten year old site VA5 is due to the presence of *Klebsormidium* crusts covering the surface and due to the representation of numerous coccal Chlorophyceae. Although the investigated plot TC9 is poorer in species diversity compared to other tertiary cypric sites, dominating *Euglena mutabilis* causes markedly high amount of biomass there. Probably the acidic character of this site favours this species typically inhabiting wet, low pH localities (Lackey, 1938; Hargreaves *et al.*, 1975; Whitton&Satake, 1996).

Coincidently high algal abundances can be monitored among the older sites, too. Those sites are characterised by great representation of fast-growing green algae. Nevertheless the high algal biomass can be limited, even decreased by the development of forest habitats in those sites. Although providing suitable conditions for many organisms, the forest vegetation

impedes the light penetration to the soil microflora and thus limits the development of algae (Lukešová&Hoffmann, 1996).

pH of freshly mined soil often reaches extreme values. Generally either very low or high pH affects markedly the further development on spoils. As the succession is proceeding, the soil properties are changing. The vegetation succession contributes to organic matter accumulation, following decomposition results in increased amount of organic carbon leading to pH changes in soils. Extremely alkaline substrates are getting more neutral or acidic over the time, which was well documented also for alkaline cypric clays in Sokolov area (Lukešová, 2001). Very acidic soils are able to increase their pH during the succession, even though it is more complicated. In some cases it is necessary to support the initial changes. For that purpose many investigations and biological tests in post-mining areas have been conducted (Vinyard, 1996; Maenpaa *et al.*, 2002; Boularbah *et al.*, 2006).

In this study the linear dependence of soil pH on age was determined. It was found that green algae colonize and initiate the successional process in the acid soils of dumps. Green algae considerably dominate the algal floras of acid habitats due to the absence of competition (Hoffmann, 1989). Early colonization of alkaline and neutral soils is realized mostly by nitrogen fixing cyanobacteria. Particularly these organisms are able to cope with the lack of nitrogen in freshly mined material (Shtina, 2000).

According to the results, prevailing pH on forty year old dumps is slightly acidic. This can be caused by both the higher content of organic carbon as well as the substrate type of older investigated plots. Since the soil of dumps in later succession has acid character, the prevailing flora consists of Chlorophyceae. The dominance of *Chlorella fusca* in almost finished forest formation (CC3) is not suprising. According to Ettl & Gärtner (Ettl&Gärtner, 1995) the species is commonly found on tree bark as well as in forest soils.

5.3. Experiments

Based on visible signs of damage caused by former mining activity in combination with information gained in previously described survey, Litov dump (CC12) was considered to be the most toxic post-mining site. The efforts to recover the area have not been successful yet (Frouz, *personal communication*).

Coal clays of Litov dump are extremely acid characterized by high conductivity, low contents of iron, lack of sodium and potassium and relatively high amounts of aluminium, and surprisingly not very high content of polyphenols. Except sporadic findings, no algae were

isolated from that substrate. *Dictyochloris fragrans* and *Chlorella* sp. in Litov dump soil were present probably only because of the former reclamation treatments.

Although all tested species showed the preference to the growth in neutral or slightly alkaline conditions, there are some representatives of Chlorophyceae capable to survive and grow in extremely acid substrates. Reduced competitive stress, the definite cell size and shape, and specific intracellular mechanisms enabling to tolerate unfavourable conditions, motility as well as alternative trophic ways are the main reasons of successful development of Chlorophyceae (Nixdorf *et al.*, 2001).

Besides all these advantages of Chlorophyceae, the requirement to reach the successfull algal developement on very acidic sites is the input of diaspores from adjacent even distant natural sources. This must be accompanied by suitable climatic conditions (Hu *et al.*, 2003) and by proper substrate texture, which is well documented by *Klebsormidium* crusts growing preferably on coarse substrates (VA13).

5.4. Amelioration tests

Despite the relative successs of green algae in acidic substrates, various techniques have been proposed in order to improve or accelerate the recovery of toxic post-mining sites. The composition of soil microflora can serve as a substrate quality indicator and thus can be used for recultivation purposes.

Shubert and Starks (1979; 1982) analysed the algal and cyanobacterial communities in relation to series of amendments. They observed rather negative effect in case of using topsoil cover. In other case they noticed the increase in algal variety over time regardless the type of soil amendment they used (leonardite, scoria and fertilizers additions). All soils they investigated were neutral or slightly alkaline.

As I recorded the limestone addition leading to pH increase has positive effect on algal development. The higher pH values contribute to algal development mainly due to the enrichment of soil algal flora by representatives of Xanthophyceae and Cyanophyceae.

This corresponds to Maxwell' study (1991) describing the increase in the diversity of Chlorophyceae and Cyanophyceae in moderately acid soils amended by lime and fertilizer.

According to the results of the laboratory tests neither the manure added to the soil in order to cotribute to total organic carbon content nor wooden coal addition aiming to absorption improvement showed positive influence on microflora development.

Field experiment proved that besides limestone powder amendment, all extra additions such as bark, manure and freshly mined cypris substrate improved the soil, and so established the appropriate conditions for colonization and development of algae and cyanobacteria.
Although being affected by many factors such as weather and natural disturbances, the field experiments seemed to be more suitable for testing the soil amendments than the laboratory tests. The substrate texture, which is neglected during the laboratory tests, as well as the input of diaspores can play a significant role in treatment effectiveness.
This study emphasizes the importance of soil microflora in post-mining sites, as the composition of algae and cyanobacteria reflects the quality of substrate, their toxicity respectively. It deals with the process of colonization and succession of barren mined soils. Biological tests conducted during this survey indicates the ability of green algae to colonize even the extremely toxic substrates. Together with amendment tests the study suggests a potential use of soil microflora as alternative source of recultivation techniques on industrially devestated sites.

6. Conclusions

The composition of algae and cyanobacteria can serve as an indicator of soil quality. The structure of algal communities varies in dependence on soil properties, especially on pH. Green algae are able to colonize and grow even in toxic, extremely acid soils, thereby being the first primary producers in post-mining areas. They represent thus a potential agens for reclamation practices. However, the increase of pH is a basic precondition for the establishment of more diverse and abundant algal flora in highly acidic sites. Both application of low-pH tolerant species and the improvement of soil properties supporting algal development offer a possibility to accelerate successional processes and recultivation of certain industrially damaged areas.

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Appendix

1. Locality

Sokolov mining district (north-western part of the Czech Republic)

2. Investigated plots

1. Chodov - coal clays
2. Chodov - volcanic ashes
3. Chodov (Vintířov) - coal clays
4. Chodov (Vintířov) - volcanic ashes
5. Sokolov (soil slippage) - volcanic ashes
6. Sokolov (Svatava) - tertiary cypric clays
7. Sokolov (Svatava) - tertiary cypric clays
8. Sokolov (Pastviny) - salty tertiary cypric clays
9. Sokolov (Pastviny) - tertiary cypric clays
10. Sokolov (Pastviny) - coal clays
11. Sokolov (Pastviny) - teriary cypric clays
12. Chlum sv. Máří (Lítov) - coal clays
13. Sokolov (Pastviny) - volcanic ashes

1.

2.

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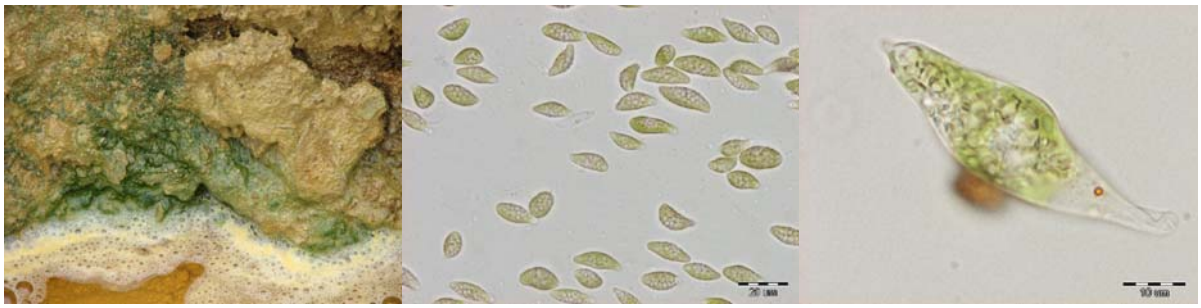
3. Substrate types

tertiary cypric clays

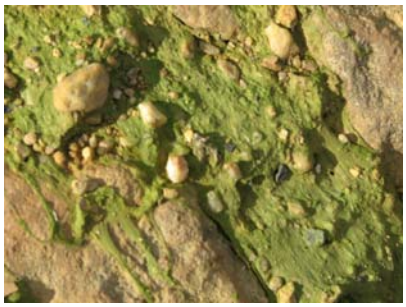
volcanic ash

coal clay

4. Some representatives of algal and cyanobacterial microflora in soils of dumps and their massive development



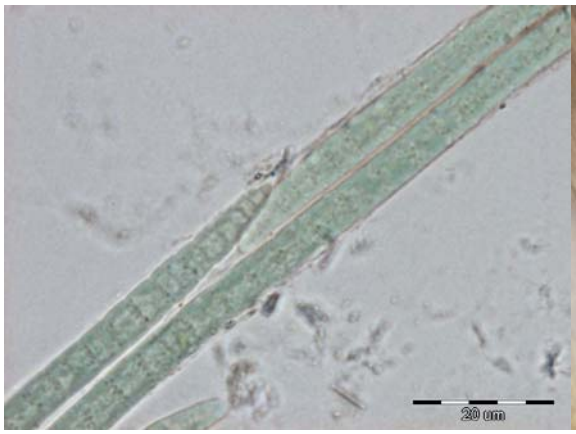
TC9 study site with population of *Euglena mutabilis*



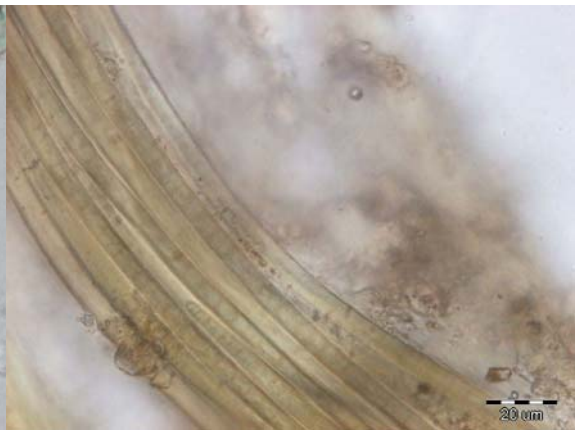
Klebsormidium crusts on investigated plots (VA13; VA4; VA5)

A. *Klebsormidium* cf. *crenulatum* freshly isolated from studied sites.

B. *Klebsormidium* cf. *crenulatum* maintained in culture under laboratory conditions.

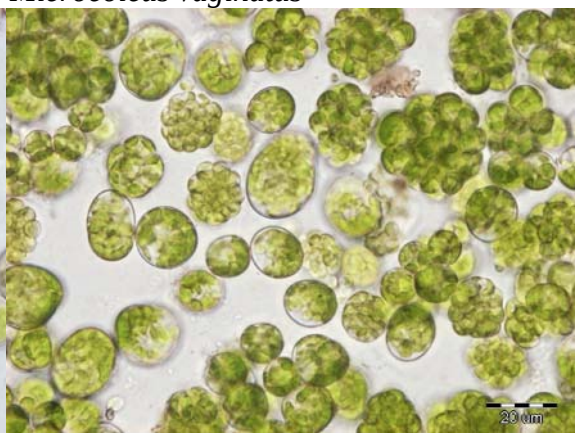


Phormidium cf. *autumnale*

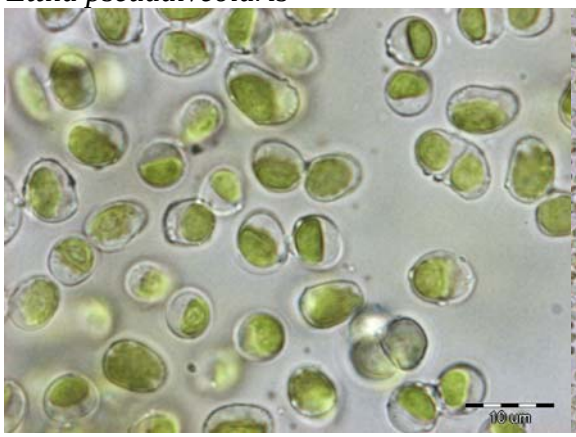


Microcoleus vaginatus

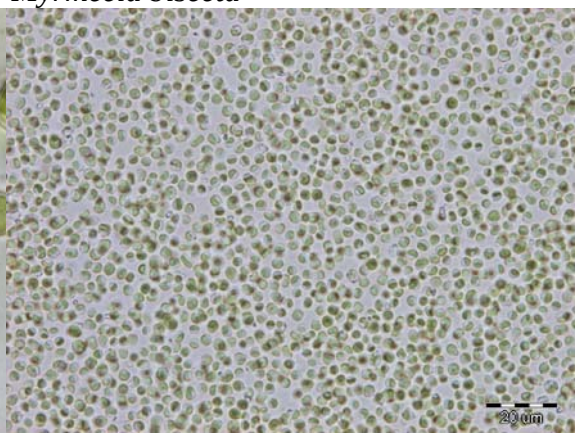
Ettlia pseudalveolaris



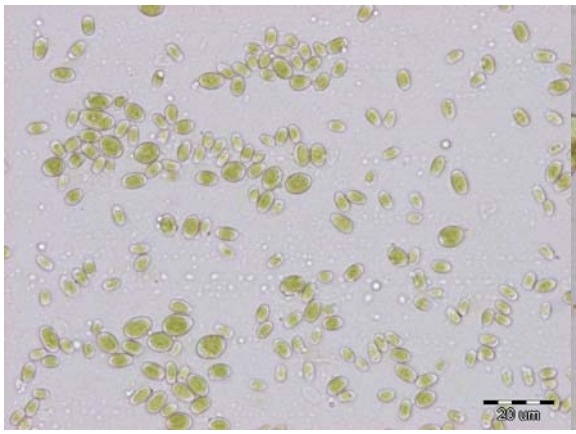
Myrmecia bisecta



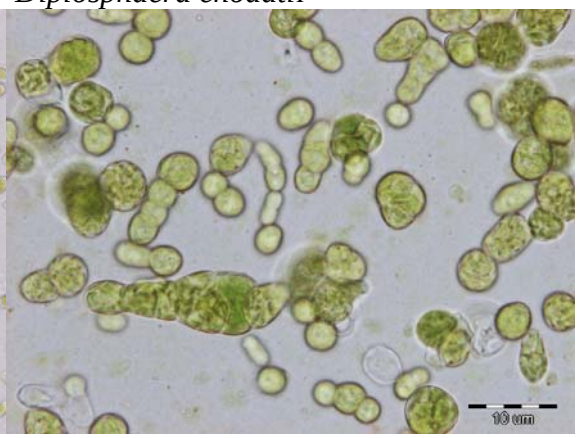
Geminella terricola



Diplosphaera chodatii



Chlorella saccharophila



Heterococcus sp.

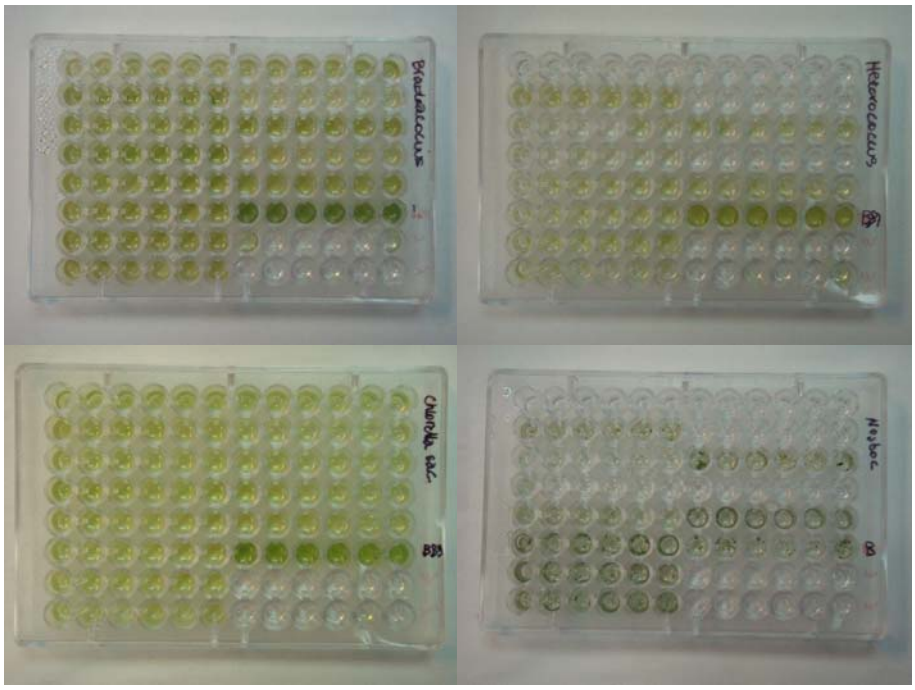
Species list

Cyanophyceae	abbreviations
Leptolyngbya sp.1	Lepto1
Leptolyngbya sp.2	Lepto2
Microcoleus vaginatus Gomont ex Gomont	Microc
Phormidium sp.1 (typ *autumnale*)	Phorm1
Phormidium sp.2	Phorm2
Phormidium sp.3	Phorm3
Pseudophormidium sp.	Pseuph
Crinalium sp. (*Tychonema* sp.)	Crinal
Nostoc sp.1	Nosto1
Nostoc sp.2	Nosto2
Bacillariophyceae	
Navicula cf.*atomus* (Kützing) Grunow	NavAt
Navicula mutica Kützing	NavMut
Navicula cincta (Ehrenberg) Ralf in Pritchard	NavCin
Navicula cf.*tantula* Grunow in Van Heurck	NavTan
Navicula ventricosa Ehrenberg	NavVen
Nitzschia ovalis Arnott	NitzOva
Nitzschia sp.1	Nitz1
Hantzchia amphioxys (Ehrenberg) Grunow in Cleve & Grunow	Hantzc
Xanthophyceae	
Actinochloris sp.	BotrIn
Botrydiopsis sp.	Botr1
Heterococcus sp.1	Heter1
Heterococcus sp.2	Heter2
Xanthonema cf.*debile* (Vischer) Silva	XantDe
Xanthonema sp. (*Stichococcus* sp.)	Xantho
Eustigmatophyceae	
Eustigmatos magnus (J.B. Petersen) Hibberd	Eustig
Chlorophyta	
Chlorophyceae	
Bracteococcus minor (Chodat) Petrová	BracMi
Bracteacoccus sp.	Bract1
Dictyochloris fragrans Vischer	Dictyoch
Elliptochloris reniformis (S. Watanabe) Ettl & Gätner	EllRen
Elliptochloris subsphaerica (Reisigl) Ettl & Gätner	EllSub
Ettlia pseudoalveolaris (Deason & Bold) Komárek	Ettlia
Fernandinella alpina Chodat	Fernan
Myrmecia bisecta Reisigl	Myrmec
Chlorella angusto-ellipsoidea Hanagata & Chihara	ChloEll
Chlorella fusca Shihira & Krauss	ChloFus
Chlorella mirabilis Andreeva	ChloMir
Chlorella minutissima Fott & Nováková	ChloMin
Chlorella saccharophila (Krüger) Migula	ChloSac
Chlorella sp.1	Chlo1
Chlorella sp.2	Chlo2
Coccomyxa sp. *(Neocystis* sp.*)*	Coccom
Coenochloris sp.	Coenoc

Gloeocystis sp.	Gloeoc
Coccobotrys sp.	Coccob
Dictyosphaerium chlorelloides (Naumann) Komárek & Perman	Dictyosp
Diplosphaera chodatii Bialosuknia em. Vischer	Diplos
Geminella terricola J.B. Petersen	Gemine
Leptosira sp.	Leptos
Monoraphidium sp.	Monora
Pleurastrum sarcinoideum Groover & Bold	Pleura
Pseudococcomyxa simplex Korschikoff	Pseudo
Scotiellopsis terrestris (Reisigl) Punčochářová & Kalina	Scotie
Spongiochloris irregularis Kostikov	Spongi
coccal green algae	green
Pseudendoclonium basiliense Vischer	Pseude
Chlamydophyceae	
Actinochloris sp.	Actino
Chlorococcum sp.	Chloroc
Chlamydomonas cf.macrostellata Lund	ChlamMa
Chlamydomonas sp.	Chlam
Chlorosarcinopsis minuta Groover & Bold	Chloros
Tetracystis sp.	Tetrac
Charophyceae	
Stichococcus bacillaris Nägeli	Sticho
Klebsormidium nitens (Meneghini in Kützing) Lokhorst	KlebsNit
Klebsormidium flaccidum (Kützing) Silva, Mattox & Blackwell	KlebsFla
Klebsormidium sp.	Klebs1
Klebsormidium crenulatum (Kützing) Ettl & Gätner	KlebsCre
Klebsormidium mucosum Lokhorst	KlebsMuc
Klebsormidium pseudostichococcus (Heering) Ettl & Gätner	KlebsPse
Zygnemaphyceae	
Cylindrocystis brebissonii Meneghini	CylinBre
Cylindrocystis crassa De Bary	CylinCra
Euglenophyceae	
Euglena mutabilis Schmitz	Euglen

5. Experiments

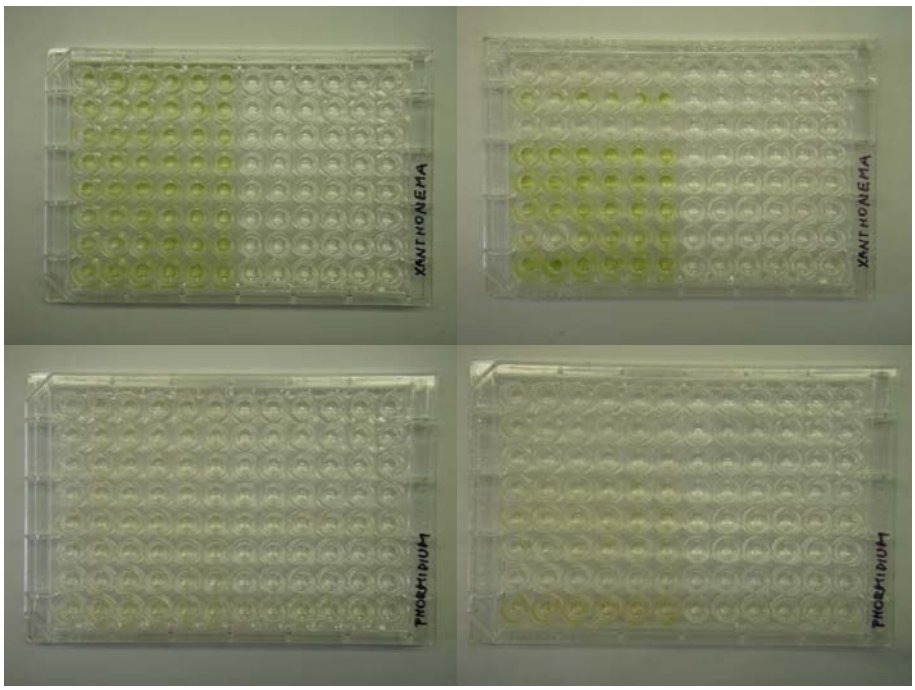
5.1. Growth and survival test



Species (*Bracteacoccus minor, Heterococcus* sp., *Chlorella saccharophila, Nostoc* sp.) tested for growth and survival in 13 water extracts in 96-well micro-plates in six replications for each species and each substrate.

5.2. Amelioration test

Before After



Species (*Xanthonema debile, Phormidium* cf. *autumnale*) tested in extremely toxic water extract and in extracts enriched with various amendments in six replications for each treatment. At the beginning of the test and after 2 weeks exposure.