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Diversity and adaptation of soil fungi in an ecosystem with contamination originating from a phosphate fertilizer plant

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Summary

In the vicinity of a former phosphate fertilizer production plant, high phosphate contents with up to 463 mg phosphate per kilogram of soil dry matter were found 13 years after closing the plant while pH is only slightly elevated at pH 7 to 8. The deposited phosphate is seen to be moved to deeper soil horizons. The effect on soil microbiota was analyzed with respect to soil respiration, fungal biomass and cultivation of benomyl resistant and ligninolytic fungi. Increasing numbers and diversity of soil fungi were found with distance from the former emittent. This was confirmed by investigation of mycorrhization rates of mycotrophic, ectomycorrhizal birch trees. We tested for adaptation by growth and phosphate acquisition on soil extract media. The best growth was seen on the media containing highest phosphate concentrations showing no *in vitro* growth inhibition. In contrast to previous findings, however, more polyphosphate granula were seen on soil extract media from distant sites, although phosphate concentration was lowest in these media.

Introduction

In the vicinity of Jena, Thuringia, Germany, a former phosphate production plant has produced fertilizer from 1957 to 1990 in Dorndorf/Steudnitz. The plant, part of the VEB Chemiewerk Coswig, produced with 100,000 t per year about 20 % of the phosphate fertilizer produced in the former GDR (Vogler and Gebauer, 1981). During the production period, high emissions of phosphate and cadmium containing dust occurred, e.g. in 1979, 3146 t of dust with high pH values of 10-11. The fertilizer plant is located in the Saale valley which led to directional deposition with the direction of wind predominately north-northeast on a steep hillside of chalcerous rocks at the left bank of the Saale river (Görner and Fröhlich, 1972). The impact on local flora was extensive (Heinrich, 1984) and heavy metal contents in remaining plants was substantially elevated (Anke et al., 1991). The phosphate concentrations in the soil were elevated with 1.2 g per kg soil dry matter (Heinrich, 1983; Langer, 2000). Phosphate is an essential nutrient, which is limiting in most soil types especially since phosphates are mostly bound in inorganic apatite, or calcium, aluminum or iron phosphates. Adsorption to soil particles like clay minerals, humic substances and organically bound forms further limit availability to plants and microorganisms. Soil microbes and plants dissolve phosphate by acidification and especially fungi have very potent high affinity uptake systems which allow the mycorrhizal fungi, for instance, to provide phosphate for the associated symbiotic plant (Beever and Burns, 1980; Harrison and van Buuren, 1995; Illmer et al., 1995; Jacobs et al., 2002; Jones et al., 2003; Gyaneshwar et al., 2002; Kothe et al., 2002; Maldonado-Mendoza et al., 2001).

Filamentous fungi are forming a mycelium which is in closest contact to a large area of soil. A very impressive example for the amount of soil colonized is given by the largest living organism, a basidiomycete, *Armillaria ostroyae*, in North America which covers 880 hectares of ground. At the same time, biomass in soils is dominated by fungi. By the close contact to soil particles, fungi are especially sensitive to soil pollution. The radioactive fallout of the Tchernobyl accident, for example, led to short-time, high enrichment in fungal fruitbodies (Linkov et al., 2000). Heavy metal pollution and other factors of soil degradation are listed as major reasons for extinction of fungal species world-wide. With more than 90,000 species described, the eumycota are showing a high biodiversity which is threatened by pollution, especially through fertilization, changing soil pH, and increasing heavy metal concentrations (Sonneborn et al., 1999; Erland and Taylor, 2002; Chander et al., 2001a,b; Kuyper, 1989). While mycorrhizal fungi generally show higher resistance to environmental stress because of the better C-source availability (Cooke, 1993), it could be shown that elevated phosphate concentrations inhibit germination of arbuscular mycorrhizal fungi (De Miranda and Harris, 1994). However, the role of phosphate in studies of environmental stress has been widely neglected.

Here, we investigate the progress of restoration of a phosphate polluted ecosystems with respect to the soil microbiota, especially higher fungi, by comparing sites with increasing distance to the former plant and therefore decreasing initial pollution 13 years after closing of the phosphate fertilizer production.

Material and methods

Cultivation

Isolation and cultivation of fungi was performed at room temperature on complete media (Czapek Dox agar). Czapek-Dox Agar (Warcup, 1950) contained per liter: 30 g sucrose, 3 g $NaNO_3$, 0.5 g $MgSO_4$ x $7H_2O$, 0.5 g KCl, 0.01 g Fe-(II)-SO_4 x 7 H_2O, 1 g K_2HPO_4, 5 g yeast extract, 13 g agar-agar and 50 mg each streptomycin and chloramphenicol to minimize bacterial growth. The pH was adjusted with HCl to 4,9-5,0.

For differentiation between lignin degrading fungi and for enrichment of basidiomycetes, agar with benomyl and guiacol was used. Lignin-guiacol-benomyl-agar (Thorn et al., 1996) contained 0.5 g KH_2PO_4, 0.2 g $MgSO_4$ x 7 H_2O, 0.1 g NH_4NO_3, 0.1 g KCl, 0.02 g Fe-(II)-SO_4 x 7 H_2O, 0.05 g $Ca(NO_3)_2$ x 4 H_2O, 2 g malt extract and 14 g agar-agar per liter. After autoclaving, 5 ml 1M KOH, 0.4 ml guaiacol, 1 g induline AT (solubilized in 10ml dioxan) and 4 mg benomyl (Benlate-50-WP; dissolved in 2 ml acetic acid 70% ethanol 1:1 were added). Antibiotics streptomycin and chloramphenicol (50 mg per liter final concentration) were used to minimize bacterial growth.

To investigate influence of the three sampling sites, soil extract medium was used. The soil extract was prepared following the instructions of the German Culture Collection, DSMZ, Braunschweig (Medium 80, Glycerol-soil-medium: http://www.dsmz.de/ media/ med80.htm) using 100 g of 2 mm maximal sized particles. The soil extract medium contained per liter: 150 ml soil extract, 30 g sucrose, 5 g yeast extract, 13 g agar-agar and 50 mg each of streptomycin and chloramphenicol. The pH was adjusted with HCl or NaOH to 7.0.

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Microscopy and staining

To stain and visualize hyphae, a sterile cover slip for microscopic slides was placed next to a growing mycelium on the agar surface and hyphae growing onto the cover slip were used. Polyphosphates were stained using 0.1 % toluidine blue at pH 1 (CHILVERS et al., 1978; BÜCKING and HEYSER, 1999). DAPI (4-6-Diamidino-2-phenylindol) was used to stain DNA (RAJU, 1982) and the observation was performed using a Zeiss Axioplan 2 fluorescence microscope.
The mycorrhization rate was determined from root samples observed after washing using a Zeiss stereoscope at 2,5-4-fold magnification counting mycorrhizal short roots per 100 short roots.

Soil characterization and respiration

The soil pH was measured following the protocol of SCHINNER et al. (1993) from three independent soil samples per horizon.
Phosphate was determined using ammonium hepta-molybdat (OLSEN and SOMMER, 1992) measuring the blue complex photometrically at 570 nm. Two independent samples were used for each point. As a standard, Na_2HPO_4 in 0.5 M $NaHCO_3$, pH 8.5, was used with three parallels. To correct for humic acids which are also visible at 570 nm, each filtrate was measured without addition of ascorbic acid and the values for these samples were subtracted from the sample measurements.
Size of soil particles was determined by sieving and sedimentation in 0.1 M $Na_4P_2O_7$ (SCHINNER et al., 1993).
Humic substances were determined photometrically at 570 nm (SCHINNER et al., 1993) using myo-inosit as a standard (116 mg equivalent to 4 %, 232 mg for 8 % and 348 mg for 12 % humic substances).
Soil respiration was determined by titration using 40 µl 0,1 % phenolphtalein in 60 % ethanol (SCHINNER et al., 1993) in three parallels for each measurement.

Fungal biomass and isolation

Ergosterol content was used to determine fungal biomass in soil samples after methanol extraction by HPLC quantification (WHEETE and GANDHI, 1996). Ergosterol (HPLC-quality, Fluka, 200 µg/ml in methanol) was used as a standard. The HPLC-system (Dionex P580 pump, AS50 autosampler, LC20 chromotography enclosure) was coupled to a UV-detector (Gyntek VVD340S) and a RP18E-column (250 x 3mm, Sepserv, Berlin, Germany). Methanol was used as solvent at 0.5 ml/min. The retention time was 20 min, software Chromeleon Client (Version 6.11) was used to determined peak volume.
The isolation of saprophytic soil basidiomycetes was performed with washed soil fractions in order to eliminate resting spores (THORN et al., 1996). Sieves pre-sterilized with 79 % ethanol with pore sizes of ca. 250 µm and 54 µm (Bückmann GmbH, Mönchengladbach) were used and after 5 min washing with tap water, soil particles in 20 ml suspension were taken from the 54 µm sieve. 5 independent plates were inoculated with 400 µl of the suspension on lignin-guiacol-benomyl-agar.
For investigation of polyphosphate accumulation on soil extract media, one of the dikaryotic basidiomycetes isolated was used. The strain 8-O-1 was isolated from site 2 and showed lignin degradation as indicated by guiacol staining.

Tab. 1: Description of sampling point soil parameters. Three sites with distances of 100 to 680 m from the former phosphate fertilizer plant were used to survey a gradient of phosphate immission.

site	depth	distance to plant [m]	pH	organic layer [cm]	humic subst. [%]	sand [%]	coarse silt [%]	fine silt [%]	clay [%]
1	O	100	7.5	1.8	-	-	-	-	-
	X		7.9	-	1.38	33	2	52	13
	U		7.7	-	0.99	65	23	9	3
2	O	400	7.0	4.3	-	-	-	-	-
	X		7.7	-	2.94	30	2	49	19
	U		7.9	-	1.87	28	4	48	20
3	O	680	7.0	3.6	-	-	-	-	-
	X		7.5	-	3.1	27	1	54	18
	U		7.6	-	1.35	17	4	55	24

Results

Plant available phosphate concentrations along a transect and in soil profiles

Three points along a traverse section on the hillside north of the former fertilizer plant along the left bank of the Saale river were sampled 100 (site 1), 400 (site 2) and 680 m (site 3) from the former plant, respectively (Fig. 1). At each site, three samples from the inceptisol soil profile were taken from topsoil at 0-4 cm (O), subsoil at 10-15 cm (X) and 20-25 cm (U) depth (Fig. 1). The content in humic substances was lowest at site 1 with app. half the amount of sites 2 and 3 and the organic layer was smaller (Tab. 1). The pH was slightly alkaline at all three positions. For site 1, a hill slide may have occurred, since the lower profile was very sandy and phosphate concentrations did not follow the general trend of highest concentration in the uppermost layers. Plant available phosphate concentrations were shown to vary between 67 and 463 mg/kg soil dry weight. While site 1 showed highest phosphate concentrations in the lowest horizon, sites 2 and 3 had shallower gradients with highest phosphate concentrations in the uppermost samples (Fig. 2). The two upper horizons at site 1 classify as low in available phosphate, the lowest horizon at site 3 shows normal phosphate contents, all other samples have excess plant available phosphate contents.

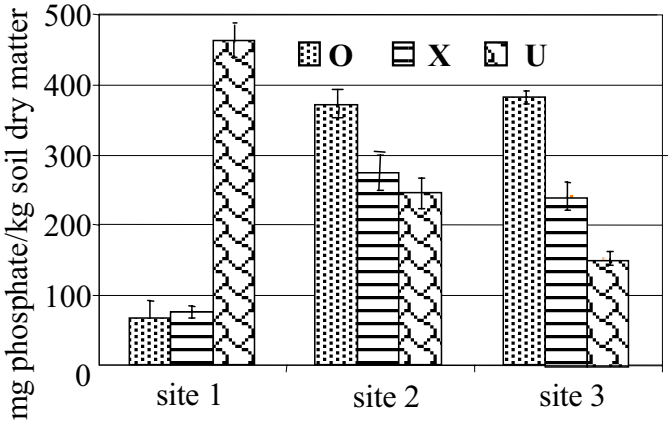

Fig. 2: Plant available phosphate at three sites with increasing distance from the former plant in three depths, (O) 0 - 4 cm, (X) 10 - 15 cm and (U) 20 - 25 cm from top.

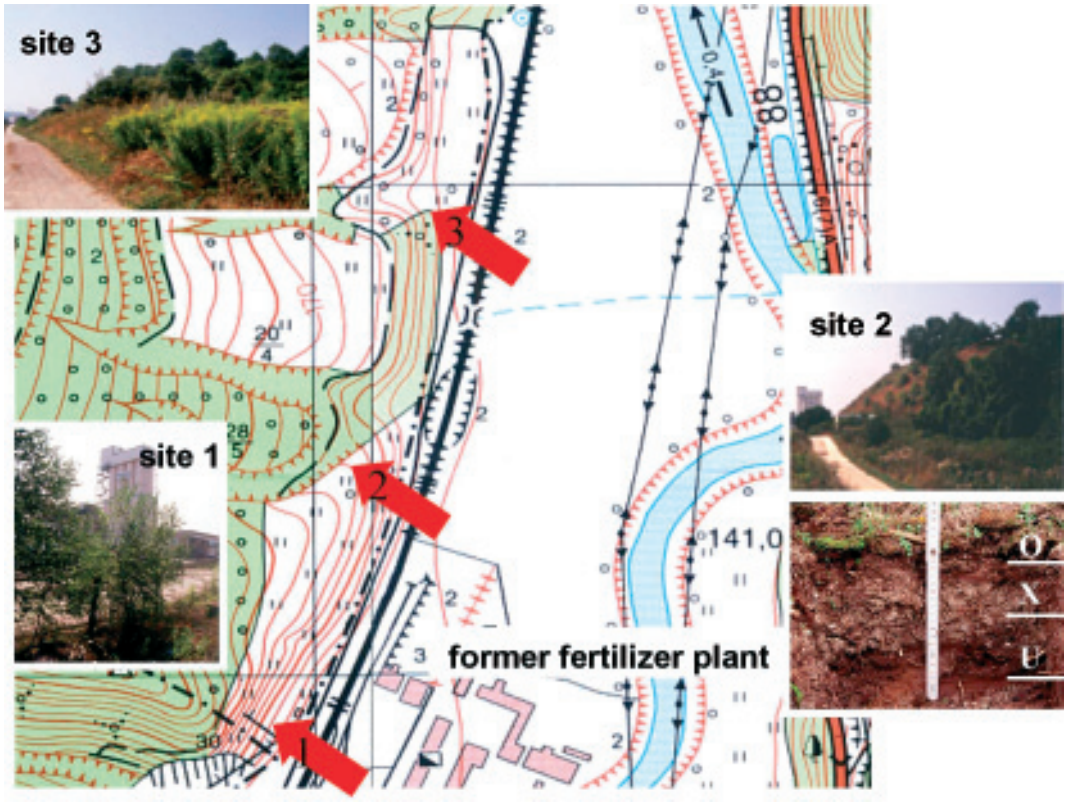

Fig. 1: Sampling sites in the river Saale valley at Dordorf-Steudnitz near Jena, Germany. Inserts show the sampling sites. The former phosphate fertilizer plant is visible in all three views at increasing distance to allow visual comparison of distance from the plant. The soil profile at site 2 is shown as an example.

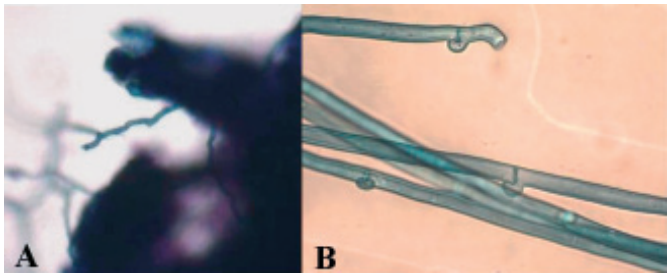

Fig. 4: Fungal hyphae (A) and basidiomycete with clamps (B) attached to washed soil particles.

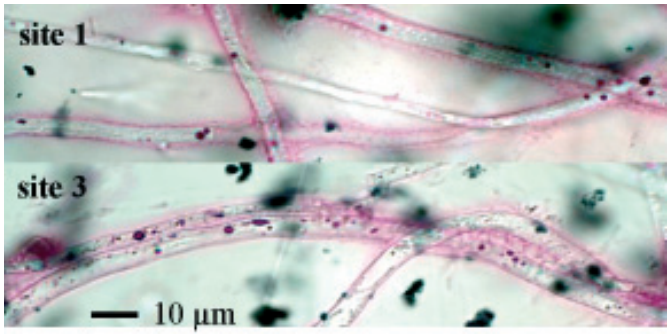

Fig. 6: Polyphosphate granula content of isolate 8-O-1 grown on soil extract media from site 1 and site 3 after staining with toluidine blue.

Microbial biomass and activity

The microbial activity was determined for all nine samples by measuring soil respiration. Since respiration rates in the deeper horizons were very low, rates over 10 µg per g of dry mass are not specified (Tab. 2). Generally, the soil respiration was lower than in uncontaminated soils. Site 1 showed elevated soil respiration in the lowest sample, U, which is unusual for normal soils. However, the highest phosphate concentrations were found in this sample which might stimulate soil respiration.

Tab. 2: Soil respiration rates (µg/g dry soil matter) at three sites (1 through 3) with increasing distance to the former emmitent

soil respiration	site 1	site 2	site 3
O	9.73 ± 0.11	≥ 12.55 ± 0.2	≥ 12.43 ± 0.14
X	1.46 ± 0.16	2.29 ± 0.39	2.38 ± 0.27
U	5.15 ± 0.19	1.28 ± 0.18	0.53 ± 0.19

Since soil respiration includes especially the diverse metabolic activity of bacteria, the fungal biomass was determined by ergosterol content, direct microscopy of washed soil particles and cultivation experiments. Ergosterol contents showed increasing fungal biomass with distance from the emmittent (Fig. 3).

The cultivation of soil without a washing step generally gives higher numbers of isolates, especially of the conidiospore forming ascomycetes. This, however, does not represent fungi that have been

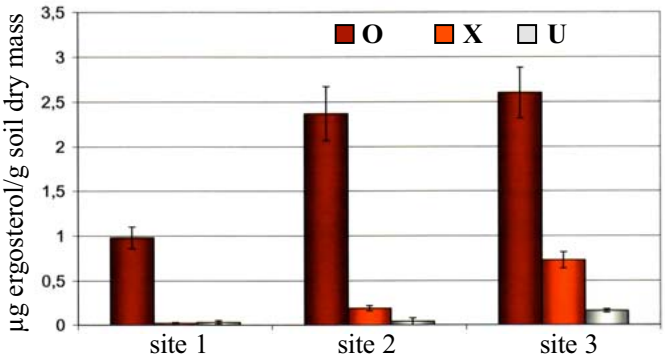

Fig. 3: Ergosterol content at three sites with increasing distance from the former plant in three depths, (O) 0 - 4 cm, (X) 10 - 15 cm and (U) 20 - 25 cm from top.

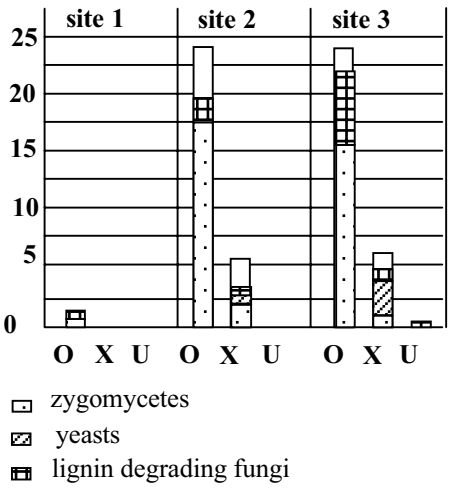

Fig. 5: Growth of fungi on selective media containing benomyl. Washed soil particles were plated from three sites with increasing distance from the former plant in three depths, (O) 0 - 4 cm, (X) 10 - 15 cm and (U) 20 - 25 cm from top. Zygomycetes, yeast colonies, and peroxidase producing fungi able to degrade lignin were scored.

growing in the contaminated soil, but rather the immission of spores from surrounding fields. In order to asses fungi which have developed soil mycelia, soil particles were size fractionated and washed examining a fraction of organic soil particles of 54 to 250 µm. Microscopically inspected particles showed adhering mycelial growth, and clamp forming basidiomycetes could be shown at all sites (Fig. 4).

The remaining particles were plated on complete media and on plates containing lignin, guiacaol and benomyl. This allows the preferential growth of basidioymcetes (and to some extend zygomycetes) which are generally more resistant to benomyl. At the same time, the lignin/guiacol shows a color reaction indicative of peroxidases activity involved in lignin decomposition, a feature of litter degrading soil basidioymcetes. For each sample, 5 replicate platings were performed. The complete medium showed the expected decrease in fungal strains from top to bottom in the profiles at all three sites with 110, 35 and 8 isolates at site 1, 105, 52 and 35 isolates from site 2 and 110, 58 and 12 isolates from site 3 for O, X and U horizons, respectively. The differences between the sites in sheer numbers on complete media are not as high as expected, if measured against the ergosterol content which was significantly lower at the contaminated site(s). However, growth on the selective plates corresponded nicely to the disturbance at site 1 with intermediate numbers at site 2 and highest number of isolates on site 3 (Fig. 5). Lignin degrading fungi were present at site 1 only in the uppermost horizon, at site 2 in horizons O and X, and only at site 3 all three horizons led to isolation of lignin degrading fungi. Some additional filamentous fungi neither identified as zygomycetes by lack of septation and fluffy growth pattern nor as lignin degrading fungi were present at sites 2 and 3 hinting at higher biodiversity.

Mycorrhization and occurrence of fungal fruitbodies

At locations 1 and 3, birch roots could be collected for determination of ectomycorrhiza formation rates. While at site 1 only 55 % of the short roots were mycorrhized, at site 3 79 % of short roots showed ectomycorrhiza formation upon microscopical inspection.

From the fuitbodies collected, at site 1 litter decomposing fungi dominated with *Hygrocybe nigrescens* (2 specimen), *Sapultario arenosa* (associated with lichen) and *Tubaria hiemalis.* Only one species of ectomycorrhiza forming fungi (*Hebeloma spec.*) was present with 2 fruitbodies. At sites 2 and 3, the ectomycorrhizal fungus *Hebeloma mesophaeum* was present with 10 to 15 fruitbodies, and at site 3 additionally *Cortinarius betuletorum* was found.

Growth on soil extract media and polyphosphate accumulation

Since the contamination was primarily with phosphate the ability of growth on soil extract media and the ability to accumulate phosphate from these soil extract media in hyphae grown under sterile conditions was tested. One basidiomycete isolate from site 2, upper horizon O was used for these investigations. The fungus is a dikaryon as visible by DAPI staining, which does not form clamps. Generally, isolates from media showed rarely clamp formation while the mycelia seen by direct microscopic observation were predominantly clamp forming basidiomycetes.

Soil extract media were prepared from the lowest horizon samples so as to provide a gradient of plant available phosphate. The phosphate concentration had been found to be 463, 245, and 152 mg/g soil dry matter. Growth occurred on all three media. While the initial pH of 7 was unchanged in soil extract medium of site 1, the pH had dropped to 6.5 and 6.3 at sites 2 and 3, respectively.

Adaptation of phosphate uptake and storage at sites with high phosphate contents

In order to test phosphate acquisition and storage, the staining of polyphosphate granula in hyphae grown on soil extract media was performed. Polyphosphate granula were stained with toluidine blue at pH 1 to allow for specificity of the staining and counted per 100 µm hyphal length. More granules and bigger granules were observed with decreasing plant available phosphate in the extract media (Fig. 6) with 6.9 granules of up to 1 µm size and 1 granule sized 1 µm or larger at site 1, 10.3 small and 1.5 larger granules at site 2 and 10.2 small and 1.9 large polyphosphate granules at site 3. Thus, the phosphate storage particles were in inverse proportion to available phosphate concentrations at these phosphate eutrophic sites.

Discussion

Characterization of a traverse along the pollution gradient

The soil type at the investigated area has been classified as part of the rendzina and sloped clay of the river Saale valley with good water

retention capacity and nutrient availability (HEINRICH, 1984; RAU, 1995; SEIDEL, 1995). The sandy lowest horizon investigated in this paper at site 1 may be due to quartz sand emission that has been mixed to the fertilizer and has shown high wind distribution (LANGER, 2000). This sandy layer is covered by loamy and more organic layers which may have resulted from a slide off the upper slopes. At the time of closing the plant, the flora consisted mainly of a monospecific grassland and high fluor contents led to bee killing (ANKE et al., 1991). The steep slopes directly situated at the northern part of the plant did show the most visible effects. Eight years after closing the plant the soil was still greatly disturbed (LANGER, 2000). We proceeded to test the capacity of natural attenuation five years after the last investigation. By now, the flora seems regenerated, constituting a grassland ecosystem with initial stages of bush and tree growth. The microflora, however, still reflects the detrimental effects of the pollution incurred during 1957 and 1990, even after 13 years have passed since production and emission have stopped.

The plant available phosphate content varied greatly as was expected among the sites along a traverse section. With increasing distance from the plant, phosphate concentrations decreased. Due to a likely slide at the first site at the steep hillside, which probably has occurred during the period when due to emission there was almost no plant cover, this was best visible in the lowest horizon of the profiles. The emitted phosphate dust was of high pH 10 - 11, and in early investigations was observed in deeper horizons only at sites very close to the plant. At distant sites phosphate and alkalinic pH was observed only in the uppermost soil layer (HEINRICH, 1984). By now, also at distant sites the deeper horizons show elevated pH and higher phosphate contents which is indicative of wash-out from the uppermost layers into deeper horizons with time. The geogenic pH on the soil types formed on the chalcerous rocks of the Saale valley are generally in the range of pH 6.9 to 7.2 (ZORN and KRAUSE, 1999), with phosphate concentrations from 28 to 117 mg phosphate per kg soil dry matter. Thus, a distinct pollution is still seen at Steudnitz. The transfer of phosphates into deeper horizons is not generally observed, as mineralization specifically with calcium, and adsorption to aluminum and iron hydroxides is high and allows retention (CURTIN and SYERS, 2001; SCHEFFER and SCHACHTSCHABEL, 1989). However, the extremely high contents may have facilitated phosphate deposition in deeper horizons in the soil types which are poor in organic layers at the polluted sites in Steudnitz which had been additionally worsened by the lack in plant cover during high emission. The soil shows regeneration from the extremely high phosphate contents with decrease from 380 - 825 mg/kg four years ago (LANGER, 2000) to now 463 mg/kg maximum. The transect still shows correlation of high phosphate contents with distance from the former pollution source.

Influence of phosphate contamination on uptake and storage in hyphae

Since the lowest profile shows the best correlation with distance from the plant, this soil horizon was used for soil extract media to determine phosphate uptake into the fungi as visible by polyphosphate granula within the cell vacuoles. The soil extract media from the site with lowest phosphate concentration showed the highest polyphosphate granula content. This implicates that phosphate storage is fostered by depletion while in an environment of excess phosphate storage seems unnecessary and is avoided by the fungi. This is in contrast to earlier investigations, where *in vitro* polyphosphate accumulation on complete media showed a direct correlation between phosphate content of media and polyphosphate granula contents (BEEVER and BURNS, 1980; JENNINGS, 1995; TASAKI et al., 2002). Since we have used soil extract media, our investigation should reflect the natural situation while on standard media with generally lower phosphate levels tested the phosphate acquisition and storage is linked directly to phosphate availability. The low accumulation of phosphate at the polluted sites is seen as a adaptive response to the phosphate eutrophy.

Another explanation to higher phosphate storage at sites with low phosphate might be inferred from molecular data. Phosphate acquisition need phosphate transporter proteins which are known from the yeast *Saccharomyces cerevisiae* and from filamentous fungi including *Neurospora crassa* and the ectomycorrhizal basidiomycetes *Tricholoma vaccinum* and *T. terreum* (PERRON et al., 1999; VERSAW and METZENBERG, 1995). At low phosphate concentration two high affinity transporter systems are operating, one with proton symport and the other by sodium symport. The proton symporter is highly pH dependent, with decreasing transporter activity at higher pH. The sodium symporter is almost independent of pH (see VERSAW and METZENBERG, 1995). Species growing on soils of neutral to alkaline pH show expression of the sodium symporter while the proton symporter is expressed in species typically found on acidic soils (KOTHE et al., 2002). However, at high phosphate concentrations, transcription of the high affinity transporters is shut down and a low affinity system is operating which uses proton symport and therefore is pH dependent. Phosphate uptake therefore can be postulated to be reduced at high pH at high phosphate sites like in Steudnitz. We corrected for pH effects by supplying the soil extract media at an artificially set pH of 7.0 such that the same initial rates of phosphate uptake would be provided for all three sites. The results therefore are not simply mirroring the pH dependence of the low affinity phosphate uptake system, but rather hint at an adaptive effect of high phosphate on the expression of phosphate transporters in a fungal isolate from this contaminated site. Further investigation of the molecular basis of phosphate uptake in these environments is needed to substantiate this hypothesis.

Influence of high phosphate contents on microflora

Soil respiration was low at the contaminated site(s). However, the high phosphate content in the lowest horizon at site 1 allowed for higher respiration as compared to the upper profiles. The contamination also led to reduced mycorrhization which has been shown to be decreased upon contamination, especially with heavy metals (ERLAND and TAYLOR, 2002). The decreased soil respiration and mycorrhization as well as low biodiversity for lignin decomposing basidiomycetes and adaptive response in polyphosphate contents can be attributed to pollution.

Fungal biomass was determined by ergosterol content, direct microscopy of washed soil particles and cultivation experiments. Ergosterol contents have been used for determination of fungal biomass (WEST et al., 1987). However, different taxa produce highly divergent ergosterol contents variable with species (HUANG et al., 1985; MARTIN et al., 1990; NYLUND and WALLANDER, 1992), cultivation (ARNEZEDER and HAMPEL, 1991; CHARCOSSET and CHAUVET, 2001; DAWSON-ANDOH, 2002) and in addition with especially zygomycetes showing low ergosterol contents in their membranes (WHEETE and GANDHI, 1996; RUZICKA et al., 2000). The ranges of ergosterol per mg fungal dry weight are given with 5-15 µg (WHEETE and GANDHI, 1996), 0.8-11 µg (CHARCOSSET and CHAUVET, 2001) and 8-15 µg (DAVIS and LAMAR, 1992). Therefore it was proposed to use a general median of 5.1 µg per mg fungal dry weight for calculations of fungal biomass (DJAJAKIRANA et al., 1996) which was proposed after comparison of 42 literature citations. Even if the exact fungal biomass therefore is not easyly determined using ergosterol extraction from soil, the results are very well comparable in one study since soil texture (GONG et al., 2001) and soil pollution (BAJARAS et al., 2002) do not influence ergosterol extraction. The examination of soil ergosterol content in the Steudnitz area showed increasing contents in all depths with

distance to the plant, correlating with the decreasing pH and phosphate contents. The contents are low if compared to grassland ergosterol contents of 5.52 µg ergosterol per g soil dry matter and rather are comparable with contents of (fertilized) agricultural sites of 2.14 µg/g (DJAJAKIRANA et al., 1996) although the flora here constitutes a grassland community and the steep hillsite had not been used agriculturally.

The determined fungal biomass using the median of 5.1 µg ergosterol per mg fungal dry mass would correspond to 200, 5 and 10 mg for O, X and U horizons at site 1, to 480, 40, and 10 mg fungal dry biomass at site 2 and to 520, 150 and 40 mg fungal biomass per kg soil at site 3, respectively. Especially at the O horizon of site 1, the discrepancy between the three methods used to determine fungal biomass is obvious. Total isolated strains on complete media from 400 µl washed soils suspension were scored with 110 isolates which is similar to uppermost horizons at sites 2 and 3, while ergosterol constents show intermediate levels with 200 mg/kg (with approx. 500 mg/kg at sites 2 and 3) and only 2 isolates were obtained on benomyl containing media (corresponding to 24 isolates for sites 2 and 3). This imlies predominance of benomyl sensititve, low ergosterol level containing fungi in thiese soil samples. Since the use of a median ergosterol content will yield good appriximations only if the funagl biodiversity is high enough to compensate for indicidual differences in ergosterol contents, theese findings imply a substantially lower biodiveristy at site 1 where highest phosphate concentrations were present. This interpretation is substantiated by the indentification of lignin degrading fungi (50%) and zygomycetes (50 %) only at site1. At site 2, idnetification of lignin-degrading fungi (8 %), zygomycetes (70 %) and others (22 %), and at site 3 ligning-degrading fungi (25 %), zygomycetes (66 %) and others (8 %) also give the impression of lowest diversity at site 1.

The isolation of fungi from all soil samples after washing to remove resting spores showed increasing diversity with distance from the plant while the numbers on complete medium varied, but did not increase generally. The selective media contained benomyl which is known to repress growth of ascomycetes stronger than growth of zygomycetes or basidiomycetes. This would lead to the conclusion that the fungal biomass at the polluted site 1 consisted more of ascomycetes as compared to the basidiomycetes that are involved in lignin degradation. Lignin degrading fungal colonies were found in substantial numbers only at site 3 where lignin degraders were present in all three horizons. Since lignin decomposition is an essential function of soil fungi involved in soil formation and formation of an organic layer, the lack in lignin degrading fungi accounts for the lower levels in organic substances found at sites 1 and 2. This increase in fungal biodiversity coupled to functional diversity in the ecosystem is typical for succession. The occurrence of fruitbodies and lower mycorrhization rates at sites close to the former fertilizer plant confirms this observation of successional stages in the fungal microflora, as ectomycorrhizal fungi are known to be late succession stage fungi (FRANKLAND, 1998).

In order to assess fungal biodiversity, soil texture and carbon source availability must be taken into consideration. While at site 1 carbon humic substances are low, site 3 has carbon contents lower than site 2. Nevertheless, site 3 shows highest fungal biodiversity and higher fungal biomass as determined by ergosterol contents. This indicates that the pollution has a higher influence on fungal biomass and diversity as compared to availability of carbon source. Nitrogen availability was not investigated in the present study. However, the area is a steep hillside which has not been fertilized and not used for any type of farming which makes it unlikely that nitrogen is a meaningful parameter at this site. Previous studies found no differences in nitrogen content with 0.4 to 0.6 % in top soil and 0.12 % in lower horizons (LANGER, 2000).

The high prevalence of zygomycetes can be explained by the low content of degradable litter or degraded, organic matter which would enhance growth of ligninolytic basidioymcets. In addition, the occurrence of a grassland plant community which foms mycorrhiza with the vesicular arbuscular mycorrhizal fungi of the order Glomerales might explain differences in ergosterol measurments with isolation of fungal saprotrophs. The VAM fungi are strictly biotrophic and cannot be cultivated axenically on agar media with a plant partner, thus providing a reservoir of ergosterol that does not correspond with fungi isolated from soil.

Phosphate, albeit an essential nutrient, inhibits germination of fungal spores at high concentrations MIRANDA and HARRIS, 1994). Thus, loss of fungal diversity as seen at this site could be attributed to the physiologically detrimental effect of the phosphate concentration itself. Phosphate uptake is at least in part promoted by acidification of the substrate (OLSSON et al., 2002).

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