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Relationships between soil parameters and physiological status of *Miscanthus* x *giganteus* cultivated on soil contaminated with trace elements under NPK fertilisation vs. microbial inoculation☆

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ABSTRACT

Crop growth and development can be influenced by a range of parameters, soil health, cultivation and nutrient status all play a major role. Nutrient status of plants can be enhanced both through chemical fertiliser additions (e.g. N, P, K supplementation) or microbial fixation and mobilisation of naturally occurring nutrients. With current EU priorities discouraging the production of biomass on high quality soils there is a need to investigate the potential of more marginal soils to produce these feedstocks and the impacts of soil amendments on crop yields within them. This study investigated the potential for *Miscanthus* x *giganteus* to be grown in trace element (TE)-contaminated soils, ideally offering a mechanism to (phyto)manage these contaminated lands.

Comprehensive surveys are needed to understand plant-soil interactions under these conditions. Here we studied the impacts of two fertiliser treatments on soil physico-chemical properties under *Miscanthus* x *giganteus* cultivated on Pb, Cd and Zn contaminated arable land. Results covered a range of parameters, including soil rhizosphere activity, arbuscular mycorrhization (AM), as well as plant physiological parameters associated with photosynthesis, TE leaf concentrations and growth performance.

Fertilization increased growth and gas exchange capacity, enhanced rhizosphere microbial activity and increased Zn, Mg and N leaf concentration. Fertilization reduced root colonisation by AMF and caused higher chlorophyll concentration in plant leaves. Microbial inoculation seems to be a promising alternative for chemical fertilizers, especially due to an insignificant influence on the mobility of toxic trace elements (particularly Cd and Zn).

1. Introduction

Due to a high demand for both food and biomass crop products (Tomlinson, 2013) farmers apply a range of agronomic techniques worldwide to improve yields and quality. One common method of yield improvement, along with plant breeding, is the application of chemical fertilisers (Hignett, 1985). The most common chemical fertilisation relies on soil treatment with nitrogen, phosphorus and potassium (NPK), in proportions that are crop and field specific (He et al., 2011; Mikkelsen and Bruulsema, 2005). Chemical fertilisers are mainly responsible for increasing the amount and availability of the major nutrients for plant growth and development. Organic fertilisers such as green manures can be effective in sustaining high

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yields (Dong et al., 2012), however, when taken from unknown sources these could expose crops to the potential presence of pathogenic fungi and bacteria, which can be harmful, not only for plants but also for humans (Strauch, 1991). In addition, decomposition of manure on the field takes considerably more time to release essential nutrients for plant growth in comparison to chemical fertilizers (Rashid et al., 2013). Nevertheless, there are disadvantages to chemical fertilisation which are primarily connected with environmental pollution. The negative effects of inappropriate fertiliser use involve leaching and runoff (N and P), and eutrophication of aquatic ecosystems (N and P) (Carpenter et al., 1998; Miransari, 2011).

One alternative to the chemical and organic fertilisation described above, particularly for nitrogen, is biological fixation of atmospheric nitrogen by bacteria (Marschner, 1996). Additionally, enhancing number of appropriate fungal and bacterial species can accelerate organic matter decomposition and increase nutrient concentrations in the soil (Rashid et al., 2013). As such, bacterial and/or fungal inocula, prepared using selected strains and species (typically existing plant symbionts) can successfully substitute chemical or organic fertilisation. This kind of treatment is not harmful to the environment and is equally, or even more, efficient at enhancing nutrient availability than chemical fertilisation (Miransari, 2011). Inocula for soil amendment usually contain sugar, macro- and micronutrients and other compounds necessary for microorganism development. Microorganisms used for inoculation are termed Effective Microorganisms (EMs) and are defined as mixed cultures of naturally occurring micro-organisms that are beneficial for the plant and soil environment, e.g. bacteria, fungi, actinomycetes and yeasts. These are applied as inoculants to change the microbial diversity and interactions within and between the soil and plant (Higa and Parr, 1995). These microorganisms are responsible for the decomposition of organic wastes and residues, which results in an increased concentration of mineralised nutrients available for plant uptake. EM inoculants can consist of Plant Growth-Promoting Rhizobacteria (PGPR) and/or Arbuscular Mycorrhizal Fungi (AMF), which alone, can be used as an inoculant. Common commercial inoculants usually consist of lactic bacteria and yeasts, though particular species are undefined due to patents and/or companies' information that is not publicly available (Adesemoye et al., 2008; Tahmatsidou et al., 2006).

Precise fertilization according to soil properties and plant requirements, is necessary for successful energy crop cultivation, particularly on low grade or contaminated soils. The Renewable Energy Directive (RED) 2009/28/EC promotes the usage of renewable energy sources in the member countries of the European Union. These countries are required to increase the share of renewable energy in their gross final energy consumption to 20% by 2020. This directive makes marginal agricultural and degraded industrial land a favourable place for cultivating energy crops, as lands with high biodiversity value and/or high carbon stock (i.e. special protection areas, semi-natural forest etc.) should be protected and high value arable lands should be reserved for food production (Scarlat and Banja, 2013).

As global warming becomes a focus of public attention and conventional energy sources are being depleted, every environment-saving technology, especially in industry, is desirable. The conversion of conventional heat production technology based mainly on fossil fuels to an increased usage of bio-based energy sources (especially biomass) is a key step in counteracting climate change. Nowadays, biomass production is focused on second generation, low input perennial bioenergy crops (e.g. *Panicum virgatum*, *Spartina pectinata*, *Miscanthus* spp.) (Clifton-Brown et al., 2002; Dohleman et al., 2012; Guo et al., 2015). Such crops have much lower input requirements, produce more energy and reduce greenhouse gas emissions compared to first generation annual food crop species which have been used previously (e.g. *Zea mays*) (Schrama et al., 2016; Sheng et al., 2012).

Miscanthus x *giganteus*, a perennial rhizomatous grass with C_4 metabolism, is a triploid sterile hybrid of diploid *Miscanthus sinensis* and tetraploid *Miscanthus sacchariflorus* species originating from Asia (Yan et al., 2012). Besides C_4 photosynthesis and highly efficient biomass production, *M.* x *giganteus* is characterized by other advantages, such as the translocation of minerals to the rhizome during winter senescence and highly efficient water use and energy conversion (Robson et al., 2012; Tang et al., 2015). However, cultivation of *M. x giganteus* especially in Europe and North America in temperate climates has a few disadvantages such as relatively high establishment costs, narrow genetic base and low hardiness in the first winter following establishment (Clifton-Brown et al., 2017; Lewandowski et al., 2000).

Industrial and post-industrial areas are frequently source of contaminants which can affect the surrounding arable lands. In regions associated with Zn, Fe, Cu and Pb mining and smelting, many 'hot-spots' are associated with trace element (TE) contaminated soils. As a result, plants grown in these areas are contaminated with TE by root uptake and/or foliar exposure (Alloway, 1990; Dudka et al., 1995; Nicholson et al., 2003). Consequently, food crop production should be restricted or forbidden in such areas, especially for root crops, such as carrot, parsley, potato etc. (Liu et al., 2013; Roba et al., 2016). Biomass production from non-food and energy crop plants could be an alternative use for such contaminated arable land, particularly when improved by specific agrotechniques such as fertilisation, tillage practices, irrigation management etc. (Kidd et al., 2015). There has already been extensive research investigating the potential of energy crop cultivation in soil contaminated with TE (e.g. Meers et al., 2010; Van Ginneken et al., 2007; Zhang et al., 2015). However, crop yield and quality in such areas can be impacted by the adverse influence of contaminants on the plants themselves, especially on the efficiency of the photosynthetic apparatus which is essential for sustained biomass production (Baszyński, 2014; Kosobrukhov et al., 2004; Parmar et al., 2013).

The aim of this field trial was to study the effect of two types of fertilisation (NPK fertilisation vs. microbial inoculation) on soil properties (physico-chemical and biological parameters) as well as growth and physiological status (photosynthesis, transpiration, chlorophyll *a* fluorescence and plant pigments content) of energy crop (*M.* x *giganteus*) cultivated on TE contaminated arable land. Concentration of selected elements in soil and plant leaves was also investigated. Relationships between physico-chemical and biological parameters of soil and plant growth and physiological status was assessed.

2. Materials and methods

2.1. Site description

The experiment was carried out on contaminated arable land in Bytom (Upper Silesia), Poland (50°20′43.0″N 18°57′19.6″E) on the experimental site of the Institute for Ecology of Industrial Areas. Soil was contaminated over the last century with TE deposition (particularly Zn, Cd and Pb) resulting from nearby Pb/Zn smelting. Total soil Pb, Cd and Zn exceed the maximum threshold values proscribed by Polish government regulation (D.2002.nr.165 poz.1369), excluding this area from food production. However, for the last 20 years, cereals have been cultivated on this arable land. The climate at the site is temperate with average temperature and total precipitation measured in Upper Silesia for July and August at 20 °C, 65 mm and 21 °C, 25 mm, respectively. Average values of

temperature and total precipitation measured during the 2014 and 2015 growing season were 17/17 °C and 455/300 mm respectively (Institute of Meteorology and Water Management, Poland). Monthly average precipitation and temperature recorded during the whole experiment are presented in Fig. S1.

2.2. Experiment design

Miscanthus x *giganteus* plants were established at the beginning of May 2014 from 45 g rhizomes (7–10 cm length) planted at 10 cm depth. On each plot 49 plants were planted over an area of 16 m^2 (3 plants per 1 m^2) with a buffer zone of 4 m between each plot which protected plants against uncontrolled fertilisation. Single plot trials with pseudo-replication were utilised due to high soil homogeneity (Table S1) on the field before trial establishment. Each plot was treated in a different way:

- **M I** - Control (without treatment);
- **M II** - NPK standard fertilization was applied directly to the soil before planting (nitrogen 70 kg ha^{-1}, phosphorus 30 kg ha^{-1} as P_2O_5 and potassium 45 kg ha^{-1} as K_2O), using commercially available fertilizers; *Polifoska* (Grupa Azoty, Zakłady Chemiczne „Police" S.A., Poland: N – 4% as NH_4; P_2O_5 – 22%; K_2O – 32%; MgO – 2%; SO_3 – 9%) and ammonium nitrate (PULAN® 34N, Grupa Azoty Zakłady Azotowe "Puławy" S.A., Poland: NH_4 – 17%; NO_3 – 17%);
- **M III** - Commercial microbial inoculum Emfarma Plus® Pro-Biotics Poland (Lactic Acid Bacteria >3.0 × 10^5 cfu ml^{-1}, Yeast < 1.0 × 10^6 cfu ml^{-1}, and Purple Non-Sulfur Bacteria >1.0 × 10^4 cfu ml^{-1} in molasses suspension). 8 L of 10% water solution of Emfarma Plus® was sprayed on the soil surface; additionally the roots of the seedlings were soaked in this solution at the beginning of the experiment. Plant leaves were treated monthly during the growing season with 10% water solution of Emfarma Plus® as aerosol treatment (8 l per plot).

The data for further analysis were collected from plots divided into three sections (Fig. S2). Within each section, two plants were selected randomly for further analysis. Plants situated at the edge of the plots were not analysed. All field measurements (plant growth, chlorophyll *a* fluorescence, RMA and gas exchange measurements) were conducted in the middle of the 2015 growing season (at the end of July-August). After the measurements plant and soil samples were collected for further analysis. Soil samples were collected during rhizosphere microbial activity measurements (see 2.5.1), air dried and sieved through 2 mm and then ground < 0.25 mm (total metal concentration) for further analysis. After field measurements, the first fully developed leaf (mostly the third from the apex) was separated from each plant for elements concentration analysis. The leaves were washed with deionized water and oven dried at 70 °C. Plant samples (as single shoot) were collected at the middle of October, washed with tap water and then with deionized water. Subsequently, shoots were oven dried at 70 °C for three days.

2.3. Soil physico-chemical parameters

All soil physico-chemical parameters were measured on soil sifted through a 2 mm sieve. Soil pH was measured in H_2O (ratio 1:2.5 m/v) with a combination glass/calomel electrode (OSH 10-10, METRON, Poland) and a pH-meter (CPC-551, Elmetron, Poland) at 20 °C. The electrical conductivity was determined by an ESP 2ZM electrode (EUROSENSOR, Poland) according to the Polish norm PN-ISO 11265:1997.

Soil texture was evaluated by the hydrometric method according to the Polish norm PNR-04032:1998. Soil dry mass and water content were measured according to Wilke (2005).

Soil organic matter content (OM) was measured by loss on ignition as follows: air dry soil was dried at 105 °C for 24 h and then (5 g) treated with 550 °C for 4 h.

2.4. Concentration of elements in soil and plant samples

The concentration of the bioavailable metals in the soil ($Me_{bioavailable}$) were obtained using extraction with 0.01 M $CaCl_2$ (for review see Peijnenburg et al., 2007). Extraction was conducted with 3 g of air-dried soil (<2 mm) and 30 ml 0.01 M $CaCl_2$ for 2 h. Bioavailable metal concentrations (Cd, Mg, Pb, Zn) were determined in extracts using a flame atomic absorption spectrometer (iCE 3500 FAAS, Thermo Scientific).

Total concentrations of metals in the soil (Me_{total}) (<0.25 mm) and leaves (Me_{leaves}) were analysed by flame atomic absorption spectrometry (iCE 3500 FAAS, Thermo Scientific) after microwave sample digestion (ETHOS 1, Milestone, Italy) according to the procedure provided by the manufacturer (concentrated HNO_3 and H_2O_2, 4:1 v/v).

The total nitrogen concentration in soil was assessed using dry combustion method (ISO 13878:1998). Available phosphorus and available potassium concentrations were assessed according to the method described by Egnér et al. (1960). Total nitrogen concentration (N) in plant leaves was measured using the titration method (Bremner, 1996), whereas total phosphorus (P) and potassium (K) concentration in plant leaves were assessed in previously mineralized samples using ICP (Liberty 220,Varian, USA).

2.5. Soil biological parameters

2.5.1. Rhizosphere microbial activity

Rhizosphere microbial activity was calculated using three compartment model which was defined by Kelting et al. (1998). For this calculation, three measurements were taken with an Infrared Gas Analyzer using a soil chamber (LCpro+, ADC Bioscientific, UK). The first measurements were conducted on bare soils without plant cover outside the plots; the second were taken from within the plots and the soil chamber put into the soil as close as possible to the stems of analysed plants. Both measurements were done at soil depth 0–15 cm. The last measurements were done on roots excised from the soil which were in the soil chamber during the second measurement. These roots were separated from the soil with a 1 mm sieve and washed with deionized water before measurement. Stabilization of chamber parameters was performed for 1.5 h for the first and the second measurement and for 20 min for the third measurement. All other soil analyses (microbiological activity, physico-chemical parameters) were conducted on soil taken from the soil chamber after the second measurements.

2.5.2. Soil microbial activity

Soil microbial activity was measured in soil samples using dehydrogenase activity (DHA) assay following the method described by Casida et al. (1964).

2.5.3. Arbuscular mycorrhiza colonization measurement

Assessment of Arbuscular Mycorrhizal Fungi (AMF) colonization of plant roots was performed using the magnified intersection method described by McGonigle et al. (1990). Total AMF (AMF_T) colonization was calculated as the sum of arbuscules, vesicles and coils (Table S2). Results and photography (Zen 2 software, Zeiss, Germany) are presented in Table 1 and Fig. S3 respectively.

2.6. Plant physiological parameters

2.6.1. Plant gas exchange measurements

Plant gas exchange parameters, such as net assimilation rate of CO_2 (A), stomatal conductance (g_s) and transpiration rate (E), were measured on the first fully developed leaf (mostly the third from the apex) in the middle of growing season. Measurements were replicated 3 times on three different leaves on each plant selected for analysis. Whole parameters were measured using an infrared gas analyzer (LCpro+, ADC Bioscientific, UK) using a narrow chamber with a set climate conditions (T = 22 °C, PAR = 1500 μmol E m^{-2} s^{-1}). In addition, water use efficiency (WUE) was calculated as a quotient of net photosynthesis to transpiration rate.

2.6.2. Chlorophyll content

Chlorophyll content was measured using chlorophyll meter (CL-01, Hansatech Instruments Ltd., UK). Measurements were conducted on the first fully developed leaf (mostly the third from the apex). Measurements were performed for three chosen leaves (on the same leaves as those taken for gas exchange measurements) from each analysed plant.

2.6.3. Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was measured in the same way as the photosynthesis measurements, on the first fully developed leaf. For each plant, three leaves were selected and measurements were performed using a Pocket Plant Efficiency Analyser (Hansatech Instruments Ltd., UK).

2.7. Plant growth measurements

For each plot the shoot height (h), number of stems (NS) and single-stem diameter (SD, at 10 cm from soil surface) of plants were measured. In addition, the average single plant biomass (BM) on the plots was determined in the middle of October. For this measurement five representative shoots from each plant were taken. To assess the average dry biomass of one plant, the biomass of five plant shoots was multiplied by the number of shoots in the analysed plant.

2.8. Statistical analysis

Data were analyzed using ANOVA with LSD post-hoc test ($P < 0.05$). Principal Component Analyses (PCA) were performed on a correlation matrix to detect any relationship between selected plant physiological parameters, soil physico-chemical and biological parameters in the plant-soil system. Statistical analyses were performed using Statistica 10 (Statsoft, USA).

3. Results

3.1. Soil characteristics

3.1.1. Soil physico-chemical parameters

The soil was classified as silty loam (Table S3). Soil pH value did not differ between M I and M III plots. However, for the M II plot the soil pH value was significantly lower by 0.24. Analysis of soil electrical conductivity (EC), humidity and organic matter content (OM) did not show any significant differences between plots (Table 1).

3.1.2. Soil biological parameters

Soil biological parameters such as dehydrogenase activity (DHA) are presented in Table 1. The DHA did not differ between M I and M III treatments, however, it was 0.5-times lower on M II plot as compared to other plots (Table 1).

Rhizosphere microbial activity increased 2-fold on the M II plot and 2.5-fold on the M III plot as compared to the control (Table 1).

Table 1
Concentration of elements, physico-chemical parameters and biological parameters of soils.

Parameters	Plots		
	M I	M II	M III
	Concentration of elements in soil		
Cd $_{total}$ (mg kg^{-1})	17.29 ± 0.98a	19.22 ± 0.60a	18.77 ± 0.76a
Pb $_{total}$ (mg kg^{-1})	411.5 ± 13.6a	460.6 ± 11.6a	424.9 ± 22.6a
Zn $_{total}$ (mg kg^{-1})	1994 ± 102a	2099 ± 117a	2066 ± 71a
Mg $_{total}$ (mg kg^{-1})	2845 ± 160b	3622 ± 355ab	4705 ± 626a
Fe $_{total}$ (mg kg^{-1})	11642 ± 95b	12196 ± 185ab	12625 ± 395a
Ca $_{total}$ (mg kg^{-1})	5148 ± 191b	4702 ± 148b	7839 ± 420a
Cd $_{bioavailable}$ (mg kg^{-1})	0.73 ± 0.05b	1.02 ± 0.02a	0.73 ± 0.06b
Pb $_{bioavailable}$ (mg kg^{-1})	1.16 ± 0.03b	1.36 ± 0.04a	1.33 ± 0.05a
Zn $_{bioavailable}$ (mg kg^{-1})	28.81 ± 5.98b	50.41 ± 4.45a	33.53 ± 2.89b
Mg $_{bioavailable}$ (mg kg^{-1})	79.57 ± 3.33c	138.42 ± 4.67b	170.91 ± 3.92a
N $_{total}$ (%)	0.14 ± 0.01b	0.16 ± 0.00ab	0.17 ± 0.01a
P $_{available}$ (mg kg^{-1})	186.8 ± 6.2a	151.8 ± 18.3a	185.0 ± 6.7a
K $_{available}$ (mg kg^{-1})	156.8 ± 16.3b	165.8 ± 16.8b	215.6 ± 4.6a
	Physico-chemical soil parameters		
pH (H_2O)	6.98 ± 0.06a	6.75 ± 0.05b	6.99 ± 0.09a
EC (μS cm^{-1})	89.03 ± 10.40a	75.64 ± 5.02a	87.33 ± 6.02a
Humidity (%)	5.7 ± 1.1a	6.63 ± 0.92a	6.42 ± 1.00a
OM (%)	4.89 ± 0.16a	4.99 ± 0.11a	5.15 ± 0.12a
	Biological soil parameters		
DHA (mg TPF h^{-1} g d.w.$^{-1}$)	0.36 ± 0.00a	0.19 ± 0.01b	0.38 ± 0.03a
RMA (μmol CO_2 m^{-2} s^{-1} g d.w.$^{-1}$)	2.14 ± 0.06c	4.17 ± 0.12b	5.47 ± 0.01a
AMF_T (%)	49.17 ± 14.91a	16.67 ± 1.27b	10.83 ± 0.00b

Values are means ± SE (n = 6). Lower case letters (a, b, c) denote significant differences among soils samples taken from different plots at $P \leq 0.05$ according to Fisher LSD test. M I - Control; M II - NPK fertilisation; M III - Microbial inoculum fertilization. EC – Electrical conductivity, OM – Organic matter, DHA - Dehydrogenase activity, AMF_T – Roots colonization rate by Arbuscular Mycorrhizal Fungi, RMA – Rhizosphere microbial activity.

Total AMF roots colonization was lower by nearly 3 times and 5 times on the M II and M III plots respectively as compared to the control (M I). However, there were no significant differences in the colonization of roots between fertilised treatments (M II and M III) (Table 1).

3.1.3. Elements concentration in the soils

Total soil concentration of Pb, Zn and Cd did not differ between plots. The NPK-treated plot (M II) had higher concentrations of bioavailable forms of Cd, Pb and Zn by +40%, +17% and +75% respectively, when compared to the control. However, there was no significant difference in the bioavailable Cd and Zn concentration between the control and M III plots. The bioavailable Pb concentration was similar in the M II and M III plot and it was higher than the control. The total Mg concentration in the soil was significantly higher on plot M III in comparison with the control. Also, the concentration of bioavailable Mg was higher in the M III and M II plots (115% and 74% of the control, respectively). The highest total soil Fe and Ca were measured in the M III plot (Table 1).

The highest N_{Total} concentration was found in the inoculum-treated soil, i.e. +17% as compared to the control (Table 1). No significant differences were observed in $P_{available}$ concentration among treatments in comparison with the control. Moreover, no significant difference in $K_{available}$ concentration between the M II and control plot was observed, however, $K_{available}$ concentration on M III was 26% higher than the control.

3.2. Plant characteristics

3.2.1. Plant growth parameters

Plant growth parameters, such as maximum shoot height (h), stem diameter (SD), number of stems (NS) and average plant biomass (BM) are presented in Table 2. No significant differences were observed between plants grown on M I and M II plots with regards to maximum shoot height, however, plants grown on the M III plots decreased in height by 8%. The number of stems per plant was similar for each plot. However, plants from the M II and M III plots had higher plant biomass and stem diameter, i.e. +55% and +9% respectively, compared to the control plants.

3.2.2. Concentrations of elements in the leaves

Inoculation of the M III plants resulted in significantly higher leaf Ca concentrations (+2081 mg kg^{-1}, respectively) in comparison with the control plants. The highest leaf Mg (+41%) and Zn (+70%) concentration was found for the M II and M III plants with no significant difference between them. Leaf Fe and Pb concentrations did not differ between the plants from each plot (Table 2). Chemical fertilization and microbial inoculation caused higher leaf concentration of N in tested plants compared with the control. The opposite, however, was observed for P and K, the result being that the concentration of both elements in leaves was lower in fertilised plants in comparison with control plants.

3.2.3. Gas exchange parameters

There was a significant difference in photosynthetic rate (A) of plants from different plots (Fig. 1a). The lowest rate was found for the control plants while significantly higher values of this parameter were measured for plants grown on M II (49%) and M III (62%) plots. Transpiration rate (E) showed the same trend (Fig. 1b). The E values were significantly higher, by 29% and 43% for the M II and M III plants respectively, as compared to the control. Plants grown on the M II and M III plots showed similar increase of stomatal conductance (g_s) and water use efficiency (WUE), by 71% and 14% respectively, in comparison to the control plants (Fig. 1c and d).

3.2.4. Chlorophyll content and chlorophyll a fluorescence

3.2.4.1. Fluorescence transient curve. Fluorescence induction curves (between F_0 and F_m) obtained on dark-adapted samples and plotted on a logarithmic time scale shows polyphasic behaviour (Fig. 2). Each step corresponds to different time of fluorescence induction and peaks are labelled as follows: 0-peak (F_0, at 20 μs), J-peak (at 2 ms), I-peak (at 30 ms) and P-peak (F_m, refer to time when maximal fluorescence is reached). Additionally, there was a suggestion of a K-peak (at 300 μs). The OJIP fluorescence curves (Fig. 2a) obtained from plants grown on the M I plot and M II plot are the same on the K, J and I steps, however, the fluorescence curve at the J and I steps obtained from plants grown on the M III plot is more flat and concave in comparison to the others. Kalaji et al. (2014) describe ΔV_t (Fig. 2b) as curves which are constructed by subtracting the normalised fluorescence values (between O and P) recorded in treated plants from those recorded in control plants. Fig. 2b shows that curves obtained from the M II and M I plants had similar shapes, however, there was a lower fluorescence yield on the ΔJ and ΔI steps of the M II plants in comparison to the control ones. Corresponding to results obtained from the M III plants there was a lower value for the ΔJ and ΔI steps in fluorescence yield, however, this parameter was higher for the ΔK step.

3.2.4.2. Fluorescence yield parameters. Fluorescence transient parameters (F_0 and F_m) obtained from fluorescence transient curves are presented in Table S4. There was a significant difference between the losses of energy through fluorescence in the antenna (F_0) obtained for plants on the M II and M III plots. Intermediate F_0 value was measured for the control plants and it was not statistically different from F_0 in plants on the M II and M III plots. A significant difference in maximal fluorescence intensity (F_m) between plants grown on the M III plot and the control was found. Conversely, there was no significant difference between plants from the M II and control plots (Table S4).

3.2.4.3. Energy fluxes per leaf cross sections. There were no significant differences in the leaf pipeline model components between the M I and M II plants, however, every parameter (ABS/CS, TR_0/CS, RC/CS, ET_0/CS) was significantly lower for plants grown on the M III plot except dissipated energy flux per light absorbed by

Table 2
Concentration of elements in leaves and plant growth parameters.

Parameters	Plots		
	M I	M II	M III
	Concentration of elements in leaves		
Cd (mg kg^{-1}d.w.)	5.08 ± 0.09b	5.12 ± 0.09b	5.51 ± 0.25a
Pb (mg kg^{-1}d.w.)	74.39 ± 3.86a	75.62 ± 1.34a	75.00 ± 3.85a
Zn (mg kg^{-1}d.w.)	85.00 ± 3.91b	145.87 ± 10.55a	155.05 ± 12.46a
Mg (mg kg^{-1}d.w.)	710.3 ± 20.4b	1032.1 ± 49.9a	979.2 ± 62.8a
Fe (mg kg^{-1}d.w.)	95.41 ± 7.18a	78.99 ± 1.34a	81.84 ± 3.84a
Ca (mg kg^{-1}d.w.)	3695 ± 132b	4367 ± 397b	6112 ± 801a
N (%)	1.65 ± 0.10b	2.22 ± 0.06a	2.32 ± 0.20a
P (g kg^{-1}d.w.)	1.18 ± 0.01a	0.98 ± 0.01b	1.01 ± 0.03b
K (%)	7.70 ± 0.96a	5.18 ± 0.32b	5.83 ± 0.72b
	Plant growth parameters		
h (cm)	258.7 ± 2.3a	257.5 ± 3.8a	237.5 ± 3.2b
SD (cm)	1.00 ± 0.01b	1.09 ± 0.01a	1.09 ± 0.01a
NS	28 ± 4a	36 ± 4a	33 ± 2a
BM (g)	1656 ± 230b	2714 ± 305a	2410 ± 224a

Values are means ± SE (n = 6), except SD (n = 30). Lower case letters (a, b, c) denote significant differences among plants grown on different plots at $P \leq 0.05$ according to Fisher LSD test. M I - Control; M II - NPK fertilisation; M III - Microbial inoculum fertilization., h – Shoot height, SD – Stem diameter, NS - Number of stems, BM – Single plant dry weight biomass.

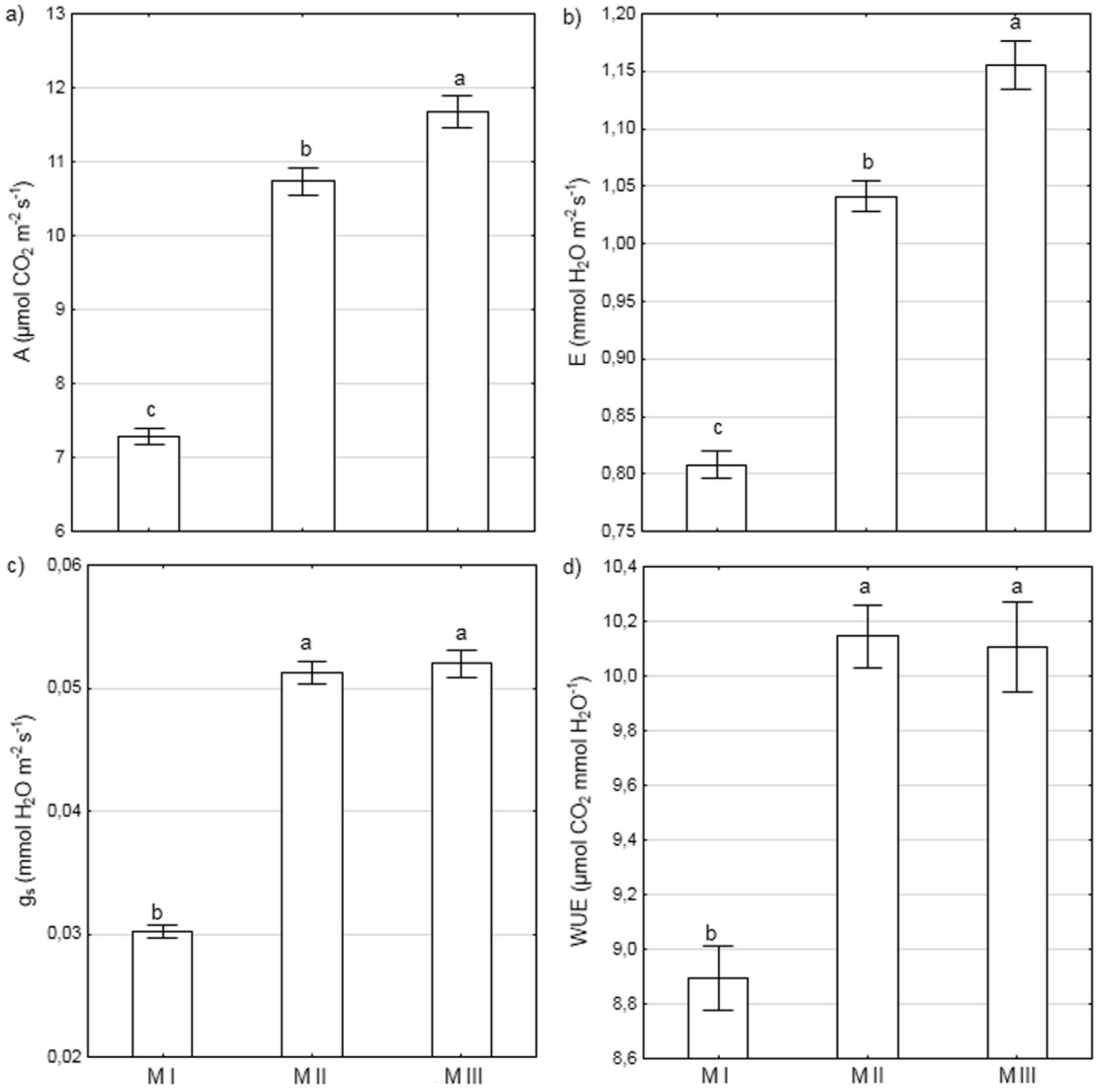

Fig. 1. Effects of NPK fertilisation (plot M II) and microbial inoculation (plot M III) on photosynthesis rate and transpiration of *Miscanthus* x *giganteus*. (a) Photosynthetic rate (A); (b) Transpiration rate (E); (c) Stomatal conductance (gs); (d) Water Use Efficiency (WUE). Values are means ± SE (n = 54). Lower case letters (a, b, c) denote significant differences among parameters in plants on different plots at $P \leq 0.05$ according to Fisher LSD test.

chlorophylls (DI_0/CS) (Fig. 3 and Table S4).

3.2.4.4. Chlorophyll content. The highest content of chlorophyll was observed in plants grown on the M II plot while significantly lower values by 20% and 8% were found for plants grown on M I and M III plots, respectively (Fig. 4a).

3.2.4.5. Photosystem II (PS II) efficiency parameters. There were no significant differences in maximum quantum yield of photochemistry (φP_o at t = 0) values between plants from the M I and M II plots, however, plants grown on the M III plot were significantly lower by a value of 0.024 (r.u.) compared with the control. Values of probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A- (ΨE_o at t = 0) for plants grown on the M III plot showed a significant difference in comparison to the control, however, there was no significant difference between M II and the other variants. There was also no significant difference in the quantum yield of electron transport (φE_o at t = 0) between plants grown on all plots (Fig. 4b).

3.3. Principal-component analysis

PCA (Fig. 5a and b) shows the multivariate relationships in the plant-soil system based on photosynthesis, transpiration, PSII efficiency parameters, chlorophyll content, plant growth parameters, leaf Cd, Zn and Ca concentrations, soil pH and soil microbial activity. Plants grown on the M III and M II plots showed higher values of gas exchange parameters, chlorophyll content (Chl), Rhizosphere

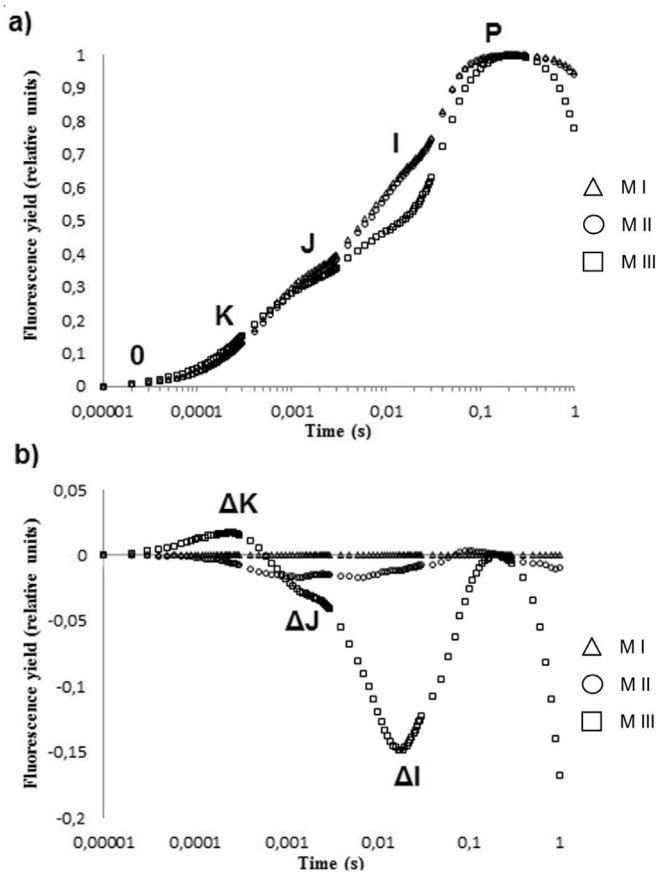

Fig. 2. Chlorophyl *a* fluorescence induction curve (a) and the relative variable fluorescence (b) ($\Delta V_t = ((F_t - F_0)/F_v - V_{tF})$ of the *Miscanthus* x *giganteus* under different treatment. 0 – Minimal fluorescence intensity (F_0), K – Peak at 0,0003 s, J – Peak at 0,002 s, I – Peak at 0,002s, P – Maximal fluorescence intensity (F_m). ΔK, ΔJ and ΔP values were obtained using formula written above. Values are means (n = 18). M I - Control; M II - NPK fertilisation; and M III - Microbial inoculum fertilisation.

Microbial Activity (RMA), leaf Cd, Zn and Ca concentrations, φE_0 and all plant growth parameters, except shoot height (h). The balance is shifted in photosynthesis parameters toward plants grown on the M III plot, whereas plants from the M II plot had higher values of plant growth parameters, Chl and Water Use Efficiency (WUE). All these parameters show strong correlation between each other and they are conditioned by Principal Component 1 (PC1), except leaf Cd concentration which is conditioned totally by Principal Component 2 (PC2) (Table S4). Variables such as dehydrogenase activity (DHA), pH, shoot height and ΨE_0 are conditioned by PC2. There is no correlation between both parameters (DHA and RMA) describing microbial activity in soil (Fig. 5a and Table S5).

4. Discussion

The impact of trace elements (TE) contamination and fertilisation on plants and microorganisms is widely described in the literature (Belay et al., 2002; Efthimiadou et al., 2010; Miransari, 2011; Rashid et al., 2016). However, there is a dearth of publications presenting plant-soil systems in a holistic context. This study reports a comprehensive analysis of the combined effect of different fertilizers on soil biological and physico-chemical parameters and the status of the photosynthetic apparatus of field-tested *M.* x *giganteus* growing on trace elements contaminated soil (TECS).

It is widely known that the proper use of fertilisers improves plant growth (Danalatos et al., 2007; Stępień et al., 2014; Xu et al., 2001). This effect was confirmed in this study, however, both fertilisers (NPK fertiliser and microbial inoculum) had the same positive effect on all measured plant growth parameters, except the shoot height, which was lower for microbial inoculum.

This study demonstrates an alternative, minimally invasive method for the assessment of rhizosphere microbial activity under perennial energy grasses, using respiration methods based on the three compartment-model (Kelting et al., 1998). Different methods for the assessment of rhizosphere microbial activity (DHA and RMA) used in the current study showed divergent results. Indirect rhizosphere microbial activity (RMA, Table 1) indicated higher activity of microorganisms at plots treated with fertilizers compared to the control, whereas direct measurements of rhizosphere microbial activity (DHA, Table 1) showed no positive effect of fertilizers on microbial activity. The positive effect of NPK and biological fertilizers on microbial activity has been documented by several authors (e.g. Majumdar et al., 2014; Stępień et al., 2014; Vafadar et al., 2014; Yu et al., 2015). Thus, the results presented in the current study for the RMA method are more compatible with published data than those obtained by the DHA method. Previous studies (e.g. Ortas, 1997) have shown that rhizosphere thickness ranges from 0.1 to 4.0 mm. Since the rhizosphere width is so small it is difficult to obtain soil samples containing solely rhizosphere soil even if the roots are taken out of the ground completely. Therefore, it seems more correct in perennial plant studies on microbial respiration to use the RMA method described in this paper rather than soil sampling by removing soil adjacent to the root system (Chaudhary et al., 2012; Grayston et al., 1998) and assessing the soil microbial activity through colorimetric methods (DHA) (Chaudhary et al., 2012).

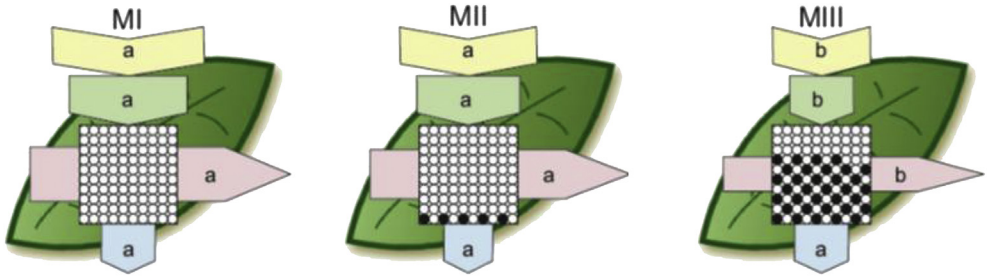

Fig. 3. Leaf pipeline model of phenomenological proportion of energy flux parameters within the *Miscanthus* x *giganteus* leaves. M I - control, M II - NPK fertilisation, M III - microbial inoculum fertilisation. Pipeline model consists of: trapped (maximum) energy flux per chlorophyll absorbance (TR_0/ABS – green arrow), dissipated energy flux per light absorbed by chlorophylls (DI_0/ABS – blue arrow), electron transport flux per chlorophyll (ET_0/ABS – red arrow). These parameters are related to equal chlorophyll absorption (ABS/CS – yellow arrow). Circles inscribed in square present 100% of reaction centres pool; open circles corresponding to active (oxidized) RC, close ones (reduced) inactive (Kalaji et al., 2011). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

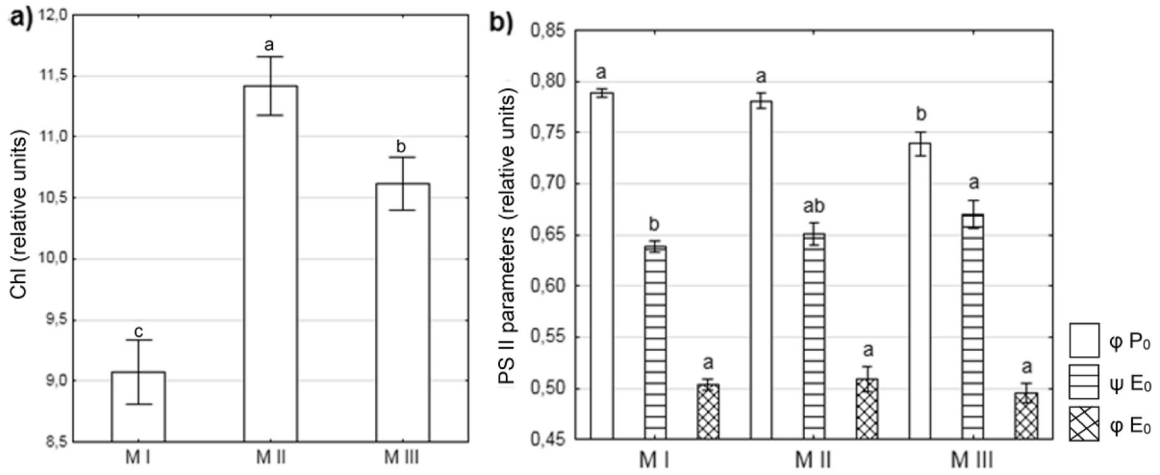

Fig. 4. Effects of NPK fertilisation (plot M II) and microbial inoculation (plot M III) on *Miscanthus* x *giganteus* (a) chlorophyll content (Chl) and (b) φP_0 - Maximum quantum yield of photochemistry, ΨE_0 – Values of probability that a trapped exciton moves electron into the electron transport chain beyond Q_A-, φE_0 - Quantum yield of electron transport. Values are means ± SE (n = 18). Lower case letters (a, b, c) denote significant differences on different plots at $P \leq 0.05$ according to Fisher LSD test.

Our results showed that the highest levels of Cd leaf concentration of plants grown on the M III plot did not correlate with the bioavailable Cd concentration in the soil, which was the highest on the M II plot. Similar results were observed for Zn. Leaf Zn concentrations were the same for plants grown on the M II and M III plots, although it was found that the concentration of Zn bioavailable forms was about 2-fold higher on the M II plot when compared to the M III plot (Table 2). This can be caused by the activity of microorganisms which could increase the mobility of Cd and Zn and their uptake from the rhizosphere. For example, the ability to enhance TE mobility by rhizospheric bacteria was described previously for Se and Hg by De Souza et al. (1999). The NPK-fertilised plot showed a significantly lower soil pH when compared to the control and inoculated plots. Furthermore, it was found that the highest concentration of Zn and Cd in bioavailable forms were present on the NPK-fertilised plot. Several studies (Hanc et al., 2012; Jenne et al., 1986; Kozdrój et al., 2007 and Komárek et al., 2008) have reported that the mobility of heavy metals can be influenced by following factors: pH, redox potential, microbiological effects and temperature. The influence of chemical fertilizer on soil pH was described by Belay et al. (2002) and Czarnecki and Düring (2015) and occurs due to the presence of NH_4^+ in chemical fertilisers as the N-source, which results in H^+ extrusion by the roots and acidification of the soil (Magdoff et al., 1997).

According to Kabata-Pedias (2011), in most plants the toxic effect is caused by 10 and 300 mg kg^{-1} of Cd and Zn respectively. Thus, the results presented in the current study show that the concentration of Zn and Cd in leaves did not exceed the toxic level for those elements in plants. However, a toxic concentration of Pb was found in *M.* x *giganteus* leaves. Nsanganwimana et al. (2016) reported that in the leaves of *M.* x *giganteus* cultivated on TE-contaminated soil concentration of Cd, Pb and Zn was about 0.3 mg kg^{-1}, 0.6 mg kg^{-1} and 50 mg kg^{-1}, respectively. The values obtained by Nsanganwimana et al. (2016) are considerably lower when compared to the Cd, Pb and Zn concentrations in leaves documented in the present study. The differences between the data presented in the current study and those presented by Nsanganwimana et al. (2016) could be driven by different concentrations of the Cd and Zn in soil. The concentration of Pb in the soil in the current study was similar to that reported by Nsanganwimana et al. (2016), however, the Pb concentration found in the leaves reported in the present study was substantially higher. Although the leaf samples were washed in tap water and then in deionized water there is a possibility that particles of soil containing Pb could remain on the leaf surface which resulted in higher Pb leaf concentration. Nsanganwimana et al. (2014) reviewed that *M.* x *giganteus* cultivated on soil contaminated with TE can accumulate Cd and Zn at the levels which were observed in the current study.

A significant decrease of AMF colonization in roots of *M.* x *giganteus* cultivated on the M II and M III plots was found (Table 1). It seems that the main cause of lower colonisation of roots by AMF was applied fertilisation. The negative effect of soil P content on AMF root colonization in different plant species is very well known (Baar, 2008; Smith and Read, 2008). However, lower root colonization by AMF fungi as a result of nitrogen fertilisation has also been described by several authors (e.g. Corkidi et al., 2002; Van Diepen et al., 2007). With regards to macroelement concentrations, N_{total} was higher in the M II and III plots when compared to the control. It is therefore reasonable to suggest that the higher concentration of this element in the soil was the main reason for the lower root colonization by AMF. The decrease in AMF root colonization in the M III plot could be also connected with competition between microorganisms derived from inoculum and autochthonous AMF (Biró et al., 2000).

The lower concentration of P and K in the leaves of plants from the M II and M III plots, which were fertilised, is an unexpected effect (Table 2). A possible explanation for a higher concentration of P and K in leaves of control plants (M I plot) could be higher root colonization of their roots by AMF. Stimulation of uptake and higher concentrations of both macroelements in plants colonized by AMF was observed (Smith and Read, 2008).

It is widely known that soil composition and soil-root interactions determine the status of aboveground plant organs like stems and leaves. In this experiment, BM and stem diameter were correlated, showing that the biomass of *M.* x *giganteus* was more dependent on the stem diameter than on shoot height. In addition, the higher *M.* x *giganteus* biomass could be related to the higher N concentration in soils on the M II and M III plots when compared to the control. Arundale et al. (2014) reported that nitrogen fertilisation significantly increased *M.* x *giganteus* yield.

Many environmental factors influence photosynthetic efficiency and intensity at different organizational levels of photosynthetic apparatus (Ashraf and Harris, 2013; Fatichi et al., 2014; Suzuki et al., 2014). In our study, chlorophyll content (Fig. 4a) was higher in plant leaves treated with inoculum and chemical fertiliser, which could

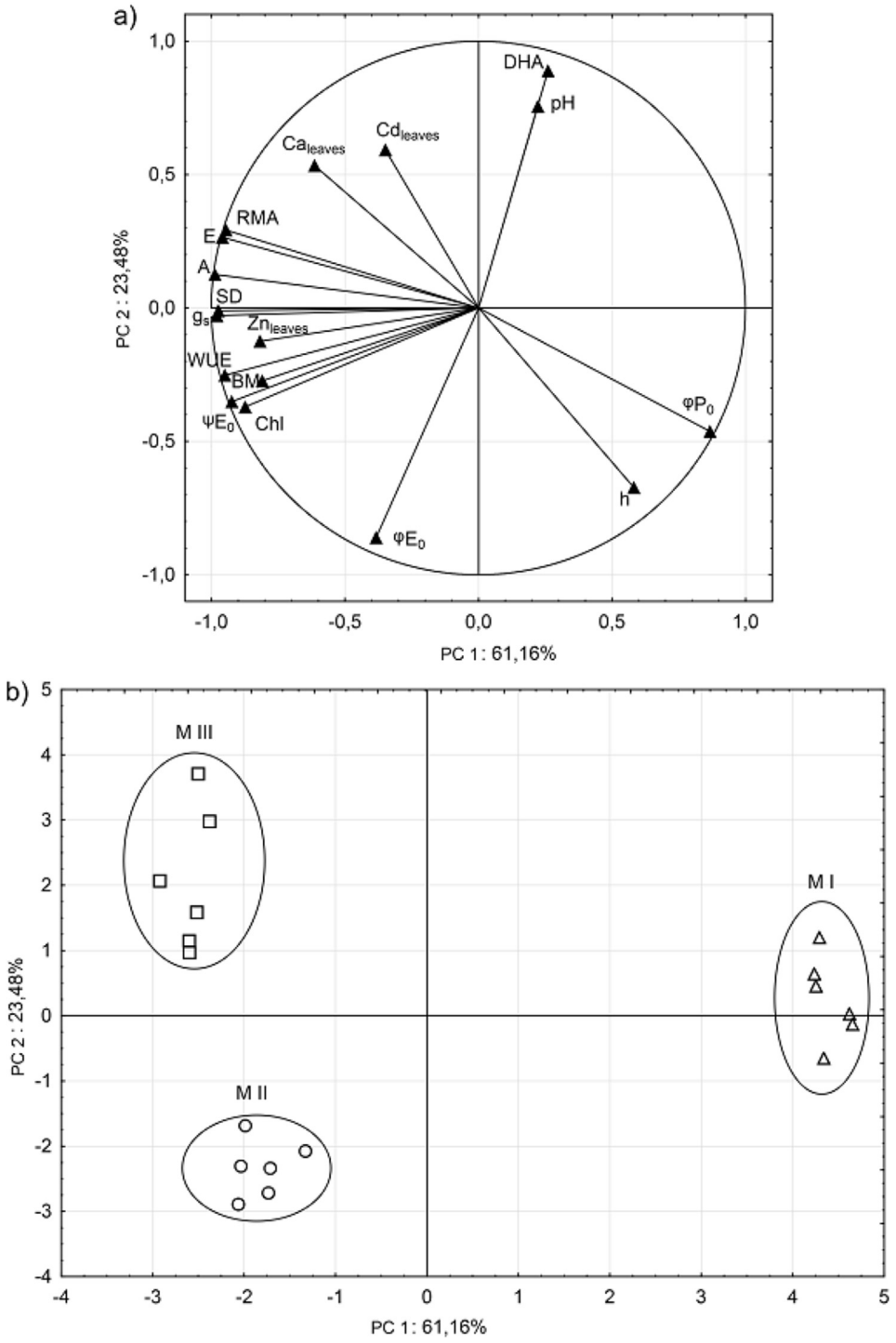

Fig. 5. Principal component analysis distinguished into two parts (a) Correlation between variables along two PCA axis (PC1 x PC1) and (b) ordination of case along two PCA axis (PC1 x PC2). Δ – M I (control); ○ – M II (NPK fertilisation); □ – M III (microbial inoculum treatment). RMA – Rhizosphere Microbial Activity, E – Transpiration rate, A – Photosynthesis rate, WUE – Water Use Efficiency, SD – Stem diameter, gs – Stomatal conductance, Chl – Chlorophyll content, BM – Single plant dry weight biomass, ΨE_0 – Values of probability that a trapped exciton moves electron into the electron transport chain beyond Q_A-,φE_0 - Quantum yield of electron transport, h –Shoot height, $\varphi P0$ - Maximum quantum yield of photochemistry, pH – soil pH (H_2O), DHA – Dehydrogenase activity, Zn $_{leaves}$ – Zn leaves concentration, Cd $_{leaves}$ – Cd leaves concentration, Ca $_{leaves}$ – Ca leaves concentration.

be caused by the higher leaf Mg concentration. In this study an inactivation of chlorophyll reaction centres in PSII was observed (Fig. 3), due to a significant decrease in the maximal fluorescence of the dark-adapted state (F_m) for plants grown on M III. Moreover, the same plants showed the highest value of minimal fluorescence of the dark-adapted state (F_0) (Table S4) which means that Light Harvesting Complex II (LHC II) could be dissociated from PS II, resulting in low energy transfer between those two components (Havaux, 1993; Kalaji et al., 2011; Strauss et al., 2006). Differences in the transient dynamics of relative variable fluorescence between the control and treated plants is possible by ΔV_t (Kalaji et al., 2014). Each presented step on ΔV_t curves (Fig. 2b) has a different meaning.

The presence of the K step in relative fluorescence transient curves could appear due to presence of a stress factor (Chen et al., 2013; Ran et al., 2015) which in this case was the TE-contaminated soil, *ipso facto* ΔK on ΔV_t curves are related to the uncoupling of the oxygen evolving complex (OEC), ΔJ step is associated with inhibition of the Q_A^- reoxidation and ΔI step shows information about inactivation of ferrodoxin-$NADP^+$ oxidoreductase (FNR) (Kalaji et al., 2014). The presented data ΔV_t curves obtained for M I and M II were similar, however, the curve obtained from M III plants was shaped differently (Fig. 2b). Inoculum treated plants, due to the same reasoning show probability of highly activated FNR and disengaged OEC in comparison to the control.

The leaf pipeline model is widely used to describe environmental and/or anthropogenic pressure to plants (Kalaji et al., 2011; Mehta et al., 2010; Zushi et al., 2012). According to the results obtained for leaf metal concentrations it can be assumed that the metals at these concentrations do not have an influence on the photosynthetic parameters presented in the leaf pipeline models. It is noteworthy that the worst pipeline model was obtained for plants cultivated on the M III plot, however, on the basis of our results we are not able to explain this phenomenon (Fig. 3).

Photosynthesis rate, transpiration and WUE (Fig. 1) increased under both types of fertilisation. Similar results were previously reported for *M.* x *giganteus* (Wang et al., 2012) and other plant species with increased photosynthesis and transpiration rate under different fertilisation regimes (Xu et al., 2001; Bondada and Syvertsen, 2003; Ferrini et al., 2005; Efthimiadou et al., 2010).

4.1. Conclusions

Application of both fertilizers diminished root colonisation by arbuscular mycorrhizal fungi (AMF). Higher AMF root colonisation of *Miscanthus* x *giganteus* in control plots could be the main cause of higher P and K leaf concentration compared to NPK fertilisation and microbial inoculation. On the other hand, microbial activity in rhizosphere was stimulated by both types of fertilisation. NPK fertilisation and microbial inoculum fertilization had a positive influence on photosynthesis rate, transpiration, water use efficiency and growth of *M.* x *giganteus*. However, application of NPK chemical fertilizer decreased soil pH and increased Cd and Zn mobility, whereas microbial inoculum did not. Given the positive effect of microbial inoculum fertilization on *M.* x *giganteus* physiological status and biomass as well as soil microbial activity, with simultaneously low mobilisation of toxic trace elements (Cd and Zn) in soil, it is proposed that this type of fertilisation could be used successfully for growing of energy crops on TE contaminated soils.

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Authors contributions are as follows: **M.P.** planned and design the research, wrote the manuscript (60%), **S.R., K.S.** performed the experiments, analysed data, conducted field work (10%), **J.K.** planned and design the research, wrote the manuscript (10%), **E.M., H.M.K.** wrote the manuscript (5%), **A.S., D.C.,** performed experiments, **S.W., J.P.M., M.M.** comments, improvements and English corrections.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2017.03.058.

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