The response of cucumber plants (*Cucumis sativus* L.) to the application of PCB-contaminated sewage sludge and urban sediment

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Background. The increasing production of sewage sludge (SS) engenders the problem of its responsible utilization and disposal. Likewise, urban sediments (SED) are deposited at the bottom of urban reservoirs and sedimentation ponds, and these require periodical dredging and utilization. However, while the SS and SED deposits often contain nutrients such as nitrogen and phosphorus; however, they also contain a variety of hazardous compounds including heavy metals, Persistent Organic Pollutants (POPs) and microbial pollutants. Fortunately, some species of Cucurbitaceae can accumulate high levels of POPs, such as polychlorinated dibenzo-*p*-dioxins (PCDD), polychlorinated dibenzofurans (PCDF) and polychlorinated biphenyls (PCB), in their tissues.

Methods. SS was collected from the Lodz Municipal Wastewater Treatment Plant and SED from the Sokołówka Sequential Biofiltration System. The SS and SED samples were added to soil in flower pots at three concentrations (1.8 g, 5.4 g and 10.8 g per flower pot), and one pot was left as an unamended control (C). Soil PCB concentrations were determined before cucumber planting, and after five weeks of growth. Also, total soluble protein, total chlorophyll content, chlorophyll a/b ratio and degree of lipid peroxidation (TBARS) were examined in the leaves of the cucumber plants (*Cucumis sativus* L.) cv. Cezar after five weeks. Antioxidative response was assessed by ascorbate peroxidase (APx) and catalase (CAT) assay.

Results. The initial PCB concentration in soil after application of SS or SED was dependent on the applied dose. After five weeks, PCB concentration fell significantly for all samples and confirmed that the dose of SS/SED had a strong effect. Soil remediation was found to be more effective after SS application. Total soluble protein content in the cucumber leaf tissues was dependent on both the type and the dose of the applied amendments, and increased with greater SS doses in the soil. The total chlorophyll content remained unchanged, and the chlorophyll a/b ratio was slightly elevated only after the application of the highest SS and SED dose. The use of SS and SED did not significantly affect TBARS content. APx activity fell after SS or SED application; however, CAT activity tended to increase, but only in the leaves of plants grown in SS-amended soil.

Discussion. The cultivation of cucumber plants reduces PCB concentration in soil amended with SS or SED; however, this effect is more evident in the case of SS. SS application also induced more intensive changes in the activity of enzymes engaged in antioxidative response and oxidative stress markers in plant tissues than SED. The levels of PCB in the SS may have triggered a more severe imbalance between pro- and antioxidative reactions in plants. Cucumber plants appear to be resistant to the

presence of toxic substances in SS and SED, and the addition of SS and SED not only acts as a fertilizer, but also protects against accelerated aging.

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Abstract

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63 **Discussion.** The cultivation of cucumber plants reduces PCB concentration in soil amended with
64 SS or SED; however, this effect is more evident in the case of SS. SS application also induced
65 more intensive changes in the activity of enzymes engaged in antioxidative response and
66 oxidative stress markers in plant tissues than SED. The levels of PCB in the SS may have
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68 plants appear to be resistant to the presence of toxic substances in SS and SED, and the addition
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70

71 **Introduction**
72 The 21st century set a new challenge for mankind, that of reconciling economic development
73 with the preservation and protection of global natural resources. This goal can be achieved
74 through adopting a sustainable development strategy, which is based on fast economic growth
75 and improvements in quality of life, coupled with the improvement of the natural environment
76 and the protection of its resources (EC, 2017). Maintaining a balance between the above is
77 possible only through the rational use of natural resources and limiting the environmental burden
78 associated with waste coming from human activity. In response to the development of urban
79 centers and the changes in consumption habits of the population, municipal waste management
80 has become a priority environmental issue in many countries. Waste administration also
81 represents a significant cost to society. As the global population grows, the amount of waste,
82 including domestic wastewater, increases with it. The development of universal sewage systems
83 requires a greater number of wastewater treatment plants, resulting in growing amounts of the
84 product of wastewater treatment, this being sewage sludge (SS).
85 Urban sediments (SED) are formed at the bottom of treatment reservoirs and sedimentation
86 ponds located in cities. One example of such structures is the Sokołówka Sequential Biofiltration
87 System (SSBS), whose sediments were used in the present study (Urbaniak et al., 2012). The
88 SSBS was constructed in the upper section of the Sokołówka River with the aim of removing
89 sediments, suspended solids, particulate pollutants, petroleum hydrocarbons, heavy metals and
90 nutrients from stormwater runoff through a system of sedimentation and filtration mechanisms.
91 In order to increase the efficiency of the system, it comprises three different zones: a
92 hydrodynamically-intensified sedimentation zone, an intensive biogeochemical process zone and
93 an intensive biofiltration zone.

94   Both SS and SED are rich organic fertilizers. They contain a number of nutrients that are
95   essential for agricultural use, such as nitrogen and phosphorus (Herzel et al., 2016; Mamedov et
96   al, 2016; Tontti, Poutiainen & Heinonen-Tanski, 2017). Organic fertilization results in an
97   increase in the organic carbon and nitrogen stock in soils and is more effective than mineral
98   fertilization (Yazdani et al., 2017; Wyrwicka & Urbaniak 2018). The presence of increased
99   amounts of soil organic matter also improves soil structure, water holding capacity and soil
100 nutrient availability, increases microbial biomass and broadens microbial community structure
101 and biodiversity (Tejada et al., 2014; Lloreta et al., 2016). However, in addition to substances
102 that bestow a beneficial effect on plants and enrich the soil, SS and SED also contain heavy
103 metals, toxic organic and inorganic pollutants, including a number of persistent organic
104 pollutants (POPs) characterized by high persistence, potential for bioaccumulation,
105 biomagnifications and toxicity, e.g. polychlorinated biphenyls (PCBs), polychlorinated dibenzo-
106 p-dioxin (PCDD), polychlorinated dibenzofurans (PCDFs), polycyclic aromatic hydrocarbons
107 (PAH); in addition, inorganic compounds, silicates and aluminates can also be found, as well as
108 pathogenic and other microbial pollutants (Oleszczuk, 2006; Urbaniak et al., 2017b; Goberna et
109 al., 2018).

110 In accordance with sustainable development strategy principles, SS and SED should be rationally
111 and responsibly managed so as not to pose a threat to human health and life. Landfilling,
112 incineration, composting and agricultural use are all used for waste control in Europe. However
113 the Landfill Directive 99/31/EC (CEU, 1999) and the statutes of Poland and other member
114 countries place very strict limits on organic matter or total organic carbon content in sludge.
115 These limits have effectively prevented the use of landfilling as a means of disposal. Hence, the
116 first choice for sludge disposal indicated by European Environmental Policy (Decision
117 2001/118/EC; Directive 2008/98/EC; CEC, 2001) is total sludge reuse, with agricultural use
118 being the obvious mode. However, due to the elevated concentrations of heavy metals in SS,
119 which are regulated at national (Journal of Laws of 2015, item 257) and EU levels (Sewage
120 Sludge Directive, 86/ 278/EEC; CEC, 1986), this approach cannot be used by agriculture for
121 food production.

122 A method that addresses the problem of increasing amounts of SS and SED is phytoremediation.
123 It is a technique based on the ability of selected plant species to remove, contain or render
124 harmless environmental contaminants (Cunningham & Berti 1993; Cunningham, Berti & Huang,

125 1995; Macek, Macková & Káš, 2000). This "green technology" is a cost-effective, non-invasive,
126 alternative or complementary technology for site restoration and partial decontamination
127 (Wiesmeier et al., 2015; Glenn, Jordan & Waugh, 2017). Phytoremediation is a promising
128 method of coping with the growing production of SS and urban reservoir sediments, particularly
129 in cities, where they could be used by direct application as soil additives on city gardens and
130 lawns. The plants used in phytoremediation should not only have a high ability to neutralize
131 pollutants, but should also be resistant to the effects of these pollutants on plant organisms.
132 Although many examples have been given of plant species capable of remediation of heavy
133 metals (Ivanov, Bystrova & Seregin, 2003; Reddy et al., 2005; Lyubenova et al., 2009), the
134 processes of phytoremediation of organic substances are not so well understood and further
135 information is still needed on this subject. The main problem associated with the potential for
136 phytoremediation of organic compounds is related to their hydrophobicity, and hence the
137 inability of plants to take up these compounds from the soil (log K_{ow} between 5.0 to 8.3).
138 Substances with a log K_{ow} higher than 3.5 are not available for most plants, because they are
139 strongly bound to soil particles and do not go into the soil solution from where they may be taken
140 up by the roots (Briggs, Bromilow & Evans, 1982; Hatzinger & Alexander, 1995). Many
141 excellent publications indicate that the plants of the Cucurbitaceae, such as cucumbers, pumpkins
142 and squashes are exceptions to this rule. These plants have a high potential to take in relatively
143 large amounts of organic compounds and to accumulate them in their tissues. In addition, it was
144 found that the collected compounds come largely from the substrate in which the plants were
145 grown rather than from the air (Hülster, Müller & Marschner, 1994; Engwall & Hjelm, 2000;
146 Mattina, Iannucci-Berger & Dykas, 2000; White, 2002; Mattina et al., 2004; Zhang et al., 2004;
147 Inui et al., 2008; Low et al., 2010; White, 2010; Matsuo et al., 2011).
148 SS and SED offer great potential as fertilizers which also improve the physicochemical
149 properties of soil due to their high organic matter content. On the other hand, the presence of
150 harmful substances in the sediments may adversely affect plant physiology and influence total
151 soluble protein content, total chlorophyll content as well as chlorophyll a/b ratio, among others.
152 However, most research on the effects of SS or SED on plants focuses mainly on growth
153 parameters such as biomass yield (Urbaniak et al., 2017a). Only a few works investigate the
154 influence of these sediments on physiological and biochemical changes occurring in treated
155 plants (Lakhdar et al., 2010).

156 Environmental stress factors have been demonstrated to result in delayed secondary oxidative
157 stress in plants (Demidchik, 2015). This state is characterized by the imbalance between the
158 formation of Reactive Oxygen Species (ROS) and their removal by the cellular antioxidant
159 systems. ROS such as superoxide anions ($O_2^{-\cdot}$), hydroxyl radicals ($^{\cdot}OH$) and peroxyl radicals
160 ($ROO^{\cdot}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) are highly reactive and can cause
161 oxidative damage to proteins, lipids, nucleic acids and other biologically important molecules;
162 this damage results in dysfunction and irreversible degradation, and thus reduced plant growth
163 and development (Gill & Tuteja, 2010). Although ROS are naturally formed under physiological
164 conditions in plant cells as by-products of photosynthesis and respiration, the homeostasis
165 between their production and elimination is not maintained under oxidative stress. One of the
166 consequences of the occurrence of oxidative stress in plant tissues is the increase in the
167 concentration of compounds reacting with thiobarbituric acid (ThioBarbituric Acid Reactive
168 Substances; TBARS). In plant cells, many elements of the antioxidant system interact with each
169 other to counteract ROS accumulation. One of the components of the enzymatic antioxidant
170 system is ascorbate peroxidase (APx), which reduces H_2O_2 to water with simultaneous oxidation
171 of the substrate, ascorbic acid. Another antioxidant enzyme is catalase (CAT), which catalyzes
172 the H_2O_2 dismutation reaction.
173 The aim of the study was to determine the effect of SS and SED contamination of soil on
174 cucumber cultivation, with regard to their potential as soil remediation agents. The study
175 examines total soluble protein and total chlorophyll content, and chlorophyll a/b ratio in the
176 plants to identify the physiological status. To determine if the given conditions affect the redox
177 equilibrium, the extent of the oxidative reaction was assessed as TBARS content, and efficiency
178 of the antioxidative system was assessed as the activities of APx and CAT.
179
180 **Materials and methods**
181 Soil preparation
182 Two sets of samples were collected from two locations in Central Poland: SS from Lodz
183 Municipal Wastewater Treatment Plant (LM WWTP) and SED from the Sokołówka Sequential
184 Biofiltration System (SSBS). The SED samples used in the experiment were collected from the
185 first zone of the SSBS, intended for accelerated sedimentation of suspended matter and
186 associated pollutants. The SS and SED samples were dried at 70 ºC for 72 hours then

187 homogenized into small particles using a mortar and used as fertilizer for the soil samples for
188 cucumber planting. The vegetable potting soil, intended for cucumber growth, used in the
189 experiment was collected from Hollas Sp. z o.o. Pasłęk. The main soil parameters used in this
190 experiment was presented in Table 1.

191 In addition to controls, in which no sludge or sediment was added (C), three treatments were
192 used: 1.8 g, 5.4 g and 10.8 g of dried matter per flower pot. The first corresponds to a dose of 3
193 tonnes ha^{-1}, the annual dosage permitted by the Ministry of the Environment of Regulations
194 (Journal of Laws of 2015, item 257); the second is the permitted dose of 9 tonnes ha^{-1} per three
195 years applied on one occasion; and the third, 18 tonnes ha^{-1} is above the permitted level.
196 Treatments are designated by the dose per pot.

197

198 PCB concentrations

199 PCB concentrations were determined in the control and SS and SED amended soils before
200 cucumber planting, i.e. the first day of the experiment, and after five weeks of cucumber growth,
201 i.e. the last day of the experiment. The collected soil samples were stored in glass tubes in the
202 dark at 4 ºC for further extraction using a PCB RaPID Assay Sample Extraction Kit (Modern
203 Water) and PCB analysis using Enzyme Linked Immunosorbent Assay (ELISA) – PCB RaPID
204 Assay (Modern Water). PCB extraction and level determination were performed according to the
205 manufacturer's instructions (https://www.modernwater.com/pdf/MW_Factsheet_Rapid-
206 Assay_PCB.pdf).

207 The PCB RaPID Assay kit applies the principles of ELISA to the determination of PCB and
208 related compounds (EPA SW-846 Method #4020). The test sample is added to a disposable test
209 tube with an enzyme conjugate, followed by paramagnetic particles bound to antibodies specific
210 to PCB. Both the PCB in the sample and the enzyme-labeled PCB (the enzyme conjugate)
211 compete for the antibody binding sites on the magnetic particles. After a short incubation period,
212 the PCB and the labeled PCB analog will bind to the particles at proportions reflecting their
213 concentrations in the suspension. A magnetic field is then applied to hold the particles in the tube
214 and the unbound reagents are decanted. After decanting, the particles are washed with Washing
215 Solution. The presence of PCB is detected by adding the enzyme substrate (hydrogen peroxide)
216 and the chromogen (3,3',5,5'-tetramethylbenzidine). The enzyme labeled PCB analog bound to
217 the PCB antibody catalyses the conversion of the substrate/chromogen mixture to a colored

218 product. After an incubation period, the reaction is stopped and stabilized by the addition of acid.

219 Since the labeled PCB (conjugate) was in competition with the unlabeled PCB (sample) for the

220 antibody sites, the color developed is inversely proportional to the concentration of PCB in the

221 sample. The absorbance was measured at 450 nm using a SDI Differential Spectrophotometer.

222 A fuller description of sample preparation was also given previously by Wyrwicka, Steffani &

223 Urbaniak, 2014.

224

225 Plant material

226 Cucumber seeds (*Cucumis sativus* L.) cv. Cezar were germinated in Petri dishes for seven days

227 and the seedlings were planted in the control or amended soil. The samples were grown in a

228 growth chamber at of 23 ± 0.5 °C under a 16 h light/8 h dark cycle, 250 µmol m^{-2} s^{-1} photon flux

229 density, during the light period, and 60% relative humidity. Five-week old plants with five fully-

230 expanded leaves were used for subsequent analysis. All biochemical analyses were carried out on

231 the physiologically second, third and fourth leaves (from the bottom) of the control and treated

232 plants. The leaves were harvested in the middle of the 16-hour light period.

233

234 Preparation of enzyme extracts from leaf tissues

235 The leaves of the cucumber plants were ground (1:10, w/v) in an ice-cold mortar using 50 mM

236 sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, 1 mM EDTA, and 1 mM sodium

237 ascorbate. The slurry was filtered through two layers of Miracloth. The filtrates of homogenized

238 cucumber leaves were then centrifuged (15000 g x 15 min). After centrifugation, the supernatant

239 was collected and APx and CAT activities as well as protein content and degree of lipid

240 peroxidation were measured.

241

242 Enzyme assay

243 APx activity [EC 1.11.1.11] was assayed following the oxidation of ascorbate to

244 dehydroascorbate at 265 nm ($\varepsilon = 13.7$ mM^{-1} cm^{-1}) according to Nakano & Asada (1981) with

245 some modifications, using a Unicam UV 300 UV-Visible spectrometer (Unicam Limited,

246 Cambridge United Kingdom). The assay mixture contained 50 mM sodium phosphate buffer pH

247 = 7.0, 0.25 mM sodium ascorbate, 25 µM H$_2$O$_2$ and the enzyme extract (5–10 µg protein). The

248 addition of H$_2$O$_2$ started the reaction. The obtained values were compared with those of another

249   reaction mixture without the enzyme extract to correct for non-enzymatic oxidation of ascorbate.

250   The enzyme activity was expressed in µmol ascorbate mg^{-1} protein.

251   CAT activity [EC 1.11.1.6] was measured spectrophotometrically according to Dhindsa, Plumb-

252   Dhindsa & Thorpe, 1981. A reaction mixture composed of 50 mM sodium phosphate buffer (pH

253   = 7.0), 15 mM H_2O_2 and the enzyme extract (5–10 µg protein) was used. The decomposition of

254   H_2O_2 (ε = 45.2 mM^{-1} cm^{-1}) was measured at 240 nm using Unicam UV 300 UV-Visible

255   spectrometer (Unicam Limited, Cambridge United Kingdom). CAT activity was expressed in

256   mmol H_2O_2 mg^{-1} protein.

257

258   Protein content

259   The protein content was determined according to Bradford (1976) with standard curves prepared

260   using bovine serum albumin. Samples were assayed using a spectrophotometer (Helios Gamma,

261   Thermo Spectronic, Cambridge, UK). The protein content was given as mg g^{-1} fresh mass of the

262   original plant tissue.

263

264   Degree of lipid peroxidation (TBARS)

265   Concentration of lipid peroxides was estimated spectrofluorometrically (F-2500 Fluorescence

266   Spectrophotometer, Hitachi, Limited, Tokyo Japan) according to Yagi (1982), by measuring the

267   content of 2-thiobarbituric acid reactive substances (TBARS). The concentration of lipid

268   peroxides was calculated in terms of 1,1,3,3-tetraethoxypropane, which was used as a standard

269   and expressed in nmol g^{-1} fresh mass.

270

271   Determination of chlorophyll content

272   Whole leaves were homogenized (1:5 w/v) in an ice-cold mortar using 50 mM sodium phosphate

273   buffer, pH 7.0, containing 0.5 M NaCl, 1 mM EDTA and 1 mM sodium ascorbate. Crude

274   homogenate obtained after filtration was assayed for chlorophyll content according to Porra,

275   Thompson & Kriedmann (1989). The absorbance of clear supernatant obtained after

276   centrifugation (5000 g × 5 min.) of 80% acetone extract was measured at 663 and 645 nm using

277   a Helios Gamma spectrophotometer (Thermo Spectronic, Cambridge, UK). The concentration of

278   chlorophyll was expressed as mg g^{-1} fresh mass.

279

280   Statistical analysis

281   Linear regression analysis was used to describe the relationships between PCB concentration in

282   soil and amendments dose separately for SS and SED. To analyze whether both regression lines

283   differed significantly, differences in slopes (b coefficient) was tested using a t-test and if the null

284   hypothesis were not rejected, the differences in intercepts (a coefficient) were also tested (t-test)

285   (Zar, 2010). The effect of cucumber plant cultivation on the PCB content in soil amended with

286   SS and SED was analyzed by two-way ANOVA (repeated measures) with type of soil

287   amendments and their dose as factors.

288   Six to eight individuals were collected from the cultivated plants and used for the experiment,

289   hence the replicate number of plant biochemical parameters varies from six to eight (n = 6-8).

290   Two-way analysis of variance (ANOVA II) was used to test the effects of both SS and SED and

291   their content in the soil on plant biochemical parameters (i.e. protein concentration, APx, CAT,

292   TBARS and chlorophyll concentration as well as chlorophyll a/b ratio). Before this, compliance

293   with ANOVAs assumptions i.e. the normal distribution and the homoscedicity of variance had

294   previously been checked by the Shapiro-Wilk test and Levene's test, respectively. If the analysis

295   of variance showed a significant effect of any of the factors, a multiple-comparison least

296   significant difference (LSD Fisher) *post hoc* test was used (Zar, 2010). All analyses were

297   performed using STATISTICA 13 software (Dell Inc., 2016).

298

299   **Results**

300   In general, the PCB concentration (average ± SD) in soil amended with SS (0.931 ± 0.284) and

301   in the soil enriched using SED (0.898 ± 0.245) did not differ significantly (t = 0.6226; df = 8; p =

302   0.5436). In both types of soil amendments, the PCB concentration was related to SS/SED dose;

303   the equations describing these linear relationships were as follows: PCB = 0.7043 + 0.0505 ×

304   amendments dose (r^2 = 0.6139; p = 0.0214) for SS; PCB = 0.6206+ 0.0507 × amendments dose

305   (r^2 = 0.8361; p = 0.0015) for SED. Covariance analysis revealed a lack of significant differences

306   in b (slope) coefficients (t = 0.01483; df = 12; p = 0.9883) and a (intercept) coefficients (t =

307   1.1113; df = 13; p = 0.2865). After five weeks of cultivation, the mean reduction of PCB was

308   38.47% for the SS-treated soil and 27.62% for the SED-treated soil (Fig. 1.). However, two-way

309   analysis of variance (repeated measures ANOVA II) showed that the reduction of PCB content in

310   soil was significant (F = 22.1443; df = 1, 8; p = 0.0015) and did not depend on the type of

311   amendments used (F = 0.2091; df = 1, 8; p = 0.6596) but confirmed the strong effect of the

312   SS/SED dose (F = 10.1274; df = 3, 8; p = 0.0042) (Fig. 2a; Fig. 2b). Moreover, there were no

313   types of significant interactions between analyzed factors (Table 2). *Post hoc* comparisons (the

314   LSD test) revealed significant reductions of PCB concentration for all doses of SS (Fig. 2a) but

315   no significant effect of remediation was observed in the case of SED (Fig. 2b).

316   Changes in soluble protein content in cucumber leaf tissues were dependent on both the type and

317   the dose of the applied amendment (Fig. 3). Two-way analysis of variance (ANOVA II) showed

318   the effect of the type of amendments used on protein content (F = 15.736; df = 1, 46; p =

319   0.000253) as well as the effect of SS/SED dose in the soil on soluble protein content (F = 9.651;

320   df = 3, 46; p = 0.000047). In the case of SS, the multiple comparisons (LSD Fisher) test revealed

321   significant effect of its dose on the leaf protein content. An increasing trend was observed

322   regarding the soluble protein content in leaf tissues together with used dose: 116% of control

323   value for 1.8, 129% for 5.4 and 136% for 10.8. However, the use of SED did not significantly

324   alter the content of soluble protein in cucumber tissues: the only significant difference was found

325   between the 1.8 and 10.8 SED doses. Moreover, a significant interaction (type of amendment ×

326   SS/SED dose) was also noted (F = 4.340; df = 3, 46; p = 0.00894). The multiple comparisons

327   (LSD Fisher) test showed that this interaction was significant: no differences in leaf protein

328   content were observed between control, low (1.8) and medium (5.4) of SED doses.

329   Two-way analysis of variance (ANOVA II) did not show the influence of the type of

330   amendments used on the total chlorophyll content value (F = 1.342; df = 1, 46; p = 0.2527), nor

331   did it indicate that SS/SED dose in the soil had any effect on the value of total chlorophyll

332   content (F = 0.531; df = 3, 46; p = 0.664) (Table 3).

333   In contrast to total chlorophyll content, the chlorophyll a/b ratio depended significantly on the

334   type of amendments used (F = 12.05; df = 1, 40; p = 0.001257) and the dose of amendments

335   added to the soil (F = 7.77; df = 3, 40; p = 0.000334). The multiple comparisons (LSD Fisher)

336   test showed that the highest dose of both SS and SED used increases the chlorophyll a/b ratio up

337   to 105.7% and to 103.1% of the control value (Table 3). An analysis of both total chlorophyll

338   content and chlorophyll a/b ratio revealed no statistically significant interactions with the type of

339   amendments used and its dose in the soil.

340   Neither SS nor SED enrichment caused any significant change in the TBARS content, which

341   represents an indicator of oxidative lipid damage (Fig. 4). Two-way analysis of variance

342   (ANOVA II) did not show the influence of the type of amendments used on TBARS value (F =
343   0.4392; df = 1, 40; p = 0.511). The analysis also did not confirm that SS/SED dose in the soil
344   influenced TBARS level (F = 2.370; df = 3, 40; p = 0.085). The multiple comparisons (LSD
345   Fisher) test revealed SS only demonstrated a significant effect at the 5.4 dose, where TBARS
346   value achieved 130% of control. There were no statistically significant interactions between the
347   analyzed factors.
348   Two-way analysis of variance (ANOVA II) did not show the influence of the type of
349   amendments used on APx activity, a key antioxidant enzyme in plants (F = 0.2915; df = 1, 40; p
350   = 0.5923). Contrary, dose-dependent changes in the activity of APx were observed after both SS
351   and SED application (F = 13.1692; df = 3, 40; p = 0.000004) (Fig. 5). The multiple comparisons
352   (LSD Fisher) test revealed that SS dose had a significant effect on enzyme activity: APx activity
353   was strongly reduced to 48%, 49% and 45% of control value in the low (1.8), medium (5.4) and
354   high (10.8) doses, respectively. Similar changes were observed after applying SED, where a
355   decrease in APx activity was observed for the medium (5.4) and high (10.8) doses, these being
356   66% and 55% of control values, respectively. There were no statistically significant interactions
357   between the analyzed factors.
358   The activity of CAT, the other enzyme involved in antioxidant defense, depended on the type of
359   soil amendment (F = 119.198; df = 1, 40; p = 0.000000) and SS/SED dose (F = 7.834; df = 3, 40;
360   p = 0.000314). Two-way analysis of variance also showed significant interaction between factors
361   (F = 6.003; df = 4, 40; p = 0.00178). However, the multiple comparisons (LSD Fisher) test
362   revealed that SS dose had a significant effect on CAT activity, but no difference was observed
363   between control and the lowest dose (1.8) and between medium (5.4) and high (10.8) doses; in
364   addition, SED dose did not significantly affect enzyme activity (Fig. 6).
365

366   **Discussion**
367   The most important task of phytoremediation is to reduce the levels of toxic substances in the
368   water, sediment, soil or air in a particular environment. Many plants can be used for
369   phytoremediation; however, their physiological condition will be affected to varying degrees by
370   the toxicity of the environment, and this in turn will influence their removal efficiency. With this
371   in mind, the members of the Cucurbitaceae are particularly good candidates for the

372   phytoremediation of PCBs from the substrate (Mattina, Iannucci-Berger & Dykas, 2000; White

373   et al., 2002).

374   Despite being banned in the 1970s because of their negative impact on the environment and

375   human health, PCBs are highly persistent and require removal from the environment. Of the

376   various PCB reclamation methods available, such as incineration and landfilling, a cost-effective

377   and environmentally-friendly approach is phytoextraction (Low et al., 2010). The ability of

378   plants to remediate PCBs from the soil is influenced by the properties of the mixture of PCB

379   congeners contained in the substrate and the possibility of their uptake by plants. It has been

380   shown that the degree of chlorination, log K_{ow} and molecular size are the most important factors

381   determining the possibility of absorption and transport for PCBs and other hydrophobic POPs

382   (Greenwood, Rutter & Zeeb, 2011).

383   Our present findings indicate that irrespective of whether SS or SED was added to the soil, the

384   PCB concentration in the amended soil at the beginning of experiment was similar and

385   dependent on the dose of applied amendments. In addition, after five weeks of cucumber

386   cultivation, lower PCB levels were observed in all soil samples; however, this effect was greater

387   in the SS amended soil, where as much as a 41% decrease in PCB concentration was found

388   compared to the initial value. These values suggest a stronger reduction in PCB content than

389   noted in previous studies. For example, Qin, Brookes & Xu (2014) report that 60-day cultivation

390   of cucumber plants reduced the PCB content by 19.5% compared to unplanted soil. The authors

391   suggest that one of the important factors determining the effectiveness of phytoremediation may

392   be the presence and composition of microbial communities responsible for PCB degradation.

393   The rhizosphere is a space in which root exudates are released to the soil, and these may

394   selectively foster the growth of PCB-degrading bacteria. The effectiveness of PCB remediation

395   may, therefore, not only be conditioned by the species, or variety of the plant involved in

396   phytoremediation, but also by the composition and origin of the sediments containing PCBs. As

397   the SS and the SED used in the present study most likely possess different microbial

398   compositions, it is not surprising that they were associated with different degrees of PCB

399   reduction.

400   Differences between the type of applied amendment (SS or SED), influencing the degree of

401   decrease of PCB concentration in the studied soil (revealed by the LSD test), may indicate that

402   the ability to remediate PCBs from amended soil by cucumber plants may depend on the origin

403   of the amendments used, and hence on the composition of the soil. It can not be ruled out that the

404   ability to remediate PCBs may depend on the presence of other toxic substances in the substrate

405   that can cause adverse effects on plants. A study of the remediation potential of zucchini

406   (*Cucurbita pepo* L.) in soil samples treated with PCDD/PCDF-contaminated SS or SED found

407   the PCDD/PCDF content to be up to 2.7 times greater in the soil treated with SS than in that

408   treated with SED (Urbaniak et al., 2016). However, the presence of these compounds in the SS-

409   treated soil did not reduce its level of phytoremediation; in fact, the level was even greater than

410   in the SED soil.

411   While the cucumber plants treated with SS demonstrated gradual increases in total soluble

412   protein content with increasing doses of the applied sludge, no significant changes were found in

413   plants growing on soil with SED. An elevated total protein content may indicate that the

414   presence of SS has a beneficial effect on the physiological condition of the plant. From the

415   physiological point of view, it is known that the soluble protein content in plants decreases

416   during the course of senescence (Camp et al., 1982; Romanova et al., 2016). This is related to the

417   initiation of the N-remobilization process, during which proteinase activity increases, leading to

418   the degradation of proteins to peptides or amino acids, which are then transported to the growing

419   organs in the phloem sap (Girondé et al., 2015). Hence, the increase in soluble protein content

420   observed in cucumber tissues growing in SS amended soil may indicate a delay of the processes

421   of senescence. Protein content in plant tissues may also increase due to abiotic stresses such as

422   heat or drought (Grigorova et al., 2011). In the case of cucumber plants studied in this work, the

423   thesis about increasing the protein content resulting from stress seems unlikely, if only due to the

424   lack of a significant increase in TBARS content in plants treated with SS and SED, which is a

425   measure of oxidative damage of lipids as well as due to the lack of lowering total chlorophyll

426   content in leaf tissues.

427   This increase in total soluble protein content in the leaf tissues of cucumber cultivated on soil

428   treated with SS, combined with the apparent lack of influence of SS and SED contamination on

429   total chlorophyll content, i.e. no decrease was observed, may be another indicator that the

430   examined plants are in good physiological condition and the applied amendments may have a

431   strong fertilizing effects. The results of both the total protein and chlorophyll content obtained in

432   this study show that senescence progresses faster in control plants than in plants fertilized with

433   SS as well as the use of SED also do not intensify the processes associated with senescence.

434   The physiological condition of the plant can also be evaluated my measuring chlorophyll a/b
435   ratio. It was found that chlorophyll a/b ratio increased slightly in the plants treated with the
436   highest dose (10.8) of SS, as well as with SED, in comparison with control. As mentioned
437   earlier, total chlorophyll content together with chlorophyll a/b ratio may be indicators of the
438   initiation of senescence process in plants and gradual changes in the biochemical processes
439   associated with them. A decrease in leaf chlorophyll content is one of the first symptoms of plant
440   senescence, which is characterized by the breakdown of thylakoid membranes and the
441   degradation of thylakoid-bound proteins. The presence of the aging process is further confirmed
442   by a depressed chlorophyll a/b ratio. For example, in a study of *Arabidopsis thaliana* plants, the
443   chlorophyll a/b ratio was found to decrease linearly with natural leaf senescence, as did total
444   chlorophyll content; differential degradation of chlorophyll a and chlorophyll b was also
445   observed during senescence, resulting in changes in chlorophyll a/b ratio (Nath et al., 2013). In
446   the case of the cucumber plants studied in the present work, it can be assumed that the addition
447   of SS and SED not only acts as a fertilizer, but also serves a protective function against the
448   accelerated aging process. Our findings also highlight another important point: cucumber plants
449   may well be resistant to toxic effects of substances contained in SS.
450   TBARS content is a commonly-used indicator of oxidative stress following environmental stress
451   (Srivastava, Sinha & Sharma, 2017). In the present study, both after applying SS and SED, there
452   were no changes in TBARS content in cucumber leaves that could indicate oxidative damage of
453   lipids. The only exception is the increase in the value of this parameter (up to 130% of control)
454   after application of SS at the medium (5.4) dose. Page et al. (2014) report lower application rates
455   of compost-like-output result in nitrate leaching, and note that this could be a limiting factor in
456   the use of sludge as a nutrient source. It is also possible that the advantages of sludge application
457   associated with nutrient content are outweighed by the presence of toxic compounds when
458   applied at low doses: Higher doses of sludge are richer in nutrients, and this compensates for the
459   effects of the presence of harmful substances. Similarly, SS was found to have a protective effect
460   on alfalfa plants (*Medicago sativa* L. cv. Aragon) subjected to additional drought stress: Its
461   addition led to a significant reduction malondialdehyde in nodules resulting from lipid
462   peroxidation, while this number was strongly increased in the nodules of untreated plants
463   subjected to drought (Antolin, Muro & Sanchez-Diaz, 2010). A similar beneficial result was
464   obtained by Tartoura & Youssef (2011) regarding the use of compost for the cultivation of

465   squash (*Cucurbita pepo* L. cv. Eskandarany) exposed to low temperature conditions, with both

466   oxidative damage of lipids and ROS generation being markedly reduced following compost

467   application.

468   Our present findings also indicate differences in the plant response to the use of SS and SED.

469   Although the soil PCB concentrations at the beginning of the experiment did not differ

470   significantly and depended only on the dose of the amendments used, the response of plants

471   growing on SS and SED-amended soil varies. It is very likely that such a situation would be

472   attributed to the composition of soil after addition of SS or SED. It can not be ruled out that SS is

473   more toxic than SED and the two can vary to a great degree regarding their remaining content.

474   Earlier studies carried out on willow plants indicate that the composition of SS and its impact on

475   plants is dependent on the size of the sewage treatment plant (Wyrwicka & Urbaniak, 2018). For

476   example, unlike the sludge produced by large, industrialized urban centers, like that used in the

477   present work, sludge from plants operating in small agglomerations does not typically contain

478   large amounts of substances harmful to plants, such as heavy metals.

479   APx is an enzyme characteristic of plant tissues. It is characterized by high substrate specificity

480   and is sensitive to substrate concentration; during its absence in the surrounding solution, the

481   enzyme is inhibited. This enzyme may also be inactivated by H_2O_2. It is also an important

482   element of the ascorbate-glutathione cycle, which maintains the reduction-oxidation balance in

483   plant cells (Asada, 1992). Of the parameters measured in the present study, APx was found to

484   display the greatest changes in activity, with decreases being observed both after applying SS

485   and SED. In the case of SS, the lowest applied dose (1.8) already inhibited the enzyme activity

486   (up to 48% of control), whereas after the SED application, the APx activity decreased only when

487   the medium (5.4) and high (10.8) doses were used. This may suggest that both SS and SED may

488   contain compounds that inhibit APx activity and it cannot be ruled out that H_2O_2 would

489   accumulate in the cells under these conditions. On the other hand, while APx activity was

490   significantly reduced, an increase in CAT activity was observed in the plant tissues exposed to

491   the same dose (5.4 and 10.8) of SS present in soil. It is possible that the increase in CAT activity

492   has a compensatory effect on inhibited APx activity in investigated cucumber tissues.

493   Furthermore, CAT activity, whose primary function it is to remove H_2O_2 from cells, in tobacco

494   cell suspension culture exposed to PCDD/Fs showed an upward trend with increasing PCDD/Fs

495   concentration (Zhang et al., 2012).

496   The decrease in APx activity observed herein may also be attributed to a lack of ascorbate, its

497   substrate, which may have been consumed elsewhere by non-enzymatic antioxidative reactions.

498   Our observations regarding APx activity and the total protein content can be compared with the

499   those of Aki, Güneysu & Acar (2009), who studied the reaction of four plant species, *viz.* tomato,

500   pepper, bean and broad bean, on industrial wastewater. Total protein content increased in all of

501   the tested plants during the experiment, while peroxidase activity decreased following exposure.

502   The elevated CAT activity observed in the present study after the application of the medium

503   (5.4) and high (10.8) doses of SS could be result of an efficiently functioning antioxidative

504   system in the cucumber tissues: It can be assumed that increased CAT activity protected the

505   tissues of the investigated cucumber plants from oxidative damage, which can be proved only by

506   a small increase in the TBARS content in one of the applied concentration variants (5.4). As it

507   appears from the results obtained, after using a higher dose (10.8) of SS, elevated CAT activity

508   was probably effective in protecting plant tissues from the effects of oxidative stress, because in

509   any of the variants elevated TBARS content was found. It cannot be ruled out that changes in

510   CAT activity after SS are related to the size of the dose of amendment. A previous study on

511   wheat (*Triticum durum*) exposed to different doses of SS found CAT activity in the leaves to be

512   unaffected when the sludge was applied in small doses, but significantly elevated at higher doses

513   (Lakhdar et al., 2010).

514   The increase in total protein content and chlorophyll a/b ratio observed in the present study, as

515   well as the changes in CAT activity and lack of the increase of TBARS content (found only in

516   one case), indicate that the plants fertilized with SS fared better than the unfertilized controls,

517   and this suggests that cucumber plants are well suited to phytoremediation of PCBs. Our results

518   suggest that cucumber plants are able to trigger a defensive response based on the action of the

519   antioxidant system, such as CAT activity, that allows them to survive adverse environmental

520   conditions and resist the effects of toxicity.

521

522   Conclusions

523   Our findings indicate that the cucumber is perfectly suitable for reducing PCB content in soil

524   treated with SS or SED in a relatively short time, while appearing relatively resistant to the toxic

525   substances present in the applied amendment. The data concerning total protein content, total

526   chlorophyll content and chlorophyll a/b ratio indicates that plants treated with SS or SED fared

527 better than untreated controls, with the greatest response displayed by those treated with SS. The
528 cucumber plants treated with SS or the SED possessed an effective, functioning antioxidative
529 system which protected the plants against the occurrence of oxidative damage. Therefore,
530 cucumber plants are ideal candidates for the phytoremediation of this type of soil pollution.
531 However, further studies are needed to integrate the knowledge regarding the mechanism of PCB
532 action and activation of plant protective systems, as well as the interdependence between the
533 plants and microbes responsible for PCB degradation, in order to improve the efficiency of the
534 phytoremediation process and adapt it to practical applications.

535

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715

The average PCB concentration (average ± SD) in the soil fertilized with SS from LM WWTP (green bars) and SED from SSBS (blue bars), before and after five weeks of cultivation

Fig. 1
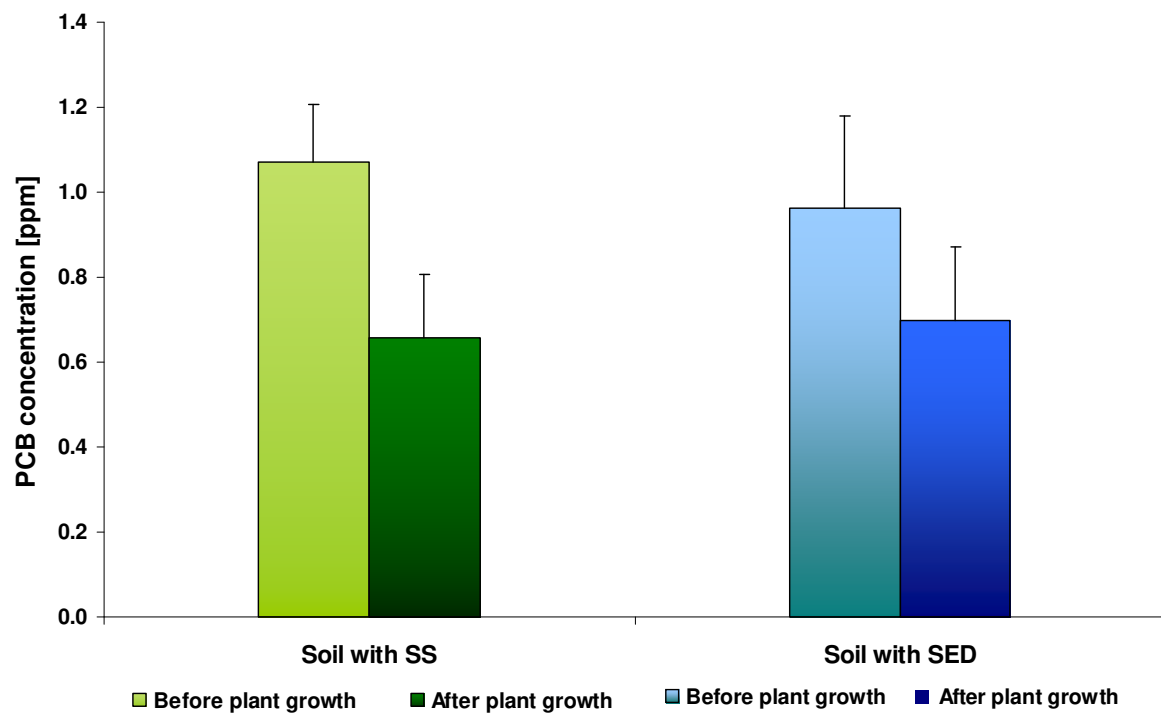
Fig. 1. The average PCB concentration (average ± SD) in the soil fertilized with SS from LM WWTP (green bars) and SED from SSBS (blue bars), before and after five weeks of cultivation.

The PCBs concentration (average ± 95% CL) in the soil fertilized with SS from LM WWTP (green bars, Fig. 2a) and SED from SSBS (blue bars, Fig. 2b), before and after five weeks of cultivation.

Asterisks denote a significant difference at the 0.05 (the LSD Fisher *post hoc* test)

Fig. 2a
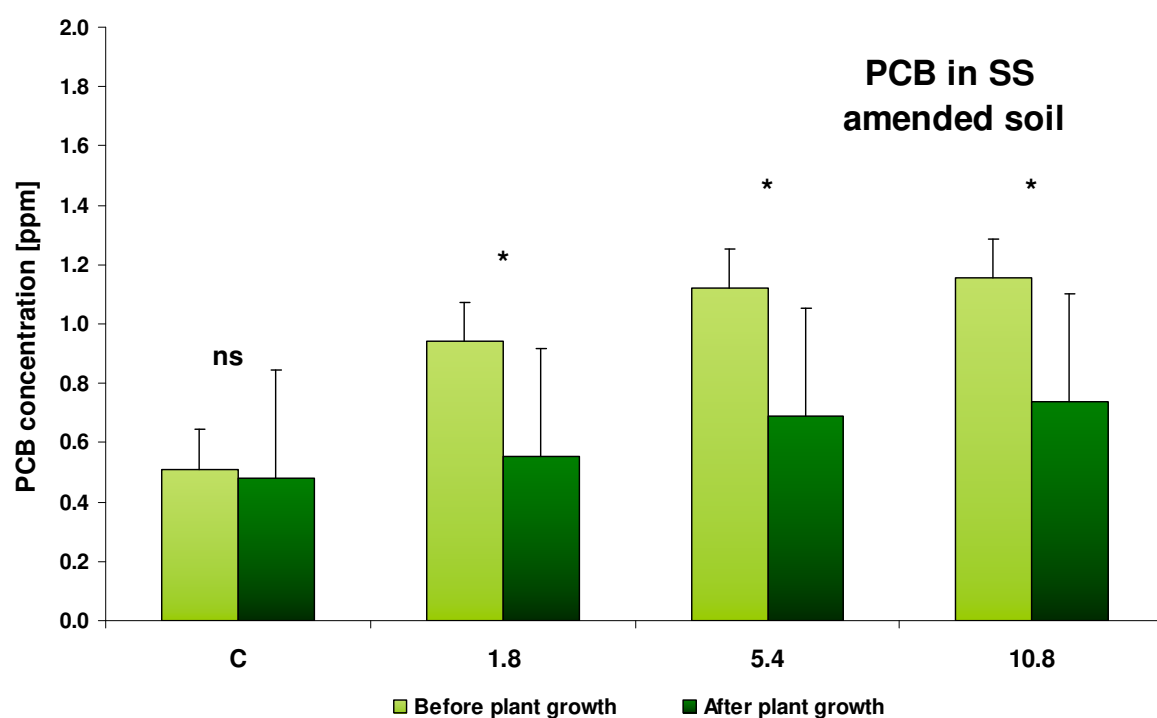
Fig. 2b
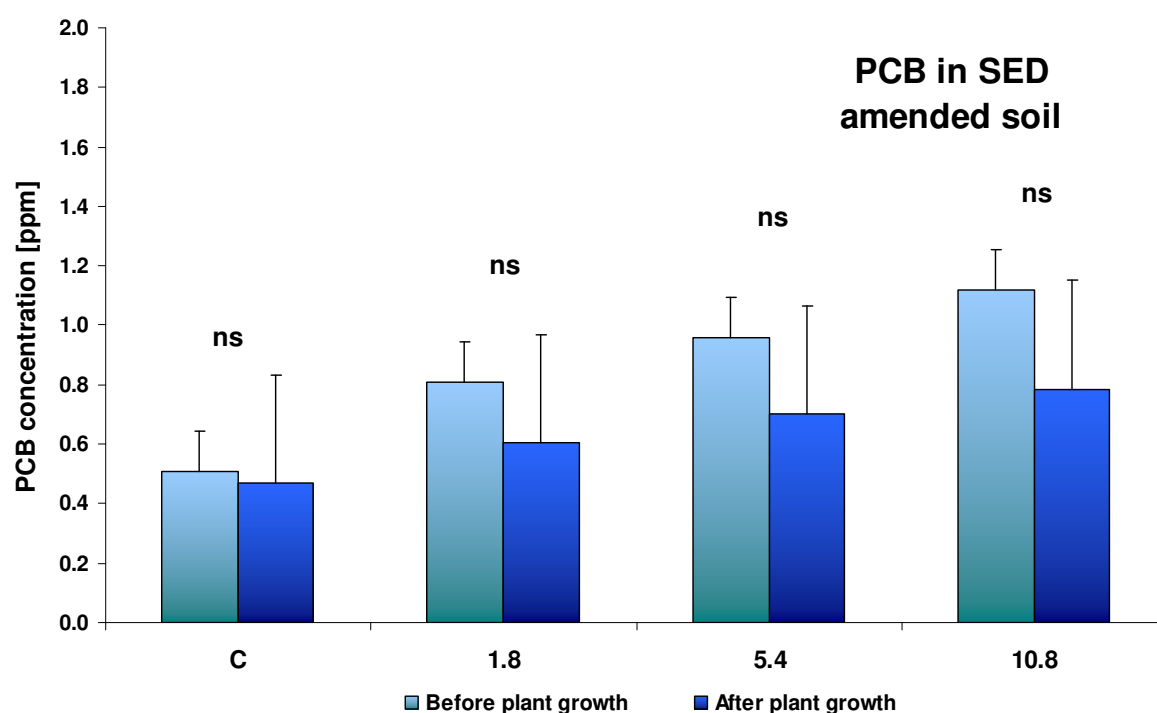
Fig. 2. The PCBs concentration (average ± 95% CL) in the soil fertilized with SS from LM WWTP (green bars, Fig. 2a) and SED from SSBS (blue bars, Fig. 2b), before and after five weeks of cultivation. Asterisks denote a significant difference at the 0.05 level and 'ns' when p > 0.05 (the LSD Fisher *post hoc* test).

Soluble protein content in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) at different doses after five weeks of cultivation

The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test)

Fig. 3
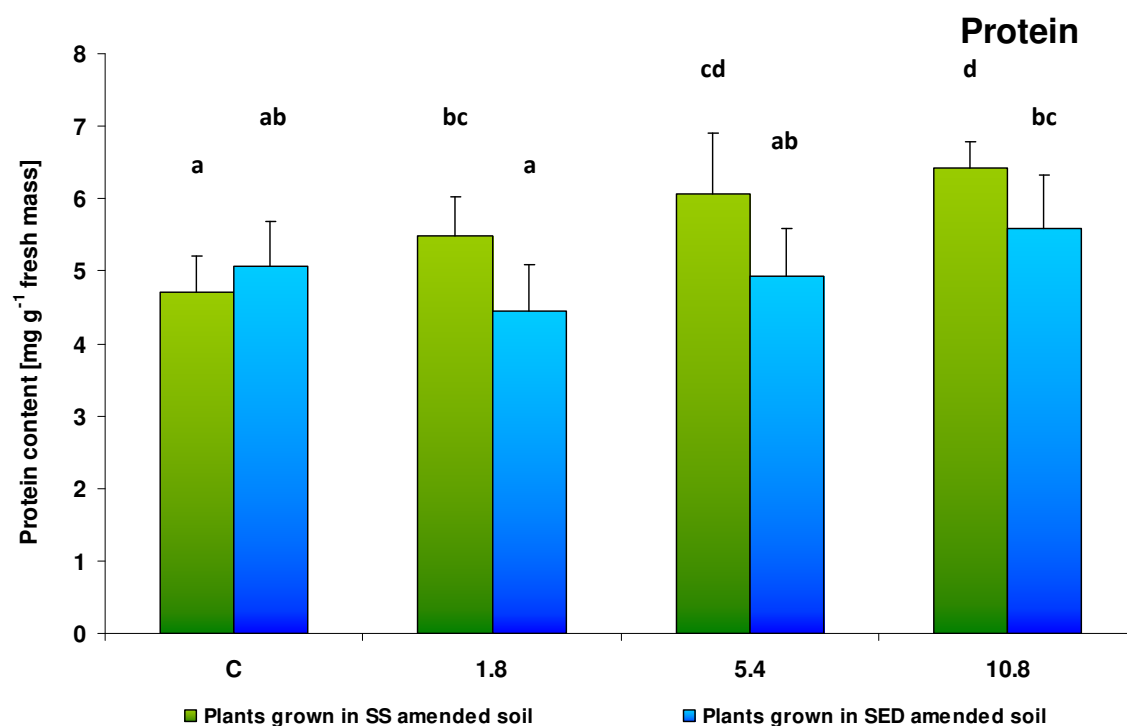
Fig. 3. Soluble protein content in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) at different doses after five weeks of cultivation. The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test).

TBARS content in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) used in different doses after five weeks of cultivation.

The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test.
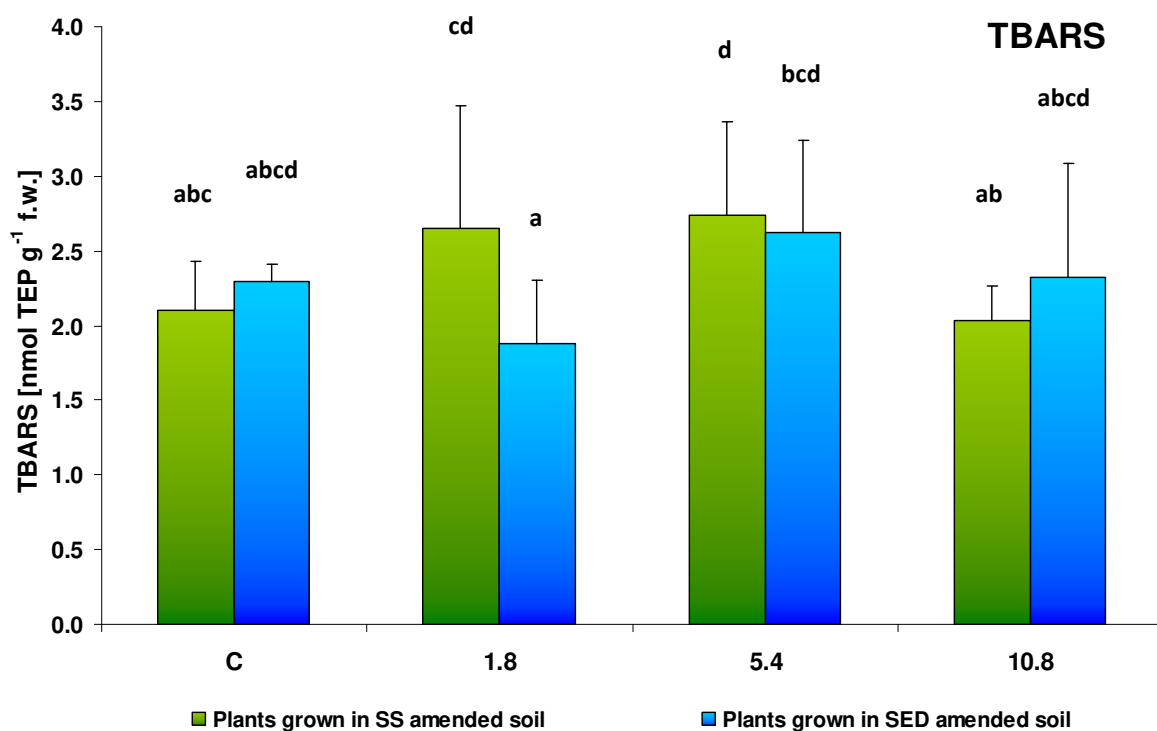
Fig. 4. TBARS content in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) used in different doses after five weeks of cultivation. The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test).

APx activity in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) used at various doses after five weeks of cultivation.

The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test.
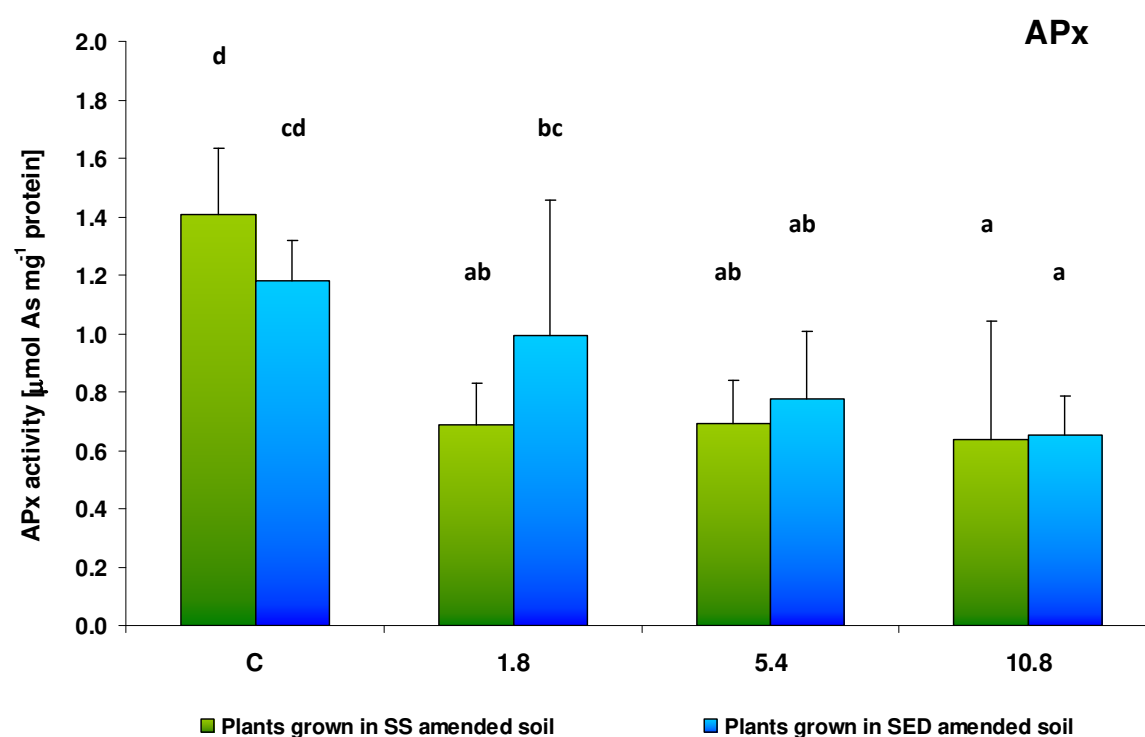
Fig. 5

Fig. 5. APx activity in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) used at various doses after five weeks of cultivation. The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test).

CAT activity in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) used in different doses after five weeks of cultivation.

The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test.

Fig. 6
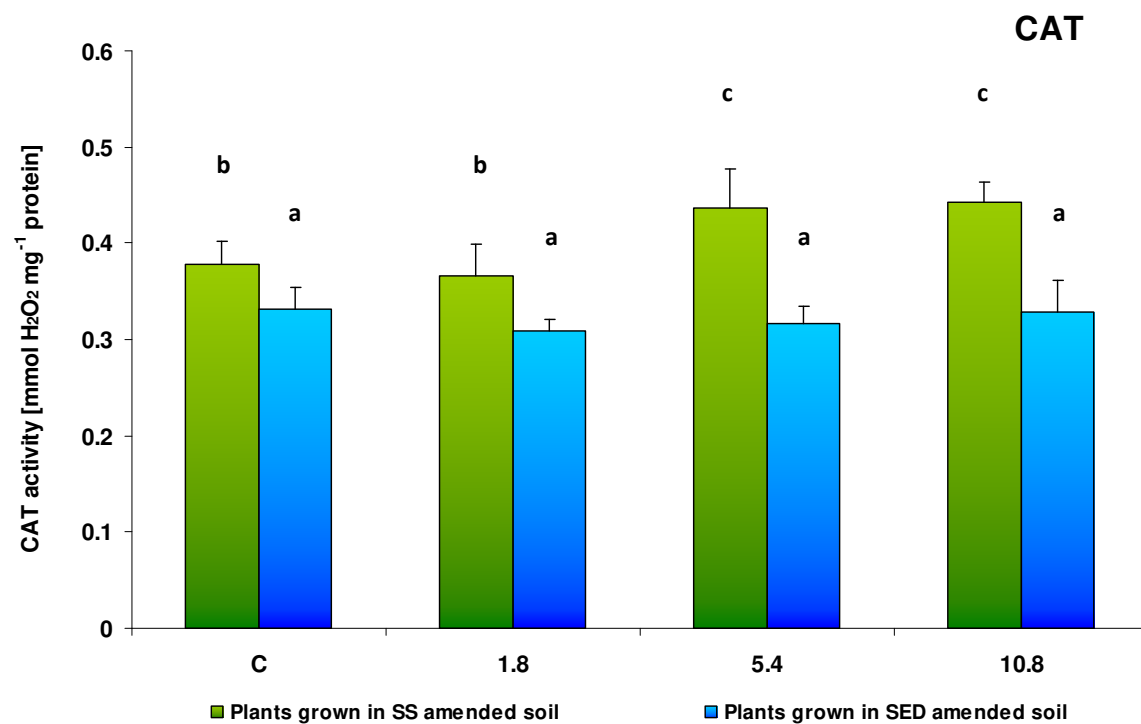
Fig. 6. CAT activity in control cucumber leaves and in leaves of plants grown on soil amended with SS from LM WWTP (green bars) or SED from SSBS (blue bars) used in different doses after five weeks of cultivation. The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test).

Physico-chemical properties of the untreated and soil amended with SS at the dose of 9 t · ha^{-1}

1 **Table 1.** Physico-chemical properties of the untreated and soil amended with SS at the dose of 9 t · ha^{-1}.

Properties	Unit	Control soil	Soil mixed with 9 t·ha^{-1} of SS
pH	-	6.02	6.4
total organic carbon	g · kg^{-1}	15.18	22.67
total nitrogen	%	0.52	0.66
N-NO$_3$	mg · kg^{-1} d.w.	782	859
P	mg · kg^{-1} d.w.	437	728
K	mg · kg^{-1} d.w.	2341	2809

Two-way analysis of variance (repeated measures ANOVA II) results to test the effects of the dose of applied amendments (0, 1.8, 5.4 and 10.8), the type of soil amendments (SS and SED) on decreases in soil PCB content after five weeks of cultivation.
1   **Table 2.** Two-way analysis of variance (repeated measures ANOVA II) results to test

2   the effects of the dose of applied amendments (0, 1.8, 5.4 and 10.8), the type of soil

3   amendments (SS and SED) on decreases in soil PCB content after five weeks of

4   cultivation.

Effect	df	MS	F	p
Dose of applied amendments (A)	3	0.3203	10.1294	0.0042
Soil amendment type (B)	1	0.0066	0.2091	0.6596
A×B	3	0.0027	0.0862	0.9656
Error	8	0.0316		
PCB decrease (C)	1	0.5544	22.1443	0.0015
C × A	3	0.0481	1.9198	0.2049
C × B	1	0.0231	0.9232	0.3648
C × B × A	3	0.0040	0.1580	0.9216
Error	8	0.0250		

The effect of the application of SS from LM WWTP and SED from SSBS on total chlorophyll content, as well as chlorophyll a/b ratio (both average ± SD) of cucumber leaf tissues after five weeks of cultivation.

The same letters denote groups that did not statistically differ (the LSD Fisher *post hoc* test).

1 **Table 3.** The effect of the application of SS from LM WWTP and SED from SSBS on
2 total chlorophyll content, as well as chlorophyll a/b ratio (both average ± SD) of
3 cucumber leaf tissues after five weeks of cultivation. The same letters denote groups
4 that did not statistically differ (the LSD Fisher *post hoc* test).

	Dose of applied amendments	Soil with	
		SS	SED
Total chlorophyll [mg g^{-1} f.m.]	C	2.380^{ab} ± 0.233	2.467^{ab} ± 0.481
	1.8	2.495^{ab} ± 0.240	2.485^{ab} ± 0.096
	5.4	2.624^{ab} ± 0.447	2.315^{a} ± 0.123
	10.8	2.656^{b} ± 0.242	2.491^{ab} ± 0.397
Chlorophyll a/b ratio	C	2.083^{a} ± 0.065	2.023^{b} ± 0.019
	1.8	2.082^{a} ± 0.024	2.067^{ab} ± 0.020
	5.4	2.075^{ab} ± 0.036	2.062^{ab} ± 0.036
	10.8	2.201^{c} ± 0.093	2.087^{a} ± 0.055