Evaluation of anaerobic digestates from sewage sludge as a potential solution for improvement of soil fertility

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Title

EVALUATION OF ANAEROBIC DIGESTATES FROM SEWAGE SLUDGE AS A POTENTIAL SOLUTION FOR IMPROVEMENT OF SOIL FERTILITY

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Declaration of Interest

Declaration of interest: none.

Abstract

Sewage sludge production in European countries has widely raised in the last decade and its fate is currently landfilling, incinerators, composting or land application. To explore its agronomic potential, the main target of this work is to understand the effects of anaerobic digestates from sewage sludge (SSAD). To this aim, four different SSADs (two liquids and two dewatered) were characterized. On the liquid ones, Germination Index was evaluated through a plate bioassay with *Lepidium sativum* L. seeds; low concentrations of SSAD (2.5%) improved GI in one case, while at higher concentrations phytotoxic effects occurred in both. Then, pot experiments were set in climate chamber with *Cucumis sativus* L. grown for 30 days on two different substrates: a sandy, alkaline and poor soil, and peat substrate. All SSADs and a mineral fertilizer were used at three increasing dosages: 85, 170, 255 kg of nitrogen per hectare (kg N/ha). Results in terms of germination, dry biomass, chlorophyll content, net photosynthesis, stomatal conductance, CO_2 concentration in substomatal cavity and root development were compared to a not treated control.

All treatments gave results significantly higher or similar to control on all the parameters evaluated. Moreover, the intermediate nitrogen dosage (170 kg N/ha) generally showed the highest results compared to other dosages, especially for dewatered SSADs. All these results were much more evident for cucumber plants grown on an the alkaline, sandy and poor soil than on peat substrate, such demonstrating that SSADs have a fertilizing effect for plants growing on this kind of soil.

Keywords: soil improver; nutrient-deficient soil; circular economy; pot experiment; climate chamber; nitrogen content.

1. Introduction

The treatment of wastewater is a process dealing with different issues, such as the engineering of innovative purification techniques, the environmental impact assessment and the effects on society and the economy (EEA, 2016). In the last thirty years in Europe, many institutions and authorities have devoted particular attention to this topic and this aspect has been sustained by European Union through the adoption of directives, e.g. Directive 91/271/EEC E.U. (European Council Directive, 1991a), which have been transposed to national laws by the different member states, such as Italian Decree Law 152/1999 and Italian Decree Law 152/2006. As a result, the quality of treated water received by groundwater bodies has improved substantially. However, this implied a considerable drawback: the dramatic increase of sewage sludge (SS), that is the principal waste coming from wastewater treatment process. The sewage sludge production of European countries raised from 5.5 (European Commission Web Sources) to nearly 10 Mtonnes of dry solid matter of sludge per year (Milieu Ltd, 2008), divided in 8.7 Mtonnes from EU-15 countries and 1.2 Mtonnes from EU-12 (Bianchini et al., 2016). According to the reports of European Commission (European Commission, 2017), these values correspond to a mean production of 17 kg per capita of dry sludge per year, but in some countries, including Italy, this ratio is below to 10 kg per inhabitant, suggesting an insufficient level of collection and treatment. SS is currently classified as a putrescible waste, which requires a proper stabilisation before its disposal or reuse. For this purpose, anaerobic digestion (AD) is one of the most exploited techniques in last generation wastewater treatment plants (WWTP), yielding biogas and anaerobic digestate from sewage sludge (SSAD). This one shows the typical pros and cons of sewage sludge: interesting agronomic features due to appreciable macronutrient content versus

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Abbreviations: A_{N}: assimilation; C: centrifuged digestate; C-85: centrifuged digestate at 85 kg N/ha; C-170: centrifuged digestate at 170 kg N/ha; C: centrifuged digestate; C-255: centrifuged digestate at 255 kg N/ha; CCI: Chlorophyll Content Index; C_i: CO_2 concentration in substomatal cavity; D: dried digestate; D-85: dried digestate at 85 kg N/ha; D-170: dried digestate at 170 kg N/ha; D-255: dried digestate at 255 kg N/ha; EC50: half maximal effective concentration; GI: Germination Index; g_s: stomatal conductance; M: mineral fertilizer; M-85: mineral fertilizer at 85 kg N/ha; M-170: mineral fertilizer at 170 kg N/ha; M-255: mineral fertilizer at 255 kg N/ha; P: primary digestate; P-85: primary digestate at 85 kg N/ha; P-170: primary digestate at 170 kg N/ha; P-255: primary digestate at 255 kg N/ha; RDI: Root Development Index; S: secondary digestate; S-85: secondary digestate at 85 kg N/ha; S-170: secondary digestate at 170 kg N/ha; S-255: secondary digestate at 255 kg N/ha; SS: sewage sludge; SSAD: anaerobic digestate from sewage sludge; T: no treated, control thesis; WWTP: wastewater treatment plant.

the presence of organic and inorganic contaminants (Antonkiewicz et al., 2018). Land and agronomic application as soil improver is currently regulated in Europe by an obsolete directive, Council Directive 86/278/EEC (European Council Directive, 1986), and, until 2015, it was mostly diffused in countries like Portugal and Spain, which adopted more stringent legislation for exploitation in agriculture (Alvarenga et al., 2015). However, according to Eurostat data updated on 2015, in other states SSAD is poorly reused and it is mostly disposed of as a solid waste through incineration (e.g. in the Netherlands and Switzerland) or landfilling (Italy, Serbia, Croatia; source: Eurostat, 2017). This solution, in particular, should be strictly limited whenever possible and exploited only as last resort, according to the Landfill Council Directive 1999/31/EC (European Council Directive, 1999).

Many works in literature have focused on this waste in order to propose different kinds of solutions both in terms of treatment and application. Contaminants removal has been shown to be possible through heavy metal leaching with physical, chemical and biological techniques (Camargo et al., 2016). However, these procedures are often expensive in terms of cost, time and sustainability. On the other hand, recovery of valuable compounds such as nitrogen (e.g. ammonia stripping, struvite precipitation or advanced processes) (Monfet et al., 2018; Siciliano et al., 2017) and phosphorus (e.g. recovery from ashes, struvite precipitation) (Ohtake and Tsuneda, 2019) seems to be quite promising, especially for what concerns the preservation of natural resources and the support of the circular economy perspective (Nesme and Withers, 2016). Other engineering approaches are based on pyrolysis, for production of syngas, bio-oil and biochar (Schulz and Glaser, 2012), or composting (Perez-Murcia et al., 2006; Xu et al., 2012) to yield soil organic conditioners. Besides these studies, many papers on the soil application of the sewage sludge (McGrath et al., 1995) and its derivatives (Andrés et al., 2011; Tarrasón et al., 2008) have also been published: in general, the main focus has been the evaluation of fertilising effects depending on the dose, species and soil studied, with experiments scaling from pots in greenhouses (Wong et al., 1996; Perez-Murcia et al., 2006) to pots in outdoor (Singh and Agrawal, 2009; Alvarenga et al., 2016), to open field applications (Hussein, 2009; Rigueiro-Rodríguez et al., 2010). On the other hand, many works have investigated the phytotoxic effects of SS and SSAD, which are principally related to the excess of heavy metals (Singh and Agrawal, 2007; Belhaj et al., 2016), organic pollutants (Erhardt and Prüeß, 2001) and ammonia nitrogen (Gulyás et al., 2012).

The targets of the present work are mainly three: 1) understanding the applicability of anaerobic digestates from sewage sludge with an agronomic approach, exploiting nitrogen dosages commonly used in field operations; 2) evaluation of their effects on sandy and poor soils; 3) studying their phytotoxicity on cress seeds (plate bioassay with *Lepidium sativum* L.) and on cucumber plants (pot experiment with *Cucumis sativus* L.) to assess the correct range for their application as soil improvers.

The main outcome of this study is thus the understanding of the reuse dynamics of this waste of our society, which is increasing more and more over years. The use of SSAD as soil improver is already known in literature (Alvarenga et al., 2016). Nevertheless, to the best of our knowledge, this is the first example of comparison of the fertilizing effects between two liquid SSADs (derived from separated anaerobic digestion of primary and secondary sludges) and two dewatered ones (centrifuged and dried SSADs) deriving from the same WWTP. Moreover, another aspect of novelty is the study of physiological parameters of cucumber plants grown in presence of different type of SSADs. with particular focus on physiological parameters.

Finally, the results of this work highlight the benefits that may derive from the application of SSADs on nutrient-deficient soils. To a brother extent, this approach could be not only a way to recycle SSAD, but also represent a potential solution to combat nutrient depletion in soils.

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2. Materials and methods

2.1 Characterization of the digestates

Anaerobic digestates used in this study came from a large-scale wastewater treatment plant (3,800,000 population equivalents) located in north-west Italy. Four different types of digestates were used: a primary liquid digestate (P), a secondary liquid digestate (S), a centrifuged solid digestate (C) derived from a mix between P and S and a dried pulverulent digestate (D), obtained by the thermal treatment at 200 °C of C. After the sampling from WWTP, digestates were stored at 4°C until use and characterized. All analyses were performed according to "Analytical Methods for Fertilizers" by the Italian Minister of Agriculture and Forestry (M.P.A.A.F. ed., 2006) and "Methods for Analysis of Sewage Sludge" by Water Research Institute of National Council of Researches (IRSA-CNR, 1985). Moreover, all analyses were performed in a laboratory meeting the requirements imposed by normative on quality management systems applied to laboratories of analysis and testing (UNI EN ISO 9001: 2008 e UNI EN ISO/IEC 17025: 2005).

2.2. Germination test on cress

The protocol of DIVAPRA et al. (1998) was used to evaluate effects on germination of liquid separates of P and S digestates which were obtained by centrifugation (15 minutes, 4000 rpm). The same tests were not conducted on C and D since it was not possible to follow the same experimental procedure, neither to obtain a proper aqueous extract due to excessive water soaking by the solid digestates.

The supernatant was used at 10 different concentrations (2.5, 5, 7.5, 10, 15, 20, 25, 50, 75, 100 %) obtained by dilution of pure supernatant (100%) in distilled water. A control with pure distilled water was prepared to compare results. Per each concentration, four replicates were set as follow: one Whatman n°1 filter paper was placed in a sterile plastic Petri dish (Ø 90 mm) where afterwards 5 ml of the abovementioned solutions were poured. At the same time, common cress (*Lepidium sativum* L.) seeds (Green Paradise Srl, Italy) were sterilized in sodium hypochlorite for 30 seconds and then rinsed abundantly with deionized water. Afterwards, seeds were incubated for 1 hour in deionized water to reach an adequate imbibition; before use, they were examined and selected, discarding discoloured, damaged or abnormally small ones (Pavel et al., 2013). Finally, each Petri dish was firstly sown with ten seeds and secondly sealed with parafilm. Plates were incubated for 72 hours at 25°C in absence of light. After 72 hours roots length (as root + hypocotyl + epicotyl) was measured (Lencioni et al., 2016) and germination index (GI) was calculated (Zucconi et al., 1981):

$$GI = (Lt * Gt / Lc * Gc) * 100$$

(where *Lt* is the treated seed average root length, *Gt* is the average number of treated germinated seeds, *Lc* is the average root length of control seeds and *Gc* is the average number of germinated seeds in the control). Seeds were considered germinated when emerging roots were longer than seed size (Bae et al., 2014). Finally, also EC50, defined as the concentration value determining a germination reduction of 50% over untreated control, was calculated.

2.3. Pot experiment on cucumber

2.3.1 Substrates

Table 1.

Physical and chemical analysis of the sandy soil and peat substrate used in this work.

C.E.C.: Cation-Exchange Capacity; AAS: Atomic Absorption Spectroscopy.

Parameter	Unit of measure	Sandy soil		Peat	
		Value	Method*	Value	Method
Stones	-	absent	Method II.1		
Sand (2.0 - 0.020 mm)	%	94%	Method II.6		
Silt (0.020 - 0.002 mm)	%	3%	Method II.6		
Clay (< 0.002 mm)	%	3%	Method II.6		
Texture	-	sandy	Method II.6		
pH	-	8.7	Method III.1	6.2	Potentiometry
Electrical conductivity	mS/cm	0.08	Method IV.1	0.722	Conductometry
Total limestone	%	36.9	Method V.1		
Active limestone	%	1	Method V.2		
Organic matter	%	0.2	Method VII.3		
N - Tot (Kjeldahl)	%	0.021	Method XIV.3	0.42	Kjeldahl
N - NO_2^-	mg/kg	<1.0	Method XIV.9	<0.05	Colorimetry
N - NO_3^-	mg/kg	1.6	Method XIV.9	30.4	Colorimetry
N - NH_4^+	mg/kg	22.3	Method XIV.7	1.3	Colorimetry
N – Org	mg/kg	186	Calculation	4000	Calculation
P	mg/kg	2	Method XV.3	8.1	Colorimetry
Fe	mg/kg	5.6	Method XII.1	0.79	AAS
Mn	mg/kg	4.2	Method XII.1	0.15	AAS
Ca	mg/kg	950	Method XIII.4	36	AAS
Mg	mg/kg	54	Method XIII.4	28	AAS
Na	mg/kg	20	Method XIII.4	16	AAS
K	mg/kg	53	Method XIII.4	41.1	AAS
C.E.C.	meq/100 g	5.48	Method XIII.1		
As	mg/kg	1.9	USEPA 2007b		
Cd	mg/kg	0.1	USEPA 2007b		
Cr	mg/kg	64.5	USEPA 2006		
Hg	mg/kg	<0.1	Direct mercury analyser		
Ni	mg/kg	57.5	USEPA 2007b		
Pb	mg/kg	8.5	USEPA 2007b		
Cu	mg/kg	17	USEPA 2007b	<0.03	AAS
Zn	mg/kg	45.5	USEPA 2007b	0.02	AAS

* Methods for sandy soil analysis are referred to "Italian Ministerial Decree, 1999", unless differently specified.

Two different growth substrates were used: a sandy soil and a peat substrate.

The soil used in this study was sampled in Grugliasco (TO), Italy (45°03'58.4"N, 7°35'32.9"E). It was collected within 20 and 100 cm depth, sieved at 2 mm and not previously sterilized. Physical and chemical soil properties (**Table 1**) were measured before the application of treatments. This soil can be classified as sandy (IUSS, 94% sand, 3% silt, 3% clay), alkaline (pH 8.7), really poor in organic matter (0.2%), high in carbonates (36.9%) but low in terms of active carbonates (1.0%) and with normal salinity (E.C. 0.080 mS/cm).

Physical and chemical analysis were performed according to the methods accepted by Italian law, which are published on Gazzetta Ufficiale n.248 of 21/10/1999 (Italian Ministerial Decree, 1999). Sonneveld method (Sonneveld et al., 2009) was used to obtain an aqueous extract, on which chemical characterization was conducted. Extraction of soil heavy metals was performed with microwave extraction method, according to U.S. Environmental Protection Agency (USEPA, 2007a); determination of heavy metals was executed according to USEPA (see **Table 1** for each method). Detection of Hg was conducted with a direct mercury analyser.

Peat substrate was mixed with perlite and then sterilised before each application. Chemical characterization (**Table 1**) of peat substrate was performed on an aqueous extract 1:2 (v/v water/peat substrate) according to Sonneveld method (Sonneveld and van den Ende, 1971). All the analytical methods exploited are specified in **Table 1.**

2.3.2 Phytotoxicity

The experiment took place in a climate chamber with controllable photoperiod and temperature, which were set at 28°C for 14 hours during the day (07:00 - 21:00) and to 20°C for 10 hours during the night (21:00 - 07:00). During the first week after sowing, shoots were irrigated from the top one time a day; after this time water level in flowerpot saucer was kept constantly between 1 and 3 cm for the purpose of guarantee always water availability.

Commercial plastic pots were used with a total volume of 1250 cm^3 and a surface area of 144 cm^2; consequently, each pot was filled with approximately 250 g of peat substrate and 2000 g of sandy soil. Ten nottreated seeds of cucumber (*Cucumis sativus* L.), cv. Marketmore (Four company, Italy) were sown in each pot. The experimental trials lasted thirty days. The position of all plants in the cell was changed every week to minimize location effects. The cultivations on peat substrate and on sandy soil were performed by using the substrate mixed with different treatments: four types of anaerobic digestates from sewage sludge (P, S, C, D), one commercial fertilizer (M) (NPK 22-5-6 + 2MgO, “Osmocote Topdress”, ICL, Israel) and one not treated control (T). All of them were tested at three increasing doses (85, 170, 255 kg N/ha and they will be called as mentioned above), with four replicates per each. The intermediate nitrogen dosage (170 kg N/ha) was selected according to the Nitrates Directive (European Council Directive, 1991b), and the lowest (85 kg N/ha) and highest (255 kg N/ha) ones were chosen to keep the same difference between the application rates.

2.3.3. Agronomic and physiologic parameters

Germination was evaluated counting germinated seeds after three to ten days; then, germination was calculated as: (germinated seeds/sown seeds) x 100

Assimilation (A_N), stomatal conductance (g_s) and CO_2 concentration in substomatal cavity (C_i) were recorded two days before the end of the experiment using an Infrared Gas Analyzer (IRGA, ADC, Hoddesdon, UK). The measurement was performed on three leaves of each sample treated with the 170 kg/ha dosage. The selected leaves were the second or the third from the top and they were the best developed and directly exposed to artificial light.

The day before the end of the test, Chlorophyll Content Index (CCI) was evaluated with SPAD 502 chlorophyll meter (CCM-200, Opti Sciences, Inc., Hudson, NH, USA). After the ordinary calibration, it was used on 5 different fullyformed leaves per pot. CCI was used as an indicator of the healthy state and the photosynthetic potentiality of plants. With the purpose of evaluating CCI, SPAD (Minolta) and CCM (Opti-Science) meters can be exploited and the second one was utilised in our investigation; to compare values obtained with results of studies that used SPAD-meter, the equations proposed by Parry and colleagues (2014) were considered.

At the end of the experiment, all plants were cut and immediately weighed to measure the fresh biomass of single pots (replicates). Determination of dry biomass was carried out weighing these samples after thermal treatment (105°C for 72 hours). In order to compare the yields of each treatment, dry biomass ratio was calculated as ratio between mean dry biomass of each treatment and control. Besides the related-to-control biomass values, even absolute dry biomasses were analysed and compared. Per each concentration, each treatment was compared to the other ones, including the control.

Root Development Index (RDI) was assigned with a proposed method for the evaluation of root apparatus. This index is based on the soil compactness and cohesion, and on the coverage intensity by the roots over the pot-shaped soil. A score between 0 (no developed) and 4 (very well developed) was given to the apparent root expansion, inspecting the upside-down soil contained in each pot.

2.4 Statistical analysis

For the germination tests on Petri dishes, the statistically significant differences between treated and untreated samples were identified with Student's *t* test, specifying the different levels of significance (*p*\0.05 = *, *p*\0.01 = **; *p*\0.001 = ***). All data about pot phytotoxicity experiment with cucumber were analysed by one-way ANOVA with a Tukey's post-hoc test ($P \leq 0.05$), after the assessment of the fundamental assumptions of ANOVA: the normality of distributions (Shapiro-Wilk test, p-value > 0.05) and the homogeneity of the variances of the residuals (Levene's test with $P(>F) > 0.05$). The statistical software R (version 3.5.1 - Feather Spray - 2018) was used for all statistical analysis.

3. Results

3.1. Characterization of the digestates

Results of characterization of the digestates are shown in **Table 2**. Dry matter content in liquid digestates was 4.4% and 4.8% (for P and S, respectively), while it reached 25.8% and 88.8% (for C and D, respectively) after dewatering processes. pH decreased throughout the different digestates from 7.7 to 6.8; total nitrogen levels ranged from 7.5% (S) to 5% (D), while NH_4^+ was up to six times higher in liquid than in solid SSADs. No consistent variation in organic matter levels was observed through the four digestates; as a consequence, C/N ratio increased from liquid to dewatered SSADs. Plant macronutrients such P and K had opposite behaviours: the first one showed appreciable concentrations, with a growing trend from liquid to solid digestates; the latter revealed highly low levels (<1%), with a slight decrease in C and D SSADs. Meso- and micronutrients (Ca, Mg, B, Zn) and some metals (Na, Cd, Ni, As) exhibited decreasing concentrations from liquid to solid digestates; the only metals which showed a diametrically opposed behaviour were Fe and Cu. No consistent difference in Pb, Cr and Hg concentrations was reported across the four digestates.

Table 2.

Physicochemical properties of the four anaerobic digestates from sewage sludge used in this work; last three columns on right specify analysis methods for sewage sludge, Italian law limits for Land application of sewage sludges (Italian Decree Law 99/1992), and Italian law limits for heavy metals in fertilizers (Italian Decree Law 75/2010).

d.m.b.: dry matter basis; E.C.: Electrical conductivity; HGAAS: Hydride Generation Atomic Absorption Spectrometry; TOC: Total Organic Carbon.

Parameter	**Unit of Measure**	**Anaerobic digestates**				**Method of analysis**	**Italian Law Land application of sewage sludge (D. Lgs. 99/92)**	**Italian Law Discipline on fertilizers (D.Lgs. 75/2010)**
		Primary (P)	**Secondary (S)**	**Centrifuged (C)**	**Dried (D)**			
pH (1:10)		7.7	7.5	7.3	6.8	M.P.A.A.F ed.2006 Method III.3		
E.C.	mS/cm	0.378	0.36	1069	1.575	M.P.A.A.F ed.2006 Method III.4		
N - Tot (Kjeldahl)	% d.m.b.	7.4	7.5	6.3	5	IRSA-CNR, 1985 Issue 64, vol 3, Method 6	>1.5	
N – Org	% d.m.b.	5.84	6.16	5.33	4.75	M.P.A.A.F ed.2006 Method IV.12		
N - NO_3^-	% d.m.b.	<0.01	<0.01	<0.01	<0.01	M.P.A.A.F ed.2006 Method IV.12		
N - NH_4^+	% d.m.b.	1.56	1.34	0.97	0.25	M.P.A.A.F ed.2006 Method IV.12		
N - org / N - Tot	%	79	82	84	94	Calculation		
Dry matter	%	4.4	4.8	25.8	88.8	Calculation		
Humidity	%	95.6	95.2	74.2	11.2	M.P.A.A.F ed.2006 Method III.1		
Organic matter	% d.m.b.	64.7	68.5	63.9	64.4	Italian Ministerial Decree, 1999 Method VII.3		
TOC	% d.m.b.	37.5	39.7	37.1	37.3	Calculation	>20	
C/N		5.1	5.3	5.9	7.4	Calculation		
Ashes	% d.m.b.	35.3	31.5	36.1	35.6	Calculation		
Ca	% d.m.b.	6.46	4.69	5.02	4.64	M.P.A.A.F ed.2006 Method VIII		
Mg	% d.m.b.	1.78	1.53	1.45	1.16	M.P.A.A.F ed.2006 Method VIII		
Na	% d.m.b.	1.05	1.03	0.34	0.19	M.P.A.A.F ed.2006 Method VIII		
K	% d.m.b.	0.55	0.69	0.39	0.18	M.P.A.A.F ed.2006 Method VIII		
P	% d.m.b.	4.16	5.75	6.74	6.26	M.P.A.A.F ed.2006 Method VIII	>0.4	
Fe	% d.m.b.	2.43	3.32	3.99	3.48	M.P.A.A.F ed.2006 Method IX		
Mn	mg/kg d.m.b.	255	190	268	228	M.P.A.A.F ed.2006 Method IX		
Cu	mg/kg d.m.b.	357	340	406	396	M.P.A.A.F ed.2006 Method IX	1000	230

Zn	mg/kg	d.m.b.	918	650	849	719	M.P.A.A.F ed.2006 Method IX	2500	500
B	mg/kg	d.m.b.	51	60	52	41	M.P.A.A.F ed.2006 Method IX		
Pb	mg/kg	d.m.b.	92	70	92	79	M.P.A.A.F ed.2006 Method IX	750	140
Cr	mg/kg	d.m.b.	245	210	245	217	M.P.A.A.F ed.2006 Method IX	*<200**	
Cd	mg/kg	d.m.b.	1	0.6	0.8	<0.1	M.P.A.A.F ed.2006 Method IX	20	1.5
Ni	mg/kg	d.m.b.	163	120	155	137	M.P.A.A.F ed.2006 Method IX	300	100
As	mg/kg	d.m.b.	2.8	2.1	0.9	<0.1	M.P.A.A.F ed.2006 Method IX	*<20**	
Hg	mg/kg	d.m.b.	<0.1	<0.1	<0.1	<0.1	HGAAS	10	1.5
Cr^{6+}	mg/kg	d.m.b.	<0.1	<0.1	<0.1	<0.1	M.P.A.A.F ed.2006 Method IX	*<2**	0.5

** Values introduced with Italian Law 130/18*

3.2. Germination test on cress

Table 3. Germination Index (GI) on *Lepidium sativum* L. using primary and secondary digestates. Observed significance levels (*p* values) from Student's *t* test (*p*\0.05 = *, *p*\0.01 = **; *p*\0.001 = ***) comparing treated with untreated seeds. Error is expressed as standard deviation (SD).

	Liquid SSAD			
	Primary (P)		Secondary (S)	
Concentration (%)	**GI**	**SD**	**GI**	**SD**
0.0	1.000	0.000	1.000	0.000
2.5	1.144 **	0.061	1.067	0.379
5.0	1.017	0.195	1.049	0.279
7.5	0.781 **	0.096	1.036	0.409
10.0	0.815	0.188	0.640 **	0.164
15.0	0.620 ***	0.076	0.459 ***	0.051
20.0	0.245 ***	0.077	0.183 ***	0.134
25.0	0.093 ***	0.023	0.081 ***	0.106
50.0	0.000 ***	0.000	0.000 ***	0.000
75.0	0.000 ***	0.000	0.000 ***	0.000
100.0	0.000 ***	0.000	0.000 ***	0.000

Results of germination test on Petri dishes are shown in **Table 3**. In both P and S, GI in 50%, 75% and 100% concentrations were 0 due to absence germination. On P the highest GI was obtained at 2.5% concentration, with a gradual decrease at higher concentrations. The 10% concentration case deserved particular attention because it showed a significantly higher value than previous and following points. Moreover, the calculated EC50 was at 17.5%.

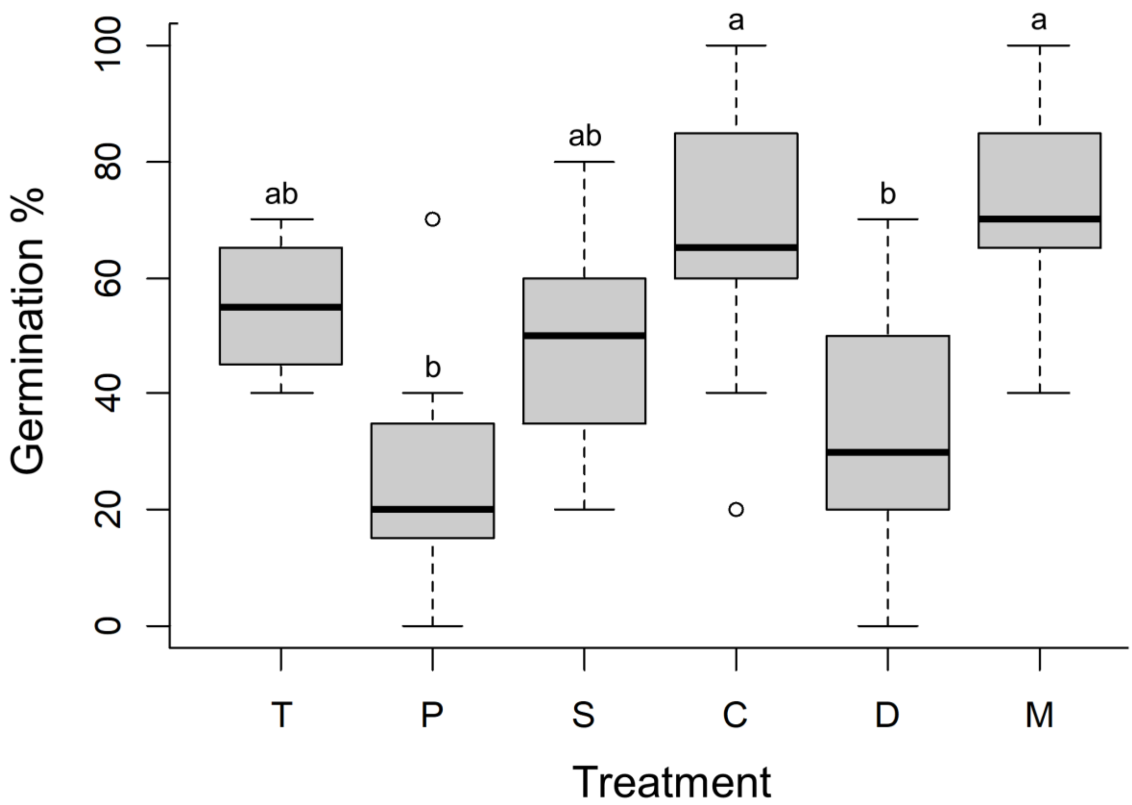
On S, GI at 2.5%, 5% and 7.5% was slightly higher than control, but not enough to affirm that GI was significantly increased from control dosage. Then, the index decreased to 0% more rapidly than P for concentrations higher than 7.5%. However, this GI value was reached at the same concentration of P treatment (50%). The calculated EC50 was at 12.5%.

3.3. Germination and phytotoxicity on cucumber

3.3.1. Germination

Figure 1.

Mean germination (%) after three days of *C. sativus* grown on sandy soil under each treatment. Different letters indicate differences between treatments that were significant at $P < 0.05$ (Tukey HSD).



Germination on sandy soil revealed significative differences only at the third day after sowing. More in detail, these differences were found only between the typology of treatment, highlighting a greater germination on C and M than on P and D (**Figure 1**). This treatment was the only one displaying significative differences even between treatment concentrations, with D255 presenting the smallest germination value (16%). Overall germination (all germinated seeds to all sown seeds ratio) after 3 days was 43% on sandy soil, while at the end of experiment it reached 80% (data not shown). On the other hand, no significative difference in germination on peat substrate emerged during the 10 days after sowing. Even in this case overall germination increased along the experiment, shifting from 83% at 3 days to 90% at 30 days after sowing.

3.3.2. Biomass

On sandy soil, all treatments, except for P255, overcame the yields of the control: C255 and D255 were considerably higher than others doubling the control biomass. The increase of biomass production was proportional with the dosages of C and D digestates as well as M; the highest dosage of the last one did not seem to cause further increase. On the other hand, P and S digestates had the highest yields at intermediate dosages (P170 and S170), while dry biomasses at lowest dosages (P85 and S85) were comparable to the highest ones (P255 and S255) and were not significantly different from control (**Figure 2**).

For what concerns the biomass yield on peat substrate, the common biomass trend is an increase going from 85 to 170 kg N/ha dosages, and a decrease moving from 170 to 250 kg N/ha. However, P digestate is the only one displaying decreasing biomass values for higher application rate of treatment. The highest biomass yield was found in C170, even doubling the control one. Moreover, P85, S170 and S255 were the only ones showing a significantly higher biomass than control. (**Figure 2**). Very important differences were found in 170 kg N/ha treatments: all yielded significantly more biomass (1.10 to 1.21 g) than the control (0.75 g) on sandy soil (**Figure 3.a**); on peat substrate, P, S and C treatments provided more biomass (2.92 g, 3.61 g and 3.95 g, respectively) than control (2.07 g), with S and C showing the top production, while D (2.82 g) and M (2.51 g) behaved similarly to the control (**Figure 3.b**).

Figure 2. Mean dry biomass related to control of *C. sativus* grown on sandy soil and peat substrate.

Each data point represents mean of replicates to mean of control replicates ratio ± standard error; different letters indicate differences between treatments and concentrations of N that are significant at P < 0.05 (Tukey HSD); upper-case letters refer to samples from sandy soil and lower-case letters refer to samples from peat substrate.

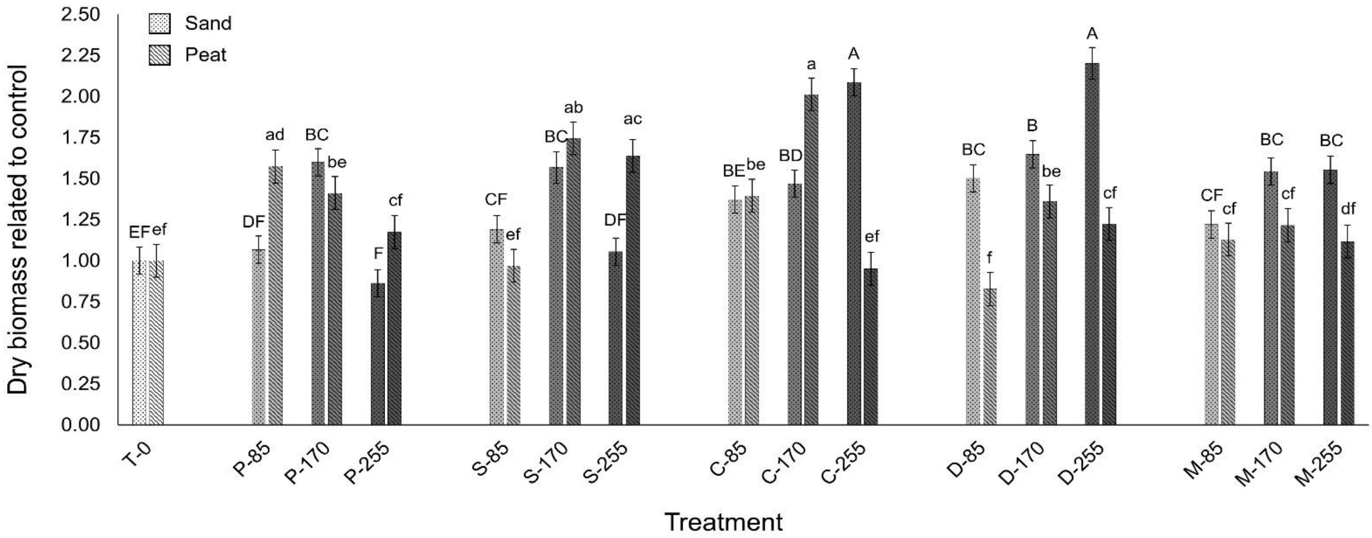


Figure 3.a. Mean dry biomass of *C. sativus* grown on sandy soil with 170 kg N/ha treatments. Different letters indicate differences between treatments that are significant at $P < 0.05$ (Tukey HSD).

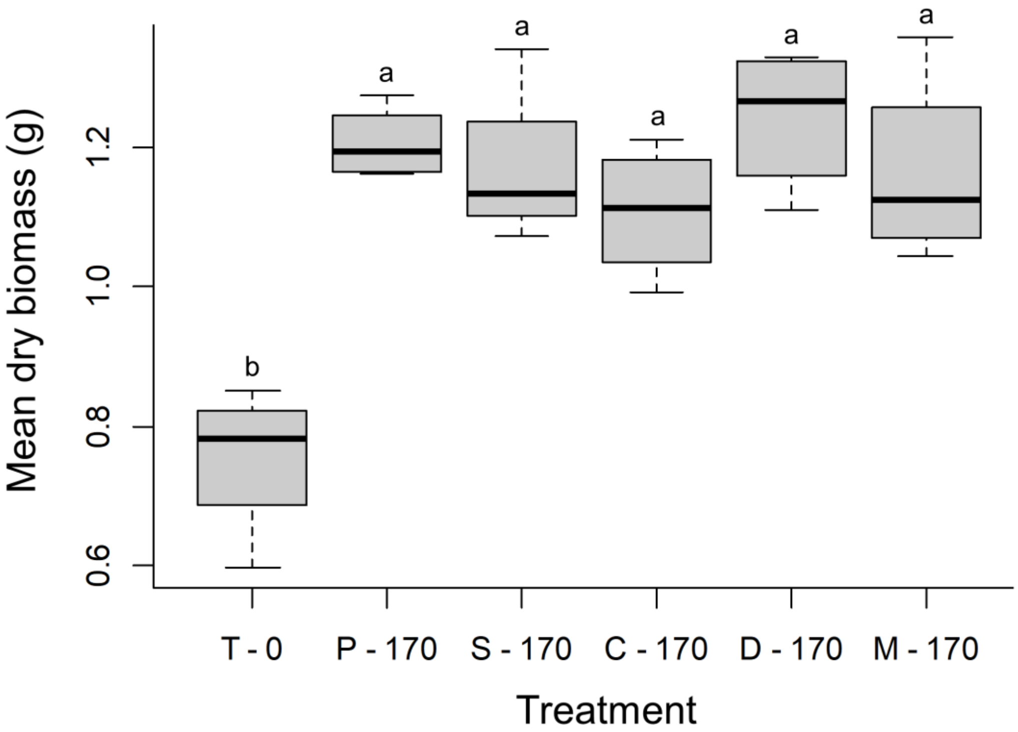
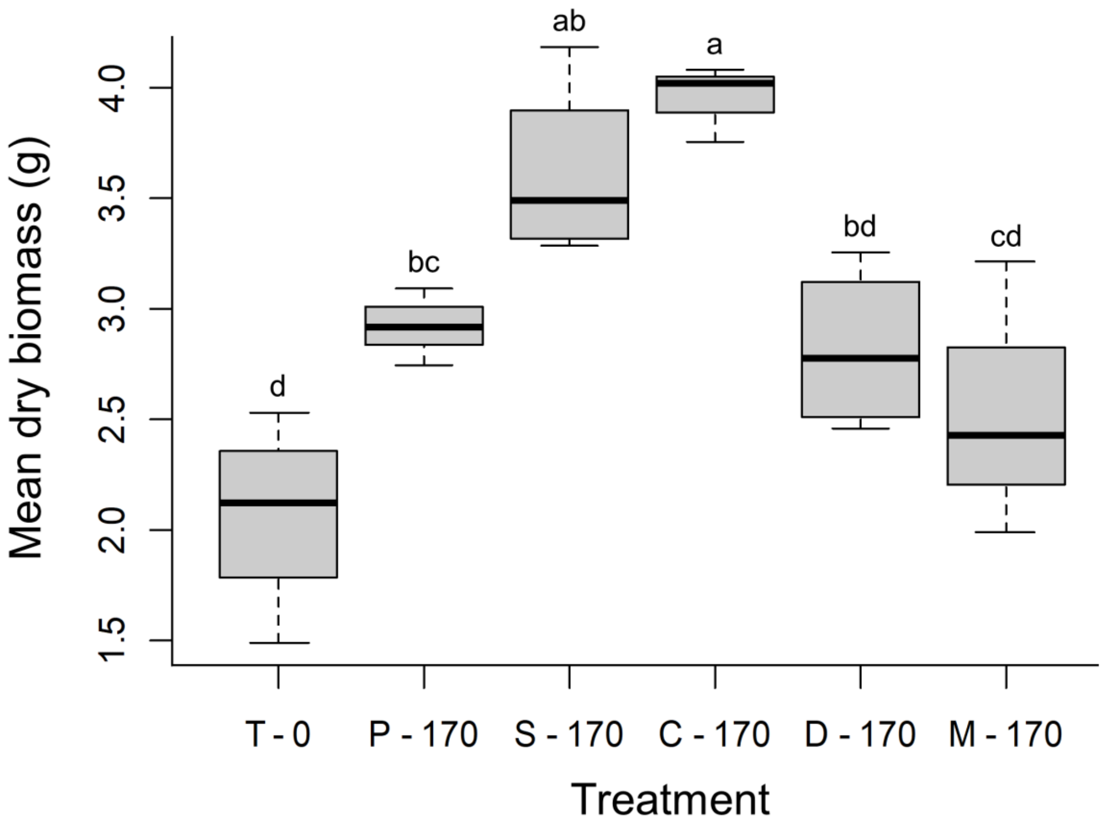


Figure 3.b. Mean dry biomass of *C. sativus* grown on peat substrate with 170 kg N/ha treatments.

Different letters indicate differences between treatments that are significant at $P < 0.05$ (Tukey HSD).



3.3.3. Chlorophyll Content Index (CCI)

CCI control mean level of plants grown on sandy soil was 26.0 (**Figure 4.a**); the chlorophyll concentration significantly higher than control were obtained in M255 (35.5), M170 (34.2), P170 (33.3), D255 (32.4) and M85 (31.95), S255 (31.3) and P85 (30.9). Moreover, the chlorophyll content was higher with the increase of the SSAD application rate. However, this behaviour was not detected for P digestate, where the increase of treatment dosage was related firstly to a CCI increment in P170, then to a CCI reduction in P255 (29.1).

On peat substrate **(Figure 4.b),** control mean level of CCI (28.2) was higher than on sandy soil. Similarly to CCI of cucumber grown on sandy soil, mineral fertilizer in M255 (36.4) and M170 (33.3) gave high results and, together with C170 (37.2), were significantly higher than control. Moreover, C170 was significantly different from other dosages within same treatment, while no significative differences among concentrations were found on P, S and D treatments.

Figure 4.a. Chlorophyll Content Index (CCI) of leaf of *C. sativus* grown on sandy soil. Different letters indicate differences between the treatments with the different concentrations of N at 85, 170 and 250 kg N/ha, that are significant at $P < 0.05$ (Tukey HSD).

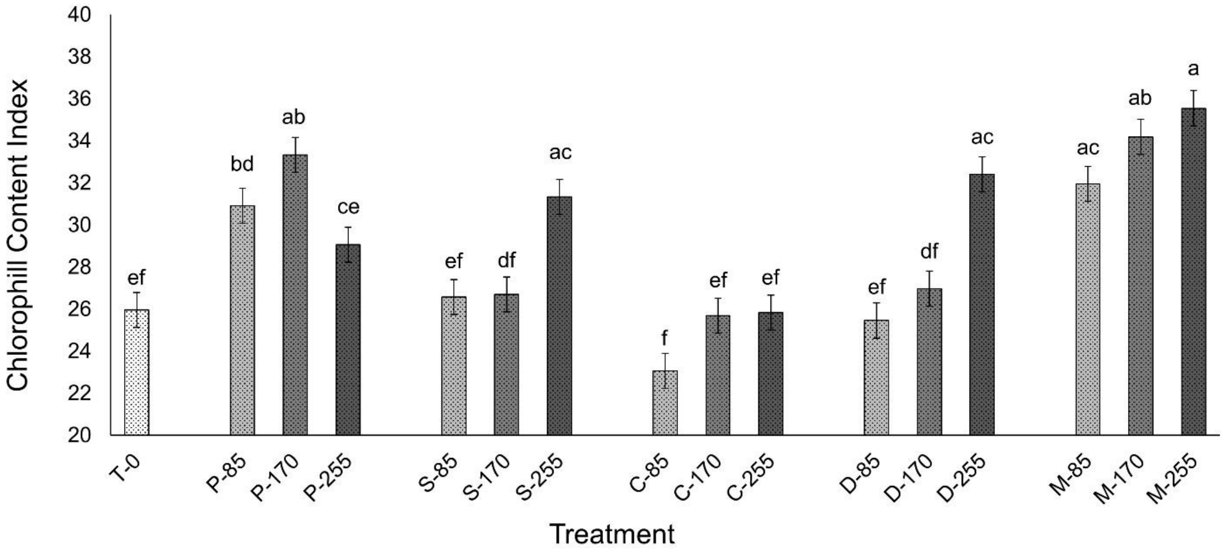
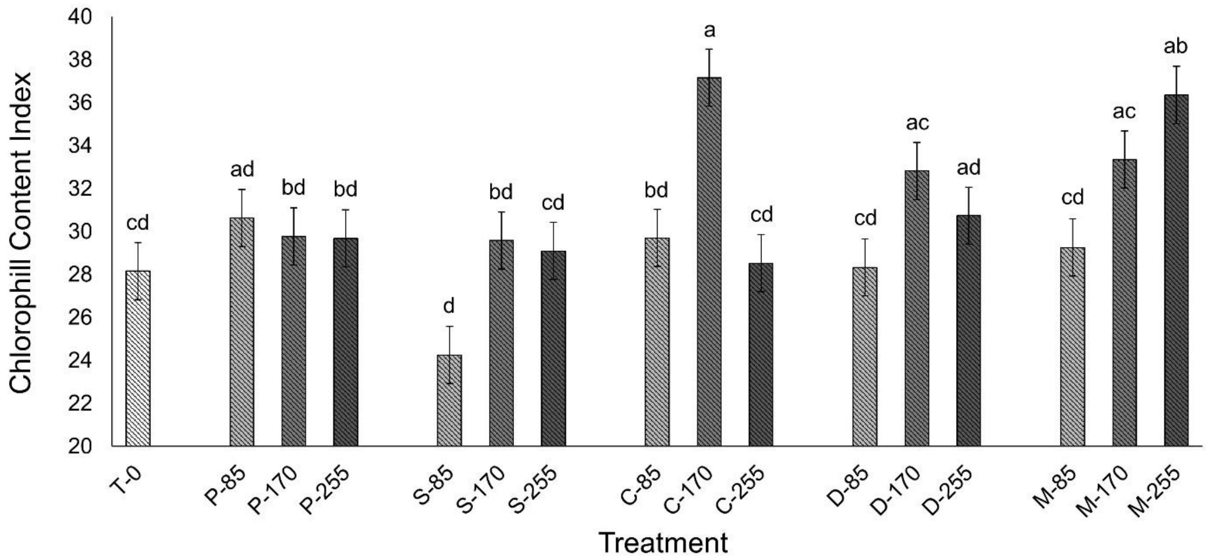


Figure 4 b. Chlorophyll Content Index (CCI) of leaf of *C. sativus* grown on peat substrate. Different letters indicate differences between treatments and concentrations of N that are significant at $P < 0.05$ (Tukey HSD).



3.3.4. Infra-Red Gas analyser (IRGA)

Treated and control cucumber plants grown on sandy soil showed significative differences in Net photosynthesis (A_N): control value (1.83 CO_2 $m^{-2}s^{-1}$) was lower than all other treatments, which however did not differ from each other (**Figure 5.a**). Therefore, it is worth underlining the value measured on P treatment (3.75 μmol CO_2 $m^{-2}s^{-1}$), which doubled control value. In order to stomatal conductance (g_s), all digestate treatments at least doubled the one of control thesis (0.098 mmol H_2O $m^{-2}s^{-1}$), while S even trebled this result (0.333 H_2O $m^{-2}s^{-1}$) (**Figure 5.b**). On the other hand, while M showed an intermediate behaviour between digestates and control as regards stomatal conductance, it reached the highest concentration of CO_2 (536 ppm) in substomatal cavity (C_i) (**Figure 5.c**).

Moving to peat substrate, differences in net assimilation of CO_2 (**Figure 5.a**) between treatments and control were few: C (4.83 CO_2 $m^{-2}s^{-1}$) and D (4.78 CO_2 $m^{-2}s^{-1}$) had a higher A_N than all other treatments (including T). However, it is important to point up that only C (0.383 mmol H_2O $m^{-2}s^{-1}$) displayed also a significantly greater value in terms of stomatal conductance (**Figure 5.b**).

CO_2 concentration in substomatal cavity revealed two different groups: the first gathering the highest C_i values, S and D (586 ppm), and the second collecting all other treatments (T included), which showed lower results (**Figure 5.c**).

Figure 5. IRGA measurements on *C. sativus* grown on sandy soil and peat substrate with 170 kg N/ha treatments. **5.a**. Net assimilation (A_n in µmol CO_2 m^{-2} s^{-1}) ± mean standard error, **5.b**. Stomatal conductance (g_s in mmol H_2O m^{-2} s^{-1}) ± mean standard error and **5.c**. CO_2 concentration in substomatal cavity (C_i in ppm) ± mean standard error.
Different letters indicate differences between treatments that are significant at $P < 0.05$ (Tukey HSD); upper-case letters refer to sandy soil and lower-case letters refer to peat substrate.

Figure 5 a. Net assimilation on sandy soil and peat substrate.

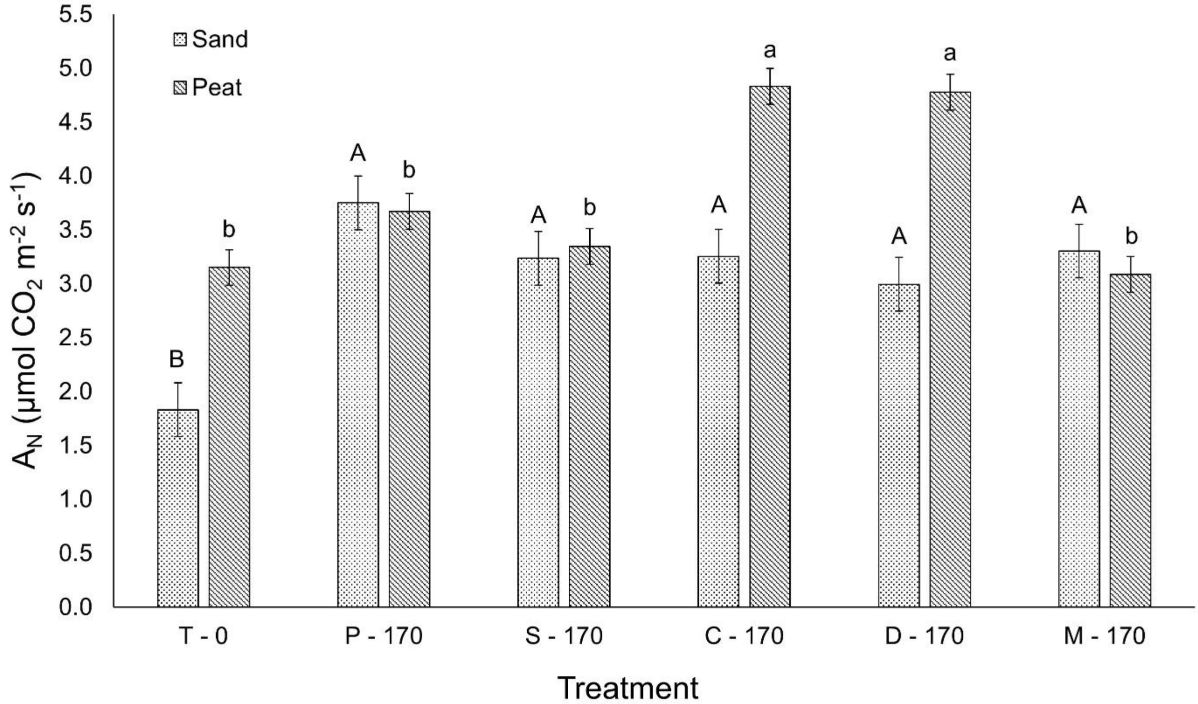


Figure 5 b. Stomatal conductance on sandy soil and peat substrate.

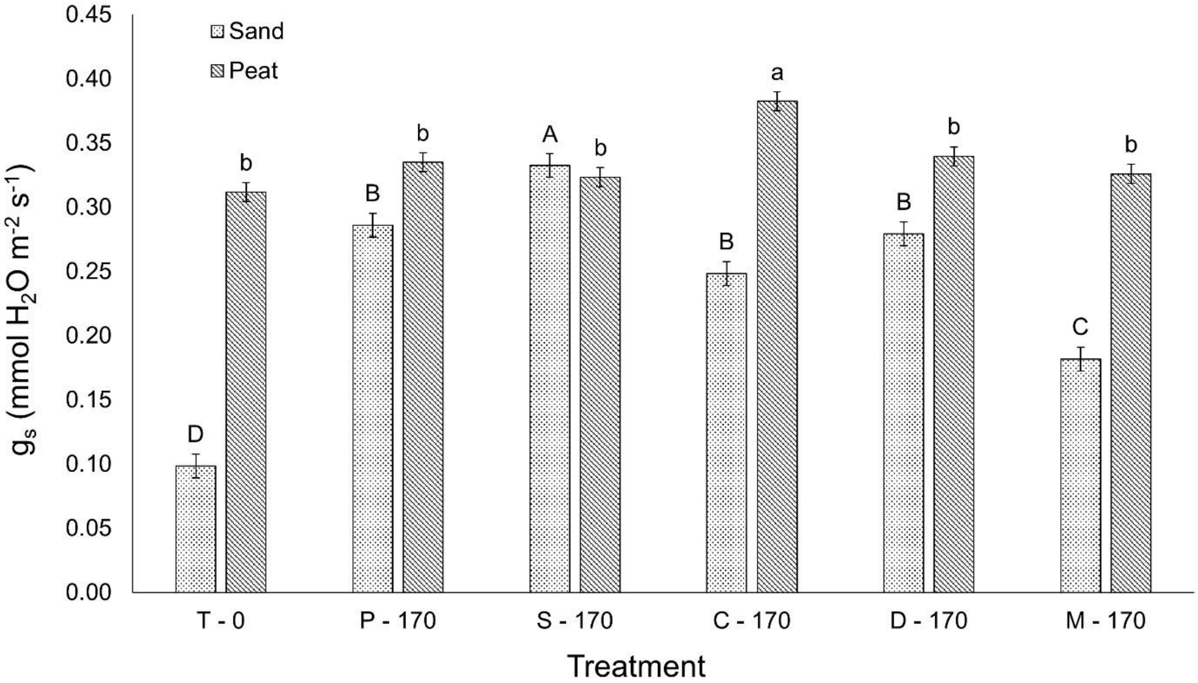
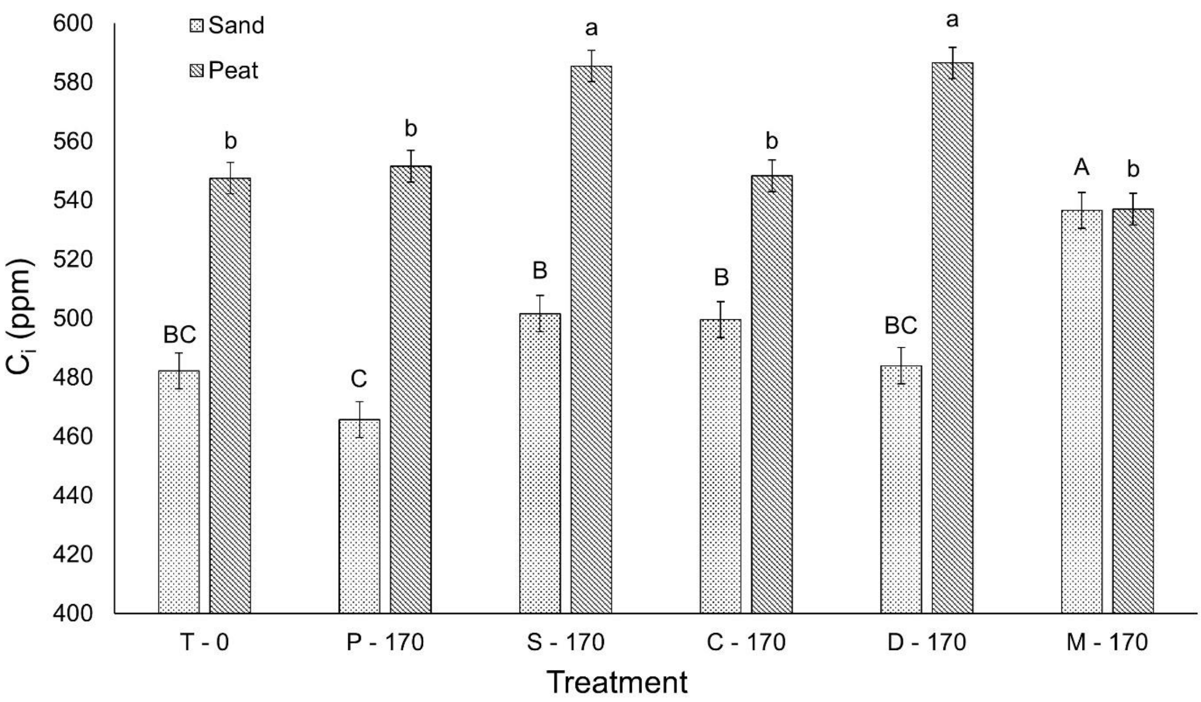


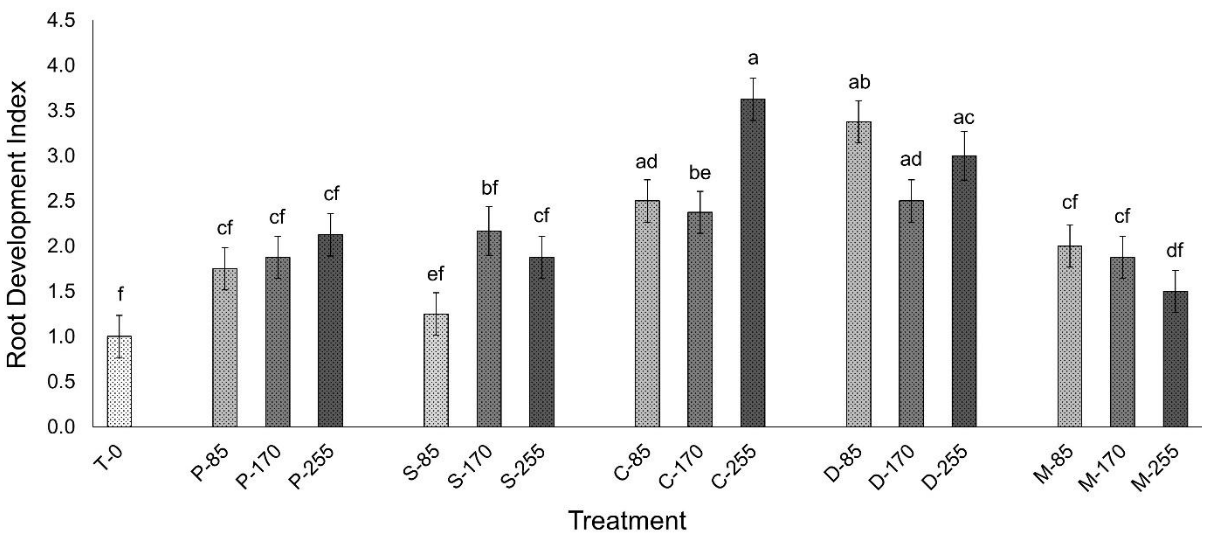
Figure 5.c. CO_2 in substomatal chamber on sandy soil and peat substrate.



3.3.5 Root Development Index

Figure 6. Mean Root Development Index of *C. sativus* grown on sandy soil.

Each data point represents mean of replicates to mean of control replicates ratio ± mean standard error; different letters indicate differences between treatments and concentrations of N that are significant at $P < 0.05$ (Tukey HSD).



Root apparatus was mostly developed in plants grown on C and D treatments on sandy soil (**Figure 6**). Indeed, C255 (3.625), D85 (3.375), D255 (3.000), D170 (2.500), C85 (2.500) and C170 (2.375) revealed an RDI significantly higher than control. Data on peat substrate did not respect the homogeneity of variances (P-value = 0.0449) (data not shown).

4. Discussion

All digestates showed interesting contents in macronutrients (N > 5% and P > 4%) as well as in meso- and micronutrients. Indeed, these values were even slightly higher than the mean ones published in other works (N_{Tot}= 3.6%; P_{Tot}= 2.5%). In the case of nitrogen, dewatering probably induced an immobilisation effect, remarked by the increasing levels of N_{Org}/N_{Tot}. On the contrary, despite K levels were a little bit low if compared to other studies (e.g. mean values of works in the references: K = 0.59 %), the applied dosage in this work was sufficient for the early growth stages (Adjei and Rechcigl, 2002; Alvarenga et al., 2016; Antonkiewicz et al., 2018; Asagi and Ueno, 2008; Belhaj et al., 2016; de Andrés et al., 2010; Singh and Agrawal, 2008; Hussein, 2009; Ferreiro-Domínguez et al., 2011; Tarrasón et al., 2008; Wang et al., 2008). However, this aspect can negatively affect the proper potassium supply when SSAD is applied as fertilizer, especially in the phase of fruit maturation (Hawkesford et al., 2012). The main disadvantage of these digestates was the presence of heavy metals. Despite all the analysed ones complied with the limits imposed by the Italian Law on Sewage Sludge Land Application (Italian Decree Law 99/1992), in some cases (i.e. Zn, Cu and Ni) the thresholds imposed by Italian Discipline on Fertilizers (Italian Decree Law 75/2010) were overcome. Moreover, heavy metals concentrations were generally lower than those published elsewhere (Cu: 413 mg/kg, Zn: 922 mg/kg, Pb: 116 mg/kg, Cd: 3.9 mg/kg, As: 3.5 mg/kg), except for Cr and Ni (93 mg/kg and 72 mg/kg, respectively) (Adjei and Rechcigl, 2002; Alvarenga et al., 2016; Antonkiewicz et al., 2018; Asagi and Ueno, 2008; Belhaj et al., 2016; de Andrés et al., 2010; Singh and Agrawal, 2008; Hussein, 2009; Ferreiro-Domínguez et al., 2011; Tarrasón et al., 2008; Wang et al., 2008).

The sandy soil was alkaline and carbonate-rich, with very low concentration of organic matter and nutrients. Several reports have shown that SS application in soils with these peculiarities can provide a good nutrient supply with a relatively small risk of pollution (Navas et al., 1998; García-Gil et al., 2004; Antolín et al., 2005).

Germination of cress increased with dilution and this trend is confirmed elsewhere (Abdullahi et al., 2008). GI of P, at 2.5% concentration, showed a significative improvement compared to 0%. In order to determine a more precise GI trend in this range, the approach proposed by Lencioni and colleagues (2016) should be applied, exploring the interval 0% - 10%, with steps of 1%. Differently, results of GI of S did not show any significative variation from not treated samples until the 7.5% concentration. At higher concentration rates, GI presents a sudden decrease. For sure, concentration of 15% for P and 10% for S were the highest ones with a germination index of at least of 60%, which is considered the GI threshold to support the absence of phytotoxic effects (Zucconi et al., 1985). Compared to other sewage sludges, the two digestates used in this study revealed higher GI values. For instance, in the work of Mañas and De las Heras (2018) a not-digested sewage sludge was utilized: at 10% concentration revealed a lower GI (1.4%) than the one obtained in the present work (81% on primary digestate and 64% on secondary digestate). Alburquerque and colleagues (2012) used twelve different kinds of anaerobic digestates from animal origin (obtained from co-digestion of different organic matrix) and only two of them showed a GI higher than 50% at concentration of 10%. Interesting results derived from the comparison of the GI of an anaerobic digestate from microalgae, and digestates from a co-digestion of microalgae with primary sewage sludges: while the former showed a GI comparable to the one obtained with P of the present work (at concentration of 10%), the latter displayed a GI of nearly 100% for the same concentration (Solé-Bundò et al., 2017). Thus, the minor toxicity of primary co-digested sludge could be justified by the synergic effect of co-digestion, which has been demonstrated to be more advantageous than mono-digestion ones due to a dilution effect of inhibitory compounds, among other factors (Tritt, 1992).

Hence, SS co-digestion could be a nice suggestion to elevate GI at higher digestate concentrations.

The germination of cucumber seedlings grown on peat substrate was higher compared to sandy soil, which may be due to pH values of the growing substrate. Optimal pH conditions for cucumber germination are between 5.5 and 6.5 (Nersisyan et al., 2017), that are values roughly similar to peat substrate, but far away from sandy soil ones (pH 8.7). Moreover, D255 induced a significatively low germination within all treatments applied on sandy soil. This effect could be explained by the high E.C. of D, which is 300% and 50 % higher than liquid and centrifuged digestates, respectively. Indeed, other authors (Sánchez-Monedero et al., 2004 and Eklind et al., 2001) demonstrated a clear correlation between the E.C. increase in soil and germination decrease.

The fertilizing effects of the digestates on cucumber were studied in previous works. However, the ones dealing with sewage sludges and derived products are mostly focused on the toxic effects derived from organic and inorganic pollutants present in this waste (Waqas et al., 2014, Wyrwicka et al., 2014). In the present work, higher biomass yields were recorded for the plants grown on peat substrate than on sandy soil due to the richness in organic matter and macronutrients of the first one. Nevertheless, this aspect likely contributed to the lower degree of differences between control and treated samples; indeed, all treatments on sandy soil at 170 kg N ha^{-1} were significantly different from the control, while the same conditions on peat substrate revealed results, for D and M, slightly comparable to T . In general, it could be inferred that fertilizing effects occurred at different levels both in terms of soil and treatment concentration. In fact, dry biomass overcame the control in all cases except four (P255 on sandy soil; S85, C255 and D85 on peat substrate). These biomass-promoting effects on cucumber grown on sandy soil have already been reported by Hussein (2009): despite the higher application rate (up to ten times greater, in terms of total nitrogen), the authors observed a crop yield improvement over control around 70%, which is in good agreement with our results. Moreover, cucumber was utilised to test the effects of sewage sludge compost applied on a sandy soil. Even in this case, the dry weight of shoot biomass almost doubled the control one (Xu et al., 2012), similarly to C255 and D255 conditions on sandy soil of the present study.

Moving to a broader perspective, other works designed with a pot experiment approach assessed the fertilizing effect of sewage sludge on different species. Asagi and Ueno (2008) and Shaheen and co-workers (2014) reported examples of komatsuna (*Brassica rapa* L. var. perviridis) grown on sandy soil, and rocket (*Eruca sativa* Mill.), grown on calcareous soil, which quintupled and doubled their dry biomass yield, respectively. Furthermore, relevant outcomes have been described on sunflower (*Heliantus annuus* L.) (Belhaj et al., 2016) and kenaf (*Hibiscus cannabinus* L.; de Andrés Parlorio et al., 2010) grown in presence of dewatered anaerobic digestates similar to C and D treatments, providing well comparable results with this study. Qasim and colleagues (2001) and Alvarenga and co-workers (2016) provided examples of cereal crops (maize and sorghum, respectively) fertilized with an unstabilized sewage sludge and a yield increase of 40% and 400%, respectively, over untreated control was reported. Even if it’s difficult to compare the behaviour of different plants exposed to diversely treated sludges, it is conceivable that weaker performances of digestates of this study may be due not only to lower application rates, but also to the nitrogen fractionation. In fact, in the present work, this is skewed in favour of organic nitrogen (N_{Org} / N_{Tot} ranging from 79% to 94%), with lower concentrations of “readily-available” nitrogen (i.e. NH_4^+ and NO_3^-).

Nevertheless, the main drawbacks of sewage sludge land application are the phytotoxic effects occurring at higher application rates, preventing the optimal growth of the plant. Indeed, this aspect has been deeply investigated as regards the presence of organic and inorganic pollutants, such as heavy metals. These ones can interfere with the biomass yield as widely reported in literature (Singh and Agrawal, 2007; Nagajyoti et al., 2010). In the present work, the decrease of dry weight with higher application rates was observed only in few cases (e.g. P255 and S255 on sandy soil, and P255, C255 and D255 on peat substrate). These reductions can be justified in part with the metal-derived toxicity, especially in the case of peat substrate. Its slightly acidic conditions maybe allowed a more sustained metal bioavailability, which was instead down modulated by high pH in sandy soil (Sukreeyapongse et al., 2002; Belhaj et al., 2016). On the other hand, another conceivable hypothesis is the ammonia-connected toxicity occurring in alkaline conditions: increasing soil pH induces higher NH_3 percentage of total ammoniacal nitrogen (Masoni and Ercoli, 2010), according to the NH_4^+/NH_3 acid-base equilibrium (Gay and Knowlton, 2005). Thus, at the pH of sandy soil exploited in this work (8.7), around 20-25% of ammoniacal nitrogen is represented by NH_3, which can negatively affect the plant growth under different aspects as described by van der Eerden (1982). This aspect has been observed mainly on plants exposed to liquid digestates, which revealed ammonia-nitrogen concentrations up to six times higher than dewatered ones. On the contrary, dehydration of SSAD might have had a positive effect on the ammonia abatement, which resulted in an overall slighter phytotoxicity exhibited by solid SSADs (C and D, in this study). In this respect, this aspect is confirmed by Alvarenga et al. (2016) and de Andrés Parlorio et al. (2010). Moreover, the last one devoted particular attention to the treatment formulation (pelletization, in this case), which can be an aspect to take into account even for future work.

Chlorophyll content can be strongly correlated to crop nitrogen content and can be sensitive to differential nitrogen nutrition in vegetable crops (Padilla et al., 2017). Nitrogen nutrition index (NNI) is an indicator of plant nitrogen status, and NNI =1 values correspond to optimal N nutrition (Lemaire and Gastal, 1997); in the case of cucumber, it was matched to CCI values between 24 and 36. In the present work, the CCI values obtained on peat substrate were in this range, likely due to the better capacity of peat substrate to retain nutrients, while on sandy soil they were lower. These values are in agreement with the ones reported by Shaaban and El-Bendary (1999), Güler and Büyük (2007), Jahromi et al. (2012) and Xu et al. (2012). High values of CCI did not coincide necessarily to high biomass yields: in fact, on sandy soil C255 had middle-low CCI, but its biomass yield was the highest. Latare and co-workers (2014) reported a similar behaviour for wheat and rice, in which yield increase was not accompanied by a significative rise in SPAD values. Moreover, M255 showed the highest CCI value on sandy soil: this result is probably linked to the mineral fertilizer formulation which ensures a long-lasting nitrogen release. Considering the typologies of treatment, many works show a general improvement of CCI values upon application of sewage sludge and its derivatives. Improvements of chlorophyll content compared to untreated controls have been recorded on cereals (Alvarenga et al., 2016; Koutroubas et al., 2014), edible plants (Asagi and Ueno, 2008) and trees (Han et al., 2004). This general behaviour indicates that sewage sludge provides a good amount of nutrients, which is an aspect that clearly emerges even in this work.

The results of gas analysis measurements were not directly comparable to other values in literature because these are strictly depending on environmental conditions (light, temperature, irrigation and phenological phase). On peat substrate, almost no difference was appreciable; just in C case, A_n and g_s values were higher than T; anyway, these differences reflect values obtained in biomasses and CCI measurements. To the best of our knowledge, no measurements of physiologic parameters and gas exchange have been performed on cucumber exposed to sewage sludge treatments with pot experiments. However, some comparisons can be done with studies on physiologic parameters of plants exposed to sewage sludge and studies on physiologic parameters of cucumber**.** Antolín et al. (2010) and Bourioug et al. (2015) carried out pot experiments with alfalfa (*Medicago sativa* L.) and European larch (*Larix decidua* L.), applying both sewage sludge rates like the ones of this study. The significative differences reported in the case of cucumber grown on sandy soil are in good agreement with A_n and g_s values of the first work, while in the second study only with A_n ones. Furthermore, similar results of A_n and g_s have been assessed using two different dosages of sewage sludge in field on rice crop (*Oryza sativa* L.) (Singh and Agrawal, 2010). On the other hand, studies with sewage sludge on beet (*Beta vulgaris* L.) (Singh and Agrawal, 2007) and okra (*Abelmoschus esculentus* L.) (Singh and Agrawal, 2009) showed lower results in terms of A_n and g_s, probably due to the higher SS doses, provoking phytotoxic effects.

Physiologic parameters of cucumber plants were studied mainly as regards metals stress, such as toxicity derived from copper (Alaoui-Sossé et al., 2004) and sodium (Chartzoulakis, 1994): their increasing concentration caused the decrease of the physiologic parameters. Anyway, in the present study, concentrations of copper and sodium were lower and, consequently, A_n and g_s values were higher. Moreover, an increase of stomatal conductance in presence of heavy metals was explained by Singh and Agrawal (2010), claiming that it may be due to high nutrient availability through SS amendment which nullified the heavy metal toxicity.

The trend of biomass production did not match always with a sustained root development (RDI). This mismatch between shoot and roots biomass in cucumber has been already reported in literature (Xu et al., 2012). Root development results clearly revealed that C and D gave best outcomes, with an RDI similar between them and higher than liquid digestates and M.

Furthermore, these findings demonstrate that the kind of treatment had a greater effect on roots growth than the nitrogen amount (except for the case of C255). This observation is in contrast to the study of Gulyás and co-workers (2012), which described a root reduction in ryegrass (*Lolium perenne* L.) treated with same dosages of SSAD, probably due to excessive ammonium content. Despite comparable nitrogen application rate, root development was not inferior than control presumably because of a lower NH_4^+/N_{Tot} ratio of the SSADs used in the present work. Lastly, this work contributed to deepen the knowledge about the agronomic recycling of SSAD. As far as we know, this was the first study conducting a systematic comparison of the fertilizing and phytotoxic effects of anaerobic digestates from primary, secondary, centrifuged and dried sludges. The significative differences between the SSADs likely indicated that the ways in which the digestate is treated at WWTP level had an effect not only on its chemical peculiarities but also on its agronomic potential. Thus, these findings can pave the way for a wiser recycling of this waste, through its usage for the improvement of nutrient-deficient soils.

5. Conclusions

Four different SSADs (two liquid and two dewatered) coming from WWTP were characterized and exploited as soil improver for promoting cucumber growth in pot experiments. Application of SSADs improved plant growth according to the exploitation of nitrogen dosages commonly used in field operations. In general, an intermediate nitrogen dosage (170 kg N/ha) showed the best results in terms of biomass, chlorophyll content, net photosynthesis, stomatal conductance and root development. All these results were much more evident for cucumber plants grown on an alkaline, sandy and poor (concerning organic matter and nutrients) soil than an acid and rich cultivation substrate, such as peat substrate. However, in some cases phytotoxicity effects occurred probably due to an excessive addition of ammonia nitrogen or heavy metals. Hence, considering these observations, SSADs share many peculiarities with improvers for nutrient-poor soils. Future work should include the study of long-term effects and of repeated applications consequences deriving from SSAD land application; on the other hand, strategies for contaminants abatement or recovery of valuable substances should be investigated, within a circular economy approach.

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