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Physico-chemical and multielemental traits of anaerobic digestate from Mediterranean agro-industrial wastes and assessment as fertiliser for citrus nurseries

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ABSTRACT

Previous researches have demonstrated the bioenergetic potential of agri-food Mediterranean wastes showing that anaerobic co-digestion is a valuable solution for Mediterranean areas. This implies a great interest for anaerobic digestates use in agriculture to replace fertilizers. The present study aimed at: i) producing knowledge on continuous anaerobic co-digestion of feedstock mixture composed by different Mediterranean agri-food wastes in terms of multielemental characterization and ii) assessing the agronomic value of industrial anaerobic digestate (AD) based on the potential as fertiliser in nursery condition for the citrus seedlings. Results have demonstrated that agro-industrial biomasses have great potentiality to be converted by anaerobic digestion in biofertilizer to be used in citrus nurseries as sustainable alternative to mineral fertilisers. Multielemental traits of the tested AD were valuable in terms of nutritional supply for the growth and development of the plant. AD was useful to replace the mineral fertilizers in terms of total N content (10.81 ± 0.32 %TS) and organic matter (43.32 ± 0.80 %TS). The seedlings nutritive status showed that no need for supplemental of nutrients was requested. Volkamer lemon highly benefited from the administration of liquid digestate, increasing the total chlorophyll level (2.97 ± 0.31 mg g^{-1} FW) presumably due to the higher ammonium content of the AD (59 ± 0.08 %TKN). Besides providing useful tools for citrus nurseries for conceiving new sustainable fertilization strategies, this study is a starting point for further in-depth works on physiological status and traits of citrus plants fertilized by using agro-industrial anaerobic digestate.

1. Introduction

Climate change has become worldwide a matter of great interest due to its effect on the entire society, in terms of sustainability of processes in all the supply chains. Building a resource-efficient, climate-change resilient economy and society is indeed one of the main societal challenges launched by the European Commission in the H2020 Programme. Focusing on the need of a more sustainable agriculture, it is absolutely urgent to mitigate CO_2 emissions deriving from conventional agriculture and increase the net primary production (NPP) by additional carbon input to the soil (Ferlito et al., 2020; Roccuzzo et al., 2018). This strategy allows the soil fertility improvement and increase the land resilience to counteract the current effects of climate change, which farmers are starting to feel worldwide. As far as the production of additional carbon is concerned, thanks to its peculiar characteristics, anaerobic digestion could contribute similarly to other sources of bioenergy such as biochar that contain about 50% of total organic carbon (Tian et al., 2012). Indeed, anaerobic digestion can i) convert carbon in biogas by applying a well-known, easy to use, free and not covered by patent biotechnology; ii) employ various feedstocks in different agricultural and ecological conditions as well as in different climatic areas; iii) allow to improve the organic fertilization at farm level, even in the absence of manure or zootechnical waste.

The use of agricultural by-products, agri-food and zootechnical wastes in addition to manures as ingredients of the diet of an anaerobic digestion plant is crucial to mitigate the emissions deriving from the incorrect use of these organic matrices, while providing a technological solution to recover energy from unused biomasses. Also, appraisal of gas

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losses at all anaerobic digestate (AD) stages has to be taken into account for the minimisation of gases emission which could also contribute to greenhouse gases (GHG) potential. At the farm level, some strategies, such as localized fertigation, could be adopted to reduce gas emissions. Focusing attention on Italy, biogas industry is significantly increasing its impact within the bioenergy sector, offering new perspectives in the agricultural field. There's evidence that, with a capacity of approximately 1,200 MW/year, equal to a production of 2.4 billion m^3/year of natural gas, Italy is one of the main producers of biogas in agriculture, fourth in the world following Germany, China and the United States. It has been estimated that, potentially, Italy could produce up to 10 billion m^3/year of biomethane by 2030, at least 8 of which from agricultural matrices (Pezzaglia, 2020). Currently, most of the Italian biogas plants are limited to the exploitation of animal wastes and/or some other dedicated industrial crops (*i.e.* sorghum, corn, etc.). The drive to the setting-up of new plants in Southern Italy inevitably passes from the exploitation of the relevant bioenergetic potential of agri-food Mediterranean wastes. Sicily is traditionally vocated to agriculture, with more than 6 million t/year of agricultural commodities (ISTAT, 2020), resulting in huge amounts of agricultural residues, which differ in quantity depending on the raw material derived from the agricultural practices. Moreover, a predictive geographical information system (GIS)-based model has recently demonstrated that the most part of Sicilian agri-food wastes is represented by citrus processing wastes followed by olive pomace, cattle manure, poultry litter and whey (Valenti et al., 2018a). Thus, there's a great need to find new sustainable solutions for the valorisation of agri-food wastes and by-products originated in Sicily.

Biomethanization, *i.e.* a process to microbiologically convert organic materials to biogas through anaerobic digestion, sounds to be a relevant cost-effective opportunity, in terms of cost: benefit ratio (Valenti et al., 2020). Several studies stressed about the potentiality of citrus wastes to be used for biomethanization. Martin et al. (2010) carried out lab-and pilot-scale experiments to test the feasibility of using orange peel waste (*pastazzo*) for bio-methanization showing that the preventive removal of residual essential oils and thermophilic conditions are to be preferred in order to increase the process efficiency. This strategy was also proposed by Ruiz and Flotats (2014) that reviewed the mechanisms of essential oils antimicrobial effects confirming that these compounds have to be removed in order to avoid process inhibition of the anaerobic digestion. Later on, Calabrò et al. (2016) demonstrated that, in batch trials without previous removal of essential oils, methane production can restart when D-limonene is partially degraded through a pathway that implies its conversion into *p*-cymene. Recently, many other studies have been performed to define how to maximise the potential yield of citrus peel bio-methanization (Siles et al., 2016), to evaluate the feasibility of bio-hydrogen production through anaerobic digestion (Torquato et al., 2017), to set-up the operative processing conditions in a semi-continuous pilot plant (Zema et al., 2018), and to evaluate the effects of waste composition and its storage on anaerobic digestion performance (Lotito et al., 2018). Steps forward have been made very recently when anaerobic digestion of multiple feedstocks, including citrus processing wastes, have been proposed to enhance biogas production in southern Italy applying batch, semi-continuous and continuous co-digestion approaches (Valenti et al., 2018b; Valenti et al., 2018c; Valenti et al., 2020) showing that anaerobic co-digestion can be a valuable solution for Mediterranean areas. Economic analysis on the use of anaerobic digestate for agronomic purposes are also available in literature, suggesting the need to provide farmers with an appropriate level of information about the digestate's attributes. Pappalardo et al. (2018) carried out a detailed economic analysis on the factors affecting purchasing process of digestate in Sicily. The results of this study showed a positive farmers' interest in digestate as an organic soil conditioner for their farms highlighting that digestate attributes significantly affect farmers' willingness to pay (WTP) for it. Indeed, a greater WTP for digestate was obtained when the participants were provided with detailed information on its agronomic attributes.

A great interest for digestates use in agriculture derives from the possibility to adopt them as a nutrient source. Both the liquid and the solid phases obtained by the digestion processes contains nitrogen, phosphorus, potassium and micronutrients in available forms for the plants absorption, therefore, they could be used in agriculture to replace fertilizers (Kowalczyk-Juśko and Szymańska, 2015). Compared to the untreated wastes, digestates presents a higher microbial stability, hygiene and higher amount of nitrogen in ammonium form (Al Seadi, 2002; Holm-Nielsen et al., 2009). Due to the organic origin, digestates could have a strong role for the organic farms for which other alternative fertilizers are not available (Vázquez-Rowe et al., 2015; Comparetti et al., 2013; Furukawa and Hasegawa, 2006). Moreover, the physico-chemical properties of the soils or growing media in nursery could be improved by digestates amendments, due to direct and indirect changes induced by organic matter supply (Oldare et al., 2008). Generally digestates contain high NH_4^+:N ratio, partially degraded organic matter, reduced biological oxygen demand (BOD), high pH values, low C:N ratio, and reduced viscosities compared to undigested animal manures (Asmus et al., 1988; Singh et al., 2010). It is well known that the available N fraction for plant is closely related to the NH_4^+ content of manures that usually is poorly available for the plant (Gutser et al., 2005). It is also often stated that degradation processes during anaerobic digestion will improve phosphorus (P) plant availability (Massè et al., 2011). Chiew et al. (2015) showed that the use of digestate as a fertilizer increases the content of macro- and micro-elements in the soil and plants. Moreover, the liquid phase of digestate can be used by sprinkler irrigation in fertigation systems. Several bioactive substances, such as phytohormones (*e.g.* gibberellins, indoleacetic acid), nucleic acids, monosaccharides, free amino acids, vitamins and fulvic acid, etc., able to increase the plant growth and the resilience to biotic and abiotic stress were found in anaerobic digestates (Liu et al., 2009; Yu et al., 2010; Möller and Müller, 2012). Some authors reported that digestate application has no phytotoxic effects (Gell et al., 2011) while others have found phytotoxic reactions (Abdullahi et al., 2008). Phytotoxicity can be related to NH_4^+ content (Drennan and Di Stefano, 2010) and to organic acids concentrations (Salminen and Rintala, 2002; Drennan and Di Stefano, 2010). The effect on crop yield are contradictory; indeed, some authors have shown its improvement in terms of harvest production while other authors have found no significant difference between AD-treated and -untreated crops (Möller and Müller, 2008). This variability depends on different factors such as: the form of the AD (liquid or semi-solid), the amount applied and the experimental conditions of the trial (pot or open-field). In general, when AD is applied in liquid form an increased nitrogen volatilization is recorded (Oldare et al., 2005). Investigations have shown that the vegetable nitrate content decreased significantly when applying digestates as an alternative to mineral fertilizers under soilless (Liu et al., 2009).

The criticisms related to the anaerobic digestate adoption for agricultural applications, are linked to potential environmental issues (Nokoa, 2014). In the first phase, the conversion of the carbonaceous compounds to methane and carbon dioxide is the most frequent reaction. As a consequence, a lower C/N ratio occur and the pH increase, since fatty acids are degraded and calcium ions are released from the degradation of organic matter. High pH and NH_4^+ concentration are conditions that favour NH_3 emission (Gutser et al., 2005; Svensson et al., 2004; Weiland, 2010). When anaerobic digestates is used for field application, nitrous oxide (N_2O) may also be significantly emitted (Vallejo et al., 2006). Also the potential contamination of surface and ground waters with excess nitrogen and phosphorus (Haraldsen et al., 2011) and the soil physical (plastics, glasses, stones) chemical (phytotoxic compounds heavy metals) and biological (pathogens bacteria such as *Salmonella, E. coli, Yersinia, Campylobacter* and the protozoa *Giardia* and *Cryptosporidium*) contaminations are matters of great interest (Nokoa, 2014). In particular, the heavy metals content, derived from the used matrices, are the main limiting factors for the AD use as fertilizer.

Among these, Zn, Cu, Mn, and microelements Ni, Pb, Cr, Cd, As are the main represented (Gosens et al., 2013). Their increase into the soil could cause phytotoxicity for the plants and an increase of the pollution for the agroecosystems. However, throughout the two-stage digestion, heavy metals can be transferred to the leachate metals and can then be removed by adsorption. This is possible when during the first stage of digestion the hydrolysis/acidification and liquefaction occurs (Selling et al., 2008).

Based on the above reported researches, this study wants to fill in some gaps lacking in literature, to the best of our knowledge. Indeed, the main aims of the present study are: i) producing new knowledge on continuous anaerobic co-digestion of feedstock mixture composed by different Mediterranean agri-food wastes in terms of multielemental characterization of intermediate fractions and final digestates and ii) assessing the agronomic value of industrial anaerobic digestate based on its potential as fertiliser in nursery condition for the citrus rootstocks production. The novelty of the study is linked to the demonstration of the feasibility of producing a valuable digestate, immediately usable as fertiliser in nursery conditions, through the joint co-digestion of organic wastes and byproduts, either of animal and vegetable origin, representative of the main agri-food supply chains in southern Italy.

2. Materials and methods

2.1. Feedstocks and continuous anaerobic co-digestion pilot-scale and industrial processes

2.1.1. Pilot scale trials

The Mediterranean biomasses selected as feedstocks (citrus processing wastes, olive mill wastewater, poultry manure, triticale silage, poultry litter, olive pomace, cattle manure, whey, straw, tomato peel), their relative percentages in the feedstock mixture, their chemical characterization in terms of total solids, volatile solids and biomethane potential of two continuous co-digestion pilot scale trials (the first performed for about 4 months, with a hydraulic retention time (HRT) equal to 50 days, and the second, started with a different bacterial inoculum, performed with a specific adaptation ramp for 54 days, with a HRT equal to 54 days) carried out at the Centro Ricerche Produzioni Animali, Reggio Emilia, Italy (CRPA Lab) are described in the recent work by Valenti et al. (2020). During the first pilot scale trial the total conversion of the organic matter into biogas was avoided presumably due to the accumulation of volatile fatty acids into the reactor. Indeed, in order to avoid the microbial inhibition of the process, the feeding diet of the second pilot plant was adapted with a ramp consisting in first replacing 1/3 of citrus pulp and olive mill wastewater by cattle manure, then gradually increasing the two vegetal by-products to partially replace the cattle manure until the process became stable. During these two continuous co-digestion pilot scale trials, intermediate (organic matter loading in daily progress) and final digestate (steady state of the AD process) fractions were sampled (100 g each) and stored at −20 °C until analysis with respect to their micro- and macronutrients profile whose results are herein reported.

2.1.2. Industrial scale trial

Based on the previous results of the pilot scale trials (Valenti et al., 2020) jointly combined with the results of the multielemental characterization herein reported, an industrial continuous anaerobic co-digestion process was carried out at AB GROUP Soc. Agr. S.r.L. (Comiso, Italy) in a 600 kWh continuous industrial biogas plant (Austep spa, Italy). The employed feedstock mixture and the operative conditions applied for the industrial production of the digestate used for the agronomic study were the same used for the second pilot scale trial. The industrial production of the digestate, realized thanks to the collaboration of AB GROUP partner, was carried out due to the need to produce the quantities of digestates needed to carry out the agronomic tests on seedlings. The final industrial semi-solid anaerobic digestate was characterized with respect to its main physico-chemical characteristics and further used for the agronomic trials herein reported. Pollutants (Cd, As, Pb) levels were measured in order to check the compliance with legal limits imposed by the Italian law (MIPAAF Decree n. 5046/2016) for agroindustrial digestate. Pb concentration was equal to 0.8 mg kg^{-1} TS (legal limit ≤ 140 mg kg^{-1} TS); Cd concentration was equal to 0.2 mg kg^{-1} TS (legal limit ≤ 1.5 mg kg^{-1} TS) while As concentration was < 0.2 mg kg^{-1} TS (no legal limits imposed by current national legislation).

2.2. Micro- and macronutrients profile of intermediate fractions and final digestates of the pilot scale processes

Intermediate weekly fractions (samples I to X for the first pilot scale trial; samples I to IV for the second pilot scale trial) and final digestates (sample XI for the first pilot scale trial; sample V for the second pilot scale trial) were analysed respect to their micro- (Fe, Zn, Mn, Cu, Li, Sr, Ba) and macronutrients (N, P, K, Ca, Mg, Na) content by Inductively Coupled Plasma Spectrometry (ICP-OES Optima 2000DV, Perkin Elmer, Italy). The sampling week interval for micro- and macronutrients analysis was specifically selected based on previous experimental trials and also taking into consideration the daily OLR (4.8 kg m^{-3} and 3.3 kg m^{-3}, for the first and the second trial, respectively) respect to the HRT of each of the two trials. Samples (10 mL) were exposed to dry digestion in a muffle furnace at 550 °C for 24 h. Ashes were then dissolved in a solution containing 4 mL of distilled water and 0.5 mL of concentrated nitric acid. The solutions were poured into a flask and made up to 50 mL with distilled water before the measurements. Nutrients levels of all the samples were expressed as mg kg^{-1} FW. Both final digestates samples were herein expressed either as mg kg^{-1} FW and %TS, in order to evaluate their potentiality to be used in agriculture.

2.3. Digestate agronomical evaluation

2.3.1. Site description, plant material, training system and experimental design

The research was carried out in the ‘San Salvatore’ experimental farm of CREA-Research centre for Olive, Fruit and Citrus Crops. The farm is located in Acireale (Catania, CT) in the East coast of Sicily, which has a warm, dry Mediterranean climate (Cicala et al., 2002). The geographical location is 37°31′ N, 15°09′ E and 200 m above sea level. Carrizo citrange (*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.) (Ccar) and Volkamer lemon (*Citrus volkameriana* Ten. & Pasq.) (Vlem) seedlings were used for the trial. The plants were produced by a commercial nursery closed to the experimental farm. Seeds were sown in nursery in March 2018 in black polyethylene (PE) seed trays filled with black peat. In mid-November 2018 seedlings were transplanted individually to round black PE container pots, each of the volume of 6.5 L (25 cm high × 20 cm diameter). In mid-March 2019, seedlings were transferred at the CREA screen-house covered with white shade-net (light transmission 35%). The plant density was 15 containers/m^2, with one seedling per pot. Irrigation was applied independently to each container. After replanting, no fertilizers were applied. The period of the trial (ca. 7 months) was from four months after the transplanting to pots on 15 November 2019 to 30 July 2019. The used growth media was that adopted by the commercial nursery that produced the seedlings. It was prepared using 50% of sandy volcanic soil and the remaining 50% being black peat (25%) and lapillus (25%). Each treatment was arranged in a completely randomized design, on 2 rows containing 12 pots each. Seven (7) index plants (7 plants × 2 rootstocks × 7 treatment = 98 seedlings) were chosen for the biometric and biomass measurements (Fig. 1). The physico-chemical properties of the growing media were analysed on three replicate samples (March 2019). Total nitrogen (N) was measured by Kjeldahl digestion (Bremner and Mulvanay, 1982) and expressed as g kg^{-1}, organic matter (OM %) was obtained by quantifying total organic carbon (TOC %) according to Springer and Klee (1954) and applying the conversion factor: OM (%) = TOC (%) × 1.72. The

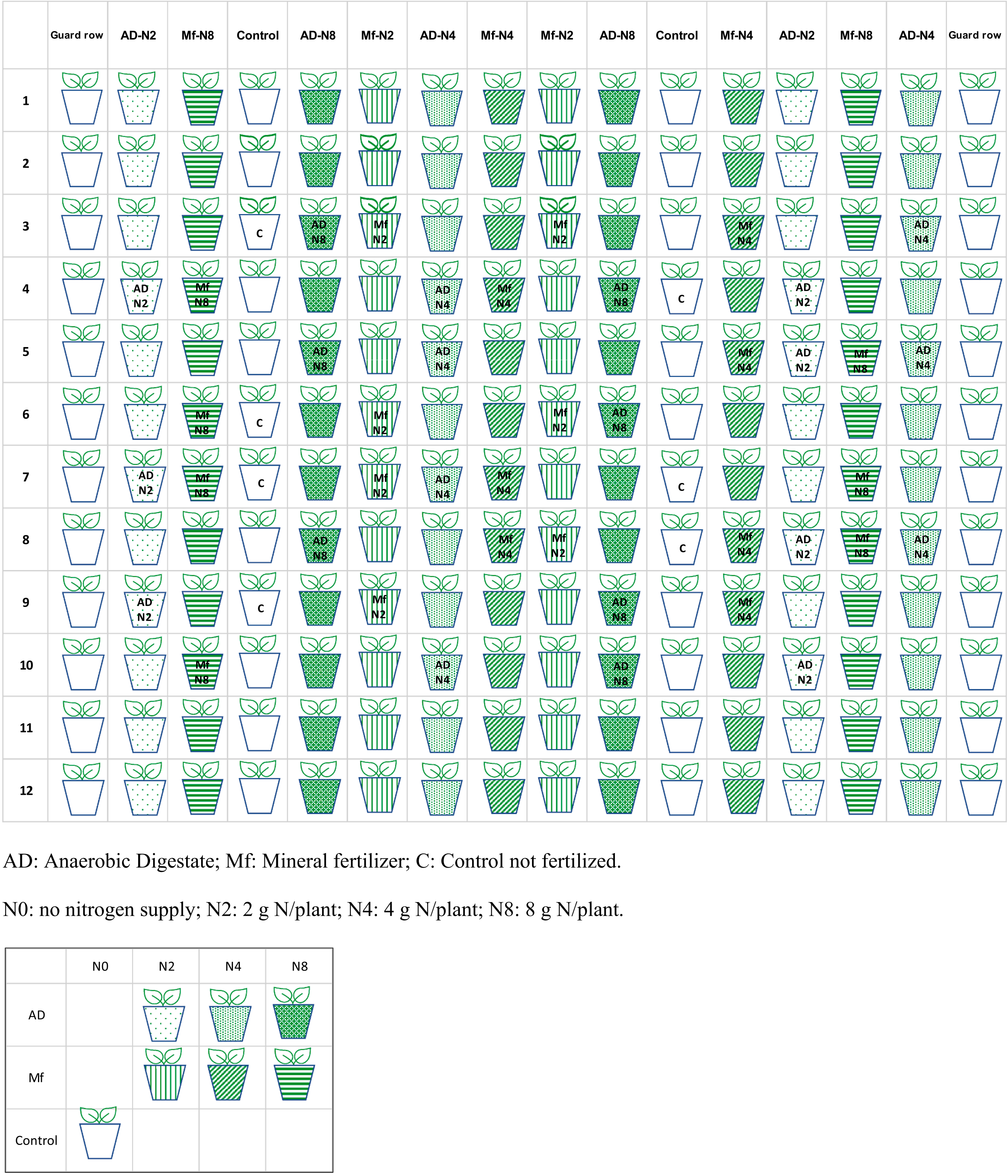

Fig. 1. Randomized experimental design. The 7 index plants of each thesis, here marked with the name of the thesis, were randomly distributed in two rows of 12 seedlings. The same experimental design was used for the two seedlings studied (Citrange carrizo and Volkamer lemon).

electrical conductivity (EC, mS m^{-1}; soil:water ratio, 1:2.5) and pH (soil: water ratio, 1:2.5) determinations were carried out using an HI 9813 portable EC meter (Hanna Instruments, Woonsocket, RI, USA) and an AB 15 pH meter (Thermo Fisher Scientific, Waltham, MA, USA) (EN 13037, 1999; EN13038, 1999), respectively. The N content was of 2.2 g kg^{-1} while 10% of OM was registered. The pH and the EC were 4.6 and 4.4 mS m^{-1}, respectively.

2.3.2. *Anaerobic digestate and mineral fertilizer*

The adopted industrial anaerobic digestate (AD) was in a semi-solid state and it was analysed for its main physico-chemical characteristics respect to N content, NH_4^+ content, TOC, OM, pH and EC by using the same methods described above. Total solids (TS), volatile solids (VS) and fixed solids (FS) were determined according to standard methods (US EPA, 2001). As mineral fertilizer (Mf), a commercial solid ammonium nitrate (NH_4NO_3) was used. Both for the anaerobic digestate and for the mineral treatments the amount of total nitrogen was 2 g/plant (N2), 4 g/plant (N4) and 8 g/plant (N8), supplied in one (21 March 2019), two (21 March and 28 March 2019) and four (21 March, 28 March, 4 April, 11 April 2019) doses of 2 g N/plant, equal to 87.42 g of anaerobic digestate and 9.6 g of ammonium nitrate, respectively.

2.3.3. Treatments

The seven (7) treatments were: for Citrange carrizo (i) Anaerobic Digestate 2 g N/plant (Ccar-AD-N2); (ii) Anaerobic Digestate 4 g N/plant (Ccar-AD-N4); (iii) Anaerobic Digestate 8 g N/plant (Ccar-AD-N8); (iv) Mineral fertilizer 2 g N/plant (Ccar-Mf-N2); (v) Mineral fertilizer 4 g N/plant (Ccar-Mf-N4); (vi) Mineral fertilizer 8 g N/plant (Ccar-Mf-N8); (vii) Control (C, not fertilized); for Volkamer lemon (i) Anaerobic Digestate 2 g N/plant (Vlem-AD-N2); (ii) Anaerobic Digestate 4 g N/plant (Vlem-AD-N4); (iii) Anaerobic Digestate 8 g N/plant (Vlem-AD-N8); (iv) Mineral fertilizer 2 g N/plant (Vlem-Mf-N2); (v) Mineral fertilizer 4 g N/plant (Vlem-Mf-N4); (vi) Mineral fertilizer 8 g N/plant (Vlem-Mf-N8); (vii) Control (C, not fertilized).

2.3.4. Seedlings biometric and biomass measurements

Seedling growth was monitored at 7-day intervals starting from mid-March to the mid-June, measuring the stem height. Destructive measurements were carried out on three seedlings per treatment to determine the dry matter content and partitioning between the main plant components: tap root, I order roots, II order roots, shoot system and leaves. These measurements were done at the end of June 2019, 7 months after transplanting the seedlings.

2.3.5. Seedlings physiological and nutritional status

The seedlings physiological and nutritional status was determined by measurements at the end of the trial. The chlorophyll index was measured using a portable chlorophyll meter soil plant analysis development index (SPAD). For chlorophyll content, aliquots of 50 mg (20–25 leaves, depending on the species) was taken and immersed in dimethyl sulfoxide (DMSO) at 65 °C, in darkness conditions until all the leaves appeared discoloured. Apparent chlorophyll content was assayed at interval of 30 to 120 min to test the effect of incubation time. Then, the chlorophyll content was analysed using a spectrophotometer. Chlorophyll “a” and “b“ content (mg g^{-1} fresh weight) was determined from absorbance at 663 nm and 645 nm using the formula of Arnon (1949) and Hiscox & Isrealstam (1979) then the chlorophyll a/b index was calculated. The seedlings nutritional status was determined by analysis of all the collected leaves (three sub-samples) of the three collected index plants. The N content analysis was carried out as described by Torrisi et al. (2013). Leaves were washed with distilled water and oven dried to constant weight at 65 °C for 24 h. A representative subsample was ground in a mill (IKA®, Werke Staufen, Germany) and the N content (g kg^{-1} dw) was determined by micro-Kjeldahl digestion. The proline content was determined on fresh leaf tissue extracting in 5 mL of 3% sulfosalicylic acid (Panreac, Barcelona, Spain) using a homogenizer (Ultra -Turrax, IKA-Werke. Staufen, Germany) at maximum speed. After a centrifugation at 4,000 × g for 35 min at 4 °C, proline was determined as described by Bates et al. (1973). Briefly, 1 mL of the supernatant was added to 2 mL of a mixture of glacial acetic acid and ninhydrin reagent (Panreac, Barcelona, Spain) in a 1:1 (v:v) ratio. The reaction mixture was incubated in a water bath at 100 °C for 1 h and then partitioned against 2 mL of toluene. Absorbance was read in the organic phase at 520 nm. A standard curve was performed with proline (Sigma-Aldrich, Madrid, Spain).

2.4. Statistical analyses

Data were statistically analyzed by using STATISTICA 6.0 (StatSoft Italia srl, Vigonza, Padova, Italy). To determine the relationships between the evaluated parameters, Pearson correlation coefficients (r) were used. The statistical differences were assessed by variance analysis (ANOVA) and means partitioning was carried out by the Tukey’s HSD test. A two-way ANOVA of the factors “treatment” and “N supply” was conducted. A Mixed factorial ANOVA design was carried out to analyse the changes in mean scores of the dependent variable ‘seedlings height’, measured at six time points: 21 and 28 March, 4, 11 and 19 April, 21 June 2019, and their variation as a function of the treatment (seven levels).

2.5. Chemicals

Micro- and macroelements standard reagents were purchased from Merck KGaA (Darmstadt, Germany). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and all were of analytical grade.

3. Results and discussion

3.1. Micro- and macronutrients profile of intermediate fractions and final digestate of the pilot scale processes

Maintaining the optimal concentrations of micro- and macroelements in anaerobic digestion allows to optimize the microbiological process and avoid slowdowns in the degradation of the organic substance. Acute micronutrient deficiencies lead to strong inhibition processes which, if badly managed, can degenerate into acute phenomena of acidosis resulting in the blockage of the biogas production. Moreover, trace elements are essential for several enzymatic reactions to produce methane. Micro- (Fe, Zn, Mn, Cu, Li, Sr, Ba) and macronutrients (N, P, K, Ca, Mg, Na) levels of intermediate weekly fractions and final digestates of the two pilot scale processes are reported in Table 1. The results of the first trial showed a decreasing trend for macro- and micronutrients during anaerobic digestion process, except for Fe, Li and Ba. It is well known that trace elements are employed by microorganisms for their growth and physiological metabolism as well as internal osmoregulators (Weiland, 2010; Wackett et al., 2004). Considering that a low nutrients availability could be detrimental for maintaining the vitality of the microbiological process, different starting conditions were applied in the second pilot scale trial in terms of initial bacterial inoculum and specific adapted ramp for the feeding of the pilot plant, as reported by Valenti et al. (2020). Conversely, the second pilot scale trial showed increasing values for all the elements, except for Li. It must be pointed out that macronutrients (N, P, K, Ca, Mg, Na) concentrations in the final digestate deriving from the second trial were significantly higher compared to those of the first trial, with more than doubled Ca and Mg values. An explanation of this trend can be found in the different strategy applied for the starting of the second pilot scale trial. Indeed, in order to avoid the microbial inhibition of the process, the feeding diet of the pilot plant was adapted with a ramp consisting in first replacing 1/3 of citrus pulp and olive mill wastewater by cattle manure, then gradually increasing the two vegetal by-products to partially replace the cattle manure until the process became stable. N content, 5.0738 ± 0.2348 %TS and 9.1067 ± 0.2421 %TS for the first and second trial, respectively, shows the great agronomic potential of digestates obtained by anaerobic digestion of agro-industrial feedstocks mixtures, also complying with legal limits imposed by the Italian law (MIPAAF Decree n. 5046/2016) for both agro-zootechnical and agro-industrial digestates to be used in agriculture (N content $\geq$ 1.5 %TS). P content of the final digestate was well above the legal limits provided by the Italian law (MIPAAF Decree n. 5046/2016) which imposes a total P content $\geq$ 0.4 %TS for anaerobic agro-industrial digestate to be employed in agriculture. Indeed, the anaerobic digestate of the pilot scale trials showed P content equal to 1,0309 ± 0.0025 %TS and 2,0402 ± 0.0026 %TS for the first and second trial, respectively. Box Plot graphical representations of the two pilot scale trials showing interquartile ranges, medians and outliers of micro- (Fe, Zn, Mn, Cu, Li, Sr, Ba) and macronutrients (N, P, K, Ca, Mg, Na) are

Table 1

Micro- (Fe, Zn, Mn, Cu, Li, Sr, Ba) and macronutrients (N, P, K, Ca, Mg, Na) profile of intermediate fractions and final digestates of the pilot scale processes.

First pilot scale trial													
Sample	**N (mg kg^{-1} FW)**	**P (mg kg^{-1} FW)**	**K (mg kg^{-1} FW)**	**Ca (mg kg^{-1} FW)**	**Mg (mg kg^{-1} FW)**	**Na (mg kg^{-1} FW)**	**Fe (mg kg^{-1} FW)**	**Zn (mg kg^{-1} FW)**	**Mn (mg kg^{-1} FW)**	**Cu (mg kg^{-1} FW)**	**Li (mg kg^{-1} FW)**	**Sr (mg kg^{-1} FW)**	**Ba (mg kg^{-1} FW)**
Intermediate fractions													
I	2929.949 ± 6.46 D	2933.11 ± 5.51 A	3968.68 ± 9.73 AB	4914.14 ± 81.36 A	907.08 ± 8.81 A	1187.54 ± 16.76 A	169.42 ± 0.19 BCD	68.99 ± 0.48 A	56.45 ± 0.11 A	9.79 ± 0.05 A	8.58 ± 0.03 A	9.52 ± 0.01 A	4.65 ± 0.02B
II	6891.33 ± 95.07 AB	1762.96 ± 2.25B	3170.13 ± 15.29 CDE	3254.53 ± 27.62B	585.55 ± 2.56B	820.03 ± 11.69 BCD	183.16 ± 0.10 BC	28.19 ± 0.09B	34.14 ± 0.00B	5.29 ± 0.05CD	2.84 ± 0.01 EF	3.6 ± 0.004 D	3.40 ± 0.01B
III	6926.72 ± 70.23 AB	1646.41 ± 1.27BC	3444.57 ± 14.28 ABCD	2899.86 ± 6.76 BC	568.36 ± 2.96B	578.72 ± 2.35 FG	213.27 ± 0.43 AB	32.24 ± 0.01B	33.82 ± 0.04B	6.96 ± 0.02B	2.29 ± 0.01F	3.97 ± 0.00 D	4.14 ± 0.01B
IV	6533.49 ± 48.68 AB	1506.21 ± 3.61C	3884.92 ± 29.44 AB	2457.82 ± 18.93CD	450.54 ± 3.66C	968.64 ± 17.20B	161.88 ± 0.00CD	21.33 ± 0.16CD	24.18 ± 0.05 CDE	6.00 ± 0.01C	3.07 ± 0.01 EF	5.65 ± 0.01 BC	4.79 ± 0.00B
V	6175.22 ± 50.39 AB	871.46 ± 2.15F	2949.50 ± 7.17 DEF	1631.37 ± 12.24 EF	336.73 ± 1.87 DE	654.41 ± 5.10 EF	122.71 ± 0.32 D	18.61 ± 0.07 DE	16.62 ± 0.06F	2.92 ± 0.01 GH	4.50 ± 0.03CD	4.03 ± 0.02 D	4.09 ± 0.01B
VI	7281.05 ± 70.75 A	985.20 ± 1.89 EF	3627.57 ± 25.19ABC	1856.31 ± 16.64 EF	306.27 ± 1.30 E	881.19 ± 9.36 BC	144.09 ± 0.18CD	15.33 ± 0.03 DE	15.61 ± 0.02 FG	4.770 ± 0.02 DE	2.96 ± 0.01 EF	4.65 ± 0.00CD	4.90 ± 0.02B
VII	4362.13 ± 64.07C	1243.35 ± 7.37 D	4025.11 ± 22.35 A	2436.92 ± 74.47CD	401.12 ± 2.01CD	912.52 ± 15.08 BC	165.74 ± 0.48 BCD	19.58 ± 0.16 DE	18.56 ± 0.11EF	5.36 ± 0.05CD	4.03 ± 0.02 DE	5.94 ± 0.01B	6.83 ± 0.01 A
VIII	6883.27 ± 21.38 AB	598.70 ± 3.06 G	2344.63 ± 2.02F	1368.60 ± 3.28F	201.37 ± 0.39F	477.31 ± 4.56 G	123.91 ± 0.11 D	14.86 ± 0.03 E	11.84 ± 0.10 G	2.44 ± 0.00*H*	2.91 ± 0.01 EF	3.67 ± 0.01 D	3.34 ± 0.00B
IX	5831.84 ± 30.27B	1023.86 ± 220.19 DEF	2783.43 ± 611.33EF	2033.52 ± 455.46 DE	313.67 ± 66.98 E	672.99 ± 149.03 DEF	210.78 ± 45.56 AB	27.75 ± 6.02 BC	25.70 ± 5.73CD	3.91 ± 0.85 EF	5.37 ± 1.19C	5.32 ± 1.16 BC	7.50 ± 1.62 A
X	6309.93 ± 38.55 AB	1126.21 ± 4.39 DE	3340.97 ± 15.60 BCDE	2460.18 ± 25.07CD	345.32 ± 3.83 DE	783.74 ± 3.15 CDE	233.99 ± 0.26 A	32.54 ± 0.03B	29.22 ± 0.10 BC	4.59 ± 0.00 DE	6.82 ± 0.04B	6.51 ± 0.02B	8.35 ± 0.03 A
Final digestate													
XI	4562.51 ± 21.11C	926.98 ± 2.21 EF	2443.92 ± 18.60F	1972.02 ± 5.62 DE	368.02 ± 3.42 DE	540.45 ± 3.96 FG	187.11 ± 0.88 ABC	15.85 ± 0.17 DE	20.87 ± 0.04 DEF	3.55 ± 0.02 FG	4.55 ± 0.05CD	3.67 ± 0.00 D	6.69 ± 0.01 A
	N (% TS)	**P (% TS)**	**K (% TS)**	**Ca (% TS)**	**Mg (% TS)**	**Na (% TS)**	**Fe (% TS)**	**Zn (% TS)**	**Mn (% TS)**	**Cu (% TS)**	**Li (% TS)**	**Sr (% TS)**	**Ba (% TS)**
XI	5.0738 ± 0.2348	1.0309 ± 0.0025	2.7178 ± 0.0207	2.1930 ± 0.0063	0.4093 ± 0.0038	0.6010 ± 0.0044	0.2081 ± 0.0010	0.0176 ± 0.0002	0.0232 ± 0.0000	0.0039 ± 0.0000	0.0051 ± 0.0001	0.0041 ± 0.0000	0.0074 ± 0.0000
Second pilot scale trial													
	N (mg kg^{-1} FW)	**P (mg kg^{-1} FW)**	**K (mg kg^{-1} FW)**	**Ca (mg kg^{-1} FW)**	**Mg (mg kg^{-1} FW)**	**Na (mg kg^{-1} FW)**	**Fe (mg kg^{-1} FW)**	**Zn (mg kg^{-1} FW)**	**Mn (mg kg^{-1} FW)**	**Cu (mg kg^{-1} FW)**	**Li (mg kg^{-1} FW)**	**Sr (mg kg^{-1} FW)**	**Ba (mg kg^{-1} FW)**
Intermediate fractions													
I	3152.85 ± 70.17 BC	806.68 ± 2.73 D	2461.62 ± 4.92C	1544.37 ± 17.22 D	391.46 ± 3.04B	308.31 ± 5.39 E	142.29 ± 0.66B	26.05 ± 0.15 D	19.14 ± 0.10C	6.78 ± 0.01 E	2.52 ± 0.00 D	5.75 ± 0.06C	2.62 ± 0.00 D
II	2571.23 ± 47.33C	824.19 ± 1.63C	2419.12 ± 22.00C	1689.38 ± 16.47B	373.96 ± 1.69 D	367.32 ± 5.17 D	110.20 ± 0.06 D	28.25 ± 0.10C	16.74 ± 0.03 E	16.84 ± 0.10 D	3.29 ± 0.01C	5.80 ± 0.00C	3.13 ± 0.00B
III	3695.01 ± 12.30 AB	863.16 ± 5.06B	3013.91 ± 14.28 A	1618.53 ± 10.11C	373.32 ± 1.23 D	513.05 ± 2.12B	106.00 ± 0.11 E	34.14 ± 0.32B	20.56 ± 0.04B	18.04 ± 0.21C	5.58 ± 0.01 A	5.77 ± 0.01C	3.04 ± 0.01C
IV	4277.78 ± 23.70C	733.72 ± 3.86 E	2604.00 ± 12.34B	1561.68 ± 21.67CD	382.33 ± 0.31C	457.35 ± 2.34C	136.56 ± 0.03C	27.56 ± 0.23C	18.60 ± 0.19 D	19.93 ± 0.05B	5.37 ± 0.06B	6.15 ± 0.02B	3.16 ± 0.00B
Final digestate													
V	6573.73 ± 37.53 A	1472.73 ± 1.87 A	2980.60 ± 6.91 A	3314.64 ± 27.43 A	642.74 ± 1.84 A	693.10 ± 1.72 A	149.02 ± 0.28 A	60.38 ± 0.36 A	35.03 ± 0.00 A	25.79 ± 0.46 A	3.37 ± 0.02C	10.80 ± 0.05 A	5.04 ± 0.02 A
	N (% TS)	**P (% TS)**	**K (% TS)**	**Ca (% TS)**	**Mg (% TS)**	**Na (% TS)**	**Fe (% TS)**	**Zn (% TS)**	**Mn (% TS)**	**Cu (% TS)**	**Li (% TS)**	**Sr (% TS)**	**Ba (% TS)**
V	9.1067 ± 0.2421	2.0402 ± 0.0026	4.1291 ± 0.0096	4,5918 ± 0.0380	0.8904 ± 0.0026	0.9602 ± 0.0024	0.2064 ± 0.0004	0.0837 ± 0.0005	0.0485 ± 0.0000	0.0357 ± 0.0006	0.0047 ± 0.0000	0.0150 ± 0.0001	0.0070 ± 0.0000

Data are expressed as means of three analytical replicates ± standard deviation; $p \leq 0.01$ – capital letters within the same column for each of the two pilot scale trials; Nutrients levels of both final digestates are herein expressed either as mg kg^{-1} FW and %TS in order to evaluate their potentiality to be used in agriculture.

shown in Fig. 2 and Fig. 3, respectively. The ranges of variations herein reported for each of the analysed elements could conveniently be used for the commercial scale-up of the anaerobic digestion process and for the optimization of the diet of biogas plants fed by agro-industrial feedstocks mixtures, also facing potential regulatory issues for the agronomic use of digestate. Valenti et al. (2020) have recently shown that the operative conditions of the second pilot scale trial - *i.e.* i) specific bacterial inoculum recovered from previous anaerobic digestion with similar agri-food feedstocks; ii) adapted ramp providing increasing percentages of agri-food feedstocks, produced better results in terms of methanogenic potential and performance in terms of volatile fatty acids (VFA) accumulation. Results of the present study confirms these findings in terms of multielemental composition of the final digestate. Indeed, taking into consideration the micro-and macronutrient data herein reported, the anaerobic digestate produced in the second pilot scale trial presents an improved quantitative composition respect to that produced with the first trial. Moreover, its nutritive characteristics makes it very interesting for agronomic purposes and applications.

3.2. *Digestate agronomical evaluation*

3.2.1. *Industrial anaerobic digestate physico-chemical traits*

The results of the physico-chemical characterization of the adopted industrial anaerobic digestate (AD) are reported in Table 2. Based on the physico-chemical analysis, the adopted AD was useful to replace the mineral fertilizers in terms of total N content (10.81 ± 0.32% TS). Moreover the high amount of organic matter content (43.32 ± 0.80% TS) could be useful for the improvement of the efficacy of mineral fertilizers suggesting the possibility to couple them with the AD. This because OM could reduce the leaching of minerals such as nitrogen ($NO3^-$) improving water retention and increasing the cation exchange capacity. Moreover, an high content of OM into the pot or soil reduce the EC and pH fast modification thus improving the stability of the soil or substrate. Both parameters are in the range of the qualitative traits imposed by the Italian law (MIPAAF Decree n. 5046/2016) for anaerobic agro-industrial digestate to be employed in agriculture (OM $\geq$ 20% TS; N $\geq$ 1.5% TS). About nitrogen, as a consequence of its predominant form as $NH4^+$ into the AD, when it is used as fertilizer, the availability in ammonium fastly occurs and it represents the main source for the plant. As far as the organic matter is concerned, it can ensure an improvement of the substrate physico-chemical characteristics and the absorption of some low mobile macro- and micronutrients such as potassium, phosphorous and iron. It is known that the organic carbon from digestate is stable in open field for about one year (Vaneeckhaute, 2016), therefore in our controlled conditions probably the OM influenced the studied seedlings. Also the moisture content and the pH value were in line with those found in AD obtained from livestock by-products and non-woody crops biomasses (Campos et al., 2019; Marti et al., 2010; Lahav et al., 2013). In particular, the pH level is optimal for the struvite of the phosphorous and its precipitation process useful for the recovery of this nutrient that become suitable for the plant (Li et al., 2016). The increase in pH depends on the increase in ammonium carbonate ($(NH4)_2CO_3$) synthesis during the anaerobic digestion and also to the volatilization of CO_2 (Webb and Hawkes, 1985; Sommer and Husted, 1995), however during the struvite process, in absence of a Mg^{2+} source, a fast pH drop is possible as a consequence of the release of H+ ions in solution (Möller and Müller, 2012; Campos et al., 2019). The electrical conductivity (26.1 mS cm^{-1}) is considered as very high compared to other values reported by Coelho et al. (2018) which ranged between 0.152 and 0.590 mS cm^{-1}. Voelkner et al. (2015) report that this parameter is of high importance when AD is used as fertiliser since it is possible an increase in electrical conductivity in loamy and sandy soils, therefore in similar conditions compared to those of the present study. The salinity symptoms are possible when AD at high salt levels are used in continuous applications (Alburquerque et al., 2012). Anyway, in the present trial, the used seedlings, both Ccar and Vlem, did not show any symptom of salinity (Fig. 1S). While for the treatments N2 and N4 one and two doses were applied, respectively, for the treatment N8 four doses were used during a period of 30 days, and also in this case, no symptoms were observed (Fig. 1S).

3.2.2. *Seedlings biometric, biomass measurements, physiological and nutritional status*

The seedlings morphological characteristics are reported in Table 3. For the Ccar all the registered parameters did not show significant difference among treatments, while the Vlem registered differences for taproot, 1st order roots and 2nd order roots. In particular, the taproot dry matter percentage was significantly higher in the treatments with mineral fertilizer at N2 and N8 concentration. The 1st order roots were similar in all the treatments except the seedlings treated with AD N8. The 2nd order roots were higher in Mf N8 and N2 compared to AD N2. On the contrary, the Vlem did not register differences. No significant differences were found for trunk and leaves dry matter percentage among all the treatments. About biochemical measurements (Table 4), concerning the SPAD index, significant differences were recorded only for Ccar, while the total chlorophyll content and the chlorophyll a/b ratio, the total nitrogen content and the proline showed several differences between seedlings and treatments. For Ccar the highest total

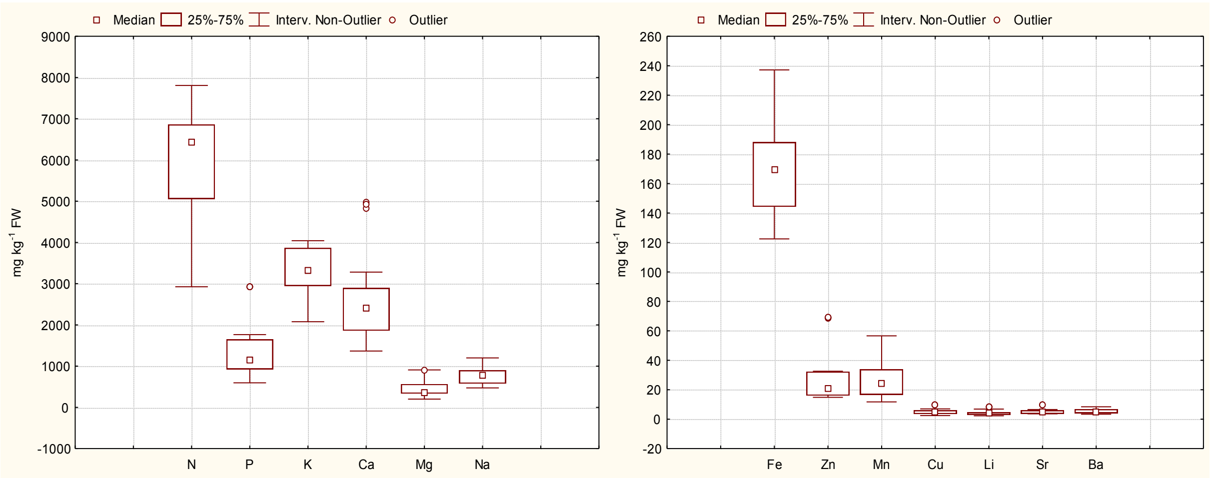

Fig. 2. Box Plot representation of macro- (N, P, K, Ca, Mg, Na) and micronutrients (Fe, Zn, Mn, Cu, Li, Sr, Ba) profile of the first pilot scale process (N = 11, three analytical replicates per each sample).

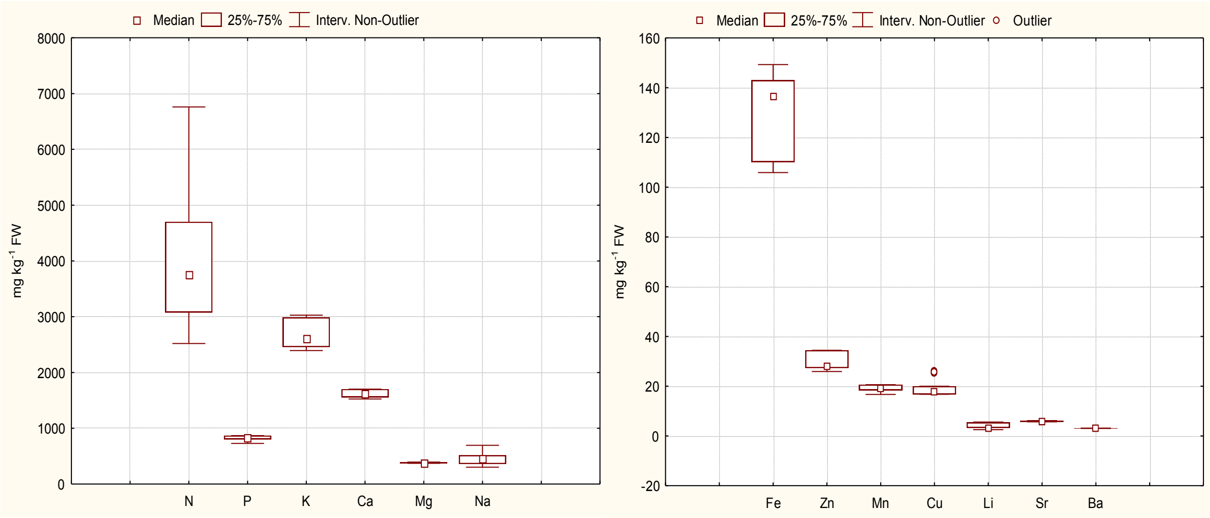

Fig. 3. Box Plot representation of macro- (N, P, K, Ca, Mg, Na) and micronutrients (Fe, Zn, Mn, Cu, Li, Sr, Ba) profile of the second pilot scale process (N = 6, three analytical replicates per each sample).

Table 2
Physico-chemical parameters of the industrial anaerobic digestate.

	Total solids (TS)	**Volatile solids (VS)**	**Fixed solids (FS)**	**Total Kjeldhal Nitrogen (TKN)**		**$NH4^+$-N**	**Total organic carbon (TOC)**	**Organic matter (OM)**		**pH**	**Electrical conductivity**
	%	**% TS**	**% TS**	**‰**	**% TS**	**% TKN**	**%**	**%**	**% TS**		**mS cm^{-1}**
Anaerobic digestate	4.34 ± 0.05	50.49 ± 2.33	49.51 ± 2.33	4.69 ± 0.14	10.81 ± 0.32	59 ± 0.08	0.94 ± 0.02	1.88 ± 0.03	43.32 ± 0.80	8.19 ± 0.26	26.1 ± 1.10

Data are expressed as means of three replicates ± standard deviation.

Table 3
Biometric and biomass measurements.

Seedlings	**Treatment**	**N supply (g/plant)**	**Taproot (g dw)**	**Roots I order (g dw)**	**Roots II order (g dw)**	**Trunk (g dw)**	**Stem (g dw)**	**Leaves (g dw)**
Carrizo citrange	Anaerobic digestate	N2	17.10 ± 4.28a	4.26 ± 0.88A	5.70 ± 2.72	43.18 ± 7.50	29.61 ± 6.78	15.71 ± 2.45
		N4	17.50 ± 1.08a	2.83 ± 0.81AB	6.28 ± 0.78	39.50 ± 9.89	33.45 ± 12.74	13.93 ± 1.45
		N8	14.89 ± 0.81ab	2.41 ± 0.99AB	6.40 ± 1.47	40.12 ± 3.91	25.58 ± 6.34	15.13 ± 2.61
	Mineral fertilizer	N2	13.39 ± 0.94ab	2.70 ± 0.36AB	5.03 ± 0.30	34.79 ± 8.77	31.90 ± 19.13	14.60 ± 1.04
		N4	16.41 ± 3.42ab	2.89 ± 1.03AB	6.26 ± 2.07	33.50 ± 5.82	24.92 ± 8.56	16.38 ± 3.06
		N8	8.30 ± 5.78b	1.23 ± 0.71B	4.52 ± 0.50	29.08 ± 2.51	19.71 ± 3.95	14.84 ± 2.92
	Control	N0	13.76 ± 0.54ab	3.02 ± 0.12AB	7.73 ± 2.34	39.52 ± 4.68	21.38 ± 1.82	13.68 ± 0.72
	F(66, 26.859) = 11.276, p = .00000							
Volkamer lemon	Anaerobic digestate	N2	11.61 ± 1.84	3.42 ± 2.31	6.56 ± 2.31	26.41 ± 9.43	14.27 ± 5.56	47.18 ± 8.02
		N4	12.58 ± 1.88	2.64 ± 2.45	7.22 ± 2.45	34.44 ± 6.52	13.89 ± 5.83	50.42 ± 15.21
		N8	11.54 ± 1.93	2.21 ± 0.60	7.35 ± 0.60	35.47 ± 3.06	19.79 ± 6.94	52.60 ± 8.92
	Mineral fertilizer	N2	12.65 ± 3.20	3.24 ± 1.89	5.25 ± 1.89	26.45 ± 10.88	14.68 ± 4.91	49.78 ± 3.20
		N4	11.30 ± 1.20	3.28 ± 0.89	5.17 ± 0.89	28.23 ± 3.39	13.49 ± 2.16	57.46 ± 19.19
		N8	8.37 ± 1.08	1.24 ± 0.46	6.77 ± 0.46	33.47 ± 5.44	15.88 ± 1.85	48.87 ± 7.99
	Control	N0	14.55 ± 2.41	2.06 ± 0.82	6.26 ± 0.82	32.85 ± 4.83	9.81 ± 4.24	43.18 ± 2.21
	F(66, 26.859) = 3.6846, p = .00019							

Data are expressed as means of three replicates ± standard deviation. Values within the same column, related to each rootstock, indicated by different letters are significantly different (capital letters $p < 0.01$; small letters $p < 0.05$) based on Tukey's HSD.

chlorophyll content was found for Mf N2 treatment, while the best performances of the plants treated with AD were those at N4 and N8 concentration. The treatment at N2 was lower and similar to the control. The performance of the Vlem was similar since the Mf N8 showed the best result but these were similar to the AD N4 and N8. Also, in this case the AD N2 was analogous to the control. The chlorophyll a/b ratio was higher in Mf N4 compared to Mf N2, while the AD treatments were similar within all the nitrogen concentration and higher compared to the

Table 4
Physiological and nutritional status.

Seedlings	Treatment	N supply (g/plant)	SPAD (%)	Total chlorophyll (mg g^{-1} fw)	Chlorophyll a/b ratio	N (g kg^{-1} dw)	Proline (μmoli/g fw)
Carrizo citrange	Anaerobic digestate	N2	62.2 ± 5.74BC	1.93 ± 0.09CD	4.43 ± 0.81ab	23.8 ± 1.9D	11.10 ± 0.05 BC
		N4	67.1 ± 4.24AB	2.37 ± 0.30BC	4.01 ± 1.40ab	29.2 ± 3.2CD	11.28 ± 0.01BC
		N8	73.9 ± 1.47AB	2.19 ± 0.04BC	4.80 ± 0.22ab	32.9 ± 2.5C	11.20 ± 0.28BC
	Mineral fertilizer	N2	73.5 ± 3.40AB	3.16 ± 0.19A	3.53 ± 0.15b	35.9 ± 3.9BC	11.60 ± 0.22B
		N4	74.9 ± 0.91A	2.70 ± 0.04AB	6.86 ± 2.20a	43.2 ± 2.7AB	10.38 ± 0.23D
		N8	78.3 ± 2.68A	2.66 ± 0.36AB	5.38 ± 0.82ab	46.0 ± 0.4A	10.88 ± 0.00CD
	Control	N0	50.6 ± 2.58C	1.25 ± 0.01D	6.03 ± 1.15ab	1.96 ± 0.14E	37.75 ± 0.09A
	F(66, 26.859) = 11.276, p = .00000						
Volkamer lemon	Anaerobic digestate	N2	43.6 ± 3.24	1.90 ± 0.23BC	3.97 ± 1.69	22.4 ± 1.4ab	12.37 ± 0.12B
		N4	53.1 ± 11.67	2.28 ± 0.08AB	2.35 ± 0.12	26.4 ± 1.1ab	13.53 ± 1.24B
		N8	52.0 ± 5.43	2.50 ± 0.12AB	3.24 ± 0.94	33.8 ± 5.6a	12.19 ± 0.14B
	Mineral fertilizer	N2	49.3 ± 5.36	1.99 ± 0.25BC	2.36 ± 0.07	33.8 ± 1.8a	13.78 ± 0.36B
		N4	53.6 ± 3.42	2.00 ± 0.51BC	3.33 ± 1.16	33.6 ± 0.7a	12.63 ± 0.01B
		N8	56.7 ± 7.99	2.97 ± 0.31A	3.08 ± 0.45	31.3 ± 1.3ab	12.86 ± 0.33B
	Control	N0	51.3 ± 8.35	1.27 ± 0.06C	2.49 ± 0.14	19.8 ± 2.3b	38.17 ± 0.86A
	F(66, 26.859) = 3.6846, p = .00019						

Data are expressed as means of three replicates ± standard deviation. Values within the same column, related to each rootstock, indicated by different letters are significantly different (capital letters $p < 0.01$; small letters $p < 0.05$) based on Tukey's HSD.

control. No differences for this ratio were found for Vlem. The total nitrogen content in Ccar seedling treated with AD was lower at each concentration compared to the mineral fertilization, despite the seedling treated with AD showed a better performance compared to the control. Our results in Ccar suggest that highest N-supply resulted in higher TKN within each material (Mf or AD), thus suggesting being responsive to the N-dosage range selected. For the Vlem the nitrogen content was similar for all the treatments. Among the AD treatments, the N8 concentration was the best. The proline content for both controls was higher respect to both AD and Mf treatments. Among the treatments, the plants treated with Mf at N2 and N4 tended to show the better status referred to the nitrogen content. On the contrary, for the Vlem, no differences among treatments were detected. Proline, in addition to being an excellent osmolyte, plays three main roles during stress, namely, as a metal chelator, as an antioxidant defence molecule and as a signalling molecule. When exposed to highly stressful conditions, plants accumulate a series of metabolites. An overproduction of proline, confers tolerance to stress while maintaining the right cellular turgor and osmotic balance. The proline level recorded in both Ccar and Vlem controls, is in line with what has been stated, confirming this amino acid as an effective indicator not only linked to water deficit conditions but also to nutritional stress. The AD-treated seedlings nutritive status showed that no need for supplemental of nutrients was requested. This doesn‘t agree with the observation of Liedl et al. (2006) that reports results in which the AD represents an incomplete fertilizer for the normal plant growth. Nitrate ($NO3^{-}$) and ammonium ($NH4^{+}$) are the main forms of available nitrogen in the soil for plants. Excessive $NH4^{+}$ accumulation is toxic in plant tissues and the exclusive administration of $NH4^{+}$ increases this effect. Ammonium toxicity syndrome commonly includes, among others, growth alterations, ion imbalance and chlorosis. In particular, when the plants have been treated with liquid digestate, it is interesting to note a positive correlation between the chlorophyll content and the nitrogen level ($r = 0.7990$, $p \leq 0.01$ for Vlem and $r = 0.3207$, $p \leq 0.05$ for Ccar). The characteristics of the nitrogen contained in the adopted liquid digestate is the ammonia component; in fact, the latter is particularly represented in the digestate (59 ± 0.08 %TKN), this being a positive trait for a fertilizer to be used in agriculture (Table 2). Therefore, chlorosis appears to be an exclusive effect of ammonium toxicity, while mild ammonium stress stimulates the accumulation of chlorophyll (Sanchez Zabala et al., 2015; Li et al., 2014). In addition, photosynthetic rates induced by high irradiation have been shown to promote ammonium tolerance by providing more carbon skeletons for the assimilation of $NH4^{+}$ (Setien et al., 2013; Ariz et al., 2011). Therefore, the improvement of the chlorophyll content in the leaf represents a valid tool for the CO_2 assimilation. A slight ammonium stress, in addition to increasing the total chlorophyll content, contributes to reduce the chlorophyll a/b ratio; this determines a greater activity of the antenna or accessory pigments. Furthermore, it has been noted that the greatest correlation between the total chlorophyll and the total nitrogen is borne by the Vlem, data confirmed by other studies (Aboutalebi and Khankahdani, 2012). This allows to deduce that the Vlem highly benefits from the administration of liquid digestate, increasing the total chlorophyll level due to the higher ammonium content. Moreover, the rapid infiltration of the liquid digestate and the presence of ammonia in the form of the hydrated ammonium ion ($NH4^{+}$), allows its rapid immobilization on the exchange sites of the soil colloids, therefore retained and not lost by leaching (Scherer, 1993). A two-way ANOVA of the factors “treatment” and “N supply” was conducted (Table 5). The main effects of the factors considered were of different magnitude for the two rootstocks. In the case of Ccar, there was a main effect of treatment on SPAD, total chlorophyll and N, significantly higher for Mf than AD, where, conversely, proline, taproot and 1st order roots, were significantly lower. This result supports the hypothesis that a lower N supply corresponds to a lower N content of the leaf tissue and a lower SPAD, while the production of proline, and the development of taproots and 1st order roots, with their role in increasing the surface area of roots to promote increased uptake of water and minerals from the soil, is stimulated to cope with the shortage (Kishor et al., 1995) As for Ccar, the treatment influenced the N content also for Vlem, which was significantly higher for Mf, but had no effect on the other parameters.

For Ccar, increased N supply was significantly associated with increased SPAD and N content and was inversely correlated with 1st order roots development. The lower proline production was found in the intermediate dose, N4, which also showed the greatest development of taproots. For Vlem, only total chlorophyll was significantly affected by N doses, increasing as the N supply increased. The ‘treatment*N supply’ interaction had effect on total chlorophyll of Ccar, where the two lowest doses, AD*N2 and Mf*N2, produced significantly different levels, higher for the latter. The higher proline level and the lower chlorophyll a/b ratio were also associated with the Mf*N2 interaction, significantly when compared to Mf*N4. Regarding Vlem, the ‘treatment*N supply’ interaction was associated only with proline levels, with Mf*N2

Table 5

Main effects of treatment and N supply and their interaction on biomass measurements and physiological and nutritional status of Citrange carrizo and Volkamer lemon rootstocks.

Seedlings	Main effects		SPAD (%)	Total chlorophyll (mg g^{-1} fw)	Chlorophyll a/b ratio	N (g kg^{-1} dw)	Proline (µmoli/g fw)	Taproot (g dw)	Roots I order (g dw)	Roots II order (g dw)	Trunk (g dw)	Stem (g dw)	Leaves (g dw)	Num.
Citrange carrizo	Treatment	AD	67,7 ± 6,25B	2,2 ± 0,25B	4,4 ± 0,89	28,6 ± 4.5B	11,2 ± 0,17 A	16,5 ± 0 A	3,2 ± 2,56 A	6,1 ± 1,14	38,7 ± 1,63	29,5 ± 12,13	14,9 ± 8,59	9
		Mf	75,6 ± 3,08 A	2,8 ± 0,32 A	5,3 ± 1,86	41,7 ± 5.1 A	11 ± 0,55B	12,7 ± 0B	2,3 ± 4,85B	5,3 ± 0,93	32,5 ± 1,35	25,5 ± 6,33	15,3 ± 12,29	9
	F(11, 2) = 9.6566, p = .09751													
	N supply (g/plant)	N2	67,9 ± 7,49B	2,5 ± 0,69	4 ± 0,72	29,9 ± 7.2B	11,3 ± 0,31 A	15,2 ± 0 ab	3,5 ± 3,44 A	5,4 ± 1,05	39 ± 1,77	30,8 ± 8,62	15,2 ± 12,9	6
		N4	71 ± 5,04 AB	2,5 ± 0,26	5,4 ± 2,27	36,2 ± 8.1 AB	10,8 ± 0,52B	17 ± 0 a	2,9 ± 2,11 AB	6,3 ± 0,63	33,2 ± 1,43	29,2 ± 13,89	15,2 ± 11,43	6
		N8	76,1 ± 3,09 A	2,4 ± 0,35	5,1 ± 0,62	39,4 ± 7.3 A	11 ± 0,25 AB	11,6 ± 0b	1,8 ± 5,16B	5,5 ± 1	34,6 ± 1,42	22,6 ± 6,72	15 ± 5,72	6
	F(22, 4) = 2.5198, p = .19130													
	Treatment*N supply	AD*N2	n.s	C	ab	n.s	AB	n.s	n.s	n.s	n.s	n.s	n.s	3
		AD*N4		BC	ab		A							3
		AD*N8		BC	ab		AB							3
		Mf*N2		A	b		A							3
		Mf*N4		ABC	a		B							3
		Mf*N8		ABC	ab		AB							3
	F(22, 4) = 2.4018, p = .20499													
Volkamer lemon	Treatment	AD	49,6 ± 8,02	2,2 ± 0,3	3,2 ± 1,2	27,5 ± 5.8B	12,7 ± 0,89	11,9 ± 0	2,8 ± 1,71	7 ± 1,79	32,1 ± 2,08	16 ± 7,33	50,1 ± 6,04	9
		Mf	53,2 ± 6,03	2,3 ± 0,58	2,9 ± 0,76	32,9 ± 5.8 A	13,1 ± 0,58	10,8 ± 0	2,6 ± 2,61	5,7 ± 1,47	29,4 ± 1,52	14,7 ± 7,06	52 ± 3,02	9
	F(11, 2) = 0.57512, p = .77928													
	N supply (g/plant)	N2	46,5 ± 5,04	1,9 ± 0,22B	3,2 ± 1,39	28,1 ± 6.4	13,1 ± 0,81	12,1 ± 0	3,3 ± 2,4	5,9 ± 1,89	26,4 ± 1,27	14,5 ± 9,11	48,5 ± 4,7	6
		N4	53,4 ± 7,7	2,1 ± 0,36 AB	2,8 ± 0,91	30 ± 4	13,1 ± 0,93	11,9 ± 0	3 ± 1,57	6,2 ± 1,69	31,3 ± 2,88	13,7 ± 5,76	53,9 ± 3,94	6
		N8	54,4 ± 6,63	2,7 ± 0,33 A	3,2 ± 0,67	32,6 ± 8.1	12,5 ± 0,43	10 ± 0	1,7 ± 2,23	7,1 ± 0,71	34,5 ± 1,19	17,8 ± 4,1	50,7 ± 5,03	6
	F(22, 4) = 2.7651, p = .16682													
	Treatment*N supply	AD*N2	n.s	n.s	n.s	n.s	ab	n.s	n.s	n.s	n.s	n.s	n.s	3
		AD*N4					ab							3
		AD*N8					b							3
		Mf*N2					a							3
		Mf*N4					ab							3
		Mf*N8					ab							3
	F(22, 4) = 2.9793, p = .14904													

significantly higher than AD*N8. Stem elongation is regulated by complex signaling pathways involving various hormones, including auxin, brassinolide, and gibberellin, and such as the other biometric data is directly related to nutritional status and plant health. The evaluation of stem elongation is a non-destructive analysis carried out to test which combination of treatment and N supply produced greater growth of the seedlings of the two rootstocks over time. A Mixed factorial ANOVA design, also known as 'Repeated measures with a between-subjects factor ANOVA' was conducted in order to relate the elongation of the stem (variable 'stem elongation') to individual responses to treatment (seven levels) over time (six time points: 21 and 28 March, 4, 11 and 19 April, 21 June). Results are reported in Fig. 4. Repeated measures ANOVA showed that seedlings height increased significantly over the growing time, due to the progressive elongation of the seedlings, differences being significantly higher in AD-N8 treatment for Volkamer lemon. The analysis was conducted separately for C. carrizo (Fig. 4a) and Vlem (Fig. 4b). As far as Ccarr is concerned, the mixed factorial ANOVA with a Greenhouse-Geisser correction showed that mean height differed significantly between time points [F (1.208, 50.746) = 48.942, p = .000], whereas no significant differences were found for treatment [F (6, 42) = 0.962, p = .462]. According to pairwise comparisons results, seedlings height increased significantly over the six time points, due to the progressive elongation of the seedlings, but it did not differ among the treatments.

The results for Vlem indicated a significant effect of time on seedlings elongation [F (1.166, 48.989) = 121.832, p = .000], as well as treatment [F (6, 42) = 2.545, p = .034]. Post hoc tests with Bonferroni correction revealed that seedlings height increased through the six time points in

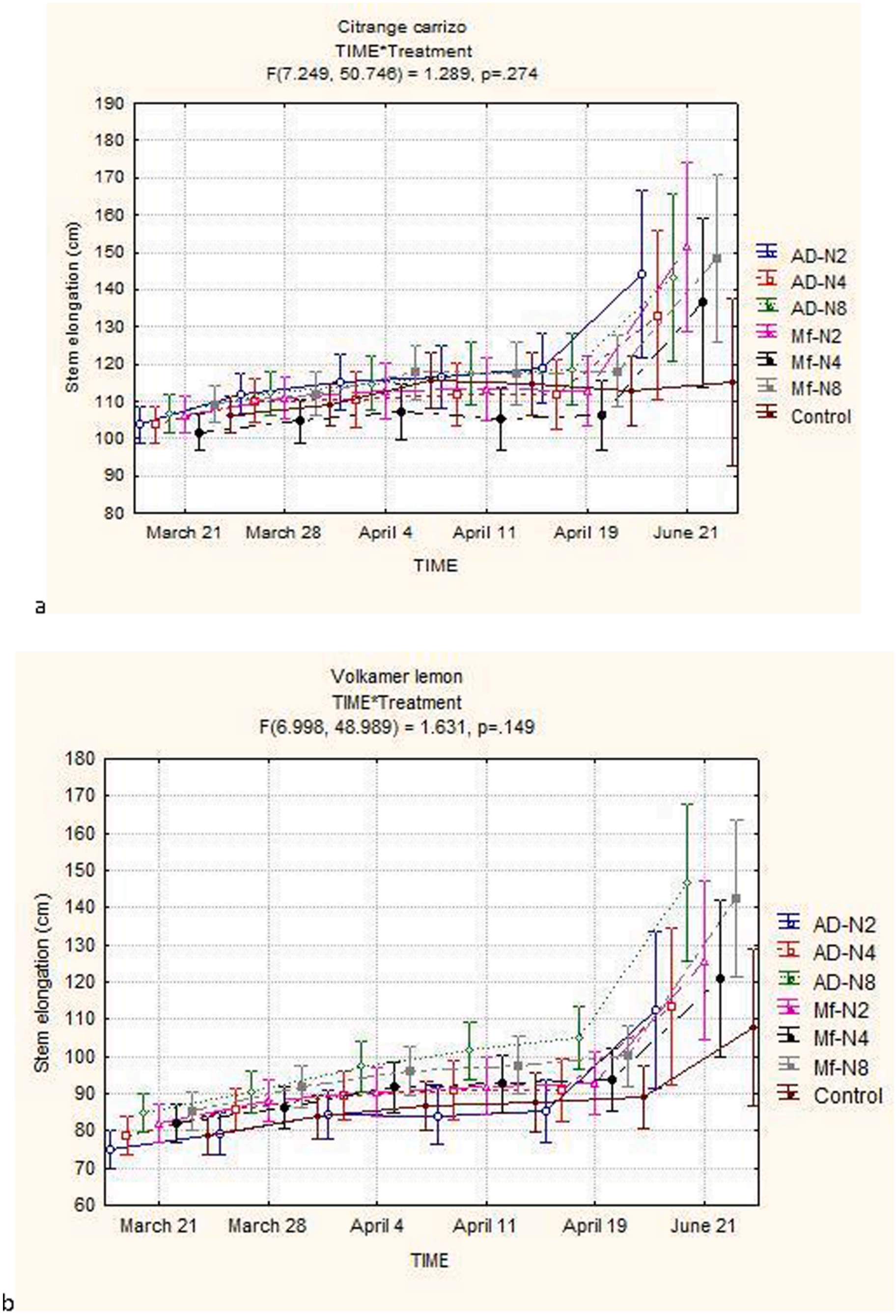

Fig. 4. Repeated measures ANOVA with Greenhouse-Geisser correction of the elongation of the stem of Citrange carrizo (a) and Volkamer lemon (b) seedlings. Bars represent 95% confidence intervals.

similar way for all the treatments. Only at time point 5 a difference between AD-N2 and AD-N8, the latter significantly higher, was found.

No significant interaction between time and treatments were found, neither for C. carrizo [F (7.249, 50.746) = 1.289, p = .274] nor for V. lemon [F (6.998, 48.989) = 1.631, p = .149]. The combination of treatment and dose did not affect elongation and, consequently, no negative impact was observed, despite the outward appearance denouncing a different state of health of the theses, better as N doses increased, regardless of the treatment (Fig. 1S).

New insights on the microelemental profile of intermediate fractions and final digestate before, during and after the digestion processes are reported and these data could be conveniently used for the optimization of the feeding diet of the pilot plant for the potential industrial bioenergetic reuse of the vegetal and animal feedstocks involved in this study. Further studies will have to be carried out to further validate these findings in different sites and operative conditions. In addition, further specific investigations on microbial communities involved in the process should be planned (Sepehri et al., 2018, 2020). Furthermore, as a results of our findings, this study provides evidence that AD obtained from Mediterranean agro-industrial wastes has a great potential for agronomic purposes and applications in citrus nurseries. Further in-depth works on physiological status and traits of tree plants, in addition to citrus seedlings, fertilized by using agro-industrial anaerobic digestate will be useful to corroborate our findings and validate their potential also for different tree crops and in different growing conditions.

4. Conclusions

With the present work it has been demonstrated that multielemental traits of the tested AD are valuable in terms of nutritional supply for the growth and development of the plant. Moreover, it must be stressed that nutrient levels of the tested AD are compliant to legal limits and requirements imposed by the Italian law (MIPAAF Decree n. 5046/2016), being able to assure relevant concentration of phosphorus, a key element for the strengthening of plant growth and chlorophyll photosynthesis performance. The present trial also showed that AD has good potentiality as bio-fertiliser since, despite of the relevant recorded electrical conductivity, it did not cause damage to plants due to the high salt content, furthermore, no other toxicity symptoms on the leaves were observed probably because AD was highly diluted and doses were appropriately spaced. It can be concluded that AD could be advantageously proposed as biofertiliser in citrus nurseries as sustainable alternative to mineral fertilisers. This study, besides providing useful tools for citrus nurseries for conceiving new sustainable fertilization strategies, can be considered as a starting point for further in-depht works on physiological status and traits of citrus plants fertilized by using agroindustrial anaerobic digestate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.wasman.2021.06.007.

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