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COLLEMBOLA IN ECOTOXICOLOGICAL STUDIES AND ENVIRONMENTAL RISK ASSESSMENT

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ABSTRACT

Ecotoxicology studies the effects of contaminants on organisms in the environment, with the final aim to protect the structure and functioning of ecosystems. The risk assessment of contaminants to soil ecosystems is generally based on the results of toxicity tests in the laboratory evaluated using many group of soil invertebrates, but only few are used as test organisms in international standard guidelines, amongst these is *Folsomia candida* (Collembola: Hexapoda). Collembola are abundant in the soil, well-investigated, play an important ecological role and are considered excellent bio-indicators because for their sensitivity to contaminants in the soil (eg. fertilizers, soil improvers, pesticides, heavy metals). In particular, *F. candida* is ideal organism for various laboratory experiments: indeed, the short life cycle, the rapid growth and the parthenogenetic reproduction make rearing easy. The ISO guidelines 11267 (1999; 2014) describe a standardized method that is based on the determination of sublethal effects of contaminated soils on *F. candida*.

In the 2014 guidelines, in addition to standard artificial soil (OECD soil), standard natural soils (e.g. LUFA soil) and reference soil were considered as substrates for the toxicity tests. Recently, some Authors affirm that existing guidelines might need to be improved mainly in order to apply them to new contaminants and in the case of contaminants produced in very small quantities might need to be reduced the amount of soil and consequently the number of springtails. One of the aims of my work is to investigate whether the protocol ISO 11267 can always be considered as "standard" and in particular, whether this toxicity test can be used for contaminants that alters soil properties such as pH and whether the outcome of toxicity tests can be affected by use of different standard soils.

The contaminants used for this study are: i) digestates, the residual organic material of anaerobic digestion of agro-industrial and urban wastes for the biogas production and that can be used in

agriculture as soil improver, and ii) cadmium, a natural contaminant widely distributed in the environment due to human industrial activities. In experiments where digestate was added to OECD soil, the soil pH values increased and a negative effect of digestate was detected on the survival and reproduction of *F. candida*. The decrease of the number of offspring was attributed mainly to the increase of pH values. Therefore, *F. candida* would not be suitable to assay contaminants that modify the soil pH. The data obtained in this study allow to attribute the decrease of the offspring at a negative effect on oogenesis. To investigate whether the results of toxicity tests were affected by type of soil (OECD and LUFA) I have used two different types of contaminants (a digestate and a metal, cadmium) added separately to the two soils. In tests in which the cadmium has been used, the toxicity test gave the same results for both soils to which was added. On the contrary, in experiments in which the digestate has been used, the toxicity test gave opposite results for the two soils. From the perspective of the environmental risk assessment, for a potential use as fertilizer, digestate showed no toxicity in tests with the OECD soil, whereas it has proved toxic when tested using LUFA soil. My study contests deeply the results of toxicity tests standardized and highlights the difficulty of their interpretation. Furthermore, another aim of my work was the miniaturization of test systems halving the amount of soil (OECD and LUFA soil) and the number of springtails using cadmium as contaminant. The halving the number of animals and simultaneously the amount of the soil does not seem to influence the outcome of the test while the halving of the amount of soil alone could influence it.

Collembola, *Folsomia candida*, ecotoxicology, ISO 11267, standard soil

INTRODUCTION

Ecotoxicology

The Ecotoxicology studies develop in the late 1960s when René Truhaut (1969) during a meeting of an ad-hoc Committee of the International Council of Scientific Unions (ICSU) in Stockholm coins the term "ecotoxicology" as the merge from the two words ecology and toxicology. Truhaut (1977) gives the following definition:

"*Ecotoxicology is the branch of Toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context*".

More recently, Walker et al., (2012) have defined Ecotoxicology as the study of harmful effects of chemicals upon ecosystems and includes effects on individuals and consequent effects at the levels of population and above. In particular, ecotoxicology studies the effects of chemical contaminants on organisms in the environment, with the final aim to protect the structure and functioning of ecosystems (Van Gestel, 2012). Toxicity tests are used to compare the hazard of one chemical to another, or for assessing the hazard of chemicals, singularly or in mixtures, to a particular test organism (Ecobichon, 1992).

The chemical contaminants can be divided into two main categories: organic and inorganic. Organic contaminants include intentional poisons such as insecticides, herbicides, fungicides, wood preservatives, unintentional poisons, e.g., degreasers, solvents, and various industrial by-products. The most important organic contaminants are: hydrochlorofluorocarbons and chlorofluorocarbons, organochlorine alkenes, polycyclic aromatic hydrocarbons, polyhalogenated benzenes, phenols, and biphenyls, polychlorinated naphthalenes, polychlorinated dibenzodioxins and dibenzofurans,

pesticides, herbicides, oxygen-demanding compounds, alkylphenols, fluorosurfactants. Other contaminants are radionuclides, genetic contaminants, nanomaterials. Inorganic contaminants are composed of intentional and unintentional poisons. Some are released for a very specific purpose (e.g., sodium arsenate as a pesticide), but others are released by a wide range of human activities. The inorganic contaminants include: metals and metalloids, organometallic compounds, inorganic gases and anionic contaminants including nutrients. They become a problem when non-target species come into contact with sufficiently high concentrations of them (Newman, 2014). All contaminants possess measurable physical and chemical properties that remain constant and do not vary under the same test conditions.

Of central importance in ecotoxicology is the relationship between the quantity of chemical to which an organism is exposed and the nature and degree of consequent toxic effects. Dose–response relationships provide the basis for assessment of hazards and risks presented by environmental chemicals (Walker et al., 2012). The first results of toxicity tests on pesticides, using earthworms and collembolans, were published in the 1960s (Ghabbour and Imam, 1967; Scopes and Lichtenstein, 1967). The first toxicity test with soil invertebrates was internationally standardized by OECD, using earthworms (OECD, 1984). This toxicity test uses the earthworm *Eisenia fetida* as a model organism for assessing the effects of chemicals on terrestrial saprotrophic invertebrates. Soil invertebrates used in toxicity tests are earthworms, e.g. *Aporrectodea calignosa* (Khalil et al., 1996), enchytraeids, e.g. *Cognettia sphagnetorum* (Sustr et al., 1997), nematodes, e.g. *Plectus acuminatus* (Kammenga et al., 1996), springtails, e.g. *Folsomia candida, F. fimetaria* (Bruus Pedersen and van Gestel, 2001; Crommentuijn et al., 1997a), beetles, e.g. *Aphodius constans* (Hempel et al., 2006), mites, e.g. *Hypoaspis aculeifer* (Owojori et al., 2014), millipedes, e.g. *Rhinocricus padbergi* (Merlini et al., 2012), and woodlice, e.g. *Porcellio scaber* (Drobne et al., 2008), but only few species are used as test organisms in international standard protocols: the springtail *F. candida* (ISO, 1999, 2011, 2014a), the earthworms *E. fetida* and *E. andrei* (ISO, 2008, 2012a, 2012b, 2014b), the enchytraeid *Enchytraeus* sp. (ISO, 2014c), larvae of the insect *Oxythyrea funesta* (ISO, 2005), the land snails of

the family Helicidae (ISO, 2006), the nematode *Caenorhabditis elegans* (ISO, 2010). Standardized testing protocols bring a measure of control that allows the comparison with other contaminants, dangerous sites or reference or control soil without contaminants that are necessary for risk assessment. Toxicity tests are based on the understanding that in the context of a set of defined test conditions, there exists a measurable and progressive relationship between dose of chemicals contaminants and effect. Toxicity tests measure an endpoint or groups of endpoints (e.g mortality, reproductive capacity, rate of growth) in a range of known concentrations of a chemical. A known number of test organisms are added to the test substrate to assess the effects of contaminants on the test organisms. Results are measured as the number of organisms surviving (or dead) or observations of sub-lethal behaviours and/or reproductive success (number of cocoons or juveniles). The results are then analysed to determine the nature of the dose-response relationship (OEHHA, 2009).

Endpoints most often measured in acute toxicity tests include a determination of the LC_{50} or EC_{50}, an estimate of the acute no-observed effect concentration (NOEC), the no observed effect dose (NOED) and lowest observed effect concentration (LOEC), and behavioural observations. The LC_{50} is a lethal concentration that is estimated to kill 50% of a test group. An EC_{50} measures immobilization or an endpoint other than death. The NOEC is the highest concentration in which there is no significant difference from the control treatment. The LOEC is the lowest concentration in which there is a significant difference from the control treatment. The NOEC and LOEC are determined by examining the data and comparing treatments against the control in order to detect significant differences via hypothesis testing. Endpoints include all the parameters of interest, i.e., egg hatchability, length, weight, behaviour, total number of juveniles produced, number of juveniles produced per adult, number of oviposition, physiological effects and survival (Hoffman et al., 2002).

The risk assessment of chemicals to ecosystems is generally based on the results of toxicity tests in the laboratory. Ecotoxicological laboratory tests are a first tool for assessing the ecological risks posed by contaminated areas (Bartlett et al., 2010; Correia and Moreira, 2010). The measurement of

endpoints in toxicity tests is singly related to the species tested in the laboratory, while the assessment endpoints include community and ecosystem structural and functional attributes in the field. An extrapolation procedure is used to connect the endpoints of measurement and assessment (Versteeg et al., 1999).

In the laboratory, organisms are generally tested in optimal condition to ensure the growth and optimal survival and / or reproduction in the controls. In the field, conditions may show considerable fluctuations and are never optimal. Temperature, soil moisture and food availability have seasonal and daily patterns. Deviations from optimal conditions can affect the susceptibility of the organism. Bioavailability of chemicals is generally lower in the field than in the laboratory. Bioassays provide a tool to bridge the gap between laboratory and the field situation, and may offer insight into bioavailability in both laboratory and field soils (Van Gestel, 1997). Furthermore, the toxicity test evaluates a specific endpoint but the effects on different life-history characteristics should be assessed to understand the effects at the population level (Crommentuijn et al., 1997b). When considering the use of invertebrates for ecotoxicological tests there are at least three criteria for selecting species. First, there may be toxicological similarities between taxonomically related species. Secondly, the ecological role of a species can be taken into account. Finally, the set of test species should include the various ways in which soil organism may be exposed to contamination. Organism can be exposed to contaminants present in soil via two major routes: oral uptake or dermal uptake of contaminants from the soil solution (Van Gestel and van Straalen, 1994). The soil invertebrates more used in toxicity tests are earthworms, enchytraeids and among microarthropod the Collembola. Several species of collembolans have been frequently used in toxicity experiments such as *Sinella communis* (Greenslade and Vaughan, 2003), *Orchesella cincta* (Van Straalen et al., 1987), *Paronychiurus kimi* (Son et al., 2009), *Sinella curviseta* (Xu et al., 2009), *Onychiurus armatus* and *O. apuanicus* (Mola et al., 1987) *Onychiurus pseudogranulosus* (Sabatini et al., 1998). Moreover, standardized tests are available for the species *Folsomia candida* (ISO, 1999, 2011, 2014a; OECD, 2009), and *Folsomia fimetaria* (OECD, 2009). The collembolans are well-investigated group of soil animals. They are

ecologically relevant species for ecotoxicological testing and represent arthropod species with a different route and a different rate of exposure compared to earthworms and enchytraeids (ISO, 2014a)

Collembola

Collembolans are an abundant group of microarthropods that play an important ecological role for ecosystem. Due to the presence of a springing organ or *furca* used for jumping, they are also known as springtails. The first descriptions of Collembola were published in 1758 (Linné, 1758). The first to use the term Collembola was sir John Lubbock in the *Monograph* in 1873:

"I have proposed for the group comprised in the old genus Podura *the term COLLEMBOLA, as indicating the existence of a projection or mammila enabling the creature to attach or glue itself to the body on which it stands"* Lubbock, (1873).

The word Collembola is a contraction of the Greek words κόλλα (glue) and ἔμβολον (peg) because Lubbock correctly considered the ventral tube, anatomic structure of collembolans, the most characteristic feature of the group. Lubbock (1873) listed a total of 130 species of Collembola; Salmon (1964) in his monograph "An index to the Collembola" listed 2603 references; Hopkin (1997) in his book "Biology of the Springtails" reports that about 6500 collembolan species have been described until that year. Today, there are ca 8500 described species worldwide (Bellinger et al.,1996-2015). The oldest fossil Collembolan is *Rhyniella praecursor* discovered in Lower Devonian chert from Rhynie (Hirst and Maulik, 1926; Whalley and Jarzembowski, 1981). In the past the Collembola were considered an order of the subclass Apterygota, class Insecta. Presently they are no longer considered insects but a lineage of superclass Hexapoda. Nardi et al. (2003) have suggested that Collembola represented a lineage separate from Hexapoda, but recent studies confirm that Hexapoda (Insecta, Diplura, Protura and Collembola) are a monophyletic group (Sasaki et al., 2013). In particular, Diplura-Insecta and Protura-Collembola are separate monophyletic groups (Bitsch and Bitsch, 2004;Beutel and Gorb, 2006; Machida, 2006; Dallai et al., 2011). In Open Tree of Life project

(2015) the superclass Hexapoda comprises a branch formed by Ellipura (Protura and Collembola) and another branch formed by Diplura and Insecta.

Börner (1904) had divided Collembola into two suborders: Arthropleona and Symphypleona. Arthropleona was replaced by two orders Poduromorpha and Entomobryomorpha (Cassagnau, 1971a). Actually Collembola are divided in four subgroups (Fjellberg, 1998):

- Poduromorpha have body elongate, clearly segmented, first segment of thorax well-developed;
- Entomobryomorpha have body elongate, clearly segmented, first segment of thorax small;
- Symphypleona have body more or less globular, segments of thorax and anterior abdomen not clearly separated. Mostly larger species with ocelli present and antennae longer than head;
- Neelipleona have body more or less globular, segments of thorax and anterior abdomen not clearly separated. Very small (0.5 mm) species, ocelli absent. Antennae are shorter than head.

The group Collembola can also be divided into different ecomorphological "life form" (Hopkin, 1997):

- epedaphic species that live on the litter and soil surface and in grass layer of forests. Body is pigmented, often covered with hairs and scales. Furca, appendices and eyes are well-developed;
- hemiedaphic species live in the litter layer. Livery is uniform. Furca and appendices are reduced, eyes are developed;
- euedaphic species that live in the lower soil layers. Pigmentation is absent. Furca is reduced or absent, appendices are reduced, eyes are reduced or absent.

Charles Darwin, in passage on Collembola at page 348 of volume I of the first edition of The descent of man and selection in relation to sex (1871), describes disrespectfully the collembolans:

"The members of this Order are lowly organized for their class. They are wingless, dull- colored, minute insects, with ugly, almost misshapen heads and bodies. The sexes do not differ; but they offer one interesting fact, by showing that the males pay sedulous court to their females even low down in the animal scale"

Collembola are small in size, from 0.25 mm to 17 mm in length (Hopkin, 1997; Steens et al., 2007); they are primitively wingless and ametabolous. The antennae consist of four basic segment with sensory organs. In some species, the segments may be divided into sub-units that make the antennae more flexible (Hopkin, 1997). The third segment often has a sensory organ, probable function chemosensory, formed by two small sensilla, covered in pores of 5nm in diameter, sometimes protected by two papillae (Altner and Thies, 1972; Massoud, 1971). Many species of Collembola have posterior to the base of each antenna a postantennal organ (PAO) (Dallai, 1971; Dallai and Sabatini, 1981). Its function could be hygro-, chemo- or thermosensitive (Altner and Thies, 1976). The structure of the PAO has long been of taxonomic importance (Hopkin, 1997). The internal architecture of the PAO is similar in all species but varies the position of the sensory cell and the morphology of the external cuticular structures (Altner and Thies, 1976). In some species are present other sensory organs such as clubbed setae or portions of cuticle modified (Dallai, 1980).

Collembola do not have compound eyes, but have a maximum of eight ocelli on each side of the head. In many species, the number of ocelli is reduced or the ocelli are absent (e.g. cave and euedaphic species). Each simple *ocellum* has a structure similar to that of a pterygote ommatidium. The dome-shaped cornea is only slightly thickened (Bitsch and Bitsch, 2005). The evolution of the eyes within the different families of Collembola has involved a series of reductions. The primitive condition is present in Symphypleona, until arriving an extreme reduction, observed in the eyes of *Anurida maritima*, in which there is to the loss of the crystalline cone (Paulus, 1979).

The mouthparts of collembolans are entognathan and the appendages are recessed within a gnathal pouch on the head capsule (Grimaldi and Engel, 2005). The five components of the mouthparts are the *labrum*, a pair of *mandibles*, a pair of *maxillae*, the *hypopharynx* and the *labium* (Hopkin, 1997).

The structure of the mouthparts are *important* taxonomic characters of Collembola (Fjellberg, 1984a, 1984b).

The thorax has three segments more or less distinct with a pair of legs each. The legs are divided in: *coxa, throcanter, femur, tibia, pretarsus* and *tarsus* undivided. *Pretarsus* has single claw and an empodium (Beutel et al., 2014; Hopkin, 1997).

The abdomen consists of six segments. However, in some species the abdomen consists of less than six segments due to a fusion secondary of the segments; in other species the sixth segment is hidden under the fifth so as it is no longer visible dorsally (Massoud, 1971). The abdomen bears the unpaired ventral tube on segment I, the small *retinaculum*, an arresting device, on segment III, and the large, jumping device on segment IV, the distally paired *furca* (Hopkin, 1997). The ventral tube (or *collophore*) arises in the embryo as paired structures; they become completely fused to be a large tubular structure (Matsuda and Kerkut, 1976). This organ is involved in balance fluid (Hopkin, 1997). The external surface has structure sensitive to water, salts and extremes of pH (Eisenbeis, 1976a, 1976b; Jaeger and Eisenbeis, 1984). The function of the ventral tube has been variously conceived: adhesion, copulation, respiration, excretion, water absorption or cleaning (Davies, 1928; Mayer, 1957; Nutman, 1941; Ruppel, 1953; Sedlag, 1952). Other Authors attribute to the ventral tube a function of transporting water and ions (Eisenbeis, 1974; Verhoef and Witteveen, 1980). In fact, in the thoracic region, the channel extends through tendon-plates interrupted. For this reason, the ventral tube is considered to be suitable for the transport of fluids (Eisenbeis, 1976b). The mechanism of water intake from ventral tube made through its apical vesicles is active (Eisenbeis, 1982; Rościszewska and Ksiazkiewicz, 1981). In the abdominal segment III there is the *tenaculum* (or *retinaculum*) which is hooked onto the *furca*, a springing organ, located in the segment IV. The *tenaculum* does not seem to be essential for the jumping (Christian, 1978). The *furca* is the most characteristic feature of Collembola. In the adult the *furca* consists of the basal *manubrium* and two slender arms; each arm consists of the dens and the *mucro* (Matsuda and Kerkut, 1976). The *furca* is

well developed in the majority of surface-dwelling springtails although in some euedaphic species is reduced or absent. The springtails use the *furca* to escape from predators. The jump is achieved by rapid flexion of the *furca* away from the body (Hopkin, 1997). In both sexes, at the posterior margin of the segment V the genital duct opens in the external genitalia represented by the simple genital plate. Instead the anal orifice is located in the sixth abdominal segment (Matsuda and Kerkut, 1976).

The cuticle does not differ much from that of insects (Dallai, 1974). The cuticular layer is arranged in a hexagonal mesh pattern with granules arranged at the vertices of the hexagons (Lawrence and Massoud, 1973). The high diversity of cuticular structures in different groups of springtails might be related to specific adaptations to life in the soil (Nickerl et al., 2013).

Collembola are ametabolic hexapods, growing through successive moults throughout the lifespan: the moult is for the animal a very important moment as 50% of the time elapsed between a moult and the other is employed to the preparation of the cuticular structures (Dallai, 1980). The number of moults is variable. Springtails moult up to 50 times, but maturity is reached after stage 6-14 (Beutel et al., 2014). Collembola stop moulting at temperatures below 3-5° C, increasing temperature also increases the frequency of the moults (Dallai, 1980).

Collembola are amphimictic or parthenogenetic. Parthenogenesis is thelytokous. It has never been reported hermaphroditism. Collembola have a reproductive strategy iteropare. The springtails have separate sexes and indirect sperm transfer. The spermatozoon has an axoneme with the typical organization with 9+2 and an acrosome with a long *perforatorium* (Dallai, 1970). Spermatozoa formation occurs through a winding process. The spermatozoa become rolled up around an extracellular core of dense material during spermiogenesis. At the end of maturation, the sperm is placed on top of the spermatophore stalk or abandoned in drops directly on the soil (Dallai et al., 2004). The spermatophore is deposited on the substrate, or placed directly on the female genital opening (Hopkin, 1997). The sperm cells will enter into the female spermatheca in this rolled-up shape (Betsch-Pinot, 1974; Cassagnau, 1971b; Dallai, 1975; Dallai et al., 2004).

The taking up of spermatophores by the females can occur in different ways, the body contact between the male and female ranging from absent to very adherent (Betsch-Pinot, 1974; Blancquaert, 1981; Döring, 1986; Joosse et al., 1972; Schaller, 1971; Stam et al., 2002). Ritual behaviours associated with spermatophore transfer have been observed. The members of the family Bourletiellidae, in particular *Deuterosminthurus bicinctus*, use their antennae, legs or heads to monopolize, stimulate, and direct female partners to spermatophores (Kozlowski and Aoxiang, 2006). Direct transfer of a drop of sperm occurs in the species *Sphaeridia pumilis* with a very complex behaviour: ventral tube is involved in cleaning operations (Blancquaert and Mertens, 1977; Hutasse-Jennenot, 1974). In some species females occasionally consume the spermatophores (Beutel et al., 2014). But especially the males in reproductive phase consume spermatophores and this behaviour was interpreted as a case of sexual competition (Harvey and Bradbury, 1991). Both male and female of the species *Orchesella cincta* are able to discriminate between spermatophores of different males. Males preferentially destroy spermatophores of other males and the females are able to discriminate spermatophores according to the level of genetic affinity so as to pick up spermatophores of specific males (Gols et al., 2004; Hedlund et al., 1990). Females that allow to choose the spermathophores produce male offspring with significantly more spermatophores. (Zizzari et al., 2009). The eggs are fertilised within the female before laying using stored sperm. Eggs may be laid individually or in small batches (Hopkin, 1997). According to the species and temperature, the collembolans may lay from 100 to 600 eggs during their life. Freshly laid eggs are oblong shaped but rapidly they assume a spherical shape seemingly smooth but at great magnification are visible ornamentation (Cappi, 1998). The eggs size varies from 100 to 300 µm and they have a colouring often pale varying from white to ochre (Dallai, 1980), that tends to become darker in the course of development. Just laid eggs are very delicate because the external envelopes are not yet hardened and the growth of the eggs takes place due to water absorption (Hale, 1965). The embryonic development varies in the different species and also depends on the temperature at which the eggs are reared. In studies conducted in the laboratory on life history of *Folsomia candida*, at a temperature of 21°C, embryonic development lasts about 9 days

(CHAPTER 2). Juveniles are similar to adults except for the absence of functional reproductive organs and secondary sex characteristics. The number of instars before reproductive maturity is about 5-8 (Hopkin, 1997).

The factors which control the ratio of males to females were not clear and they were probably related at least partially to climate (Hopkin, 1997). Previous studies demonstrated that Symphypleona, but not Arthropleona, carry out a post-zygotic sex-determination mechanism resulting from a precocious elimination of chromosomes during the zygote cleavage. The result of this process are males with two chromosomes less respect to females (Dallai et al., 1999, 2000, 2001, 2004; Fanciulli et al., 2013). Dallai et al. (2000) interpret the possibility for females to choose the sex of their offspring as an evolutionary step toward parthenogenesis. In this case, males are rare or completely absent. Petersen (1980) - studying populations of springtails of a beech forest - found eight species in which males were not present. The majority of populations of the species *Folsomia candida* are parthenogenetic, although bisexual populations were described (Frati et al., 2004; Goto, 1960). The unfertilized egg of *F. candida* is able to develop without a male contribution by means of newly assembled centrosomes from which the zygotic spindle organizes (Riparbelli et al., 2006). The reproduction by parthenogenesis is widely believed to result from Gram-negative α-Proteobacterium *Wolbachia* infection. Infection by the *Wolbachia* was found in parthenogenetic populations of *F. candida* whereas it was absent in bisexual populations (Frati et al., 2004) and Czarnetzki and Tebbe (2004) put evidence forward that *Wolbachia* is responsible for parthenogenesis in Collembola. However, Riparbelli et al., (2006) affirm that parthenogenesis induced by *Wolbachia* seems to be restricted to haplodiploid species, where males are haploid and females diploid. Collembola are not haplodiploid, and there is evidence of other Exapoda species that reproduce parthenogenetically without being infected by Wolbachia. These Authors thus exclude that the presence of *Wolbachia* induces parthenogenesis in *F. candida*. A possible alternative hypothesis is that *Wolbachia* is an opportunistic association with an organism that reproduces parthenogenetically in a non-endosymbiont-induced manner.

In Collembola sexual dimorphism is rare. They have no specific copulatory organs. Females may be larger than males if they are full of eggs. However, to identify the sex of springtails it must refer to the genital plate which can only be seen at high magnification (Hopkin, 1997). The genital plate in the males is circular, surrounded by setae, with a longitudinal slit; in the females is elliptic, surrounded by setae, with transverse slit (Matsuda and Kerkut, 1976). The setae grow after moulting that leads to the reproductive maturity (Massoud, 1971). Only for a few species there is a clear sexual dimorphism. In some species of the Symphypleona, sexual dimorphism is more evident, and this character affects antennae of the males used to engage females during copulation (Massoud and Betsch, 1970). Among the Entomobryomorpha, *Guthriella muskegis* (Isotomidae) presents modifications in the end of the body, in particular the presence of spines and modifications of the setae. Males present ornamentation in order to attract females. For the Entomobryidae, the main sexual differences are in the colour patterns, and in the modification of mucro and genital aperture (Palacios-Vargas and Castaño-Meneses, 2009).

The adult Collembola, mainly in the families Hypogasturidae and Isotomidae, may be characterised - through one or more stages in their life cycle - by reduced activity and respiration and distinct morphology as a result of environmental factors and intrinsic genetic factors (Hopkin, 1997). Three types of polymorphism are recognized: ecomorphosis, epitoky and cyclomorphosis. The ecomorphosis is characterised by morphological changes and termination of feeding as an adaptation to extreme climatic conditions (Cassagnau, 1974). If the stage is part of reproductive cycle it is called epitoky (Cassagnau, 1985; Chimitova and Potapov, 2011; Fjellberg, 1988; Greenslade and Potapov, 2012). When the conditions are part of a regular cycle the process is called cyclomorphosis (Waltz and Hart, 1995; Zettel and Zettel, 1989).

Collembola in more than 400 million years have evolved to fill a huge variety niches. Their success is due to the small dimensions that allow them to colonize confined spaces and the physiological and behavioural adaptability (Hopkin, 1997). The springtails live in hot and polar deserts (Somme, 2012),

in the intertidal zones (Joosse, 1966), in fresh water (Deharveng et al., 2008), in cave at a depth of 1980 m (Sendra and Reboleira, 2012), in anthills and termite mounds (Richards and Davies, 1977), on the bark of trees (Ponge, 1993). In Antarctica, Collembola are in a higher proportion of the total fauna compared with most other habitats on the Earth (Peterson, 1971; Sømme, 1985; Teets and Denlinger, 2014). Collembola have evolved physiological mechanisms to survive in low temperatures, in particular avoid freezing by supercooling through elimination or masking of potential ice nucleators in the body and accumulation of cryoprotective substances (Hopkin, 1997).

Collembola are a group of soil animals that plays an important role in the transformation of organic matter and their feeding activity promotes the decomposition processes in soils (Cragg and Bardgett, 2001). They consume a wide variety of food material, especially fungal hyphae. The fungi are an important sources of nutrients which are released from plant material by fungal digestive enzymes which collembolans are not able to produce themselves (Bakonyi et al., 1995). Collembola also feed on arbuscular mycorrhizal fungi but this does not seem to decrease the positive effect of mycorrhizal fungi on the plant biomass, not reducing their biocontrol capacity (Innocenti et al., 2009a). On the contrary, springtails also feed on plant pathogens fungi (Innocenti et al., 1997; Sabatini and Innocenti, 1995, 2000a, 2000b; Sabatini et al., 2004) and this action appears to have a beneficial effect on plant growth (Innocenti et al., 2009b). The species of springtails of larger size stimulate mineralization processes by selective feeding on fungi. Smaller springtails contribute to humification by non-selective scavenging and mixing of organic material and mineral soil particles (van Amelsvoort et al., 1988).

Agronomic practices certainly improve the quality and production capacity of soil but they also cause alterations in the structure and composition of the community of soil organisms (Curry, 1994). In cultivated land, balance between soil fauna, vegetation and organisms is continuously altered from chemical and mechanical human actions. The effects of mineral fertilizers on soil fauna may be a consequence of the effects that these substances have on vegetation. In high doses they can determine

a decrease in the number of animal species and in the number of organisms (Marshall, 1977). The use of a balanced fertilization, using organic amendment (e.g., crop residues, manure, compost), helps to maintain organic matter level in soils (Miller and Wali, 1995). In the last years, the use of organic wastes to produce organic fertilizers has grown a lot. The organic residues of agricultural are a possible renewable energy resources, and their potential can be exploited through an anaerobic digestion process (Lehtomäki and Björnsson, 2006; Møller et al., 2009). The anaerobic digestion is a promising technology enhancement to treat wastes producing a methane-rich biogas. The anaerobic digestion option can play a particular role in global warming savings, first, by substituting the use of fossil fuels with the produced methane-rich biogas, and second, by storing carbon in the soil and limiting the consumption of mineral fertilizers with the remaining digested substrate. This latter, called digestate, is used as a fertilizer and a soil improver (Caramiello et al., 2013; Carchesio et al., 2014; Møller et al., 2009). In my work, I have studied the effects of digestates on springtails (CHAPTER 1, 2, 3).

An agronomic practice with significant effects on soil fauna, in particular on springtails, is the use of pesticides (Frampton, 2002). The majority of these products consists of biologically active substances that may have adverse effects also on no-target organisms (Thompson and Edwards, 1974). although some pesticides have effects on springtails only at doses higher than the recommended agricultural dose (Rebecchi et al., 2000; Sabatini et al., 1998).

Other factors that can affect the abundance of different groups of pedofauna are the vegetation cover, the type of cultivation and the amount of water available (Madge, 1981). Past landscape patterns and processes like land-use conversion and subsequent succession have a considerable impact on the present day pattern of species richness and community composition of Collembola within a landscape (Chauvat et al., 2007).

In recent years, it has increased the interest in using animals as bio-indicators for monitoring the health of an environment. The abundance and diversity of Collembola have been used widely to

assess the environmental impact of a range of contaminants on soils (Fountain and Hopkin, 2005). Collembola are excellent bio-indicators of the health of the soil because they are the most abundant microarthropod, along with the mites, in the soil. Collembola have characteristics anatomical, physiological and ecological well known and they are sensitive to contaminants in the soil (fertilizers, soil improvers, pesticides, heavy metals). Soil-quality assessment is a complex issue because it depends on the combination of the physical, chemical and biological properties that contribute to soil functions (Knoepp et al., 2000). The chemical contamination of the environment has been implicated in the decline or disappearance of many soil populations. Methods for soil quality assessment can based on the general evaluation of soil microarthropods (Parisi, 2001; Parisi et al., 2005; Sabatini et al., 1979) or on the evaluation of a single taxon (Iturrondobeitia et al., 1997; Paoletti, 1999; Parisi, 2001; Rebecchi et al., 2000; Sabatini et al., 1997, 1998). Collembola can be used to obtain information about soil quality, using species richness and diversity, classification of species according to life-history attributes, classification according to ecophysiological preferences and the structures of food webs (Van Straalen, 2004). Soil biological quality can be also estimated by applying the QBS-ar index (Parisi et al., 2005) or the QBS-c index (Parisi and Menta, 2008) that consider the presence and morphological characteristics of microarthropods or only springtails respectively. For important role played by springtails in the soil, the QBS-c index, based exclusively on Collembola, is now often applied. The QBS indexes combine two important features of microarthropods: biodiversity and the level of adaptation to the soil (reduction or loss of pigmentation and visual apparatus, reduction of appendages e.g. setae, antennae and legs, reduction or loss of flying, jumping or running adaptations). Another approach to obtain information about soil quality is the application of the ecotoxicological tests for biomonitoring the environmental pollutions, and for the risk assessments of environmental chemicals. Ecotoxicology deals with the harmful effects of chemicals in the environment, especially where the chemicals may be related to adverse changes at the population, community, or ecosystem level (Walker et al., 2012). The organisms utilized in ecotoxicological test for soil quality are, principally, earthworms and collembolans. The springtails can be exposed to contaminants via the

soil and/or food in a battery of tests that examine life-history parameters, bioaccumulation, and/or effects on behaviour (Fountain and Hopkin, 2005). The springtail *Folsomia candida* Willem, 1902 is considered the most suitable microarthropod for soil quality assessment (Ronday and Houx, 1996) and it was utilized for tests assessing the toxicity of a wide range of organic and inorganic soil contaminants (Crommentuijn et al., 1997b; Crouau et al., 1999, 2002; Domene et al., 2007; Waalewijn-Kool et al., 2013, 2014; CHAPTER 1, 2, 3). Moreover, the species *Folsomia candida* can be considered representative of Collembola and soil arthropods in general.

Folsomia candida and ISO 11267

The genus *Folsomia* of the family Isotomidae, includes species that have a well-developed *furca*, no anal spines, and an abdomen with the posterior three segments fused (Potapov, 2001). *Folsomia candida* is a euedaphic collembolan with no pigment and eyes. The original description of *F. candida* by Willem in 1902 was based on a single specimen found on a puddle in a cave at Rochefort in Belgium:

"Un seul exemplaire, une femelle adulte, dans la grotte de Rochefort, à la surface d'une flaque d'eau. Absolument incolore, pas de tache oculaire. Le caractère le plus saillant de cette forme nouvelle réside dans la soudure des quatre derniers anneaux abdominaux : la séparation entre les terga des segments 6 et 7 est encore représentée par un sillon qui n'atteint pas la région ventrale ; le segments 7,8 et 9 forment un 'ensemble indivis et ramassé.

The most distinctive characteristic that separates *F. candida* from other members of the genus is the presence of many (at least 16) stout *setae* on the ventral side of the *manubrium* of *furca* (Potapov, 2001). This species has been found in most regions of the world except for Africa, India and Malay Peninsula (Bellinger et al., 1996-2015). However, many populations are derived from human introductions (Hopkin, 1997). Populations of *F. candida* consists above all of females that reproduce by thelytokous parthenogenesis, but populations with males and females were described (Frati et al.,

2004; Goto, 1960). In *F. candida*, *Wolbachia* may be the cause of parthenogenesis (Czarnetzki and Tebbe, 2004; Frati et al., 2004).

Folsomia candida is a well-adapted species to dry soil conditions (Hilligsøe and Holmstrup, 2003). Oxygen uptake is via the cuticle. Because collembolans do not possess respiratory pigments, the oxygen capacity of the extracellular fluids is low. However, some individuals can survive for up to 18 h in completely anaerobic conditions (Fountain and Hopkin, 2005).

These springtails feed on fungal hyphae and in laboratory cultures are bred with Brewer's yeast. In our laboratory they were maintained in 100 mL glass containers with a diameter of about 5 cm containing clay, mainly consisting of kaolinite and smectite, bottom saturated with deionized water and kept in a thermostatic chamber at 20±1°C with a light-dark cycle of 16:8 h. The dark background of clay facilitates observation of the white springtails.

The subsequent description of the life history of *F. candida* is based on observations of parthenogenetic specimens maintained in the Department of Life Science, University of Modena and Reggio Emilia. At 20 ± 1 °C about 20 to 60 eggs are laid in each batch. Eggs freshly laid are spherical and white, after become yellow-brown. Hatching occurs 8-12 days after oviposition. The juveniles develop into adults directly: they become sexually mature when reach the sixth instar. The first deposition occurs on average 16 days after hatching. The average lifespan is 86 days during which eggs are laid six times (Fig. 1), whereas the oldest specimen of *F. candida* observed lived 185 days, having undergone 28 moults and laid eggs for eight times. In particular, I have studied life history traits of the specimens of *F. candida* for a period of 28 days, which corresponds to the time of test ISO 11267 (2014a) utilizing animals 12 days old. During the study period they laid eggs four times but only the eggs of the first three oviposition have hatched within 28 days (CHARTER 2).

Folsomia candida for the ease of maintenance in the laboratory, the rapid growth and short life cycle, and parthenogenesis, is an ideal organism for various laboratory experiments (Beresford et al., 2013; Chamberlain et al., 2005; Yuan et al., 2013). In particular, many laboratories worldwide use this

species to assess the effects of contaminants on no-target soil arthropods (Crouau et al., 1999; Domene et al., 2007, 2008; Fountain and Hopkin, 2001, 2004a, 2004b).

The International Organization for Standardization (ISO) has published guidelines 11267 in 1999, (ISO, 1999), and a second edition in 2014, (ISO, 2014a) for the use of *F. candida* in ecotoxicological test that has reproduction as endpoint. Several authors have suggested alternative collembolan species to be used in standard toxicity tests because *F. candida* has limited ecological relevance due to its absence from many natural or agricultural habitats (Krogh, 2009). This has led to suggestions of species such as *Paronychiurus kimi* (Son et al., 2007), *Sinella communis* and *Proisotoma minuta* (Greenslade and Vaughan, 2003) as appropriate test species. However, so far the International Organization for Standardization has published international standardized methods only for *F. candida*. The ISO 11267 guideline (ISO, 1999, 2014a) describes a method that is based on the determination of sublethal effects of contaminated soils to adult Collembola of the species *F. candida* by dermal and alimentary uptake. The ISO guideline (ISO, 1999) has included only the use of the standard artificial soil as recommended for the earthworm standard test (OECD, 1984). The artificial soil (OECD soil) was composed of 70 % quartz sand, 20 % kaolinite clay and 10 % *Sphagnum sp.* peat, air-dried, finely ground and with no visible plant remains. A small amount of $CaCO_3$ is mixed in to bring the pH up to 6.0 ± 0.5. Deionized water was added during mixing to reach about 60 % of the maximum water-holding capacity. The second edition of ISO 11267 guideline (ISO, 2014a) includes the use of standard natural, field-collected soils (e.g. LUFA soil) in addition to standard artificial soil. Furthermore, the ISO guideline (2014a) extends the use of the test also for soil contaminants such as waste materials: in this edition the term "pollution" of the title has been replaced with the term "contaminants".

The test is carried out in test containers with a diameter of about 5 cm and a volume of 100 mL. At least four replicates for each concentration plus eight controls are recommended. The more replicates are used, the more the test is valid statistically (Van Der Hoeven, 1998). Ten *F. candida* 10-12 days

old are placed into each container. At the beginning of the test and after a period of 14 days, granulated dry yeast is added in all containers. The test containers are kept in a thermostatic chamber at 20±1°C with a light/dark cycle of 16:8 h. After four weeks, the soil is flooded with water and the adult and their offspring float to the soil surface. Counting can be done manually under light microscope or photographing the surface of each container and later counting the specimens on enlarged print or projected slides. The adults can be removed and weighed and analysed to determine the concentrations of the test substance in their bodies. The results are considered to be valid if in the controls the mortality of the adults should exceed 20%, the reproduction rate should reach a minimum of 100 juvenile springtails per control containers, and the coefficient of variation of reproduction in the control should not exceed 30%.

The ISO test 11267 on *F. candida* reproduction is among the most widely used standardized toxicity test applied to obtain information about the effects of contaminants in soil, however, some Authors (Crouau et al., 2002; Van Gestel, 2012; Filser et al., 2014) consider necessary adjustment to make them more efficient to evaluate new contaminants. In fact, the ISO guideline (1999) describes a method for testing the effects of chemicals on the reproduction of *F. candida* in artificial soil. The ISO guideline (2014a) extends the use of the test also for amended soils, soils after remediation, industrial, agricultural, or other sites of concern and waste materials. Fountain and Hopkin, (2005) affirm that the test results are sensitive to changes in pH, cation exchange capacity, and organic matter (OM) content: these soil properties can be modified by adding fertilizers, soil improvers and waste materials. The use of standardized soils does not represent realistic field conditions, because soil properties can have an important influence on the bioavailability and toxicity of soil pollutants. Several soil properties significantly influenced reproduction: number of offspring decreases with decreasing moisture content, decreasing coarse texture, and increases with decreasing nitrogen content (Domene et al., 2011). Furthermore, soil pH highest or lowest respect to pH 5.6, at which *F. candida* reach the highest level of reproduction (Fountain and Hopkin, 2005), causes a decrease in the number of offspring (Greenslade and Vaughan, 2003; Van Straalen and Verhoef, 1997;

CHAPTER 1, 2). Comparing artificial soils prepared according to OECD standardized procedures from different laboratories, high variability was found in the properties of the soils, such as total organic carbon, pH, cation exchange capacity, size of clasts and it was revealed variability with respect to contaminants tested behaviour (Bielská et al., 2012; Hofman et al., 2014). Therefore, ecotoxicological tests of a same contaminant performed in different laboratories may produce different results due to the variations of properties of OECD artificial soil. The ISO 11267 (ISO, 2014a), in contrast to the previous edition (ISO, 1999), provides for ecotoxicological tests the possibility of using, in addition to the standard artificial soil, a standard natural soil (eg. LUFA soil). The advantages of using a standard natural soil are the not soil preparation, mixing the various components, its long-time availability and therefore the comparability of the testing of several years with different contaminants, by different laboratories. However, the possibility to use different types of standard soils can determine a different interpretation of the results in terms of environmental risk assessment (CHAPTER 3). Furthermore, the ISO guideline could be used to assay contaminants with synthesis process very expensive and available in low quantities and this perspective I have evaluated the possibility of reducing the number of animals and the amount of soil provided for by the ISO guidelines (ISO, 2014a) for toxicity test (CHAPTER 3).

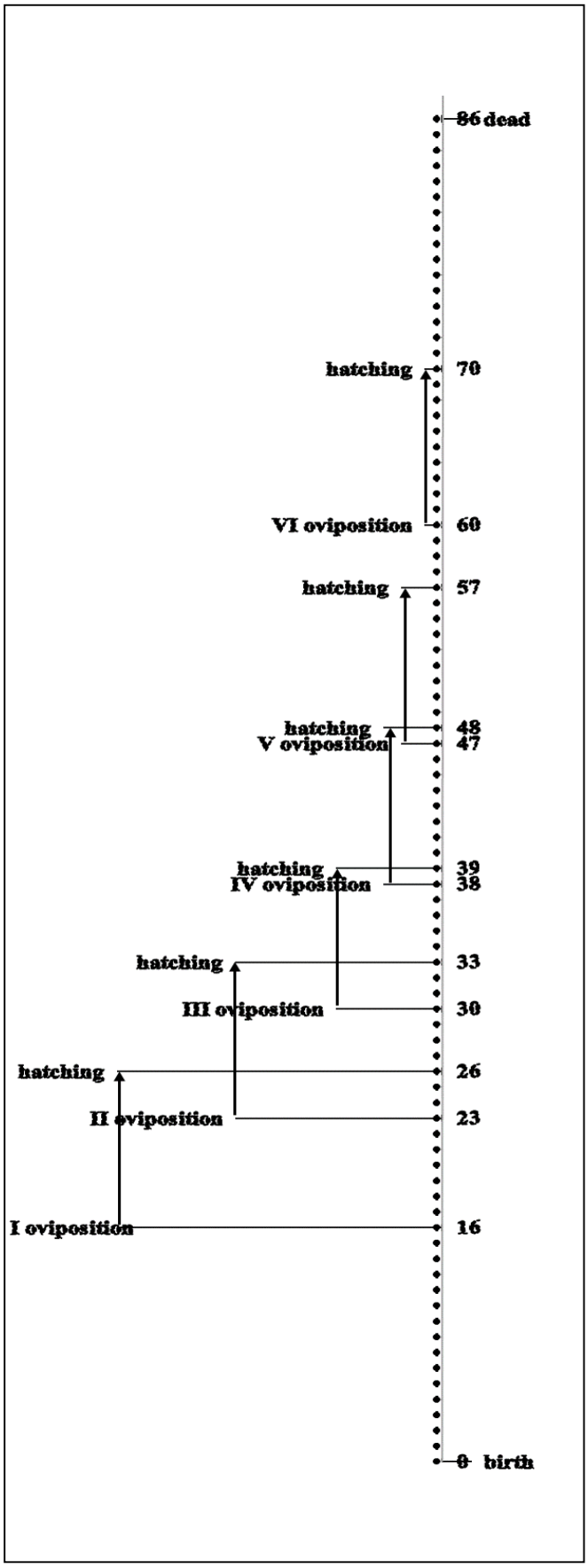

Figure 1. *Folsomia candida* life history traits of 15 females The continuous arrows indicate the time from oviposition to hatching. The time is reported as mean days.

Study aims

The ISO test 11267 on *F. candida* reproduction is among the most widely used standardized toxicity test applied to obtain information about the effects of contaminants in soil, however, some Authors consider necessary adjustment to make them more reliable and to evaluate new contaminants. The second edition of ISO (2014a) makes changes to the guideline allowing the use of standard natural soil (e. g. LUFA soil) in addition to the standard artificial soil (OECD soil) and extending the use of the toxicity test also to soil contaminants such as waste material that could modify soil properties influencing the outcome of the toxicity test. The aim of my study is to evaluate the effects on the survival and reproduction of the collembolan *F. candida* of a digestate obtained from anaerobic digestion of biomass, when added to soils. Since the digestate can change the pH of the soil, the study was conducted using standard artificial soils, with different pH values, in addition to that provided by the ISO guideline, in order to obtain information on soils to which can be added the digestates.

Some Authors affirm that the only disadvantage of the test is that reproduction cannot be observed directly, and cannot be separated from juvenile mortality and success hatching. In order to discriminate among different traits of the reproduction, another aim of my study is the detection of size-age classes, in relation to oviposition, to build a correlation table observing life history traits of *F. candida* in controlled conditions. The data obtained can be used to identify what traits of reproduction (oogenesis, embryonic development or post-embryonic development) are sensitive to the addition of the digestate to soil.

Another aim of present study was to compare the use in toxicity test of the standard artificial soil (OECD, 1984) and the standard natural soil LUFA 2.2 both proposed in the guideline ISO 11267 (ISO, 2014a) studying the effects of two contaminants (a heavy metal, such as cadmium, and an organic compound such as a digestate), on the survival and reproduction of *F. candida* separately added to the two soils types.

In the perspective to make the test applicable for determining the toxicity of new and emerging contaminants, often produced in very small quantities and with synthesis process very expensive another aim of the study is investigate the effects on *F. candida* reproduction of cadmium in two different soils (OECD and LUFA 2.2), reducing the number of springtails and the amount of soil provided for by the ISO guideline (ISO, 2014a).

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CHAPTER 1

Effects on *Folsomia candida* Willem, 1902 of products resulting from anaerobic digestion of biomass tested at different soil pH

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Abstract

In recent years, it has become increasingly important to reduce the production and impact of wastes on the environment to save and restore natural resources. A way of disposing industrial, agro-industrial and urban wastes is their treatment by anaerobic digestion, with the production of biogas and residual organic material. The latter is commonly called digestate and can be used in agriculture as fertilizer, soil improver or adsorbent material, but only scarce or no evaluations of its biological effects on soil organisms are presently available. The aim of the present research was to study the

effects of digestate, when added to soils with different pH values, on the survival and reproduction of the collembolan *Folsomia candida*. The digestate investigated in this study was obtained from agro-industrial wastes mixed with biological sludge. We exposed springtails to two different concentrations of digestate (2.5 %, 5 %) in two artificial soils with pH values of 6 and 4.5. The addition of digestate resulted in an increase of experimental-soil pH values, depending on the concentration and original pH value of the artificial soil used. The negative effects of digestate detected on the survival and reproduction of *F. candida* was mainly attributed to pH values > 6. The high pH value, however, does not explain by itself the strong decrease in number of juveniles in the experimental soil when the highest concentration of digestate was combined with the highest pH value reached. In this case it is possible to assume a combined effect of pH and other digestate characteristics, such as nitrogen content and salinity, currently under study.

Keywords: Collembola | digestate | toxicity test | survival | reproduction

1. Introduction

In recent years waste disposal has significantly increased as a result of the current economic system, based on continued growth in the production and consumption of goods and services. By 2025 it is estimated that the urban population will reach 4.3 billion people, which will generate 2.2 billion tons of waste per year (Hoornweg & Bhada-Tata 2012). It is therefore important to reduce waste production and impact on the environment, and to save natural resources by waste recovery. A way of disposing waste is its treatment by anaerobic digestion, a promising technology enhancement to treat industrial, agro-industrial and urban wastes, obtaining two categories of products: biogas and the remaining digested substrate, commonly called digestate. The digestate can be further refined into

concentrated fertilizer and organic amendment, all suitable for recycling (Tambone et al. 2009, Dolan et al. 2011).

The addition of digestate to soil could contribute to solving one of the most important problems of agricultural soils, the alteration of their structure, and could increase the retention of the nutrient levels and microbial activity, thus promoting soil fertility (Tambone et al. 2009, Makádi et al. 2012). However, the presence of digestates in soil could also alter physical-chemical properties, such as pH (Gómez et al. 2007, Pognani et al. 2009), or buffer capacity and, by modifying the environmental conditions, could cause ecotoxicological effects, leading to a decrease of soil-organism population fitness. Therefore, this practice could exacerbate the decline of biodiversity in agricultural soils rather than improving it. Chemical analyses seem inadequate to reveal the biological long-term effects of materials added to soil because of a lack of knowledge about potential interactions between contaminants and toxicity for soil organisms. Thus, chemical analysis has to be combined with ecotoxicological tests (Van Gestel 2012). Ecotoxicological studies have been conducted to test the effects of sewage sludge and organic waste compost on soil organisms (Domene et al. 2007, 2008, 2010, 2011, Fuchs et al. 2008, Moreira et al. 2008, Pivato et al. 2014). However, only scarce (Fuchs et al. 2008) or no evaluations of the biological effects of digestates are available. The aim of present research was to study the effects of digestate, when added to soils with different pH values, on the survival and reproduction of the collembolan *Folsomia candida* Willem, 1902. This springtail is among the most widely used standard test organism for terrestrial ecotoxicology (Fountain & Hopkin 2005, Filser et al. 2014), due to its widespread distribution in different soil types, its role in the decomposition of organic matter, regulation of microbial activity and nutrient cycling (Luo et al. 2014) and its tolerance to a wide range of important soil properties (Amorim et al. 2005). A digestate obtained from the biomethanization of biomass, produced in the Emilia Romagna region in Italy in large amounts, was used.

2. Material and methods

2.1. Digestate

The digestate used in this study was obtained from agro-industrial wastes mixed with biological sludge and it is characterized by a high pH value (pH = 8). The pH value was measured in a suspension 1:2.5 digestate:$CaCl_2$ following standard procedures (MiPAF 1999). The digestate was dried at 110°C for 24 h, milled and used in the soil in particles of < 500 µm.

2.2. Test organisms

The utilised Collembola belonged to the species *Folsomia candida* Willem, 1902. The specimens were reared in the laboratory for several generations. They were maintained in 100 mL glass jars with a diameter of about 5 cm containing clay, mainly consisting of kaolinite and smectite, bottom saturated with deionized water and kept in a thermostatic chamber at 20±1°C with a lightdark cycle of 16:8 h. Animals were fed on Brewer's yeast.

2.3. Toxicity test

According to ISO guideline (ISO 11267: 2014), the artificial soil was composed of 69 % quartz sand, 20 % kaolinite clay and 10 % *Sphagnum* sp. peat, air-dried, ground and sieved to 0.05 mm. Deionized water was added during mixing to reach about 60 % of the maximum water-holding capacity (WHC measured using guideline ISO 11267). Following the ISO guideline, pH value was adjusted to 6.0 ± 0.5 by adding $CaCO_3$. The artificial soil was also used to prepare soils with different pH values (4.5 and 7.0) by adding adequate amounts of CaCO3.

Four different experimental soils were obtained with the addition of the digestate at two different concentrations (2.5 % and 5 %), namely:

• 2.5 % H: digestate 2.5 % added to artificial soil with pH = 6 (High level pH)

• 5 % H: digestate 5 % added to artificial soil with pH = 6 (High level pH)

• 2.5 % L: digestate 2.5 % added to artificial soil with pH = 4.5 (Low level pH)

• 5 % L: digestate 5 % added to artificial soil with pH = 4.5 (Low level pH)

Experimental soil with pH = 6 without digestate (0 pH 6) was used as control soil. Experimental soil with pH = 7 without digestate (0 pH 7) was used for comparison, because preliminary studies revealed that the addition of digestate causes an increase in soil pH. The experimental soils were prepared at least three days prior to the start of the test in order to equilibrate acidity. The chosen concentrations of digestate of 2.5 and 5 % correspond to 27 and 54 ton/ha (dry weight) respectively. The lower dose is in line with recommendations for Italian agricultural soils. The conversion was made using the density of artificial standard soil of 1.1 g/cm3, measured according to ISO 11272 (1998) and assuming a mixing in the first 10 cm of soil. The pH value of experimental soils was measured at the start of the toxicity test, in two replicate samples of each experimental soil, in accordance with standard procedures for soil characterization (MiPAF 1999). The soils were shaken with distilled water (1:2.5) for 2 h at 200 rpm. After settlement of the particles, the pH of the soil solution was measured using a Crison BASIC20 pH meter. Eight replicates were prepared for each experimental soil utilizing glass containers (100 mL capacity, 5 cm diameter). According to ISO guideline 11267, ten 10-day- old springtails were placed in each tightly closed test container and were maintained at a temperature of 20±1°C with a light-dark cycle of 16:8 h for 28 days. The containers were opened twice a week for aeration and feeding with yeast (4 mg in 28 days). At the end of the experiment, water was added to each container: the animals sorted by floating were grouped into adults and juveniles and counted manually under a stereomicroscope.

2.4. Statistical analysis

All statistical analyses were carried out using SPSS software release 21.0. One-way Analysis of Variance (ANOVA) was performed to compare the survival and reproduction levels of *F. candida* among control soil, the four experimental soils with digestate and an experimental soil without digestate at pH = 7. When significant statistical differences were detected ($P < 0.05$), a post hoc Student-Newman-Keuls test (SNK) was used. Percentage survival data were arc-sin transformed before statistical analysis. In order to evidence possible interactions between digestate concentrations and soil pH values on survival and reproduction of *F. candida*, the Generalized Linear Model (GLM) procedure was used.

3. Results

The pH values of experimental soils with and without digestate are reported in Figs 1 and 2.

The number of surviving adults of all experimental groups at the end of the test is reported in Fig. 1. The number of surviving adults of the experimental group with 5 % digestate and pH = 7.7 (5 % H) was statistically lower than the number of surviving adults of all other experimental groups (SNK; $P < 0.05$), while the numbers of surviving adults of the other experimental groups did not significantly differ among themselves (SNK; $P > 0.05$). The GLM analysis highlighted a significant effect of pH and digestate on survival, but no interactions between digestate and pH (pH, $P < 0.001$; digestate, $P < 0.05$; digestate*pH, $P = 0.088$).

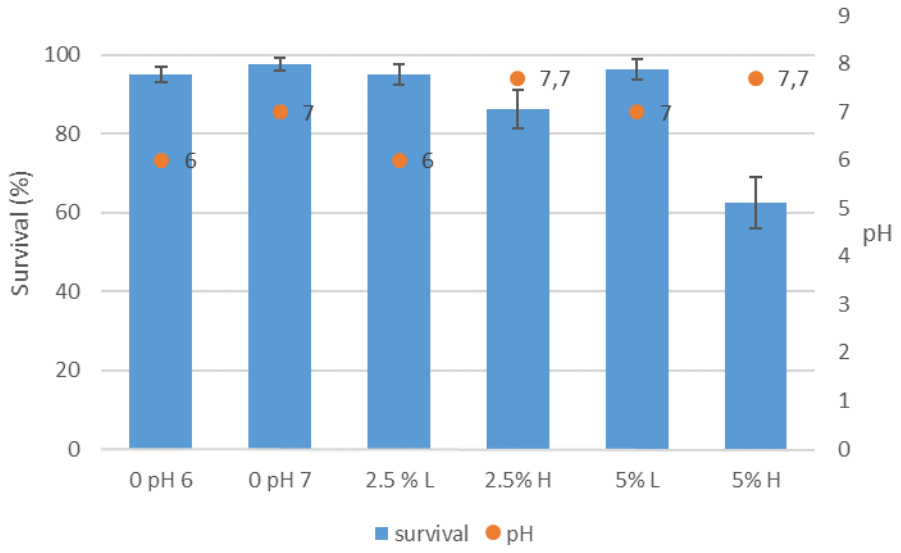

Figure 1. Percentage survival of *Folsomia candida* exposed for 28 days to different concentrations of digestate. pH values of experimental soils are also indicated. Survival values represent the mean number of eight replicates ± SE. Survival data were arc-sin transformed before statistical analysis. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test. H: artificial soil with pH = 6; L artificial soil with pH = 4.5.

The number of juveniles of all experimental groups at the end of the test is reported in Fig. 2. The number of juveniles of the experimental group with 2.5 % digestate and pH = 6 (2.5 % L) was not statistically different from the number of juveniles of the control group 0 pH 6 (SNK; $P > 0.05$). The numbers of juveniles of the experimental groups with 2.5 % digestate and pH = 7.7 (2.5 % H), with 5 % digestate and pH=7.0 (5 % L), without digestate and pH = 7.0 (0 pH 7) are not different among themselves (SNK; $P > 0.05$), but are significantly lower than the number of juveniles of the control group 0 pH 6 (SNK; $P < 0.05$). The number of juveniles of the experimental group with 5 % digestate and pH 7.7 (5 % H) is significantly lower compared with the numbers of all other experimental groups ($P < 0.05$). In particular, the numbers of juveniles of this group (5 % H) is significantly lower compared to the numbers of juveniles of the group of experimental soil with the same concentration of digestate, but with pH = 7.0 (5 % L) and of the group of experimental soil with the pH = 7.7, but a lower digestate concentration (2.5 % H). The GLM analysis highlighted a significant effect of pH on reproduction and interactions between digestate and pH (pH, $P < 0.001$; digestate, $P = 0.257$; digestate*pH, $P < 0.001$).

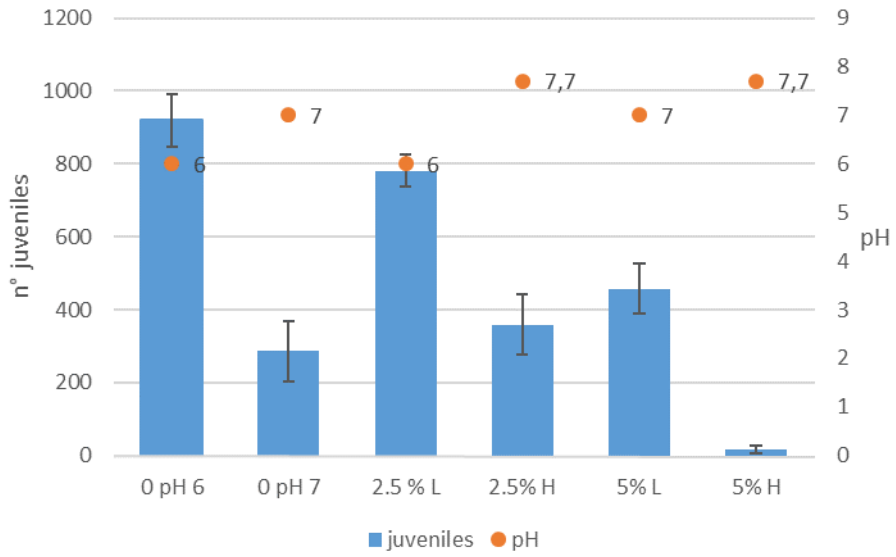

Figure 2. Reproduction, shown as number of juveniles, of *Folsomia candida* exposed for 28 days to different concentrations of digestate. pH values of experimental soils are also indicated. Numbers of juveniles are indicated as a mean number of eight replicates ± SE. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test. H: artificial soil with pH = 6; L artificial soil with pH = 4.5.

4. Discussion

The addition of the studied digestate resulted in an increase of experimental-soil pH values depending on digestate concentration and original pH values of the artificial soil. Reproduction seems to be the collembolan population characteristic mainly affected by the treatments. The data obtained indicate that the negative effects of digestate on survival and reproduction of *F. candida* can be mainly attributed to the pH increase of experimental soils to a value above 6. In fact, a negative impact on reproduction was found in all experimental soils with pH > 6, with and without digestate. The high pH value, however, does not explain by itself the strong decrease in the number of juveniles in the experimental soil where the highest digestate concentration is associated with the highest pH value: in this case it is possible to assume a combined effect of pH and other digestate characteristics, such as nitrogen content and salinity, currently under study. The experimental soil characterized by the

highest digestate concentration and the highest pH value is also the only one that showed a significant decrease of springtail survival compared to all the other experimental soils. Our results are in line with data on the sensitivity of *F. candida* to pH reported by other authors. Fountain & Hopkin (2005) report for this species a mild preference to settle on weakly acidic soils (pH = 5.6), whereas Greenslade & Vaughan (2003) affirm that the highest rates of reproduction were established at pH = 5.4 and a sharp decline was found at higher pH with few, if any, offspring being produced above pH = 7.0. Our study pointed out that the biological effects and possible ecological sustainability of materials used for the improvement of soil structure and/or fertility, such as the digestate in study, do not depend only on the characteristics of the material itself, but also on the results of its interaction with the characteristics of the soil to which it is added. Preliminary studies of the soil characteristics are therefore needed before a widespread use of organic fertilizers or soil improvers, in order to avoid or limit disturbance on the soil populations and, in the long term, a decrease in biodiversity. The addition of digestate in soils should in particular be discouraged when this practice strongly increases soil pH values. On the other hand, soil acidification, which can cause degradation with an increase in the toxicity of some metals and deficiency of nutrients for the plants reducing crop yields (Russell et al. 2006, Zhang et al. 2008), could be countered by using digestates. Soils differ greatly in pH according to the geographical location and thus the pH ecological optimum of their communities varies. In Emilia Romagna (Italy), soil pH values generally range from 7.3 to 8.4 (ARPA Emilia Romagna, 2010), and ecotoxicological tests useful for regulatory decisions regarding acidic soils may not be appropriate for these types of soils.

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CHAPTER 2

Ecotoxicological evaluation of a digestate for an agricultural use: effects on survival and reproduction of *Folsomia candida* (Collembola: Hexapoda).

Submitted to Chemosphere

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Keywords: anaerobic digestion; digestate characterization; *Folsomia candida*; ecotoxicological test; ISO test.

Abstract

The organic fractions of agricultural and industrial wastes and residues are considered as possible renewable energy resources, and their potential can be exploited through an anaerobic digestion process with the production of biogas and residual organic material commonly called digestate that could be used in agriculture as soil improver. Nevertheless, scarce or no evaluation of its effects on soil and soil organisms are available. The aim of present study was to characterize a mixture of three different digestates obtained from agro-industrial wastes mixed with biological sludge and to evaluate the effects on chemical and physical properties of the soil and on some traits of life history of the collembolan *Folsomia candida*.

We detected that the digestate investigated has negative effects on survival and reproduction of *F. candida* depending on concentration. In particular, only the highest concentration tested has a negative effect on the survival of *F. candida*, while the negative effects on reproduction, evident also at lower concentrations, increase with increasing concentration of digestate. These effects can be mainly attributed to the pH increase of experimental soils caused by digestate added. The high pH value, however, does not explain by itself the strong decrease in the number of juveniles in the experimental soil with the highest digestate concentration: in this case it is possible to assume a combined effect of pH and other digestate characteristics such as a cumulative or synergistic effect of the metals and salinity. Thanks to the measurement of all juveniles and their attribution to age size classes built previously, it was possible to attribute the adverse effects on reproduction of the digestate to effects on oogenesis.

1. Introduction

The agricultural industry played an important role in waste production with high amounts of CO_2 emitted: 5.381.510 Gg (5.4 Pg) CO_2 eq in the world, 407.040 Gg (0.4 Pg) CO_2 eq in Europe and 32.101 Gg (0.03 Pg) CO_2 eq in Italy (FAOstat, 2012). The total amount of agricultural biomass waste produced annually is around 5 billion tons (5 Pg)– the thermal equivalent of approximately 1.2 billion tons (1.2 Pg) of oil or 25 percent of current global oil production (UNEP, 2011). Bentsen et al. (2014) have found a global production of residues from six most important crops of 3.7 billion tons (3.7 Pg) dry matter yr^{-1} and, because of development towards high input agriculture, the global residue production can increase by about 1.3 billion tons (1.3 Pg) dry matter yr^{-1}. The agricultural residues generally are managed with elevated costs of transport and disposal. The most important biomasses are the residues from woodworking or forest activities, the wastes from farms and agro-business, the organic fraction of municipal solid waste, and the plants deliberately grown for energetic purposes. At present, agricultural by-products are mainly used as combustion feedstock for biofuels (Chandra et al., 2012; Demirbas et al., 2011). The Directive 2009/28/EC of the European Union promotes as target for 2020 the use of energy from renewable sources and biomasses are expected to contribute about two-thirds of the share of renewable energy. In 1997 Italy covered only 0.3% of energy needs through the use of biomass, in 2014 covered 6.4% of energy needs through biomass (AEEGSI, 2015). Moreover, Italy was, in 2013, almost in line with the aim for 2020 to reach the 17% share of renewable energy in gross final consumption of energy, one of the headline indicators of the EU's growth strategy for the next decade (Eurostat, 2015). In this context, the use of biomass as a source for renewable energy can play an important role to reach the target set for 2020. The organic fractions of agricultural and industrial wastes and residues are in principle considered as possible renewable energy resources, and their potential can be exploited through an anaerobic digestion process (González-Sánchez et al., 2015; Mata-Alvarez et al., 2014).

The anaerobic digestion is a promising technology enhancement to treat industrial, agro-industrial and urban wastes producing a methane-rich biogas. The anaerobic digestion option can play a

particular role in global warming savings, first, by substituting the use of fossil fuels with the produced methane-rich biogas, and second, by storing carbon in the soil and limiting the consumption of mineral fertilizers with the remaining digested substrate, called digestate used as a soil improver (Caramiello et al., 2013; Carchesio et al., 2014). In particular, the digestate can have very good fertilizing properties because of the high nutrient content (N, P, K) in available form and could contribute to solving the problem of the alteration of soil structure, to the retention of the nutrient levels and could increase to the enhancement of the microbial activity promoting soil fertility (Dolan et al., 2011; Makádi et al., 2012; Tambone et al., 2009, 2010; Weiland, 2010).

However, the digestates present physical-chemical properties, depending on process conditions, that may allow to modify some physical-chemical characteristics of soils, when added, such as pH (Gómez et al., 2007; Pognani et al., 2009; Stefaniuk et al., 2015), or buffer capacity. Modifications of the environmental conditions may induce alterations of soil population fitness and then increase the decline of biodiversity in soil rather than improving it. Long-term ecotoxicological effects of soil handling are not exhaustively highlighted only by chemical analysis, that don't make to reveal the effective impact of contaminants, environmental conditions and toxicity to soil organisms, and has to be combined with ecotoxicological tests (Van Gestel, 2012). The inhibition of reproduction of Collembola (*Folsomia candida*) test (ISO 11267: 1999, 2014) is suitable for this purpose and was applied also to test the effects of sewage sludge from wastewater treatment plants, and compost produced from organic waste on the soil community (Domene et al., 2007a, 2007b, 2008, 2010, 2011b; Fuchs et al., 2008; Natal-da-luz et al., 2009; Pivato et al., 2014). Nowadays, we still have little information on the biological effects of digestates obtained from the anaerobic digestion of organic residues on soil organisms (Fuchs et al., 2008; Moreira et al., 2008; Stefaniuk et al., 2015).

Thus, the aim of present study was to characterize a mixture of three different digestates and to evaluate the effects of different concentrations of this mixture on chemical and physical properties of the soil and the effects on survival and reproduction of the springtail F. candida, according to ISO guideline (ISO 11267: 2014). The agricultural residues of grape seeds and plum stones were used as

the original substrates to be tested through anaerobic digestion because the Italian regions of Emilia-Romagna and Marche produce in large amounts both residues (Caramiello et al., 2013; Carchesio et al., 2014). Since a preliminary research (d'Errico et al., 2015) showed that mixtures of digestates affects mainly F. candida reproduction, observations to highlight what traits of reproduction (oogenesis, embryonic development or post-embryonic development) are sensitive to the addition of the material in object to soil are performed.

2. Material and Methods

2.1 Digestates

A 1:1:1 mixture of three digestates described in a previous work (Carchesio et al., 2014) was studied. The three digestates were obtained from a laboratory-scale anaerobic digestion system, with the process conducted in batch mode under mesophilic operating conditions (Caramiello et al., 2013), which treated: i) biological sludge, ii) 1:1 mixture of grape seed and the sludge used as the inoculum, iii) 1:1 mixture of plum stone and the sludge used as the inoculum. The biological sludge was obtained from the anaerobic digestion stage at a large wastewater treatment plant (WWTP) located in central Italy near the Adriatic Sea. The original grape seed material was obtained from a cooperative wine-growers association located in the Emilia-Romagna Region, whereas the original plum stone material was collected from a fruit processing plant located in the same region.

The mixture of the three digestates, dried at 110°C for 24 h, was milled and used in particles of < 500 μm to obtain homogeneous samples.

2.2 Digestate mixture characterization

The solid fraction of the digestate mixture was characterized in terms of X-ray fluorescence (XRF ARL ADVANT'XP) spectrometry, using argon as inert gas, and elemental analysis. Calcium

carbonate content, total and organic carbon were determined following standard procedures (APHA, 1992). The inorganic element contents were evaluated on the basis of the residue after a thermal treatment at 600°C.

The carbonate content was measured by using a Dietrich-Fruhling calcimeter on 1 g samples. The samples react with HCl and the quantity of CO_2 released from the reaction of the carbonate with HCl was used to determine the percentage of $CaCO_3$ in the samples. The calcium carbonate content was used to evaluate the inorganic carbon (C_{inorg}), while the organic carbon percentage ($C_{organic}$) was calculated as: $C_{organic}$ (%) = [(C_{tot} – C_{inorg}) / C_{tot}] x 100.

The pH value of the digestate mixture was measured in a suspension 1:2.5 digestate:$CaCl_2$ following standard procedures (MiPAF, 1999).

2.3 Experimental soils

According to ISO guideline (ISO 11267: 2014) the standard artificial soil was composed of 69% quartz sand, 20% kaolinite clay, 10% *Sphagnum* sp. peat air-dried, ground and sieved to 0.05 mm. Deionized water was added during mixing to reach about 60% of the maximum water-holding capacity (WHC measured using guideline ISO 11267). Following the ISO guideline, pH value was adjusted to 6.0 ± 0.5 by adding $CaCO_3$.

Four different experimental soils were obtained with the addition to standard artificial soil of the digestate mixture, herein after called digestate, at different concentrations. The chosen concentrations of digestate of 1, 2.5, 4 and 5% correspond to 10.8, 27, 43.2 and 54 ton/ha (dry weight) respectively. The 1% concentration is well below that recommended for Italian agricultural soils. The conversion was made using the density of standard artificial soil of 1.1 g/cm^3, measured according to ISO 11272 (1998) and assuming a mixing in the first 10 cm of soil. Experimental soil with pH = 6.5 without digestate (identifiable as “0 pH 6.5”) was used as control soil because at this soil digestate mixture

was added. The artificial soil was also used to prepare an experimental soil with pH value 7.2 (identifiable as "0 pH 7.2") by adding adequate amounts of $CaCO_3$: this experimental soil, without digestate, was used for comparison, because preliminary studies revealed that the addition of digestate causes an increase in soil pH. All six experimental soils were prepared three days prior to the start of the toxicity test in order to equilibrate acidity.

2.4 Experimental-soils: pH and salinity analysis.

The pH_{H_2O} and electrical conductivity (EC) of experimental soils were measured, in two replicate samples of each experimental soil, following standard procedures for soil characterization (MiPAF, 1999.). Suspensions 1:2.5 soil: deionized water were prepared and shaken for 2 h at 200 rpm. After settlement of the particles, the pH and EC of the soil solution were measured using a Crison BASIC20 pH meter. The conductivity values are related to the salts content of the soils, i.e. the salinity. The measurements were conducted three days after the experimental soils preparation, immediately before the start of the toxicity test.

2.5 Test organisms

The utilised Collembola belonged to the species *Folsomia candida* Willem, 1902. The specimens were reared in the laboratory for several generations. They were maintained in 100 mL glass containers with a diameter of about 5 cm containing clay, mainly consisting of kaolinite and smectite, bottom saturated with deionized water and kept in a thermostatic chamber at 20±1°C with a light-dark cycle of 16:8 h. Animals were fed on Brewer's yeast.

2.6 *Folsomia candida* life history traits

Firstly, a study to evaluate some traits of life history of *F. candida* during 28 days, corresponding to the duration of the toxicity test ISO 11267 (2014) was carried out.

Eggs laid within 12 h were isolated and maintained in a thermostatic chamber at 20±1°C, and fifteen animals that had hatched within 6 h were transferred singly in 100 mL glass containers, with a diameter of about 5 cm containing clay, mainly consisting of kaolinite and smectite, bottom saturated with deionized water and regularly moistened. All containers were checked every day; animals were fed with yeast (5.7 mg in 40 days).

The observations were carried out for 40 days from hatching, because as reported by the ISO 11267 guideline it is necessary the use of animals 12 days old for a period test of 28 days. The time of the first oviposition was recorded for each female and the eggs laid by each female were counted. After oviposition, the females were transferred into other similar containers and checked every day to record the time of further oviposition; after each oviposition the females were transferred into other similar containers. The containers with eggs alone were also checked every day in order to record the hatching time. At the end of the study, all containers were put in freezer at – 20 °C. Successively the animals of each container were defrosted and digitally photographed with Nikon Coolpix S8000 (image quality fine; macro-mode). The animals of each oviposition were counted and the length of animals, from the end of the posterior abdominal segment to the anterior margin of the head, was measured through on-screen viewing by means of the ImageJ software. The obtained data were used to identify the size-age classes corresponding to the juveniles of the different oviposition made within 28 days of the test.

2.7 Toxicity test

The test was carried out according to ISO guideline (ISO 11267: 2014). Eight replicates were prepared for each experimental soil utilizing glass containers (100 mL capacity, 5 cm diameter). Ten 12-day-old springtails were placed in each tightly closed test container and were maintained at a temperature of 20±1°C with a light-dark cycle of 16:8 h for 28 days. The containers were opened twice a week for aeration and feeding with yeast (4 mg in 28 days). At the end of the test all containers were put in freezer at – 20 °C. Successively, water was added to each container: the animals sorted by floating were digitally photographed with Nikon Coolpix S8000 (image quality fine; macro-mode). The animals were grouped into adults and juveniles, counted and their length, from the end of the posterior abdominal segment to the anterior margin of the head, was measured by on-screen viewing by means of the ImageJ software. The juveniles were divided in size classes according to classes identified in the *F. candida* life history study (see above) and counted.

2.8 Statistical analysis

All statistical analyses were carried out using SPSS software release 22.0. Regarding data of life history traits, one-way Analysis of Variance (ANOVA) was performed to compare the time interval among different oviposition, the time of hatching and the length of juveniles among different oviposition. When significant statistical differences were detected ($P < 0.05$), a post hoc Student-Newman-Keuls test (SNK) was used.

Regarding data of toxicity test, one-way Analysis of Variance (ANOVA) was performed to compare the survival and reproduction levels of *F. candida* among control soil, the four experimental soils with digestate and the experimental soil without digestate at pH = 7.2. Percentage survival data were arc-sin transformed before statistical analysis. Number of juveniles was square root transformed to

obtain normal distributions of observations. When significant statistical differences were detected ($P < 0.05$), a post hoc Student-Newman-Keuls test (SNK) was used. Kruskal-Wallis test was performed to assess the effect of the digestate concentration on the number of juveniles found in different experimental soils, divided according to the size classes previously identified. When significant statistical differences were detected ($P < 0.05$), a U Mann-Whitney test was then carried out.

The data on survival, reproduction, soil pH and salinity were compared by means of a Principal Components Analysis (PCA) and Single Linkage Cluster Analysis

3. Results

3.1. Digestate mixture characterization

The chemical characteristics of the solid fraction of digestate are given in Table 1. The high calcium (Ca) content obtained by XRF spectrometry is in agreement with the high calcium carbonate content revealed by the calcimeter analysis results shown. The nitrogen content is higher than the maximum nitrogen load for Italian agricultural soils (Nordberg, 1999). The X-ray fluorescence spectrometry, highlighted the presence of heavy metals such as chromium (Cr), copper (Cu), zinc (Zn), lead (Pb), and important micronutrients for the growth of microorganisms such as iron (Fe), nickel (Ni), manganese (Mn), magnesium (Mg) (FNR, 2010; Weiland, 2010). The digestate is characterized by a pH value of 8.0.

Table 1. Inorganic chemical analysis, obtained by X-ray fluorescence (XRF) spectrometry, of digestate mixture after a thermal treatment at 600°C and expressed as % (not volatile solid =52.6%), calcium carbonate, elemental analysis (as C, N, and H contents) of the dry digestate, the calculated organic C and pH value of digestate.

Parameter	Units	Value
pH	$CaCl_2$ 1:2.5 (V/V)	8.0
$CaCO_3$	g kg^{-1}	138.5
C_{tot}	% (w/w)	27.09
C_{org}	% (w/w)	25.43
H	% (w/w)	3.42
N	% (w/w)	2.81
Ca	% (w/w)	34.52
Si	% (w/w)	10.23
Fe	% (w/w)	23.43
Al	% (w/w)	7.06
K	% (w/w)	3.06
P	% (w/w)	4.50
S	% (w/w)	4.52
Cl	% (w/w)	3.12
Na	% (w/w)	3.59
Mg	% (w/w)	0.91
Sr	% (w/w)	1.11
Zn	% (w/w)	1.07
Ti	% (w/w)	0.78
Ba	% (w/w)	0.53
Cu	% (w/w)	0.37
Mn	% (w/w)	0.35
Ni	% (w/w)	0.06
Cr	% (w/w)	0.18
Pb	% (w/w)	0.13
Br	% (w/w)	0.10

In Table 2 are reported the concentrations (µg/g) of heavy metals present in experimental soil with 5% digestate compared with concentrations that affected reproduction and eggs hatching of *F. candida* reported in literature.

Table 2. Concentration of heavy metals present in experimental soil with 5% digestate compared with literature data regarding no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), median effect concentration (EC_{50}) regarding *Folsomia candida* reproduction (28 days exposure) and hatching (10 days exposure).

	Concentration (µg/g) in soil with 5% digestate	**NOEC (µg/g)**	**LOEC (µg/g)**	**EC_{50} (µg/g)**	**Reference**
Reproduction					
Chromium	47	560	1000	604	Lock and Janssen 2002a
Copper	97			700	Sandifer and Hopkin 1996
				658	Løkke and van Gestel 1998
Zinc	281			900	Sandifer and Hopkin 1996
				391	Lock and Janssen 2003
Nickel	16	320	560	476	Lock and Janssen 2002b
Manganese	92	1067	1100	1663	Kuperman et al 2004
Lead	34			2970	Sandifer and Hopkin 1996
Barium	139	211	375	478	Kuperman et al 2006
Hatching					
Copper	97			625	Xu et al 2009
Zinc	281			1763	Xu et al 2009
Lead	34			2361	Xu et al 2009

3.2 Experimental soils: pH and salinity analysis

Values of pH and salinity of experimental soils with different digestate concentrations (1%, 2.5%, 4% and 5% in weight) and without digestate at the start of toxicity test, are shown in Fig. 1. The addition of alkaline digestate resulted in an increase of the pH of the experimental soils depending on the digestate concentration: in particular, the highest values were found in the samples with 4% and 5% in weight of digestate. The addition of digestate to the experimental soils affected the salinity values, expressed as electrical conductivity (µS/cm). Higher salinity values were measured at higher digestate concentration (4 and 5% wt).

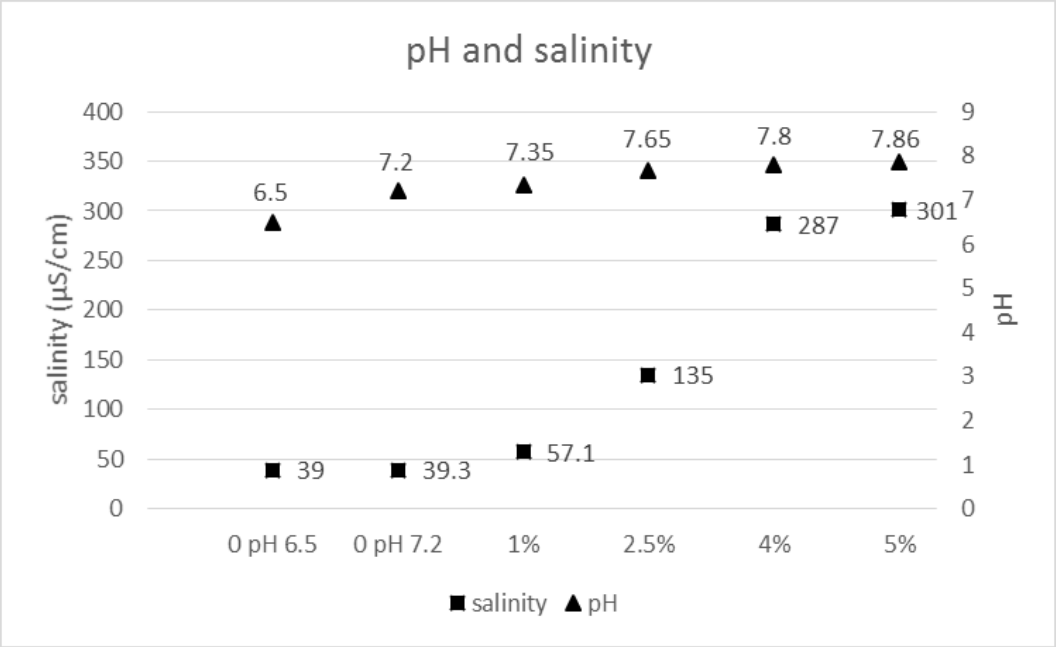

Fig.1 pH and **s**alinity of the experimental soils with different concentrations of digestate and without digestate at the start of the toxicity test (measured immediately before the start of the toxicity test)

3.3 *Folsomia candida* life history traits

The preliminary study revealed that at a temperature of 20 ± 1 ° C specimens of *F. candida* 12 days old in a period of 28 days, which corresponds to the time of test ISO 11267 (2014), have laid eggs four times but only the eggs of the first three oviposition (I, II, III oviposition) have hatched during the study period (Fig. 2). The I oviposition occurred 16.1 ± 1.3 days after hatching (average ± SD), the II oviposition 23.2 ± 1.4 and the III oviposition 30.4 ± 1.4 days after hatching. The fourth oviposition occurred on average 38.3 ± 1.6 days after hatching. The intervals among the different oviposition times were not significantly different except that between the third and the fourth oviposition that is longer. (SNK; $P<0.05$). The eggs hatched 9.5 ± 0.5 days (average ± SD) after the I oviposition, 9.5 ± 0.5 days after the II oviposition and 9.3 ± 0.6 days after the III oviposition. The length of embryonic development is not significantly different for the three oviposition times (ANOVA; $P>0.05$). The juveniles of the three different oviposition were measured: the length of

juveniles of the I oviposition was 1.30 ± 0.16 mm (average ± SD), 0.51± 0.10 mm of the II oviposition and 0.31± 0.06 mm of the III oviposition (Fig. 2). The average lengths of the juveniles of the three oviposition were significantly different among themselves (SNK; $P<0.05$). The data were used to identify the size-age classes corresponding to the three different oviposition (I, II, III oviposition) that occurred within 28 days of the test.

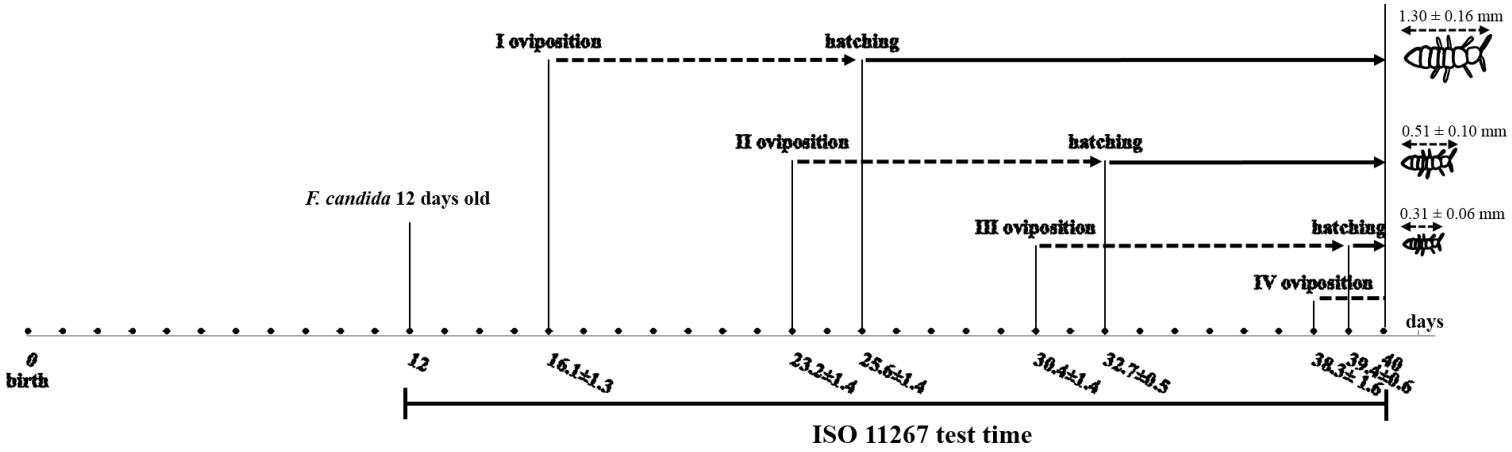

Fig. 2 *Folsomia candida* life history traits. Data obtained from 15 females observed for 40 days from hatching (the ISO guideline states the use of animals 12 days old and the test period is 28 days).The dotted arrow indicates the time from oviposition to hatching and continuous arrow indicate the time from hatching and the end of the study. The time is reported as mean days ±SD; the juvenile length is reported as mean length ±SD.

3.4 Toxicity test

The number of the surviving adults of all experimental groups at the end of the test is reported in Fig. 3. The number of surviving adults of the experimental group with 5wt% of digestate was significantly lower than the number of surviving adults of all the others experimental groups (SNK; $P<0.05$), while the numbers of surviving adults of the other groups did not significantly differ among themselves (SNK; $P>0.05$).

The number of juveniles of all experimental groups at the end of the test is reported in Fig. 4. The number of juveniles of the experimental groups with 1 and 2.5wt% of digestate and without digestate (0 pH 7.2) did not significantly differ among themselves (SNK; $P>0.05$), but their number was significantly different as compared with the number of juveniles of the control group (SNK; $P<0.05$). The number of juveniles of the experimental group with 4wt% digestate was significantly different as compared with all the other experimental groups, and also the number of juveniles of the experimental group with 5wt% digestate was significantly different as compared with all the other experimental groups (SNK; $P<0.05$).

The length of juveniles found at the end of the toxicity test, allowed us to divide them into three size classes (I, II, III) according to the size-age classes obtained from the *F. candida* life history study (Fig. 5).

Only the number of juveniles of the class size I of the experimental group treated with the highest concentration of digestate (5wt%) was significantly lower than the number of juveniles of the same class of the control group (Fig. 5). The number of juveniles of the class size I of the all other experimental groups treated with digestate did not significantly differ among themselves and with the control group (Mann- Whitney test; $P<0.05$). The number of juveniles of the II and III classes of the experimental groups with digestate and of the experimental group without digestate and with pH 7.2 was significantly lower than the number of juveniles of the same classes of the control group, except

for the number of juveniles of the II class size of the experimental group with 1wt% digestate that was not significantly different as compared with the number of juveniles of the same size class of the control group (Fig.5). The number of juveniles of the classes size II and III of the experimental groups treated with 1% and 2.5wt% of digestate and the experimental group without digestate and with pH 7.2 did not significantly differ among themselves (Mann- Whitney test; $P<0.05$).

The PCA highlights that the first principal component PC1 (eigenvalue 2.78; % variation 69.59) allows to separate three clusters: i) experimental groups with 4wt% and 5wt% of digestate, ($PC1 \geq 0.85$; right half plane); ii) experimental groups with 1wt%, 2.5wt% of digestate and without digestate pH 7.2 ($-0.50 \leq PC1 \leq 0.51$; vertical strip); iii) control group ($PC1 \leq -1.49$; left half plane). The second principal component PC2 (eigenvalue 0.68; % variation 17.1) allows to separate the experimental group with 4% of digestate from the experimental group with 5% (Fig. 6).

Cluster Analysis has divided the experimental groups in a dendrogram with three branching (Fig. 7). The first branching separates the experimental groups with lower digestate concentrations (1wt%: 2.5wt% digestate) and experimental group without digestate and pH 7.2, from experimental groups with higher digestate concentration (4wt % and 5wt %). Another branching separates the control group from all the other experimental groups.

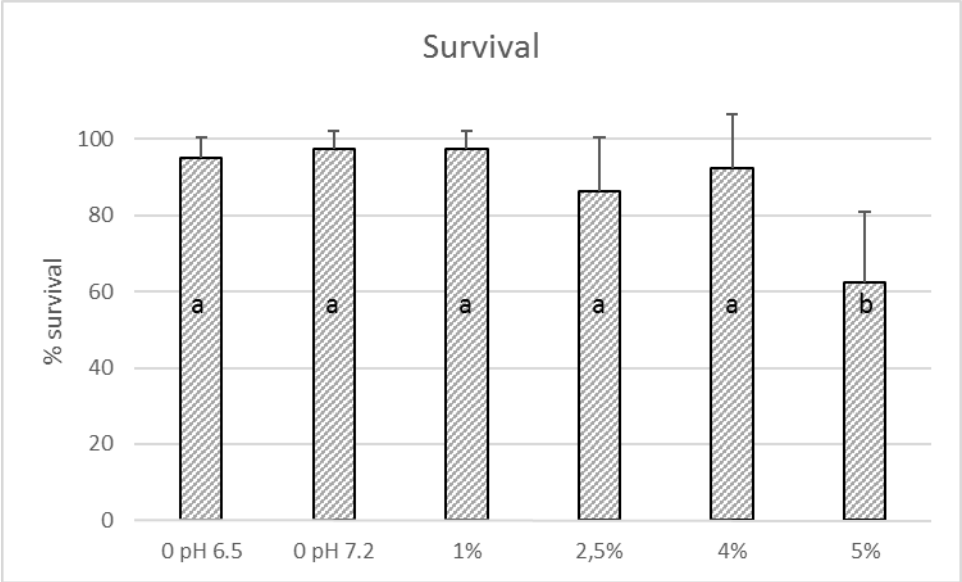

Fig. 3 Percentage survival of *Folsomia candida* exposed for 28 days to different concentrations of the digestate and different experimental soil pH values. Survival values represent the mean number of eight replicates ± SD. Survival data were arc-sin transformed before statistical analysis. Different letters indicate significantly different means at P<0.05 according to the Student-Newman-Keuls' test.

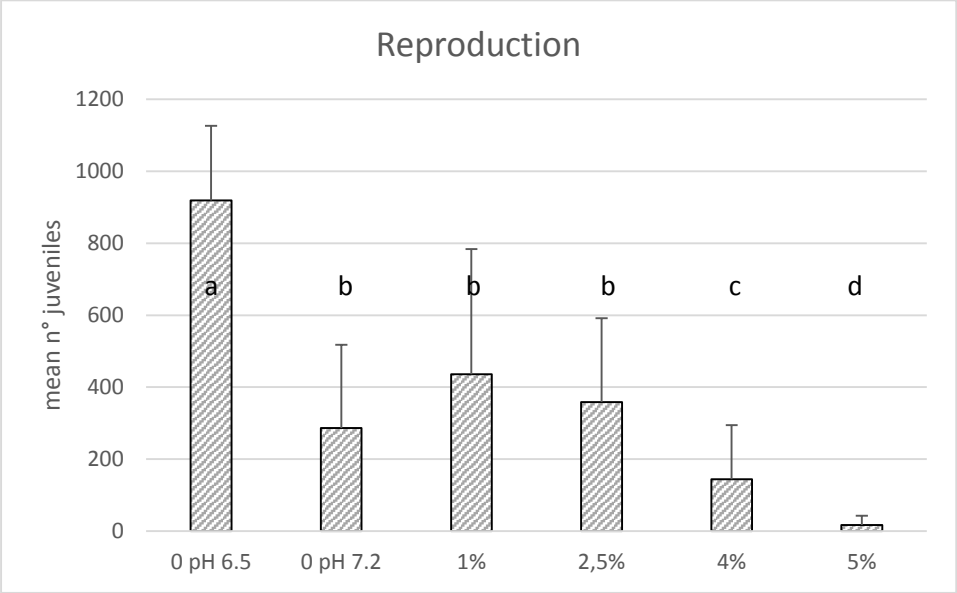

Fig. 4 Reproduction, shown as number of juveniles, of *Folsomia candida* exposed for 28 days to different concentrations of the digestate and different experimental soil pH values. Numbers of juveniles are mean number of 8 replicates ± SD. Data were square root transformed to obtain normal distributions of observations. Different letters indicate significantly different means at P<0.05 according to and the Student-Newman-Keuls' test.

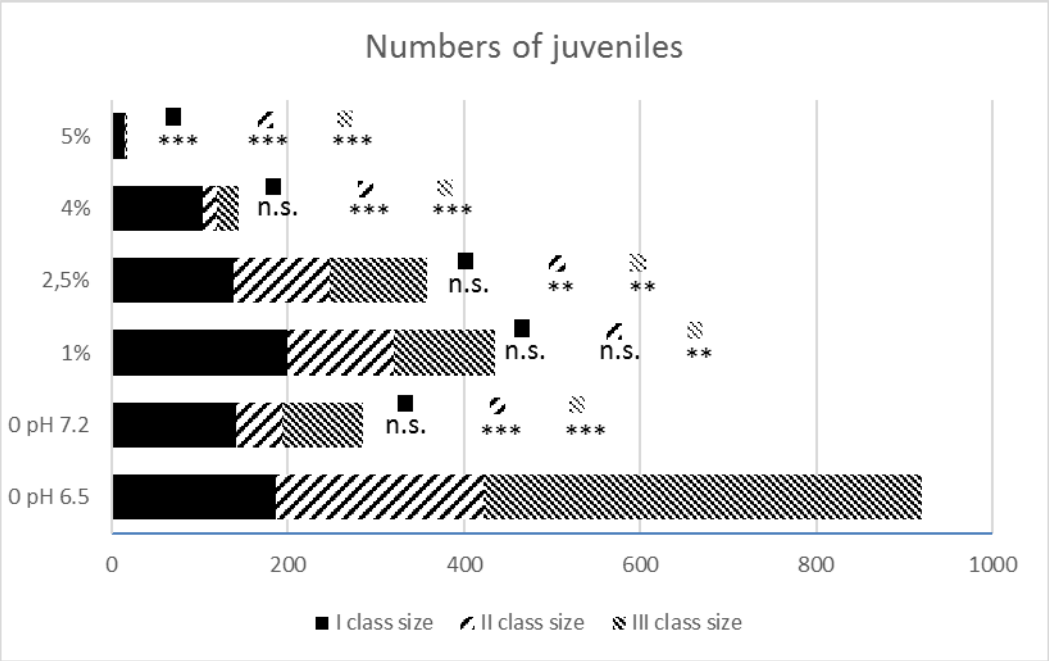

Fig. 5 Number of juveniles, of *Folsomia candida* exposed to different concentrations of digestate and different experimental soil pH values. Numbers of juveniles are mean number of 8 replicates. Each bar in the graph is divided according to the three size classes (I, II, III) that correspond to the three age-size classes recorded during the preliminary study Stars indicate significantly significant differences as compared with control group according to the Mann-Whitney U test (n.s. not significant, ∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001)

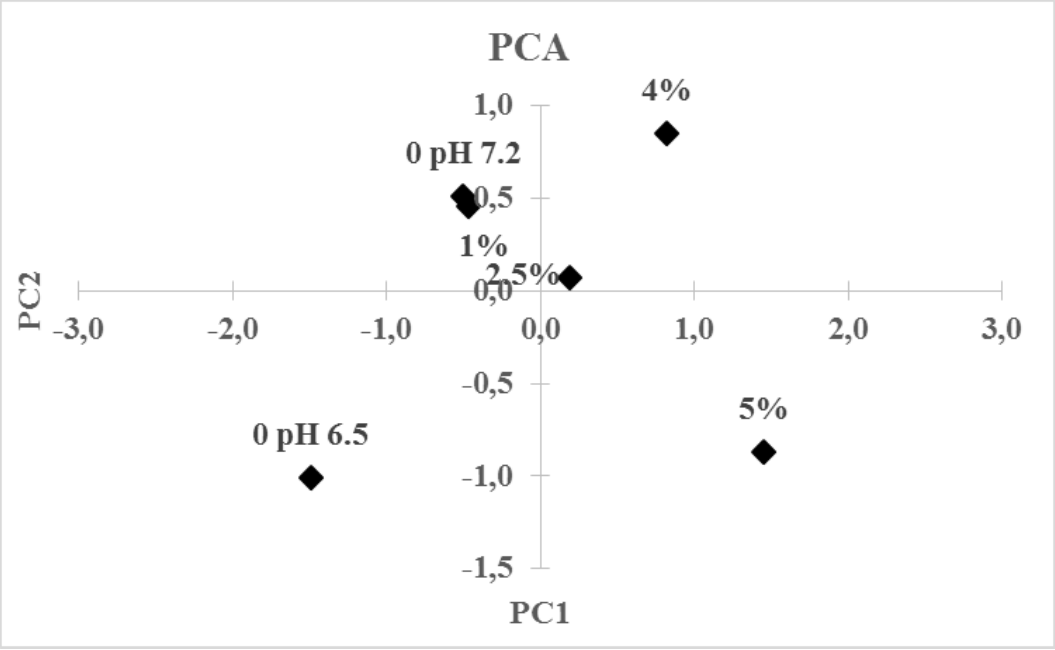

Fig. 6 Principal Component Analysis (PCA) of experimental groups characterized by data of survival, reproduction of *Folsomia candida* exposed for 28 days to different concentrations of digestate and to different soil pH and the pH and salinity values of their experimental soils (PC1: eigenvalue 2.78, % variation 69.59; PC2: eigenvalue 0.68; % variation 17.1)

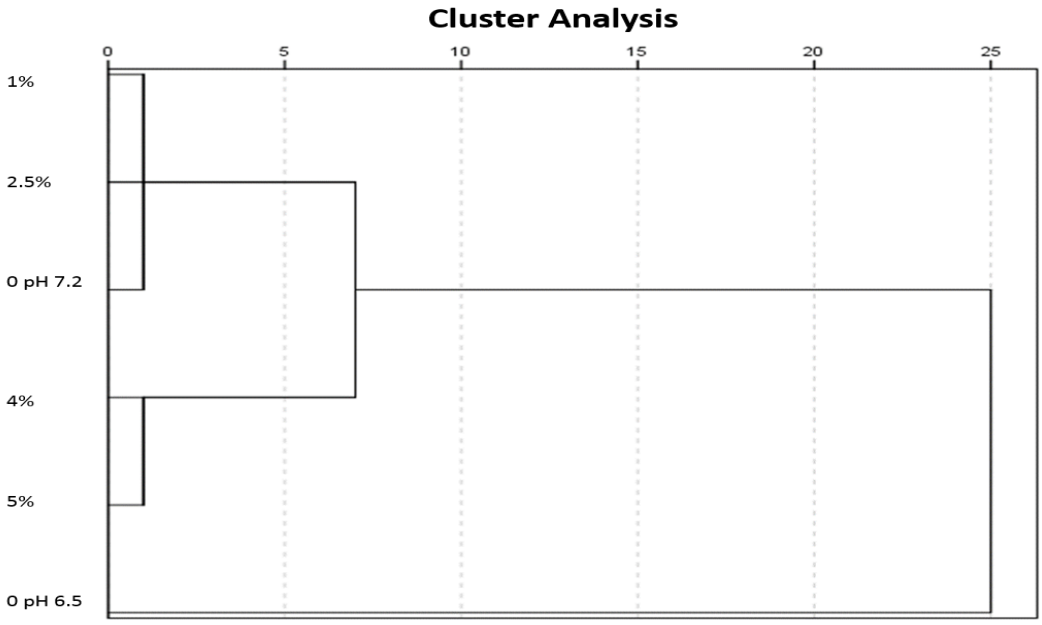

Fig.7 Clustering dendrogram resulting from the classification of groups according to soil pH, soil salinity, digestate concentrations, survival and reproduction of *Folsomia candida*, exposed for 28 days to different concentrations of digestate and to different soil pH.

4. Discussion

In this study we detected that the digestate investigated has negative effects on survival and reproduction of the springtail *F. candida* depending on digestate concentration. In particular, only the highest concentration tested has a negative effect on the survival of *F. candida,* while the negative effects on reproduction are evident also at lower concentrations tested and increase with increasing concentration of digestate. The addition of alkaline digestate to the soil resulted in an increase of the experimental soil pH depending on the digestate concentration The pH value of digestate was alkaline because before starting the anaerobic digestion process at the lab-scale it was adjusted to a level above a threshold of 8 with a small amount of Na_2CO_3 powder to prevent a detrimental decrease during the early phase of the process- (Carchesio et al., 2014; Esposito et al., 2012; Weiland, 2010).

In our previous work (d'Errico et al., 2015), we demonstrated that the effect of the digestate on *F. candida* survival at the concentration of 5wt% was attributable only to an increase in pH caused by

the addition of digestate to the soil. As regarding reproduction, the negative effect of the lowest concentration of digestate tested was attributed to increase in pH alone, while the effect of the higher concentration was attributed to a combined effect of pH and other characteristics of the digestate not identified (d'Errico et al., 2015). The negative effects of soil pH on the reproduction of *F. candida* is in line with the data obtained in the present work. In fact, we did not detect differences between the experimental groups treated with lower digestate concentrations and the experimental group without digestate (0 pH 7.2): PCA and Cluster Analysis confirm the similarity among these experimental groups. Regarding pH, our results are in line with data of Greenslade and Vaughan (2003) that found the highest rates of *F. candida* reproduction at pH = 5.4 and a sharp decline at higher pH with few offspring produced above pH = 7.0. In experimental soils treated with the highest concentrations of digestate the effects on reproduction could be caused both by the increase in pH and by other digestate characteristics such as nitrogen content, salinity and heavy metal concentration considered in this study. On the base of the data obtained, we cannot exclude a negative effect of nitrogen on survival and reproduction of *F. candida.* However, the content of nitrogen in our experimental soils is lower than those tested by other authors who affirm that nitrogen can have a negative effect on the survival, feeding behavior and reproduction of *F. candida* (Domene et al., 2007a, 2007b, 2011a).

The presence of heavy metals such as zinc (Zn), copper (Cu) and chromium (Cr), observed in digestate, can be related to the anaerobic sludge, but also to the residues submitted to the digestion process. However, the concentration of the majority of heavy metals in all experimental soils tested in present study was below the concentrations reported as toxic in different traits of the life history of *F. candida*, and in many cases even below the no effect concentration as reported in Table 2 but cannot be excluded a cumulative or synergistic effect of the metals. Some authors have suggested that reducing soil pH, increases metal toxicity. Fountain and Hopkin (2005) affirm that, the lower the pH of the soil, the more negative the effect of metals on reproduction of *F. candida* at a given concentration; but in our study the negative effects on reproduction of *F. candida* increased with

increasing soil pH. We also cannot exclude the additive toxic effect of other toxicants present in the sludge used as inoculum and as reported by Natal-da-luz et al., (2011) for another sludge.

The high Ca content of digestate can be connected with the bioconversion of organic matter into biogas, it causes an expected release of compounds as Ca that reacts with carbonate or phosphate and precipitates (Carchesio et al., 2014; Chen et al., 2008; Marcato et al., 2009). The Ca content could have influenced the uptake and effects of copper in *F. candida* in line with results of Ardestani et al (2013).

In line with what reported for other digested materials by other Authors (Voelkner et al., 2015), the addition of digestate to the experimental soils affected the electrical conductivity due to the high amount of inorganic salts present in the digestates such as NaCl, as reported by Caramiello et al. (2013). Consequently, a proportion between conductivity and digestate addition is pointed out. Higher salinity increase was measured at high digestate concentrations, probably because oxic microbial decomposition of organic fragments contained in the digestates releases cations and anions (Yilmaz and Alagöz, 2010). The salinity level may lead to a decrease in seed germination and may reduce microbial activity (Teglia et al. 2011; Voelkner et al., 2015). Some authors highlighted effects of soil salinity on survival and reproduction of the collembolan *F. candida* (Hutson, 1978; Owojori et al., 2009), however, the soil salinity reached in our experimental soil after addition of the highest concentration of digestate was lower than the salinity that in the work of these authors has a significant effect on *F. candida* reproduction, and also below the no effect value.

5. Conclusions

The present and previous our study (d'Errico et al. 2015) showed that the toxic effect of the addition of digestate to soil, occurs mainly on the reproduction of *F. candida*, and even at the lowest concentration we found a decrease in the number of juveniles. The low number of juveniles could be attributed to the negative effect on oogenesis, embryonic and post-embryonic development, or on all three. In order to understand at what stage of the springtails life cycle the digestate had a negative effect, we built the age size classes on the base of the data obtained for each oviposition in our study on *F. candida* life history traits and that are in line with data reported in literature (Snider, 1973). All the juveniles born during the toxicity test were divided into the three size classes (I, II, III) corresponding to three oviposition (I, II, III) that we suppose have occurred during the 28 days of the test. Based on the data obtained we suppose that, with the exception of the higher concentration tested, it is possible to exclude a negative effect of digestate on embryonic and post-embryonic development of *F. candida* because the juveniles of the class size I have made embryonic and post embryonic development in presence of digestate and their numbers did not differ from that of control. The eggs of the I oviposition were laid soon after the start of the toxicity test while the eggs of the II and III oviposition were laid by females that were in the presence of the digestate for at least 10 days and it can be hypothesize an effect of digestate on oogenesis. Regarding the experimental group treated with the highest concentration of digestate we can hypothesize a negative effect of digestate also on embryonic and/or postembryonic development. Our approach could be used to obviate to the impossibility to directly observe, during the test, the reproduction and to separate from juvenile mortality and hatching success. In conclusion the tested digestate has negative effects on reproduction also at lower concentrations and this effect was attributed to increase in pH alone. Thus this digestate could be used as soil improver at low concentrations to counter soils acidification.

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CHAPTER 3

Influence of soil type in standardized soil ecotoxicological tests with *Folsomia candida* (Collembola: Exapoda).

In preparation

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Abstract

ISO 11267 guideline describes a method that is based on the determination of sublethal effects to Collembola of the species *Folsomia candida* Willem, 1902 of contaminated soils or of substances added to standard soils. ISO guideline provides for the possibility of using a standard artificial soil (OECD soil) or a standard natural soil (e.g. LUFA soil) to test both organic (e.g. waste) and inorganic (e.g. heavy metal) contaminants. However, artificial soil does not represent realistic field conditions, because many soil properties are important factors that determine the bioavailability of contaminants to soil organisms. The aim of present study was to compare the use of OECD and LUFA 2.2 soils to evaluate the effects on the *F. candida* reproduction of two contaminants, a heavy metal (cadmium) and a digestate, residual organic material obtained from an anaerobic digestion process. The effect of cadmium on the reproduction of *F. candida* resulted not different in the two standard soils. In a perspective of environmental risk assessments, the use of two different standard soil do not influence the outcome of the toxicity test. The effects on reproduction of the digestate detected in the two soils were different: the test carried out with OECD artificial soil did not detect effect of the digestate on

the reproduction of *F. candida*, while the juveniles in LUFA soil treated with digestate were very scarce or absent. One of the causes of the strongly decrease of the number of juveniles could be the increase of soil pH caused by addition of the digestate in LUFA soil. In this work, we evaluated the possibility to utilize a miniaturized version of the *F. candida* reproduction test. The halving of the amount of the soil and simultaneously the halving of the number of animals would seem to be a valid alternative to the standard *F. candida* reproduction test. However, more in-depth studies are necessary before using the miniaturization of the reproduction test for environmental risk assessment

1. Introduction

Ecotoxicological tests are used to obtain information on the effects of contaminants in the soil and are proposed to complete conventional chemical analysis (Van Gestel, 2012). ISO 11267 guideline (1999, 2014) describes a method that is based on the determination of sublethal effects to Collembola of the species *Folsomia candida* Willem, 1902 of contaminated soils or of substances added to standard soils. This microarthropod species is common and widespread, easy to maintain in the laboratory and has parthenogenetic populations. The first edition of ISO 11267 guideline was published in 1999 and in 2014 was published the second edition. The main differences between the two editions are the substitution in the title of the word "pollution" with the word "contaminant" and the possibility of using, in addition to a standard artificial soil (OECD, 1984), standard natural, field-collected, soils e.g. LUFA 2.2 (Schinkel, 1985). Several soil toxicity tests are carried out using the OECD soils, which allow comparisons between laboratories. However, artificial soil does not represent realistic field conditions, because many soil properties are important factors that determine the bioavailability of contaminants to soil organisms (Bielská et al., 2012; Waalewijn-Kool et al., 2013) and significantly influence reproduction of *F. candida* (Domene et al., 2011; Römbke et al, 2006). Furthermore, Bielská et al., (2012) and Hofman et al., (2014) compared artificial soils prepared

according to OECD (1984) standardized procedures in different laboratories and revealed variability with respect to behaviour of contaminants tested. Therefore, the OECD standard artificial soil cannot be considered always as "standard". Hofman et al., (2008) highlighted that the results measured in the artificial soils could underestimate the situation in natural soils almost by one order of magnitude. In this perspective, the use of a standard natural soil as LUFA 2.2 in ecotoxicological tests allow to expose the organisms to contaminants in conditions more similar to those present in the field. In the literature there are several studies on the toxicity test with LUFA 2.2 soil (Ardestani and van Gestel, 2013; Broerse and van Gestel, 2010; Römbke et al., 2006) but few studies have compared the effects of heavy metals added to artificial and natural soils (Amorim et al., 2005a; Luo et al., 2014; Römbke et al., 2006; Smit and van Gestel, 1998; Van Gestel and Mol, 2003) and even fewer studies have compared the effects on *F. candida* of organic compounds added to the two different soils (Amorim et al., 2005b). Some Authors have used toxicity tests with *F. candida*, carried out in standard artificial soil OECD to evaluate the effects of organic wastes (Crouau et al., 2002; Domene et al., 2007; Natal-da-Luz et al., 2009; Scheffczyk et al., 2009) and/or derivates such as digestates (d'Errico et al., 2015; Moreira et al., 2008; Stefaniuk et al., 2015) but to date there are no studies comparing OECD and LUFA soils.

The aim of present study was to compare the use in ecotoxicological test of the standard artificial soil (OECD, 1984) and the standard natural soil LUFA 2.2 both proposed in the guideline ISO 11267 (ISO, 2014). We studied the effects of two contaminants (a heavy metal and organic compounds) on the survival and reproduction of the collembolan *Folsomia candida* Willem, 1902, separately added to the two soils types. The tests have been carried out with cadmium (Cd), a natural contaminant of zinc ores and widely distributed in the environment due to human industrial activities, and a digestate. We used cadmium because it is one of the most tested metals on *F. candida* (Ardestani et al., 2014; Crommentuijn et al., 1997; Nota et al., 2013; Sandifer and Hopkin, 1996; Spurgeon et al., 1994; Van Gestel and Mol, 2003) and a digestate, obtained from the anaerobic digestion of biomass, produced in the Emilia Romagna region in Italy in large amounts and that could be used as soil improver.

Furthermore, Van Gestel, (2012) affirm that the currently available toxicity test may need adjustment to make them applicable for determining the toxicity of new and emerging contaminants, often produced in very small quantities and with synthesis process very expensive. In this perspective Filser et al., (2014) proposed the miniaturization of test systems and reported the results of tests carried out with LUFA 2.2 soil without contaminants.

Another aim of this work is investigate the effects on *F. candida* reproduction of two concentrations of cadmium in two different soils (OECD and LUFA 2.2), reducing the number of springtails and the amount of soil provided for by the ISO guideline (ISO, 2014).

2. Material and Methods

2.1 Test organisms

The utilised Collembola belonged to the species *Folsomia candida* Willem, 1902. The specimens were reared in the laboratory for several generations. They were maintained in 100 mL glass containers with a diameter of 5 cm containing clay, mainly consisting of kaolinite and smectite, bottom saturated with deionized water and kept in a thermostatic chamber at 20±1°C with a light-dark cycle of 16:8 h. Animals were fed on Brewer's yeast. To obtain synchronised animals, adults were transferred in new containers to lay eggs and after two days, adults were removed. The eggs were checked every 12 h to record the time of hatching that occurred about after 9 days. In all experiments were used springtail 10 days old, born within 12 h.

2.2 Test soils

Tests were performed with a standard artificial soil according to OECD (1984) and ISO (2014) and LUFA 2.2 standard natural soil (ISO, 2014). The OECD soil is composed of 69% quartz sand (dominant fine sand with more than 50 % of particle size 0.05 mm to 0.2 mm), 20% kaolinite clay and 10% *Sphagnum* sp. peat, air-dried, ground and sieved to 0.05 mm, with no visible plant remains. Following the ISO guideline, pH value was adjusted to 6.0 ± 0.5 by adding calcium carbonate ($CaCO_3$, pulverized, analytical grade). LUFA 2.2 soil was purchased from the LUFA-Institute at Speyer, Germany and the initial pH of 5.4 was not manipulated. The amounts of deionized water used were sufficient to reach about 60% of the maximum water-holding capacity (WHC) of the two soils type measured using guideline ISO 11267 (2014). The particle size distribution (sand, clay, silt), organic matter content (OM) and pH_{CaCl_2} were measured in accordance with standard procedures for soil characterization (MiPAF, 1999). The density of soils was measured according to ISO (1998). The characteristics of the two soils are shown in Tab. 1.

Table 1 Main characteristics of the test soils, showing approximate values for: particle size distribution (sand, clay, silt), organic matter content (OM), pH and density

Soil	pH	O M	Sand	Clay	Silt	Density
OECD	6.13	9%	79%	12%	9%	1.1 g/cm^3
LUFA 2.2	5.43	3%	77%	15%	8%	1.2 g/cm^3

2.3 Experimental soils with cadmium

Six different experimental soils were obtained with the addition to OECD and LUFA 2.2 soils of a solution of cadmium ($Cd(NO_3)_2 \cdot 4H_2O$; SIGMA-ALDRICH, ≥99% pure in deionized water), at nominal concentrations of 0-70-140 µg Cd/g dry soil. These concentrations were selected on the basis

of literature results of ecotoxicological tests of cadmium effects on *F. candida* reproduction (Crommentuijn et al., 1997; Crouau et al., 1999; Van Gestel and Mol, 2003). The amounts of water used were sufficient to moisten the soils to the moisture level following guideline ISO (2014). All six experimental soils were prepared three days prior to the start of the toxicity test. The pH_{CaCl_2} value of experimental soils was measured at the start of the toxicity test, in two replicate samples of each experimental soil, in accordance with standard procedures for soil characterization (MiPAF, 1999). The soils were shaken with $CaCl_2$ solution 0.01 M (1:2.5) for 2 h at 200 rpm. After settlement of the particles, the pH of the soil solutions was measured using a Crison BASIC20 pH meter. The measurements were conducted three days after the experimental soils preparation, immediately before the start of the toxicity test.

2.4 Experimental soils with digestate

The digestate (Tab. 2) used in this study was obtained from processing waste of maize mixed with manure. The pH value and electrical conductivity (EC) of digestate were measured following standard procedures (MiPAF, 1999). Suspensions 1:2.5 digestate:$CaCl_2$ were prepared and shaken for 2 h at 200 rpm. After settlement of the particles, the pH and EC of digestate were measured using a Crison BASIC20 pH meter. The conductivity values are related to the salts content of the soils, i.e. the salinity. The organic matter content (OM) was measured following standard procedures (MiPAF, 1999). The digestate was dried at 110°C for 24 h, milled and used in the soil in particles of <500 μm.

Table 2 Main characteristics of the tested digestate, showing values for: pH, electrical conductivity (EC) and organic matter content (OM),

	pH	EC (mS/cm)	OM (%)
digestate	7.36	13.55	76.7

Six different experimental soils were obtained with the addition to OECD and LUFA 2.2 soils of digestate at concentrations 0-2-4%. The chosen concentrations of digestate of 2 and 4% correspond to 22 and 44 tons/ha (dry weight) respectively. The lowest dose is lower than the recommended for

Italian agricultural soils. The conversion was made using the density of standard artificial soil of 1.1 g/cm^3 and the density of LUFA 2.2 soil of 1.2 g/cm^3, measured according to ISO (1998) and assuming a mixing in the first 10 cm of soil. The amounts of water used were sufficient to moisten the soils to the moisture level following guideline ISO (2014). All six experimental soils were prepared three days prior to the start of the toxicity test.

The pH_{CaCl_2} value of experimental soils was measured at the start of the toxicity test, in two replicate samples of each experimental soil, in accordance with standard procedures for soil characterization (MiPAF 1999). The soils were shaken with $CaCl_2$ solution 0.01 M (1:2.5) for 2 h at 200 rpm. After settlement of the particles, the pH of the soil solution was measured using a Crison BASIC20 pH meter. The measurements were conducted three days after the experimental soils preparation, immediately before the start of the toxicity test.

2.5 Toxicity test

Eight replicates were prepared for each experimental soil utilizing glass containers (100 mL capacity, 5 cm diameter). According to ISO guideline 11267(ISO, 2014), ten 10-day-old springtails were placed in each tightly closed test container and were maintained at a temperature of 20±1°C with a light-dark cycle of 16:8 h for 28 days. The containers were opened twice a week for aeration and feeding with yeast (4 mg in 28 days). At the end of the test all containers were put in freezer at – 20 °C. The adults of *F. candida* of the toxicity test with cadmium were counted and removed from the frozen containers for the analysis of cadmium concentration.

For counting, water was added to each frozen container: the animals sorted by floating were digitally photographed with Nikon Coolpix S8000 (image quality fine; macro-mode). The animals were counted by on-screen viewing by means of the ImageJ software.

2.6 Cadmium analysis

For the cadmium analysis the frozen adults of *F. candida* were lyophilized and accurately weighted. Samples were added with 100 µL of hydrogen peroxide (H_2O_2) and 500 µL of nitric acid (HNO_3) and then maintained in pre-digestion for at least 12 h. Digestion were completed using a microwave mineralization system (CEM Mars-6, CEM Corporation, Matthews NC). Quality assurance and quality control were assayed by processing blank samples and standard reference material (SRM NIST-2977, National Institute of Standards and Technology, Gaithersburg, MD, USA). Cadmium was measured by atomic absorption spectrophotometry (AAS) using graphite furnace atomization and Zeeman effect (SpectrAA 300 Zeeman, Varian, Mulgrave, VIC, Australia). A palladium solution (1 g/L, 20% nitric acid, 10% citric acid) was added as chemical matrix modifier and the standard addition technique was applied for resolution of matrix effects. The concentrations were expressed as µg/g dry weight (d.w.). Three replicates were used per exposure concentration. The values obtained for the standard reference material were always within the 95% of confidence interval of certified values.

2.7 Miniaturization test

For the miniaturization test we used OECD standard artificial soil and LUFA 2.2 standard natural soil and we varied soil mass (20 or 10 g dry weight) and numbers of springtails (10 days old) introduced at start of the test (10 or 5 animals) for a total of eight experimental series (Tab. 3). For each experimental series tree experimental groups were prepared with the addition to standard soils of a solution of cadmium ($Cd(NO_3)_2 \cdot 4H_2O$; SIGMA-ALDRICH, ≥99% pure in deionized water), at nominal concentrations of 0-70-140 µg Cd/g dry soil. The amounts of water used were sufficient to moisten the soils to the moisture level following guideline ISO (2014). All experimental soils were

prepared three days prior to the start of the toxicity test. Eight replicates were prepared for each experimental group for a total of 192 containers test.

Table 3 Experimental series prepared to miniaturization test

experimental series with OECD soil	experimental series with LUFA soil
5 animals in 10 g soil	5 animals in 10 g soil
5 animals in 20 g soil	5 animals in 20 g soil
10 animals in 10 g soil	10 animals in 10 g soil
10 animals in 20 g soil	10 animals in 20 g soil

Tests were performed in glass containers of 100 mL diameter of 5 cm for 20 g soil mass and in glass containers of 50 mL, diameter 2.5 cm for 10 g soil mass maintaining the surface-to-volume ratio constant. Depending on the experimental series, 5 or 10 springtails 10 day sold were placed in each tightly closed test container and were maintained at a temperature of 20±1°C with a light-dark cycle of 16:8 h for 28 days. The containers were opened twice a week for aeration and feeding with yeast (4 mg for experimental groups with 10 animals and 2 mg for experimental groups with 5 animals in 28 days). At the end of the test all containers were put in freezer at – 20 °C. Successively, water was added to each container: the animals sorted by floating were grouped into adults and juveniles and were digitally photographed with Nikon Coolpix S8000 (image quality fine; macro-mode). The animals were counted by on-screen viewing by means of the ImageJ software.

2.8 Statistical analysis

All statistical analyses were carried out using SPSS software release 22.0. One-way Analysis of Variance (ANOVA) was used to assess the effects of cadmium and digestate on the survival and reproduction levels of *F. candida*. When significant statistical differences were detected ($P < 0.05$), a post hoc Student-Newman-Keuls test (SNK) was used. Percentage survival data were arc-sin transformed before statistical analysis. Data of cadmium analysis were analysed by Mann-Whitney test.

The Generalized Linear Model (GLM) procedure was used for analyse the effects of the amount of soil and of the number of introduced animals considering separately the experimental groups with the same type of soil (OECD or LUFA) and the same concentration of cadmium (0-70-140 µg Cd/g dry soil)

3. Results

The percentage of surviving adults and the number of juveniles of the controls of all experimental series meets validity criterion of the ISO guideline (ISO, 2014).

3.1 Toxicity test: cadmium

The pH values of OECD standard artificial soil treated with different concentration of cadmium (0-70-140 µg Cd/g dry soil) are reported in Figs. 1 and 2. The number of the surviving adults of all experimental groups at the end of the test is reported in Fig. 1. The number of surviving adults of experimental group treated with 140 µg /g Cd was statistically different from the number of surviving adults of control group and of experimental group treated with 70 µg /g Cd (SNK; $P<0.05$). The number of surviving adults of experimental group treated with 70 µg /g Cd was not statistically different from the control group (SNK; $P>0.05$).

The number of juveniles of all experimental groups at the end of the test is reported in Fig. 2. The number of juveniles of experimental groups treated with 70 µg /g and with 140 µg /g Cd was statistically different from the number of juveniles of control group and among themselves (SNK; $P<0.05$).

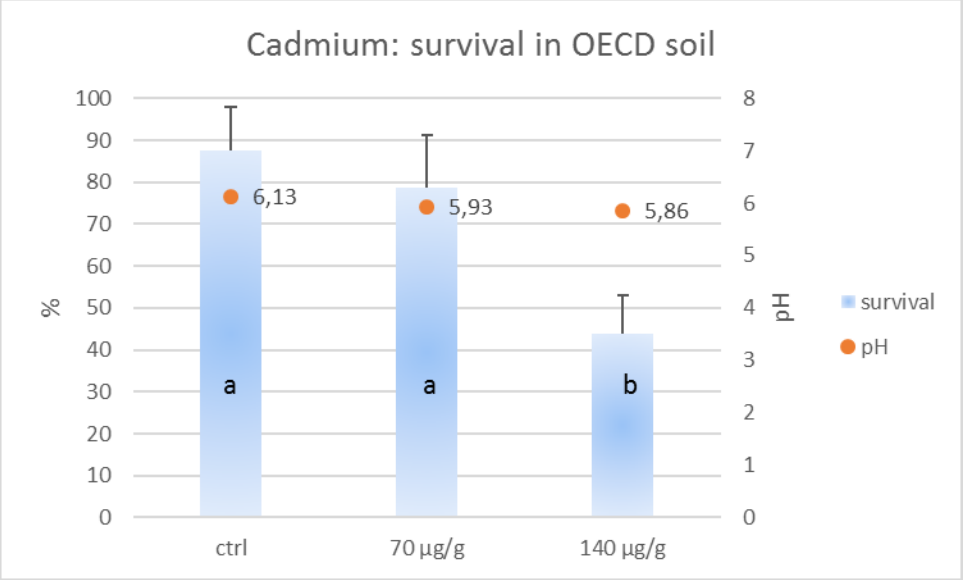

Fig. 1. Percentage survival of *Folsomia candida* exposed for 28 days to different concentrations of cadmium in OECD soil. pH values of experimental soils are also indicated. Survival values represent the mean number of eight replicates ± SD. Survival data were arc-sin transformed before statistical analysis. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test.

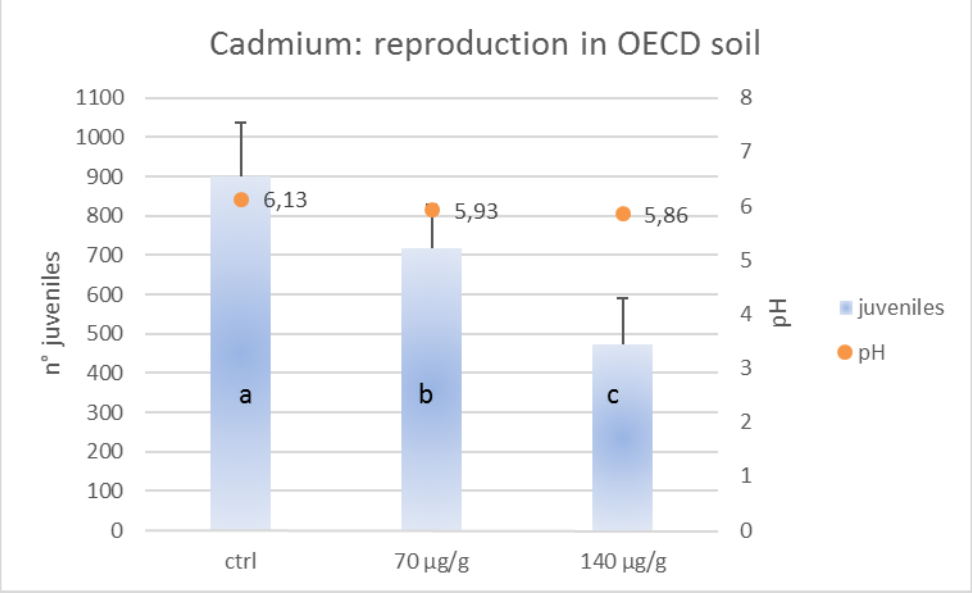

Fig. 2. Reproduction, shown as number of juveniles, of *Folsomia candida* exposed for 28 days to different concentrations of cadmium in OECD soil. pH values of experimental soils are also indicated. Numbers of juveniles are indicated as a mean number of eight replicates ± SD. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test.

The pH values of LUFA 2.2 standard natural soil treated with different concentration of cadmium (0-70-140 µg Cd/g dry soil) are reported in Figs. 3 and 4. The number of the surviving adults of all experimental groups at the end of the test is reported in Fig. 3. The number of surviving adults of all experimental groups did not significantly differ among themselves (ANOVA; P>0.05).

The number of juveniles of all experimental groups at the end of the test is reported in Fig. 4. The number of juveniles of experimental groups treated with 70 µg /g and 140 µg /g Cd was statistically different from the control group and among themselves (SNK; P<0.05).

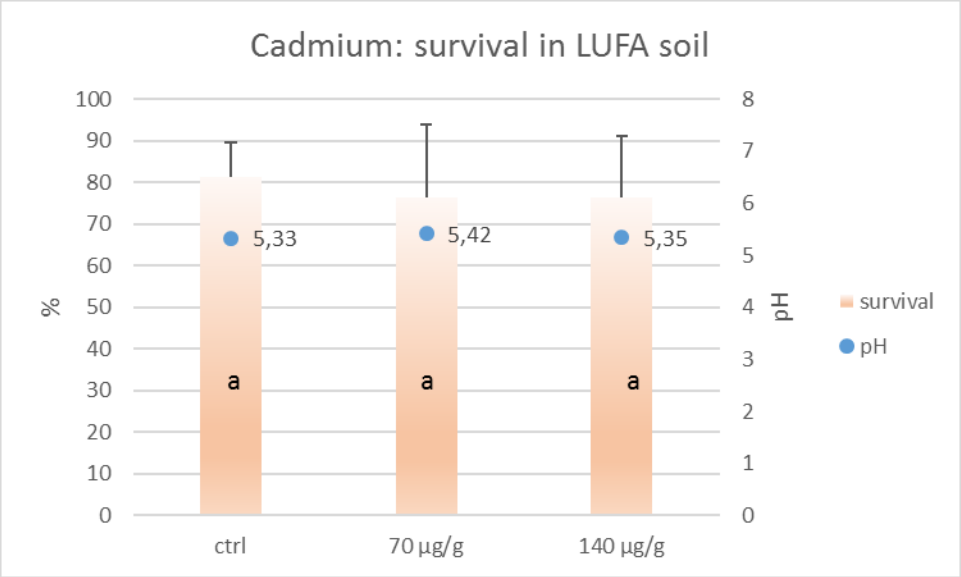

Fig. 3. Percentage survival of *Folsomia candida* exposed for 28 days to different concentrations of cadmium in LUFA soil. pH values of experimental soils are also indicated. Survival values represent the mean number of eight replicates ± SD. Survival data were arc-sin transformed before statistical analysis. Different letters indicate significantly different means at P < 0.05 according to the Student-Newman-Keuls' test.

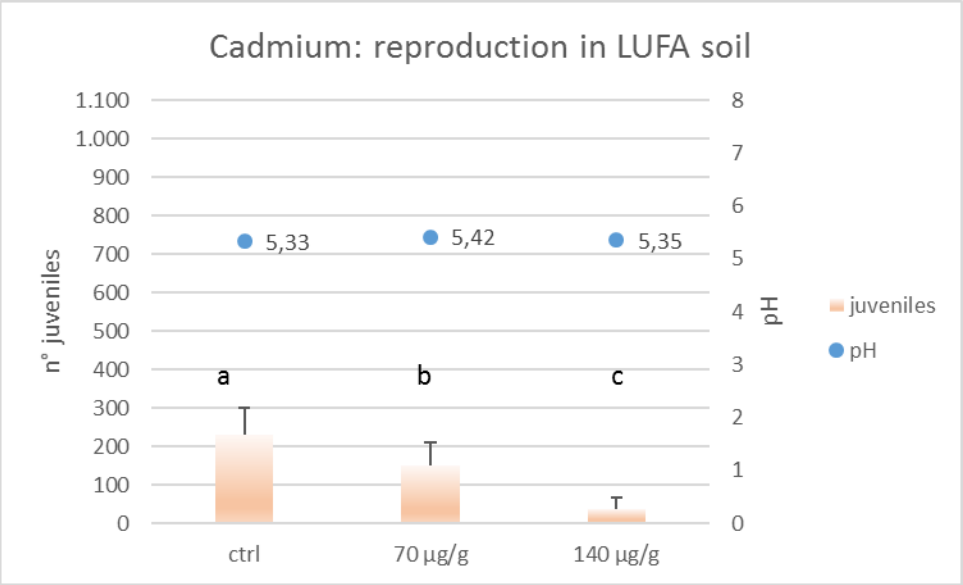

Fig. 4. Reproduction, shown as number of juveniles, of *Folsomia candida* exposed for 28 days to different concentrations of cadmium in LUFA soil. pH values of experimental soils are also indicated. Numbers of juveniles are indicated as a mean number of eight replicates ± SD. Different letters indicate significantly different means at P < 0.05 according to the Student-Newman-Keuls' test.

3.2 Cadmium analysis

Cadmium analysis carried out with atomic absorption spectrophotometry (AAS) revealed that cadmium concentrations in adult of *F. candida* increased with increasing cadmium concentrations in the soil for both soils tested (Fig. 5). The cadmium concentration in animals grown in OECD soil and in LUFA soil treated with 140 µg /g Cd was significantly different (P<0,01; Mann-Withney test) on the contrary the concentration of cadmium did not significantly differ in animals grown in soils treated with 70 µg /g Cd (P>0,05; Mann-Withney test).

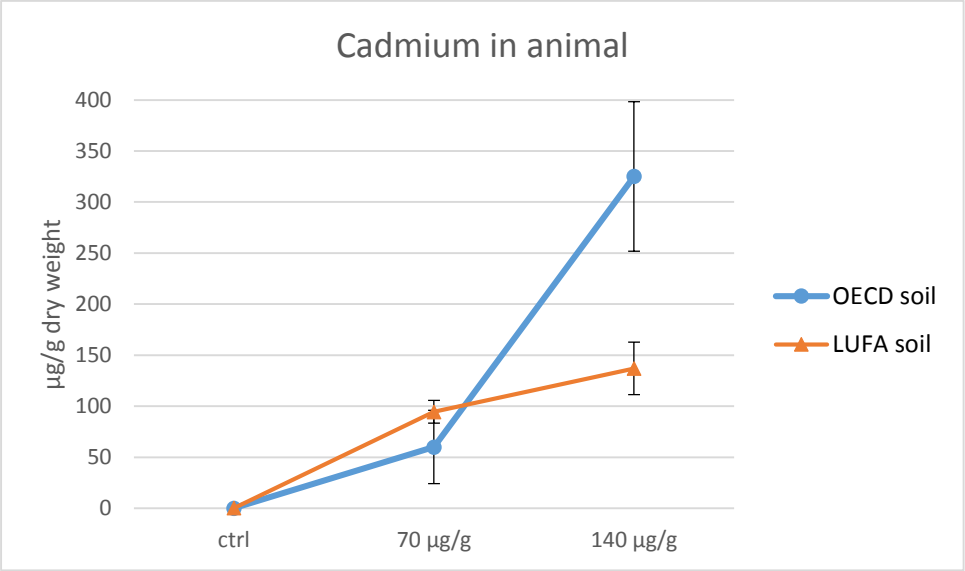

Fig. 5. Cadmium concentrations in adult of *Folsomia candida* after 28 days of exposure to cadmium in OECD and LUFA soils related to total soil concentrations (µg Cd/g dry soil). Cadmium concentrations are indicated as a mean number of three replicates ± SD.

3.3 Toxicity test: digestate

The digestate is characterized by a pH value of 7.4 and an electrical conductivity of 13.55 mS/cm. The organic matter content in the digestate was 76.7%. The pH values of OECD standard artificial soil treated with different concentration of digestate (0-2-4%) are reported in Figs. 6 and 7. The number of the surviving adults of all experimental groups at the end of the test is reported in Fig. 6. The number of surviving adults of all experimental groups did not significantly differ among themselves (ANOVA; $P>0.05$).

The number of juveniles of all experimental groups at the end of the test is reported in Fig. 7. The number of juveniles of all experimental groups did not significantly differ among themselves (ANOVA; $P>0.05$).

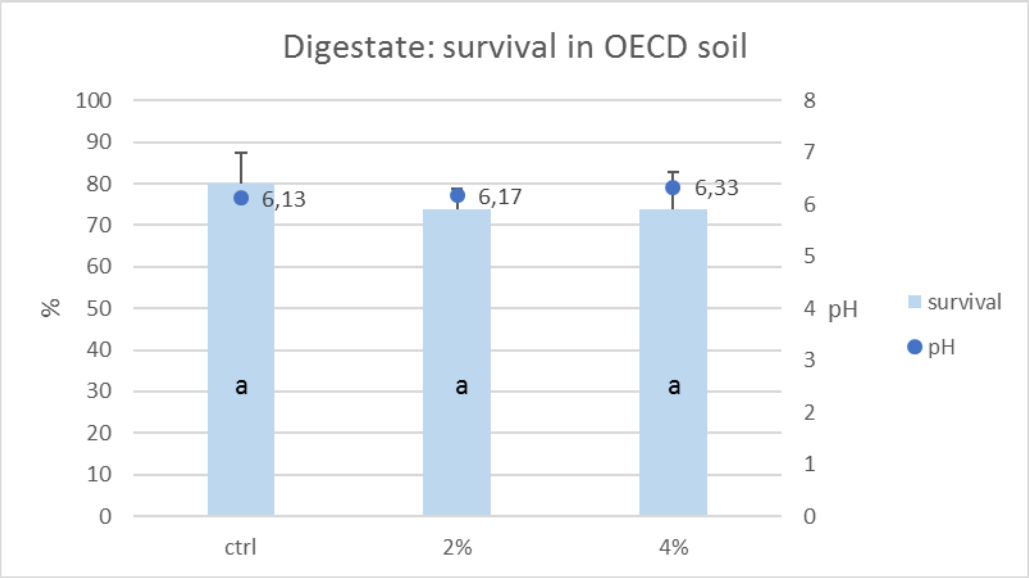

Fig. 6. Percentage survival of *Folsomia candida* exposed for 28 days to different concentrations of digestate in OECD soil. pH values of experimental soils are also indicated. Survival values represent the mean number of eight replicates ± SD. Survival data were arc-sin transformed before statistical analysis. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test.

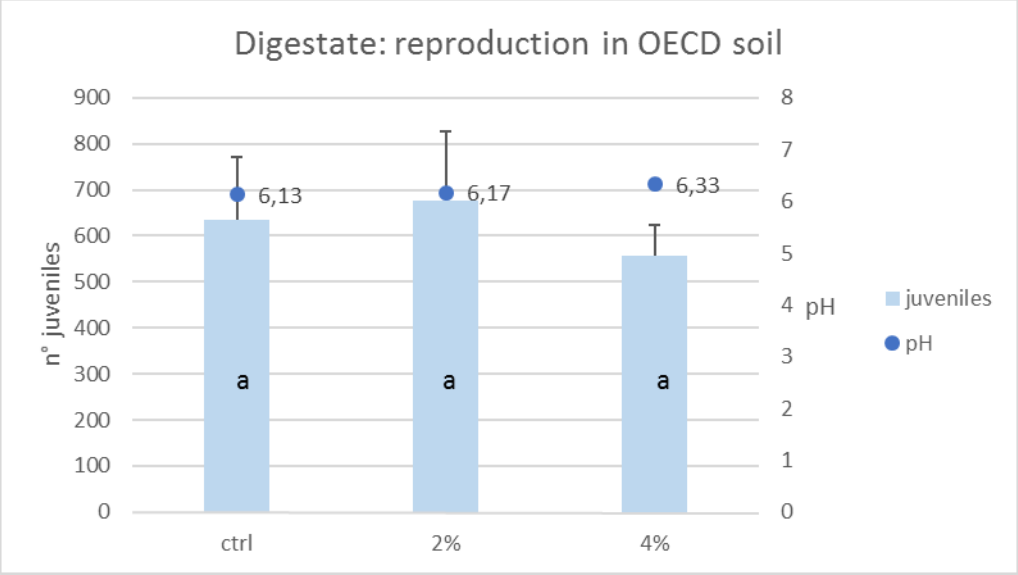

Fig. 7. Reproduction, shown as number of juveniles, of *Folsomia candida* exposed for 28 days to different concentrations of digestate in OECD soil. pH values of experimental soils are also indicated. Numbers of juveniles are indicated as a mean number of eight replicates ± SD. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test.

The pH values of LUFA 2.2 standard natural soil treated with different concentration of digestate (0-2-4%) are reported in Figs. 8 and 9. The number of the surviving adults of all experimental groups at the end of the test is reported in Fig. 8. The number of surviving adults of all experimental groups was not significantly different among themselves (ANOVA; $P>0.05$).

The number of juveniles of all experimental groups at the end of the test is reported in Fig. 9. The number of juveniles in experimental group with 2% digestate was very scarce. Juveniles were absent in experimental soil with digestate 4%.

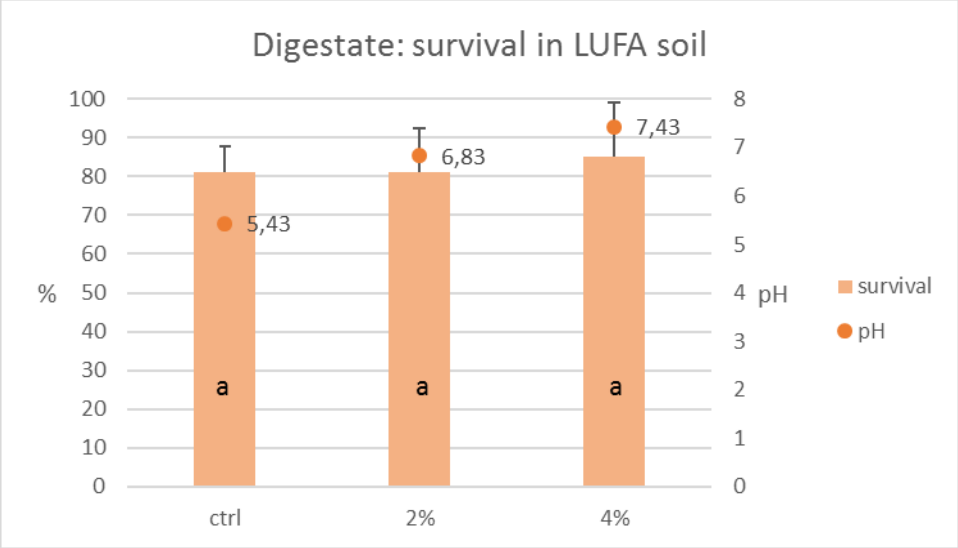

Fig. 8. Percentage survival of *Folsomia candida* exposed for 28 days to different concentrations of digestate in LUFA soil. pH values of experimental soils are also indicated. Survival values represent the mean number of eight replicates ± SD. Survival data were arc-sin transformed before statistical analysis. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test.

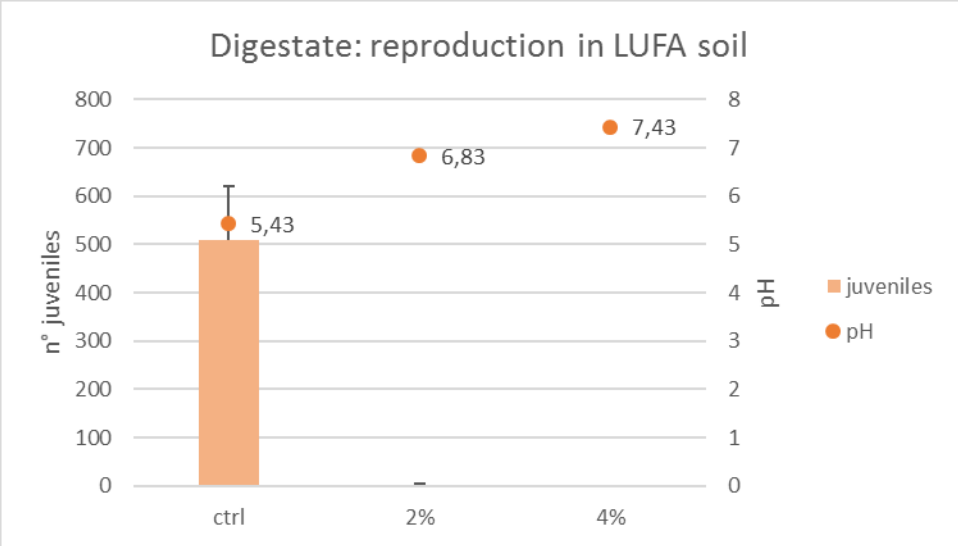

Fig. 9. Reproduction, shown as number of juveniles, of *Folsomia candida* exposed for 28 days to different concentrations of cadmium in LUFA soil. pH values of experimental soils are also indicated. Numbers of juveniles are indicated as a mean number of eight replicates ± SD. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test.

3.4 Miniaturization test

The data of the miniaturization test are reported in Figs. 10, 11 and 12. The number of juveniles per introduced adult of all experimental groups at the end of the test is reported in Fig. 10. In general, the number of juveniles of the experimental groups with the OECD soil was higher than the number of juveniles of the corresponding experimental groups with LUFA soil. In all experimental series the number of juveniles of the groups treated with 140 µg /g Cd was statistically different from the number of juveniles of control group and of the experimental group treated with 70 µg /g Cd (SNK; $P<0.05$). The number of juveniles of the groups treated with 70 µg /g Cd was statistically different from the number of juveniles of control group of all experimental series with 10 animals and of experimental series with 5 animals and 10 g of soil (SNK; $P<0.05$). The number of juveniles of the groups treated with 70 µg /g Cd with 5 animals and 20 g of soil did not significantly differ from the number of juveniles of control group (SNK; $P>0.05$).

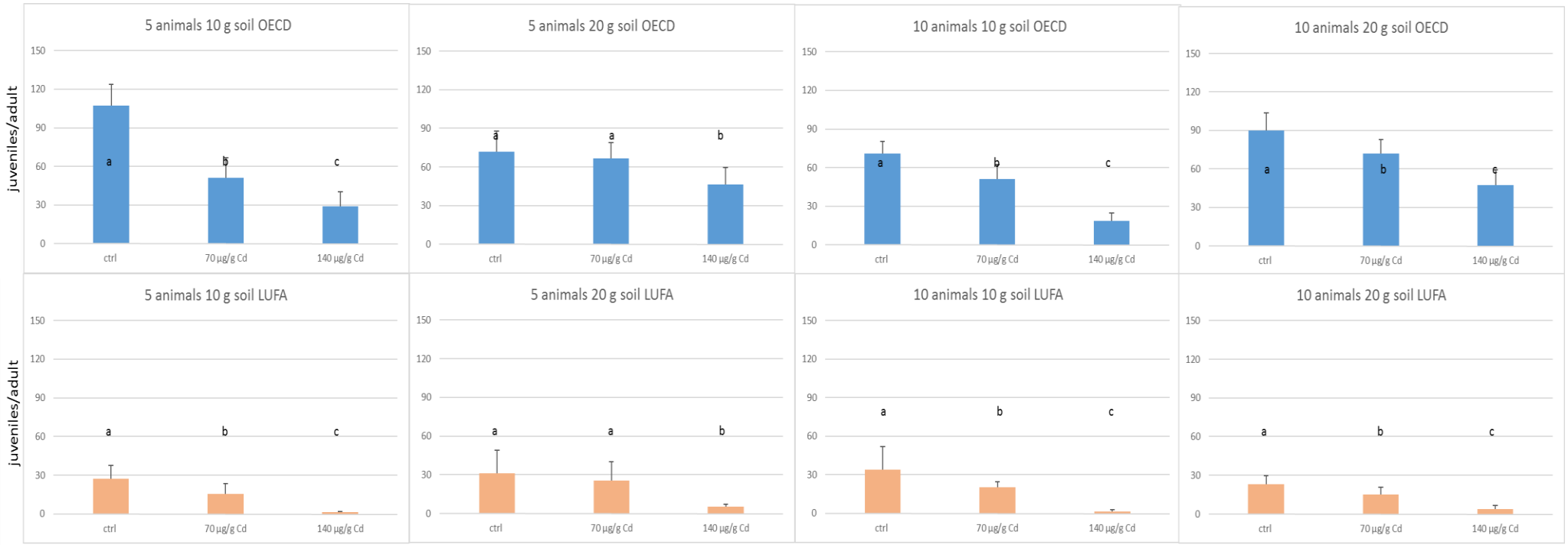

Fig. 10. Reproduction, shown as number of juveniles per introduced adult, of *Folsomia candida* exposed for 28 days to different concentrations of cadmium OECD (above) and LUFA (below) soils in the miniaturisation experiment with varied soil mass (10g or 20g) and individual numbers (5 or 10). Numbers of juveniles are indicated as a mean number of eight replicates ± SD. Different letters indicate significantly different means at $P < 0.05$ according to the Student-Newman-Keuls' test.

The GLM analysis of data regarding survival of *F. candida* in experimental series carried out with OECD soil pointed out that the different number of introduced adults affected survival of the control groups (GLM; adult numbers: p=0.003, soil mass: p=0.854, interaction: p=0.342) and of the experimental groups treated with 140 µg/g Cd (GLM; adult numbers: p=0.000, soil mass: p=0.133, interaction: p=0.218); in particular the survival of the experimental groups with ten animals introduced was lower than the survival of the experimental groups with five animals, instead the soil mass had no substantial effect on the number of surviving. In the experimental group treated with 70 µg/g Cd the different number of introduced adults and soil mass did not affected survival (GLM; adult numbers: p=0.111, soil mass: p=0.767, interaction: p=0.787). The GLM analysis of data regarding survival in LUFA 2.2 soil pointed out that the different number of introduced adults and soil mass did not affected survival of the control groups (GLM; adult numbers: p=0.411, soil mass: p=0.873, interaction: p=0.497), of the experimental groups treated with 70 µg/g Cd (GLM; adult numbers: p=0.903, soil mass: p=0.626, interaction: p=0.193), and with 140 µg/g Cd (GLM; adult numbers: p=0.732, soil mass: p=0.862, interaction: p=0.233; Fig. 11).

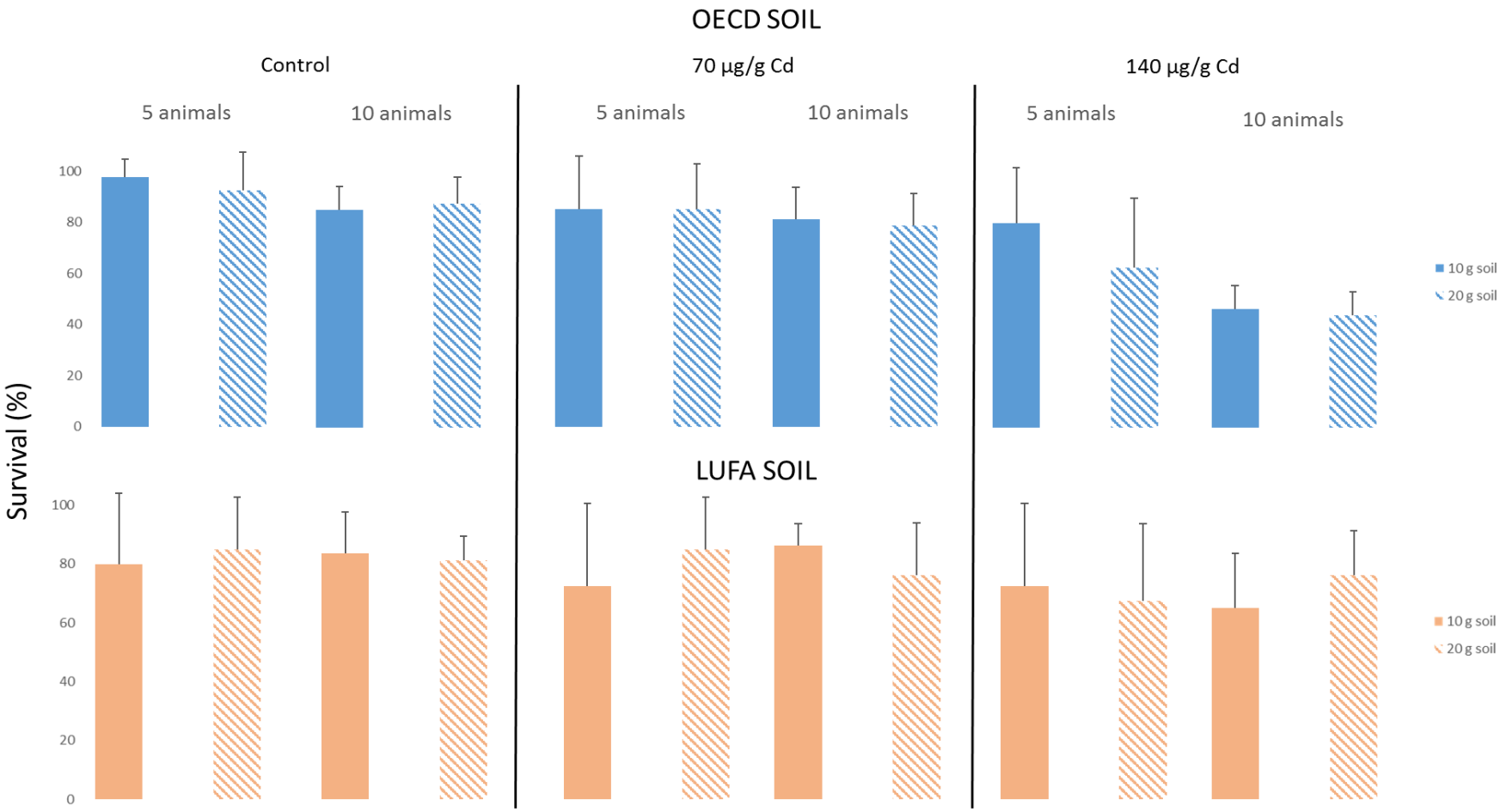

Fig. 11. Percentage survival of *Folsomia candida* exposed for 28 days to different concentrations of cadmium in OECD (above) and LUFA (below) soils in the miniaturisation experiment with varied soil mass (10g or 20g) and individual numbers (5 or 10). Survival values represent the mean number of eight replicates ± SD.

The GLM analysis of data regarding reproduction in OECD and LUFA soils pointed out that the different number of adults introduced did not affect the reproduction of *F. candida* in all experimental groups. On the contrary mass soil reduction resulted in a decrease in the number of juveniles: i) in the experimental groups prepared with the OECD soil treated with 70 µg/g Cd (GLM; adult numbers: p=0.529, soil mass: p= 0.000, interaction: p= 0.565) and with 140 µg/g Cd (GLM; adult numbers: p=0.228, soil mass: p= 0.000, interaction: p=0.161). ii) in the experimental groups prepared with the LUFA soil treated with 140 µg/g Cd (GLM; adult numbers: p= 0.399, soil mass: p= 0.000, interaction: p= 0.419). The GLM analysis highlighted interaction between the number of adults introduced and the mass of soil in i) the control groups prepared with the OECD soil (GLM; adult numbers: p=0.082, soil mass: p=0.125, interaction: p=0.000); ii) the experimental groups prepared with the LUFA soil treated with 70 µg/g Cd (GLM; adult numbers: p= 0.422, soil mass: p= 0.479, interaction: p= 0.024; Fig. 12).

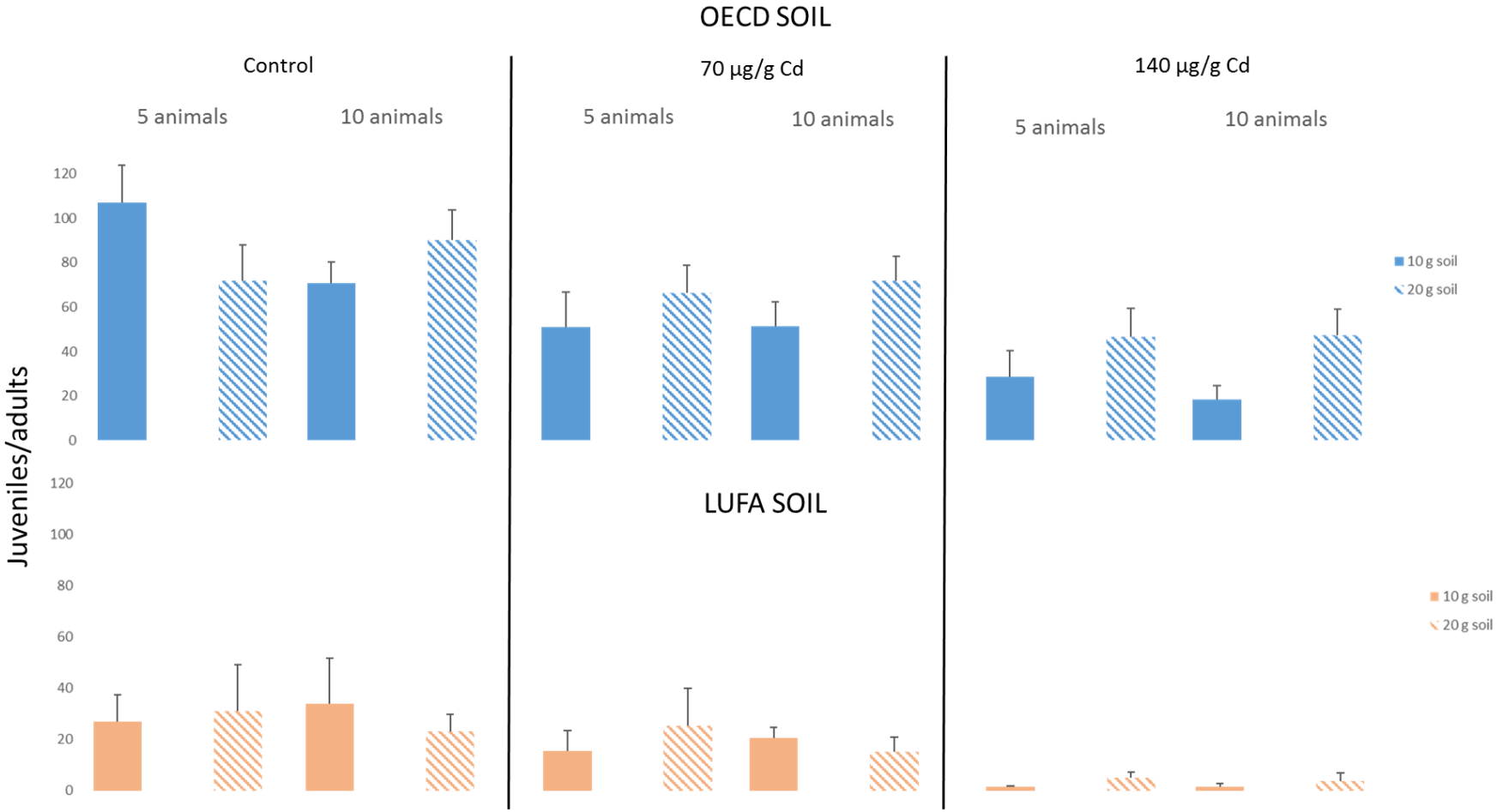

Fig. 12. Reproduction, shown as number of juveniles per introduced adult, of *Folsomia candida* exposed for 28 days to different concentrations of cadmium in OECD (above) and LUFA (below) soils in the miniaturisation experiment with varied soil mass (10g or 20g) and individual numbers (5 or 10). Numbers of juveniles are indicated as a mean number of eight replicates ± SD.

4 Discussion

The toxicity tests carried out to evaluate the effects on the survival and reproduction of the collembolan *F. candida*, of different concentration of cadmium utilizing two standard soils, OECD (1984) and LUFA 2.2 soil, both provided for by ISO 2014, have given different results regarding the survival of adults. In fact, only in the experimental group with OECD soil has been detected a negative effect on adult survival. This result can be explained by the greater accumulation of cadmium detected by us in adults maintained on OECD soil treated with the highest concentration of Cd, as compared with adults maintained in LUFA soil. A greater accumulation of cadmium in adults of *F. candida* maintained in OECD soil as compared with field soils treated with cadmium was detected by Vijver et al., (2001) that correlated Cd accumulation pattern mainly to solid–phase soil characteristics. However, in the present study, the effect of cadmium on reproduction of *F. candida* resulted not different in the two standard soils and in a perspective of environmental risk assessments, the use of two different standard soil do not influence the outcome of toxicity test in line with Van Gestel and Mol (2003).

The second edition of ISO 11267 (2014) as well as include the use of the standard natural soils as LUFA 2.2, has also extended the use of toxicity tests for assessing the effects of waste materials. In this study, we evaluated a digestate obtained from agricultural waste. In a perspective of the ecotoxicological risk assessments of organic contaminants, our results deeply question the interpretation of standardized toxicity tests. The effects on reproduction of the digestate detected in the two soils were different: the test carried out with OECD artificial soil did not detect any effect of the digestate on the reproduction of *F. candida*, while the juveniles in LUFA soil treated with digestate were very scarce or absent. In a previous work (d'Errico et al., 2015) designed to assess the effect of another digestate on survival and reproduction of *F. candida*, has been possible to attribute the negative effect detected on reproduction, to the change of the soil pH caused by the addition of

the alkaline digestate to the OECD artificial soil. The digestate utilized in this work was less alkaline and its addition to the OECD soil did not cause important changes in pH, while its addition to the LUFA has caused a considerable increase of pH values. One of the causes of the strongly decrease of the number of juveniles could be attributed to the increase of the pH in line with what was stated by Greenslade and Vaughan, (2003) and d'Errico et al., (2015). The differences in behavior of the two soils could be attributed to a different buffer capacity. A thorough knowledge of the soil characteristics is needed before using contaminants such as digestate that can affect soil properties.

In our miniaturization test the GLM analysis, carried out by analysing separately the experimental groups with the same type of soil (OECD or LUFA) and the same concentration of cadmium showed that the variation of the number of animals has no effect on the number of juveniles per adult of *F. candida* in any experimental group, while the variation of the amount of soil has an effect on number of juveniles in the experimental groups treated with Cd. Our experiment, aimed at halving the amount of the soil and the number of animals used in toxicity test, highlighted that the standard and the miniaturized reproduction test give the same outcome in both soil types at least in tests with heavy metals. Other Authors comparing the standard and the miniaturized *F. candida* reproduction tests, in differently fertilized natural soils, founded a significant difference in the outcome (Schröder et al., 2015). Different results could be attributed to the different type of contaminants tested, heavy metal and organic material. Therefore, the miniaturization test carried out halving the amount of the soil and simultaneously the number of animals would seem to be a valid alternative to the standard *F. candida* reproduction test to assay contaminants available in low quantities although it needed further intensive studies to use the miniaturization test reproduction for the environmental risk assessment.

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Conclusion

My study revealed that the ISO guideline 11267 (2014) can be considered "standard" when used to test contaminants that do not alter the soil properties, and the soil type, standard artificial or natural, did not affect results. On the other hand, when the guideline is adopted to carry out toxicity tests on contaminants that alter the soil properties, it appears fundamental to carefully investigating the interactions between the contaminant and the soil as well as carefully choose the standard soil to be used among those provided the by ISO 11267 (2014). Based on the data obtained in this study has been possible to exclude, with the exception of the higher concentration tested, a negative effect of digestate on embryonic and post-embryonic development of *F. candida* and to hypothesize an effect on oogenesis. My approach could be used to obviate to the impossibility to directly observe, during the test, the reproduction and to separate from oogenesis, hatching success and juvenile mortality.

In particular, my study highlighted that the toxic effect on the *F. candida* reproduction of a digestate is attributable to increasing in soil pH caused by its addition. Therefore, it reveals that the *F. candida* reproduction test used to evaluate a contaminant that increases soil pH may be useful for regulatory decisions regarding acidic soils but not appropriate for alkaline soils.

Furthermore, I compared the use of the standard artificial soil (OECD, 1984) and of the standard natural soil LUFA 2.2 - both proposed by the recent ISO 11267 guideline, to test an organic contaminant as a digestate: I obtained different results in the two soils. The test carried out with OECD artificial soil did not detect any effect of the digestate on the reproduction of *F. candida*, whereas the test conducted with LUFA 2.2 soil showed a strong negative effect. The differences in the behavior of the two soils could be attributed to a different buffer capacity. The effect of cadmium on *F. candida* reproduction resulted not different in the two standard soils. Therefore, in a perspective of environmental risk assessments, the use of two different standard soils does not influence the outcome of toxicity test of heavy metals such as cadmium, but affects the outcome of toxicity test of contaminants such as the digestate, the latter modifying soil properties.

A thorough knowledge of the soil characteristics, thus, is needed before adding to soil these contaminants as soil improvers.

Furthermore, because the ISO guideline could be used to assay contaminants with synthesis process very expensive and available in low quantities I have evaluated the possibility of reducing the number of animals and the amount of soil. The results found highlight that halving the amount of the soil and simultaneously halving the number of animals would seem to be a valid alternative to the standard *F. candida* reproduction test useful to assay new and emerging contaminants available in low quantities. However, further in-depth studies are needed to broadly validate the use of the miniaturization as an alternative reproduction test for environmental risk assessment.