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MSc. in Sustainable Agriculture

EFFECTS OF TRADITIONAL AND BIODYNAMIC FARMYARD MANURE APPLICATION ON SOIL PROPERTIES.

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Abstract

Biodynamic agriculture is a potentially sustainable method of farming developed in the 1920s by Rudolph Steiner. It was proposed to tackle the growing problem of soil erosion that was occurring at the time. The practice incorporates the idea that agriculture is holistic: a collective spiritual, ethical and ecological approach to food production. Biodynamic farming disallows the use of chemical pesticides and fertilisers and instead opt to use some peculiar preparations. These are composed of natural substances derived from the farm. This preparation is applied to the farm according to a rigorous calendar with a growing season and lunar cycles. In this work, we analysed the effects of traditionally composted farmyard manure (FYM) applications with two types of biodynamic additions that had been applied for over 27 years on the same trial soils. The following parameters were analysed: soil dry mass, soil water holding capacity, soil pH, soil basal respiration, soil carbonates, soil ergosterol content, soil microbial biomass carbon, soil microbial biomass nitrogen, and C/N ratio microbial biomass. Four treatments were applied to the experimental plots: (1) no manure application ('control'), (2) application of manure without the addition of biodynamic compost preparations ('manure'), (3) application of manure with biodynamic compost preparation of yarrow (Achillea) blossoms ('manure+yarrow'), (4) application of manure with biodynamic compost preparation of flowers of yarrow, chamomile, dandelion, stinging nettle shoots, oak bark and valerian extract (manure+All preps). The experiment was conducted near Bonn on a Fluvisol using a randomised complete block design (n=6). Results showed that: a) repeated manure supplementation increased soil microbial biomass carbon; b) the addition of the six biodynamic preparations to manure counteracted the above effect; c) the addition of the single preparation from yarrow (*Achillea millefolium*) to manure did not affect the increase of soil microbial biomass carbon due to manure, and its mean value higher than that of manure alone, d) known microbial communications models from quorum sensing and other cell-to-cell signalling notions are consistent with the explanation of how the observed effects can be explained on a quantitative basis.

Introduction

Over the past century, agricultural researchers have widely recognised that sustainable agricultural production systems are becoming increasingly more important as land becomes more scarce and population increases. It highlights a need to develop more suitable methods and definitions to measure sustainability within the agricultural field (Pacini *et al*., 2003). The development of "cleaner" and "low input" farming methods are required to produce enough food for the world's population by conserving land and habitats. Currently, only 4% of the UK's land is farmed based on the concept of sustainability (Pacini *et al.,* 2003).

Presently, the human population is increasing. As it increases, the demand for food will also rise in direct correlation. It is estimated that the global food demand will double over the next 50 years (Tilman *et al.,* 2002). This could result in substantial environmental damage; a study by Carpenter *et al.* (1998) states that agriculture can lead to the loss of natural ecosystems and adds globally significant and environmentally harmful amounts of nitrogen and phosphorus to terrestrial ecosystems. The damaging impacts caused by agriculture has led to the desire for sustainable farming systems to increase in accordance with the movement to protect wildlife, prevent land degradation and decrease environmental harm caused by farming practices (European Commission, 2006) . Fundamentally, sustainability is based upon three pillars; economic, social and environmental. If one pillar is weak, the whole system is deemed unsustainable. A universal definition for sustainability does not currently exist, so this definition of sustainable development will be used for the purposes of this research.Sustainable development is defined as "*development*

that meets current needs without jeopardizing future generations' ability to meet their own" (Sustainable Development Commission, 2012).
Currently, there are many different variations and theories to "sustainable agriculture". For the purpose of this research, the definition provided by The Brundtland Commission of the UN (1987) will be used:
"The management and conservation of the natural resource base, and the orientation of technological and institutional change in such a manner as to ensure the attainment and continued satisfaction of human needs for present and future generations. Such development... conserves land, water, plant and animal genetic resources, is environmentally non-degrading, technically appropriate, economically viable and socially acceptable."
The development of a universal definition for sustainability would aid agriculturalists all over the world to measure sustainability. This would be of value as the scale of agriculture is increasing to meet food demand. Presently half of the global usable farming land is already in pastoral or intensive agriculture (Tilman *et al.,* 2002); by having a universally agreed and globally recognised sustainability measurement, farmers would be able to develop sustainable farms. For a universal definition to be established, the implementation of sustainable development needs to occur first.

1.2 Conventional Agriculture

The concept of conventional agriculture was developed to justify and provide a comparative foundation for alternative methods of agriculture (Hansen, 1996) . "Conventional agriculture is characterised as capital-intensive, large-scale, highly mechanised agriculture with monocultures of crops and extensive use of artificial fertilisers, herbicides and pesticides, with intensive animal husbandry" (Knorr & Watkins, 1984, p. 148). This type of agriculture became very popular during the Second World War. The UK government encouraged its people to utilise land to its full potential when faced with food shortages (Robinson & Sutherland, 2000) . The "Dig for Victory" campaign encouraged people to transform parks, gardens and even ditches into the land to grow vegetables (Education Scotland, 2014). This intensive, industrialised farming caused a large amount of degradation to valuable land and contributed to the population decrease of many species of farmland birds (RSPB, 2009) . The decline of the raptor species *Falco columbarius* (or Merlin) occurred because of the heavy use of organochlorine pesticides during and after the Second World War (Boatman *et al.,* 2004). These problems are widely associated with "conventional" or "industrial" agriculture, which is perceived to be unsustainable by many researchers (Dahlberg, 1991). It is generally understood that this method of agriculture is not a sustainable option because it causes environmental damage, does not aim to conserve habitats, nor does it consider future human needs. Furthermore, a study by Rasul & Thapa (2004) states that this method of farming is known to degrade land and water resources, in addition to producing smaller yields after chemical fertilisers are used, thereby showing that conventional farming has exceeded the carrying capacity of the land once yields decrease. Nevertheless, this method of farming (conventional or industrial agriculture) is currently widely used across the world on large-scale farms.

1.2.1 The Green Revolution

From the 1940s to the 1960s, the development of the Green Revolution movement began. The Green Revolution is a series of research, technological advances and high investment into the agricultural sector in an effort to maximise crop yields and decrease diseases susceptibility within crops to combat the risk of human starvation and malnutrition (Pingali, 2012) . "The success of the Green Revolution was caused by the combination of high rates of investment in crop research, infrastructure, and market development and appropriate policy support that took place during the first Green Revolution" (Pingali, 2012, p. 12302). The most notable breakthrough to come from this movement was the invention of genetically modified organisms (GMO's) by Norman Borlaug

(Briney, 2014) . This development of improved agronomy: hybridised high yielding crops and modernised chemicals fertilisers and pesticides (International Food Policy Research Institute, 2002) greatly helped developed and developing countries all over the world to produce enough crops to feed their population. In some countries, such as Mexico, they were able to produce more than what they needed for their population and were able to export to other countries (Briney, 2014). Initially, the production levels and crop variety did increase; however, it did not take long for people to realise that this was a short-term solution (King, 2008). The three main varieties of seeds: wheat, rice and millet, that were developed for the Green Revolution required heavy irrigation and applications of chemicals fertilisers in order for the crops to be successful (Sebby, 2010). It meant that crops failed if there was reduced access to fertiliser or water. In the 1960s, India suffered back-to-back droughts that damaged food production (Pingali, 2012).

Conventional agriculture does not prohibit the use of chemical fertilisers or the use of GMOs except in states where legislation prevents them specifically. In fact, chemicals and GMOs are used widespread (Pimentel *et al.,* 2005). In order to achieve higher yields, this system requires high-energy inputs, which results in the heavy use of fossil fuels and technological innovations to supplement the required energy needed (Gomiero *et al*., 2011). GMO crops were developed to help increase yields, decrease costs for food production and help crops become resistant to pests and diseases (Phillips, 2008) . However, a study conducted by Gurian-Sherman, (2009) found that the use of GMO crops does not increase yields. His study showed that the yields of corn and soybeans did increase, although not due to genetically engineered traits but rather traditional selective breeding.
Furthermore, conventional farming also requires high-energy inputs to achieve high yields (Food and Agricultural Organisation for the United Nations, 1999) . Consequently, The Common Agricultural Policy (CAP) was introduced to help "the agricultural sector to meet strategic food requirements and to reduce poverty" (Donald *et al.,* 2002, p.171). Shortly afterwards, The Wildlife and Countryside Act 1981 was introduced to help protect wildlife and habitats from the exploitation of the Second World War. Since these policies introductions, alternative and potentially sustainable agricultural practices have become popular and developed further, including organic, hydroponics, and biodynamic agriculture. Biodynamic agriculture will now be considered further.

1.3 Biodynamic agriculture

Biodynamic agriculture was introduced as a possible solution to farmers' concerns about their weakening soils and their fields and crops (McCullough *et al.,* 2012, p. 1364). Biodynamics is an innovative sustainable method of farming that philosopher Rudolph Steiner developed in the 1920s. It is fundamentally based on his philosophy of anthroposophy. Biodynamic farmers view a farm as a "total" organism and attempt to develop a sustainable system where everything within the system is respected and has a proper place (Biodynamic Association, 2014; Mason, 2003:14). The Biodynamic Association (2014) states that biodynamic agriculture incorporates the idea that agriculture is holistic: a collective spiritual, ethical and ecological approach to the production of our food.
Rathore *et al.,* (2014) and Pfeiffer (1940) state that the main principles of Biodynamic Agriculture are:

- To create a diverse and balanced farm ecosystem that can support itself from within the farm (Mason, 2003)
- To restore the soil through the incorporation of organic matter
- To treat soil as a living system
- To create a system that brings all factors which maintain life into balance
- To encourage the use and importance of green manure, crop rotation and cover crops

- Treat manure and compost in a biodynamic way, and have knowledge of enzymes and hormones.

From these principles, it can be recognised that no artificial materials or harmful chemicals are used in the practices of biodynamic agriculture, as mentioned in the previous section. This is also stated by Reganold *et al.* (1993). Instead of chemicals, only natural substances sourced on the farm are used in the preparations of fertilisers, sprays, and manures. In doing this, it allows minimal outside inputs creating a closed system as all the inputs are retrieved from within the farm itself (Carpenter-Boggs, 2011). To ensure stewardship of the Earth is maintained, it is the belief in biodynamic farming that the restoration and harmonisation of the farm's life forces are reinforced to enhance the quality, flavour and nutrition of the farm's produce (Mason, 2003) . A study conducted by Woodward-Clyde (2000) highlights that there has been a decline in the public's confidence in modern industrialised farming and processing methods; the study also states that it is due to an increase in the consumer's awareness of food-borne hazards such as pesticides, antibiotics, hormones and artificial ingredients. The public also commonly perceives that organically and biodynamically farmed foods are healthier than conventional ones; however, there is little scientific evidence to support this theory (Tassoni *et al.,* 2013). Biodynamic farming is viewed as the first alternative method of farming (Chalker-Scott, 2014), which incorporates a sustainable system that can organically produce quality crops without using any harmful chemicals (Diver, 1999).

1.3.1 Preparations

Biodynamic farming shares many practices with organic farming methods, including soil building, crop rotation and compost (Diver, 1999). However, the key aspect of biodynamic agriculture is to work closely with nature with the use of special *"preparations"* that are applied to the soil, crops and composts (Reevea *et al*.. 2010). They are considered to be the most important feature of biodynamic farming and are probably the most difficult part of biodynamics to understand (Ellis, 2010). As shown in Table 1, the preparations themselves are unique to biodynamic agriculture and only consist of specific minerals or plants, which are treated or fermented with animal organs, water, and/or soil (Stenier, 1974) . The preparations are applied to the farm in manures and sprays following a strict planting calendar. This calendar incorporates lunar cycles and the seasons; planting and spraying in relation to this will increase the growing capability of the crops (Thun, 2015). It is thought that the use of preparations "produce compost that develops faster with less loss of nitrogen, fewer odour problems, and greater nutrient holding capacity, by stimulating organisms present in the feedstock" (Klett, 2006, p. 34). The preparations are conveyed as supposedly having a positive impact on the environment in terms of energy use and efficiency (Turinek *et al.,* 2009). This preparation aims not to add nutrients to the ground but to stimulate the soil's energy and nutrient cycling natural process (Carpenter-Boggs *et al*., 2000b). Some research suggests that the soil quality of a biodynamic farm could be higher than that of a conventional farm because of the preparations used. Reganold *et al.* (1993) found that soils from biodynamic farm's had a higher biological and physical quality and a considerably greater organic matter content and microbial activity than that of soil from conventional farm's. That study concluded that the use of preparations on the soil decreases the soil density, which increases penetrability, and the thickness of the topsoil is also greater.

Preparation	Main ingredient	Use
500	Cow (*Bos taurus*) manure	Field spray
501	Ground silica from quartz or feldspar	Field spray
502	Yarrow (*Achillea millefolium* L.) blossoms	Compost additive
503	Chamomile (*Matricaria recucitata* L.) blossoms	Compost additive
504	Stinging nettle (*Urtica dioeca* L.) shoot	Compost additive
505	Oak (*Quercus robur* L.) bark	Compost additive
506	Dandelion (*Taraxacum officinale* Weber) flowers	Compost additive
507	Valerian (*Valeriana officinalis* L.) extract	Compost additive
508	Horsetail (*Equisetum arvense* L.)	Field spray

Table 1 – Preparations used in Biodynamic Agriculture (Steiner, 1924)

Biodynamic farming has received a lot of criticism for its credibility, and Turner (2014) and Saltini have referred to it as pseudoscience (2010). The Oxford Dictionary defines pseudoscience as "a collection of beliefs or practices mistakenly regarded as being based on scientific method." According to Reganold (1995), many biodynamic agricultural practices are of questionable scientific quality. His study concludes that it is due to the lack of additional secondary reviewing and verification from other scientists, which is crucial in modern scientific research. Preparations and the effects of the cosmos and lunar cycles are also a cause for criticism. These preparations and the association with the spiritual science of anthroposophy sets biodynamic farming apart from other agricultural practices and is the only agricultural practice that believes that lunar cycles and cosmic forces can influence the whole farm. The creation of the preparation methods was not "developed through scientific methodology, but rather through Steiner's own self-described meditation and clairvoyance" (Chalker-Scott, 2004, p. 1), which means the methodology is not scientifically proven through modern scientific techniques. Currently, the underlying natural science of the preparations is still under investigation (Turinek *et al.,* 2009). Steiner himself believed that his spiritualistic founded methods did not need to be confirmed through traditional scientific reviewing, as they were “true and correct” unto themselves (Kirchmann, 1994). In terms of research, Biodynamic farming is still in its infancy, as there is a lack of additional and scientifically reviewed research to suggest that these preparations do have any benefit to the soil and the farm's produce.
Nevertheless, thirty published, scientifically certified, and peer-reviewed studies suggest that these preparations do have a recognisable impact on the farm, produce, and soil quality (Turinek *et al.,* 2009). For further reading, please consult (Carpenter-Boggs *et al.,* 2000a; Rathore *et al.,* 2014; Reeve *et al.,* 2005; Villanueva-Reya *et al.,* 2014). Research had found that there is an increased microbiological movement within the soil, in addition to having a higher level of nutrients and an increased rate of crop development when biodynamic preparation was used in comparison to a conventional farm (Reeve *et al.,* 2010; Reganold *et al.,*1993; Carpenter- Boggs *et al.,* 2000a; Koepf 1993). However, in contrast, Carpenter-Boggs *et al.* (2000b) and Tassoni *et al.* (2013) found there to be no benefits of using the preparations.

1.4 Organic agriculture

Organic agriculture is a production system that avoids or largely excludes the use of synthetically produced fertilisers, pesticides, growth regulators and livestock feed additives, relying instead on crop rotations, crop residues, animal manures, legumes, green manures, and aspects of biological pest control to maintain soil productivity and tilth, to supply plant nutrients and to control insects, weeds and other pests (Lampkin 1990). Some studies showed that organic farming leads to higher soil quality and more biological activity in soil than conventional farming (e.g. Reganold 1988; Alföldi et al. 1993; Drinkwater et al. 1995; Droogers and Bouma 1996). These organic systems have also been shown to use fertilisers and energy more efficiently than conventionally managed systems (Mäder et al. 2002) and to be just as economically viable as conventional farms (Reganold et al. 1993; Reganold and Palmer 1995). Biodynamic agriculture has many similarities to other

organic agricultural systems and relies heavily on composted farmyard manure (FYM) as a fertiliser. Additionally, biodynamic farming uses field sprays and compost preparations consisting of specific minerals or plants treated or fermented with animal organs, water and/ or soil (Steiner 1924). Since biodynamic preparations are added to composting organic material in very low doses of a few grams per ton of compost material, the primary purpose of these preparations is not to add nutrients but to stimulate the processes of nutrient and energy cycling, hasten decomposition and improve soil and crop quality (Koepf 1993). Generally, biodynamic compost additives are made from six different plant species (Steiner 1924): flowers of yarrow (*Achillea millefolium* L.), chamomile (*Matricaria chamomilla* L.), dandelion (*Taraxacum officinale* Web.) and valerian (*Valeriana officinalis* L.), bark of oak (*Quercus robur* L.) and whole plant of stinging nettle (*Urtica dioica* L.). Several studies demonstrated that biodynamically treated composts maintained a significantly higher temperature throughout the composting period, suggesting more thermophilic microbial activity and/or faster development of compost with biodynamic treatment (von Wistinghausen 1984; Koepf 1989; Carpenter-Boggs et al. 2000b). As a fertiliser, biodynamic FYM has been shown to increase soil organic C and N (Abele 1978), microbial biomass and biological activity (Mäder et al. 1995), and decrease extractable P (Penfold et al. 1995) compared to fertilisation without biodynamic preparations. However, Carpenter-Boggs et al. (2000a) did not find effects of biodynamic preparations on selected soil parameters. The functional relationships between biodynamic compost preparations and the composting process are still not fully understood. The objectives of the current work were to determine whether the application of traditionally and/or biodynamically composted cattle manure can affect chemical, biochemical and biological soil parameters, root production and yields in an organically managed six-course crop rotation design. The experiment was established in 1993 and has been maintained ever since by applying the same amount of differently biodynamically prepared FYM. Data presented here are from the eighth and ninth year after starting the experiment.

2. Materials and Methods

2.1 Site description and experimental design

The experimental site is located on the Wiesengut certified organic research farm of the Institute of Organic Agriculture, University of Bonn (65 m a.s.l.; 7°17′E, 50°48′N). The mean annual air temperature at this location is 9.5°C, and the mean annual precipitation is about 700 mm. Twenty-four 6 m×10-m experimental field plots were established in 1993 on a Fluvisol and maintained within the research farm's six-course crop rotation design. The rotation consists of the six main crops grass-clover, potatoes, winter wheat, field beans, spring wheat, and winter rye with an undersown red clover-grass mixture. Four treatments (see below) were arranged in a randomised complete block design with six replicates. Soil cultivation, sowing and mechanical weed management (e.g. harrowing), which are not part of the experimental design, were identical among all experimental plots and were performed on similar dates and in a similar manner to adjacent fields. No substances to raise soil pH levels had been applied to the experimental plots to avoid interactions with the treatments under study. Soil conditions at the location were similar before the establishment of experimental plots (Table 1). Soil texture was : Sand: 45,7 %; Silt: 44,2 %; Clay: 10,2 %.

2.2 Farm Yard Manure (FYM) treatment

Cattle manure from the research farm (FYM) was composted each autumn in a straw-covered windrow composting system, beginning in November 1992. Four treatments were applied to the

experimental plots: (1) no manure application ('control'), (2) application of manure without the addition of biodynamic compost preparations ('manure'), (3) application of manure with biodynamic compost preparation of yarrow (Achillea) blossoms (‘manure+yarrow’), (4) application of manure with biodynamic compost preparation of flowers of yarrow, chamomile, dandelion, stinging nettle shoots, oak bark and valerian extract (manure+All preps). On average over the experimental years, about 3-month-old composts (the rotting period between 60 days and 110 days) were manually applied to field plots in February or March of each year, beginning in 1993 at 30 Mg fresh mass ha−1 (average dry matter content 25%). The three types of composted FYM contained similar concentrations of nutrients: on average 394±8 g organic C kg−1, 22±1 g total N kg−1, 216±2 g available K kg−1 and 46±4 g available P kg−1. Biodynamic FYM was prepared as reported in Koepf et al. (1993). Briefly, after heaping up three similar piles of thoroughly mixed cattle manure (about 1.5 Mg fresh mass each), we bored six 50-cm-deep holes into one compost pile using a rod and poured each preparation into a separate hole. The valerian preparation is a liquid and was stirred into 8 l tap water before being poured on top of the compost pile (FYM+all preps). The FYM+Achillea treatment was prepared by pouring the yarrow preparation into a single 50-cm deep hole of the second compost pile. The holes in the compost piles were then filled with cattle manure. The third compost pile did not receive any preparation (FYM without preps). In total, 9–10 g preparation was added to about 1.5 Mg cattle manure. All compost piles received the same amount of water as was applied with the valerian preparation

The following methods are redacted as laboratory protocols for easier performing

2.3 Determination of soil moisture and water content

2.3.1 Background and principle of the method

The principle of the method is drying soil samples to a constant mass at 105°C and using the difference in the mass amount of soil before and after the drying procedure to calculate the dry matter and water contents on a mass basis. This gravimetric method is in accordance with international standards (ISO 11465:1993).

Although the determination of soil dry matter looks quite simple and easy to work out, it is definitely the most important determination in a soil lab. All soil properties, no matter if they indicate chemical or biological conditions of soil or a soil sample, will be related to the soil dry matter (e.g. the nitrogen content of the soil in mg g-1 dry soil). Therefore, it is very important to be accurate and precise in determining the water content and the dry matter of the soil. All errors derived from insufficient dry matter determination will affect all later investigations.

Note: Soil moisture or water content of a soil is always related to the dry matter of a soil sample. Therefore, the water content of a 100 g soil sample with 30 g water and 70 g dry soil is not 30% but 42.8% (30 g water / 70 g dry soil).

2.3.2 Materials

Small paper envelopes or paper bags
Oven adjusted at 105 °C
Scale

2.3.3 Procedure

The bags should be labelled with sample codes and the group number. From each sample one or two spoons of soil are weighed into the bags. The weight of the fresh (wet) soil (+ bag) has to be recorded. The soil samples were dried to constant dry weight at 105 °C for no less than 17 h. After 17h the soil samples were transferred into a desiccator filled with desiccant for returning to room temperature. The weight of the dry soil samples is recorded in a table. To be sure to refer the loss of water from the soil and not to the used bags, 3 empty bags are taken as references and applied to the

whole procedure. The average weight of the empty bags before and after drying should be discounted from the fresh and dry soil samples, respectively.

2.3.4 Calculations

Calculation of the water content (WC %) of a soil sample:

WC (%) = (soil before drying (g) – soil after drying (g)) / soil after drying (g) x 100

Calculation of the soil dry matter (soil dm) of a soil sample:

Soil dry matter (%) = soil after drying (g) / soil before drying (g) x 100

2.4 Determination of the water holding capacity

2.4.1 Background and principle of the method

The water holding capacity is defined as the amount of water that a soil column can hold against gravity. It is given as the corresponding water content. The higher the portions of small pore volumes in a soil, the higher the water holding capacity. Hence, coarse-textured soils have low water-holding capacities. Comparing different soils, the number and biomass of microorganisms is usually increasing with increasing water-holding capacities. The optimum moisture content for microbial investigations is between 40 to 60% of the maximum water holding capacity. Further increases of water contents in the soil samples result in decreasing microbial biomass contents.

A number of soil biological methods require the adjustment of water content relevant to biological activity, which is comparable in terms of the availability of water to soil organisms. However, the numeric water content does not help here since the water availability is a function of both water content and texture.

2.4.2 Materials

20 glass tubes with porous membrane
Oven adjusted at 105 °C
Scale

2.4.3 Procedure

The glass tubes should be labelled with the code of your samples. Put about 10 g fresh soil from each sample into the corresponding glass tube (see figure beside). About one half of the glass tubes should be filled with soil. Take the weight of the glass tubes with fresh soil and transfer them into a water bath. The filling level of water should be about 1 cm in height. Then, add water until the soil is completely covered with water.

The tubes have a porous membrane that allows the water infiltration. The tubes should be closed to avoid losses by evaporation.

Take the tubes out of the water after 2 – 4 hours and place them onto some absorbent wet tissue paper. About 10 min is enough time to let the spare water runoff onto the paper. The weight of the tubes with the wet (saturated) soil is recorded again before the tubes are put in the oven at 105 °C another 24 hours. From now on, the tubes are handled like the envelopes for dry matter determination (see 4.1). The water content determined for the wetted soil corresponds to the water holding capacity. To prevent that the water contained in that porous membrane of the glass tubes are imputed in the soil water, both, the tare weight of the glass tubes without water and with water in the membrane were determined for all tubes.

2.4.4 Calculations

For calculation, please see 4.1.4. Instead of discounting the envelope tare weight, now discount the tare of the glass tubes. Relate the tare of the wet tubes to the wetted soil and the tare of dry tubes to the dry soil.

Calculation of the water holding capacity (WHC %) of a soil sample:

WHC (%) = (saturated soil (g) – soil after drying (g)) / soil after drying (g) x 100
Example: If a soil sample has a water content of 29% and a WHC of 66%, hence, the adjustment to 50% of the max. WHC corresponds to a water content of 33%! Practical course Methods
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2.5 Determination of soil pH

2.5.1 Background and principle of the method
The pH-value (abbr. potential of hydrogen) is the negative logarithm of the measured concentrations of hydrogen ions in a solution (pH = -log (H+)) and, therefore, a characteristic of their acidity or alkalinity. In aquatic systems, the hydrogen ion concentration is dictated by the dissociation constant of water (Kw = 1.011 × 10-14 at 25 °C) and interactions with other ions in solution. Due to this dissociation constant a neutral solution (hydrogen ion concentration equals hydroxide ion concentration) has a pH-value of approximately 7 (pH 7 = 10-7 g H+/l). Aqueous solutions with pH values lower than 7 are considered acidic, while pH values higher than 7 are considered alkaline.
Soil pH is one of the most common measurements in soil laboratories and an indicator for soil fertility. The range of pH normally found in soils varies from 3 to 9. The significance of pH lies in its influence on the availability of soil nutrients, the solubility of toxic nutrients in the soil, physical breakdown of root cells, the cation exchange capacity in soils whose colloids (clay/humus) are pH-dependent, and biological activity. Acid soils are not commonly found in semi-arid dryland areas of the world. They tend to occur in temperate and tropical areas where rainfall is frequent throughout the year.
Many nutrient cations, such as zinc (Zn2+), aluminum (Al3+), iron (Fe2+), copper (Cu2+), cobalt (Co2+), and manganese (Mn2+), are soluble and available for uptake by plants below pH 5.0, though their availability can be excessive and thus toxic in more acidic conditions. They are less available in more alkaline conditions, resulting in nutrient deficiency symptoms such as thin plant stems, yellowing (chlorosis) or mottling of leaves, and slow or stunted growth.

Levels of pH also affect the complex interactions among soil chemicals. Phosphorus (P) for example requires a pH between 6.0 and 7.0 and becomes chemically immobile outside this range, forming insoluble compounds with iron (Fe) and aluminium (Al) in acid soils and with calcium (Ca) in calcareous soils.
Common categories of pH ranges are: <5 = strongly acid, 5 - 6.5 = moderately to slightly acid, 6.5 - 7.5 = neutral, 7.5 – 8.5 = moderately alkaline, >8.5 = strongly alkaline.
Measurement of soil pH is done with pH glass electrodes. The potential of H+ in a solution is detected against an internal standard. To learn more about glass electrode devices and details to the detection you can visit: http://en.wikipedia.org/wiki/PH_glass_electrode.
Fig.: Influence of soil pH on soil processes and nutrient availability.

2.5.2 Materials
Beakers (50 ml)
Water (distilled)
Glass rod
pH glass-electrode

2.5.3 Procedure
Label the beakers according to the coding of your soil samples. Fill in about 10 g fresh weight of each sample and pad 25 ml of distilled water to every soil sample. Then stir the suspension with a rod and wait for half an hour to allow deposition on the bottom of the beakers again. Now carefully measure the pH values of soil. First, the pH meter has to be calibrated using two standards related to

pH of 7 and 9 (or 4 if it is expected to go in the acid direction). To rinse and equilibrate the electrode again put it in distilled water before putting it into the soil suspensions. In both procedures, rinsing and measuring, always wait for constant values so that the potential of the solution can be detected and no further changes of values will occur. Always put back the electrode to its rack after use.

2.6 Determination of inorganic carbon (carbonate) in soil

2.6.1 Background and principle of the method

The Scheibler apparatus is used to determine the amount of inorganic carbon in the soil, specifically the carbonates (see figure below). Following an excess addition of HCl to the soil, the carbonates in the sample react according to the following equation:

$CaCO3 + 2\ HCl \rightarrow H2O + CaCl2 + CO2 \uparrow$

The amount of $CO2$ released from a soil sample after the addition of HCl is measured volumetrically inside the closed Scheibler apparatus. The calculation of the $CaCO3$ content in the soil sample in relation to the $CO2$ released after HCl addition could be done after the previous calibration of the apparatus with pure $CaCO3$ (per analysis).

2.6.2 Materials

Scheibler apparatus
Scale
Glas bottels (100 ml)
Beakers (40 ml)
HCl (~14%)
$CaCO3$ (per analysis: p. a.)
$H2O$ (aqua dest.: a. d.)

2.6.3 Procedure

The amount of soil needed for this procedure depends on the expected carbon content in the soil. 0.5 to 10 g soil is weighed into the reaction vessels of the Scheibler apparatus. A beaker containing 15 ml HCl (40 ml) is placed carefully into the reaction vessels. Avoid any contact of the HCl with the soil before the system is totally closed! Connect the reaction-vessels to the Scheibler apparatus. To balance the pressure inside and outside of the system, open the equalising valve (valve 1). The water inside the system (*communicating tubes*) has to be equal and adjusted to the zero-line. Close valve 1. Remove water from the system by opening the outlet valve (valve 2) as $CO2$ volume is expected to be released from the soil. Shake the reaction-vessel carefully until HCl gets in contact with the soil. If the water from the system impends to flow over, more water needs to be let out of the system by opening the outlet valve again. Depending on the carbonate content of the soil it needs 5 to 10 min until the chemical reaction is completed. If $CO2$ liberation ceases, the volume of released gas (ml) is noted and calculated according to the calibration. The calibration of the apparatus is done in the same way as described for the soil samples by using 200 mg $CaCO3$ (p. a.).

2.7 Quantification of soil microbial biomass: The chloroform-fumigation-extraction method

2.7.1 Background and principle of the method

The soil microbial biomass responds much more quickly than most other soil fractions to changing environmental conditions such as changes in substrate inputs (e.g. Powlson et al. 1987) or increases in heavy metal contents (Brookes and McGrath 1984). This and much other similar research supports the original idea of Powlson and Jenkinson (1976) that the biomass is a much more sensitive indicator of changing soil conditions than, for example, the total soil organic matter

content. Thus, the biomass can serve as an 'early warning' of such changes, long before they are detectable in other ways. Biomass measurements are certainly useful in studies of soil protection. They have the advantage that they are relatively cheap and simple as well as being rapid. There is now a considerable amount of literature to show that these measurements are useful in determining the effects of stresses on the soil ecosystem. Measurements of the soil microbial biomass by the fumigation extraction method have been used to estimate the environmental effects of pesticides (Harden et al. 1993) and antibiotics (Castro et al. 2002). It has been repeatedly used to monitor successfully the bioremediation process of fuel oil-contaminated soil (Joergensen et al. 1994ab, 1995, 1997; Plante and Voroney 1998; Franco et al. 2004).
Linked parameters (e.g. biomass specific respiration or biomass as a percentage of soil organic C) are also useful as they have their own intrinsic 'internal controls' (see Barajas Aceves et al. 1999 for a discussion). This may permit interpretation of measurements in the natural environment, where, unlike in controlled experiments, there may not be suitable non-contaminated soil (for example) to provide good 'control' or 'background' measurements (Brookes 1995).
The biomass of a microbial community can be quantitatively determined by fumigation and extraction in a large variety of soils developed under very different environmental conditions, especially in contaminated and remediated soils.
Soils are fumigated with chloroform, incubated for 24 h, and extracted. Different components can then be measured in the extracts. Non-fumigated soil is also extracted to correct for extractable non-biomass compounds.
Following chloroform fumigation of soil, there is an increase in the amount of various organic and inorganic components coming from the cells of soil microorganisms (Jenkinson and Powlson 1976). The membranes of living soil microorganisms are partially lysed by the fumigant chloroform. After a 24 h incubation period to allow autolysis, a large part of the soil microbial biomass can be extracted from fumigated soil. The amount of additionally rendered extractable C and N from the fumigated microorganisms is proportional to the original microbial biomass C and N. Organic C (Vance et al. 1987), total N and NH4-N (Brookes et al. 1985), and ninhydrin-reactive N (Joergensen and Brookes 1990) can be measured in the same 0.5 M K2SO4 extract (Alef and Nannipieri 1995). Organic C (Joergensen 1995) and total S (Wu et al. 1993) can be measured after extraction with 0.01 M CaCl2 and phosphate or total P after extraction with NaHCO3 (Brookes et al. 1982).

2.7.2 Materials

Soil

Moist and adjusted to 40-50% WHC, sieved (2 mm)

Devices

Room, incubator or water-bath adjustable to 25 °C
Implosion-protected desiccator
Vacuum-line (water-pump or electric pump)
Horizontal or overhead shaker
Deep-freezer at -15 ºC
Folded filter papers
Glass flasks (100 ml)
Balance
Extractor

Reagents

Ethanol-free CHCl3 (toxic)
Soda lime
0.5 M K2SO4 (87.1 g l-1, p.a.)

2.7.3 Procedure

1. Divide a moist soil sample of 20 g into two sub-samples of 10 g.
2. Place the non-fumigated control samples in 100 ml flasks, extract immediately with 40 ml 0.5 M K_2SO_4 (extractant-to-soil ratio of 4:1) for 30 min by oscillating shaking at 200 rev min-1 (or 45 min overhead shaking at 40 rev min-1), filter through a folded filter paper.
3. For the fumigated treatment, place glass vials containing the moist soil into a desiccator containing wet tissue paper and a vial of soda lime, add a beaker containing 25 ml ethanol-free $CHCl_3$ and a few boiling chips and evacuate the desiccator until the $CHCl_3$ has boiled vigorously for 2 min.
4. Incubate the desiccator in the dark at 25 °C for 24 h. After fumigation, remove $CHCl_3$ by repeated (6-fold) evacuation and extract with 0.5 M K_2SO_4 as described above.
5. Store 0.5 M K_2SO_4 extracts at -15 °C prior to analysis of organic C, total N or ninhydrin-reactive N.

2.7.4 Notes and points to watch

The desiccator must be kept under vacuum for 24 h to ensure the presence of a $CHCl_3$ atmosphere, which kills virtually all soil microorganisms.
Ethanol-free $CHCl_3$ must be used to measure microbial biomass C because ethanol cannot be completely removed from the soil after fumigation. Ethanol-stabilized $CHCl_3$ can only be used if solely microbial biomass N or ninhydrin-reactive N will be measured (DeLuca and Keeney 1993).
The soil must be sieved only if homogeneous samples are required (Ocio and Brookes 1990).
Soil weight can range from 200 mg (Daniel and Anderson 1992) to 200 g (Ocio and Brookes 1990).
Soil microbial biomass is extracted by 0.5 M K_2SO_4. The high potassium concentration flocculates the soil and prevents adsorption of NH_4^+ released by fumigation. The relatively high salt concentration also inhibits decomposition of the microbial material extracted after fumigation. However, if the extracts have to be stored for a long period, they must be frozen.
Upon thawing of frozen K_2SO_4 soil extracts, a white precipitate of $CaSO_4$ occurs in near-neutral or alkaline soils. However, this causes no analytical problems in either method and may be safely ignored (Joergensen and Olfs 1998)
Soil water content can fluctuate widely, but must be higher than 30% water holding capacity (WHC). Microbial biomass C and biomass N of soils at 40-50% WHC were similar to those in saturated soils (Widmer et al. 1989; Mueller et al. 1992).
Problems arise for fumigation and extraction in very compressed soils, which cannot be dispersed. Young living root cells are also affected by $CHCl_3$ fumigation. Consequently, in soils containing large amounts of living roots, a pre-extraction procedure must be carried out (Mueller et al. 1992).
In substrates containing more than 20% organic matter, e.g. compost, the ratio soil-to-extractant should be increased to 1:25 or more (Joergensen et al. 1997).

2.8 Basal respiration

2.8.1 Background and principle of the method

The respiration of living cells is the process in which the chemical bonds of energy-rich molecules such as glucose are converted into energy usable for life processes. Oxidation of organic material — in a bonfire, for example — is an exothermic reaction that releases a large amount of energy rather quickly. The equation for the oxidation of glucose is:
$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$ + Energy released
In a fire, there is a massive uncontrolled release of energy as light and heat. Cellular respiration is the same process, but it occurs in gradual steps that result in the conversion of the energy stored in glucose to usable chemical energy in the form of ATP.
The basal respiration of soil is defined as the respiration without addition of organic substrate to soil at 22 °C. It is taken as an indicator for microbial activity and carbon turnover in soil. The soil

respiration or soil CO2 release is related to the catabolic carbon turnover of cells to gain energy in soil. Its ratio to the anabolic turnover in soil, i.e. the microbial biomass, gives detailed information on the metabolic status of the soil microbes. The so-called metabolic quotient (CO2 release/soil microbial biomass; *q*CO2) increases with decreasing availability of carbon in the organic compounds used by the soil microorganisms. However, not only the quality of substrates can affect *q*CO2 but also environmental stress, resulting in higher energy turnover to maintain a certain amount of biomass. There are several proved procedures to detect the CO2 emission from soil samples. In this course, a modification of the *Isermeyer*-method is used. The principle is that the emitted CO2 is trapped in a base causing neutralisation to a certain extent. After precipitation of carbon as carbonate the rest of alkaline activity (= free OH- ions) is detected by titration with HCl.

2.8.2 Materials

Devices

Incubator adjustable to 22 °C

Burette or automatic titrator

Chemicals and solutions

0.5M NaOH (p.a.)

0.5M HCl (Titrisol)

saturated BaCl2 x 2H2O (p.a.)

0.1 % phenolphthalein solution in 70 % Ethanol

2.8.3 Procedure

Put 50 g sieved soil at 40-50 % WHC into a 500 ml Blue Cap Bottle

Pipette 5 ml of 0.5M NaOH into a test tube and place it on the bottom of the bottle (see figure below).

· Don't forget the blanks, 3-5 Blue Cap Bottles with NaOH in the test tubes but without any soil. The blanks refer to the amount of CO2 trapped in the base without respiration activity. The blank results have to be discounted later from the results in the soil bottles.

Incubate all Bottles 5 or 7 days at 22 °C

After finishing the incubation take the test tubes with the base carefully out of the bottles. Use tweezers to ensure a safe transition.

Transfer the NaOH into a beaker that will be used as the titration vessel. Rinse the test tubes with distilled water and add the rinse water to the titration vessel.

<u>Titration to pH 8.3 in the presence of BaCl2 (Anderson 1982):</u>

The 0.5 M NaOH is placed in a beaker (titration vessel). The trapped CO2 is absorbed in the NaOH-solution as Na2CO3.

$2\ NaOH + CO_2 \rightarrow Na_2CO_3 + H_2O$

5 ml saturated BaCl2 solution is added to precipitate the carbonate as insoluble BaCO3.

$2\ NaOH + BaCl_2 + CO_2 \rightarrow 2NaCl + BaCO_3 + H_2O$

A few drops of phenolphthalein are added as an indicator. Then, not reacted NaOH is brought to pH 8.3 by slowly adding 0,5M HCl under magnetic stirring (disappearance of the colour). The acid must be added slowly to avoid any possible dissolution of the precipitated BaCO3.

2.8.4 Calculations

Calculation of evolved CO2-C (titration to pH 8.3 in the presence of BaCl2).

$CO_2\text{-}C\ (\mu g\ g^{-1}\ soil) = ((B - S) \times M \times E / DW) \times 1000,$

where

B is the amount of acid needed to titrate the NaOH in the blank (ml) (take the average of the blanks),

S is the amount of acid needed to titrate the NaOH in the samples (ml),
M is the molarity of the HCl (mol),
E is 6 (g mol-1), the equivalent weight of carbon to OH- ions used in the titration reaction. From CO2 and OH- to CO32- and H2O one C equals two OH-. Hence, the neutralisation of one OH- with H+ equals one half C with a mol weight of 12 g mol-1 (12/2 = 6)
DW is the dry weight of the soil in gram.
CO2-C (µg g-1 soil d-1) = CO2-C (µg g-1 soil) / days of incubation

2.9 Ergosterol content in soil

2.9.1 Background and principle of the method
Bacteria and fungi are the two largest functional microbial subgroups in soil. Separating the microbial community in soil into fungi and bacteria is important due to their different functional behaviour in soil. For example: Fungi play an important role in the decomposition of recalcitrant organic matter such as cellulose and lignin in soil. For estimating the proportion of the fungal biomass in soil, the determination of ergosterol has been increasingly used as a sensitive and reliable indicator of fungal biomass in soil. The organic compound ergosterol is the predominant sterol in fungal cell membranes. Ergosterol controls the permeability, micro-viscosity of cell membranes and thus the activity of membrane-bound enzymes. There is a linear correlation between the ergosterol content and the fungal surface area. The quantification of ergosterol in soil is also in good correspondence to other fungal markers such as fungi specific phospholipid fatty acids (PLFA). Djajakirana et al. (1996) found ergosterol contents in soil ranging from 0.75 to 12.94 µg g-1. In the upper soil layer of Mediterranean grasslands ergosterol content varied from 0.6 to 3.8 µg g-1 (Lopez-Sangil et al., 2011). The ergosterol content of fungal cells is not constant; it varies depending on species and environmental conditions (Djajakirana et al., 1996).
The method used here is based on an ethanol-extraction of ergosterol from soil, followed by quantitative determination using HPLC followed by UV detection at 282 nm. Detection limit of ergosterol is 0.1 µg g-1 of soil.

2.9.2 Materials
Devices
HPLC equipment, precision pump, solvent delivery system, UV detector operating at 282 nm and automatic injector
C-18 reverse-phase analytical column, 125 x 4.6 mm (e. g.: hypersil 5 µm, Gynkotek, Munich, Germany)
Rotary evaporator
Round-bottom flasks (100 ml)
Pipettes
Horizontal or overhead shaker

Chemicals and Solutions
Mobile phase for HPLC separation (methanol)
Ergosterol standards (Sigma Chemicals)
Methanol Li Chrosolv
Ethanol 96% dest.

2.9.3 Procedure
Weigh 2 g of fresh soil into the brown flasks and add 100 ml of distilled ethanol. Distillation of ethanol ensures a high quality and purity. For extraction of the samples the soil-ethanol mixture has to be shaken for ½ hour at 250 rounds min-1 using an agitator (shaker). Then, filter the soil-ethanol

suspension into a round bottom flask using a Buchner-funnel, a Whatman GF/A glass fibre filter, a desiccator and a water jet pump (see figure below).
Putting this desiccator to a pressure below the atmosphere, the ethanol solution will be sucked into the round bottom flask. Filter paper and the extracted soil can be dumped. Still under vacuum, the *Buchner*-funnel has to be rinsed 3 times with 5 ml ethanol to wash all ergosterol from the filter into the extract. This way no further cleaning of the funnel is needed before the next filtration. Fix the round bottom flask with the filtered solution to the rotary evaporator. Evaporate all the ethanol to nearly dryness using a water bath with a temperature not higher than 40°C since ergosterol is not stabile at higher temperatures. The extracted compounds (including ergosterol) are precipitated to the walls of the round bottom flask. Add 3 ml of methanol into the flask, shake the flask slightly and transfer the methanol solution into 10 ml balloons using a pipette. To receive all the present ergosterol that is in the flask, repeat the addition of methanol and the transfer 2 more times. Be careful in using methanol since it is more toxic than ethanol. Fill up the balloons to the 10 ml mark with methanol. Be very careful with this, any uncertainties in the total volume of the methanol will cause errors in the calculation after detection of ergosterol. To remove any particles, the methanol solution is filtered by pressing it through a filter (< 0.45 µm) into labelled (use numbers or the sample coding) plastic cups using a syringe. Transfer an aliquot in brown HPLC vials. HPLC analysis has to be performed in not more than 3 days since ergosterol is not stable. Details on the principles of HPLC detection will be given orally in front of the machine by one of our team members.

2.9.4 Calculations
Using the ergosterol standards, a calibration curve is prepared with ergosterol concentrations in methanol of 1, 0.5, 0.25, 0.1, 0.05 and 0.025 µg ml-1. The relationship between peak area of ergosterol detection and concentration should be linear. The HPLC equipment used allows one to do the calibration directly with the output device. Hence, the output of data will be in µg ml-1. Then, using the rule of proportion, the absolute weight of ergosterol in 10 ml methanol extract can be related to the dry weight of soil that was extracted
Example: If the output is 0.7 µg ergosterol ml-1 extract then 7 µg ergosterol are in 10 ml methanol extract. If these were extracted from 2 g fresh soil or 1.75 g dry soil according to a water content of 14%, 7 µg ergosterol are in 1.75 g dry soil. Related to 1 g dry soil, these are 3.99 µg ergosterol g^{-1} soil.

2.10 Statistical analyses

Upon verifying normality of distribution (Shapiro-Wilks, Kolmogorov-Smirnoff, Anderson-Darling and Jarque-Bera) and equality of variances (Levene's test) , the correspondingly appropriate tests for equality of means were performed, followed by Dunn's, or Tukey's HSD or Mann Whitney's Post Hoc tests, for differences significance. Data were analysed using the SAS Statistical Package.

3. Results

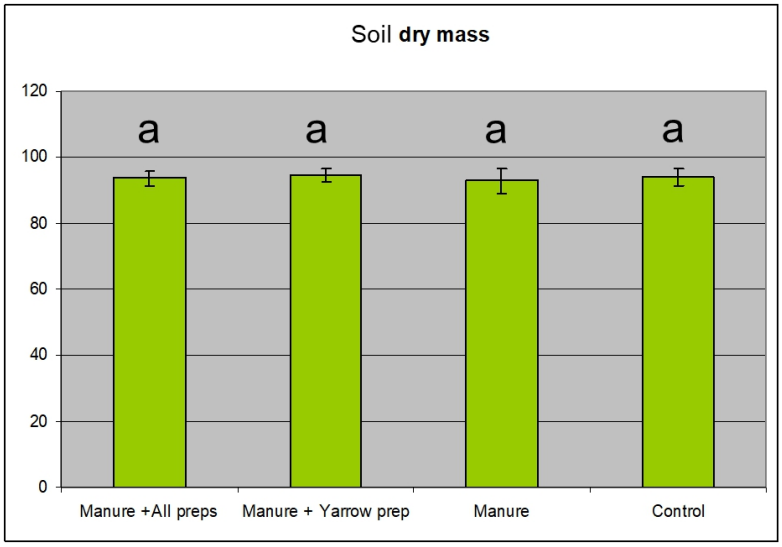

Fig. 1 Comparison of the soil dry mass values (grams) among the four treatments.
Values sharing the same letter are not statistically different for p< 0.05 significance.

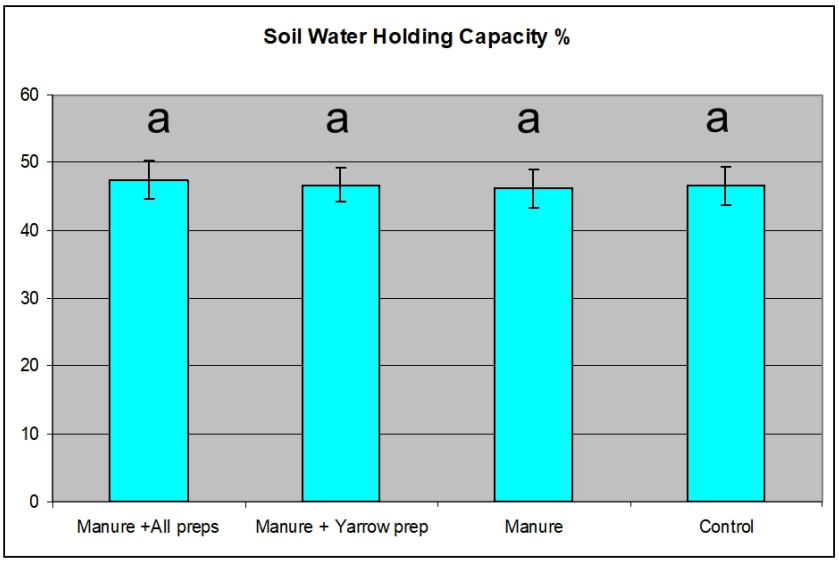

Fig. 2. Comparison of the soil water holding capacity % among the four treatments.
Values sharing the same letter are not statistically different for p< 0.05 significance.

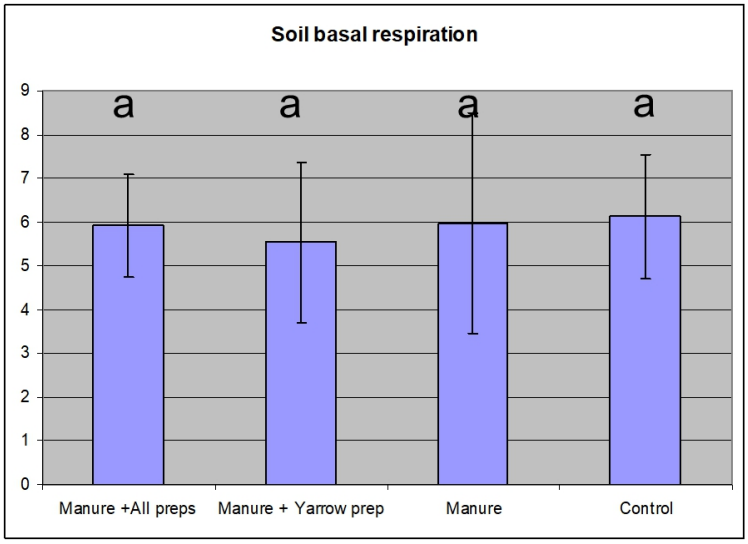

Fig.3 Comparison of the soil basal respiration values (µg/g soil dw/day) among the four treatments. Values sharing the same letter are not statistically different for p< 0.05 significance.

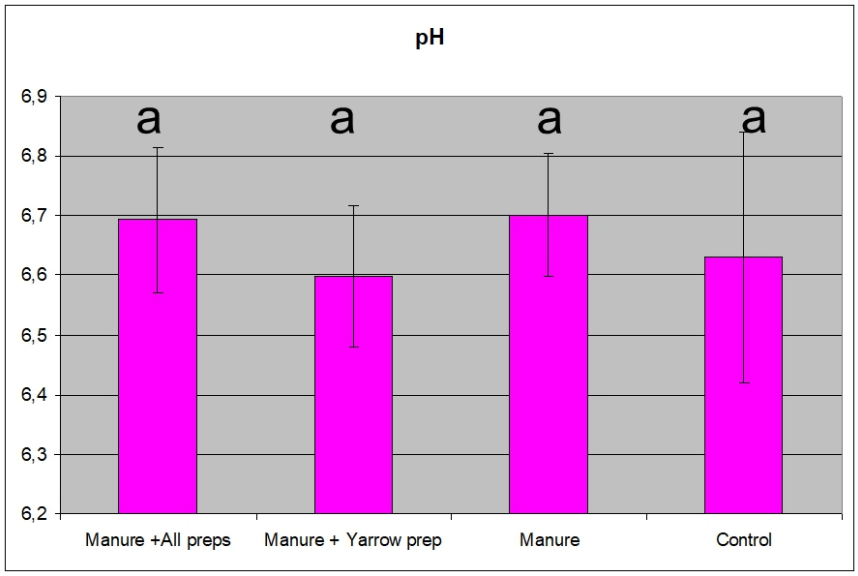

Fig. 4. Comparison of the soil pH among the four treatments.
Values sharing the same letter are not statistically different for p< 0.05 significance.

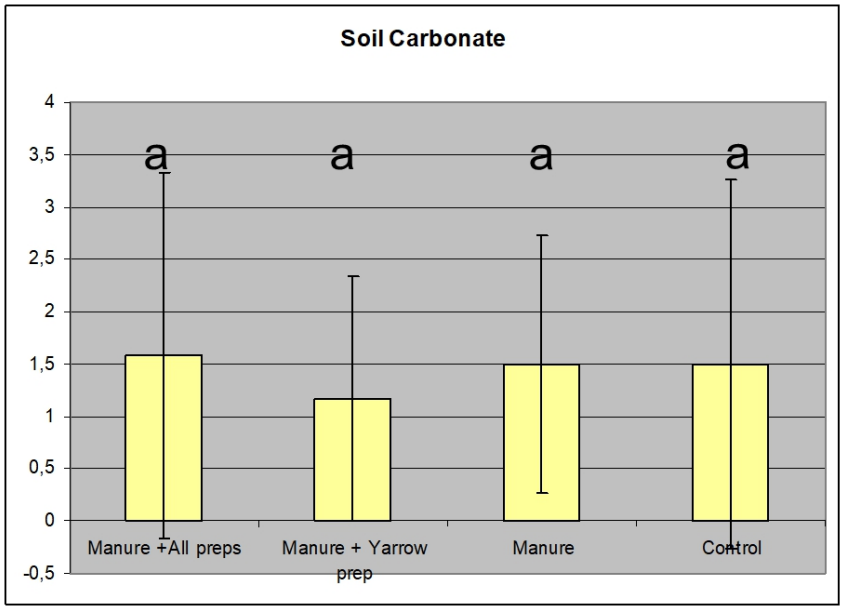

Fig.5 Comparison of the soil inorganic carbon (ml of liberated CO_2) among the four treatments. Values sharing the same letter are not statistically different for p< 0.05 significance.

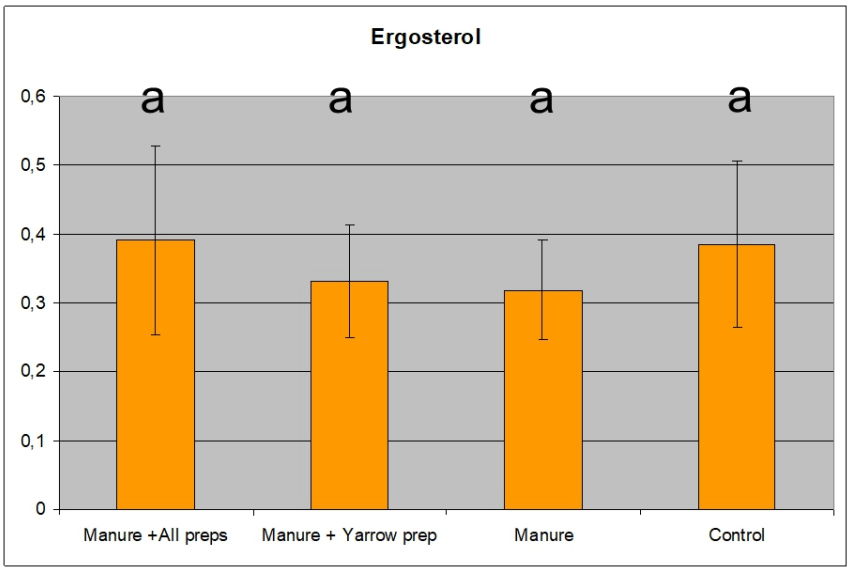

Fig. 6 Comparison of the fungal proxy soil ergosterol content (µg/g soil DW) among the four treatments. Values sharing the same letter are not statistically different for p< 0.05 significance.

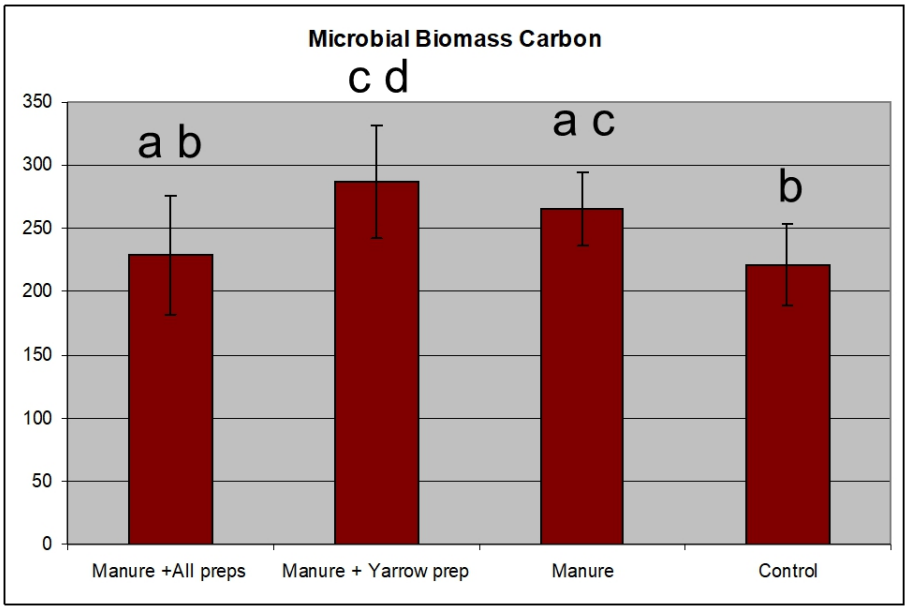

Fig.7 Comparison of the soil microbial biomass carbon (µg/g soil DW) among the four treatments. Values sharing the same letter are not statistically different for p< 0.05 significance.

Dunn post hoc test p values

Significant ones p < 0.05 are highlighted

	+All	Manure	Control
+All Manure	0,1416		
Control	0,5956	0,04546	
+Yarrow	0,04546	0,5956	0,01137

Fig. 8. p values resulting in the post-hoc test for the significance of differences in the comparison of means of the soil microbial biomass carbon analysis.

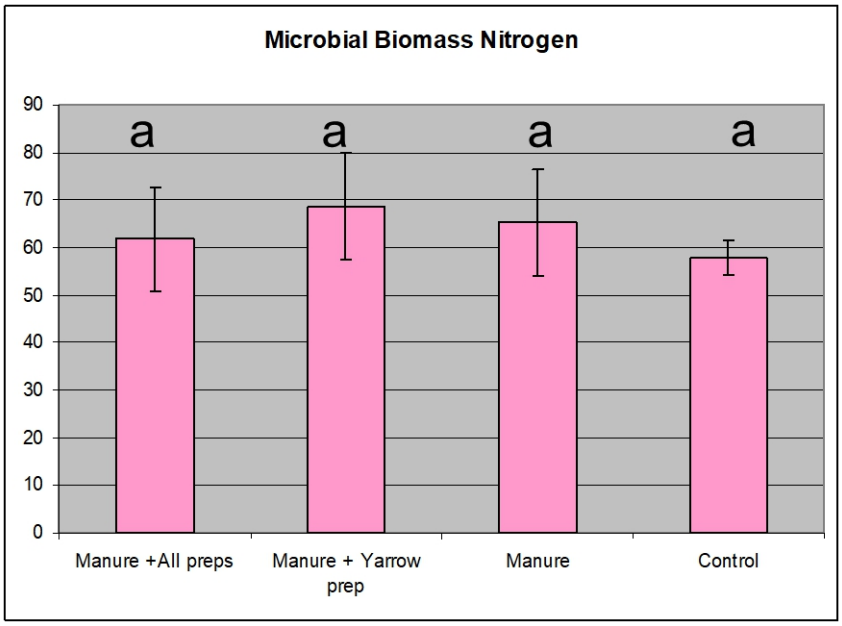

Fig. 9. Comparison of the soil microbial biomass nitrogen (µg/g soil DW) among the four treatments. Values sharing the same letter are not statistically different for p< 0.05 significance.

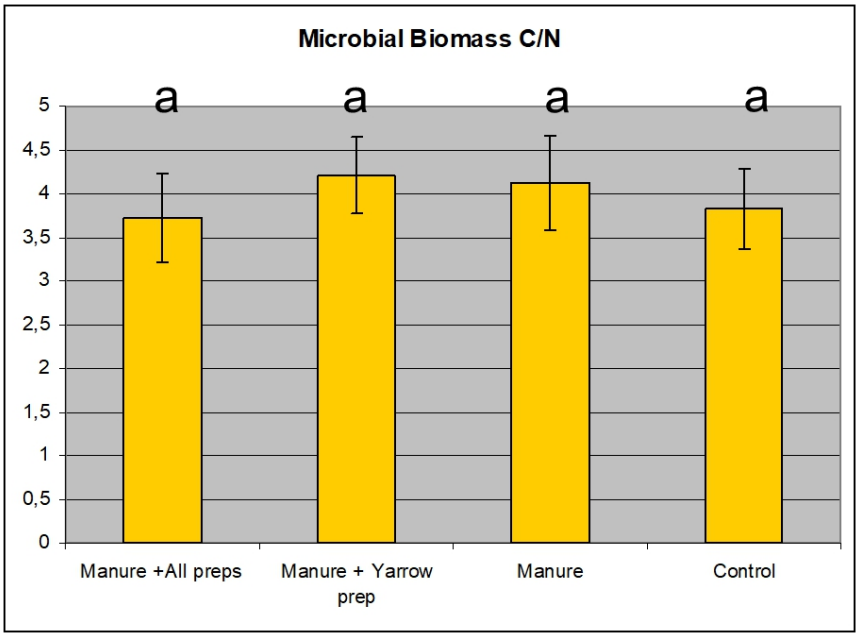

Fig. 10. Comparison of the ratio between soil microbial biomass carbon and nitrogen among the four treatments. Values sharing the same letter are not statistically different for p< 0.05 significance.

4. Discussion

The above analyses showed that the only parameter that presented statistically significant differences was the microbial biomass carbon. In that comparison (Fig. 7) the following evidences arose:

Before commenting the biodynamic aspects, a consideration on the organic fertilisation efficiency in itself can be made. The application of farmyard manure increased microbial biomass carbon over the untreated control. This is a basically expected outcome that confirms the notion that manure is not ending up in a total decomposition by microbial respiration to CO_2, but it can result in an increment of the same biomass of about 20%. While this may seem an efficient balance, it needs to be placed in temporal perspective to evaluate the actual benefits of the process. In this trial, manure was yearly applied for 27 years at a dosage of 30000 kg/Ha. About 1/3 of manure weight is made by live bacterial cells. In one hectare of agricultural soil, microbes amount to about 5000 kg/Ha. The treated fields, over 27 years, received cumulatively 27 x 30000 = 810000 kg of manure, which is 182-fold the weight of the soil microbes. The fact that this overload has built only a 0.2-fold increase of microbial biomass in comparison to the plots that in the same 27 years did not receive any manure, is showing the limits in this practice when assuming that it could foster carbon stocking in a fast way.

Coming to the effects of the biodynamics preparations, a major point is that when the whole set of six preparations, containing chamomile, dandelion, stinging nettle shoots, oak bark, valerian extract and yarrow blossoms, were added to the manure heaps at the recommended dosage of 10g in 1500 kg of composting manure, their addition counteracted the manure effect in terms of soil microbial biomass carbon. This is shown by the fact that the plots receiving manure + all six preps had a mean of soil microbial carbon that was no longer significantly different from that of the untreated control without manure and very close to it (229,2 vs. 221,2 ug/g). This annihilation of the manure effect could seem surprising if one looks at the ponderal ratio of dilution of the preparation into the manure heaps (150000-fold for those 10 g in 1500 kg) but considering the mechanisms of action of signal molecules, the phenomenon results instead well within the average biological range. Molecules as rhizobium nod factors are effective from as low as 10^{-10} M and some hormones are even effective at 10^{-15} M (D'Haeze et al., 2000). This means that if dilutions of ten billion-fold up to 100 trillion-fold from a 1M solution are still perfectly efficient in the biological world, a step like the one of the biodynamic preps in the manure heap) (150000 fold), leaves ample room for the active molecules within those 10 g to be even a minimal part of the mix and still qualify as a potentially high concentration as biological effects are concerned. In living cells, it is well demonstrated that a signal starting off with a single molecule, as e.g. adrenaline, can undergo an amplification of 10000 in few seconds within the same cell by becoming sequentially processed by four enzymes each encountered in a subsequent step in which the number of units processing the incoming product is exponentially higher than the ones in the prior step (Lodish et al., 2000)., In bacteria, a signal amplification to cover the whole volume of manure is easily explained by quorum sensing autoinduction and in slime molds by cyclic AMP-mediated amplification where billions of cells within diffusion distance gradients can be activated by a single starting cell, and in turn begin to send the same cAMP second messenger signal which exponentially will trigger novel gene expression within the whole environmental boundaries of the existing population (Haeger et al., 2015)

Extending this to the field, in which the actual effects are recorded, if 30000 kg, of this prep-conditioned manure were delivered every year, what is the ultimate dilution factor involved? Considering that the weight of a hectare of land, considering a depth from zero to 20 cm as useful to plant roots, is about 2000 tons, i.e. 2000000 kg. The ponderal dilution of this manure in the soil is therefore merely 66-fold and, also in this case there is a wide window of possible actual concentration of the active signals to be still fully active on their potential microbial targets to explain the observed impacts on the microbial biomass carbon variation result observed. Since biological signals work by cascades of transduced signal amplification, if we consider that in a gram of soil, we have up to a billion of live bacteria, the exponential kinetics that apply can easily account for a plain signal-mediated explanation of the observed effects.

While the supplementation of manure with all six biodynamics preps gave a reduction in soil microbial biomass carbon, the most interesting finding of this whole experimentation was that when manure was treated just with one of them, i.e. the yarrow (*Achillea millefolium*) preparation, the result was very different, the manure effect was not reversed as observed with the six preps together, but it was on the contrary increased in a statistically significant manner and reached the highest value observed, with a mean that was also higher than the one given by the manure alone. It is worth noticing that the value of significance (Fig. 9) between the yarrow-treated manure and control (0.011) is even more robust than the one between the manure treatment and control (0.045).
It is worth commenting that microbial biomass nitrogen instead was not significantly different, but looking at the corresponding graph (Fig. 8), the same trends can be independently spotted. The fact that also the C/N ratio was not affected either (Fig. 10), shows that there is no C-N uncoupled effect (acting on microbial carbon only) and that the differences commented seem to pertain to the microbial biomass in general and not to its differential enrichment in C-containing biochemicals devoid of N, as sugars or lipids, but rather to its increase as a whole.

The above observations call for their possible interpretations. In the first instance, it could be interesting to speculate how would the addition of the whole array of six biodynamic preparations bring about the reduction of microbial biomass carbon. The issue could be linked to mechanisms of self-regulation, as often the case in microbial quorum sensing phenotypes. The signals contained in the preps and transduced throughout the maturing manure heap could be carrying an information coordinating the reduction of cell density, which is common in situations in which signals are the result of high metabolic activity after intense microbial growth. In fact, in contexts of position-based sensing as those of cells in composting materials or in soil, signals could be interpreted as carrying a message triggering a more spaced repositioning of cells to ensure an ideal biofilm architecture. Upon attaining the proper space partition, the longer intercellular distances would automatically bring the action to a halt, as cells would be beyond reciprocal gradient reach. Indeed, an exogenous signal molecules supply to given mutants restores correct cell spacing (De Kievit et al., 2001), showing that the signal's actual effect is a 'spread-away'-type order. Since bacterial cell-to-cell signals occur where there has been profuse cell growth, it can be postulated that biodynamic preparations are signal-rich. An intense catabolic process occurs in fact within the fermentation during the confined incubations leading to the six biodynamic compounds.

The parallel interesting evidence is that instead when the manure treatment was done with the sole yarrow preparation, that one alone was not affecting negatively the biomass C content but rather fostering its increase in a statistically significant fashion. It is not known whether only one or more of the other five preparations (or the synergy of all six together), were responsible for the reduced soil microbial C content, since the experiments with single preparations involved the sole case of yarrow, but this one showed effects that were opposite to those exerted by their mix.
There is another important confirmation in literature on both facts observed here, In a prior campaign of analyses on the very same soil but as early as after 9 years of treatments two authors reported that the same biodynamic preparation of farmyard manure with fermented residues of six plant species significantly decreased soil metabolic quotient compared to manure without preparations or manure prepared with yarrow only. (Zaller & Kopke 2004). This shows that the same combinations that we found active in reducing soil microbial biomass carbon after 27 years of treatments had been reported to be active in reducing soil metabolic quotient after 9 years of treatments.

5. Conclusions

1. Repeated manure supplementation increased soil microbial biomass carbon.

2. The addition of the six biodynamic preparations to manure counteracted the above effect

3. The addition of the single preparation from yarrow (*Achillea millefolium*) to manure, did not affect the increase of soil microbial biomass carbon due to manure and its mean was higher than that of manure alone.

4. Known microbial communications models from quorum sensing and other cell-to-cell signalling notions are consistent with the explanation of how the observed effects can be explained on a quantitative basis.

6. Literature cited and further References

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