3. Materials and Methods

3.1. Location

Information about the sampling area had been collected mainly by interviews with the farmers during sampling time.

3.2. Soil sampling

At each vineyard, fallow and meadow, four diagonal sampling points per field were laid out with a standardized metal frame (0.5 × 0.5 m, 20 cm depth) and soil was carefully excavated (about 60 kg soil). The sampling points were between the grapevine rows and evenly distributed over the field (Fig. 2). The distances between the four sampling points depended on the location. For example, a vineyard on a steep slope had to be sampled differently than a vineyard near a river. As a rule, a distance of 10 m was always maintained between the diagonal sampling points. The influence from the field edge was placed in such a way that edge effects could be excluded.

All soil and earthworm data refer to 312 sampling points (78 area x 4 sampling point per area).

Only for the eCEC and microbial communities, the soils of the 4 sampling points were mixed together to one composite sample per area, i.e. 78 samples. It is known that there is a lot of heterogeneity in the sampling area. Although the microbial community was not the focus of the study, the analyses of the composite soil sample can still provide additional information on the effect of copper.

The methods used for the soil analysis and surveys on the occurrence of earthworms and microorganisms are OECD or ISO compliant.

3.3. Physicochemical soil analysis

Soil of about 2.5 kg per sampling point was stored in plastic buckets. In the laboratory, the soil samples were air-dried at room temperature, sieved at 2 mm and stored until analysis. All soil parameters are related to dry matter (DM).

Fig. 2. Soil excavation of a single sampling point.

The pH value of the soil was determined at room temperature according to A5.1.1 (VDLUFA 1991). For this purpose, 10 g soil was weighed into a 50 ml beaker, mixed with 25 ml of 0.01 M $CaCl_2$ solution and stirred twice within one hour with a glass rod (WTW inoLab 720 pH meter).

The analysis of total carbon and nitrogen content was carried out according to DIN ISO 10694 (1998) and DIN ISO 13878 (1998), respectively. The air-dried soil samples were ground in a ball mill. 30 mg soil plus three times the amount of tungsten were weighted in tin boats. The determination of the total carbon and nitrogen content has been done by combustion in an elemental analyzer (CHNS Analyzer Vario EL I, Elementar Analysensysteme GmbH) and expressed in percent by dry weight.

The determination of the soil organic matter in humus-rich, carbonate-free sandy soils and peat was carried out according to DIN 19684-3 (1998). For analysis 10 g of soil was weighed into ceramic crucibles and dried first at 105°C to constant weight. Subsequently, the samples were ashed at a temperature of 550°C. Soil organic matter was determined by loss on ignition and the ignition residue.

The particle size determination (properties of sand, clay and silt) was carried out according to DIN 19683-2, 1997 by wet sieving to 0.063 mm and sedimentation analysis for grain fractions $0.002 < d < 0.02$ mm after pretreatment with sodium pyrophosphate. The determination of the soil type was carried out according to DIN 4220 (2008) using the grain triangle KA5.

The effective cationic exchange capacity (eCEC) was determined according to DIN ISO 11260 (1997) with $BaCl_2$. Therefore 2.5 g of soil was weighed and mixed three times with 30 ml of 0.1 M $BaCl_2$ solution, shaken for one hour and centrifuged for 10 minutes at 4100 U min^{-1} (Megafuge 16, Thermo Scientific). After filtration of the extracts, the K^+, Ca^{2+} and Mg^{2+} concentrations were determined by inductively coupled optical plasma emission spectroscopy (ICP-OES, IRIS Intrepid®, Thermo Scientific). To determine the Mg excess concentration, 30 ml of 0.0025 M $BaCl_2$ solution was added to the samples, shaken overnight and centrifuged. The supernatant solutions were discarded. This was followed by the addition of 30 ml of 0.02 M $MgSO_4$ solution. The samples were shaken overnight and then centrifuged. The Mg^{2+} concentration of filtered extracts were determined by ICP-OES. For eCEC determination soil samples of the 4×0.25 m^2 sampling points per area were put together to one mixture sample (MS) and analysed as one sample per area.

The determination of total heavy metal and nutrient concentration was carried out by digestion of 2 g soil with 10 ml ($HCl:HNO_3$, 3:1) 2 hours at 130°C [A 2.4.3.1, VDLUFA, 1991]. The digested solutions were diluted to 50 ml with distilled water, filtered and analysed by ICP-OES.

Two extraction solutions were used for assessing element mobility from the vineyard soils, 1 M NH_4NO_3 and 0.001 M $CaCl_2$: The determination of the mobile heavy metal contents in the ammonium nitrate extract was carried out according to DIN 19730 (1997). 20 g of soil was mixed with 50 ml of a 1 M NH_4NO_3 solution and shaken for two hours. Prior to filtration, the samples were allowed to stand for 15 minutes. The soil extracts were filtered in a vacuum filter unit with a 0.45 μm membrane filter. The mobile element contents of the soil solution were determined by ICP-OES.

For the extraction with $CaCl_2$ [DIN CEN ISO/TS 21268-2] 5 g soil were mixed with 50 ml 0.001 M $CaCl_2$ solution and shaken for 24 hours. The soil extracts were centrifuged by 25000 g for 30 minutes at room temperature (Sorval Lynx, Thermo Scientific). The soil extracts were filtered in a vacuum filter unit with a 0.45 μm membrane filter. The mobile element contents of the soil solution were determined by ICP-OES.

For quality control of ICP-OES analysis, an in-house validated reference soil from the JKI experimental field and reagent blanks were digested with each batch. The methods were validated using certified reference materials BCR 142 and BCR 143 as reference soil. The relative standard deviation between the single element measurement with ICP-OES during the whole sampling period was less than 11 % for aqua regia digestions, less than 20 % for ammonium nitrate and calcium chloride extraction for relevant elements. The metal recoveries varied between 72 and 123 % depending on analysed element.

3.4. Earthworm parameters

Earthworms were sampled by hand selection of excavated soil at each sample point and by formalin extraction in the remaining hole according to ISO 23611-1 (2006). The earthworms were collected separately and stored in plastic containers in cooling boxes before being transported for taxonomic determination, counting all individuals separately according to development stage, and weighing fresh mass in the laboratory. The taxonomic determination of adult and juvenile individuals down to the species level was done using identification literature [5–7] or expert knowledge. The nomenclature is based on international standards [8–10].

To determine the heavy metal content in earthworms, in 2010 and 2011, the earthworms were kept in petri dishes as mixture samples (MS) per ecological group and per area after taxonomic determination of the sampling points. That is why the data of the heavy metal content of single earthworm tissues were only given in the data set for MS. From 2012, the earthworms were kept in petri dishes according to their ecological group and the heavy metal content was analysed as single earthworm per sampling point. The earthworms were given time to empty themselves over night using the "filter paper method" [11]. The filter paper was changed as needed and not rinsed out. The subsequent determination of element content in the earthworm tissue was performed after pressure digestion with HNO_3 according to A1, UBA Texte 10/95 (1987). Therefore, a single earthworm was weighted into Teflon vessels and 5 ml of 69 % HNO_3 was added. The Teflon vessels were sealed by hand in high pressure vessels heated under pressure at 160°C for 10 hours (Loftfields, 1986). Then the extracts were filtered and made up to 25 ml with distilled water. Total element concentrations of earthworm tissues were determined by ICP-OES. For expression in dry weight, the analysed element content (mg kg^{-1} FM) were converted by 6.25. This conversion factor is based on a middle water content of 84 % at the earthworm tissue [12].

3.5. Microbial parameters

To activate microbial populations, soils were moistened to approximately 50 % of maximum water holding capacity one week before the start of the analysis and stored in the dark at 20°C ± 1°C for 7 days. At the end all parameter are related to dry matter (DM).

Microbial long term soil respiration (LSR) was determined by pressure measurement in a static system according to ISO/DIS 16072 (2001). Therefore, 100 g of mixed soil samples from the sampling area was put into a 500 ml SCHOTT DURAN® bottle. An absorption vessel containing 1 ml of 1M KOH was then added into the flask which was then sealed shut by the manometer. Pressure changes were measured for 24 hours with 5 replicates for every sample. Soil volume was determined by adding 100 g of soil to 100 ml of distilled water in a measuring cylinder, the amount of the water level rising being equal to the soil volume. The rates for O_2 consumption and for CO_2 evolution were then calculated as described in the norm.

Substrate-induced respiration (SIR) was determined by a maximum SIR according to ISO 14240-1(1997). Therefore, 100 g of mixed soil of the four sampling plots was put into a 500 ml SCHOTT DURAN® bottle. An absorption vessel containing 1 ml of glucose was then added into the flask which was then sealed shut by the manometer. Pressure changes were measured for 8 hours using 5 replicates for every sample. The CO_2 evolution rate was calculated as described in the norm.

LSR and SIR are expressed in mg O_2 kg^{-1} DM h^{-1}. Using a factor of 1.375, oxygen consumption could be converted to carbon dioxide formation (R) expressed in mg CO_2 kg^{-1} DM h^{-1} according to ISO 14240, 1997.

Using the maximum SIR data, microbial biomass (Cmic) was calculated as follows:

Cmic= 40 R + 0.37 [mg C kg^{-1} DM]; R = CO_2 evolution rate [ml CO_2 kg^{-1} DM h^{-1}] (DIN ISO 16072, 2001).

The metabolic quotient (qCO_2) was calculated from the ratio between CO_2 formation in LSR and Cmic and expressed as mg CO_2 h^{-1} g^{-1} Cmic.

Dehydrogenase activity (DHA) was analysed using iodonitrotetrazolium chloride (INT) according to DIN EN ISO 23753-2, 2011. Therefore, 5 g of mixed soil samples was mixed with 5 ml of INT solution. After 18 hours incubation at 30°C 20 ml acetone was added to stop the enzyme activity. The samples were then further incubated in the dark at room temperature for 2 hours. Afterwards, all samples were filtered and measured at 485 nm wavelength using Specord 205 UV/VIS spectrophotometer. The whole procedure was repeated five times for every soil sample using 3 samples without INT solution as a blank sample. The DHA was then calculated using an iodonitrotetrazolium violet-formazan (INTF) calibration curve.

Ethics Statements

The authors state that our surveys on invertebrate soil organisms do not refer to the objectives of the "EU Directive 2010/63/EU on the protection of animals used for scientific purposes", based on Article 1(3).

CRediT Author Statement

Nadine Herwig: Conceptualization, Writing – original draft, Writing – review & editing, Methodology, soil data curation; **Bernd Hommel:** Writing – original draft, Writing – review & editing, Methodology, earthworm data curation; **Dieter Felgentreu:** Formal analysis, microbial data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships which have or may have influenced the work reported in this article.

Data Availability

Copper distribution in German vineyards and its impact on soil organisms – Dataset of a field survey from 2010 to 2014 (Original data) (Open Agrar).

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