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Original Articles

Defining a reference system for biological indicators of agricultural soil quality in Wallonia, Belgium

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ABSTRACT

Tools that will enable the assessment of agricultural soil quality and include measurements of biological indicators, such as soil respiration or nitrogen mineralisation, are increasingly in demand. Such tools require the establishment of reference systems to provide comparative 'baseline' or 'normal' values. In this study, we measured the spatial and seasonal variability of eight biological indicators (including two eco-physiological quotients) in order to establish a reference system at the regional level of Wallonia (Southern Belgium).

Respiration potential, microbial biomass carbon, microbial C/N ratio, net nitrogen mineralisation, metabolic potential of soil bacteria, earthworm abundance, microbial quotient, and metabolic quotient were measured at 60 sites across contrasting agricultural regions (different soil types and climate) in both grasslands and croplands. Additionally, the same biological indicators were measured four times during the vegetation period (April, June, August, and October) in 11 cropland sites to assess seasonal variability. Reference ranges were defined for each biological indicator, based on the addition of variances (seasonal and spatial) and the calculation of cumulative distribution functions.

Land use was the most useful classification variable to define a reference system in Wallonia. Two separate reference systems, one for grasslands and one for croplands, were thus appropriate for Wallonia. Sampling season had a significant effect on all biological indicators. The inclusion of seasonal variability resulted in reference ranges 1.1–5.7 times wider than ranges accounting only for spatial variability. The reference system provides a basis for a first comparative assessment of soil quality for most agricultural soils of Wallonia, independent of sampling period.

1. Introduction

Tools for the assessment of soils are needed to evaluate the effects of agricultural practices and support sustainable soil management. Farmers generally rely on a combination of informal observations and chemical analyses to assess the state of their soils (Wood and Litterick, 2017). In Wallonia, a federal entity in the southern part of Belgium, a network of provincial laboratories provides soil analyses for farmers. So far, soil assessment routinely includes a range of different chemical and physical parameters (Genot et al., 2011), but an increasing demand for measures linked to the biological activity of soils has been noted (Genot, personal communication). Furthermore, the possibility of future legal obligations to report on biological parameters of agricultural soils motivates laboratories to prepare for this eventuality and expand their offer.

Biological indicators, such as soil respiration or earthworm abundance, are commonly used in comparative studies, for instance to measure the effects of different soil management practices (D'Hose et al., 2014; Van Leeuwen et al., 2015) or to monitor the recovery of degraded soils (Gil-Sotres et al., 2005). These indicators have been integrated into soil monitoring networks (SMN) across Europe (van Leeuwen et al., 2017). A pilot study in Wallonia allowed the collection of a first data set on biological indicators (Krüger et al. 2017), but routine measurements of biological indicators are not yet commonly available for farmers. In order to offer such measurements, agricultural laboratories need to select indicators that are economic in terms of running costs, initial investment, and required measurement times (Doran and Zeiss, 2000). Furthermore, the ecological meaning of the data needs to be easily interpretable by managers and farmers. Thus, we considered the use of well-established methods with clear links to soil functions and easily interpretable data, such as microbial

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biomass (Vance et al., 1987) or nitrogen (N) mineralisation, as preferable to novel measurements such a molecular microbial diversity that require scientifically demanding data analysis (Hermans et al., 2017).

While scientific studies include control sites, these are not commonly available for commissioned measurements. Evaluation of results thus requires a comparative reference system, where values are compared to those for other sites across the same farming region. The concept of critical limits (i.e. distinction between safe and unsafe conditions in toxicological contexts; Chaisuksant et al., 1999) cannot be applied to biological indicators. As biological indicators are subject to inherent site factors, such as soil texture, mineralogy (Sparling, 1997) and climate (Wienhold et al., 2009), reference values need to be defined according to a hierarchy of site and landscape classification variables. Following the definition of soil quality as 'fit for a purpose', a classification of reference systems according to actual or intended land use is a logical choice (Sparling, 1997). For some biological indicators, areas might be further divided based on their driving factors (texture, pH, soil organic carbon content) (Dequiedt et al., 2011; Griffiths et al., 2011). In the context of SMNs, criteria to divide the data into subsets vary between European countries: for instance, the online database of ranges of biological indicators measured on 47 sites in the French "Bioindicator program" allows division of sites by land use, contamination level, texture, pH, and soil organic carbon content (Cathelineau et al. 2014, Pérès et al., 2011), whereas the French SMN RMQS bases its approach on land use, management system, fertilisation intensity, tillage, use of pesticides (Cluzeau et al., 2012), and the Dutch SMN BISQ (Biological Indicator system for Soil Quality) uses a stringent combination of soil type and land use to stratify its sampling sites (Rutgers et al., 2009). Given the similarity in pedoclimatic conditions between Wallonia and its neighbouring countries, a similar approach to stratification could prove useful.

Spatio-temporal variability presents a challenge for defining value ranges in reference systems. Biological indicators reflect complex interactions between different environmental parameters (Ritz et al., 2009), and, generally show a higher spatiotemporal variability than physical and chemical indicators. Spatial variability can be attenuated to some degree through the use of composite samples, sometimes made up of several hundred cores (Bloem et al., 2005). In Wallonia, soil assessment for farmers is generally provided for composite samples, representative for homogenous farm plots (similar in colour, texture, rock content, humidity, etc.), following ISO norms (Genot et al., 2012).

While shorter reaction times to environmental changes is one of the motivations for the inclusion of biological indicators in SMNs (Dale and Beyeler, 2001), seasonal variation of soil conditions (substrate availability, temperature, moisture etc.) can hamper their interpretation (Schloter et al., 2003). In SMNs, the sampling moment is generally set, taking into account management practices and meteorological conditions in order to reduce inter-annual variation. Samplings should be performed before ploughing, in the absence of recent frost or fertilization, in humid, but not waterlogged soil. As such, both sampling in spring or autumn are considered as suitable (Bloem et al., 2005). In practice, the sampling period often depends on the farmers' wishes that soils are sampled before seedlings might be disturbed. Nonetheless, the variability of weather conditions in the Atlantic climate (Zveryaev, 2004) might result in highly different conditions between years and subsequent differences in biological indicator measurements, even if criteria for suitable sampling conditions are clearly defined. Soil fauna indicators are generally very sensitive to meteorological conditions, whereas soil samples for molecular analysis are sampled year-round in France. Samples for microbial analysis can be pre-incubated to mitigate the effect of weather conditions preceding sampling (Bloem et al., 2005), but this might also impact results due to substrate depletion in pre-incubated samples. Additionally, changing crop rotations and related management practices result in soils where the underlying dynamics are difficult to disentangle, if the land management history can be documented at all. While it is possible to define reference plots without taking into account the variability in that location for comparison (Rutgers et al., 2009), we considered the added value of including spatiotemporal variability in the reference system important for its practical use as a meaningful assessment tool.

The main goal of a SMN is to track the long-term evolution of soil quality at specific sites over years or even decades (Mol et al., 1998). Measurements from representative sites can also provide reference values against which data from other sites can be compared. Representativeness of data sets collected within a SMN is defined, first through the selection of sampling sites (generally chosen through a regular grid or a stratification approach (Morvan et al., 2008)), the standardisation of sampling and measurement procedures (including sampling period), and through the mathematical approach used to express the ranges. To facilitate diagnosis, ranges are expressed as quartiles (as done in France, (Cluzeau et al., 2012)), or through calculation of 95% confidence ellipsoids referred to as the Normal Operating Range (NOR) (used in the Netherlands, (Kersting, 1984; Pereira e Silva et al., 2013)). NOR also present the advantage of mathematically combining several measurements. These approaches account for spatiotemporal variability of biological indicators, without giving extreme values power to skew the data distribution. Such methods require large data volumes that are so far not available in Wallonia.

The development of a reference system for biological indicators of soil quality for Wallonia was the main aim of this study. Specifically, the objectives of this study were to (1) assess the relevant classification variables for defining reference values (landscape classification and soil chemical parameters); (2) quantify the relative importance of seasonal and spatial variability; (3) define a representative reference system, accounting for spatial and seasonal variability.

2. Material and methods

2.1. Site selection and soil sampling

Spatial variability of biological soil quality indicators was studied at the regional scale of Wallonia, south Belgium. Sixty sites from the CARBOSOL network, monitoring soil organic carbon stocks and dynamics in Wallonia (Goidts and van Wesemael, 2007), were selected among 10 landscape units (LSU) through the Latin hypercube method (Minasny and McBratney, 2006) and sampled in spring 2015 (Fig. 1). Soils within each LSU are homogenous with regard to land-use (grassland or cropland), soil type (texture, rock fragment content, and drainage), and belong to the same agricultural region (a proxy for climatic conditions, Table 1). The ten selected LSU represent an area that covers about 47% of the agricultural land of Wallonia and present different conditions along environmental gradients (Chartin et al., 2017). Sites are situated at altitudes of about 60 to 440 m asl. Agricultural practice and current crop are not defined in the thus created CARBIOSOL network for biological indicators, resulting in diverse management situations at the time of sampling.

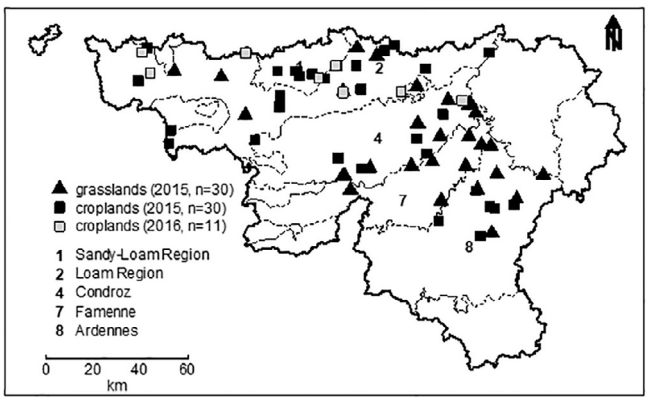

Fig. 1. Location of sampling sites in Southern Belgium (Wallonia). Samples taken to study spatial variability in 2015 are shown in black and samples taken to study seasonal variability in 2016 are shown in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Characteristics of landscape units (LSU). Following the legend of the Walloon soil map (Bah et al., 2007), texture is given in the classes A: light clayey soils, G: stony soils (> 15% stones), L: loamy-sand soils; drainage is given in two classes 1 = good to moderate drainage; 2 = imperfect drainage (Goidts and van Wesemael, 2007), for chemical parameters, minimum, mean, and maximum values are given.

LSU	Agricultural region	Land use	Texture	Drainage		C_{org} %	N_{tot} %	C/N	pH_{KCl}
1	Sandy loam region	Crop	L	1	Min	0.97	0.10	9.3	5.5
					Mean	1.11	0.11	10.4	6.8
					Max	1.45	0.14	11.6	7.4
3	Loam region	Crop	L	1	Min	0.94	0.09	9.7	5.1
					Mean	1.16	0.11	10.7	6.8
					Max	1.34	0.12	11.9	7.3
4	Loam region	Crop	A	2	Min	0.90	0.10	9.3	5.1
					Mean	1.27	0.12	10.3	6.8
					Max	1.77	0.15	11.7	7.4
5	Loam region	Grass	A	1	Min	2.19	0.19	9.5	5.7
					Mean	3.93	0.37	11.0	5.9
					Max	5.05	0.48	14.0	6.2
6	Condroz	Crop	A	1	Min	0.92	0.11	8.8	5.0
					Mean	1.24	0.13	9.9	6.2
					Max	1.60	0.17	11.2	6.8
9	Condroz	Grass	A	1	Min	1.88	0.10	9.2	3.7
					Mean	3.13	0.30	11.3	5.7
					Max	4.46	0.42	18.0	6.5
11	Condroz	Grass	G	1	Min	2.80	0.33	8.6	4.2
					Mean	4.14	0.40	10.2	5.9
					Max	5.36	0.46	11.9	6.6
13	Famenne	Grass	G	1	Min	2.30	0.27	8.5	4.8
					Mean	3.69	0.40	9.2	5.2
					Max	5.45	0.53	10.4	5.6
14	Ardenne	Crop	G	1	Min	1.99	0.24	8.2	4.6
					Mean	3.13	0.35	9.0	6.1
					Max	4.03	0.43	9.6	6.7
15	Ardenne	Grass	G	1	Min	3.35	0.33	8.7	4.3
					Mean	4.13	0.44	9.5	4.8
					Max	4.63	0.51	10.1	5.3

Seasonal variability was investigated at 11 additional cropland sites, which were sampled at two monthly intervals in 2016 (April, June, August, and October). These sites were selected according to current (winter wheat) and previous (sugar beet) crop, which represents the most common crop rotation in Wallonia.

At each of the 60 sites (spatial variability), a composite soil sample (0–10 cm soil depth) was taken in spring 2015. In the additional croplands (seasonal variability), four replicate composite samples were taken at each site at the corners of a 10 * 10 m square in 2016. For both sampling campaigns, the composite samples consisted of five individual samples taken between one and four meters from the central point with a manual auger, following Goidts et al. (2009). Fresh soil samples were sieved (4 mm) and stored at 4 °C until analysis (performed within a month of soil sampling). Soil moisture was determined after drying at 105 °C for 3 h (Allen et al., 1989). Water holding capacity (WHC) of sieved soil was measured using a Haines-funnel system, where 50 ml of water was added to 50 g of fresh soil for 30 min. Excess water was collected and its volume measured (Jenkinson and Powlson, 1976). Before analyses of biological indicators, soil samples were adjusted to 50–60% water holding capacity. pH was measured in a suspension (1:1; m:v) with 1 m KCl with a pH meter (HI2550 HANNA instruments, USA). C and N were measured by element analysis (VarioMax CN dry combustion Analyzer, Elementar GmbH, Germany).

2.2. Earthworm abundance

One earthworm sample was collected at each of the 60 sites and at each sampling for the 11 additional croplands close to the marked sites, but outside the sampling radius defined for soil samples. Earthworms were extracted by two consecutive applications of 4 l mustard solution (3 and 6 g l^{-1} of mustard, Sinapis Albae Seminis Pulvis, Pharmaflore, respectively) (Gunn, 1992; Lawrence and Bowers, 2002) on a 30 * 60 cm surface marked with a wooden frame. All earthworms leaving the soil were collected and conserved in 70% ethanol. Earthworms were counted and their mass measured (g biomass saturated with ethanol).

2.3. Net nitrogen mineralisation

Net nitrogen mineralisation was measured through a 29-day aerobic laboratory incubation at constant temperature (25 °C) in the dark (Hart et al., 1994). At the beginning and at the end of the incubation, inorganic nitrogen was extracted with a 1 M KCl solution (1:5; w:v) (Allen et al., 1989) and analyzed colorimetrically using a continuous flow analyzer (Auto-Analyser3, BranLuebbe, Germany). The net nitrogen mineralisation rate was calculated by dividing the net increase in inorganic nitrogen ($N\text{-}NH_4^+$ and $N\text{-}NO_3^-$) during the incubation period by the number of incubation days.

2.4. Microbial biomass

Soil microbial biomass C (MBC) and N were determined by the chloroform fumigation extraction method (Vance et al., 1987), consisting of 0.5 M K_2SO_4 extraction of both fumigated and unfumigated soils. Fumigations were carried out for three days in a vacuum desiccator with alcohol-free chloroform. Fumigated and unfumigated extracts were filtered (Whatman Filter Papers 42, CAT No. 1442-150). In both extracts, dissolved organic carbon was measured with a Total Organic Carbon Analyzer (Labtoc, Pollution and Process Monitoring limited, UK) and total N was measured colorimetrically using a continuous flow analyzer equipped with a UV digestion unit (Autoanalyser3, BranLuebbe, Germany). Soil microbial biomass C and N were calculated by dividing the difference of total extract between fumigated and unfumigated samples with a conversion factor of

0.45 for biomass C (Jenkinson et al., 2004) and 0.54 for biomass N (Joergensen, 1996).

2.5. Respiration potential

The respiration potential (Robertson et al., 1999) was measured as CO_2 accumulation in the headspace (250 ml) of an amber bottle (Supelco, USA) from 20 g fresh soil, at 15 °C in the dark after an overnight pre-incubation. Gas samples (4 ml) were taken at 0, 120, 150, and 180 min (samples taken in 2015) or 0, 120, 240, and 360 min (samples taken in 2016) with an air-tight syringe (Hamilton Model 1005) and analyzed with an infrared absorption gas analyzer (EGM-4, PPsystem, UK). The respiration potential estimated by linear regression of CO_2-C against time (mg kg^{-1} h^{-1}).

2.6. Metabolic potential of soil bacteria

BIOLOG ECOplates (BIOLOG™, California) with 32 wells each containing one of 31 different carbon substrates and one control well with water were used to assess metabolic potential of soil bacteria. Each well contained an oxidized tetrazolium dye, changing from colourless to purple when bacterial respiration oxidized the carbon source provided. 1 g of fresh soil was extracted with 9 ml of 0.1% sodium cholate and diluted to three dilutions (10^{-2}, 10^{-3}, 10^{-4}) with 0.85% NaCl to determine the number of CFU (colony forming units). An aliquot of 100 µl of the dilution corresponding to 1000–2000 CFU was incubated in the Ecoplates for 72 h at 20 °C. Number of substrates used by bacteria were detected through visual observation of coloured wells after incubation (Buysse et al., 2013).

2.7. Eco-physiological quotients

The metabolic quotient (q_{CO2}) represents the quantity of respired CO_2-C per unit of soil microbial biomass and was calculated by dividing respiration potential by soil microbial biomass C (Anderson and Domsch, 1990). The microbial quotient (q_{mic}) represents the availability of soil C and was calculated by dividing microbial biomass C by soil organic carbon (Anderson and Domsch, 1990).

2.8. Data analyses

In order to select relevant classification variables for a common reference system, we analysed the data from the spring 2015 sampling (60 sites) by permutation-based linear models from the 'lmPerm' package, R (Wheeler and Orphaned, 2014). Models including up to three parameters defining landscape units (land use, texture, and agricultural region, see Table 1) and/or chemical soil parameters (soil organic carbon, total nitrogen, and pH_{KCl}) and their interactions were constructed. Land use was included in the majority of models given the close links between soil quality and land use (Sparling, 1997). Nonetheless, a model including only chemical soil parameters tested the possibility of classification based on commonly available chemical indicators (Genot et al., 2011). Soil organic carbon was included in the models with chemical indicators given its primordial importance for soil biology (Koch et al., 2013) except for the model including the soil C/N ratio. Eight models were considered for each biological indicator (β_i is the estimated coefficient for each parameter and ε is an error term):

$$M_o: biologicalindicator = \beta_0 + \varepsilon(\text{nullmodel})$$

$$M_1: biologicalindicator = \beta_0 + \beta_1 landuse + \varepsilon$$

$$M_2: biologicalindicator = \beta_0 + \beta_1 landuse * \beta_2 agriculturalregion * \beta_3 texture + \varepsilon$$

$$M_3: biologicalindicator = \beta_0 + \beta_1 landuse * \beta_2 C_{org} + \varepsilon$$

$$M_4: biologicalindicator = \beta_0 + \beta_1 landuse * \beta_2 C_{org} * \beta_3 N_{tot} + \varepsilon$$

$$M_5: biologicalindicator = \beta_0 + \beta_1 landuse * \beta_2 \mathrm{C/N} + \varepsilon$$

$$M_6: biologicalindicator = \beta_0 + \beta_1 landuse * \beta_2 C_{org} * \beta_3 pH_{KCl} + \varepsilon$$

$$M_7: biologicalindicator = \beta_0 + \beta_1 C_{org} * \beta_2 N_{tot} * \beta_3 pH_{KCl} + \varepsilon(\text{Chemicalmodel})$$

Akaike information criterion (AIC), an estimator of the relative quality of statistical models, was calculated with the 'MuMIn' package (Barton, 2009). The model with the lowest AIC_c was selected as best or most parsimonious and all models with a difference in their AIC values (ΔAIC_c) superior to 2 were considered as less suited (Burnham and Anderson, 2004). Seasonal differences were analysed (2016 sampling, 11 croplands) by permutation-based ANOVA with repeated measurements (site) using the RM function from the R "MANOVA.RM" package (Friedrich et al., 2018).

The magnitude of seasonal and spatial variability was assessed through: (a) coefficients of variation (CV: SD/mean * 100, %), (b) max/min-ratios, (c) relative ranges, i.e. the range of a data subset as the percentage of the range of the 30 sampled croplands in Wallonia (2015). Relative ranges were calculated as $\max(X_i)$-$\min(X_i)$/$\max(X_{2015})$-$\min(X_{2015})$ * 100 where X_i is the observed subset of the data and X_{2015} includes the 30 croplands sampled during the 2015 campaign. For spatial variability, these measures were calculated for the following data subsets: land use (30 cropland or 30 grassland, samples taken in 2015, n = 30); LSU (6 samples for each of the five cropland LSU and five grassland LSU, samples taken in 2015, n = 5), and site (for 4 samples within 400 m^2 areas that were considered homogenous in soil characteristics in the field for each of the 11 cropland sites, calculated separately for each of the four sampling times, samples taken in 2016, n = 11). For seasonal variability, we first calculated a mean value for the 4 samples taken at each site for each sampling time, and then calculated a separate CV for each of the 11 cropland sites sampled in 2016 (n = 11). No relative ranges were calculated for earthworm abundances as division by the minimum value (min(x) = 0) is not possible.

For cropland soils, reference ranges, accounting for both spatial and seasonal variability were calculated, based on the mathematical addition of variances (Fisher, 1919), and on the quantiles from a cumulative distribution function calculated from the standard deviation (and the median value of the whole data set (Schmidt, 2001). MBC, microbial quotient, C_{mic}/N_{mic}, net nitrogen mineralisation, and respiration potential data were log transformed to meet the assumptions of cumulative distribution functions. Variances were calculated for the data collected from croplands in 2015 (n = 30 sites, spatial variability) and for each of the 11 sites for the data sampled in 2016 (n = 4 sampling times, seasonal variability). Total variance was then calculated as the sum of the spatial variance and the highest seasonal variance (of one of the 11 cropland sites). We assumed that the highest seasonal variability found for the 11 sites could be used as a proxy for seasonal variability in Wallonia. Variances can be added if they are independent (Steyer, 2003). Here we assume that seasonal variance would be identical across Wallonia, and thus independent from spatial variance. The overall standard deviation was calculated for each indicator as the square root of the calculated variance. Finally, cumulative distribution functions were computed using the median value of the whole data set (n = 106) and the calculated overall standard deviation. Subsequently, the 5th, 25th, 50th, 75th, and 95th quantiles were calculated for each biological indicator and re-transformed to their original unit. The range between the 5th and the 95th quantiles was assumed to include 90% of the values expected across Wallonia at any time during the vegetation period (April to October). To illustrate the relative importance of spatial and seasonal variability, cumulative distribution functions for each biological indicator were also calculated with spatial variances only (median value of the whole dataset and the standard deviations of the 30 cropland sites sampled in 2015).

For grassland soils, seasonal variability was not assessed, and the reference system considered only spatial variability. The calculation required log transformation of MBC, microbial quotient, C_{mic}/N_{mic} ratio, respiration potential, and earthworm abundance and reciprocal transformation for metabolic potential and metabolic quotient. Cumulative distribution

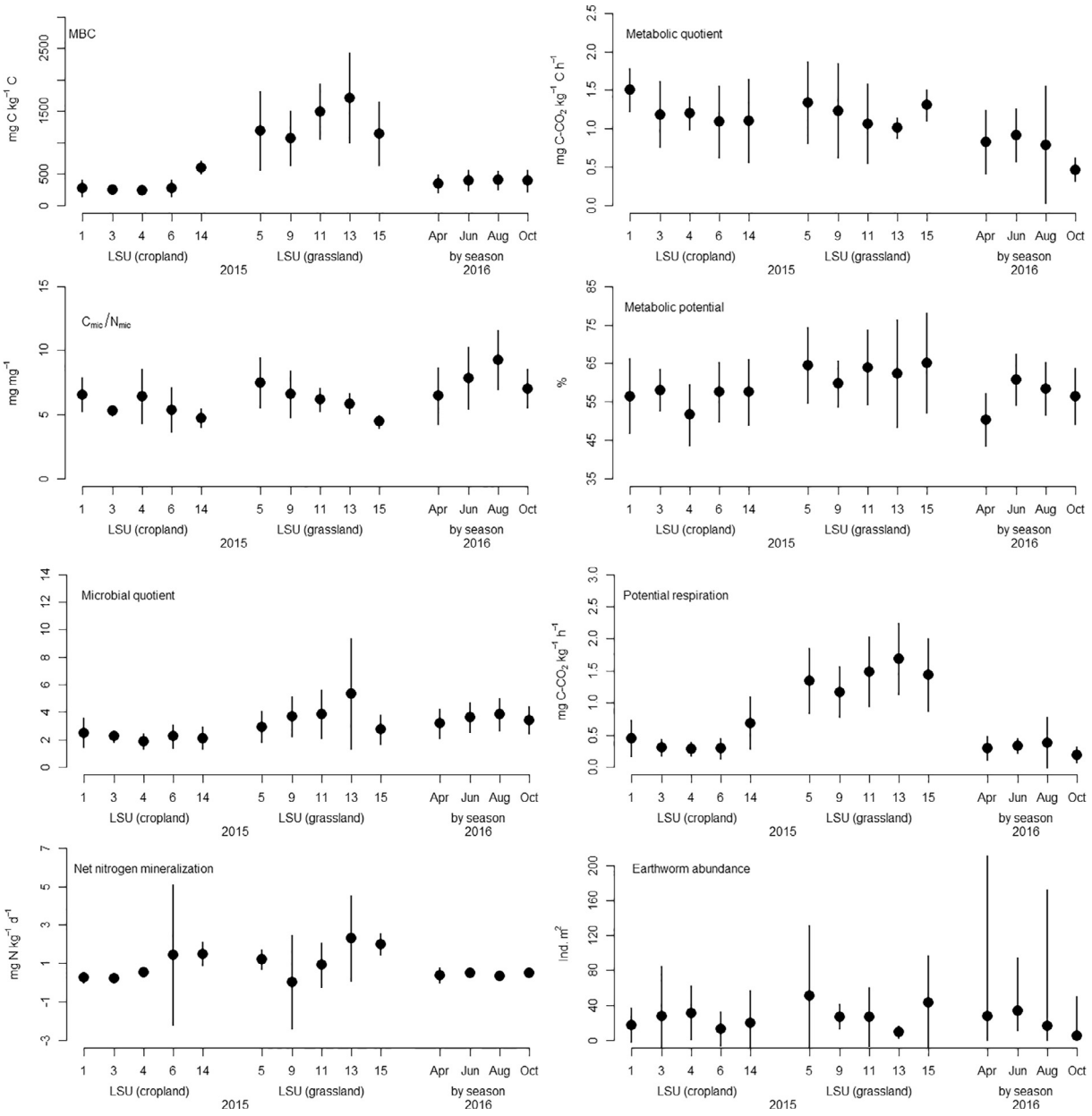

Fig. 2. Mean values for eight biological indicators by LSU (land use unit, see Table 1; samples taken in 2015) or sampling month (cropland samples taken in 2016). Error bars give the standard deviation (for cropland samples taken in 2016, here the standard deviation is calculated from the mean values of the 11 sites). MBC: microbial biomass carbon; C_{mic}/N_{mic}: microbial biomass carbon/microbial biomass nitrogen.

functions were computed using the standard deviations and median values of the 2015 data set (n = 30).

Data were displayed in graphic form as radar plots using the R package 'fmsb' (Nakazawa and Nakazawa, 2015), using the 5th to 95th quantiles of the calculated cropland reference ranges (including spatial and seasonal variance) for each biological indicator as inner and outer edge of the polygon. All four radar plots used the same unitless axis. For the croplands, one radar plot represented calculated reference ranges for croplands, showing the median value in black and the area between the 25th and the 75th quantile in dark grey. Another radar plot displayed the reference ranges for croplands calculated with spatial variability only. Here the values between the 5th and 95th quantile are shown in light grey, the values between the 25th and 75th quantile are shown in dark grey, and the median in black. A third radar plot displayed the measured distribution for croplands soils (data of both samplings, n = 106). The light grey area corresponds to the ranges and the dark grey area to quartile ranges. Finally, one radar plot displayed the calculated reference ranges for grasslands with a colour code corresponding to the radar plot for croplands without seasonal variability. All analyses were performed using R 3.4.1.

3. Results

Soil organic carbon contents ranged between 0.9 and 5.4%, total nitrogen ranged from 0.09 to 0.52%, and pH_{KCl} values were between 3.7 and 7.4 (Table 1) in the ten landscape units (LSU), including croplands and grasslands, sampled in Wallonia in 2015. Mean values of eight biological indicators by LSU and their standard deviations are shown in Fig. 2. Among the individual parameters defining the LSU, only land use improved the model fit compared to the null model, for some biological indicators (Table 2). Also, soil organic carbon, soil nitrogen, pH or C/N ratio, did not improve the model fit. Land use was in

Table 2
Comparison of models to explain variability in eight biological indicators where AICc is Aikake's information criterion and ΔAICc the difference in AICc from the best model. The model with the lowest AICc (e.g. the best model) is shown in bold.

	Model	AICc	ΔAICc
MBC	0	951,11	47,77
	1	903,34	0
	2	1029,61	126,27
	3	917,54	14,20
	4	911,22	7,88
	5	974,95	71,61
	6	981,34	77,99
	7	992,66	89,32
C_{mic}/N_{mic}	0	226,41	0
	1	230,67	4,26
	2	349,24	122,83
	3	244,15	17,74
	4	244,67	18,26
	5	298,83	72,43
	6	303,23	76,83
	7	296,72	70,32
Respiration potential	0	122,82	51,99
	1	70,84	0
	2	201,83	130,99
	3	86,19	15,36
	4	87,08	16,24
	5	145,58	74,74
	6	153,17	82,34
	7	170,16	99,33
Earthworm abundance	0	616,32	0
	1	620,86	4,54
	2	757,27	140,95
	3	637,32	21
	4	638,92	22,6
	5	705,78	89,47
	6	700,5	84,18
	7	697,68	81,36
Net nitrogen mineralisation	0	235,87	0
	1	239,97	4,1
	2	369,87	133,99
	3	256,38	20,51
	4	256,15	20,28
	5	324,42	88,54
	6	322,98	87,11
	7	322,66	86,78
Metabolic quotient	0	67,99	0
	1	73,36	5,37
	2	207,63	139,64
	3	88,25	20,26
	4	81,53	13,54
	5	151,22	83,23
	6	151,19	83,19
	7	148,91	80,92
Metabolic potential	0	445,85	2,82
	1	443,03	0
	2	581,02	137,99
	3	460,88	17,86
	4	458,37	15,35
	5	525,61	82,58
	6	527,14	84,12
	7	529,03	86,01
Microbial quotient	0	245,35	6,19
	1	239,16	0
	2	369,81	130,65
	3	247,46	8,3
	4	253,9	14,74
	5	306,93	67,77
	6	313,14	73,98
	7	325,08	85,93

Table 3
Permutation based linear models on variables related to spatial variability for biological indicators (samples taken in 2015, n = 60) for which Model 1 ($biologicalindicator = \beta_0 + \beta_1 landuse + \varepsilon$) was most parsimonious.

Biological indicator	Variables	Estimate	p-value
MBC	F(1,58) = 82.76, adj. R^2 = 0.58		
	land use	−495.9	< 0.0001
Respiration potential	F(1,58) = 93.03, adj. R^2 = 0.61		
	land use	−0.51	< 0.0001
Metabolic potential	F(1,58) = 8.56, adj. R^2 = 0.11		
	land use	−3.44	< 0.01
Microbial quotient	F(1,58) = 12.4, adj. R^2 = 0.16		
	land use	−0.76	< 0.0001

the most parsimonious models for four biological indicators (MBC, respiration potential, metabolic potential, and microbial quotient). Values of these four biological indicators were significantly higher under grasslands than under croplands (Table 3, Fig. 2). For the other four biological indicators (earthworm abundance, metabolic quotient, net nitrogen mineralisation, C_{mic}/N_{mic}), the null model had the lowest AIC_c, suggesting that the parameters used in this study could not explain the spatial variability of these indicators (Table 2).

There was a significant seasonal effect on all biological indicators, except earthworm abundance, under croplands, for the sites sampled in 2016 (Table 4). Depending on the biological indicator, values were lowest in either April (metabolic potential, C_{mic}/N_{mic}) or October (respiration potential, metabolic quotient), and peaked in June (metabolic potential, metabolic quotient) or August (C_{mic}/N_{mic}, microbial quotient, respiration potential) (Fig. 2). Although significant, seasonal differences in mean values across the 11 croplands were low for microbial biomass carbon and net nitrogen mineralisation (Fig. 2).

To compare seasonal and spatial variability at different scales, CVs, relative ranges, and max/min ratios were calculated (Table 5). Variability within grasslands and croplands (CV grasslands and CV croplands) was generally higher than variability among LSU within each land use type. For croplands, the difference was most important for net nitrogen mineralisation and least important for the microbial quotient. Seasonal variability (CV_{season}) was generally in the same order of magnitude as the variability at the scale of sites (CV_{site}). Earthworm abundance and respiration potential had the highest CV_{season}. Seasonal differences between the highest and lowest measured values of biological indicators at the same site corresponded to 4% (net nitrogen mineralisation) to 73% (C_{mic}/N_{mic}) of the ranges found for the 30 croplands sampled in 2015, showing substantial differences in the importance of seasonal variability compared to spatial variability between biological indicators. Net nitrogen mineralisation had the highest mean factor (19.6) between the lowest and highest measurement of the four campaigns of 2016. For the other biological indicators, this factor was much lower (1.26 (metabolic potential) to 3.28 (metabolic

Table 4
Seasonal differences (ANOVA) for eight biological indicators (samples taken in 2016).

Biological indictaor	df	F-value	p-value
MBC	2.87	2.67	< 0.05
C_{mic}/N_{mic}	2.81	14.0	< 0.0001
Respiration potential	1.31	7.16	< 0.01
Earthworm abundance	1.63	3.11	0.06
Net nitrogen mineralisation	2.73	3.91	0.01
Metabolic quotient	1.86	8.98	< 0.001
Metabolic potential	2.79	23.1	< 0.0001
Microbial quotient	2.78	3.96	< 0.01

Table 5
Coefficient of variation (CV), ranges relative to the range of croplands [%], and max/min ratios, for grasslands (n = 30, 2015), croplands (n = 30, 2015), landscape units (LSU, within grasslands (n = 5) and croplands (n = 5) separately, 2015), and by site (n = 11) and season (n = 4) (samples taken in 2016). MBC: microbial biomass carbon; Cmic/Nmic: microbial biomass carbon/microbial biomass nitrogen.

	CV			Relative Range [%]			Max/Min		
	Min	Mean	Max	Min	Mean	Max	Min	Mean	Max
MBC									
Grasslands		43			438			10.1	
Croplands		51			100			5.48	
LSU (within grasslands)	29	42	53	184	232	309	2.29	3.55	6.21
LSU (within croplands)	18	33	48	21	41	58	1.64	2.34	3.22
Site (croplands)	10	27	66	11	25	37	1.32	1.63	1.84
Season (croplands)	19	35	52	9	32	79	1.25	2.31	10.4
C_{mic}/N_{mic}									
Grasslands		26			82			2.54	
Croplands		26			100			2.94	
LSU (within grasslands)	11	19	28	20	44	67	1.38	1.64	2.00
LSU (within croplands)	8	21	33	16	49	90	1.24	1.83	2.48
Site (croplands)	9	28	66	22	43	63	1.25	1.55	1.69
Season (croplands)	11	20	32	22	73	175	1.25	2.40	10.8
Respiration potential									
Grasslands		36			153			3.90	
Croplands		68			100			11.0	
LSU (within grasslands)	33	36	39	94	114	133	2.21	2.64	3.23
LSU (within croplands)	37	51	63	27	47	89	3.23	4.06	5.30
Site (croplands)	6	26	76	2	6	16	1.36	1.93	3.72
Season (croplands)	27	50	100	2	10	26	1.12	2.31	12.8
Earhworm abundance									
Grasslands		144			146				
Croplands		155			100				
LSU (within grasslands)	53	106	158	12	68	146			
LSU (within croplands)	98	151	206	27	57	100			
Site (croplands)									
Season (croplands)	38	89	160	1	54	119			
Net nitrogen mineralisation									
Grasslands		132			112			13.8	
Croplands		210			100			85.4	
LSU (within grasslands)	28	2383	11,620	12	37	63	2.74	6.38	13.8
LSU (within croplands)	25	109	255	3	27	100	1.98	10.7	37.7
Site (croplands)	3	58	247	1	3	7	1.49	7.74	35.4
Season (croplands)	20	49	90	0	4	9	1.08	19.6	309
Metabolic quotient									
Grasslands		36			98			3.94	
Croplands		33			100			4.78	
LSU (within grasslands)	13	33	50	26	65	94	1.52	2.52	3.81
LSU (within croplands)	18	33	49	28	65	98	1.45	2.69	4.74
Site (croplands)	10	43	130	5	50	79	1.26	1.95	2.81
Season (croplands)	16	33	50	5	10	26	1.26	3.28	10.6
Metabolic potential									
Grasslands		16			110			1.79	
Croplands		14			100			1.83	
LSU (within grasslands)	10	17	22	50	80	110	1.29	1.51	1.79
LSU (within croplands)	9	14	17	50	70	90	1.31	1.50	1.69
Site (croplands)	0	10	23	0	39	50	1	1.26	1.39
Season (croplands)	5	11	17	0	39	80	1	1.26	1.73
Microbial quotient									
Grasslands		60			380			11.6	
Croplands		34			100			3.75	
LSU (within grasslands)	39	48	75	89	151	325	2.79	3.33	4.62
LSU (within croplands)	18	33	44	35	58	86	1.75	2.37	2.69
Site (croplands)	9	27	65	29	50	79	1.33	1.66	1.97
Season (croplands)	9	21	33	19	63	153	1.19	2.33	10.1

quotient)). The comparison of reference ranges for croplands accounting for spatial and seasonal variability (Fig. 3a) and spatial variability only (Fig. 3b) showed that the ranges for the latter were narrower. The differences were small for net nitrogen mineralisation (5th quantile: −1.06 and −0.96 mg N kg^{-1} d^{-1}; 95th quantile: 3.00 and 2.72 mg N kg^{-1} d^{-1} for the ranges with and without seasonal variability respectively) and most important for C_{mic}/N_{mic} (2.4–21.3 vs. 3.7–7.8 for the 5th and 95th quantile) and respiration potential (0.06–0.81 vs. 0.12–4.01 mg C $kg^{-1}h^{-1}$ for the 5th and 95th quantile).

The reference system was defined to exclude 10% of the values predicted from the cumulative distribution function including spatial and seasonal variability (Supplementary Material A). For the samples under croplands taken in 2015 and 2016, 2.6% were outside the reference range (5th to 95th quantile) (Fig. 3c). All measured values were

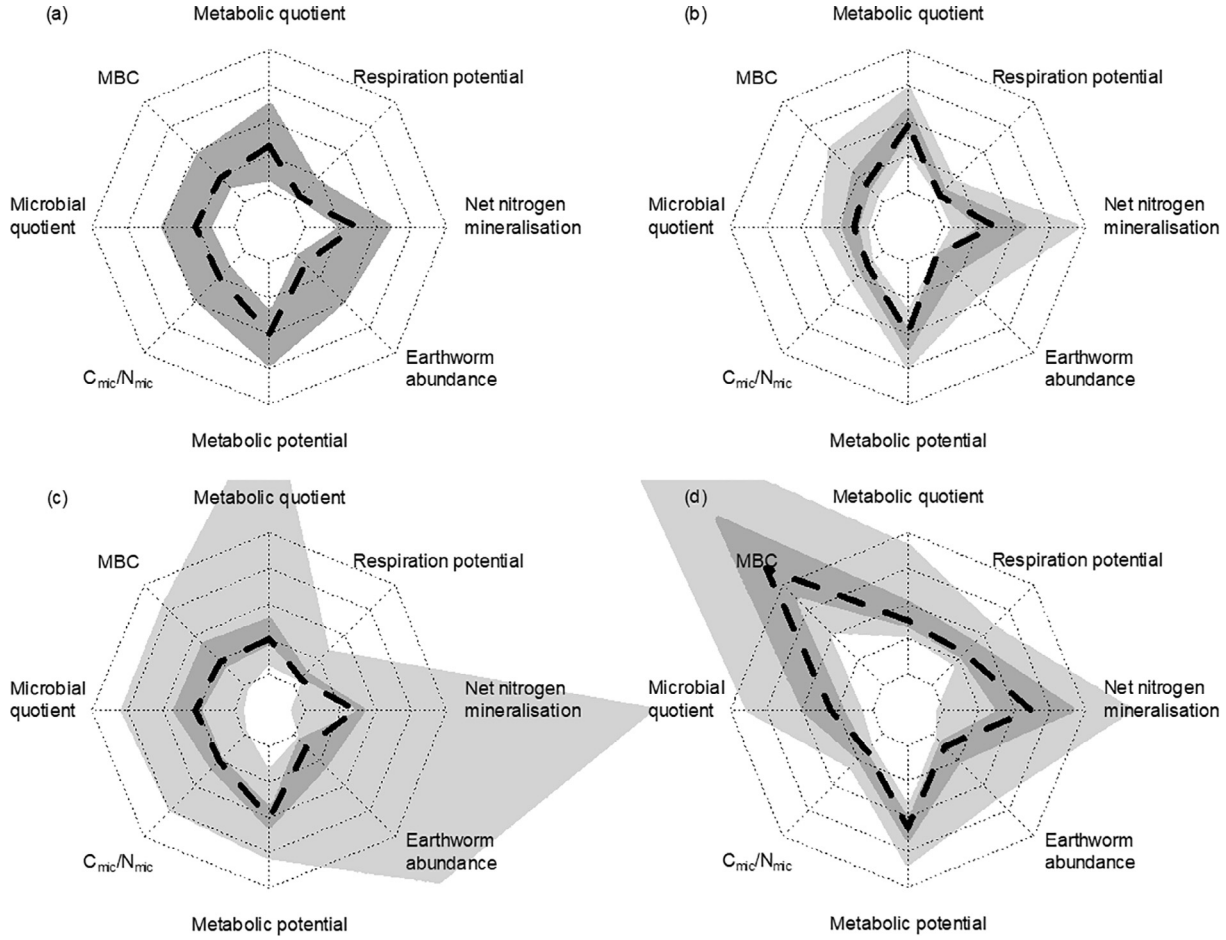

Fig. 3. Radar plots illustrating the calculated reference range values. The axes represent the range between the 5th quantile (inner edge of the plot) to the 95th quantile (outer edge of the plot) of the calculated distribution for croplands including spatial and seasonal variability for each biological indicator. The black line gives the median and the dark grey area shows the quartile ranges (25%–75%) of the data displayed. (a) calculated distribution of values for croplands including spatial and seasonal variability, (b) calculated distribution of values for croplands with spatial variability only (light grey area: 5th -95th quantile), (c) distribution of measured values under croplands (samples taken in 2015 and 2016; light grey area: minimum -maximum), and (d) calculated distribution of values for grasslands with spatial variability (light grey area: 5th -95th quantile). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

within the reference ranges for two biological indicators (respiration potential and metabolic quotient) (Fig. 3c). The defined ranges corresponded to the measured range in one case (earthworm abundance, lower value, 0 ind. m^2). For six biological indicators, some measurements were outside the reference system. Values below the reference ranges were measured for net nitrogen mineralisation, MBC, microbial quotient, and C_{mic}/N_{mic}. Net nitrogen mineralisation, earthworm abundance, and metabolic quotient had values above the reference ranges. A radar plot was further used to visualize the differences between the reference systems defined for croplands and for grassland (with spatial variability only). The radar plot emphasized the differences between the land uses in MBC (5th to 95th quantile for grasslands: 578–2724 mg C kg^{-1} (with spatial variability only)) (Fig. 3d).

4. Discussion

4.1. Spatial variability and classification variables

Land-use, soil type (texture, rock fragment content, drainage), and agricultural region (a proxy for climatic conditions) form the basis for the stratification of Wallonia into landscape units (LSU). They have previously been used in the sampling strategy of the CARBOSOL network. Here, the sampling approach according to LSU allows a clear definition of the areas to which the reference system applies (10 LSU, e.g. about 50% of agricultural area of Wallonia) and guarantees that the sampling sites are equally distributed across the different soil types and climatic conditions in Wallonia (Chartin et al., 2017). Among the parameters defining the LSU, land use was the only explanatory variable retained in the models. Half the tested biological indicators showed significant differences between grasslands and croplands, highlighting the importance of management for biological indicators, probably related to the lower level of human disturbance under grasslands (Fan et al., 2018). The results also support the distinction between land use types within the definition of soil quality (Sparling, 1997). While Wallonia shows a northwest to southeast climatic gradient and a shift in soil types from Haplic Luvisol to Dystric Cambisol (Chartin et al., 2017), no further explaining factors were identified. At other scales and for other regions, additional factors might be relevant for the definition of ranges. For example, climate, soil texture, and soil drainage, have been shown to influence soil functioning in the context of SMNs of neighbouring countries (de Vries et al., 2012; Dequiedt et al., 2011; Schulte et al., 2015), covering a larger scale and ranges in environmental conditions. Cross-country comparisons of value ranges for biological indicators are difficult due to differences in reporting units and methodologies. For instance, the ranges for nitrogen mineralisation under croplands from the French Bioindicators programm (0.96 to 31.17 mg N kg^{-1}; https://ecobiosoil.univ-rennes1.fr/ADEME-Bioindicateur/bdu.php) are given in a unit that is not convertible into ours (mg N kg^{-1} d^{-1}), and microbial biomass is estimated from DNA extractions. Compared to references established for the Netherlands

(Rutgers et al., 2008), the average nitrogen mineralisation rate measured in our study are slightly higher than ranges given for arable lands on clay and slightly lower than ranges given for arable lands on sand.

Soil fauna is generally considered to be very sensitive to management practices. The impact of land use on earthworm abundance has been shown for various European countries (Postma-Blaauw et al., 2010; Smith et al., 2008). Nonetheless, earthworm abundance did not show significant differences between grasslands and croplands in this study. We attribute this to the dry weather conditions in the weeks preceding the 2015 sampling. Measured earthworm abundances are not only impacted by the decline of earthworm populations following unfavourable conditions (Curry, 2004), but also by methodical constraints. Hot mustard extraction relies on the infiltration of a relatively huge amount of aqueous solution (about 40 l per m^2) in a relatively short time (< 30 min) (Gunn, 1992). During both dry and wet periods, common under Atlantic climate conditions, this prerequisite might not be fulfilled by many agricultural soils, resulting in low measures in earthworm abundances at the regional scale (Bouché and Aliaga, 1986). Moreover, chemical extraction works best for anecic worms, but it is less reliable for endogeic species, which represent the largest part of the earthworm community under croplands (Bartlett et al., 2006; Pérès et al., 2011). For a thorough analysis of earthworms, hand-sorting should thus complete the chemical extraction methods. However, hand-sorting is not only time consuming but also destructive, and possibilities of routine implementation are restricted. While earthworms present one of the biological indicators whose importance for soil quality is most demonstrative for farmers, these restrictions limit their suitability for quantitative assessment of soil quality. Subsequently, a complete absence of earthworms falls within the “mean range” of our reference system (see below). Several authors also showed that micro- (nematodes), meso- (mites and collembola) and macrofauna (beetles, spiders, centipedes etc.) communities present further biological indicators of interest, based on taxonomical and functional community structure (Postma-Blaauw et al., 2010; Velasquez et al., 2007). They were not considered here, due to time constraints and the lack of expertise in the laboratories performing the routine analyses. Currently, functional indicators (e.g. traits-based approaches; (Pey et al., 2014) and integrated approaches of contrasted biological indicators still present too many major challenges to be routinely implemented (Postma-Blaauw et al., 2012; Vincent et al., 2018). The same applies to a number of novel microbial indicators like enzyme activities (Paz-Ferreiro and Fu, 2016) or taxonomical and functional microbial community structure (Mbuthia et al., 2015).

4.2. Seasonal variability

All biological indicators but earthworm abundance showed significant differences between four sampling moments throughout one year. Seasonal differences in biological indicators are a result of fluctuations in substrate availability (Bossio and Scow, 1995; Nunan et al., 2000; Waldrop and Firestone, 2006), plant growth (Franzluebbers et al., 1994), weather conditions (Curry, 2004), and management (tillage, application of fertilizers etc.). These factors do not influence all biological indicators equally, as shown by the individual patterns and the differences in magnitude of seasonal variability of each biological indicator. For some biological indicators such as net nitrogen mineralisation and MBC, seasonal variability was low and thus only contributed slightly to the total width of the ranges (see below). A general trend towards higher values during the summer months, when substrate availability is highest, is nonetheless noticeable for most biological indicators. Despite the occurrence of a major disturbance to the ecosystem between the June and August samplings (harvest), differences between these two sampling periods were smallest for most biological indicators.

Options to standardize sampling dates according to climate conditions are limited. Sampling schemes of most SMNs clearly define when soil samples should be collected - usually either in spring or in autumn (Stenberg, 1999) – yet campaigns stretch over several months (Cluzeau et al., 2012; Rutgers et al., 2009), as treatment and analysis of biological indicators are time consuming and sensitive to storage, limiting the number of samples that can be collected within any time frame. Given these limits, it is essential to account for seasonal variability when defining a reference system used to assess independently collected samples. However this approach also has its limitations, as it might not be satisfactory for biological indicators particularly vulnerable to temporal trends. MBC and the microbial quotient had the slightest seasonal variability. These biological indicators might thus present a good compromise between very sensitive indicators and the chemical indicators with a dynamic that can only be measured at the scale of decades (Goidts and van Wesemael, 2007).

4.3. Definition of a reference system

We applied the principle of addition of variances to create a reference system accounting for both spatial and seasonal variability. This approach enabled us to compute ranges, despite the relative low amount of data on biological indicators available in Wallonia, compared to the demands of many mathematical methods reported in the literature (Pereira e Silva et al., 2013). We showed that the inclusion of seasonal variability resulted in ranges up to 5.7 times (respiration potential) wider, compared to spatial variability only. The reference system considered the highest measured seasonal variability among 11 croplands in the loamy region in its calculation; on average, seasonal variability at most sites was smaller than spatial variability at the scale of Wallonia. Relative to spatial variability, seasonal variability was highest for C_{mic}/N_{mic}, which, as it relates to stoichiometry, varies within narrow natural limits (Cleveland and Liptzin, 2007). Consideration of seasonal variability had only a slight effect on the range for net nitrogen mineralisation, despite a significant peak in measurements in the June. We estimated seasonal variability for croplands from the main agricultural region in Wallonia (loamy region), situated at lower altitude (50–200 m, for our sites), while some croplands included in the study of spatial variability were situated in the Ardennes up to an altitude of 440 m. Thus the application of the reference system to croplands situated at higher altitudes might require the inclusion of data on seasonal variability within this area. We did not consider seasonal variability under grasslands. As the majority of factors causing fluctuations in biological indicators in cropland soils also affect grassland soils, a reference system that includes seasonal variability for grasslands is likely to have wider ranges than the ones presented here.

The graphic display of an interval derived from cumulative distribution helps to focus on the ranges most interesting for the assessment. Similar plots with axes ranging from minimum to maximum values of measurements (Krüger et al., 2017) could result in extreme stretching of the axes due to single outliers, reducing the visibility of less extreme differences between sites. Obviously, a continuous refinement of the ranges, if further representative data become available, can help to expand the spatial range and to account for long-term trends. To facilitate the visual assessment of soil quality, we chose to display quantile ranges. Any measurement can be placed into one of five groups (< 5th quantile: very low, 5th quantile to 25th quantile: low, 25th to 75th quantile: mean, 75th to 95th quantile: high, and > 95th quantile: very high). This offers important new reference points for the assessment of soil quality through biological indicators. The inclusion of 90% of the values expected across the Wallonia in the graphics means that, theoretically, assuming the eight considered biological indicators are independent variables, any site has an overall likelihood of 57% that at least one biological indicator measurements cannot be displayed on the axes. Although each biological indicator measures a distinct aspect of biological soil quality, existing links between biological indicators should result in a larger number of sites for which all measurements can be displayed. Furthermore, it should be kept in mind that a ‘very high’ or ‘very low’ value is not necessarily a reason for alarm. Depending on the biological indicator considered and the context, high values, for example, can be interpreted as a sign of good or bad soil quality. Furthermore, comparing data from croplands to those from grasslands may help with the interpretation of data, as grasslands represent a less management intense land use form.

5. Conclusions

This study created a basis for a reference system of biological indicators of soil quality for croplands in Wallonia, which will be useful for farmers and decision makers. The reference system takes into account spatial and seasonal variability, both contributing to the overall variability of biological indicators. Spatial variability was generally larger than seasonal variability, but the addition of seasonal variability widened reference ranges up to 1.1 to 5.7 times. The reference system can thus be applied to any cropland in Wallonia, independent of season and location. Differences between grasslands and croplands were confirmed and called for separate reference systems, but a further subdivision by soil texture or organic carbon content, for example, was not required. The variability of soil biological indicators was not well predicted by field-based site classification variables and soil chemical parameters, underlining the need for the inclusion of biological indicators in routine soil testing for farm management. Practical implementation will require further developments of communication and information tools. The definition of such a reference system is an important step forward towards the routine use of biological indicators for the assessment of soil quality in Wallonia. Similar approaches could be applied in other regions and for other indicators for which little data are available.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ecolind.2018.08.010.

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