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A rapid flow cytometry method to assess bacterial abundance in agricultural soil

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ABSTRACT

Soil microorganisms play key roles in ecosystem functioning. Finding tools to accurately measure, quantify and understand this component of soil is crucial to establish relevant indicators of the state of soil. This study proposes an optimized methodology using flow cytometry (FCM) for the quantification of bacteria in soil. This rapid and reproducible methodology was validated in two steps: (a) the recovery of spiked cells from sterilized soil matrix backgrounds and (b) the determination of the total number of bacteria from silty native soils (grassland and crop field). The ratio of counted SYBR Green-stained cells *versus* expected values amounted to 0.83 on average by FCM, compared to 0.64 by plate counts. We used this FCM method to compare bacterial abundance measured in four types of crop management systems: organic, integrated with (TRC) or without tillage (RTRC) and conventional. The FCM count ranged from 3.91×10^8 to 5.69×10^8 cells g^{-1} soil for crop field and was 6.69×10^8 cells g^{-1} soil for grassland. Similarly, two other frequently used descriptors of the bacterial community (quantification of the 16S rRNA gene by real-time PCR and total culturable heterotrophic bacteria by plate count analysis) were analyzed for all soils and compared with the FCM data. FCM counts were relevant enough to report significant differences between the four crop management systems. Higher bacterial counts were observed in conventional and integrated RTRC systems in comparison with organic and integrated TRC. The same tendency was observed when considering 16S rRNA gene abundance. As expected, a slight but significant correlation was observed between FCM counts and 16S rRNA gene quantification when considering all soil samples. No difference between crop management systems was noticed if we considered only colony forming unit (CFU) count. A calculated ratio between culturable and total bacterial counts (CFU/FCM) suggests some differences in the culturable proportion according to soil management.

In the context of evaluating the state of soil, our FCM method offers a quick and simple assessment of total bacterial abundance in numerous samples. Combined with other biological indicators, FCM contributes to understanding soil bacterial biomass and could represent a useful complement in a larger panel of indicators to evaluate and detect changes in the structure of the soil microbial community.

1. Introduction

The demand for rapid and reproducible indicators to study and evaluate soil has increased over the last decade. As the state of soil cannot be measured directly, it must be inferred by well-chosen physical, biochemical or biological indicators reflecting major ecological processes (Garbisu et al., 2011; Pankhurst et al., 1997; Schloter et al., 2003). In soil, the bacterial community is a complex and variable assemblage of populations with high taxonomic diversity and metabolic potential (Acosta-Martínez et al., 2008; Fierer and Jackson, 2006; Roesch et al., 2007; Torsvik and Øvreås, 2002). Among quantifiable microbial variables, bacterial abundance in soil has been conventionally determined using selective or non-selective heterotrophic plate counts. However, only a small unrepresentative fraction – less than 1–0.1% of the total soil microbial community – can be recovered by these classic methods (Amann et al., 1995; Hill et al., 2000). Other methods implicate direct counts of extracted bacteria by microscopy (Bittman et al., 2005; Mulder et al., 2005) or global biomass measures (Jenkinson et al., 2004; Wardle et al., 1999). However, these quantitative analyses are laborious and require incubation times. To overcome these inherent limitations, microbial ecology is increasingly turning to direct molecular approaches. Qualitative molecular surveys, such as DNA- or RNA-based fingerprint techniques, have

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provided insight into the structure and composition of whole soil bacterial communities (Hill et al., 2000). The abundance of a bacterial community can be determined by real-time PCR targeting 16S rRNA gene (Shen et al., 2010; Wessén et al., 2010). However, all of these molecular techniques are technically demanding, expensive, time-consuming and sometimes difficult to interpret (Gangneux et al., 2011; Kirk et al., 2004).

Flow cytometry (FCM) offers the advantage of simultaneously analyzing in a high-throughput way multiple parameters at a single-cell level (Czechowska et al., 2008; Nebe-von-Caron et al., 2000). This technique provides a direct and rapid determination of cell numbers, size related scatter signal and fluorescence (Davey and Kell, 1996; Mueller and Nebe-von-Caron, 2010). FCM may be a useful and complementary approach to culture-based and molecular methods for the study of the soil structure community. In recent years, FCM has become a valuable tool for the detection and quantification of bacteria in complex environments, such as natural water, sediments and sludge (Amalfitano and Fazi, 2008; Bombach et al., 2011; Foladori et al., 2010; Lavergne et al., 2014). Some studies also reported the use of FCM in soil to quantify native soil bacteria or monitor inoculated isolates (Elväng et al., 2001; Maraha et al., 2004; Unge et al., 1999; Whiteley et al., 2003). FCM assays are based on the suspension of single cells, whereas in soil matrices bacteria are closely associated with physical components. Specific techniques for cell extraction and purification from this complex matrix are often applied, such as Nycodenz® density centrifugation (Gougoulias and Shaw, 2012; Whiteley et al., 2003). However, these methods are known to present significant biases. Holmsgaard et al. (2011) noted a drastic reduction in bacterial diversity in soil samples after Nycodenz® density centrifugation. Moreover, the recovered bacterial populations were not representative, as Nycodenz extractions appeared to be biased toward certain bacterial phyla. The efficiency of this method also largely depends on the physical and chemical characteristics of soil (Maron et al., 2006). Nevertheless, this technique is very time-consuming and not suitable for routine analyses that requires the handling of numerous samples.

Therefore, the aim of this study was to modify a technique of extraction using a rapid, simple and economic protocol based on serial dilutions, avoiding density gradient centrifugation and purification stages for total soil bacteria counting. This technique was tested and applied to a range of agricultural soils (crop field and grassland), collected in different farming systems (conventional, integrated and organic crop management systems). FCM and other selected biological descriptors (viable plate counts for culturable heterotrophic bacteria, 16S rRNA gene abundance for total bacterial community) were then used to examine and compare bacterial biomass in these different farming systems.

2. Materials and methods

2.1. Bacterial strains and growth conditions

The following strains were used to calibrate FCM cytograms: *Pseudomonas fluorescens* (SBW25), *Staphylococcus aureus* (ATCC6538), *Micrococcus luteus* (ATCC4698), *Bacillus megaterium* (ATCC14581), *Bacillus polymyxa* (ATCC7070), and *Enterococcus faecium* (ATCC6569). All organisms were grown in rich Luria–Bertani (LB) medium. Aliquots were taken at the end of log phase. Cells were collected by centrifugation for 5 min at 2600 × *g*. Pellets were re-suspended in NaCl 0.85% (filtered 0.22 μm).

2.2. Soil background spiking

Before spiking, a representative farm soil was gamma-sterilized with an amount of radioactivity between 25 and 50 kGy (Ionisos, Dagneux, France) to avoid the influence of indigenous microbes. This soil was sampled in a crop field in Yvetot, in northwestern France (Normandy). This region is characterized by an oceanic and temperate climate with temperatures between 5 and 17 °C, with a mean rainfall of 800–900 mm yr^{-1} and narrow seasonal ranges. The soil, representative of the Paris Basin, is classified as a silty soil containing 15 % clay, 65 % silt, and 20 % sand. Physical and chemical characteristics are summarized in Table 1. To assess the influence of soil particles on bacterial count, 0.5 g of this soil was spiked in a NaCl (0.85%) suspension of *P. fluorescens* (SBW25) to a range from 5×10^8 to 6×10^{10} cells ml^{-1} to a final volume of 5 ml, in same ratio than the procedure of bacteria extraction from soil but scaled to a ten-fold reduction to facilitate manipulation. The ratio of the counted *versus* the expected cell numbers in the presence of the soil matrix was tested under a possible real environmental abundance of bacteria. The bacterial count was then estimated by FCM analyses, as described below.

2.3. Collection of agricultural soil samples

Four contrasted farming systems were studied: conventional (Conv) with statutory high chemical inputs, integrated with tillage and reduced chemical inputs (Int TRC), integrated with reduced tillage and reduced chemical inputs (Int RTRC), and organic (Org) with tillage but without chemical inputs. We identified four natural farms of interest in the Normandy region of France that presented comparable silty soils. Locality and GPS coordinates of the chosen fields are presented in Table 2. The two chosen farms with integrated systems belong to the French DEPHY network, pooling voluntary farms and experimental sites under the aegis of Ecophyto 2018 plan, to test and demonstrate the suitability of reducing chemicals use in agriculture. Soil sampling was carried out in October 2012 for each crop management system in three similar wheat fields belonging to the same farmer. Similarly, soils

Table 1
Basic physico-chemical characteristics of studied soils.

	C_{org} (mg g^{-1} dry soil)	N_{tot} (mg g^{-1} dry soil)	C/N	pH	CEC ($cmol^+$ kg^{-1} dry soil)	Clay content (%)
Yvetot soil	31.00	2.70	8.92	6.90	8.92	16.3
Grassland	27.82	2.40	13.31	6.03	13.76	18.2
Crop field						
Conv	10.40	1.04	10.16	7.17	11.66	18.7
Int RTRC	10.25	1.01	10.34	7.93	11.51	18.1
Int TRC	10.07	0.95	11.01	7.70	10.27	19.9
Org	9.56	0.90	10.94	7.00	9.89	20.1

Table 2
Localization and management of sampled fields.

Management system	Field	Locality	Geographic coordinates
Conventional	1	Tourny	N 49° 10.454′ E 1° 32.151′
	2	Tourny	N 49° 10.351′ E 1° 32.284′
	3	Tourny	N 49° 10.924′ E 1° 33.162′
Integrated RTRC	1	Guitry	N 49° 12.460′ E 1° 32.333′
	2	Guitry	N 49° 12.355′ E 1° 32.435′
	3	Guitry	N 49° 12.207′ E 1° 32.484′
Integrated TRC	1	Richeville	N 49° 15.742′ E 1° 32.357′
	2	Richeville	N 49° 15.810′ E 1° 32.475′
	3	Richeville	N 49° 15.310′ E 1° 33.080′
Organic	1	Combon	N 49° 06.491′ E 0° 51.185′
	2	Sacquenville	N 49° 04.467′ E 1° 04.639′
	3	Sacquenville	N 49° 04.469′ E 1° 04.654′