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A fluorescence-based assay for measuring the viable cell concentration of mixed microbial communities in soil

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ABSTRACT

Microbial cell concentration is a particularly important bioindicator of soil health and a yardstick for determining biological quotients which are likely to gain in ecological significance if they are calculated in relation to the viable, rather than total, microbial density. A dual-staining technique with fluorescent dyes was used for the spectrofluorimetric quantitative determination of the concentration of viable microbial cells present in three different soil types. This is a novel and substantially modified application of the dual-staining procedure implemented in the LIVE/DEAD™ *Bac*Light® viability kit which has never been successfully applied to the quantification of naturally occurring soil microbial communities. Indigenous microbial cell concentrations were quantified using an internal standard, i.e. spiking environmental samples with suspensions containing different concentrations of live *E. coli* cells, and external calibration, by comparing fluorescence emission by indigenous bacteria and known concentrations of *E. coli* in nutrient saline. Two types of environmental samples were tested: bacterial preparations obtained by density gradient centrifugation and soil suspensions. In both cases, prior dilution of the sample was necessary to minimise fluorescence quenching by soil particulate matter. Spectrofluorimetric measurements of indigenous cell concentration in bacterial preparations were in close agreement with those found using epifluorescence microscopy. Limits of detection of 5×10^6 for the soil bacterial preparations and 8×10^7 for the soil suspensions were estimated. Deviations observed when soil suspensions are dealt with are likely due to the selection of a unique bacterial strain for standardisation and calibration. Thorough testing of a variety of reference bacteria and fungi is suggested to determine a more accurate average fluorescence enhancement per microbial cell or mass unit.

1. Introduction

Determining the bacterial density or biomass in soil is a major concern for soil microbiologists. Culture-independent techniques, i.e. fumigation-based (Jenkinson and Powlson, 1976; Vance et al., 1987) and substrate-induced respiration approaches (Anderson and Domsch, 1975) have provided innovative methods for determining the total amount of microbial carbon. Specific activity coefficients (e.g. metabolic quotient, qCO_2, death rate quotient, etc.) (Anderson, 2003) enhance ecological significance by examining the ratio of physiological performance to viable, rather than total, microbial biomass or density. Vital staining procedures with fluorescent dyes may be used for that purpose. They have been essentially implemented in connection with laborious enumeration using epifluorescence microscopy (Porter and Fraig, 1980; Lopez-Amoros et al., 1997), not directly applicable in routine analysis. MacDonald (1986) presented a spectrofluorimetric technique for measuring fluorescence enhancement by DAPI-stained microbial cells in a suspension of mixed populations of soil microorganisms. He concluded that fluorescence emission did not offer a substantial advantage over conventional light absorption. Since then, dual staining with fluorescent dyes, as revealed by the LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit (Invitrogen), commonly used to determine the proportion of viable cells within a bacterial culture, has opened up new prospects in this field. Over 20 bacterial species have been successfully tested (Molecular Probes, 2004). The method is based on two fluorescent stains: SYTO9, which penetrates all bacterial cells (optimum excitation and emission: 480 and 530 nm), and propidium iodide, PI, (optimum excitation and emission: 490 and 635 nm), which penetrates cells with damaged membranes. In dead cells, PI displaces SYTO9 from DNA due to higher affinity for nucleic acids. When both dyes are used in combination, viable cells fluoresce in green whereas dead or damaged cells fluoresce in red. This selective staining procedure has been successfully applied to the enumeration of viable and total bacteria, using membrane filtration and epifluorescence microscopy in drinking water (Boulos et al., 1999), lake or deep sea sediments (Haglund et al., 2003; Queric et al., 2004), hypersaline environments for extremophilic archeas living in a wide range of pH (Leuko et al., 2004), probiotic preparations (Alakomi et al., 2005) or, using confocal laser scanning microscopy, to determine the

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Table 1
Main physico-chemical properties of the soils

	Soil A	Soil C	Soil E
WHC (g/100 g)	10.3	3.12	18.9
Clay (g/kg)	112	24	178
Loam (2/20 µm) (g/kg)	65	19	74
Loam (20/50 µm) (g/kg)	56	18	48
Sand (50/200 µm) (g/kg)	122	59	86
Sand (200/2000 µm) (g/kg)	645	880	614
OC (g/kg)	12.4	5.95	18.4
Total N (g/kg)	0.934	0.365	1.71
C/N	13.3	16.3	10.7
OM (g/kg)	21.5	10.3	31.8
pH (water)	6.51	6.89	8.13
CEC (cmol+/kg)	7.44	1.74	9.88

spatial distribution of viable and nonviable bacteria in dental plaque (Hope et al., 2002). A similar preparation of SYTO13 and SYTOX Orange applied to activated sludges yielded promising results (Biggerstaff et al., 2006). As recently reported (Chen and Séguin-Swartz, 2002), staining is not specific to bacteria and may be used to evaluate the viability of fungal spores. A similar product (LIVE/DEAD® Funga*Light*™ Yeast Viability Kit; Molecular Probes, 2005) with the same combination of stains was devised for testing yeast viability.

The initial objective of this study was to define experimental conditions for optimum application of the dual-staining procedure to living microbial communities in soil. A fluorescence-based microplate assay was designed to measure the concentration of indigenous living microbial cells and applied using two types of microbial communities: soil bacterial preparations obtained by density gradient centrifugation (Lindahl, 1996; Courtois et al., 2001; Caracciolo et al., 2005; Maron et al., 2006) and whole microbial communities in soil suspensions. Live indigenous bacteria in bacterial preparations were quantified using internal standards, by spiking the preparation with known concentrations of live and dead *Escherichia coli* cells in varying proportions, and calibrated, by comparing fluorescence emission due to indigenous bacterial cells with that of known concentrations of *E. coli* cells in normal saline. A simplified protocol for soil suspensions was based on the addition of live *E. coli* as internal standard and for calibration. Results were validated by comparison with enumeration by conventional epifluorescence microscopy.

2. Materials and methods

2.1. Sample preparation

Soil samples were collected from vineyard plots at three locations in an INRA experimental plot at Couhins near Bordeaux (France). Soil properties are reported in Table 1. Fresh soils were sieved (Ø<2 mm) and stored at 4 °C for a maximum of 2 months. Soil samples (1 g dry weight) were dispersed by shaking overnight (16 h at 25 °C) in a reciprocal shaker in centrifuge tubes (15 ml) in a solution (9 ml) of sodium hexametaphosphate (35 g l^{-1}) and sodium carbonate (7 g l^{-1}). After mechanical dispersion, soil samples were sonicated using an Ultrasonic Processor (Heat Systems, Farmingdale, N.Y.), set as recommended by the manufacturer (20% of max output power). Microbial cells were sonicated in an ice bath in two runs (30 s) separated by cooling (30 s) to avoid membrane disruption.

2.2. Dislodging microbial cells from soil particles

Initially, a "simplified" soil bacterial preparation was obtained by mechanical shaking of the soil suspension for an additional 30 min in the presence of PVPP (Polyclar AT) (100 mg g^{-1} soil) and centrifugation on a density gradient consisting of different aqueous solutions of Optiprep (Abcys, Paris). Optiprep was diluted in SW1 buffer (Saccharose 0.25 M; EDTA 6 mM; Tris–HCl 60 mM, pH 7.4) to obtain solutions at three different densities: 1.30, 1.15, and 1.10 g ml^{-1}. These solutions (2 ml) were gently deposited one on top of the other in a glass centrifuge tube (15 ml) and the soil suspension (5 ml) loaded on top of the gradient. After centrifugation (2600 *g* at 4 °C for 1 h), the fraction of the solution under the separation limit between densities 1.15 and 1.10, containing heavy particles, was discarded. The supernatant was washed and re-suspended in normal saline (2 ml, 0.9% NaCl solution) to obtain suspensions of soil microorganisms ready for enumeration and fluorimetric assays. As revealed by frequent microscopic observations, these preparations essentially contained bacterial cells and are thus referred to as "bacterial preparations".

2.3. Enumeration of soil microorganisms by epifluorescence microscopy

The determination of the concentration of viable cells by epifluorescence microscopy was made using the Chemunex system (Ivry-sur-Seine, France), a reference counting system (Cools et al., 2005) routinely used in our laboratory (Divol and Lonvaud-Funel, 2005). One millilitre of the 10^{-2} dilution (soil bacterial preparation) or 10^{-3} dilution (soil suspension) was filtered through a black polyester membrane filter (Cycloblack™ CB04, 25 mm diameter, 0.4 µm pore size, Chemunex). The ChemFilter was placed on a cellulose pad (Millipore) saturated with a labelling solution containing 5 µl ChemChrome V6 (fluorescein diacetate) in 550 µl ChemSol B16 (both from Chemunex). After incubation at 30 °C for 30 min, the substrate had penetrated the cells and was hydrolysed into fluorescein (excitation 488 nm, emission 520 nm). The ChemFilter was placed between a slide and cover slip and observed under a microscope (Olympus BX51, 1000 times magnification, interferential green filter) under UV light (Hg lamp) in low-fluorescence immersion oil. Initial sample dilutions were selected to obtain 10 to 100 cells per microscope field. For each sample, 10 fields were observed and mean counts determined.

2.4. Preparation of live/dead mixtures of E. coli

E. coli was grown overnight (16 h, 25 °C in LB). The density of viable cells was determined by epifluorescence microscopy of 1 ml *E. coli* culture (10^{-3} dilution), as described for soil bacterial preparations. *E. coli* cultures (producing between 4.0×10^8 and 6.4×10^8 cells ml^{-1}) were adjusted to the desired cell density in normal saline for further experiments.

Two aliquots (1.5 ml) of *E. coli* cell suspensions adjusted to 5×10^7 cells ml^{-1} in normal saline were centrifuged (10,000 *g*, 15 min) and the supernatants discarded. One of the cell pellets was directly re-suspended in the same volume of normal saline (1.5 ml). Bacterial cells in the second pellet were killed in 70% isopropanol (1.5 ml, 1 h at ambient temperature). After removal of the biocide, dead cells were washed, centrifuged (10,000 *g* for 15 min), and re-suspended in normal saline (1.5 ml). Suspensions of live and dead *E. coli* cells at the same concentration (5×10^7 cells ml^{-1}) were mixed to give different proportions of live cells (0%, 25%, 50%, 75%, and 100%).

The same procedure was repeated to prepare bacterial mixtures of live and dead *E. coli* cells for spiking the soil bacterial preparations obtained by density gradient centrifugation. Samples (1.5 ml) of the different mixtures with increasing proportions of live *E. coli* cells in normal saline were centrifuged (10,000 *g* for 15 min) and re-suspended in the same volume (1.5 ml) of the soil bacterial preparations, diluted when required.

2.5. General microplate reader protocol

Assays were performed using 96-well microtiter plates (Microwell™ F96 black, Nunc; Fisher) according to the manufacturer's instructions for the L 13152 viability kit (Molecular Probes, 2004).