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TECHNICAL NOTE

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A fluorescence-based microplate assay to quantify DOM-induced catabolic activity

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Abstract This note describes a novel method to quickly quantify the dissolved organic matter (DOM)-induced catabolic activity from low-volume samples. The concept is based on the catabolic response profiles (CRP) assay and is described as an inverse CRP, where the reactivity of a complex and diverse mixture of organic compounds towards single strains of bacteria is quantified. A strain of *Pseudomonas fluorescens* was grown and then transferred to an organic carbon-free mineral salt medium. 90 μL of a fluorogenic redox indicator was added to 90 μL of the bacterial suspension in a well on a 96-well microplate. The DOM sample (90 μL) was added to the well and the fluorescence emitted by the reduced indicator was read over the period of incubation. Only 0.8 mL of the DOM sample, including controls and replicates, was required to quantify the activity of each sample. Results are presented for a surface soil DOM sample and they were compared to glucose samples of various concentrations. The detection limit was reached for samples containing as little as 55 μM of glucose (0.3 mg C L^{-1}). The assay showed that only 9% of the total carbon of the soil surface DOM sample was readily biodegradable.

Introduction

The dissolved fraction of natural organic matter (DOM) is an ubiquitous concept that can be applied to marine water, soil, groundwater and surface water [1]. This fraction originates from plants and organic waste and, importantly, has the capacity to bind metals and cotransport them down watersheds, soil profiles and streams to water catchments [2]. Water quality is therefore partly dependent upon the ability of DOM to serve as a substrate for environmental bacteria. Furthermore, the quality of DOM is partly responsible for the bacterial diversity in the environment [3–7]. However, DOM is highly variable in composition and evolution. It is a complex mixture, with constituents ranging from low molecular weight sugars, amino acids and organic acids to structurally unknown high molecular weight multiacidic compounds, polyphenols, proteins, and aminopolysaccharides for example [8–10]. Therefore, the extent and rate of catabolism derived from such a variety of compounds will vary greatly depending on the nature of the DOM (fresh, intermediate or mature). Compounds like simple sugars and amino acids are usually rapidly catabolised by bacteria, whereas hydrolysis reactions are needed to slowly break down high molecular weight compounds for further biodegradation [11]. Finally, some compounds can be recalcitrant to biodegradation, and are therefore found in a wide variety of DOM samples [12].

Various methods are available to quantify the interactions between DOM and bacteria, and most have been developed for surface waters, including respiratory-based methods where the CO_2 evolution from incubations is monitored [13]; prediction from a biochemical description of DOM [14]; direct monitoring of the decrease in DOM during incubation [15, 16], and plate-counting of bacterial growth linked to DOM catabolism [17]. However, these all require time-consuming operations and rather large sample volumes, which are not always available, which is often the case for soils [11]. Therefore, the objective of the method presented in this note is to quickly screen and quantify the interactions between a low-volume DOM sample and single strains of bacteria.

The concept is based on the catabolic response profiles (CRP) assay [18], where mixtures of bacteria are screened for their catabolic activity on single organic substrates placed in each well of a microplate. The assay presented here is developed as an inverse CRP, where the reactivity of a mixture of organic compounds towards single strains of bacteria is quantified. The test is designed for use with a 96-well fluorescence-compatible microplate and it uses a

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fluorescent redox indicator to monitor the extent of the DOM-induced catabolic activity. The single strain bacterium is grown and then transferred to an organic carbon-free mineral salt medium prior to its use in the assay.

Materials and methods

Reagents

The bacterium strain used for the assay was *Pseudomonas* fluorescens AK15 [19]. It was provided by the Laboratory of Microbial Ecology in Université Claude Bernard, Lyon, France, where it was grown and stored on LB EZ mix medium (Sigma). To prepare the assay, a sample of this culture was incubated on a Petri dish at 30 °C for 24 hours on LB EZ mix medium and then transferred to 100 mL organic carbon-free mineral salt medium (MSM), based on a phosphate buffer, pH 7.15 [20]. Ten millilitres of the bacterial suspension was washed twice to remove all soluble organic carbon by resuspending the supernatant obtained after centrifugation of the suspension at 10,000×g for 10 min, and then counted by dilution on a Petri dish. A three-hour lag time was imposed before using the bacterial suspension in order to minimise the signal corresponding to the catabolism of bacterial carbon stocks.

The redox indicator used for the assay, Alamar Blue (BioSource Europe, Belgium), was selected from a number of indicators [21, 22] due to its cell stability and its high sensitivity. It consists of a special formulation of the well-known formazan salt which has both chromogenic and fluorogenic properties and is stabilised in the cell [23–27]. The redox potential of Alamar Blue is +380 mV at pH 7.0, 25 °C and so it can be reduced in the cell by metabolic intermediates like NADPH (E_0=−320 mV), FADH (E_0= −220 mV), NADH (E_0=−320 mV), and cytochromes (E_0= −290 mV to +80 mV) [28]. Redox indicators are therefore very useful for monitoring catabolic activities involving at least one of these electron acceptors. Alamar Blue is used in the assay at 25% of the concentration of the commercial solution. The reduced form of the indicator is monitored at λ_{ex}=460 nm to λ_{em}=560 nm.

The DOM sample was obtained from a crop-free agricultural topsoil (0–30 cm) from the Mediterranean region. The soil is a hypercalcaric cambisol (FAO classification) and contains 90% carbonates, 5% clay, 2.7% silt, and 2.3% sand. The soil pH is 8.8 and its organic carbon content is 20.04 g kg^{-1}. Thirty grammes of dried soil (40 °C for 72 h) was shaken vigorously for 15 minutes with 60 ml of ultrapure water (giving a resistivity of 18.0 MΩ cm^{-1} at 25 °C and a total organic carbon content of <20 μg L^{-1}) in a 100 mL amber glass bottle. The slurry was then centrifuged at 10,000 rpm for ten minutes. Then the supernatant was filtered through 0.45 μm membrane filters (Durapore, Millipore). The carbon content of the sample was measured with a total organic carbon analyzer (TOC 5050A, Shimadzu, Japan). Artificial samples containing only glucose were prepared at concentrations of 1–1,000 mg L^{-1} to calibrate the response given by the redox indicator.

Microplate preparation and reading

White opaque 96-well polypropylene microplates were used (OptiPlate, Perkin-Elmer, USA) in order to minimise the microplate-borne fluorescence. The fluorescence emitted by the microplate is negligible at the specific wavelengths of the redox indicator. The assay is performed by mixing 90 μL of the bacterial suspension with 90 μL of the redox indicator solution and 90 μL of the sample in a microplate well (Fig. 1). The microplate is incubated in a Thermomixer (Eppendorf, Germany) at 25 °C and the fluorescence is monitored every 30 minutes or on a shorter timescale for a highly biodegradable sample. Three wells are dedicated to controls on each plate (Fig. 1), to check for: (i) nondegradation of the redox indicator when mixing the indicator with the MSM; (ii) the absence of fluorescence interference when mixing the sample and the bacterial suspension, and; (iii) bacterial activity in the absence of organic substrate when mixing the indicator with the bacterial suspension. Therefore, the 96-well microplate can be used for a great number of samples or to quantify the reactivity of DOM with different bacteria. Fluorescence readings are performed on a Perkin-Elmer luminescence spectrometer (LS 55) equipped with the Perkin-Elmer plate reader accessory.

Results and discussion

Sample concentration range

The concentration range tested with glucose was very large (from 1 to 1,000 mg L^{-1}). As expected, the kinetics of the redox indicator reduction are directly related to this concentration (Fig. 2). The response is apparent for high concentration samples after just 30 minutes of exposure (see results for 500 mg L^{-1}), but it takes almost three hours to differentiate the 5 mg L^{-1} sample from the control

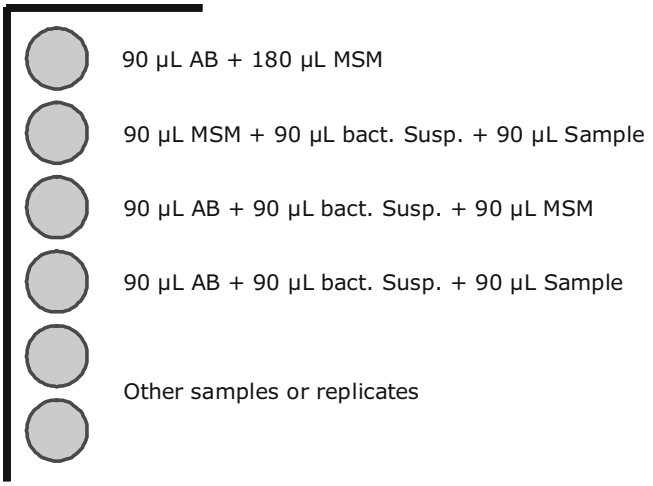

Fig. 1 Contents of the wells in the assay: *AB* stands for the redox indicator Alamar Blue, *MSM* stands for mineral salt medium, *bact. susp.* stands for bacterial suspension

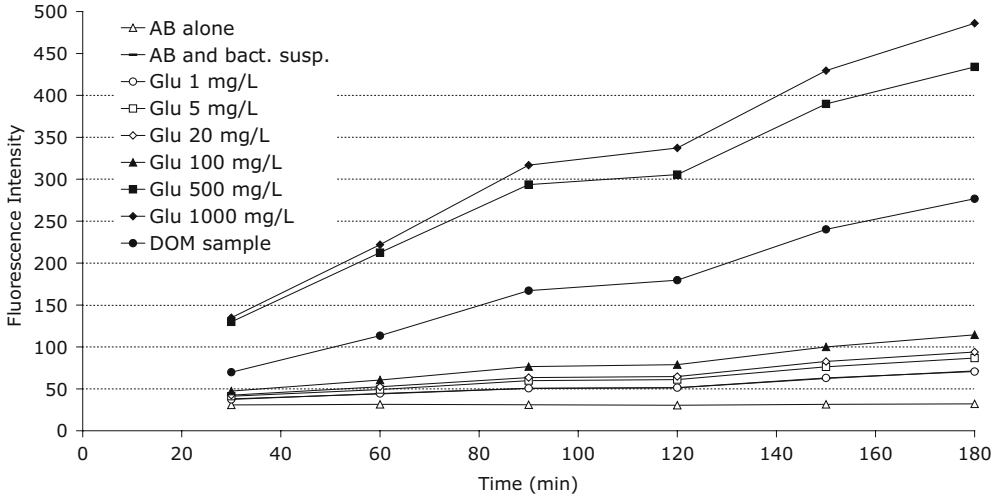

Fig. 2 Kinetic evolutions of the fluorescence emitted by the reduced redox indicator for the control wells, the DOM sample well and for the wells corresponding to various glucose concentrations. The "AB and bact. susp." line is confounded with the "Glu 1 mg/L" line

(which does not contain any organic substrate). The assay was able to differentiate between the response of the bacteria alone (control) and the response of the bacteria in the presence of 5 mg Glu L^{-1}, but not when only 1 mg L^{-1} of glucose was added to the well. This response corresponds to a detection limit of 1.5 μg of organic carbon in each well and to a concentration of 0.33 mg L^{-1} of organic carbon. Therefore, it allows the assay to be used for a very wide range of environmental samples. However, mixing appears to be a critical step for higher concentration samples, since it can ensure that oxygen limitation does not occur during biodegradation within the aqueous phase. Results for 1,000 mg L^{-1} glucose show this limitation in comparison to 500 mg L^{-1} (Fig. 2). Concentrated samples can also saturate the fluorescence detector if the assay is incubated for too long. It is then useful to compare the samples to a known substrate (glucose or any other organic bacterial substrate) and to further dilute the highly concentrated ones. Another possibility is to use the assay with a more dilute bacterial suspension (these results were obtained with a suspension containing 3.7×10^8 cfu/mL), or to incubate and monitor the microplate for much shorter times (a few minutes). Anyhow, the assay is analysed kinetically and more complex samples (organic wastes, crop residues, and so on) may lead to response curves showing peaks of substrate use.

Assay calibration

The relationship between the fluorescence intensity and the glucose concentration (from 1 to 500 mg L^{-1}) was analysed for various incubation times (from 30 to 180 min). All of the regression curves obtained gave regression coefficients >0.98 (results not shown). Furthermore, the evolutions of these regression curves (fluorescence versus substrate concentration) over the period of incubation were linear (Fig. 3). This result shows that the assay can be performed

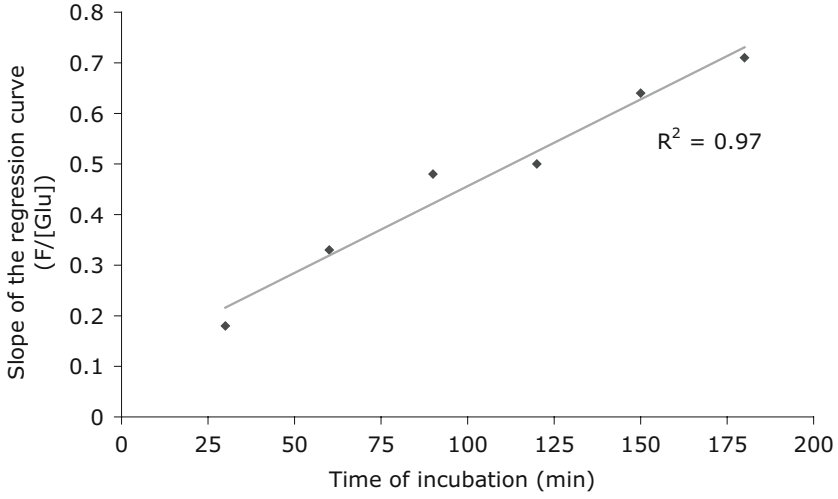

Fig. 3 Evolutions of the regression curves obtained for glucose at various incubation times. Each *dot* represents a regression with R^2>0.98

over any incubation time so long as there is no physical limitation to the bacterial activity, as stated earlier (saturation of the fluorescence intensity after increased incubation time, or limited gas–liquid oxygen transfer for high substrate concentration).

Environmental sample

The soil surface water extract tested in the assay had a carbon content of 226 mg C L^{-1}. The bacterial catabolic activity from this sample gave a response intermediate between the 100 and the 500 mg L^{-1} of glucose (Fig. 2). However, to be able to compare the responses of the samples, their concentrations have to be expressed in the same units; 100 and 500 mg Glu L^{-1} are equivalent to 6.7 and 33.3 mg C L^{-1} respectively. Inversely, the concentration of the DOM sample of 226 mg C L^{-1} is equivalent to 3,390 mg Glu L^{-1}. Therefore, the DOM sample contains much more carbon than the glucose samples, but only a fraction of this carbon is catabolised by the bacteria during incubation. This fraction can be evaluated by dividing the equivalent glucose concentration (20 mg C L^{-1}), deduced from the calibration curve, by the actual concentration of the sample (226 mg C L^{-1}), leading to a value of 9%. Similar levels of easily mineralisable DOM have been observed by others using different methods [11, 13, 29]. This result can be related to the properties of the DOM sample, notably its fluorescent pattern [30–32]. Figure 4 presents the fluorescence excitation–emission matrix (EEM) of the sample. Two major peaks are observed (at λ_{ex}=230 nm to λ_{em}=440 nm, and λ_{ex}=330 nm to λ_{em}=440 nm), which are associated with "humic-type" materials, or mature DOM in contrast to fresh DOM (lower excitation and emission wavelengths) [32]. The humification index (HIX) calculated for this sample is close to 10, which is quite high and much greater than the index for fresh material (usually close to 1, [12]). Therefore, the value of 9% seems to accurately describe the content of easily mineralisable compounds in this DOM sample.

The method presented in this note is faster (requiring a few hours of incubation), more sensitive (detects catabolism at 5 mg/L of organic carbon and above) and uses far less material (only a few hundreds of microlitres) than the existing methods. It will be useful for screening and differentiating various types of dissolved organic matter for their potential reactivity with bacteria.

Perspectives

The assay is currently being developed in two directions. First, other bacterial strains are being tested to evaluate how DOM can act as a source of substrates for various metabolisms, including anaerobic metabolisms, by placing a drop of paraffin into selected wells. Secondly, the original bacteria used are being lyophilized to facilitate further use in other samples (as in the Microtox assay for example).

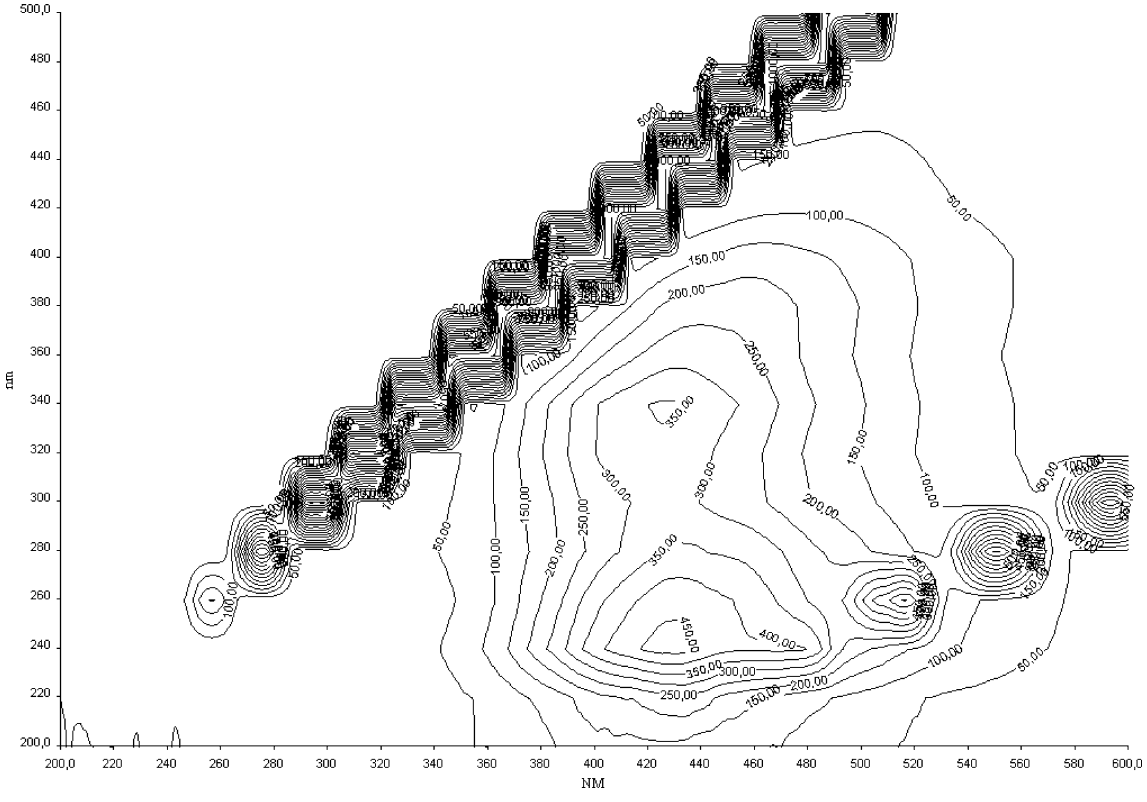

Fig. 4 Fluorescence excitation–emission matrix (EEM) profile of the DOM sample

This process will allow us to quantitatively compare the responses obtained from various environmental samples and at different times.

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