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Optimisation for assay of fluorescein diacetate hydrolytic activity as a sensitive tool to evaluate impacts of pollutants and nutrients on microbial activity in coastal sediments

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ABSTRACT

Fluorescein diacetate (FDA) assay has been widely applied in coastal research to quantify microbial activity in sediments. However, the present FDA assay procedures embodied in sediment studies potentially include operational errors since the protocol was established for studies of terrestrial soil. In the present study, we optimised the procedure of FDA assay using sandy and cohesive sediments to improve experiential sensitivity and reproducibility. The optimised method describes quantitative measurement of the fluorescein produced when 1.0 g of fresh sediment is incubated with 50 mM phosphate buffer solution (pH: 7.3) and glass beads (2 g) at 35 °C for 1 h under a rotation of 50 rpm. The covariation coefficient of the optimised method ranged from 1.9% to 3.8% and the method sensitivity ranged from 0.25 to 1.57. The improved protocol provides a more reliable measurement of the FDA hydrolysis rate over a wide range of sediments compared to the original method.

1. Introduction

Due to the rapid development of urbanisation, industry and agriculture, anthropogenic pollutants (e.g., heavy metals and persistent organic pollutants) and excessive nutrient loadings (e.g., nitrogen and phosphorus) have increased significantly in coastal systems on a global scale (Heim and Schwarzbauer, 2013; Reed and Harrison, 2016). These pollutants and nutrients are intensively circulated within coastal sediments. As active reactors, sediments in coastal zones have large potentials in a series of biogeochemical reactions (Huettel et al., 2014), such as denitrification, chelation, aerobic/anaerobic degradation. Subsequently, sediments may decrease the ecological risks of these contaminants to coastal ecosystems via these pathways. Notably, most biogeochemical reactions in sediments are mediated by microbial communities (Jeon and Madsen, 2013). As a consequence, microbial activity in coastal sediments has been regarded as an important index in the evaluation of retention and circulation of pollutants and nutrients (Ramakrishnan et al., 2011).

Microbial activity can be directly determined by quantification of adenosine triphosphate (ATP) content and activity of coenzyme F_{420} (Rozzi and Remigi, 2004). Indirect measurements, such as the quantification of microbial biomass and population sizes, and titrimetric bioassays (Adam and Duncan, 2001), were also applied in previous research. However, these assays are often time consuming, labour intensive and highly equipment-dependent (Green et al., 2006). Thus, environmental researchers have looked for appropriate alternatives for determining microbial activity in order to reduce time usage while standardising measurement procedures and increasing the uniformity of analysis protocols to reduce costs. Fluorescein diacetate [3′, 6′-diacetyl-fluorescein (FDA)] is a colourless compound. Compared to other substrates used in microbial activity analysis, such as acetate and nitrate (Rozzi and Remigi, 2004), FDA can be hydrolysed by a wide spectrum of extracellular enzymes and membrane bounded enzymes, such as protease, lipase, and esterase (Adam and Duncan, 2001). The end product of this hydrolysis reaction is fluorescein, which is yellow-green in colour. Fluorescein exhibits strong absorption in the visible range (490 nm) and therefore can easily be determined by spectrophotometry. FDA assay has long been regarded as a standard approach to determine bacterial/cell activity in the biochemistry field. In 1982, Schnürer and Rosswall (1982) introduced FDA assay for determining microbial activity in soils and litters. Extension of FDA assay for measurements of microbial activity in soils and sediments has several operational advantages compared to traditionally employed methods. Specifically, the analysis only requires a small amount (approximately 1 g) of soil/sediment samples (Green et al., 2006), particularly beneficial for field campaigns that incorporate a large number of sampling sites. Furthermore, the test is rapid due to the short incubation period (1 to 3 h) and spectral quantification (Adam and Duncan, 2001), while being cost-effective. As a direct result, it is widely applicable and affordable for the wider scientific community. In addition, results of FDA assay correlate well with other microbial indices, such as biomass, ATP content and cell density (Stubberfield and Shaw, 1990). Due to these advantages, the FDA test has been frequently used in soil research (reviewed by Green et

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al. (2006)), and coastal sediment studies for the last two decades (El-Tarabily, 2002; Simpson et al., 2004; Costa et al., 2007; Brito et al., 2009; Gillan et al., 2012; Nogaro and Hammerschmidt, 2013; Gómez et al., 2015).

Indeed, FDA assays are easy to carry out, while the reducibility and sensitivity of FDA assay depend on operational settings, like the pH of the buffer solution, rotation speed and incubation period (Green et al., 2006). Improper experimental conditions may decrease sensitivity and precision, leading to incorrect interpretation of the impacts of pollutants or excessive nutrients on benthic microbial metabolism. As a result, the optimisation of the assay procedure is essential. Previous studies mainly focused on the optimisation of FDA assays for terrestrial soils (e.g., Adam and Duncan, 2001; Green et al., 2006). In contrast, documented support on the optimum test conditions for coastal sediments is limited. Compared to terrestrial soils that are mainly oxic, sediments in coastal regions are usually anaerobic (Davis, 2012). In addition, owing to high primary productivity, the sediments, specifically cohesive (muddy) sediments, are enriched in organic matter, potentially leading to large microbial population sizes. Consequently, the reaction potential of FDA hydrolysis in sediments may be significantly higher than that of terrestrial soils. Furthermore, due to the higher proportion of clay/silt particles in cohesive sediments, the adhesion between sediment particles tends to be strong. Given the presence of these differences, it might be difficult to achieve a reliable measurement of FDA hydrolysis rates in coastal sediment samples using the existing protocol because it is optimised for terrestrial soils. Therefore, the objective of this work was to optimise the current procedure and further develop a precise and sensitive FDA assay protocol for coastal sediments in order to meet the needs of biogeochemical and biological research.

2. Materials and methods

2.1. Sediment collection

Four different sediment samples, including both sandy and cohesive types, were used in the development of this method. They were collected from four distinct sites (Table 1) in the Jiulong Estuary, Fujian Province, China. Prior to collection, the top 5 cm of surface sediments were removed. The sediments were collected using PVC corers. The core liners were pushed into the sediment at low tide, retrieved and kept in cooler boxes. In the laboratory, all these samples were sieved with a 0.5 cm pore size sieve to remove algae/seagrass debris and bivalve shells. The sediment samples were stored in acid-washed glass beakers prior to experiments; a portion of these sediments were divided for freeze-drying and air-drying. To facilitate assays, physical and chemical properties of these sediment samples were analysed, as presented in Table 1.

2.2. Reagents

Phosphate buffer, acting as the incubation solution, was prepared by dissolving phosphate sodium ($Na_3PO_4 \cdot 12H_2O$; analytical grades) in deionised water (produced with a Millipore™ purification system) to a final concentration of 50 mM. The pH of the buffer solution was adjusted by adding 2 M hydrochloric acid. The FDA ($C_{24}H_{16}O_7$, purchased from Sigma-Aldrich, U.K.) substrate stock solution was prepared by dissolving 0.2 g of FDA powder in 200 mL acetone (analytical grades). The fluorescein ($C_{24}H_{14}O_6$, Sigma-Aldrich, U.K.) standard solution was prepared by dissolving fluorescein into phosphate buffer to a concentration of 1.0 g L^{-1}. To avoid a decrease in concentration due to degradation, both solutions were stored in amber bottles in a fridge at 4 °C.

2.3. Original method

The original method was adopted from protocols described by Adam and Duncan (2001) and Green et al. (2006). In particular, 1 g air-dried sediment was placed into a 50 mL conical flask containing 15 mL phosphate buffer (pH: 7.6). Subsequently, 0.3 mL of FDA substrate stock solution was added to start the reaction. The control sample was prepared by injecting 0.3 mL acetone in place of the substrate. The flasks were sealed with Parafilm and placed in a shaker (TS-111D, Baixin Incorporation, China) at 40 °C for 1 h. The rotation rate was set to 200 rpm. The reaction was terminated by adding 2 mL acetone. Subsequently, the sediment–water matrix was transferred into a 30 mL glass centrifuge tube and centrifuged at 5000 × *g* at 4 °C for 5 min (320R, Hettich™, Germany). The supernatant was then filtered through Whatman No.2 filter paper and the florescence intensity of the liquid was determined by spectrophotometry (UV-1800, Shimadzu™, Japan) at wavelength 490 nm. The concentration of fluorescein released from hydrolysis was calculated on the basis of a standard curve. For preparation of the working standards, 0.025, 0.05, 0.1, 0.2, and 0.4 mL of fluorescein standard stock solution was injected into 50 mL volumetric flasks. In order to compensate for the liquid composition of the sample assay, 6 mL acetone was added into the flasks. The solutions were then filled to the set volume with the prepared sodium phosphate buffer.

2.4. Optimisation procedure

As illustrated in the introduction, the reproducibility and sensitivity of the FDA assay can be influenced by a variety of experimental parameters. In this study, we focused on factors in the following three categories due to their importance in enzymatic reactions: (1) factors that can directly influence the sediment enzyme activity, including pH of the buffer solution, incubation temperature and sediment pre-treatment; (2) length of incubation time, i.e., a factor that is related to the sediment enzymatic potential; and (3) rotation rates and absence/presence of glass beads, factors that determine the separation of sediment particles during the incubation. Detailed information for each optimising factor is shown in Table 2. Identification of the optimised condition of each

Table 1
Physical and chemical properties of sediment samples involved in the optimisation study, LOI indicates loss on ignition. The sediment order (S1 to S4) in the following test is consistent with the order in this table.

Sediment (order)	pH	LOI (%)	Organic carbon (%)	Organic nitrogen (%)	Water content	Location
Sandy (S1)	6.3	0.9	0.5	0.1	0.32	24°26′05.7″N 118°05′45.3″E
Sandy (S2)	6.4	1.2	0.6	0.1	0.34	24°26′30.0″N 118°03′37.5″E
Muddy (S3)	6.6	4.8	2.2	0.2	0.56	24°24′16.2″N 117°57′20.7″E
Muddy (S4)	6.8	5.5	2.6	0.3	0.59	24°23′41.1″N 117°54′36.8″E

Table 2
Detailed information of each factor within optimization for both sterilised and non-sterilised sediment samples.

Order	Factors	Operations
1	Buffer pH	7.0, 7.2, 7.4, 7.6, 7.8, and 8.0
2	Incubation period	0.25, 0.5, 1, 2, 4, and 8 h
3	Incubation temperature	25, 30, 40, 50, and 60 °C
4	Shaking	0 (Static), 50, 100, 150, 200 rpm
5	Physical separation	Absence/presence of glass beads (0.5 mm diameter)
6	Sediment pre-treatment	Air-dried, freeze-dried, fresh

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