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Short Communication

Shift of low to high nucleic acid bacteria as a potential bioindicator for the screening of anthropogenic effects in a receiving river due to palm oil mill effluent final discharge

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ABSTRACT

The microbiological effects of palm oil mill effluent (POME) final discharge upon a receiving river were assessed in this study by using the nucleic acid double staining assay based on flow cytometry. The functional status of the bacterial community at the single-cell level was determined with regards to their abundance, viability and nucleic acid content to monitor the effects of POME final discharge on the affected river. The effluent resulted in the increment of the total cell concentration (TCC) and viable cells which were correlated with the increment of biological oxygen demand (BOD_5) and total organic carbon (TOC) concentrations in the receiving river. The shift of low nucleic acid (LNA) to high nucleic acid (HNA) bacterial cells in the affected river suggested the transformation of dormant to active cells due to the POME final discharge. This is the first study to report on the shift of LNA/HNA ratios which may serves as a potential bioindicator in the screening of the anthropogenic effects due to POME final discharge in river water with originally high LNA proportions. Monitoring the effluent discharge at low trophic level using flow cytometry is a rapid and sensitive approach when compared to the current physicochemical assessment method. This approach allows for the screening of river water contamination caused by POME final discharge prior to a full assessment using the recently proposed specific bacterial indicators.

1. Introduction

The palm oil industry is expected to continuously grow as it is currently being actively promoted in Southeast Asia and is also known be expanding in West Africa and Latin America (Hansen et al., 2015). Malaysia alone has recognised the palm oil industry as one of the main economic-driving industries in the country (Alam et al., 2015). However, the generation of liquid waste from the mills known as palm oil mill effluent (POME) has become a major problem to the aquatic system, as most of the mills discharge the treated wastewater into nearby rivers (Rupani et al., 2010). Since the final discharge still contains a certain amount of organic load, the introduction of POME final discharge into natural water system causes an increase of the BOD level due to the biodegradation process (Ado et al., 2015). This in turn changes the physicochemical properties and affects the microbiological status of the water system (Ibrahim et al., 2012).

Up to date, knowledge on the bacterial community shift caused by the anthropogenic effects of POME in the effluent receiving rivers is still limited. As bacteria are phylogenetically and functionally diverse (Faure et al., 2015), information on how bacterial populations responded to external disturbance could be utilized as a key indicator to determine the effects of pollution (Essahale et al., 2010). Moreover, the quantification of microbiological changes had been shown to be more sensitive than the physicochemical characterisation of wastewater (Muela et al., 2011). However, the effects of effluent discharge in many cases are moderate and not easily detected, hence a rapid and sensitive monitoring strategy needs to be carefully chosen (Harry et al., 2016).

The application of flow cytometry as an automated, cultivation independent diagnostic tool is regarded as a feasible approach which allows the visualisation and quantification of bacterial clusters based on staining using fluorescent nucleic acid dyes. Bacteria with high nucleic acid content (HNA) could be further quantified and discriminated from low nucleic acid (LNA) bacterial cells, hence nucleic acid content could be a good indicator of the fraction of active cells (Lebaron et al., 2000).

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This approach could be applied as a rapid screening tool to complement the full assessment method using specific bacterial indicators which had recently been proposed based on the emergence of *Chromatiaceae* and *Alcaligenaceae* in the affected river to indicate river water contamination caused by POME final discharge (Sharuddin et al., 2017).

This is among the first few reports to reveal the effects of treated POME final discharge on the functional status of the bacterial community in effluent-receiving rivers through the application of flow cytometry, in relation to the changes of BOD_5 and TOC concentrations. For the evaluation, the effects of POME final discharge were correlated by comparing the upstream (before the effluent discharge point itself) and to the downstream (after the effluent discharge point) of the river. The shift of the nucleic acid contents of bacterial cells categorised into HNA and LNA cells could be proposed as a potential bioindicator for the screening of the anthropogenic effects due to POME final discharge prior to a full assessment using the specific bioindicators. Measurement of the HNA/LNA cell ratio changes was found to be a rapid and sensitive approach to screen and monitor the effects of POME final discharge in the receiving river as compared to the current physicochemical assessment of the final discharge which is considered to be inaccurate and time consuming.

2. Materials and methods

2.1. Sampling sites

POME final discharge was collected from a typical palm oil mill in Malaysia which adopted the secondary (aerobic and anaerobic) and tertiary (biopolishing plant) treatment system. The POME final discharge flowed through the plantation channel before entering the receiving river located approximately 3 km from the palm oil mill. Water samples were taken from the downstream (after the effluent discharge point) and the upstream (before the effluent discharge point) of the river with a distance between them of about 100 m to minimize spatial differences including sunlight intensity, soil condition and fertilization activities of nearby areas and other external factors. Samplings were carried out monthly over a one year period starting March 2015 to February 2016, in order to take into account the variability due to the unpredictable rainy and dry seasons in a tropical climate. Grab samples of 2 L were collected in a pre-cleaned plastic container, kept in an ice box during transportation and processed in the laboratory within six hours of collection.

2.2. Physicochemical characterisations

Water samples were analysed for BOD_5 and TOC to correlate the changes of the functional status of the bacterial community with the basic pollution parameters. The BOD_5 analysis was conducted according to the procedure in the Standard Method (APHA, 2002) and the TOC was analysed using a Shimadzu TOC-V_{CSH} Analyzer (Tokyo, Japan).

2.3. Flow cytometry sample preparation

Water samples were diluted at least 1:10 (v/v) to have a measurement range of less than 1000 cells/second (Falcioni et al., 2008). The samples were filtered using a 40 µm Nylon cell strainer (Falcon) to minimise the likelihood of causing a clog in the flow cytometer.

2.4. Fluorescent probes

In order to enumerate the bacteria in the water samples, a nucleic acid double staining assay based on the work of Barbesti et al. (2000) was applied. A 42 µmol/L of thiazole orange diluted in dimethyl sulfoxide (Buzatu et al., 2014; Matos and Da Silva, 2013) and 4.3 mmol/L propidium iodide diluted in water (BD™ Cell Viability Kit) were used to distinguish between viable and dead bacterial cells, respectively for analysis by flow cytometry. Thiazole orange is a permeable dye that emits green fluorescence and stains all cells (Ex 510 nm, Em 530 nm), while propidium iodide is an impermeable dye that emits red fluorescence and stain cells with compromised membrane (Stiefel et al., 2015) i.e. dead cells (Ex 536 nm, Em 617 nm).

2.5. Staining procedure

The filtered samples were vortexed for at least five minutes and were divided into two sets. The first set was further divided into two subsamples, named as unstained and stained samples. The unstained set of samples was used as a control to eliminate the assay background noise (Allegra et al., 2008). Afterwards, 5 µL of each dye was added into 500 µL of cell suspension (stained samples) to achieve a final concentration of 420 nmol/L for thiazole orange and 43 µmol/L for propidium iodide, respectively while 5 µL of EDTA (1 mM, pH 8) was added into both the stained and unstained samples. The staining process was carried out following the manufacturer's protocol of the BD™ Cell Viability Kit (Cat. No. 349483, Becton Dickinson, San Jose, CA). The samples were incubated for one hour in an ice box on an orbital shaker.

2.6. Heat treatment

The gating control for the characterization of the membrane-compromised cells was done by heat-killing the cells, whereby 50 mL of the second set of samples was heated using a water bath for 45 min at 80–90 °C (the temperature and time were modified from Grégori et al. (2001)).

2.7. Flow cytometric analyses

The flow cytometric analyses were done using a BD Accuri® C6 cytometer (Becton Dickinson UK Ltd., Oxford, UK) equipped with blue (488 nm) and red (640 nm) lasers, forward-scattered (FSC) and side-scattered light (SSC) and four fluorescence detectors. The thiazole orange fluorescence was collected at the FL1 channel while the propidium iodide fluorescence was collected at the FL3 channel, with 20,000 cells counted (Foladori et al., 2007; Matos and Da Silva, 2013) for each sample. A gating on a dot plot of red versus green fluorescence after excluding the auto-fluorescence allowed the differentiation of viable and dead cells. The HNA and LNA cells were distinguished by plotting the thiazole orange fluorescence (FL1) against SSC after excluding the dead fraction. The visualisation of the plots and the gating process were done using the built in CFlow Plus software.

2.8. Statistical analyses

The UNIVARIATE procedure of SAS version 9.2 was used to describe the distribution of the data. The data were tested for normality and skewness for all variables by applying the Shapiro-Wilk test (W), Kolmogorov-Smirnov (D), Cramer-von Mises (W-Sq) and Anderson-Darling (A-Sq) tests. The null hypothesis of normality was rejected when $p < 0.05$. Non-normally distributed data were log-transformed before proceeding with the Welch's *t*-test at a confidence level of 95% to analyse the physicochemical properties data between the upstream and downstream points of the river. The repeated measurements ANOVA were also conducted to test for significant differences of total cell concentrations (TCC) at different sampling points collected in different months. The correlation test between parameters was done using Pearson's correlation coefficient.

3. Results and discussion

Fig. 1 shows the effects of POME final discharge on the increments of BOD_5 and TOC concentrations at the downstream as compared to the