Bioresource Technology 218 (2016) 1115–1122

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

[1]H nuclear magnetic resonance-based metabolomics study of earthworm *Perionyx excavatus* in vermifiltration process

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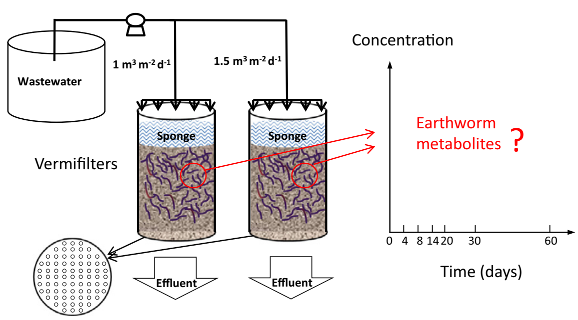
HIGHLIGHTS

- Earthworm physiology in vermifiltration was characterized using NMR metabolomics.
- Significant differences of NMR spectra appear in response to hydraulic loading.
- 13 metabolites were significantly different among control, VF1 and VF1.5 groups.
- Metabolic activity of earthworms in VF1 increased within 20 days, then decreased.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form 18 July 2016
Accepted 19 July 2016
Available online 21 July 2016

Keywords:
Vermifiltration
Metabolomics
NMR
Earthworm
Perionyx excavatus

ABSTRACT

In this study, [1]H nuclear magnetic resonance (NMR)-based metabolomics approach was used to characterize the metabolic response of the earthworm *Perionyx excavatus* in continuous vermifiltration for two months under hydraulic loading rates of 1 $m^3\ m^{-2}\ d^{-1}$ (VF1) and 1.5 $m^3\ m^{-2}\ d^{-1}$ (VF1.5). Both VF1 and VF1.5 showed higher removal of chemical oxygen demand and total nitrogen than the biofilter without earthworms. Principal component analysis of the NMR spectra of earthworm metabolites showed significant separations between those not subjected to wastewater filtration (control) and VF1 or VF1.5. Temporal variations of earthworm biomass, and the identified metabolites that are significantly different between control, VF1 and VF1.5 revealed that worms underwent increasing metabolic activity within 20 days in VF1 and 14 days in VF1.5, then decreasing metabolic activity. The use of NMR-based metabolomics in monitoring earthworm metabolism was demonstrated to be a novel approach in studying engineered vermifiltration systems.

1. Introduction

Vermifiltration (VF) is a relatively new, decentralized wastewater treatment technology (Sinha et al., 2008) with low construction, operation and maintenance costs. It is a gravity-fed filtration system that employs filter materials (e.g. soil, sawdust, sand and ceramsites), earthworms and microorganisms to remove or degrade organic matter and nutrients in wastewater. It has been proven by a number of researchers to be capable of treating domestic wastewater (Sinha et al., 2008; Taylor et al., 2003), swine wastewater (Li et al., 2008), herbal pharmaceutical wastewater (Dhadse et al., 2010) and wastewater sludge (Zhao et al., 2014, 2010). A full-scale vermifilter in Shanghai, China (Liu et al., 2013) demonstrated successful operation for treatment of rural domestic sewage during a 17 month period.

Compared with biofilter without earthworms, vermifilter exhibited around 25% higher removal efficiency in terms of volatile suspended solids reduction in sludge (Zhao et al., 2010), as well as

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http://dx.doi.org/10.1016/j.biortech.2016.07.079
0960-8524/

30% higher removal efficiency of total suspended solids (TSS), chemical oxygen demand (COD) and 5-day biochemical oxygen demand (BOD_5) in domestic sewage (Sinha et al., 2008). Xing's group (Li et al., 2014; Liu et al., 2012; Xing et al., 2014; Yang et al., 2013; Zhao et al., 2010) found that earthworms extended the food web, significantly regulated microbial biomass, and enhanced microbial and metabolic activities through predation and burrowing in VF systems for treating liquid-state excess sludge. These demonstrate that earthworms play significant roles in the vermifilter ecosystem.

The operation conditions for earthworms to acclimatize in the vermifilter environment for wastewater treatment require optimization. Earthworms in VF suffer from much higher hydraulic loading than in the natural soil environment. Their physiological responses toward excessive hydraulic loading stress may inhibit their growth and reproduction, affecting earthworm-microorganism interaction and possibly resulting in reduced removal efficiency of organic matter. In literature reports to date, most vermifilters that have been designed for treatment of wastewater sustained hydraulic loading of no more than 1 m^3 m^{-2} d^{-1}. The underlying reason may be due to the earthworm's inability to survive in higher hydraulic loading rates. This weakness results in enlarged occupied space in large-volume wastewater treatment. Therefore, efforts are needed to enhance the ability of earthworms to survive in higher hydraulic loadings within the vermi-bed. Apart from optimizing the bedding medium in the earthworm layer, understanding how earthworms respond to excessive hydraulic loading is of great significance, as this knowledge serves as the first step to further improvement strategies. However, to date, few studies have systematically explored this topic. A very recent research (Li et al., 2015) studied the acclimation process of earthworms after introduction into VF through their antioxidant and behavior responses. However, there is still a lack of understanding of the metabolic processes of earthworms in response to environmental changes.

Metabolomics is an "omics" approach for studying organisms in response to external stressors by comprehensive analysis of small molecule metabolites in cells, tissues, or biofluids (Lankadurai et al., 2013a). Metabolomics can be used to study the perturbations in different metabolic pathways and identify novel modes of action of new contaminants or complex environmental changes. It has demonstrated applicability on various earthworm species for toxicology studies of heavy metal (Bundy et al., 2008) and organic pollutants exposure (Ji et al., 2013; Lankadurai et al., 2013b; Mudiam et al., 2013; Yuk et al., 2013). Earthworms in VF process suffer from a new mixture of exposures composed of organic matter loads and flooding, warranting the use of metabolomics methodology.

In this study, ^1H nuclear magnetic resonance (NMR)-based metabolomics was adapted as a novel approach in analyzing the earthworms' time-series metabolic response under high hydraulic loading VF process. The effect of wastewater loading on temporal variation of metabolites was investigated. The objectives of this work include: (1) to gain an in-depth and comprehensive understanding of the perturbation of the earthworms' metabolic pathways due to VF stress; (2) to explore metabolomics as a monitoring method for characterizing the earthworms' health condition in VF processes.

2. Materials and methods

2.1. VF setup and operation

Four PVC reactors (20.8 cm in diameter and 34 cm in depth) in parallel were packed with the artificial substrate to a height of 25 cm. The artificial substrate is composed of a mixture of peat moss, coconut chips and volcanic sand (Hua Hng Trading Co Pte. Ltd., Singapore) with a volume ratio of 2:2:1. Besides providing a comfortable habitat for earthworm burrowing activity, the peat moss also possesses a water-retaining property that may increase the hydraulic retention time and therefore enhance wastewater treatment efficiency. The coconut chips have a surface area of 1–6 cm^2 per unit and serve as potential food source for earthworm feeding. The volcanic sand has a diameter ranging from 1 mm to 4 mm. It provides porosity that facilitates biofilm attachment and also prevents excessive packing of the medium that may result in clogging. As the purpose of this study is to investigate effect of wastewater loading on earthworm's physiology, the other layers (e.g. sawdust, sand, ceramsites, gravels, river bed material, wood coal, glass balls, mud balls, detritus, cobblestones etc), which are usually constructed in practical VF but contains no earthworm, were omitted for simplicity. A number of small holes with a diameter of 3 mm are evenly distributed on the bottom surface of reactors. These holes allow effluent sampling and drainage. A 4 cm highly hydrophilic and porous sponge was placed on the top of each vermifilter to ensure even distribution of influent and to avoid direct hydraulic shock on earthworms. Synthetic wastewater containing 250 mg L^{-1} glucose, 80 mg L^{-1} urea, 15 mg L^{-1} NaH_2PO_4, 1.5 mg L^{-1} KH_2PO_4, 5 mg L^{-1} $CaCl_2$ and 2 mg L^{-1} $MgSO_4$ was transferred continuously into each vermifilter with a peristaltic pump. The four vermifilters were set up as follows: one under 1 m^3 m^{-2} d^{-1} hydraulic loading without earthworm (biofilter, BF), one with earthworm but no wastewater treatment (control), one with earthworm under 1 m^3 m^{-2} d^{-1} hydraulic loading (VF1), and one with earthworm under 1.5 m^3 m^{-2} d^{-1} hydraulic loading (VF1.5). We used *Perionyx excavatus* as earthworm species, as it is widely distributed in tropical areas. This species has demonstrated applicability for use in the VF process (Rajpal et al., 2014). The vermifilters were continuously operated for 2 months at 20–21 °C.

2.2. Water quality analysis

The wastewater influent and effluent were sampled every one or two days for chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP) analysis. COD, TN, and TP were determined following the Hach standard protocols (http://www.hach.com/wah#C) using specific Hach reagents (COD digestion vials, total nitrogen reagent set and total phosphorus reagent set, respectively) and a DR5000 spectrophotometer.

2.3. Earthworm biomass and reproduction analysis

The first generation of earthworms was bought from an earthworm farm in Singapore. Worms were fed with fruit peel in a container kept in a lab environment for 2 weeks before introduction into vermifilters. For each of the three vermifilters, 85 g of mature worms were inoculated to give a density of 10 g L^{-1}. At the time points of 4 d, 8 d, 14 d, 20 d, 30 d and 60 d, all the worms were picked out of the filters, gently rinsed and weighed for total biomass. The juvenile worms were also counted. Eight mature worms were randomly taken at each time point for metabolites extraction. The rest of the worms were placed back into the vermifilters. The peristaltic pump was paused for only one hour at each time point for this procedure, hence the VF operation is still considered "continuous".

2.4. Earthworm metabolites extraction

Eight mature worms taken at the abovementioned time points were placed in a beaker with a wet filter paper at the bottom. The beaker was covered with aluminum foil on the top and surrounding, and placed statically for 24 h to empty the worm gut.