Journal of Environmental Management 207 (2018) 192–202

Contents lists available at ScienceDirect

Journal of Environmental Management

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

Research article

Bacterial nitrogen fixation in sand bioreactors treating winery wastewater with a high carbon to nitrogen ratio

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ARTICLE INFO

Article history:
Received 3 May 2017
Received in revised form
20 October 2017
Accepted 5 November 2017

Keywords:
Bacterial nitrogen fixation
High C:N wastewater
Winery wastewater
Biological sand filters

ABSTRACT

Heterotrophic bacteria proliferate in organic-rich environments and systems containing sufficient essential nutrients. Nitrogen, phosphorus and potassium are the nutrients required in the highest concentrations. The ratio of carbon to nitrogen is an important consideration for wastewater bioremediation because insufficient nitrogen may result in decreased treatment efficiency. It has been shown that during the treatment of effluent from the pulp and paper industry, bacterial nitrogen fixation can supplement the nitrogen requirements of suspended growth systems. This study was conducted using physico-chemical analyses and culture-dependent and -independent techniques to ascertain whether nitrogen-fixing bacteria were selected in biological sand filters used to treat synthetic winery wastewater with a high carbon to nitrogen ratio (193:1). The systems performed well, with the influent COD of 1351 mg/L being reduced by 84–89%. It was shown that the nitrogen fixing bacterial population was influenced by the presence of synthetic winery effluent in the surface layers of the biological sand filters, but not in the deeper layers. It was hypothesised that this was due to the greater availability of atmospheric nitrogen at the surface. The numbers of culture-able nitrogen-fixing bacteria, including presumptive *Azotobacter* spp. exhibited 1–2 log increases at the surface. The results of this study confirm that nitrogen fixation is an important mechanism to be considered during treatment of high carbon to nitrogen wastewater. If biological treatment systems can be operated to stimulate this phenomenon, it may obviate the need for nitrogen addition.

1. Introduction

Nitrification, denitrification and nitrogen fixation (NF) are the main processes responsible for the biogeochemical cycling of N, represented by the N cycle (Ray et al., 2014). NF is the process whereby N_2 gas is converted into ammonia by heterotrophic bacteria or photosynthetic N-fixing bacteria (NFB) and the reaction is catalysed by an intracellular nitrogenase enzyme system (Olivares et al., 2013). In soil environments, particularly in plant rhizospheres, NFB naturally supply N for the growth of crops and indigenous plants (Barua et al., 2012; De Salamone et al., 2012). Bioaugmentation of N-deficient soils with NFB can increase crop yield, although negative effects have also been demonstrated (Barua et al., 2012; De Salamone et al., 2012). N is also an essential nutrient for all bacteria, and consequently high concentrations of organics in the soil environment (e.g. from petroleum oil or olive mill waste) have been shown to result in the selection of NFB which provide N for the carbon (C)-degrading microbial populations (Balis et al., 1996; Chronopolou et al., 2013).

N is also important in biological wastewater treatment plants, where the general premise is that unless exogenous N is added, performance decreases when the influent N to C ratio falls below 0.05 (Sakar et al., 2016; Tchobanoglous et al., 2003). However, it has been demonstrated that in some instances, natural NF increases the amount of N available in systems treating high C:N wastewater. It has been suggested that in these circumstances, exogenous N should not be added as it suppresses NF (Clark et al., 1997; Dennis et al., 2004; Kargi and Özmıhçı, 2004).

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https://doi.org/10.1016/j.jenvman.2017.11.015

Abbreviations

BN	Basal nutrients
BSF	Biological sand filter
NF	Nitrogen fixation
NFB	Nitrogen-fixing bacteria
SWW	Synthetic winery wastewater

A number of publications have described the use of N-fixing systems for the successful treatment of pulp and paper mill effluent in suspended growth laboratory-scale (e.g. Dennis et al., 2004; Kargi and Özmıhçı, 2004) and full-scale systems (e.g. Clark et al., 1997; Dennis et al., 2004). For example, approximately 600 kg N/day was fixed in an aerated stabilisation basin with a capacity of 120000 m^3 and hydraulic retention time of 1.2 days when treating kraft mill wastewater (Clark et al., 1997). More recently, it was shown that NF occurred at the anode of a bioelectrical system fed with glucose (Wong et al., 2014). Apart from the obvious cost and labour savings, a major advantage of these self-regulating systems over those where exogenous N is added, is that the final effluent N concentrations are low and stable (Clark et al., 1997; Pratt et al., 2007). In addition, granular morphology, sludge settle-ability, and dewater-ability may be improved, as demonstrated in laboratory scale sequencing batch reactors (Clark et al., 1997; Pratt et al., 2007). Despite the fact that nitrogenases are sensitive to oxygen, it has been established that high dissolved oxygen concentrations created via aeration do not negatively affect the functional NF population (Pratt et al., 2007; Slade et al., 2003). This is probably because of well described intrinsic intracellular and extracellular mechanisms which protect the enzymes from oxygen exposure (Pratt et al., 2007; Slade et al., 2003).

Bioaugmentation is the practice of supplementing an environment with exogenous microorganisms in order to stimulate bioremediation. In the case of biological wastewater treatment systems, bioaugmentation is controversial (Gentry et al., 2004). Not only is there a cost consideration, but the introduced organisms may not be suited to the prevailing conditions and will be competitively eliminated by the existing flora (Gentry et al., 2004). However, in laboratory-scale experiments, in which reactors are not exposed to an environmental microbial pool, inoculation with known diazotrophs is an option. For example, in flask experiments, the treatment of N-deficient wastewater was improved by the addition of *Azotobater vinelandii* to activated sludge (Kargi and Özmıhçı, 2004), and inoculation with reference strains of *Azotobacter* spp. resulted in the reduction of the polyphenolic content of olive mill wastewater (Aquilanti et al., 2014). The latter finding was supported by Piperidou et al. (2000), who found that inoculation of a pilot-scale fed-batch reactor with *A. vinelandii* resulted in the elimination of potentially phytotoxic organics from olive mill wastewater.

In a previous study, the performance of two biological sand filters (BSFs) treating high COD:N wastewater were compared. Both systems were used to treat synthetic wastewater with the same composition, but in one replicate, nutrients were added to provide an 'ideal' COD:N:P ratio. During these experiments, satisfactory COD removal was achieved in the nutrient-deprived system (approx. 80% removal with an influent COD of 7587 mg/L). However, the COD removal rate and nitrate reductase activity was significantly higher in the nutrient-supplemented system (Rodriguez-Caballaro et al., 2012). It was hypothesised that the organic substrate was being utilised as an electron donor for heterotrophic denitrification. In the BSF that was not nutrient-supplemented, a thick layer of slime developed on the surface. A Gram stain and culture of the layer revealed an abundance of Gram negative bacteria, with the distinctive microscopic and colonial morphology of *Azotobacter* spp. Identity was confirmed by sequencing 16S rRNA gene amplicons as *Azotobacter chroococcum* (J.B. Ramond, unpublished data).

Azotobacter spp. are diazotrophs that fix nitrogen at high rates and are known to produce copious amounts of extracellular polysaccharide slime to protect the sensitive nitrogenase enzyme system from oxygen toxicity (Pratt et al., 2007). It was speculated that N limitation led to the natural selection of *Azotobacter* spp. over time. Using culture-dependent methods, it was determined that there were significantly lower numbers of *Azotobacter* spp. in the nutrient-supplemented BSFs (P.J. Welz, unpublished data).

In a separate study, the *nifH* gene was used as a marker to monitor the changes in the NFB population in BSF systems exposed to a phenolic cocktail with a final COD concentration of 5842 mg/L and a high C:N ratio (100:0.1) (Ramond et al., 2013). Analysis of terminal restriction fragment length polymorphism (T-RFLP) and denaturing gradient gel electrophoresis (DGGE) results showed that there was a significant structural change in the NFB population after exposure to the phenolic cocktail, with the selection of various *Azotobacter* spp. and *Beijerinckia indica*. In terms of the NFB populations, these results differed from those previously found with kraft-mill wastewater, and in a bioelectrochemical system, where coliforms and *Clostridium* spp., respectively, were thought to be responsible for NF (Gauthier et al., 2000; Knowles et al., 1974; Wong et al., 2014).

In light of these findings, a study was designed to specifically focus on the NFB population in BSFs treating high C:N wastewater. The main aim of the study was to ascertain whether there would be a qualitative and/or quantitative adjustment of the nitrogen-fixing bacterial population in sand bioreactors in response to exposure to synthetic winery wastewater (SWW). It was hypothesised that there would be a structural and functional adaptation of this population. Physicochemical and microbiological results of replicate samples from BSF systems were compared with control replicate samples before and after addition of SWW over the period of 3 months. Changes in the community structure of bacteria harbouring the *nifH* gene were analysed using T-RFLP, next generation sequencing (NGS), and culture-based enumeration techniques.

2. Materials and methods

2.1. Set-up and operation of biological sand filters

Three identical BSFs were used in this study, one control (designated C) and two experimental systems (designated 1 and 2). The systems were installed in an outdoor, undercover environment. The BSFs consisted of sturdy polyethylene containers (1.73 m in length, 1.06 m in width) containing sand to a depth of 0.3 m, with a total volume of 0.502 m^3, and void volume of 0.13 m^3. The sand was a mix from two quarry sites, with a final density of 1.60 ± 0.03 kg/L and porosity of 258 ± 22 L/m^3sand (measured before feeding/amendment). The hydraulic conductivity of 0.15 mm/s (determined by the constant head method) ensured that the systems were capable of draining rapidly, so that the average saturated permeation rate of effluent outflow from the systems before the start of the experiment was 21.1 ± 2.7 L/hr.m^3sand^{-1}. The physicochemical characteristics of the sand are given in Table 1. A more detailed explanation of the set-up and operation is provided in Welz et al. (2014) and Welz and Le Roes-Hill, 2014.

In order to rapidly establish an appropriate microbial population, the BSFs were pre-inoculated with sand taken from other systems previously amended with winery wastewater and/or SWW