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Practical approach to the evaluation of industrial wastewater treatment by the application of advanced microbiological techniques

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ABSTRACT

In cork industry, the operation of boiling raw cork generates large volumes of wastewater named Cork Boiling Wastewater (CBW). The main characteristics are the low biodegradability and medium to low acute toxicity, resulting in the necessity of designing advanced biological treatments by possible conventional activated sludge adaptation. In order to evaluate the variation of bacterial population along that process, a study based on optical microscopy, plate count, DNA extraction, qPCR and massive sequencing techniques was performed. Results showed a diminution of the total and volatile solids (TSS and VSS), jointly with a decrease in DNA concentration, general bacteria (16 S) and ammonia-oxidizing bacteria (AOB). After a few hours of testing, diverse microbiological species died while others showed a possible adaptation of the biological system, accompained by a dissolved organic carbon (DOC) reduction. In addition, toxicity tests based on activated sludge showed the development of chronic toxicity through the contact time. Combination of classical and advanced microbiological techniques, such as quantitative real time Polymerase Chain Reaction (qPCR) and metagenomics, was essential to predict the variation of species during the experiment and to conclude if effective biological adaptation could be finally attained for the target complex wastewater.

1. Introduction

Cork is the outer bark of *Quercus suber*, a common tree species in the Mediterranean region. Due to its properties, cork has a large variety of applications, such as the production of stoppers for wine and other alcoholic drinks bottles. Cork industrial processing involves a boiling step focused on the cleaning, disinfection and moistening of the raw material. This step generates about 400 L of wastewater per ton of cork (Mendonça et al., 2004). The produced effluent, named cork boiling wastewater (CBW), presents low biodegradability and medium acute toxicity values (inhibition percentages between 30% and 60%) (Mendonça et al., 2007). CBW is normally being reused between 20 and 30 times in the own industry, becoming highly concentrated in corkwood organic extracts including relevant contaminants (phenolic acids, tannins, 2,4,6-trichloroanisol, pentachlorophenol, etc.) (Mazzoleni et al., 2005) and so, it results recalcitrant enough to make conventional biological systems unable to treat it. In consequence, the need to search for solutions based on advanced processes to tackle the treatment of this type of wastewater has arisen in the last years (Dias-Machado et al., 2006).

Nowadays, biological treatment based on activated sludge is one of the most common and preferred technology employed for wastewater treatment mainly due to its low operating costs compared to physicchemical oxidation processes, and the high versatility and ability to get adapted not only to different kinds of wastewater (more or less complex) but also to changes in the plant operating parameters. Therefore, big efforts should be done on the design and definition of new decontamination techniques based on advanced biological treatments when possible.

From a general point of view, the possible adaptation of a conventional biological system for attaining the complete depuration of CBW presents several problems related to their low biodegradability and somehow significant acute toxicity coming from the specific contaminants contained in this wastewater, such as polyphenolic compounds (Benitez et al., 2003). The importance and role of the microfauna community in the purification process of activated sludge biological reactors has been well documented in literature (Curds and Vandyke, 1966; Curds, 1973; Curds, 1982; Klimowicz, 1970; Madoni and Ghetti, 1981; Madoni, 1986). The microbial structure of biomass depends on the type of technological system used but, it is possible to

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favor different species in the bio-structure of activated sludge that supports stability and high efficiency process against specific wastewater with special complex characteristics (Ng et al., 1989; Galil and Sheindorf, 2006). Therefore, control and/or influence on variations in the microbial community structure appears as key parameter to find out an efficient advanced biological treatment for facing complex industrial wastewaters.

Up to date, knowledge of microbial community compositions and their dynamics in the wastewater treatment system usually comes from studies employing traditional culture methods, community fingerprinting techniques, and molecular approaches. Optical microscopy observation and culture-based studies help to identify the predominant microbial populations present in WWTPs due to the susceptibility of bacteria growing on microbiological media (Cydzik-Kwiatkowska and Zielińska, 2016). In addition, quantitative real time Polymerase Chain Reaction (qPCR) has been demonstrated to be a useful tool for quantitative analysis of specific microorganisms present in environmental samples. Nevertheless, the design of advanced biological treatments able to tackle with complex industrial wastewater, such as CBW, would require the identification of arisen microbial populations present in the adapted biomass responsible of specific wastewater purification actions (including the degradation of specific recalcitrant compounds). With this objective, emerging molecular biology and new genetic techniques are starting to be applied nowadays. Novel techniques based on genomic sequencing are being developed with the aim of determining dominant population in complex microbial communities (Ansorge, 2009). These new methods will also be useful for studying the diversity of microbial life in organisms inhabiting common environments, (referring to the totality of genomes found) such as activated sludge and marine samples (Hugenholtz and Tyson, 2008).

In this work, the use of microbiological techniques based on the combination of simple optical microscopy observation, culture-based studies with quantitative PCR (Handelsman et al., 1998; Dionisi et al., 2002a,2002b) and microbial genomics (applying metagenomics accomplished at high taxonomic resolution) (Hugenholtz and Tyson, 2008), have been carried out in order to study the adaptation process followed by microbial population from conventional WWTP activated sludge when the system is fed with CBW. The main objective was to define and predict the effectivity of the developed adapted biological system as well as clearly identify the limitations that it should face to attain this complex wastewater decontamination.

2. Materials and methods

Real cork boiling wastewater used in this study was collected at a cork processing plant located in San Vicente de Alcántara (Extremadura, Spain). Conventional activated sludge was provided by the municipal WWTP of El Toyo (Almería, Spain). Main characteristics of CBW and Mixed liquor received from the urban treatment plant are presented in Table 1 (Results and Discussion section).

2.1. Aerobic biological experiments

Biological assays simulating a Batch (Bio)Reactor were performed in a 5 L stirred flask reactor at laboratory scale. The system consisted of a cylindrical PVC reactor tank (17.5 cm depth and 16.5 cm internal diameter) provided with a porous air diffuser placed at the bottom of the reactor. The average temperature was 25 °C, and aeration was directly injected in order to keep dissolved oxygen concentration close to saturation values (~8 mg L^{-1}), providing also a correct agitation of the whole system.

Initially, activated sludge (mixed liquor) taken from the municipal WWTP was maintained in aeration for 24 h in order to attain endogenous phase. Then, and after adjusting CBW pH to about 7, the required volume of wastewater to get an initial Dissolved Organic Carbon (DOC) value of 200 mg L^{-1} in combination with the mixed liquor in endogenous phase was added to the flask. With such initial DOC, influent operating conditions employed in the real municipal WWTP were replicated with the aim of avoiding shock effects on the biological system provoked by higher organic loads and trying to simulate the possible dilution of this complex wastewater when managed in a conventional biological treatment.

Table 1
Cork boiling wastewater and mixed liquor characterization.

Parameters (mg L^{-1})	Mixed liquor	CBW
DOC	12.0	1059
COD	32.0	2968
TSS	7.1	–
VSS	5.6	–
Total nitrogen	0	9.5
Na^+	483.8	32.8
K^+	46.7	353.2
Cl^-	745.7	90.8
NO_3^-	0.7	2.7
NO_2^-	N.d.	N.d.
SO_4^{2-}	275.9	2.5
PO_4^{2-}	22.5	41.9
NH_4^+	N.d.	N.d.
Mg^{2+}	67.7	13.5
Ca^{2+}	102.6	67.9
Trimethylamine	N.d.	N.d.
CH_3COO^-	N.d.	N.d.
Short term biodegradability	–	0.1 (non-biodegradable)
Acute toxicity (inhibition)	–	48%

N.d. non detected.

The complete procedure for microbial population adaptation to CBW took 19 days (458 h). During this period of time, two feeding of the system (operated as a Bath (Bio)Reactor) with CBW were performed. Samples were taken throughout the experiment at different contact times. Total Solids (TSS) and Volatile Suspended Solids (VSS), optical microscopy, plate count, DNA extraction, and Polymerase Chain Reaction (qPCR) were performed in each sample. Massive sequencing techniques were also applied for a group of selected samples.

2.2. Analytical determinations

For analytical purposes, samples from the biological treatment were centrifuged and filtered through 0.22 μm syringe-driven Millex nylon membrane filters (after 30 min of activated sludge settlement). Organic matter was measured as chemical oxygen demand (COD) using Merck® Spectroquant kits, and as dissolved organic carbon (DOC) in a Shimadzu TC-TOC-TN analyzer (model TOC-V-CSN). Total dissolved nitrogen was measured in the same TC-TOC-TN analyzer coupled to a TNM-1 unit. TSS and VSS were determined according to American Standard Methods (American Public Health Association, American Water Works Association, Water Pollution Control Federation, and Water Environment Federation, 1915). Ions and carboxylic acids were quantified by ion chromatography using Metrohm 872 Extension Modules 1 and 2 configured for gradient analysis. Cations and amines were determined using a Metrohm 850 Professional IC configured for isocratic analysis.

2.3. Microbiological analyses

Each taken sample was measured by triplicate in each one of the microbiological analytical techniques reported in this work. Statistical analysis was done by one-way ANOVA and results were highly reproducible ($p < 0.05$). The results showed in graphs are the average of both replicates with standard deviation as error bars.

2.3.1. Heterotrophic plate count (HPC)

The heterotrophic plate count (HPC) or standard plate count