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# Morphology and physiology of anaerobic granular sludge exposed to an organic solvent

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ABSTRACT

The use of quantitative image analysis techniques, together with physiological information might be used to monitor and detect operational problems in advance to reactor performance failure. Industrial organic solvents, such as *White Spirit*, are potentially harmful to granular sludge. In preliminary batch assays, 33 mg $L^{-1}$ of solvent caused 50% relative biomass activity loss. In an expanded granular sludge blanket reactor fed with 40 mg $L^{-1}$ of solvent, during 222 h, the reactor performance seemed to be unaffected, presenting COD removal efficiency consistently >95%. However, in the last days of exposure, the biogas production and the methane content were inhibited. Afterwards, already during recovery phase, the COD removal efficiency decreased to 33%, probably because the reactor was underloaded and the biomass became saturated in solvent only at this stage. In the first hours of exposure the specific acetoclastic and the specific hydrogenotrophic methanogenic activities decreased 29% and 21%, respectively. The % of aggregates projected area with equivalent diameter ($D_{eq}$) > 1 mm decreased from 81% to 53%. The mean $D_{eq}$ of the aggregates ≥0.2 mm decreased, as well as the settling velocity, showing that the granules experienced fragmentation phenomenon caused by the solvent shock load. The ratio between total filaments length and total aggregates projected area (LfA) increased 2 days before effluent volatile suspended solids, suggesting that LfA could be an early-warning indicator of washout events.



## 1. Introduction

Organic solvents comprise a group of various liquid hydrocarbons obtained from the intermediate products of crude distillation. They are used in the chemical industry as solvent for extraction, cleaning, and degreasing, and, in aerosols, paints, wood preservatives, lacquers, varnishes, and asphalt products. The destiny of petroleum type pollutants in the environment has been investigated in many studies [1]. Also, the biodegradation of many components of petroleum hydrocarbons has been reported in a variety of terrestrial and marine ecosystems [2]. Organic solvents are flammable, malodorous and potentially toxic to aquatic organisms and thus require complete elimination by wastewater treatment systems [3]. Due to the hydrophobic nature of hydrocarbons they are mostly bound to the sludge and escape aerobic treatment in a wastewater treatment plant making them present in the anaerobic post treatment [4,5]. It is nowadays undeniable that various toxic organic compounds, such as surfactants and hydrocarbons, like the organic solvent *White Spirit* [6] are found in sewage and industrial wastewaters. A review of the toxicology of mineral spirits, expanding the existing database on the toxicology of this group of hydrocarbon solvents can be found in [7]. Wastewater containing solvents poses a risk to biological treatment systems [8], with emphasis in anaerobic digesters because of their high biomass adsorption capability.

Less than 20 years ago it was a generally accepted idea that hydrocarbons, except for comparably reactive ones such as acetylene, could not be degraded in the absence of molecular oxygen [9]. Meanwhile, studies have reported about the degradation of hydrocarbons in anaerobic systems. Spormann and Widdel [10] present a review focused on the anaerobic degradation of aromatic and saturated hydrocarbons. An anaerobic completely stirred tank reactor fed with pharmaceutical wastewater, experienced a dramatic deterioration in performance in terms of chemical oxygen demand (COD) removal efficiency (almost none) and acetoclastic methanogenic activity (less than 10 mL$CH_4$ g$TVS^{-1}$ $d^{-1}$) and a significant increase in volatile fatty acids (VFA) concentration [11]. Enright et al. [12] used expanded granular sludge bed reactors for the treatment of solvent contaminated wastewater at 15 °C, and achieved COD removal efficiencies of 60–70%. However, despite current knowledge about the anaerobic digestion process little is known about the effects caused by solvents in anaerobic granular sludge.

Combination of accurate morphological parameters, given by quantitative image analysis at micro- and macro-structure levels, with physiological activity of anaerobic granular sludge and

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reactor performance, may provide pertinent information about the stability of high rate anaerobic reactors in the presence of potential disturbances. The usefulness of this methodology in the detection of operational problems was already demonstrated for anaerobic granular sludge in contact with detergent/surfactant [13]. Like surfactants, solvents can disrupt the efficient functionality of anaerobic digesters when a redundant failure occurs because of its widespread use in industry. This work focuses on the study of effects caused by the exposure of anaerobic granular sludge to the organic solvent *White Spirit*, which is the most widely used solvent in the paint industry.

## 2. Methods

### 2.1. Solvent characterization

The solvent tested is a paraffin-derived transparent liquid, which is a common organic solvent used in extraction, cleaning and degreasing industrial processes. It is a mixture of saturated aliphatic and alicyclic C7 to C12 hydrocarbons with a maximum content of 25% of C7 to C12 alkyl aromatic hydrocarbons. The solvent presents a density (15 °C) of about 785 kg $m^{-3}$.

### 2.2. Toxicity assays

The effect of an inhibitor in the methanogenic activity of a specific trophic group may be determined following the increase of biogas, in sealed vials.

After biomass acclimation at 37 °C and 150 rpm, toxicity batch experiments were performed by adding sodium acetate (30 mM) and increasing solvent concentrations (1.6, 7.9, 39.3, and 78.5 mg $L^{-1}$) to the sludge, in batch vials. Working volume was 12.5 mL, and total volume was 25 mL. Fifty percent inhibition concentration ($IC_{50}$) was defined as the solvent concentration that caused 50% relative acetoclastic activity loss. All batch experiments were performed in triplicate assays.

### 2.3. Experimental set-up

A Plexiglas expanded granular sludge blanket (EGSB) reactor was used. The reactor had a height of 1.95 m and internal diameter of 21 mm. The working volume was 1.15 L and the up-flow velocity was 4.0 m $h^{-1}$. Temperature was kept at 37 ± 1 °C by means of an external jacket for water circulation.

### 2.4. Inoculum and substrate

The EGSB reactor was inoculated with 400 mL of granular sludge. The biomass was characterized in terms of specific methanogenic activity (SMA) with sodium acetate (212 ± 27 mL$CH_{4@STP}$ g$VSS^{-1}$ $d^{-1}$) and $H_2/CO_2$ (910 ± 85 mL$CH_{4@STP}$ g$VSS^{-1}$ $d^{-1}$) as substrates. The morphological characteristics assessed by quantitative image analysis were: filaments length/total area of aggregates, LfA = 24 $mm^{-1}$; total filament length/volatile suspended solids = 1800 m g$VSS^{-1}$; volatile suspended solids/total projected area = 13 g $m^{-2}$. The volatile suspended solids (VSS) were 26.5 g $L^{-1}$ and settling velocity was 26 ± 14 m $h^{-1}$.

The reactors were fed with 1.5 gCOD $L^{-1}$ of ethanol. Sodium bicarbonate was added as the alkalinity source (2 g $L^{-1}$). Micro- and macro-nutrients were added [14].

When the reactor was operating in steady-state, with stable values, either in terms of COD removal efficiency, SMA or morphological parameters, the solvent (40 mg $L^{-1}$) was mixed with the feeding, with constant agitation, during 222 h. The recovery phase was followed through 7 days.

### 2.5. Routine analysis

The COD and VSS were determined according to Standard Methods [15]. Biogas flow rate was measured by a Ritter Milligascounter (Dr. Ing. Ritter Apparatebau GmbH, Bochum, Germany). Methane content of biogas was determined by gas chromatography using a Porapack Q (100–180 mesh) column, with helium as the carrier gas at 30 mL $min^{-1}$ and thermal conductivity detector. Temperatures of the detector, injector, and oven were 110, 110, and 35 °C, respectively. VFA and ethanol were determined by high performance liquid chromatography using an HPLC (Jasco, Japan) with a Chrompack column (6.5 mm × 30 mm); sulfuric acid (0.01N) at a flow rate of 0.7 mL $min^{-1}$ was used as mobile phase. Column temperature was set at 60 °C. Detection of VFA and ethanol was made sequentially with an UV detector at 210 nm and a RI detector, respectively.

### 2.6. Biomass sampling

One of the most critical steps in this methodology is the sludge sampling. A sampling device was used to take biomass from the reactor without disturbing its morphology [16]. It was introduced at the top of the reactor and biomass was collected to the tube, along the reactor to get homogeneous and representative sample, avoiding mechanical stress. All the sludge samples were characterized by image analysis, SMA assays, settling velocity, and VSS content.

### 2.7. Specific methanogenic activity assays

The SMA tests were performed using a pressure transducer technique [17]. The specific acetoclastic activity (SAA) was measured in the presence of sodium acetate (30 mM), and the specific hydrogenotrophic methanogenic activity (SHMA) was measured in the presence of $H_2/CO_2$ 80:20 (v/v), at 1 bar. No trace-nutrients were added. Methane was measured by gas chromatography with helium as the carrier gas and a TCD detector.

### 2.8. Biomass dilution

Biomass samples must be diluted for image analysis using an optimized dilution factor. When the dilution is excessive the observer may unconsciously search objects over estimating them. If the dilution is insufficient, the objects will be overlaid. The optimal dilution value was determined as the lowest dilution that enabled the maximum percentage of objects to be recognized. The percentage of recognition is the ratio between the area of objects that are completely inside the image and the total area of objects in the image including those that are at the boundaries and cannot be completely recognized. In these experiments, the optimal dilution was 1:5.

### 2.9. Image acquisition, processing, and analysis

For the acquisition of filaments and micro-aggregates (equivalent diameter ($D_{eq}$) < 0.2 mm) images, a volume of 35 μL from the diluted sample was distributed on a slide and covered with a 20 mm × 20 mm cover slip for visualization and image acquisition. This volume was exactly covered by the cover slip. Each image corresponded to a volume of 0.0445 μL. Then, more than 120 images were acquired. Image acquisition was obtained by dividing the cover slip into 42 identical fields and taking a photo in each imaginary square. At least three slides were examined to minimize sampling errors. Concerning to macro-aggregates ($D_{eq} \geq 0.2$ mm) images, an arbitrary volume was transferred to a Petri dish for visualization and image acquisition. All the aggregates present in that