# ENHANCING THE MICROBIAL REMOVAL OF ORGANIC MICROPOLLUTANTS IN WASTEWATER TREATMENT PROCESSES

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## ABSTRACT

The discharge of trace levels of organic micropollutants (OMPs) to water bodies from wastewater treatment plants has raised concerns over the adverse effects on the health of aquatic and terrestrial organisms. Operational costs of current OMP removal technologies (e.g. reverse osmosis and UV treatment) are prohibitive expensive for many water treatment facilities. Previous studies have demonstrated that oxidative stress can induce the synthesis of oxidoreductases, which can catalyze OMP hydroxylation and further biodegradation. We present the results of an experimental study to promote OMP removal from farm and municipal wastewater by inducing enzyme production using dynamic oxygen control. Microbial consortia from dairy farm runoff sludge was cultivated in fed-batch reactors (FBR) given synthetic wastewater containing acetate as carbon source and a mixture of ten OMPs (trimethoprim, sulfamethoxazole, tylosin, carbamazepine, ibuprofen, naproxen, triclosan, sucralose, atrazine and nonylphenol) at a concentration of 0.1 mg/L. Removal of OMPs was caused by a combination of biotic and abiotic factors. Triclosan, atrazine and trimethoprim were largely removed by non-biological processes. Cyclic perturbation of dissolved oxygen concentration enhanced the biodegradation of ibuprofen, sulfamethoxazole, tylosin, nonylphenol and naproxen. These results indicate that cyclic DO perturbation in biological treatment processes can enhance OMP removal, however the specific mechanism behind this behavior is yet to be elucidated.

## KEYWORDS

**Wastewater treatment, emerging contaminants, organic micropollutants, whole-cell biocatalysts, oxidoreductases, biodegradation,**

## 1 INTRODUCTION

The world's fresh water resources receive thousands of modern, man-made organic chemicals including pharmaceutical compounds and agrochemicals (Luo et al., 2014; Oulton et al., 2010). New chemicals are continuing to be developed to satisfy the demand for new applications and products. For instance, the USA registry of chemical compounds currently references more than 72 million chemicals with 15,000 new compounds added daily (Kolvenbach et al., 2014). Inevitably, a large number of these chemicals will be released into the environment during their life cycle.

Over the past decade, the rapid increase in instrument sensitivity has led to the widespread detection of many man-made chemicals in fresh waterbodies at ng/L levels. These xenobiotic compounds include pharmaceuticals, personal care products, steroid hormones, industrial chemicals, and pesticides (Galloway et al., 2010; Oulton et al., 2010; Petrie et al., 2014a; Vandenberg et al., 2013). At low concentrations, (e.g. nano-gram per litre) these organic micro-pollutants (or OMPs) can reduce the quality of receiving waters, their associated ecosystems services and human health (Roig et al., 2013; Vos et al., 2000). Discharges from wastewater treatment plants are acknowledged as a major source of these contaminants into receiving environments (Loganathan et al., 2009; Spongberg and Witter, 2008). Various studies have highlighted the need to remove the ng/l levels of these

contaminants prior to the discharge of treated wastewater. For example, a recent critical review concluded that doses of Bisphenol A up to four orders of magnitude lower than the reported effect level of 50 mg/kg/day adversely affected mammals (Vandenberg et al., 2013) and another study reported changes in sex hormones associated with exposure to Bisphenol A in men (Galloway et al., 2010). Nevertheless, ecotoxicity data is available only for a few micropollutants, so the real dimension of the effect of these contaminants on water resources is yet to be fully assessed. Additionally, the cost of treating impacted environments can be large. For instance, the cost of cleaning up London's waterways contaminated with excreted contraceptive pill residues is estimated to exceed £30bn (McKie, 2012). Therefore development of efficient and cheap technologies is needed to convert wastewater into a water resource, potentially even for meeting potable needs.

# 2 TECHNOLOGIES TO REMOVE ORGANIC MICROPOLLUTANTS FROM WASTEWATER

Current technologies to remove OMPs from wastewater can be placed three categories: a) advanced oxidation technologies; b) filtration technologies; and c) biological degradation technologies. Ozonation and UV treatment are examples of advanced oxidation processes; ultra-filtration, reverse osmosis and membrane bioreactors (MBR) are examples of filtration technologies; and the activated sludge processes is a biodegradation base technology. In general advanced oxidation and filtration technologies are prohibitively expensive while biological degradation is often ineffective in removing the majority of OMPs (Table 1). Oulton et al. (2010), found that the variability of OMP removal performance of different treatment technologies is very large. For instance, the average (± st.dev.) fraction of OMP remaining from primary and secondary treatments is 0.39±0.25, for membrane bioreactors is 0.13±0.6 while for ozonation is 0.06±0.1 and reverse osmosis is 0.02±0.0 (Oulton et al. 2010). In another study, Luo et al., (2014) found that the efficiency of 12 different wastewater treatment plants to remove 36 different OMPs was also highly viable. For instance, micropollutants like Atrazine, Carbamazepine, Erythromycin, Diclofenc and Sulfametoxazole are very poorly removed (i.e. 20 – 30 % of removal efficiency) during conventional wastewater treatment and therefore reach fresh water bodies.

*Table 1: Current technologies to remove organic micropollutants, advantages and disadvantages*

| Technology | Advantages | Disadvantages | Approximate Cost ($USD/L) | Reference |
|---|---|---|---|---|
| Advanced oxidation | Efficient and easy to operate | High energy consumption and cost | $15-0.14 | Chong et al., (2012) |
| Membrane filtration | Compact, selective separation, no chemical addition | Membrane fouling and costly | $7-0.3 | Guo et al., (2012) |
| Biological degradation | Already used in the majority of wastewater treatment facilities | Not efficient (50-60% removal efficiency). Not designed to deal with OMPs | $0.15-0.008 | Oulton et al., (2010) |

# 3 ENHANCING BIOLOGICAL DEGRADATION OF MICROPOLLUTANTS

While biological treatment processes are significantly cheaper to build and operate than advanced oxidation or filtration based processes, current designs are not capable of delivering high quality water free of OMPs. Biological systems are designed to effectively remove substances present at mg/L concentrations but not at the µg or ng/L range. This explains the high variability of OMP removal efficiency commonly observed during this kind of wastewater treatment. Consequently, biological treatment processes have to "evolve" in order to achieve

efficient and consistent OMP removal from wastewater. We envision that there are three general plant configurations to do this upgrade (Figure 1) (Singhal and Perez-Garcia, 2016): i) Enhance OMP biodegradation in an existing reactor/process; ii) Implement an additional reactor/processes dedicated to biodegrade OMPs in the effluent of a secondary treatment, this advanced treatment process would replace or complement a advanced oxidation or filtration treatment; and iii) Implement additional reactors/processes specifically dedicated to produce catalytic agents to biodegrade OMPs (e.g., specific microbes or enzymes). Recent studies indicated that it is possible to maximize OMP removal of the conventional biological treatment (i.e. activated sludge process) by increasing the retention time (SRT, above 15 days) and the hydraulic retention time (HRT, above 18h) (Petrie et al., 2014b). This enhanced OMP degradation behaviour is ostensibly related to the concomitant reduction in food: microorganism ratio. However, extending HRT compromises process efficiency and increases the concentration of aqueous metals in effluent, which compromises Government regulations compliance (Petrie et al., 2014b). Therefore high efficiency plant configuration most likely will involve the implementation of a new ON- or OFF- line process specifically dedicated to enhance OMP biodegradation. Micropollutant removal indeed occurs through the whole wastewater treatment operation. However processes specifically focusing on the removal of recalcitrant contaminants should complement or replace the advanced treatment stages. The new process inputs should come from waste streams of the treatment facility and take advantage of the infrastructure and resources already in place (Singhal and Perez-Garcia, 2016).

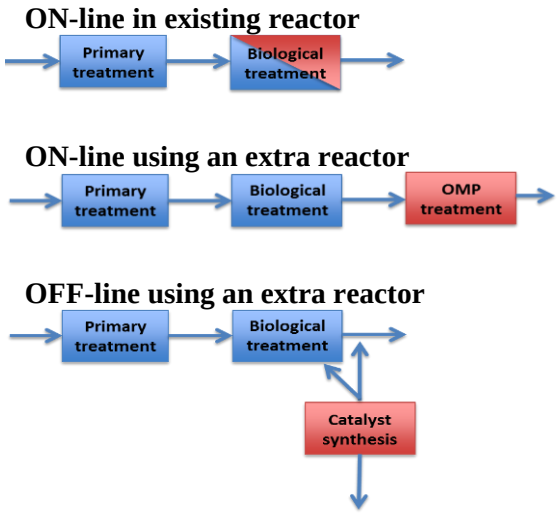



*Figure 1: Conceptual modifications of wastewater treatment to achieve OMP removal using biological treatments. Red boxes indicate unit operations dedicated to enhance the biological degradation of OMP in the wastewater treatment plant.*

Complex microbial consortia which allow interactions among bacteria-bacteria, bacteria-archaea and bacteria-fungi can be developed to enhance biological degradation of micropollutants. Also enzymes such as laccase and Cytochrome P450 have been shown to efficiently degrade a vast array of organic micropollutants in pure enzyme assays (Lah et al., 2011). Despite the demonstrated effectiveness of these enzymes, their use in wastewater treatment is just starting to be investigated and has not been implemented in pilot or full scale (Lah et al., 2011). Fungi can be activated by inducing the production of high levels of oxidase enzymes and at the same time stimulating enzyme activity (e.g. by providing $H_2O_2$ as cofactor for bacterial Cytochrome P450). By controlling the carbon source and electron acceptor regimens, it is possible to induct biocatalyst expression and activation (Chubukov et al., 2014; Price et al., 2013).

Singhal and Perez-Garcia, (2016) recently proposed an approach to induce the synthesis of OMP degrading enzymes by exposing microbes to cycles of stressing and non-stressing environmental conditions. High dissolved oxygen concentrations (> 6 mg/L) and hard to degrade organics (e.g. starch, lignin or cellulose) impose physiological stress to microbial cells. Stress responses involve the synthesis of OMP degrading oxidoreductase enzymes (i.e. peroxidases, cytochromes and laccase). Stressing conditions stimulate an initial synthesis of oxidoreductase. However, long term stress compromises cell growth and eventually biocatalyst

synthesis. Therefore a non-stressing period is required to replenish cells with energy and carbon to continue further oxidoreductase synthesis and activation. In this paper we present the first results of a multidisciplinary project investigating this oxidoreductase induction mechanism.

# 4 RESEARCH APPROACH AND METHODS

## 4.1 RESEARCH APPROACH

Our research approach involves four main research areas, all of them around the concept of induction and regulation of oxidoreductase synthesis (Figure 2): i) process development using mixed microbial cultures; ii) pure microbial culture to investigate fundamental mechanisms of oxidoreductase induction; iii) development of methods for wide identification and quantification of oxidoreductase and their catalytic activity; and iv) development of methods to quantify oxidoreductase on-line using biosensors In this paper we will present only our preliminary results in the area of process development using mixed microbial cultures.

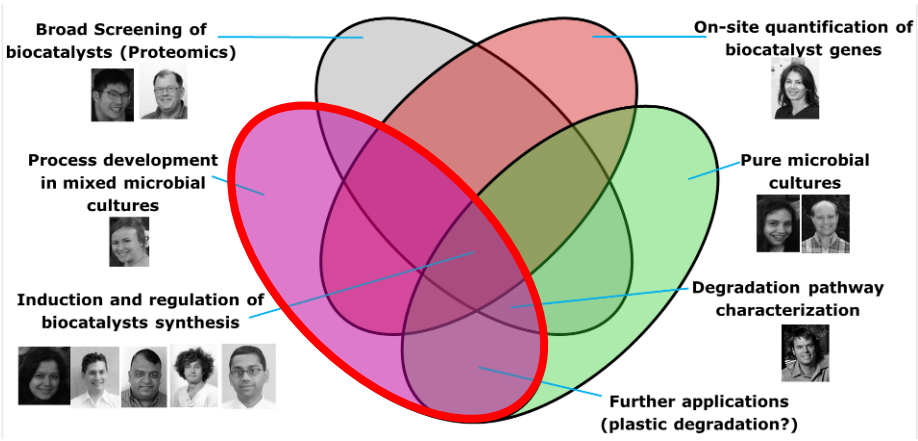



*Figure 2: Multidisciplinary research approach and collaborators*

## 4.2 METHODS

Our pipeline of research methods, depicted in Figure 3, involves the selection and collection of specific biocatalyst sources, the cultivation of microbes in laboratory scale bioreactors where we expose the microbes to cycles of environmental perturbations and the posterior analysis of culture samples using different analytical and molecular biology techniques which are described below.

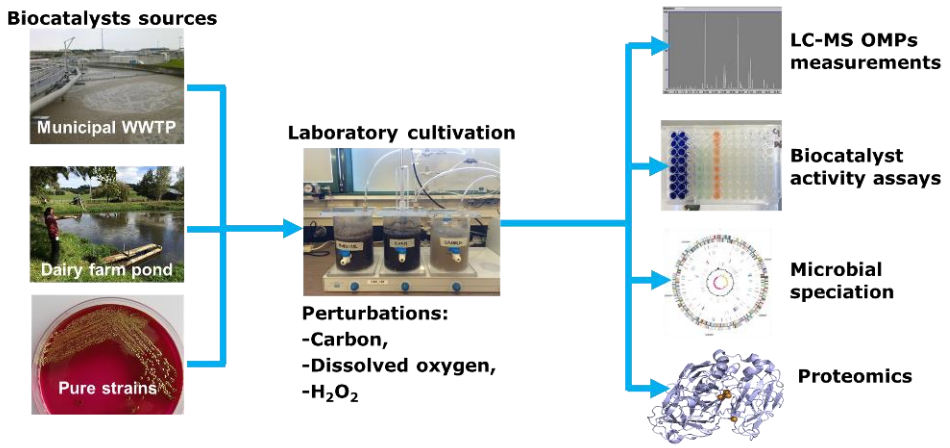
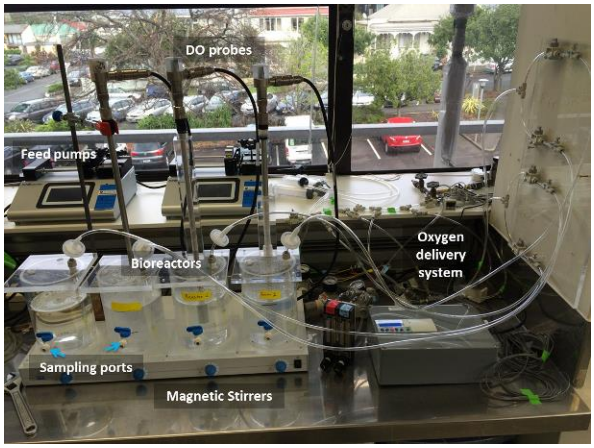



*Figure 3: Pipeline of methods used to investigate biocatalyst synthesis and OMP removal.*

### 4.2.1 EXPERIMENTAL SET UP

We investigated oxidoreductase activation and OMP removal using laboratory scale mixed microbial cultures. Culture's tested conditions were 14 different regimes of ON-OFF dissolved oxygen (DO) supply cycles. The fourteen conditions were the result of combinations of different durations of high DO perturbation (5 or 30 min) and different perturbation frequencies (4, 2, 0.5, 0.25 and 0.16 ON-OFF cycles per hour). Non-perturbed (constant), high (6-7 mg/L) and low (0.1-0.5 mg/L) DO concentration conditions were tested as control experiments. Also, cultures of autoclaved biomass were tested to quantify the removal of OMPs through biomass absorption. The cultures were done in 1 L bioreactors inoculated with 100 mL of washed microbial activated sludge collected from a dairy farm pond containing run-off wastewater from stables and milking areas. The cultures were operated in fed-batch mode using concentrated synthetic wastewater with a mixture of OMPs as influent supplied at a rate of 0.034 mL/min during 48 hours. This operation mode was selected to maintain a steady supply of nutrients for a prolonged time (48h) without implementing and additional settling tanks and pumps for returning activated sludge. An electronic DO supply and monitoring system was implemented to control the cycles of DO oxygen exposure (Photograph 1). The synthetic wastewater contains 600 mg/L of acetate as carbon source, 60 mg/L $NH_4^+$ as nitrogen source and 0.1 mg/L of each of the ten following OMPs: the veterinarian and human antibiotics Trimethoprim (TMP), Sulfamethoxazole (SMX) and Tylosin (TYL)); the pharmaceuticals Carbamazepine (CBZ), Ibuprofen (IBP) and Naproxen (NPX); the disinfectant Triclosan (TCS), the artificial sweetener Sucralose (SCL), the agrochemical Atrazine (ATZ) and the industrial chemical Nonylphenol (NP). These OMPs were selected as they are recalcitrant, have eco toxicological effects and are commonly found in municipal and farm wastewater




*Photograph 1: Experimental set up used to evaluate the removal of organic micropollutants and oxidoreductase production*

### 4.2.2 MEASUREMENT OF OMPS USING LC-MS

OMP concentration measurements were done using liquid chromatography coupled with mass spectrometry (LC-MS) method in the following way. At the end of the cultivation cycles 200 mL of culture's liquid phases (without biomass) were sampled and centrifuged to remove suspended particles (18,000 g for 20 minutes at -4°C). OMPs in centrifuged samples were extracted by solid phase extraction (SPE) using 500 mg hydrophilic-lipohilic balance (HBL) cartridges from Waters Corp (USA) and recovered in 10 mL of methanol following the method described in Vanderford et al., (2003). Then, the latter was quantified via LC-MS analysis using Shimadzu 2020 Series LC-MS (Shimadzu, Japan) equipment with an Agilent ZORBAX Eclipse Plus C18 column (Agilent Technologies, Germany) following the method described in Ferrer et al., (2008).

### 4.2.3 ENZYME ACTIVITY ASSAYS

The activity of oxidoreducases in cultures' biomass was measured spectrophotometrically using colorimetric assays in 96-well micro-plate format. Culture samples (30 mL) were harvested, centrifuged (13,000 rpm for 20

minutes at -4°C) and resuspended in 10 mL of 10 mM acetate and 100 mM phosphate buffer. Then, 100 µL aliquots of resuspended biomass were incubated with 200 µL of a chromogenic dye in micro-plate wheels for 24 hours. Table 2 provides the details of the target oxidoreductases and the dyes used to detect their activity. A Victor X3 Multimode Plate Reader (PerkinElmer, USA) was used to register changes in absorbances generated by chromogenic reactions between the biomass and the dyes.

*Table 2: Targeted enzymatic activities and dyes used to detect targeted oxido-reductases.*

| Enzymatic activity targeted | Dye |
|---|---|
| Lignin Peroxidase (LiP) | Methylene blue,<br>Azure B |
| Horse Radish Peroxidase (HRP) | L-DOPA,<br>ABTS |
| Laccase (Lacc) | ABTS,<br>Sudan orange |
| Cytochrome p450 (CytP450) | pNP-dodecanoate, Indole |
| β-glucosidase (β-glu, not oxidoreductase) | pNP-Acetylglucosaminide, pNP-glucopyranoside |

### 4.2.4 MICROBIAL SPECIATION

Microbial speciation of cultures' biomass was analysed to identify bacterial species capable of synthetizing oxidoreductases. Species identification was done by sequencing and comparing 16S ribosomal RNA (rRNA) gene copies in biomass following the method described by Lane, (1991). Total genomic DNA was isolated using the standard protocol of the PowerSoil DNA isolation kit (MoBio, Carlsbad, USA). All extractions were done in duplicate. Quantification of extracted DNA purity was done spectrophotometrically with a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) at A260/280nm wavelength ratio. 16S rRNA genes were amplified from extracted DNA using the polymerase reaction mixture KAPA HiFi Hot Start Ready Mix PCR kit) together with the universal 16S Illumina forward and reverse V3 and V4 ultramer primers (Klindworth et al., 2013), in a 9700-PCR thermocycler machine. All PCR reactions were run in duplicate. Qualitative and quantitative evaluation of 16S rRNA gene amplicons was performed by agarose gel electrophoresis and Qubit Assay on a Qubit 2.0 Fluorometer (Invitrogen, USA). The sizes of 16S rRNA amplicons were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Amplicons were sequenced using the Illumina MiSeq System of the University of Auckland. Finally, phylogenetic classification (genus or species) of bacterial community based on variable regions in 16S rRNA gene were assessed using Qiime, Usearch and Mothur bioinformatic software (The University of Michigan, USA).

# 5 RESULTS AND DISCUSSION

## 5.1 CYCLIC DISOLVED OXYGEN PERTURBATIONS

Cyclic perturbations of oxygen supply generated transitions between aerobic (>3 mg-DO/L) and micro-aerobic (0.1-0.5 mg-DO/L) conditions in cultures. Although, this oscillatory behavior was only observed using perturbation frequencies below 0.5 cycles/h. Figure 4 shows that the low frequencies allowed the biomass to completely deplete the dissolved oxygen (DO) added during the oxygenation phase of the perturbation cycle. DO consumption rates were not influenced by different frequencies, which suggests that cell metabolism is not affected by periods of exposure to micro-aerobic conditions.

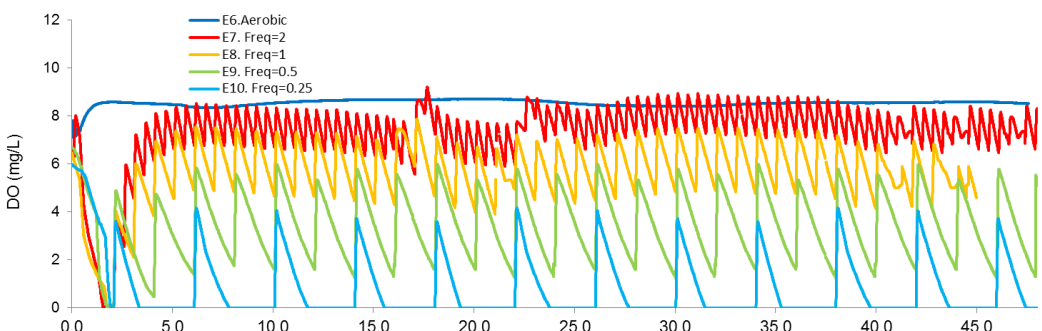


*Figure 4: Profile of dissolved oxygen in cultures with different frequencies of cyclic oxygen perturbation*

## 5.2 ORGANIC MICROPOLLUTANT REMOVAL

The tested DO supply regimens modified the OMP removal efficiency of cultures. The removal of Ibuprofen, Sulfamethoxazole, Tylosin, Nonylphenol and Naproxen was enhanced by exposing cultures to cyclic DO perturbations which indicates that the perturbing DO cycles actively enhanced their biodegradation (Figure 7, shocked experiments) although, there is no clear correlation between the perturbation frequencies and the removal of these compounds. The net amount of OMP removed from the synthetic wastewater was combination of biotic (biodegradation) and abotic (sorption) factors as control cultures without biological activity (autoclaved biomass) also resulted in decreased concentrations of OMPs. Triclosan, Atrazine and Trimethoprim were removed non-biologically as controls experiments using autoclaved biomass presented removal efficiencies similar to the ones with active biomass. A removal efficiency of 58±13 % was observed in cultures under continuous microaerobic conditions (non- perturbed cultures) while that observed in continuous aerobic conditions was of 39±24 %.

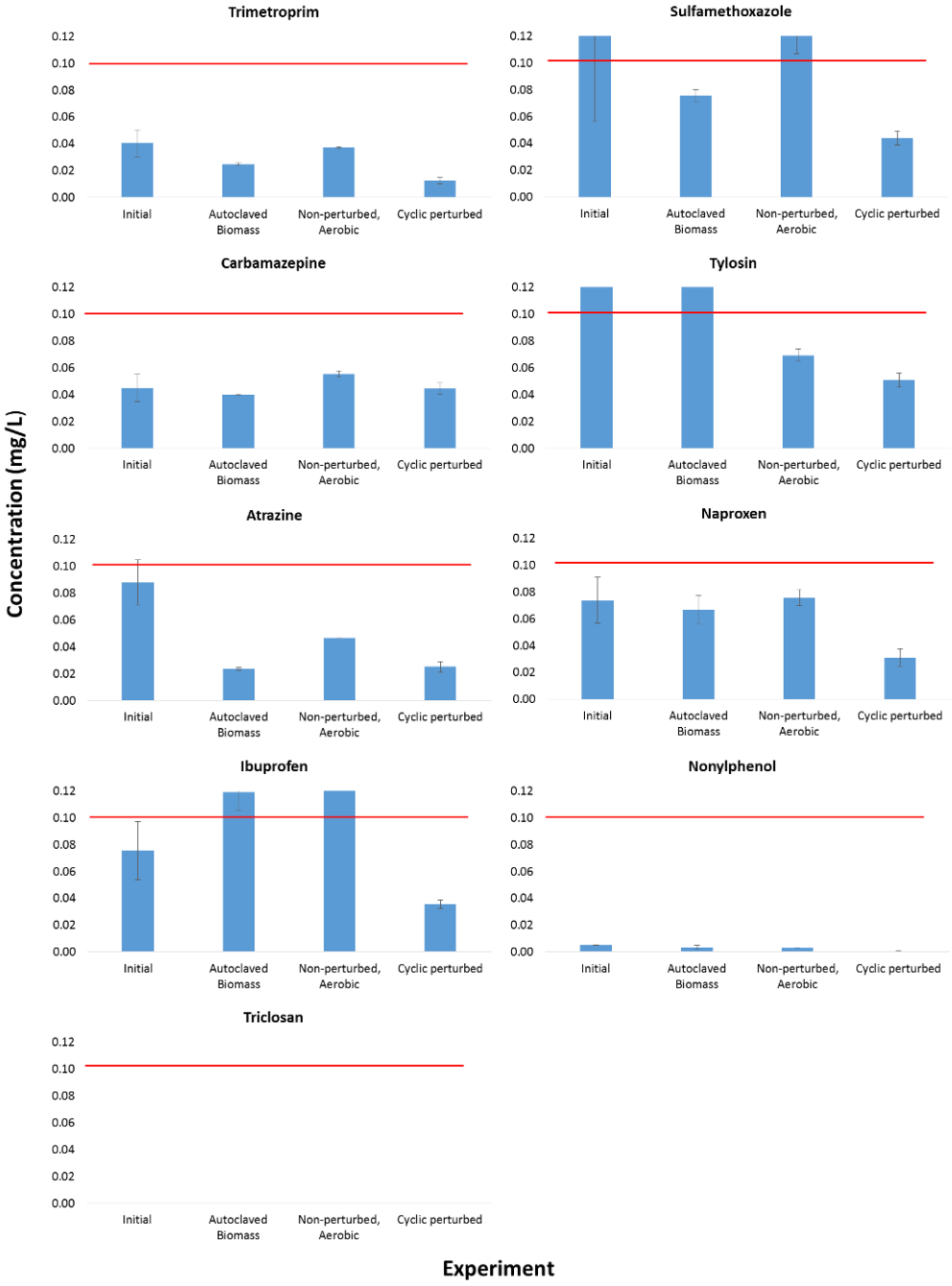


*Figure 7: Concentration of OMP measured in cultures effluent at the end of the 48 hour cultivation cycle. Results from initial measurement (hour 0) are also shown for comparison. The redline indicates OMP concentration initially added to the reactor*

## 5.3 ENZYME ACTIVITIES

Cyclic DO perturbations affected the enzyme activity profile of biomass. In general, cyclic perturbations induced the expression of Cytochrome P450 activity, cultures with lower perturbation frequency (0.25 and 0.16) presented the highest CytP450 activity, which indicates that oscillations of cells redox states activates the activity of this enzyme. Activity of Lignin Peroxidase and Horse Radish Peroxidase was observed in all tested conditions except in autoclave biomass indicating that biomass from farm wastewater ponds continuously express and activates these enzymes independently of the environmental dissolved oxygen.

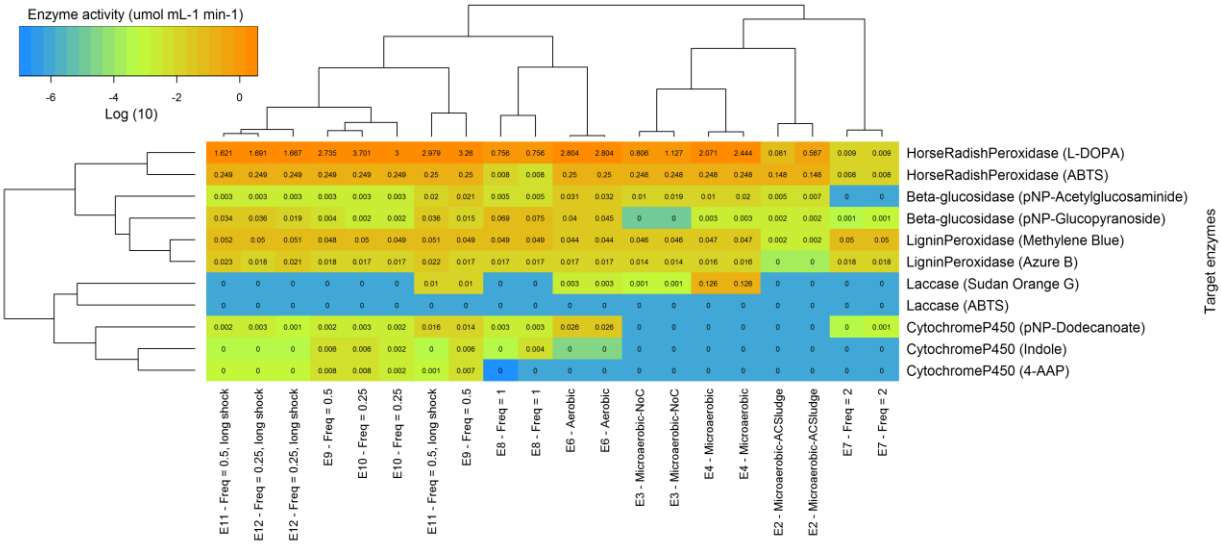



*Figure 8: Results of biomass enzyme activity assays*

## 5.4 MICROBIAL SPECIATION (PRELIMINARY FINDINGS)

Quantification of extracted Genomic DNA (A260/280nm) showed the highest concentration in perturbed sludge samples, which indicatesgreater cell growth under these conditions in comparison to non-perturbed experiments. Figure 9a shows the agarose gel electrophoresis of PCR products indicating that 16S rRNA genes were successfully amplified in all samples. Fgure 9a shows that the PCR products have the same length (500 bp) as the one commonly reported for this biomarker gene (Klindworth et al., 2013). This result was further verified by the bioanalyzer test (Figure 9b), which confirmed that the amplicons sizes were of ~550 bp.

Amplicon band intensity further revealed that perturbed sludge has more 16S rRNA gene copy numbers than both the unperturbed (continuous aerobic and micro-aerobic conditions) and the sludge used as a culture inoculum. The nucleotide sequences of amplified 16S rRNA gene are currently being analysed for phylogenetic classification and species identification.

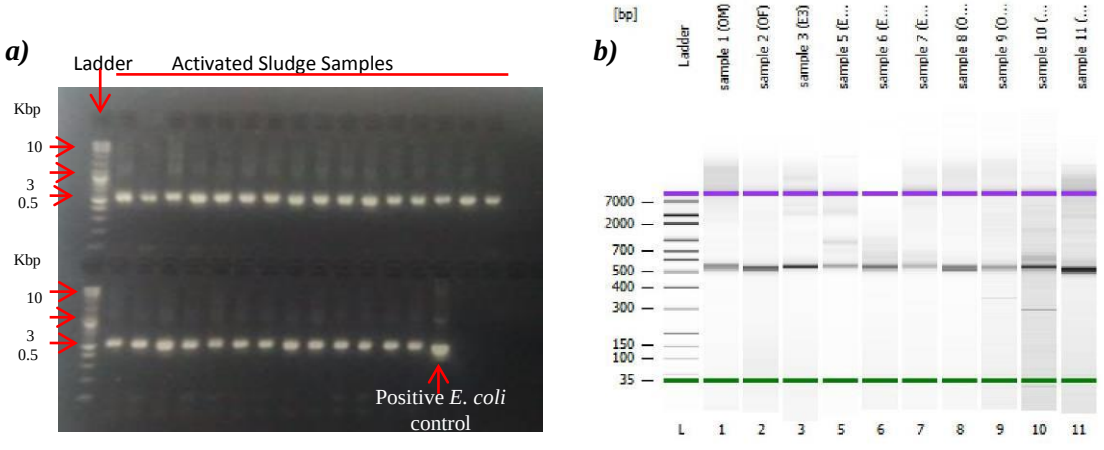


*Figure 9: Microbial speciation results: a) Agarose gel electrophoresis of the PCR products showing 16S rDNA gene fragments in DNA extracted from cultures biomass, b) Bioanalyzer Trace results showing DNA fragments of 550 lengths obtained after the PCR amplification step.*

# 6 CONCLUSIONS

- Removal of OMPs is given by a combination of biotic and abiotic factors. Each of these factors has a major influence depending on the specific organic pollutant. In general, Triclosan, Atrazine and Trimethoprim are removed non-biologically.

- Cyclic DO perturbation enhanced the biodegradation of specific OMPs, this effect was clearly observed for the pollutants Ibuprofen, Sulfamethoxazole, Tylosin, Nonylphenol and Naproxen.

- Different frequency of DO perturbation did not significantly impacted the removal of OMPs, although they did affect the Cytochrome P450 activity of biomass.

- Activity of Lignin Peroxidase and Horse Radish Peroxidase was observed in all conditions, which indicates that these oxidoreductase are commonly synthetized in microbial sludge from wastewater farm ponds, independently of the environmental DO. These results indicate that microbes in wastewater ponds of farms can be a potential source of valuable catalytic compounds.

- Ongoing analysis of protein and microbial species profile will further reveal which microbes and enzymes could be playing a major role in the biodegradation process.


## ACKNOWLEDGEMENTS

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