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Treatment of micropollutants in municipal wastewater: study of a sequential batch biofilter

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Abstract

All the experimental part of this final project was done at Laboratoire de Biotechnologie Environnementale (LBE) from the École Polytechnique Fédérale de Lausanne (EPFL), Switzerland, during 6 months (November 2013- May 2014).

A fungal biofilter composed of woodchips was designed in order to remove micropollutants from the effluents of waste water treatment plants. Two fungi were tested: *Pleurotus ostreatus* and *Trametes versicolor* in order to evaluate their efficiency for the removal of two micropollutants: the anti-inflammatory drug naproxen and the antibiotic sulfamethoxazole,.

Although *Trametes versicolor* was able to degrade quickly naproxen, this fungus was not any more active after one week of operation in the filter. *Pleurotus ostreatus* was, on contrary, able to survive more than 3 months in the filter, showing good removal efficiencies of naproxen and sulfamethoxazole during all this period, in tap water but also in real treated municipal wastewater.

Several other experiments have provided insight on the removal mechanisms of these micropollutants in the fungal biofilter (degradation and adsorption) and also allowed to model the removal trend.

Fungal treatment with *Pleurotus ostreatus* grown on wood substrates appeared to be a promising solution to improve micropollutants removal in wastewater.

Introduction

Many organic micropollutants (pharmaceuticals, personal care product, biocides and pesticides) found in municipal wastewater are poorly removed in conventional wastewater treatment plants, resulting in a constant input into the aquatic environment. As these compounds are designed to be biologically active, they can affect sensitive aquatic organisms even at low concentrations (Margot, Bennati-Granier et al. 2013). To reduce the release of these compounds into surface water, complementary treatments are necessary (Margot, Kienle et al. 2013).

Some white rot fungi produce very powerful oxidative exoenzymes, such as laccase, lignin and manganese peroxidases when they grow on wood chips (Hakala, Lundell et al. 2005), and whole fungi have even higher oxidative potential than exoenzymes alone (Harms et al, 2011). Because of this oxidative power, fungi are able to degrade lignin (Leonowicz, Matuszewska et al. 1999) to access to cellulose and hemicelluloses, using these last two compounds as a substrate. By using the same enzymatic tools, they are also able to degrade a wide range of micropollutants.

1 Goals

Taking benefit of all the characteristics of white rot fungi, the goal of this study was to develop an innovative pilot biofilter in order to remove micropollutants from waste water. The biomass of the system was white rot fungi using wood chips as a support. Two kinds of fungi were tested: *Trametes versicolor* and *Pleurotus ostreatus*. Several experimentations were done with this system adding micropollutants in the medium in order to check the efficiency of the system, the life span, etc.

2 Material and methods

2.1 Selection of micropollutants

Two micropollutants were selected for this experimentation due to (i) their low removal rate (around 40%) in conventional wastewater treatment plants (WWTPs) (low bacterial degradability), (ii) their ubiquity in WWTP effluents at significant concentrations (100-500 ng/L) (Margot, Kienle et al. 2013), close to their limit of aquatic toxicity (600-1700 ng/L[1]) and (iii) their potential to be degraded by white-rot fungi. These two pollutants were the anti-inflammatory drug naproxen and the antibiotic sulfamethoxazole. These two compounds cannot be degraded by exoenzymes such as laccase produced by white rot fungi (previous experiences done at LBE) but can be degraded intracellularly. It was decided to use micropollutants that cannot be degraded by extracellular laccase in order to assess the

[1] Source: http://www.oekotoxzentrum.ch/expertenservice/qualitaetskriterien/vorschlaege/index_EN

potential of the whole fungal cells, which is less known but possibly more promising than the potential of extracellular enzymes alone (Harms H, et. al 2011).

The initials concentrations of micropollutants used during the experimentation were of 10 mg/L or 5 mg/L because it was not possible to detect values of concentration below 0.3 mg/L.

2.2 Characteristics of the reactor

As a reactor, a biofilter made of wood chips colonized by fungal mycelium was used as it will be described more in detail in this section.

First decision was to choose the best filter configuration: two possible options of filters were considered to be implant for the experimentation: trickling filter and sequential batch filter.

Trickling filter was the first filter configuration tested. All the elements of this system were: a glass bottle to collect the filtered water, a glass column with the inoculated wood with fungal mycelium, a pump, to impulse the water from the glass bottle to the top of the column, and pipes, to make possible to drive the water from the glass bottle to the top of the column. The operation was this: the medium from the bottle was driven through pipes to the top of the column with the power of the pump. Once the water reached the top, it went down trickling through the filter (unsaturated conditions) until reaching again the bottle. This was a continuous system and the water was always reused (**Figure 1**).

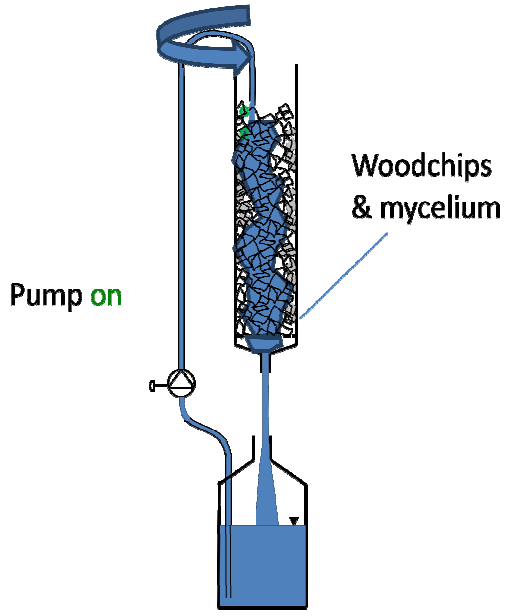

Figure 1: Trickling filter with a rotator system for the water spread.

This kind of configuration had a lot of problems due mainly because of preferential pathways of water in the filter. To avoid that, a rotating system was designed, so the water was spread to the top of the column in a circular way (**Figure 1**). Even with this new system, the

problems because of preferential pathways of water were still not solved and of preferential pathways of water, foaming problems and clogging of the system were also detected.

Sequential batch filter: this other configuration was more complex and it had better results.

The elements were the same as explained in trickling filter but with two more devices: an electronic valve system and a programmer to be able to determine when the pump was working and when the valve was opened or closed.

In **Figure 2** you can see the scheme of this new configuration:

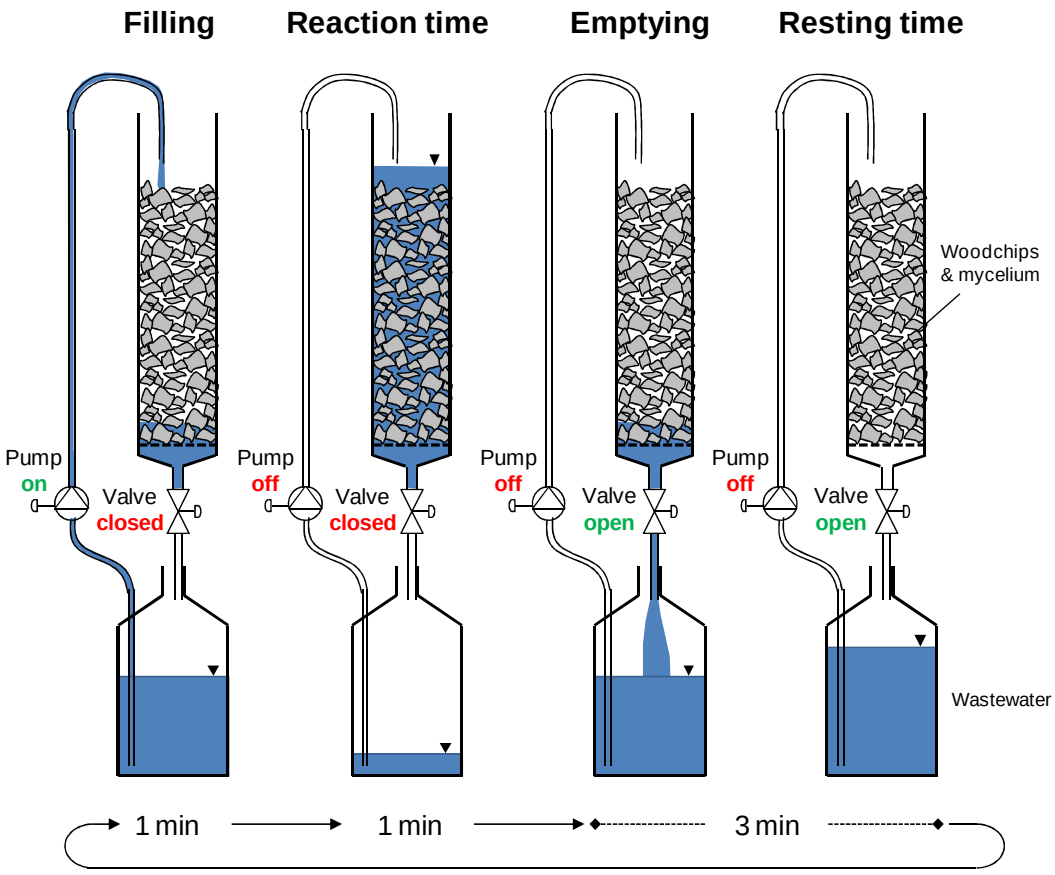

Figure 2: configuration of the sequential batch filter. Illustration of the same filter during different moments of the process.

In this case, water followed exactly the same route as explained in trickling filter but the pump was working just for one minute (filling). After this time, the filter was completely full of water because the valve was closed. When the pump stopped, the valve kept closed one minute more to ensure the good contact between the mycelium and the micropollutant (reaction time). After this time the valve was opened again for three minutes and the liquid medium returned to the bottle (emptying) and stayed again inside the glass bottle until reached the three minutes (resting time in unsaturated conditions). After that, the process was repeated again.

The trickling system was working worse than the sequential batch filter, and the results were more reproducible in the second system described.

Reactor characteristics

The dimensions of the each column were of 3.6 cm of diameter and 25 cm of high. The flow of the pump was 66.67 mL/ minute and volume of liquid treated in each cycle was 150 mL.

It was a batch reactor because the medium was the same during 2-3 days (it was recycled until the complete degradation of the micropollutant). It was also a sequential reactor because the system works with following repetitive processes and also because of the operation, it required to change the liquid manually when the micropollutants were degraded.

The electricity consumption for the water recirculation for a treatment time of 48h was up to 0.35 kWh per m^3 of water treated, similar to the electricity consummed in conventional WWTPs (around 0.37 kWh/m^3 in Switzerland).

Operation

The general way to operate the system was to remove the old medium and add a fresh one when the micropollutant was completely (or almost) degraded. Sometimes the medium was change before complete degradation due to the fact that it was not possible to analyse the concentration of micropollutant immediately or because there were two different rates of degradation. In this case the medium was changed in all the replicates when complete degradation was observed in the fastest one. Usually the elapsed time since the addition of fresh medium until it was removed (cycle) was around 24-48 hours; it depended on the micropollutant but also on the age of the column.

2.3 Composition of the biofilters

The biofilters were composed of mycelium of white-rot fungi immobilized on woodchips. Two species of fungi were tested: *Trametes versicolor* and *Pleurotus ostreatus*. The preparation of the mycelium and the inoculation of the woodchips are presented below.

Gorwing mediums

For the preparation of white-rot fungi mycelium these mediums were required:

- Malt extract medium (MEM) at 20 g/l adjust to pH 4,51 with HCl 1 M and autoclaved.

- Malt extract – agar Petri plate (MEMAP): a medium with malt extract 20g/l and agar 15 g/l was adjusted to pH 4,5 with HCl 1 M and autoclaved. When the medium was

still warm from the autoclaved process it was transferred to the petri dishes under sterile conditions and stored at 4°C once it was solidify.

Subculture of white-rot fungi in malt extract agare plate:

White-rot fungi were maintained on (MEMAP), and subculture every 1-2 months. New inoculations in the Petri plates were done from the old Petri plates that already contained the mycelium of the white-rot fungus. After the inoculation, the plates were incubated at 25°C during 7 days, until the mycelium covers the whole plate. Finally, the plate was stored at 4°C during 1-2 months until the next subculture.

White-rot mycelium preparation

Under sterile conditions, 6 squares (0.5 x 0.5 cm) of MEMAP containing 7 days old (or older) mycelium of the white-rot fungus were added to 200 ml of MEM. After that the medium was incubated at 25°C, 130 rpm, during 5 to 7 days to let grow the fungus (in pellet form).

When the fungal pellets were big enough (usually it was around 5-7 days) the pellets were collected, rinsed with sterile water and drained under sterile conditions.

The pellets were then broken and homogenized within the same volume of NaCl solution (8 g/l) with a sterile mixer at 10000 rpm and then stored at 4°C until they were used as an mycelium inoculum (storage time: a few months).

Wood chips inoculation with white-rot fungi

Wood chips of a size around 5-10 mm, produced for dry beech branches collected in the forest around Lausanne, were cleaned with tap water to remove the dust with the help of a sieve to retain the wood. This was repeated as many times as necessary to have relatively clear water at the end. The wood was let soaked in the water during 30 min to 1 h to saturate it, and then the water was removed with the help of a sieve. After this process, the wood was autoclaved and once it was sterile and at ambient temperature, 4 % (v/v) of white-rot fungus mycelium preparation was inoculated under sterile conditions.

The mycelium and wood were mixed and incubated at 25°C during 5 to 7 days for *Trametes* or 2-3 weeks for *Pleurotus ostreatus* under sterile conditions too. When the wood was completely colonized by the mycelium (all white) it was mixed and transferred to (non-sterile) columns. In each column was added 65 g (around 20 g dry weight) of the inoculated wood (approximately). Once the column had the inoculated wood, the column was shacked slightly in order to distribute well the wood supports and to avoid large voids. Then, after 2-4 days (1-2 weeks for *Pleurotus ostreatus*) the mycelium was developed again and the column was ready to treated water.

Number of replicates

For each experiment, the number of biofilters per similar conditions (replicates) were at least 2 and in some cases 3. This number of replicates was dependent of the number of available biofilters (8 could run at the same time). In almost all the experimentations, there was a biofilter composed of wood without inoculation acting as a control to check the removal due to adsorption on the wood or other possible factors (e.g. bacterial degradation). In this case, first experimentations were done with two controls, but it was checked that results were reproducible and after these first experiments just one control was kept in order to be able to install biofilters with different conditions.

2.4 Experiment with pure submerged cultures

For one experiment, fungal biofilters were not used and all the work was done with pure cultures in liquid media under sterile conditions. The experiment consisted on 7 different mediums, but in all of them there was a common mineral medium. The composition of this mineral medium was adapted with minor changes from Blánquez et al. (2004), and personal communication from staff of the Universitat Autònoma de Barcelona (UAB). The composition of this mineral medium (**UAB medium**) was:

Nitrilotriaceticacid (NTA, chelating agent)($C_6H_9NO_6$) – 0.015 g/L , $MnSO_4$·H2O – 0.005 g/L , NaCl - 0.01 g/L , $FeSO_4·7H_2O$ - 0.001 g/L , $CoSO_4·7H_2O$ – 0.00181 , $ZnSO_4·7H_2O$ – 0.001 g/L, $CuSO_4·5H_2O$- 0.0001 g/L , $AlK(SO_4)_2$ ·$12H_2O$ – 0.0001 g/L , H_3BO_3 – 0.0001 g/L , $NaMoO_4·2H_2O$ – 0.00012 g/L, KH_2PO_4 - 2 g/L, $MgSO_4·7H_2O$ – 0.53 g/L, $CaCl_2·2H_2O$ 0.1335 g/L and $(NH_4)_2SO_4$ – 2.368 g/L.

Below it is detailed the composition of the 7 mineral mediums used:

Medium A: only UAB medium

Medium B: UAB medium with glucose at 8 g/L

Medium C: UAB medium with naproxen at 10 mg/L

Medium D: UAB medium with sulfamethoxazole at 10 mg/L

Medium E: UAB medium with methanol at 1% (v/v)

Medium F: UAB medium with methanol at 1% (v/v) with naproxen at 10 mg/L

Medium G: UAB medium with methanol at 1% (v/v) with sulfamethoxazole at 10 mg/L

For each medium there were two replicates for both fungi (*Pleurotus ostreatus and Trametes versicolor*). For medium "E" there were two extra controls without the fungi in order to check the evaporation of methanol. The initial volume of medium was 90 ml (without taking into account the inoculation). Before the inoculation of fungi, all the media were sterilize by filtration at 0,2µm in 250-ml sterile Erlenmeyers. They were then inoculated

with 4% (v/v) of white–rot fungal mycelium, except the 2 control mediums as explained just before. All the Erlenmeyers were then incubated in the dark (to avoid photodegradation) at 25 °C, shaking at 130 rpm.

2.5 Sampling and analyses

2.5.1 Sampling

For those experimentations with biofilters, 2 ml of medium were collected each time of sampling. The frequency of sampling was shorter at the begging of each cycle: from 3-4 samplings per day because more changes were produced: fast removal of micropollutant and fast drop of pH. At the end of the cycle, it was enough with just 1 sampling per day because the changes in the medium were slower. All the parameters checked in each sampling were the next ones:

- pH
- laccase activity
- concentration of micropollutant (naproxen or sulfamethoxazole)

For the experiments in pure submerged cultures, the frequency of sampling depended on the stage of the experimentation and it was always in sterile conditions

At the beginning, when the degradation was faster and more changes happened in the medium, it was daily. After this fast process of degradation the sampling was less often, after 2-3 days. Sampling process required to remove 1.5 ml from the medium to be able to analyze all the parameters. In this case, all the parameters checked in this experimentation were the next ones:

- Concentration of micropollutant in the medium (naproxen or sulfamethoxazole)
- Concentration of glucose in the medium
- Concentration of methanol in the medium
- laccase activity
- pH

Once this experiment was finished, the determination of biomass took place in order to know if there was growth in some of the mediums. To do that, it was necessary to centrifuge the liquid (5000 revolutions per minute during 10 min) to collect the biomass and measure the dry weight (one night in a oven at 105 °C) and the mineral weight (2h of calcination at 550°C).

Laccase activity

The activity of the extracellular enzyme laccase, produced by the fungus, was regularly monitored in the liquid medium to evaluate the fungal activity in the filter. Laccase activity was determined using a colorimetric assay by measuring the oxidation of 0.5 mM ABTS in oxygen-saturated acetate buffer (0.1 M) at pH 4.5 and 25°C. The sample containing laccase

was added to the solution and the increase of absorbance at 420 nm was monitored with a temperature controlled spectrophotometer (U-3010, Hitachi, Tokyo, Japan). One unit of activity (U) was defined by the oxidation of one µmol of ABTS per min, using the extinction coefficient ε420nm of 36,000 M^{-1} cm^{-1} (Margot, Bennati-Granier et al. 2013).

Micropollutant analysis

Determination of naproxen and sulfamethoxazole concentrations was carried out by reverse phase liquid chromatography with a diode-array detector (HPLC-DAD) (LC-2000plus, Jasco, Tokyo, Japan, equipped with Bondapack-C18 column, 15–20 µm, 3.9 mm× 300 mm, WatersTM, Milford, USA). 50 and 100 µl of sample were injected for naproxen and sulfamethoxazole determination, respectively. Separation of the compounds was conducted under isocratic conditions during 20 min, at 1 ml min^{-1}, with a ratio of pure H_2O and methanol (%H_2O:%Methanol, v/v), containing both 0.1% acetic acid, of 53:47 (H_2O:Methanol) and 86:14 (H_2O:Methanol) for naproxen and sulfamethoxazole determination, respectively. Detection of the compounds was done by DAD at 232nm for naproxen and 268 nm for sulfamethoxazole. The limit of detection (LOD) was around 0.3 mg l^{-1} (~1 µM).

Methanol and glucose analysis

Methanol and glucose concentrations were determined by HPLC equipped with a ORH-801 column (from Transgenomic) and with a refractive index (RI) detector (RI-2021plus, Jasco, Tokyo, Japan). 20 µl of sample were injected and separation of the compounds was conducted under isocratic condition with H_2SO_4 5mM in pure water, during 18 min, at 0.5 ml/min and 35°C. Limits of detection were at 0.1 g/l (3 mM) and 0.01 g/l (0.05 mM) for methanol and glucose, respectively.

Filtration correction

To avoid the clogging of the HPLC column, all the samples were filtered at 0. 2 µm with glass microfiber filters of 13 mm diameter (GMF-13mm) in order to remove wood residues or particles present in the water. Due to this filtration, important loses of naproxen were observed, especially at low pH, which was then absolutely necessary to correct. To be able to correct these loses, it was done a test with different pH to determine the loss. In Figure 3 is showed the loss for naproxen at different pH. This curve was used to correct the concentration of naproxen.

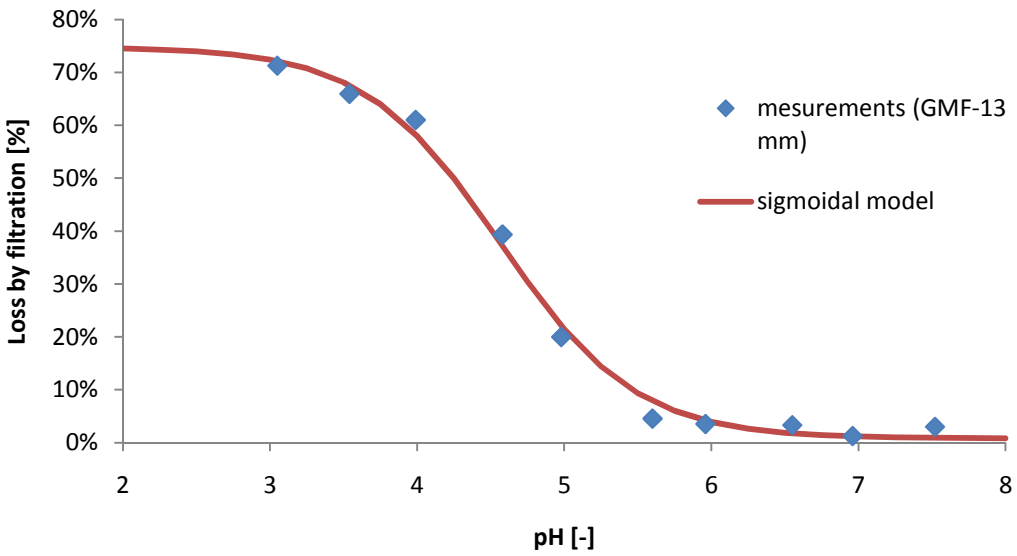

Figure 3: Loss of naproxen by filtration at different pH. The results fit a sigmoidal model.

For all the other compounds analysed by HPLC the same test was done, but in these cases, there were not significant loses and no correction was done.

3 Experimental work

3.1 Comparison of *Trametes versicolor* and *Pleurotus ostreatus* growth in different kinds of wood.

The goal of this experimentation was to check the influence of different kind of wood chips on the growth of *Trametes versicolor* and *Pleurotus ostreatus*.

The test was done with 4 different kinds of wood (dry branches collected in the forest):

- Beech
- Spruce
- Ash
- Birch

For each kind of wood two replicates were done with each fungi (two replicates for *Trametes versicolor* and two more for *Pleurotus ostreatus*). The procedure was to inoculate 4 ml of fungal mycelium in all the replicates in 16 grams (approximately) of each sterilize wet wood. These solid media were then incubated at 25°C during more than one month. The growth was evaluated just with visual observation.

3.2 Competition test between *Trametes versicolor* and *Pleurotus ostreatus* in a solid medium with beech wood

A glass column was the support for that; in on side of the column wet beech woodchips inoculated with *Trametes versicolor* was added and in the other side wet beech

woodchips inoculted with *Pleurotus ostreatus*. In the middle fresh (wet) wood was added without any inoculation of fungi. This experiment was conducted in replicates. The columns were incubated at 25°C in dry condition. All the changes in this column were evaluated just visually.

3.3 *Pleurotus ostreatus* biofilter in a liquid medium with methanol and naproxen

This experimentation consisted on 3 fungal biofilters composed of beech woodchips running at ambient temperature (20-25°C). The three filters were inoculated with *Pleurotus ostreatus* mycelium. During all the experimentation, the initial composition of the medium in each cycle was: naproxen at 10 mg/l and methanol at 1% (v/v) in tap water. The duration was around 14 days.

3.4 *Pleurotus ostreatus* and *Trametes versicolor* biofilters in a liquid medium with naproxen

Composed of 6 fungal biofilters. Three of them with an inoculation of *Pleurotus ostreatus* and the other three ones with an inoculation of *Trametes versicolor*. In this case, the initial composition of the medium was : naproxen at 10 mg/l in tap water. Another two filters were installed in the same time but in this case without inoculation of any fungi. They acted as a control, to check the removal of naproxen just due to adsorption on the wood.

3.5 Inhibition test with sodium azide

After 9 days of experimentation (experiment number **3.4**), sodium azide with a concentration of 10 mM was added in the medium of 1 replicate with *Pleurotus ostreatus* and also in another replicate with *Trametes versicolor*. Sodium azide is a strong toxic that cause a completely inhibition of the fungus in the concentration that was added. The goal in this case was to check the degradation versus adsorption for both fungi.

3.6 Long term experimentation in *Pleurotus ostreatus* biofilters

The two replicates with Pleurotus ostreatus in experiment **3.4** (the ones where sodium azide was not added), were still active after 14 days. To check for how long they were able to work we decided to keep them running for a long time (more than 3 months).

3.7 Influence of the resting time in the removal kinetic

The temporization chosen for the system was decided based in a previous experiences but in an arbitrary way. As explained in the introduction, the usual temporization for the system was: 1 minute of feeding, 1 minute of reaction and 3 minutes of resting. It was decided to program a shorter resting time in order to check if it could have a significant impact on the naproxen removal. The new temporization was this one: 1 minute feeding, 1 minute of reaction and 1 minute of resting. This new temporization was kept just for one cycle to check if there were significant differences.

3.8 Treated waste water with naproxen

Two biofilters inoculated with *Pleurotus ostreatus* were used for this experimentation. In this case, instead of working with tap water, the system was working with real treated wastewater from the wastewater treatment plant from Lausanne. The initial medium for each cycle was waste water with addition of naproxen at 10 mg/l. Another filter was installed in the same time but in this case without inoculation of the fungus (wood control). The liquid medium for this filter was exactly the same as the other two ones. The goal of this filter was to check the removal of naproxen due to adsorption on the wood and maybe in this case due to degradation by bacteria from the wastewater too.

3.9 Sulfamethoxazole in tap water

Sulfamethoxazole was tested with tap water. Again two biofilters inoculated with *Pleurotus ostreatus* were used in this experimentation. The initial composition of the medium in each cycle was sulfamethoxazole with a concentration of 10 mg/l or 5 mg/l dissolved in tap water. Another filter was installed in the same time but without inoculation of the fungus. The liquid medium for this filter was exactly the same as the other two ones. The goal of this filter was again to check the removal of sulfamethoxazole on the wood.

3.10 Comparison of micropollutants removal by pure cultures of *Trametes versicolor* and Pleurotus ostreatus in different liquid media

This work was in sterile conditions with pure cultures of both fungi. All the mediums described in **section 2.4** were used to check deeply the capacity of degradation of different micro pollutants and also the growing of fungi and the degration of other easy substrates (glucose and methanol). This experiment is detailed in **section 2.4**.

4 Modelling

For a better understanding of all the phenomena in the biofilter two models were fitted to the experimental results.

The first phenomenon modelled was the removal of micropollutant due to adsorption on the wood chips. When the micropollutant is in the system, it is present in two phases: the liquid one and the solid one. The trend of the system will be to reach equilibrium between both phases in terms of micropollutant concentration.

When there is just removal due to adsorption on the wood (without inoculation of fungi in the system), it was assumed that our system in the equilibrium point followed a Freundlich isotherm, described by next equation:

$$q = \frac{x}{m} = Kc^n$$

Equation 1: Freundlich isotherm

Where:

q= solid concentration (mg/g)

X= mass of adsorbate (sorbed pollutant) (mg)

m= mass of adsorbent (solid phase, such as wood) (g)

c= Equilibrium concentration of adsorbate in solution (mg/l)

K ($l^n mg^{1-n}/g$) and n (-) are constants for a given adsorbate and adsorbent at a particular temperature.

The coefficient of this model were determined by linear regression of the log(c) versus log(q) of the data. The slope of the regression line give the coefficient n and the y-intercept gives log(K) (log(q)=log(K)+nlog(c)).

Before reaching the adsorption equilibrium or when there was removal by degradation, kinetic models are necessary. In this case, a pseudo-first order model was used as presented in **Equation 2**:

$$\frac{dc}{dt} = c.k'.x$$

Equation 2: Pseudo first order kinetic model for naproxen removal by degradation.

Where:

c= pollutant concentration in the liquid (mg/l)

k'= constant rate of removal, in this case it is a mixture between adsorption and degradation (l/(g d))

x = concentration of fungal biomass in the column (g/l)

t= time (d)

In this system, there was no variation of biomass during the process and it was assumed that parameter "x" was a constant with the time. To simplify the equation, it was defined a new constant rate:

k= xk' (1/d)

Solving the previous equation a first order model equation is obtained:

$$\frac{C}{\mathrm{Co}} = e^{-kt}$$

Equation 3 First order kinetic model for naproxen removal by degradation, assuming k=xk' (x=constant)

This model was fitted to the data by minimizing the sum of the square of the difference between the model and the measures (Solver tool in Excel).

5 Results and discussion

5.1 Comparison of *Trametes versicolor* and *Pleurotus ostreatus* growth on wood chips

In all the cases, the growth of *Pleurotus ostreatus* (**Figure 4 a**) was slower than the growth of *Trametes versicolor* (**Figure 4 b**).

Figure 4: beech wood chips with an inoculation of Pleurotus ostreatus (left) and beech wood chips with an inoculation of Trametes versicolor (right) both after 21 days of incubation.

The two fungi were able to grow in all supports, but in a different speed depending on the kind of wood:

Beech wood chips: good growth of both fungi, *Trametes versicolor* clogging completely chip wood in 21 days while *Pleurotus ostreatus* was developed just enough, without clogging the wood (**Figure 4 a**).

Spruce wood chips: Both fungi grew but it was insufficient to use as a biofilter.

Ash and birch wood chips: the growth in these two kinds of wood was similar. The growth was faster than in spruce wood chips but slower than in beech wood chips.

According to these observations, beech wood chips are the best support for the fungal growth and it is one of the most common kind of woods in Switzerland (place where the study was done). It was for this reasons that all the biofilter experimentations were done with beech wood chips.

In order to compare growth of *Trametes versicolor* and *Pleurotus ostreatus*, the competition test was done as described in **section 3.2**.

With this test it was possible to check these points (**Figure 5)**:

- *Trametes versicolor* grew faster than *Pleurotus ostreatus*: in only 1 week the wood was completely clogged with *Trametes versicolor* but not with *Pleurotus ostreatus*.
- The growth of *Trametes versicolor* is completely different in comparison with *Pleurotus ostreatus.* The first one covered completely the chips and the second one produced less biomass but could colonise more wood surface without clogging (mycelium propagation).
- *Pleurotus ostreatus* was more competitive against a green fungi contamination. Green fungi appeared in the fresh wood zone. These fungi were able to colonise the place where *Trametes versicolor* was inoculated but not the place with *Pleurotus ostratus*. At the end of the experimentation, *Pleurotus ostreatus* was able to colonise the place where the green fungi contamination appeared.
- In terms of ecology, and because of all the details explained in the previous points, *Trametes versicolor* growth follows a “r” strategy (fast growth, low competitiveness) and *Pleurotus ostreatus* follows a “k” strategy (longer lifespan, slower growth, higher competitiveness) (Begon, Harper L. 1996)

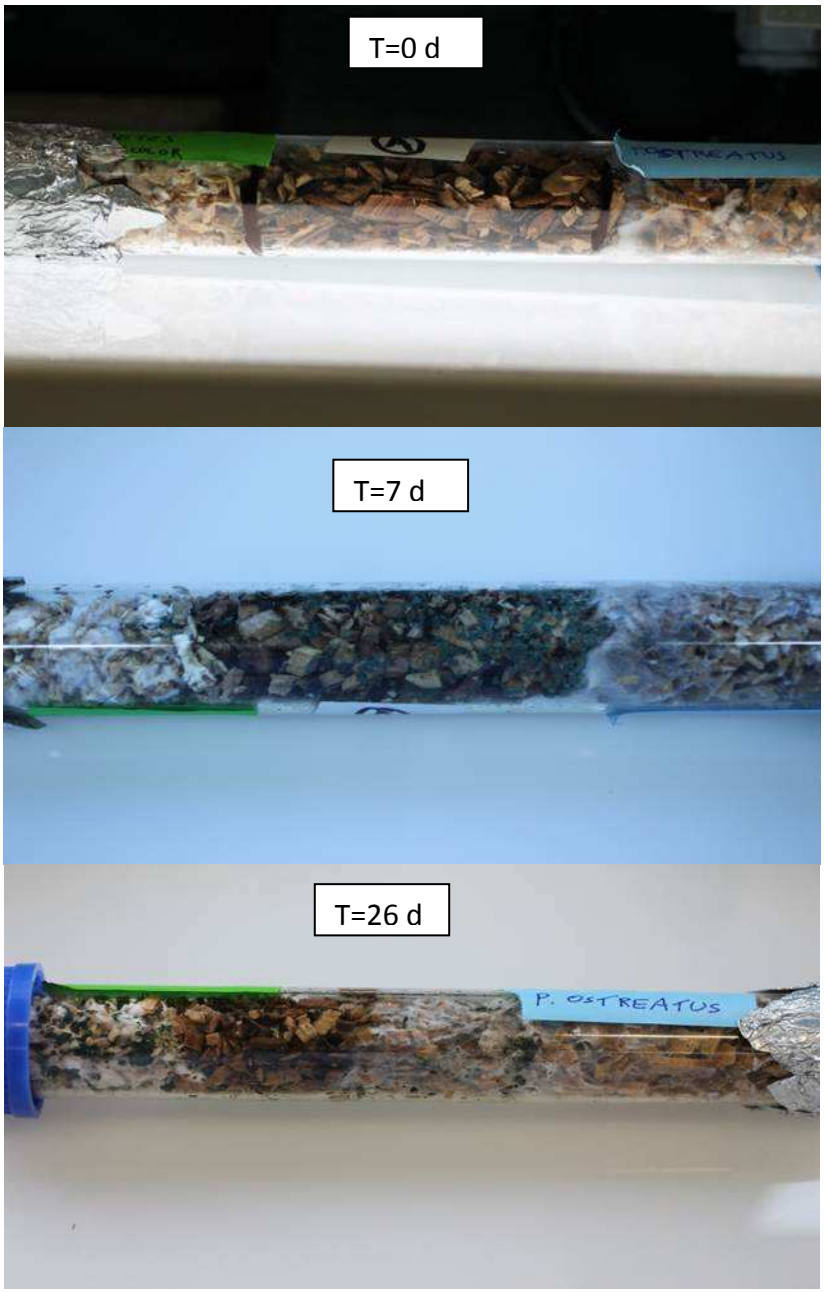

Figure 5: Evolution of the competition test. On the left side, inoculation with *Trametes versicolor*, in the middle fresh wood and in the right side, inoculation with *Pleurotus ostreatus*. From up to down: 0 days of incubation time, 7 days of incubation time, 26 days of incubation time.

5.2 Optimal filter configuration

The batch sequential reactor was working better than trickling filter because in general, in batch there were not preferential water path flow and it was possible to ensure a good contact between the mycelium and the liquid phase. Besides, there were not foaming problems, less problems due to clogging and results were reproducible. Thus, all the experiments were then conducted with batch sequential reactors.

5.3 Efficiency of the fungal filters to remove naproxen in tap water with methanol

Three filters inoculated with *Pleurotus ostreatus* were installed with the same conditions, replicate 1, 2 and 3 as described in **section 3.3**. The medium was tap water with naproxen at 10 mg/l. Methanol, in concentration of 1% (v/v), was added during the spike of naproxen from a stock solution of 1 g/l prepared in methanol.

In this system, *Pleurotus ostreatus* was able to survive and the fungal filter was working during all the experimentation (14 days or more). This was the first time reported. Indeed, previous experiments done with similar systems but with different kind of fungi (*Trametes versicolor*) never succeed.

The experiment had 7 cycles (**Figure 6**). On the first cycle, naproxen was completely removed from the medium in about 24 hours. During the whole experiment of 14 days, the time of each cycle varied from 24 hours to almost 3 days, on the weekends.

Laccase activity increased strongly during the firsts three cycles, reaching the maximum value of 565 U/L. This value is comparable to the level produced by *Trametes Versicolor* cultures in waste water after only 2-3 days of incubation (Margot, Bennati-Granier et al. 2013).

At the end of each cycle, just before changing the water, laccase activity was highest. The maximum value was achieved during the third cycle and then started to decrease, reaching less than 60 U/L after 7 cycles.

Each time the water was changed pH was around 7,7. In all the cycles, pH dropped quickly, especially at first three cycles. In the first cycles, pH decreased from pH around 7,7 to pH around 5 (depends on the replicate). In each following cycle, the pH decreased slower.

There was a clear correlation between the behaviour of pH and activity: when pH was decreasing, the laccase activity was increasing.

In the first cycle pH dropped very quickly until reaches the value of 4,8 to 5. This can be explained by these following reasons:

- Some white rot fungi, like *Trametes versicolor*, are able to produce oxalic acid in the extracts from the solid state spruce wood chip cultures (Mäkelä, M. Et al. (2002)). After the third cycle, coinciding with the drop of lacasse activity, the pH remains higher than the previous cycle.

- As it is well known, wood also contains some organic compounds, like humic and fluvic acids. When the water passes through the filter they can be solubilised acidifying the medium. Probably this is also the reason why in the first cycles the acidification is stronger.

- Another factor that may affect pH decrease but probably with less importance is the role of CO_2 produced due to the degradation of organic matter by white rot fungi. This CO_2 can be dissolved in the water and because of deprotonating process can acidify the medium (cycle of carbonates). To check if this phenomenon had an important role in our experimentations, the filtrate of one biofilter (of another experiment) with pH 3.4 was collected. Afterwards, nitrogen gas was bubbled in the filtrate for 5 min in order to strip the CO_2. No increase in pH was observed, meaning that CO_2 is not the cause of the pH decrease.

In the first cycles (especially cycle 1 and cycle 2) naproxen removal was very fast mainly due to the adsorption phenomena.

After a few days, a layer of biofilm was observed on the wall of all biofilters, probably because of growth of some bacteria due to the presence of methanol (easy substrate), which means that fungi had to face competition against other species. The decrease in activity with the time is possibly due to this competition with other organisms.

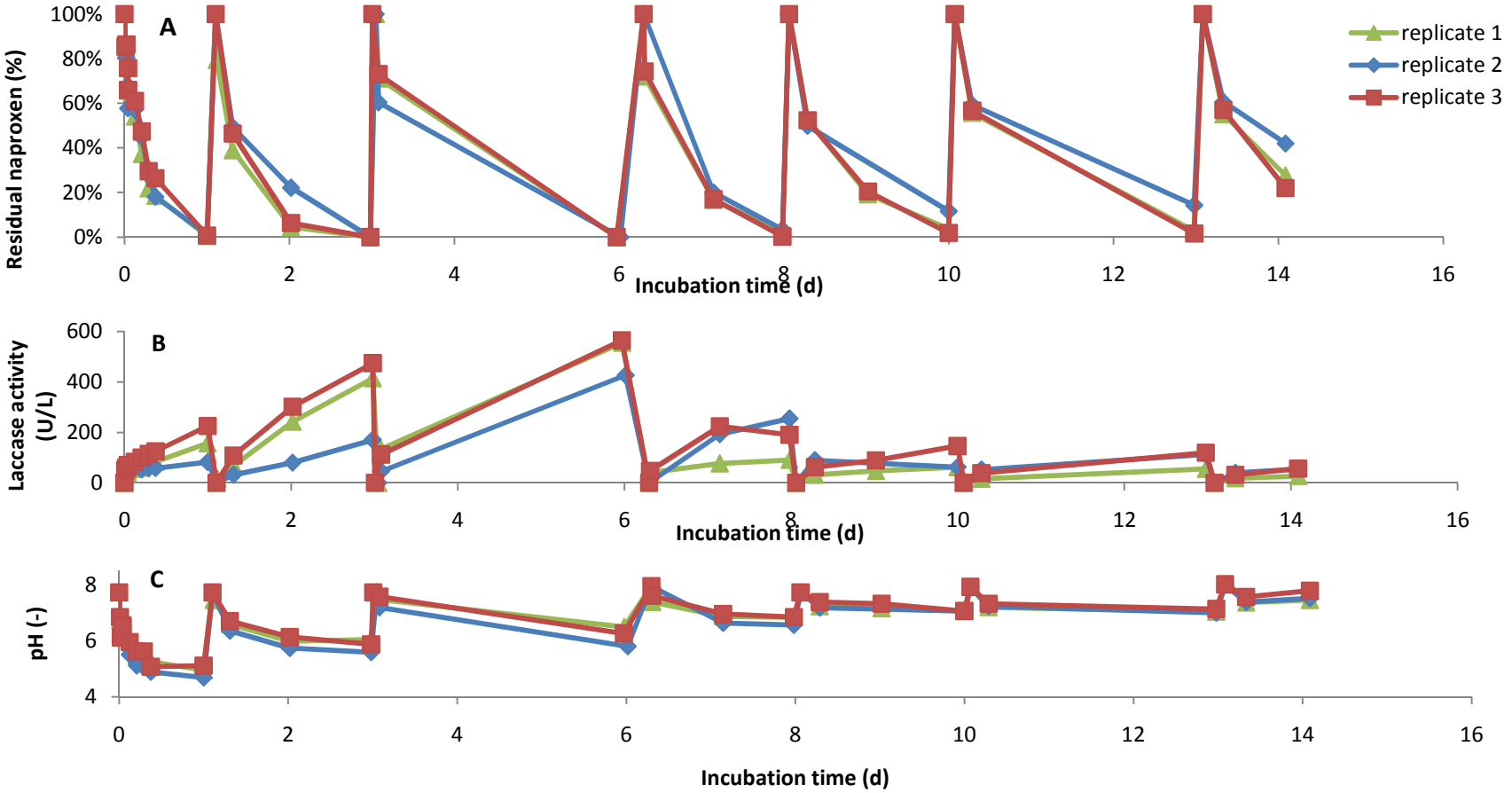

Figure 6: (a) Residual naproxen concentration in three replicates of fungal filter with *Pleurotus ostreatus*, (b) evolution of laccase activity and (c) evolution of pH for the same period. Characteristics of the initial medium in each cycle: methanol 1% (v/v), naproxen 10 mg/l, dissolved in tap water.

5.4 Efficiency of the fungal filters to remove naproxen in tap water without methanol

5.4.1 *Trametes versicolor* versus *Pleurotus ostreatus*

In order to avoid the methanol effects in the medium (growth of other microorganisms), next experiments were done without methanol, by dissolving the pollutant directly into the water.

Both fungi were able to remove naproxen in biofilter under these conditions (**Figure 12** and **Figure 13**). Despite a maximum laccase activity 2-3 times higher than with *Pleurotus* during the second cycle, *Trametes versicolor* was not active longer than one week. Indeed, after four cycles, no significant laccasse activity was observed anymore (**Figure 12**). This was confirmed also by other experiments (data not shown).

However, *Pleurotus ostreatus* was active without a visible biomass increase and without clogging the system until the end of the experimentation (**Figure 13**). No biofilm appears on the wall of the reactor, confirming that the microbial growth observed in the previous experiment was due to the presence of methanol. Much lower laccase activity (max 80 U/L) was observed in this experiment compared to the previous one (max 565 U/L). This suggests that methanol stimulates the production of laccase by *Pleurotus ostreatus*. Despite these higher values of laccase activity, naproxen removal was not faster with the experiment where methanol was added in the medium.

In first four cycles, a quick removal of naproxen was observed especially in the two first cycles. Adsorption phenomena may have an important role in these first cycles: in less than 24 hours naproxen was completely removed from all the replicates, but also important removal was observed in the controls.

In cycle 3 and 4, with *Trametes versicolor*, the complete removal of naproxen was not reached in any of the replicates. It was also in cycle 3 that laccase activity started to decrease compared to the previous cycles (**Figure 12**). It was exactly in this point when the fungi started to be less efficient: in cycle 3, around 90% of naproxen was removed, in cycle 4 just around 70% and in cycle 5, less than 30%. There was thus a strong correlation between laccase activity (indicator of fungal activity) and naproxen degradation.

In biofilters inoculated with *Pleurotus ostreatus*, the activity was more or less similar during all the experimentation and naproxen was completely removed at each cycle.

The comparison shows than *Trametes* can produce higher amount of laccase than *Pleurotus* but is not able to survive longer than one week in the system. Despite lower laccase activity, *P. ostreatus* was active during a much longer period, keeping the same ability to degrade efficiently naproxen (**Figure 13**). *Pleurotus oestratus* seems thus to be more adapted for this kind of fungal filters.

5.4.2. Degradation versus adsorption

As described in **Section 4,** adsorption phenomena is an important factor, especially at the beginning of the experiment, with fresh wood chips because the wood is completely free of pollutant and has a lot of adsorption capacity. In **Figure 7** a modelization of Freundlich isotherm with real values (measured in the control column) is presented. In this case, the filter was not inoculated, so all the micropollutant (naproxen in this case) removal was due to adsorption on the wood.

In **Figure 7**, the relation between naproxen concentration in liquid phase and naproxen concentration in solid phase, at equilibrium, is presented. Points from 1-8 on the curve correspond to the same points 1-8 as in **Figure 8** each of which presents last naproxen concentration of the cycle. These naproxen concentrations were assumed to be in the equilibrium in liquid and solid phase. The concentration in the solid phase was calculated based on the mass of pollutant removed from the liquid phase during each cycle. The Freundlich model fitted very well to these data suggesting that adsorption of naproxen on wood follows a Freundlich isotherm.

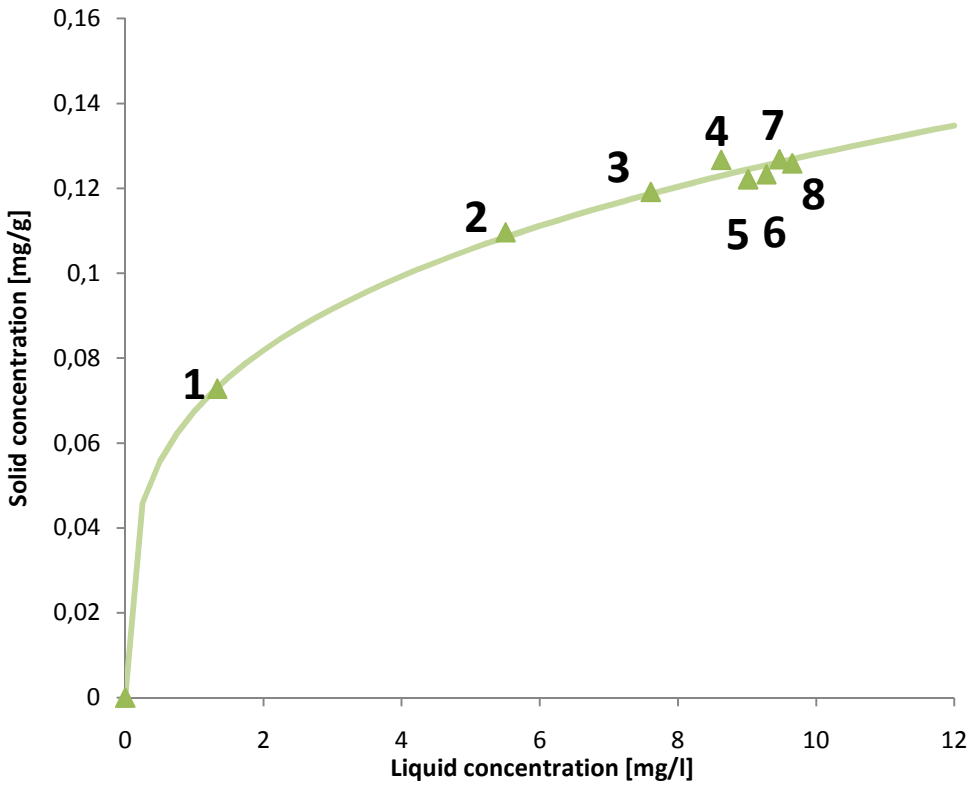

Figure 7: Modelization of Freundlich isotherm (full line) with experimental values (points) in filter without fungi inoculation.

All the values are in a chronological way, in this case there were 8 cycles and the initial concentration in the medium for each cycle (as it can be checked in **Figure 8**) was around 10

mg/l of naproxen. In the course of the experiment, the naproxen removal was less efficient. In the last cycle, there was no naproxen removal in the liquid phase and the naproxen concentration on the solid phase reached the highest values. Both facts are related: the concentration in the solid phase due to adsorption is probably at the equilibrium with the initial concentration in the liquid phase and there was not adsorption anymore and therefore no more naproxen removal. In order to check if adsorption in the wood could be reversible in the second series of experiments, in cycle 5 naproxen was omitted in the initial medium (tap water without micropollutant) (**Figure 10)**. In this point, the equilibrium between the liquid phase and the solid phase was not reached, and naproxen from the solid phase was released to the liquid phase to reach the equilibrium between both phases. This can be seen clearly in **Figure 9**, where the concentration in the solid medium decrease from cycle 4 to cycle 5 due to naproxen desorption from solid phase. The adsorption on the wood was thus reversible. When tap water was added in the biofilter without naproxen, it was removed from the solid phase to the liquid phase in a short time (**Figure 11**).

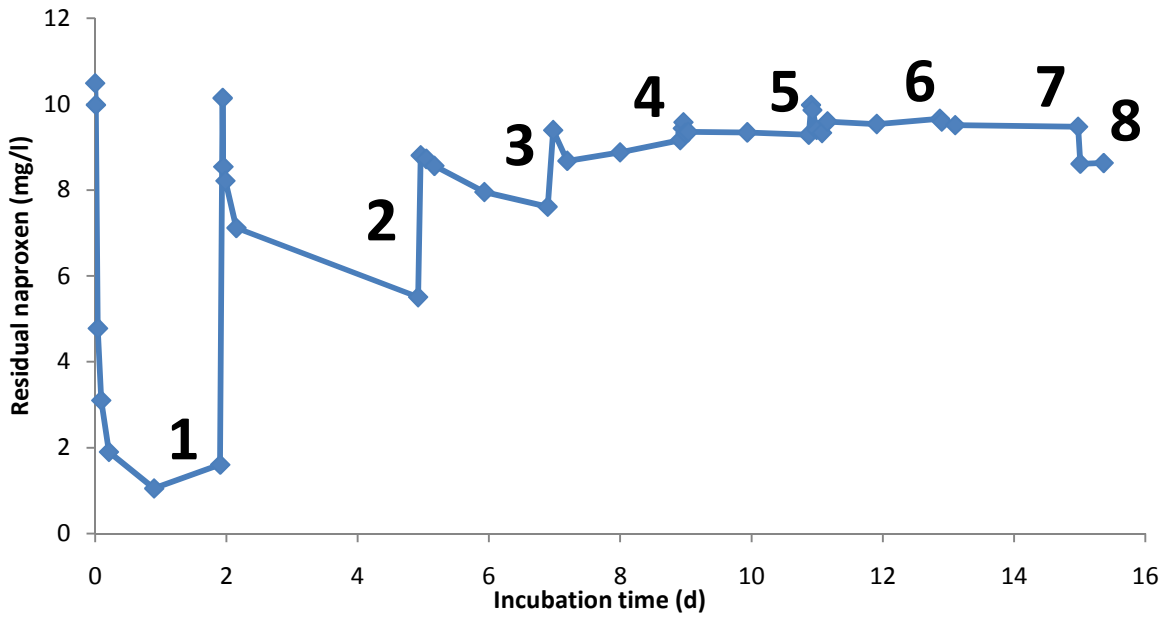

Figure 8: real values of residual naproxen in the liquid phase observed in control 2 (filter without inoculation of fungi). Numbers (from 1-8) corresponds in to the same values in the liquid phase for **Figure 7**.

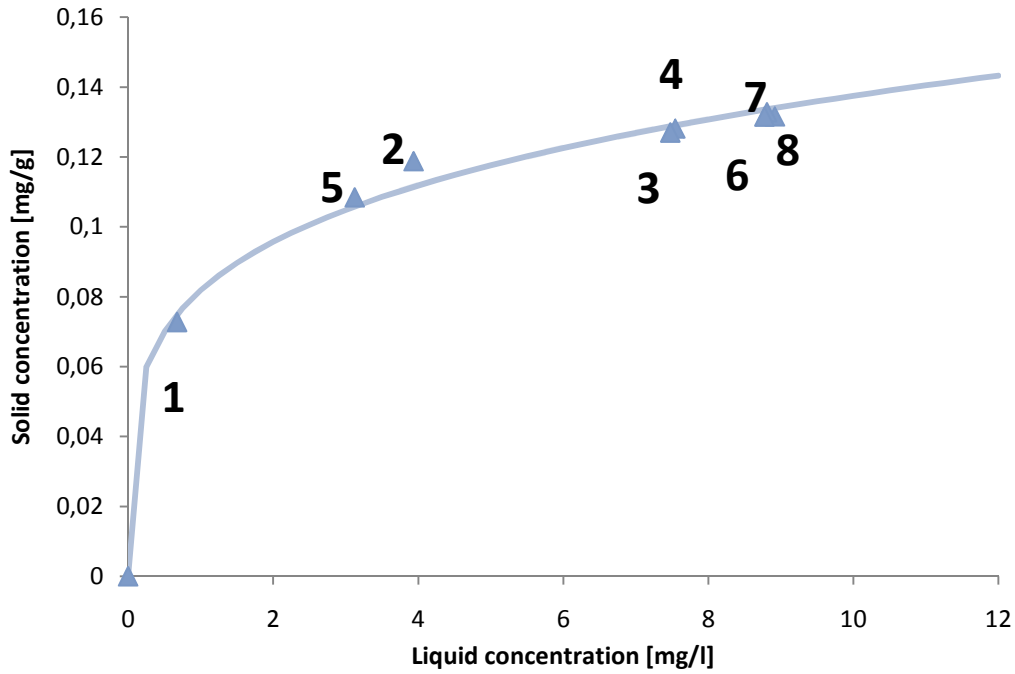

Figure 9: Modelization of Freundlich isotherm (full line) with experimental values (points) in filter without fungi inoculation. Point 5 presents naproxen concentration in liquid and solid phase equilibrium when the initial medium in the cycle was tap water without micropollutant.

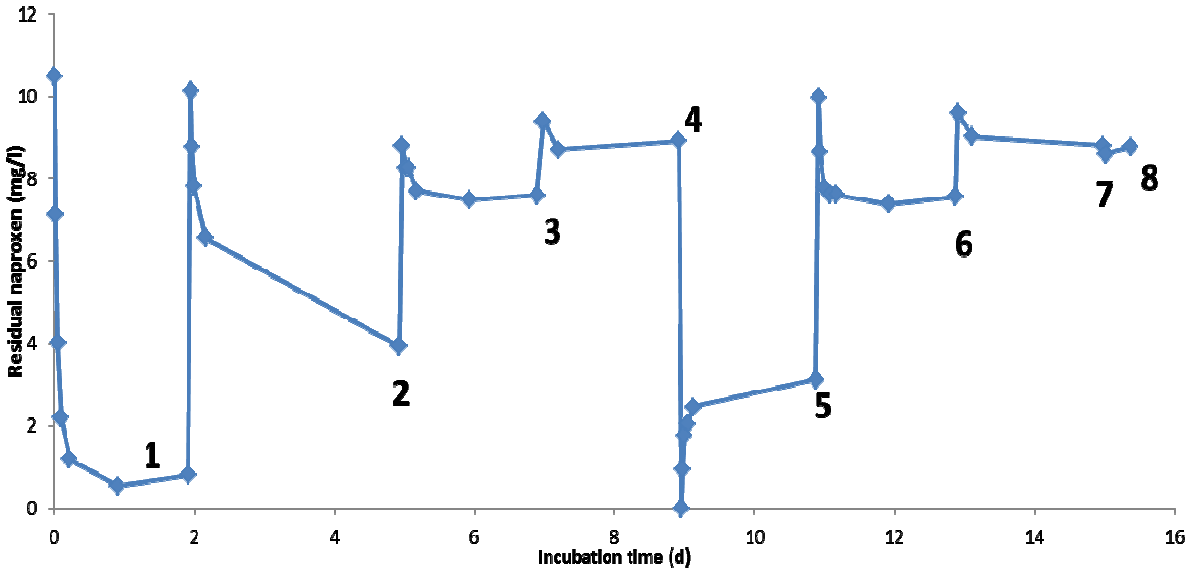

Figure 10: real values of residual naproxen in the liquid phase observed in control 1 (filter without inoculation of fungi). Numbers (from 1-8) corresponds in to the same values in the liquid phase for **Figure 9**.

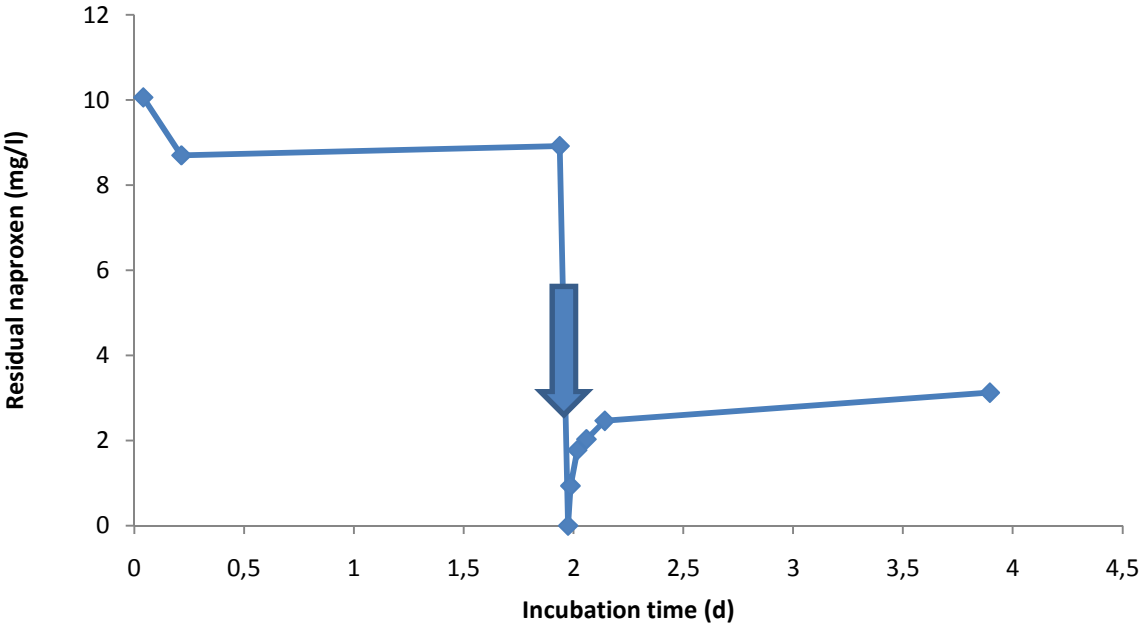

Figure 11: Zoom to cycle number 5 of Figure . The arrow is the moment when tap water without naproxen was added in the medium.

Adsorption on the wood vs on adsorption on wood covered with mycelium

It is not exactly correct to say adsorption phenomena has the same influence in the removal of micropollutant in wood filters or in filters containing fungal mycelium. Indeed, the mycelium covers the wood, limiting possibly the access of the pollutant to the adsorption site of the wood. But, on the other hand, mycelium contains also potentially sites of adsorption for the pollutant.

To have a real proof that removal of naproxen was not only because of adsorption on the mycelium or on the wood but also due to degradation phenomena, an inhibition test was done. After 9 days of incubation, sodium azide[2] was added in one filter inoculated with *Trametes versicolor* and also in one inoculated with *Pleurotus ostreatus* (**Figure 12** and **Figure 13**). Before the addition of sodium azide in the filter inoculated with *Trametes versicolor*, the fungus was already not active, so the addition of the toxic in the medium did not implicate any changes in terms of naproxen removal.

All the replicates with *Pleurotus ostreatus* were still active after 9 days (**Figure 13 b**). After this point, it can be seen a clearly decrease in naproxen removal in the filter containing sodium azide in comparison with the previous cycles, meaning that degradation was a real fact (**Figure 14 a**)

Differences in the naproxen removal between the biofilter where sodium azide was added and the other two replicates without the addition of the toxic were observed too. This

[2] Sodium azide inhibits immediately the fungal activity in the concentration added

proved that fungal degradation is responsible for the long term removal of naproxen in the filters.

The final naproxen concentration in the liquid medium in cycle number 4, reached the value of 0 mg/l. It can be assumed, that the concentration in the solid phase was also 0 mg/g because of the equilibrium in between liquid and solid phases. Maybe this assumption is not completely true because with the analytical devices it was not possible to detect concentrations lower than 0,1 mg/l, but in any cases for sure that when sodium azide was added in the medium, the solid phase was almost completely free of pollutant, like in the first cycle.

Comparing cycles 1,2,3 from the control and cycles 5,6,7 from the replicate were sodium azide was added, it is possible to have an estimation between the adsorption due the wood and adsorption due the mycelium. This estimation will not be completely realistic, because as said before, it is not possible to ensure that concentration in cycle 5 on the solid phase was 0 mg/g in the column were sodium azide was added. Another weakness of this assumption is the fact that the comparison of data is in a different moments of the experimentation and maybe the constants of adsorption in the wood chips and in the mycelium are not exactly the same in all the experimentation. But in any cases, probably there are not big differences.

In cycle number 1,2,3, (total duration of 7 days) the naproxen removal for the filter without fungi inoculation were, respectively: 90%, 40% and 10%. After this cycle, biofilter was already at the equilibrium with the initial concentration. For the filter with fungi inoculation, in cycles 5,6 the naproxen removal in liquid medium were of 60%, 30% (3 days in total) and after this cycle the concentration in both phases were at the equilibrium with the initial liquid concentration and no removal of naproxen was observed anymore. In column with *Pleurotus ostreatus*, adsorption of naproxen in the solids (mycelium/wood) is thus slightly lower in comparison of adsorption on the wood alone (faster “saturation” of the solids when the fungus is present).It is thus possible that the mycelium, which occupies an important surface on the wood, have a lower adsorption capacity than the wood. It can explains why adsorption produced by the combination of chips wood and mycelium is lower than when the filter is not inoculated with the fungi.

In *Trametes versicolor* it is not possible to do this kind of comparison, because in the first moment that laccase activity was not detected (7 days of incubation time) the naproxen in the liquid medium was not completely degraded and the concentration in the solid phase for sure it was not 0 mg/g, making impossible to do a comparison with the control.

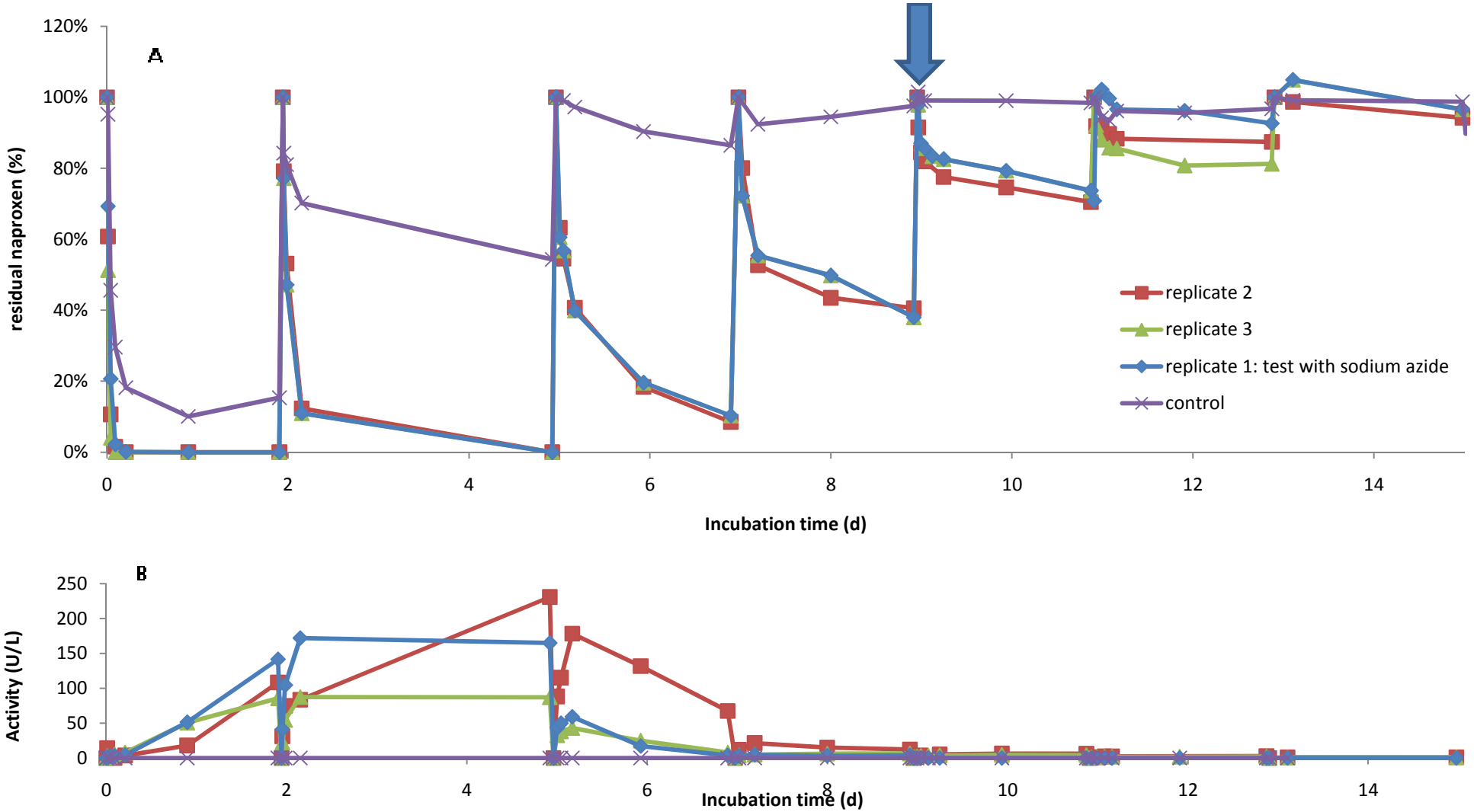

Figure 12 (a) residual naproxen in three replicates of fungal filter with *Trametes versicolor*. The blue arrow corresponds to the addition of sodium azide in the medium of replicate 1, (b) evolution of laccase activity for the same time. Characteristics of the initial medium in each cycle: naproxen 10 mg/l, dissolved in tap water.

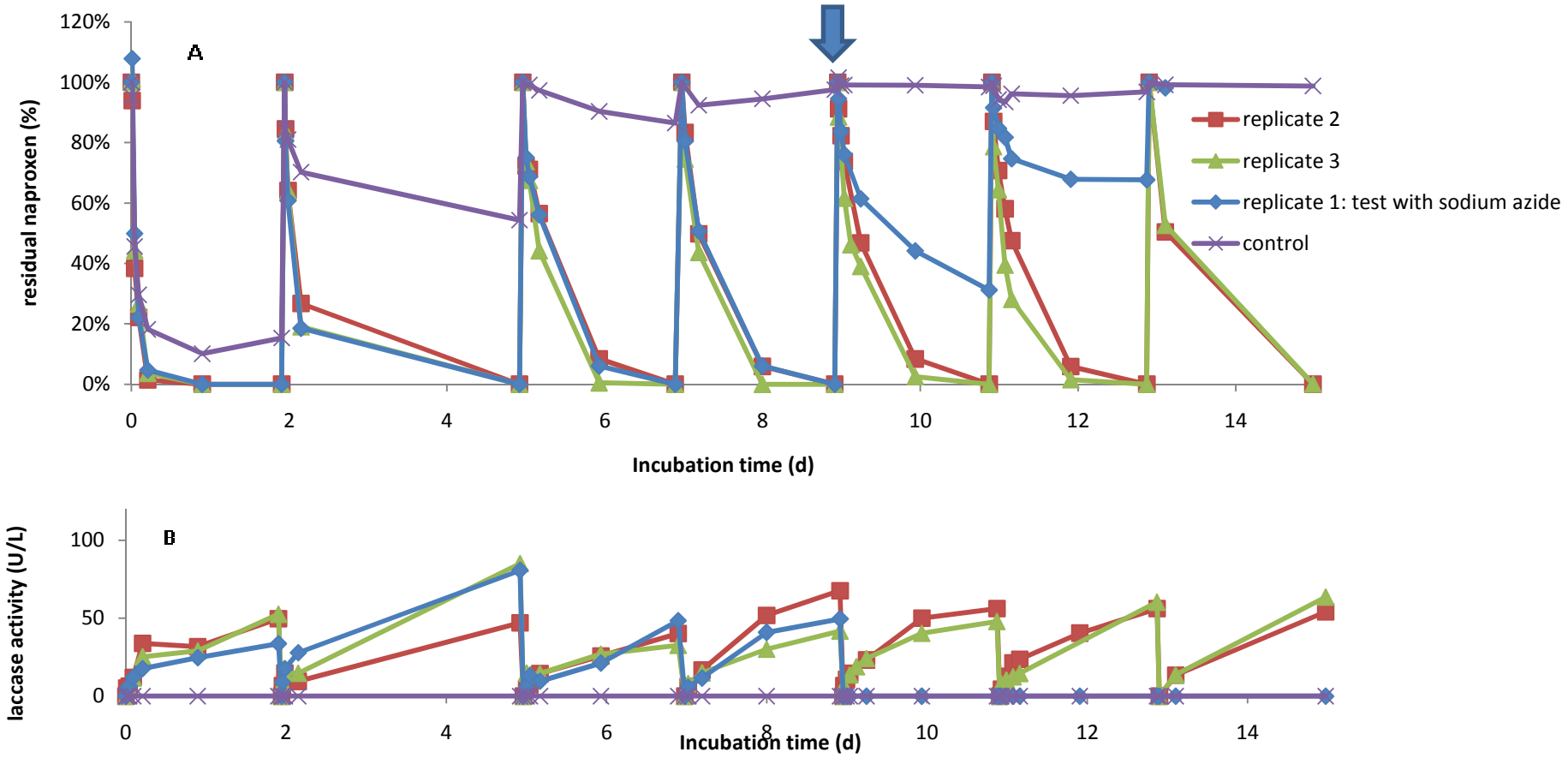

Figure 13 (a) Residual naproxen in three replicates of fungal filter with *Pleurotus ostreatus*. The blue arrow corresponds to the addition of sodium azide in the medium of replicate 1, (b) evolution of laccase activity for the same time. Characteristics of the initial medium in each cycle: naproxen 10 mg/l, dissolved in tap water.

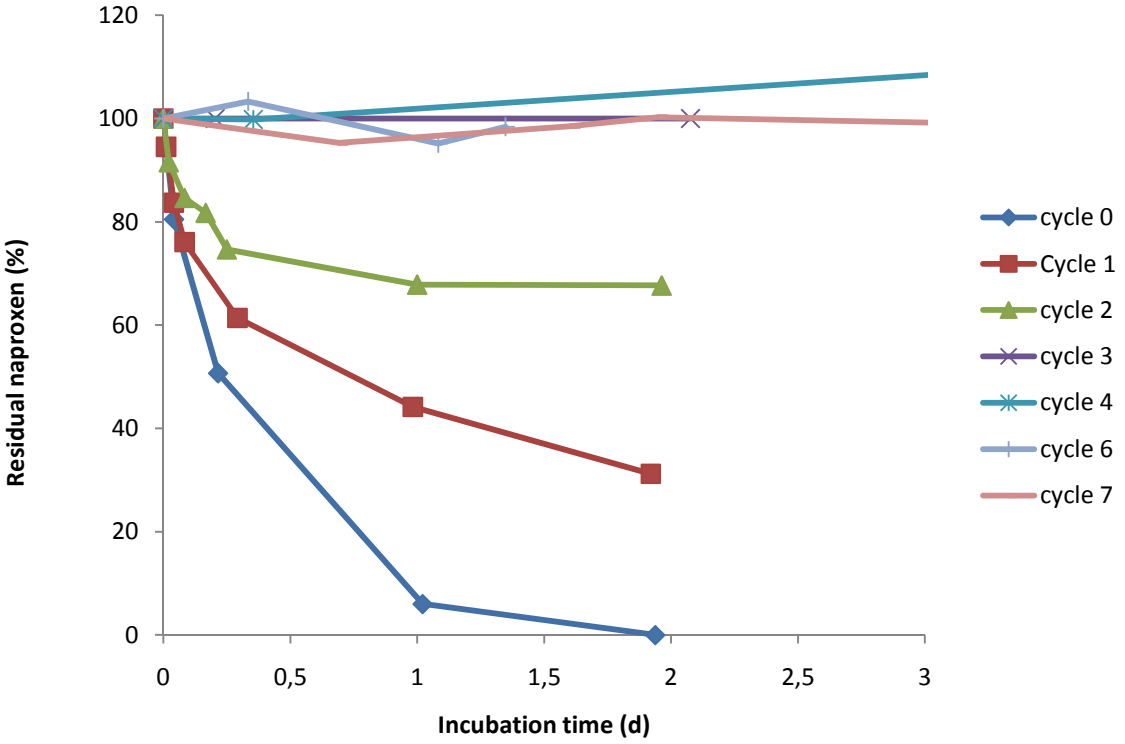

Figure 14 Residual naproxen during the inhibition test with sodium azide 10 mM in the medium. Cycle 0 is the cycle before of the addition of sodium azide.

5.4.3 Long term operation of the filter with *Pleurotus ostreatus*

To check out how long biofilters inoculated with *Pleurotus ostreatus* were able to work without re-inoculation or addition of fresh wood chips, long term operation experiment with two replicates were performed.

During the firsts 15 days naproxen removal from both replicates was very similar (**Figure 16**): in less than 48 hours naproxen was completely removed from the medium. After 15 days there was an omission of data (during 13 days it was not possible sampling). On day 28, it was observed that one column was completely clogged (replicate 1); it means that the column was completely immerged, without having any resting time. After that point the behaviour of two replicates were completely different. The one that was not clogged, replicate 2, could reach 53 days with almost the same efficiency. The efficiency of naproxen removal from the column that was clogged, replicate 1, started a strong decrease, after 30 days. This was probably because of too stressful conditions. In both cases, the values of activity started to decrease after 28 days, but more evident in replicate 1 (the one that was immerged) in which after 58 days no laccase activity was detected.

Excellent results were reached in replicate 2: until 78 days the naproxen removal was about 95% (**Figure 16 a**) and laccase activity was still significantly detected (**Figure 16 b**).

Until 78 days, the replicate 2 had almost the same efficiency as at the beginning. After that point the efficiency started to decrease.

5.4.4 Influence of the resting time on the removal kinetic

All the temporization (time of feeding, reaction and resting of the system) was chosen based on previous experiments with similar systems but in an arbitrary way. In order to check if a shorter resting time could have significant influence on the naproxen removal, a test of one cycle was done. The initial assumption was that with a shorter resting time the naproxen removal could be faster because there is more time for the reaction. There were not significant differences between two resting times (**Figure 15**). A shorter resting time implies also more stressful conditions for the fungi because of longer submersion and also more energy consume for pumping. That is why the longer resting time of three minutes was kept.

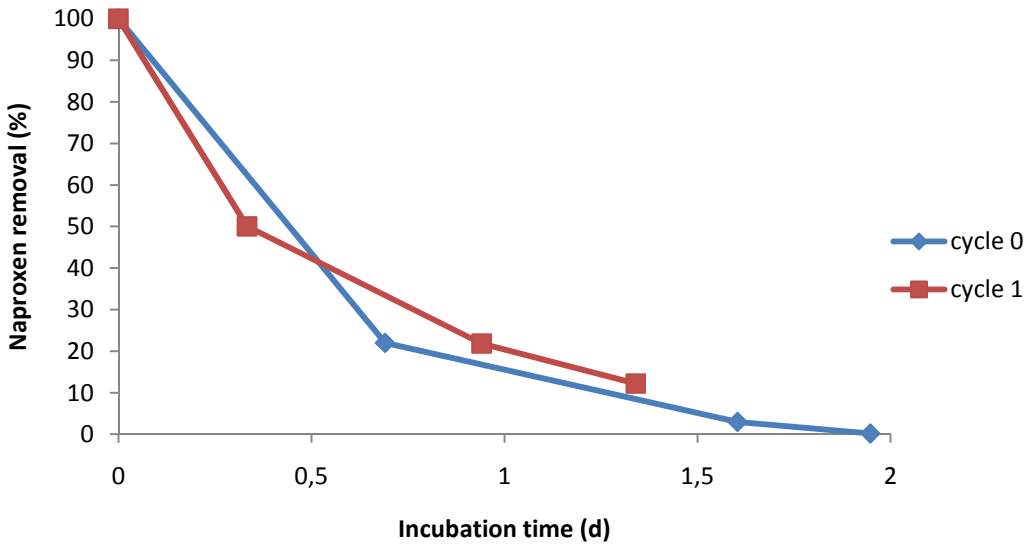

Figure 15: Naproxen removal with two different temporizations. Cycle 0 (5' total): 1 min feeding – 1' reaction - 3' resting. Cycle 1(3' total): 1' feeding – 1' reaction - 1' resting.

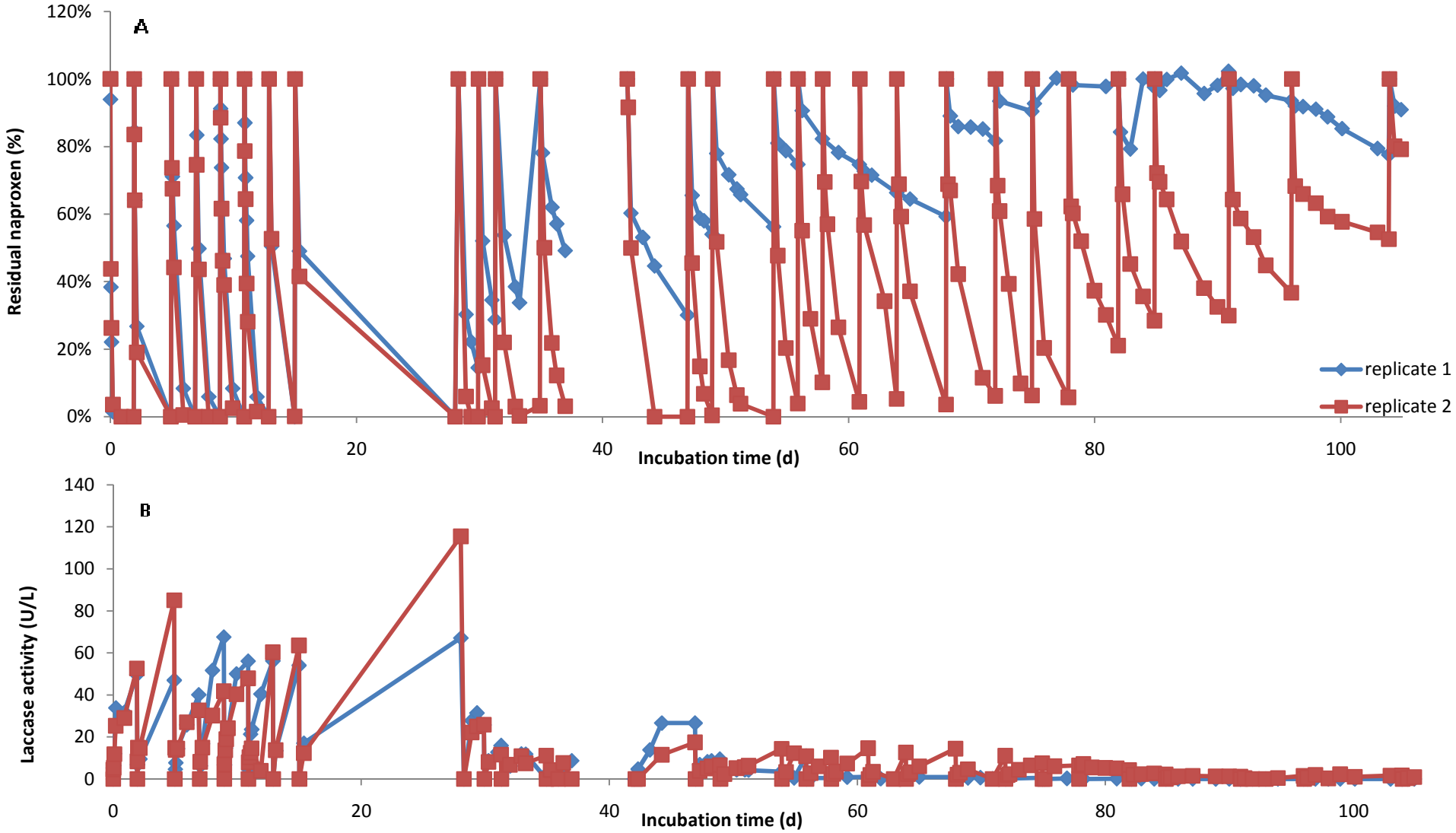

Figure 16: (a) Naproxen removal in two replicates with an inoculation with *Peurotus ostreatus* for a period longer than 100 days (b) laccase activity for the same time. Initial medium composition of each cycle: 10 mg/l of naproxen in tap water.

5.5 Efficiency of the fungal filters to remove naproxen in real treated waste water

As described in **section 3.8**, one experiment was done with real waste water. Waste water taken for the experimentation was sampled from the effluent of the Lausanne waste water treatment plant. In

Table 1, the composition of tap water from Lausanne (from publish data) and of the treated wastewater from the effluent of the Lausanne waste water treatment plant (measured in this study by ion chromatography (IC) and DOC/TOC analyser) are compared.

Compound	Concentration in the effluent of treated waste water	Concentration in tap water[a]	Units
Ammonium	6,59		mgN/l
Nitrate	8,03	2,7	mgN/l
Nitrite	0,48		mgN/l
Total Nitrogen	15,16		mgN/l
DOC	10,84		mgC/l
Phosphate	Non detected (n.d.)		mgP/l
Sodium	64,27	5,8	mg/l
Magnesium	9,86	6,4	mg/l
Potassium	12,29	1,5	mg/l
Calcium	76,99	47,1	mg/l
Fluoride	0,16		mg/l
Chloride	112,23	7,8	mg/l
Sulfate	53,40	49	mg/l
Bromide	n.d.		mg/l
Bicarbonates		104,3	mg/l
Aerobic mesophilic organisms	100000	156(maximum value detected)	CFU/ ml

Table 1: composition of tap water in Lausanne (published data) and composition of 24h composite sample (200ml/15 min), effluent of the moving bed bioreactor in Lausanne waste water treatment plant, from 15.01.2014 9.07 to 16.01.2014 9.07 (measured data). DOC: dissolved organic carbon, CFU: colony forming unit. [a]Source:http://www.lausanne.ch/lausanne-officielle/administration/travaux/eauservice/espace-didactique-et-loisirs/aventure-au-fil-de-l-eau/mainArea/00/col1/00/links/02/linkBinary/Info_04_La%20composition%20de%20l'eau.pdf

All the compounds were more concentrated in treated waste water than from the tap water. Which is consistent, because the second one is water for the human consumption and requires more accurately treatment.

The highest differences observed between the compounds are with sodium and chlorides: The value for sodium in tap water is 5.8 and in the waste water treatment plant of 64.27.

Chlorides are also very different between both kind of waters: the one from the tap water has a concentration of 7.8 mg/l and the one from the waste water treatment plant of 112.23 mg/l.

The maximum DOC value allowed for the effluent in Switzerland is 10 mgC /l, the values detected was slightly higher. In other similar waste water treatment plants, values of DOC are so much lower, around 3-4 mg C/l (Imai, Fukushima et al. 2002). DOC can be a source of carbon and energy for microorganisms, and thus growth of microorganisms in our filters due to this organic supply cannot be excluded.

In real treated waste water there are a lot of microorganisms, mainly bacteria, meaning that, with this medium, there are possibly more competition against the fungus than in tap water medium. The composition of this water provides also more facilities for bacteria to survive in it (higher DOC content). The treated wastewater contains about 10^5 CFU/ml of aerobic mesophilic organisms; per comparison, the limit for drinking water is at 300 CFU/ml and the maximum detected in Lausanne drinking water at 156 CFU/ml.

With these more adverse conditions for fungi it could be checked that naproxen could be also degraded in treated waste water with the fungal system.

Naproxen was removed from treated waste water with a similar trend than when the micropollutant was added in the medium with tap water until approximately 45 days (**Figure 17**).

In this experimentation, after about 50 days, a strange behaviour was detected in the naproxen removal in replicate 2 and in the control. From there, in replicate 2, naproxen was removed faster than the cycles before but even faster than from the beginning of the experimentation. Also in this point, the laccase activity of replicate 2 was increasing significantly. From this point, a strong removal in the control was also observed. As, in the previous cycles, the control was already at the equilibrium with the initial concentration of each cycle, it was not possible that this removal was due to adsorption on the wood. This strange behaviour should not exist if the system was working in habitual conditions. It seemed that a new organism, able to degrade naproxen, developed in the control.

The medium used from the new cycle when the incubation time was around 50 days was kept longer in the storage room (at 4°C) than the other ones. Maybe a specific bacteria able to degrade naproxen had time to develop in the medium, possibly explaining why the stronger degradation in replicate 2 and degradation in the control. Assuming that this stronger degradation was coming from some specie contained in the water, it cannot be explained the increasing of activity in replicate 2. Visually, it was observed that medium from replicate 2 became more yellow (approximately at the same time of fast degradation). It was assumed that this yellow colour came from the wood, maybe from some organic compounds released into the medium such as fluvic acid. It was not a proof, but the pH dropped strongly in this moment maybe because of this acidic compounds. Just as an assumption and to try to

understand this increasing of activity, maybe this specie that was able to degrade the micropollutant could also degrade cellulose and leave more compounds available also for the fungi, leading to a slow recovery of the fungus.

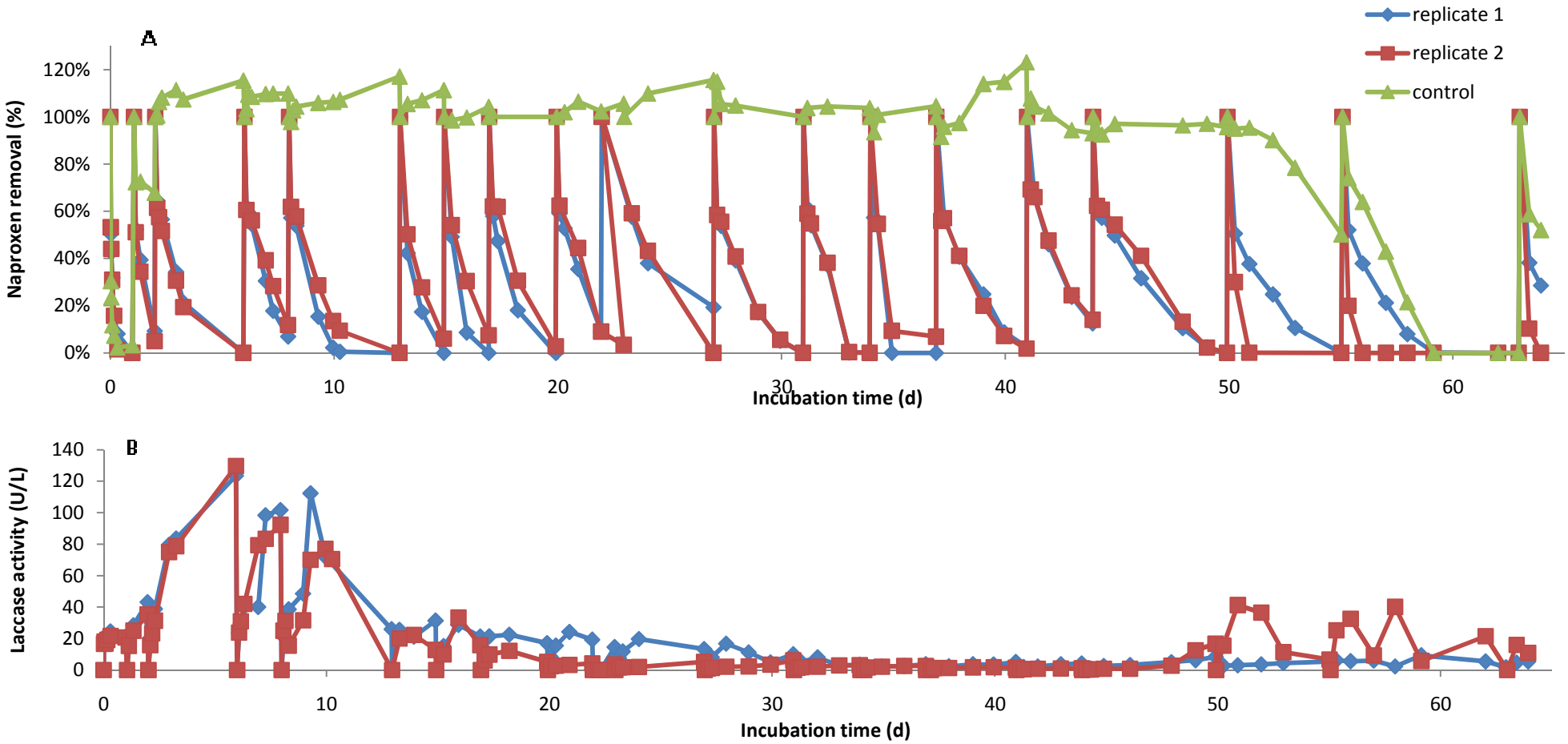

Figure 17 (a) Naproxen removal in two replicates with an inoculation of *Peurotus ostreatus* and in one filter (control) without inoculation. (b) laccase activity for the same samples at the same time. Initial medium composition of each cycle: 10 mg/l naproxen with real waste water effluent.

5.6 Efficiency of the fungal filters to remove sulfamethoxazole from tap water

The other micropollutant tested, sulfamethoxazole, was also degraded in the fungal filter by *Pleurotus* in tap water (**Figure 18**). One important fact to be noticed is that in replicate 1, sulfamethoxazole was not as well degraded than in replicate 2, and from cycles 4 to 8 the efficiency decrease in each cycle but not in replicate 2. It was assumed that sulfamethoxazole could be toxic in a concentration of 10 mg/l. The different of behaviour in replicate 1 and replicate 2 would be because the initial concentration of 10 mg/l was the limit of toxicity for fungi. The fungus in replicate 2 was, from the beginning, slightly more active and sulfamethoxazole was removed quite fast in each cycle, limiting the exposition to sulfamethoxazole. The fungus in replicate 1 was slightly less active and the pollutant was not completely removed in each cycle. This fact also means that the time of exposition to higher concentrations of sulfamethoxazole was longer in replicate 1 and it was possibly more toxic for the fungus. This assumption was based also in another previous test done with *Trametes versicolor* in a pure culture (data not shown) where it was possible to check a complete inhibition of growth at 10 mg/l of sulfamethoxazole. Because of these reasons, it was decided after 22 days to change the initial concentration of sulfamethoxazole from 10 mg/l to 5 mg/l (notice that there is one cycle again at 10 mg/l but it was just an operational mistake).With this new concentration, sulfamethoxazole was removed completely from the medium in both replicates.

Another interesting result from this experimentation was that sulfamethoxazole seemed to be a little degraded also in the control. To check if it was really degradation instead of adsorption on the wood, the cycle of the control was kept longer than usual and the concentration decreased from 10 mg/l until around 2.5 mg/l. This fact was meaning that sulfamethoxazole removal was probably due to degradation.

10 mM of sodium azide was added in the control (second blue arrow in **Figure 18 a**) in order to check if this degradation was due to the presence of any microorganisms in the water. After this addition degradation stopped, meaning that it was probably due to the activity of microorganisms in the water.

Sulfamethoxazole can be also degraded by photolysis (Niu, J. Et al. , 2012). That is why the system was covered by aluminium sheet to avoid the light contact (the third blue arrow is the moment where aluminium was put). There was no observed difference in sulfamethoxazole removal before and after this moment, meaning that photolysis was not responsible for the degradation.

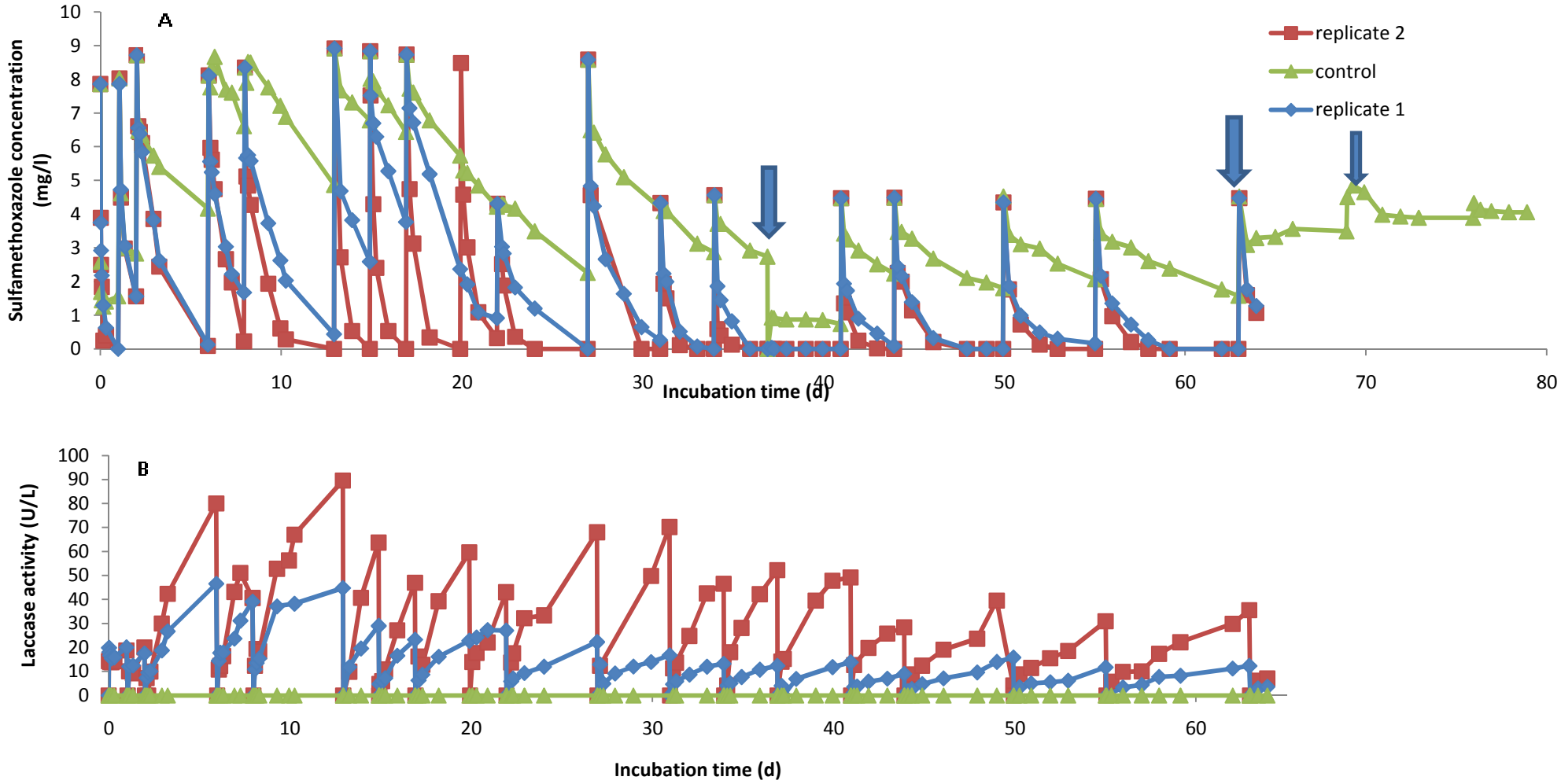

Figure 18 Residual concentration of sulfamethoxazole in two replicates with an inoculation of *Pleurotus ostreatus* and in one filter without inoculation (b) laccase activity for the same samples at the same time. Initial medium composition of each cycle: 10 mg/l or 5 mg/l of sulfamethoxazole with tap water. Arrows: from left to right: desorption test in control (1), addition of sodium azide in control(2), isolation from light (3).

5.7 Modelling of degradation and adsorption in the filters

The removal of micropollutant in a filter inoculated with *Pleurotus ostreatus* was modelled in order to try to predict the degradation in the medium and also to check if it was possible to fit a model (as described in **section 4**) to calculate and compare removal constant rates for each cycles. The degradation of micropollutant fits a pseudo-first order model as it can be checked in **Figure 19**.

Fast removal in the beginning of the experimentation was due mainly to adsorption phenomena as it has been discussed during all the report and when the incubation time increase the efficiency of each cycle decrease; because the pollutant concentration on the wood is in the equilibrium point with the liquid concentration but also because the fungi are less active.

In **Figure 20** can be checked the constant rate of removal for the same experimentation. This value gives an idea of the efficiency of the micropollutant removal (speed of the reaction).

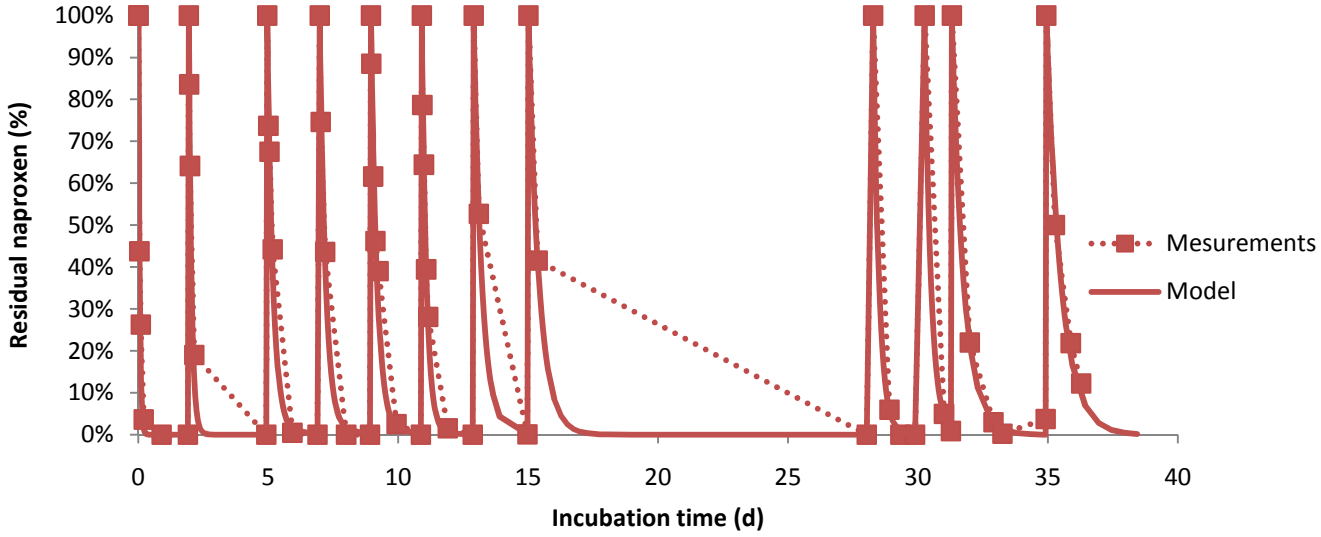

Figure 7: real values of naproxen removal in a biofilter with an inoculation of Pleurotus ostreatus with the values of modelling overlapping.

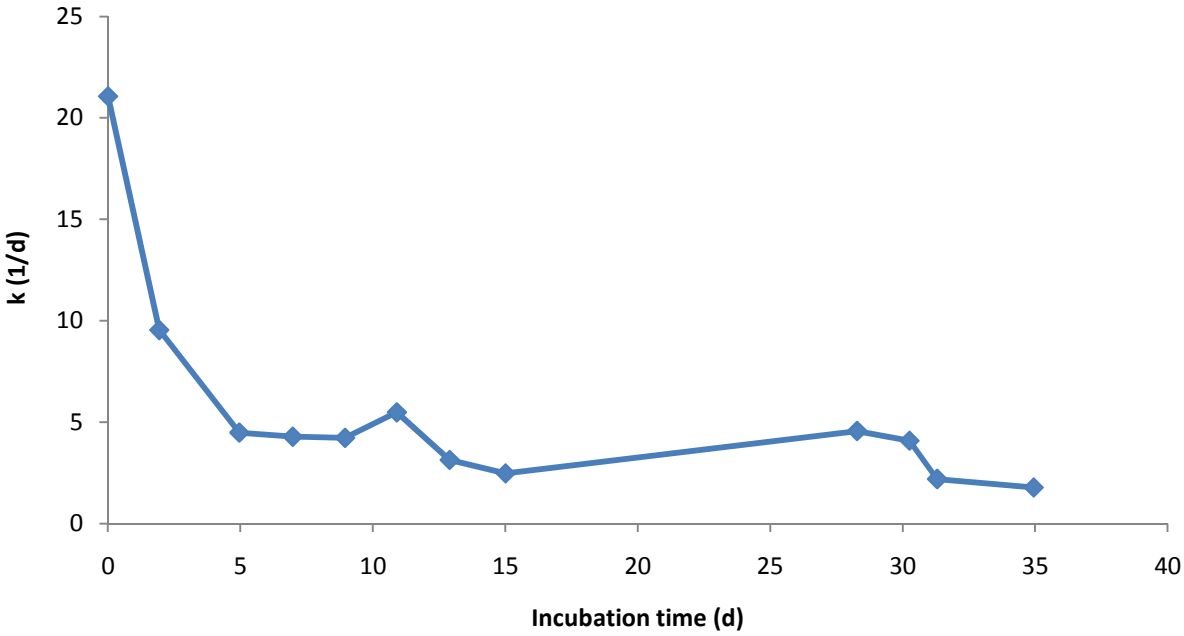

Figure 20: constant rate of removal k (mixture between adsorption and degradation) from the same experimentation as **Figure 19**.

In firsts cycles k value is high because of adsorption phenomena. Then, the general trend is to decrease because of the reasons explained just before. After 35 days, the removal rate is more than 10 time lower than at the beginning, and two time lower than after one week. An important fact to be noticed again in **Figure 19** and **Figure 20** is that from the day 17 to 28 of incubation time, there was a long cycle without changing the medium. When the cycle is longer, there is more time to desorb the micropollutant from the solid phase to the liquid phase. It means that there is more free space again on the wood to adsorb more quantity of pollutant. If you check when the incubation time is 28 days in both graphs, this fact is confirmed because the naproxen removal is faster than the previous cycle (in **Figure 19**) and also the k value is faster than in the previous cycle (**Figure 20**).

5.8 Comparison of micropollutants removal by pure cultures of *Trametes versicolor* and *Pleurotus ostreatus* in different liquid media

As described in **section 3.10**, experimentation with pure submerged batch cultures was done in order to check the influence of the medium composition with both fungi (*Trametes versicolor* and *Pleurotus ostreatus*).

Degradation of naproxen and naproxen mixed with methanol

Naproxen was well degraded in the medium by *Pleurotus ostreatus* in both conditions, with and without methanol. In around three days naproxen was completely removed from mediums containing *Pleurotus ostreatus*. In the biofilter, naproxen was removed from the medium in around 2 days when naproxen was added with methanol and also when naproxen was added alone. With *Trametes versicolor*, in both cases, naproxen was less

degraded than with *Pleurotus ostreatus* and significantly better degraded with methanol than when methanol was not added (**Figure 21 a**). It also must be noticed that laccase activity is not responsible of naproxen degradation, but is due to intracellular enzymes.

Pleurotus ostreatus reached the highest value of lacasse activity (55 U/L) in mediums containing naproxen with methanol, and also always higher activities in other medium with methanol compared to the ones without (**Figure 22 b**). This means that, probably, methanol alone or when naproxen (or sulfamethoxazole) is combined with methanol, stimulates the laccase activity, like it was observed in the experimentation with the entire biofilter (**Figure 6b**).

Both fungi can probably use methanol as a substrate or co-substrate when it is alone in the medium or combined with naproxen because, when there were these conditions, methanol was slightly, but significantly removed compared to the control without fungus (**Figure 22 c** and **Figure 22 d**). Methanol was, however, not used by the fungi as an easy growth substrate.

In the medium were methanol was added without inoculation of fungi, this alcohol was removed significantly due to evaporation, meaning that it is absolutely necessary to take it into account to calculate the methanol removal due to fungi degradation.

Degradation of sulfamethoxazole and sulfamethoxazole mixed with methanol

In all the cases sulfamethoxazole was not significantly removed. The only medium where there was a slight degradation was when sulfamethoxazole was added with methanol in mediums with *Pleurotus ostreatus* (**Figure 21b**).

Methanol seems to stimulate laccase production by *Pleurotus Ostreatus* but not with *Trametes versicolor*. (**Figure 21 b**).

Sulfamethoxazole is probably toxic at 10 mg /l for the fungal growth, like it was checked in one replicate in experimentation with the entire biofilter, because sulfamethoxazole was not significantly removed here compared to in the biofilters (**Figure 21 b**).

Degradation of glucose

Glucose was completely degraded in 5 days by *Trametes versicolor* but only much slower by *Pleurotus ostreatus* (**Figure 21c**). Another fact to be noticed is that, in presence of glucose, pH dropped very fast with *Trametes versicolor* but with *Pleurotus ostreatu*s the decrease was less significant and later. The drop of pH correlates well with the degradation of glucose. The acidification was very important with both fungi, reaching pH around 3; contrary in all other conditions tested, where pH was always around 6 (**Figure 21 d**). With *Trametes versicolor*, a part of this strong acidification, very high levels of laccase activity were detected, up to 384 U/L (**Figure 22a**) meaning that glucose can be used very easily for this kind of fungi.

However, the activity of *Pleurotus ostreatus* was not that high, which is correlated with the lower efficient degradation of glucose (**Figure 22 b**).

Biomass

The inoculum of *Pleurotus ostreatus* is denser than the one of *Trametes versicolor* because the same volume of inoculation has more biomass: 69 mg for *Pleurotus ostreatus* and 43.2 mg for *Trametes versicolor*.

As only the initial and the final values of biomass were taken, it is not possible to link the biomass with the real growth in each medium because in some mediums there was a fast growth at the beginning of the experimentation and the final biomass was less in comparison with the moment of the highest growing because of cellular degradation.

The final biomass of *Pleurotus ostreatus* does not have significant differences between the followings mediums: control (UAB medium), naproxen without or with methanol, sulfamethoxazole without or with methanol and methanol without micropollutant. In *Trametes versicolor*, the final biomass from those mediums were the micropollutant was added with methanol were significantly higher in comparison with the mediums with pollutants without methanol. However, when methanol was added without the micropollutant, the biomass did not have significant differences.

Mediums with glucose were the only mediums were a clear increased of biomass had place in both fungi. *Trametes versicolor* increased 181% the value of the initial biomass and *Pleurotus ostreatus* 295% of the initial biomass. Probably the biomass in *Trametes versicolor* when the maximum degradation took place was even higher than this value, because at the end of the experimentation less biomass was observed (**Figure 23**).

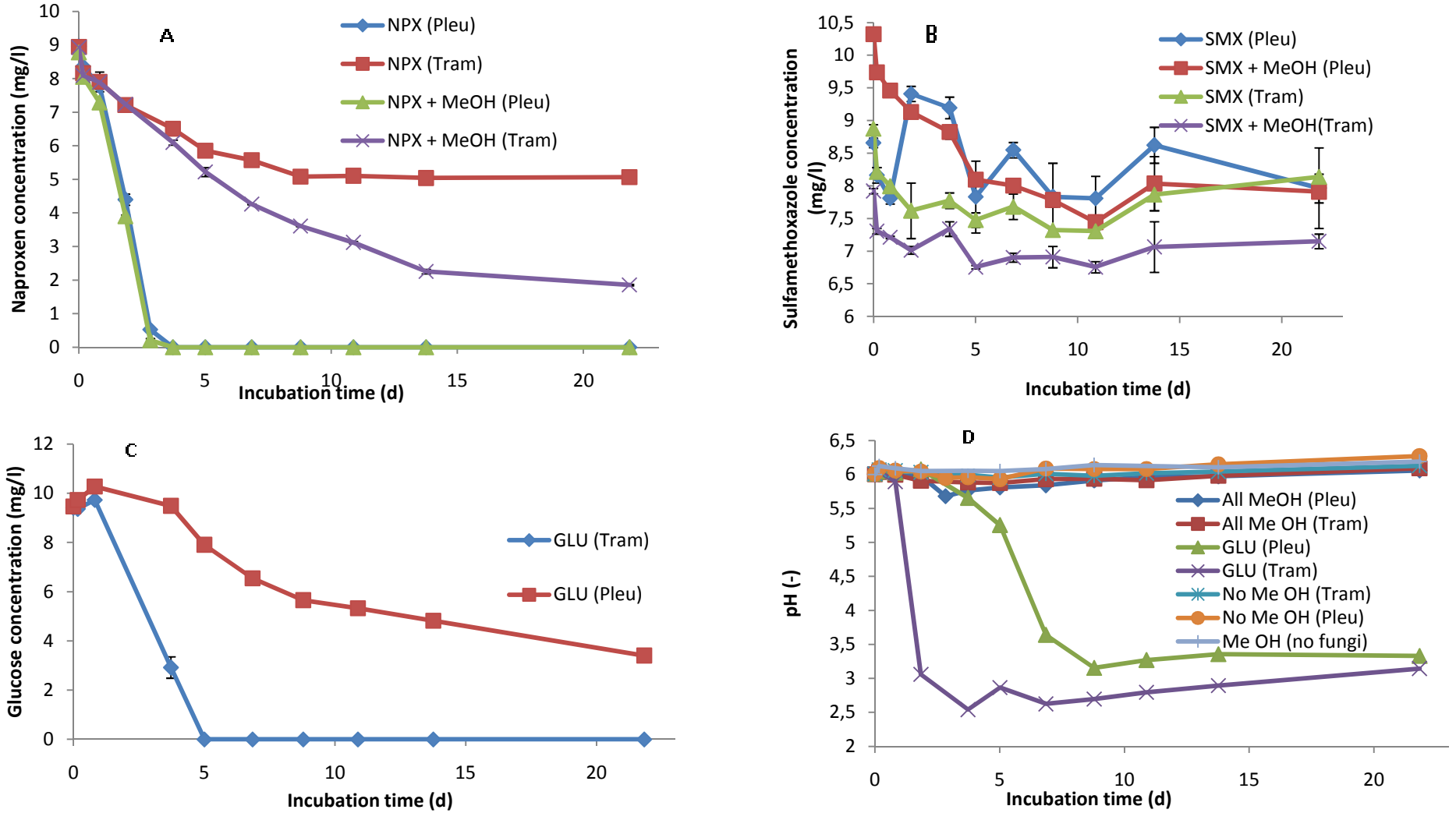

Figure 21: Experimentation in pure cultures. NPX: naproxen, SMX: Sulfamethoxazole, MeOH: methanol, GLU: glucose, Tram= *Trametes versicolor*, Pleu: *Pleurotus ostreatus*, no fungi: without inoculation. All the graphs contain the error bare.

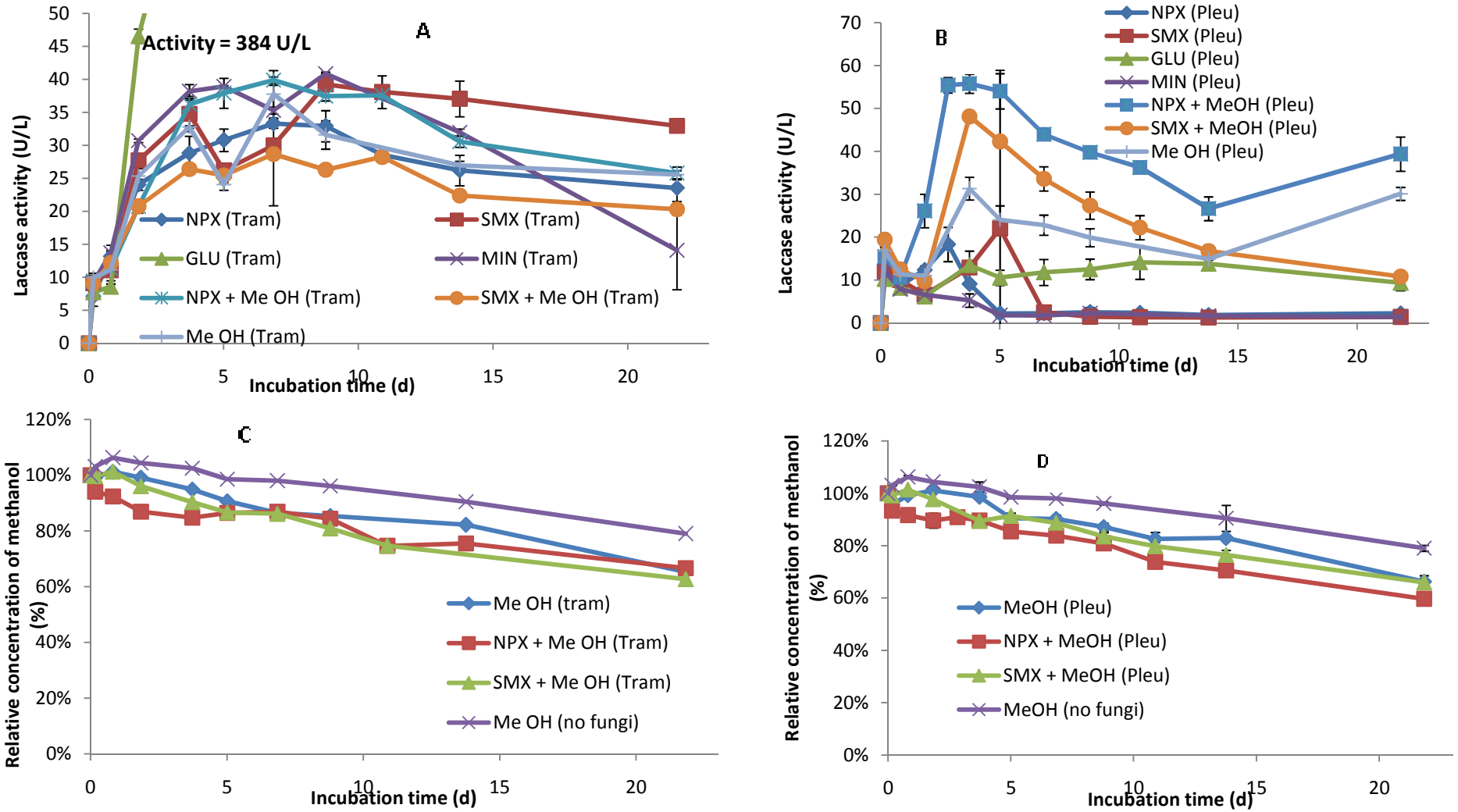

Figure 22: Experimentation in pure cultures. NPX: naproxen, SMX: Sulfamethoxazole, MeOH: methanol, GLU: glucose, Tram= *Trametes versicolor*, Pleu: *Pleurotus ostreatus*,All MeOH= average of all the samples containing methanol, No MeOH= average of all the samples without containing methanol, no fungi: without inoculation. All the graphs contain the error bare.

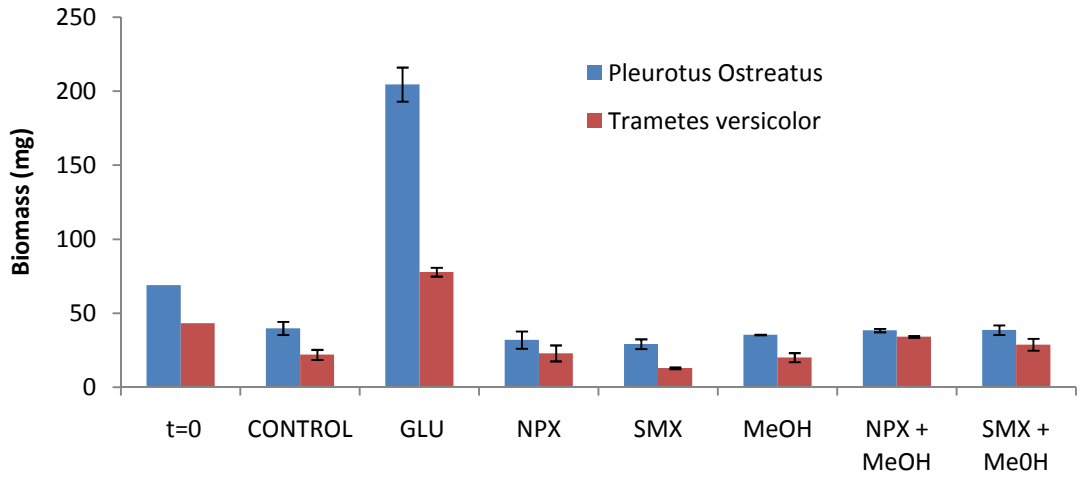

Figure 23: collected biomass at the end of the experimentation in pure culture test. Legend: T=0: Before starting the experimentation, control=just with mineral medium, GLU= mineral medium with glucose, NPX= mineral medium with naproxen, SMX= mineral medium with sulfamethoxazole.

6 Conclusions

This study highlighted several important points for the future development of a fungal filter for micropollutant removal in municipal wastewater:

Pleurotus ostreatus is better than *Trametes versicolor* for the system designed. *Trametes* did not survive more than one week in the filter.

Fungal treatment with *Pleurotus ostreatus* grown on wood substrates is a promising solution to improve micropollutants removal in wastewater:

- Naproxen and sulfamethoxazole are well degraded in the conditions tested.
- Cheap and widely available substrates (woodchips) are used.
- There is no need to add other external carbon source. The system is simple and requires only low maintenance (only pumps and valves)
- Long term operation (>3 months) without renewing the substrate is possible.
- Results are reproducible and fit a pseudo-first order model, meaning it is possible to make predictions for the biofilter management.
- Addition of methanol as a carbon source stimulates laccase production, but does not influence the rate of naproxen removal, and impact the survival of the fungus (growth of competitive organisms). Thus addition of external carbon source is not recommended.

Still some research has to be performed before the development a fungal filter suitable for small waste water treatment plants:

- Design optimization to reduce the treatment time (24-48h), the electricity consumption for recirculation (now up to 0.35 kWh/m^3) and the space needed for the filter (now up to 0.5-1 m^2/capita)
- Strategy for wood inoculation and replacement (long term operation)
- Reduction of input of organic compounds leaking from the wood
- Test a wider range of pollutants (just tested 2 micropollutants with the experimentation).
- Test with real concentration of micropollutants

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