PHAREM
BIOTECH

Summary report on PCure pilot project at Uppsala University Hospital

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Abstract

This study was conducted to demonstrate how a toilet rimblock containing enzymes (PCure), can be used for the degradation of pharmaceutical residues that are emitted through consumption, and further released into the toilet in a hospital environment. The test environment was developed to emphasize testing in real environment at Uppsala University Hospital (UUH) focusing on the use of PCure at daily hospital environment. A section of 8 toilets was included in this study with the goal to show a reduction of 30 – 50 % of the targeted pollutants. The result showed high removal rates but low statistical relevance and has been redacted from this summary at the request of UUH with the agreement on first right of publication. The decision was made to instead publish data on the future large scale implementation at UUH.

Introduction

Through daily usage and passage through the body, pharmaceutical residues will end up in the sewage through the toilet. This constitute to the largest input of pharmaceuticals to waste water treatment plants (WWTPs) (Thomas et al. 2007). The WWTPs are generally not designed to remove pharmaceuticals and most of the substances pass the plant unaffected (Hansen et al., 2016) and can reach concentrations in the effluent water of ng/L - µg/L (Falås et al., 2012). The reports on the harmful effect from pharmaceutical residues in the environment are increasing and pose a major risk for aquatic and terrestrial ecosystems. A highlighted case concern the sex change and sterility in fishes and amphibian animals caused by hormones found in birth control pills (Metcalfe et al., 2001). The study of the changed behavior of perch when exposed to oxazepam received attention (Brodin et al., 2013). The discovery of the high death rate of indian vultures connected to the usage of diclofenac in farm animals (Cuthbert et al., 2014) has raised the concern of pharmaceuticals effect on the environment. One major concern is the possibilities of the development of antibiotic resistance connected to the spread of antibiotics into the environment (Bengtsson-Palme & Larsson, 2016)

Municipal waste water is the main contributor to pharmaceuticals in WWTPs, but hospitals constitute an important point source (Hansen et al 2016). In a study of a Norwegian hospital, same size as the study site Uppsala university hospital (UUH), pharmaceutical concentrations in effluent waste water from the hospital were measured in 24-hours samples (means: Ciprofloxacin 23336 ng/l, Doxycycline 124 ng/l, Diclofenac 819 ng/l, Metoprolol 1072 ng/l and Trimethoprim 4302 ng/l , compared to the influent water of a WWTP in Norway (means: Ciprofloxacin 1480 ng/l, Doxycycline <5 ng/l, Diclofenac 362 ng/l, metoprolol 717 ng/l and Trimethoprim 835 ng/l), demonstrating hospitals as an important point source of pharmaceutical residues to the sewer system (Thomas et al 2007).

There are examples on hospitals around the world with installment of water treatment with examples in Germany, Netherlands and Switzerland (Pills, 2012) and instalments done in Denmark (Nielsen et al., 2013. Common approach is the use of membrane bio reactor (MBR) followed by activated carbon,

nanofiltration or oxidation processes to remove pharmaceuticals (Casas et al., 2015).

These technologies all pose extensive infrastructure changes and additional operative costs to be considered for implementation at a broad level at hospitals. With concern for the risk of pharmaceuticals in the environment it is thus needed to identify methods to degrade pharmaceuticals at the point source. Therefore the project performed a pilot study to evaluate PCure, a method to distribute enzymes directly to the water in toilets.

Enzymatic degradation

The use of enzymatic transformation of persistent pollutants show great promise as eco-friendly alternative to conventional physiochemical methods (Hai et al., 2013). Compared with most chemical catalysis processes, enzymatic degradation can be done without added supplements and energy and thus produce less waste. Enzymatic processes can be performed under mild conditions with high reaction rate and specificity at an industrial scale.

Enzymes has been reported in many cases for degradation of pharmaceuticals. For example the use of laccase has been reported to degrade synthetic hormone disruptors that are considered being behind the environmental effect seen in fishes (Lloret et al., 2012) and has also shown effect on a wider arrange of pollutants (Gasser et al., 2014) and tetracycline classed antibiotics (Suda et al., 2012). The enzymes within the group of peroxidases also show effect on a wide arrange of pollutants (Bansal et al., 2013) and are seen as important candidates for industrial applications. Enzymes has been used for degradation of carbamazepine and diclofenac (Zhang et al., 2010) considered to be some of the more severe pharmaceutical pollutants. There also exist wide arrange of enzymes that target different kind of antibiotics, for example macrolides by esterases (Morar et al., 2012), tetracycline by lignin peroxidase (Wen et al., 2009) and ampicillin, sulfathiazole by glutathion S-transferase (Park et al., 2007).

The wide arrange of publications concerning enzyme degradation of pharmaceutical and the established industrial use of enzyme form a foundation where the use of enzymes can be used for treatment of pharmaceutical pollutants.

PCure toilet block

PCure is a toilet block placed over the rim of the toilet, and it contains enzymes capable of degrading pharmaceuticals. The block consists of 40 % starch, 40 % cellulose, 5 % magnesium stearate, 10 % Tween-20 and 5 % enzymes. The mixture is pressed to form a block and then put in a plastic casing. Every time the toilet is flushed, an expected amount of 2 % of the total block volume is released into the water by flushing over the block. When the enzymes enter the water, they will diffuse into the water volume and start reacting with the pharmaceuticals targeted by the enzymes. The enzymes are expected to stay latent in the still toilet water between flushes and follow the water down the drain. Since enzymes are proteins, they will quickly be degraded by the active biological environment in the sewage for nutrients, but is expected to keep the activity until then. Three different enzymes were developed by Pharem Biotech for this project and put into PCure. The enzymes has been going through targeted mutations for optimized functionality in the toilet environment. The enzymes are also selected by their functionality to target certain chemical functional groups in the substances of interest. The enzymes where produced by fermentation of recombinant E. Coli BL21(DE3). By targeting functional groups, the selected substances can be degraded into smaller inactive parts, causing them to not keep their biological activity and have no toxic effect on the environment.

Experimental set-up

Uppsala University Hospital (UUH) is a full-scale tertiary/university hospital with 1000 beds. The study took place in a 7 floor building housing several inpatient wards and day care clinics. The sewage pipes at the study site are vertical and one of them was connected to a tank in the basement, so that a portion of the sewage flow ended up in the tank, filling approximately half of its 200 liter volume in 24 hours (Figure A). Six toilets were connected to this sewage pipe and included in the study. The toilets served eight patient rooms in three wards (geriatrics, gastroenterology and renal, and infectious diseases) and one dermatology outpatient clinic (Figure A).

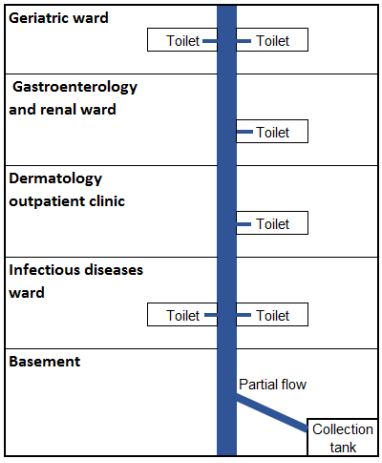

Figure A. The experimental set-up displaying the sewage pipe, toilets included in the study and the collection tank in the basement.

Every morning, around 8 o'clock, the partial flow was stopped to the collection tank, and the contents were mixed. A 50 ml sample was taken from the tank and put in a -20 degree Celsius freezer. After sampling the tank was emptied and cleaned, and then the partial flow was opened for another 24 hours. The study was performed during six weeks in August to October 2016. Every other weeks PCure was installed and not installed, respectively. The goal is to show a reduction of selected substances to 30 – 50 %.

Measured substances were Ciprofloxacin, Diclofenac, Doxycycline, Metoprolol, Tazobactam, Tramadol and Trimetoprim.

Estimation of used amounts of pharmaceuticals

To estimate the use of the studied pharmaceuticals, the nurses at the included wards marked a checklist every time they dispensed any of the drugs to a patient using a toilet included in the study. The checklist contained information of the substances, such as the name(s) of different formulations of the pharmaceutical and different dosages. The checklists was changed once a week, and notes on the doors to study rooms and regular visits by a member of the research team were employed to remind the nurses of the checklists.

During the study, the patients in the rooms with the studied toilets only got to use that particular toilet. One of the studied toilets was located at the dermatology outpatient clinic, and thus it was impossible to get access to the history of pharmaceutical use from the patients using this toilet.

Analysis of pharmaceuticals in collected wastewater

To analyze the concentrations of the substances in the sampled water, the company Metasafe AB were employed that measured the concentrations using a standard addition technique and Ultra Performance Liquid Chromatography (Waters Acquity) and Mass Spectrometry (Sciex API 5500 triple quadrupole) (UPLC/MS).

Instruments

UPLC
Waters Acquity

Column: Waters Acquity, 50 x 2.1 mm, 1.7 µm, 0.3 mL/min, Phase A: water 0.1 % FA

Phase B: ACN 0.1% FA

Gradient

Time, min	0	3.5	3.55	4.0
Phase A, %	95	0	95	95
Phase B, %	5	100	5	5

Mass spectrometry
Sciex API 5500 triple quadrupole

MRM mass transitions, positive mode, turbospray

Compound	Q1 mass	Q3 mass	Dwell time, ms	Collison energy, eV
Ciprofloxacin	332	231	50	45
Doxycycline	445	428	50	24
Trimetroprim	291	239	50	30
Diclofenac	296	215	50	25
Metoprolol	268	116	50	23
Tazobactam	301	168	50	18
Carbamazepin ^{13}C, ^{15}N	239	194	50	24
Tramadol	264	58	50	19

Injection volume 10 µL.

Solutions
Prepare a 2 µg/mL solution of Carbamazepine ^{13}C, ^{15}N (internal standard) in acetonitrile.

Prepare the following solutions in acetonitrile for all reference compounds 200, 20, 2, 0.2 and 0.02 µg/mL.

Analytical procedure
Pipette 1.5 mL of WW into a 1.5 mL Eppendorf tube and centrifuge at 20000 g for 10 minutes.

Pipette 1 mL of the centrifuged sample into a 2 mL glass injection vial and add 10 µL internal standard solution.

Prepare calibration samples in DD water covering the expected concentration range of drug compounds in the WW. Make calibration standards in 2 mL glass vials by adding the same amount of internal standard as used for the WW samples. Analyse all samples using UPLC/MS.

Matrix effects on the mass spectrometric response
The mass spectrometric response varies depending on the composition of the matrix (background substances in the WW). The matrix effects can be either positive, ion enhancement or negative, ion suppression. Since the matrix will vary from sample to sample depending on the exact composition of background substances in each given sample it is not possible to prepare calibration standards in a matrix which is equal to the WW samples. Therefore, a standard addition technique was used where the matrix is kept constant and thus eliminating possible matrix effects.

Pre-quantitation of drugs in WW
Analyse the calibration standards and all WW samples by UPLC/MS and quantitate them. This gives a rough estimate of the concentration of the drugs.

Final quantitation of drugs in WW
Add a known amount of drug to the WW sample in the injection vial. The amount added should be selected so that the mass spectrometric response for each drug is roughly doubled. The sample is then reanalysed by UPLC/MS. This procedure is repeated once more. The concentration of each drug is determined by the standard addition technique.

Discussion

The study indicated a high removal rate of the targeted substances above the goal of 30 - 50% reduction. The results did not show sufficient statistical certainty of pharmaceutical reduction in hospital wastewater due to fluctuation in data. The decision from UUH is to continue with PCure at a larger scale of implementation and wait with any publication of data until such a project has been conducted. Thus this report will not contain result details

of the project until agreed upon by UUH and Pharem Biotech.

The input data was dependent on information from the nurses giving patients medicine which contained the targeted substances. The collection of these data were occasionally forgotten could show significant fluctuations in in-put of data and measured substance concentrations. During certain periods during the project there were substances not used in sufficient amount giving low analytical responses. The conclusion from this is that the scale of eight toilets as testing pool was not sufficient to give statistical data of high significance to fully demonstrate the full effect of using PCure in the toilets. The formal decision for a large-scale implementation at UUH for further investigation of using PCure as full-scale solution for removing pharmaceutical pollutants in hospital sewage, show that there is a consensus within the project that the functionality and the possibilities of PCure is promising. The conclusion within the project indicate that using enzymes, which are released through a toilet rim block, can be used as a solution at hospitals for pharmaceutical degradation.

The work of this project has led to promising future prospect for PCure as a contributing solution to the issue of pharmaceutical pollutants. The already promising results and future development of PCure has the potential of making it a commercially available product for hospitals in the near future.

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