Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Institutionen för energi och teknik

Application of 2D fluorescence spectroscopy on faecal pigments in water

– Characterization of wastewater fluorescence and potential indication of faecal pollution

Bärbel Daub

Master's Thesis 45 credits
Second cycle, A2E
Uppsala 2017

Institutionen för Energi och Teknik
Department of Energy and Technology

Examensarbete 2017:01
ISSN 1654-9392
Uppsala 2017

Title:	Application of 2D fluorescence spectroscopy on faecal pigments in water
Supervisor:	Annika Nordin, Swedish University of Agricultural Sciences, Department of Energy and Technology
Assistant Supervisor:	Martin Wagner, DVGW Technologiezentrum Wasser (Water Technology Center), Germany, Außenstelle Dresden
Examiner:	Björn Vinnerås, Swedish University of Agricultural Sciences, Department of Energy and Technology

Credits: 45 ECTS
Level: Advanced level, A2E
Course title: Independent project in Environmental Science – Master's thesis 45.0 credits
Course code: EX0828
Programme/education: Soil and Water Management – Master's programme

Series title: Examensarbete (Institutionen för energi och teknik, SLU), 2017:01
ISSN: 1654-9392

Place of publication: Uppsala
Year of publication: 2017
Online publication: http://stud.epsilon.slu.se

Keywords: wastewater, faecal pollution, faecal pigment, fluorescence, urobilin, stercobilin

Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Natural Resources and Agricultural Sciences
Department of Energy and Technology

Abstract

Drinking water pollution by faeces and associated enteric pathogens can cause serious health issues and outbreaks of diseases. A fast and reliable indication of faecal pollution is necessary to prevent the consumption of polluted water. This work aims at identifying faecal pigments in wastewater and discusses the possibility of using on-line fluorescence monitoring of faecal pigments in water as a tool for the detection of faecal pollution. Three faecal pigment standards, urobilinogen, urobilin, and stercobilin, as well as wastewater in- and outflows from five German wastewater treatment plants (WWTPs) were characterized by 2D fluorescence spectroscopy (using Excitation Emission Matrices), and by high performance liquid chromatography (HPLC) coupled with absorption (DAD) and fluorescence detection (FLD), as well as mass spectrometry (MS). Furthermore, tests on faecal pigment stability, reaction to zinc addition, kinetics, and pH influence on faecal pigment fluorescence were performed. With the obtained fluorescence data, a parallel factor analysis (PARAFAC) model for the detection and quantification of urobilin and stercobilin in real water samples was developed. An addition of zinc to the pigments in real water lead to a time-dependent fluorescence intensification of a factor >30 and red shift of the pigments' fluorescence spectra, which can be used as a tool to detect low concentrations of faecal pigments in water. Urobilin and stercobilin were identified in all examined WWTP inflows. The results and literature study indicated that a degradation of faecal pigments during wastewater treatment may have taken place. In the wastewater of one treatment plant, fluorescein was detected. Fluorescence detection and quantification of faecal pigments in wastewater was possible with the help of zinc addition or prior enrichment, but more studies are needed to enhance the sensitivity of the method to be sensitive enough to detect faecal pollution in concentrations relevant for drinking or surface water monitoring. It was concluded that fluorescence detection of faecal pigments in water is promising as an early warning system, but in this study it did not prove sensitive enough to be used as a stand-alone method.

Keywords: wastewater, faecal pollution, faecal pigment, fluorescence, urobilin, stercobilin

Popular Science Summary

It is not new that one can get sick by the consumption of polluted water. Many of the diseases spread with polluted water are caused by organisms living in faeces, which are present in the water because there has been some faecal contamination. Understandably enough, ingesting faeces and pathogens with water is not a nice thought. For this reason it is very important to detect any faecal pollution of drinking or recreational water as fast as possible. For the detection, the yellow-brownish pigments that determine the colour of urine and faeces can potentially be used as indicators. Some of these pigments are fluorescent, which means that they emit light with a characteristic colour when they are illuminated and this can be used to identify them.

There are already other methods to detect faecal pollution of water, but the established methods take a lot of time and labour is necessary to handle water samples in the laboratory. To detect faecal pollution with the help of fluorescence techniques would have the advantage of being very fast and requiring little handling. Especially a fast result is important to warn people before they drink polluted water or swim in it.

In this study, it was investigated how well the fluorescence detection of faecal pigments works under different conditions. One aim was to find the pigments in wastewater with the help of fluorescence techniques. Also, an unidentified fluorescence signal that had been found in an earlier study in wastewater was examined. For this purpose, faecal pigments (urobilin, urobilinogen and stercobilin standards) were characterized in different water media with fluorescence spectroscopy as well as with some reference methods. Samples from 5 different wastewater treatment plants were taken and also characterized with the same methods as the pigment standards. Then the results were compared. To make the identification of faecal pigments easier, a model was developed that was supposed to recognize faecal pigments in water by interpreting fluorescence data.

It was found out that the unidentified fluorescence signal in wastewater was caused by several substances, both faecal pigments and also another substance, which is called fluorescein. As the name says, this is a chemical which has a strong green fluorescence and can be used to mark the flow of water, for example when leaching from a pipe is suspected (it is also said to have been used earlier to dye the Chicago river green on St. Patrick's Day, but that is another story). The faecal pigments could be found in the inflow to all wastewater treatment plants, but they could not be found in the cleaned outflow of the plants. It was not possible to recognize the pigments by fluorescence only, because wastewater contains many other substances. An enrichment of the wastewater, meaning that it was concentrated, could help. Adding zinc to the wastewater also improved the fluorescence signal and might make an enrichment unnecessary. However, the fluorescence intensity after zinc was added decreased again, so more tests need to be done to get a reliable method. Fluorescein was mainly found in water from one of the treatment plants. It looks very similar to faecal pigments in its fluorescence, causing a risk to confuse the substances. The model was able to solve this problem and could distinguish between faecal pigments, their zinc complexes and fluorescein. However, the model needs to be tested on more data to ensure its reliability. In conclusion, there are still some uncertainties and the method is not yet sensitive enough, but after some more development, fluorescence detection of faecal pigments in water has a great potential.

Table of contents

Glossary

λ_{em}	Emission wavelength [nm]
λ_{ex}	Excitation wavelength [nm]
Aqualog	Horiba Aqualog fluorescence spectrometer
DAD	Diode array detector (UV-Vis absorption)
EEM	Excitation emission matrix
Gain factor	Gain in fluorescence intensity in relation to the start intensity
FLD	Fluorescence detector
HOWI	Drinking water after active coal filtration from the drinking water plant Hosterwitz, Germany
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
Matrix	Refers in this case to the excitation emission matrix (EEM) of fluorescence intensities resulting from a fluorescence measurement
Medium	Refers to the liquid, in which the pigment standards were measured
MilliQ	Ultrapure water (filtrated through a membrane filter)
MS	Mass spectrometry
NMF	Non-negative matrix factorization
PARAFAC	Parallel factor analysis
Phosphate buffer	If not stated otherwise, this refers to phosphate buffered MilliQ water at pH 6.6
SB	Stercobilin
SSE	Sum of squared error
UB	Urobilin
UBGN	Urobilinogen
WWTP	Wastewater treatment plant

1 Introduction

Faecal pollution in water is not only an unpleasant thought, but often the cause of pathogen presence in drinking or surface water. Especially in regions with lacking or insufficient sanitation, severe disease outbreaks can be caused by faecal pollution of drinking water (Ashbolt, 2004). Pathogen detection, however, is difficult and the analysis of microbial or molecular indicators of faecal pollution can be a time consuming and complicated effort (Jones-Lepp, 2006). Minimizing the time that passes between the pollution event and its detection is critical to take measures for preventing the consumption of polluted water by people. Thus, in order to minimize health risks, there is a need for fast and reliable indication of faecal pollution in water.

Optical methods like fluorescence spectroscopy provide a good tool for water quality assessment because they do not require a lot of effort in handling and the results are visible immediately. A further advantage is the possibility of on-line monitoring, allowing for continuous data collection. Thereby, already small changes in the water quality can be detected, as well as quality problems reported immediately. There are already some common applications of fluorescence spectroscopy for environmental monitoring, including e.g. classification and quantification of dissolved organic matter (DOM) in marine water and freshwater, as well as wastewater analysis (Hudson *et al.*, 2007; Henderson *et al.*, 2009).

Fluorescence detection of bile pigments has been used for medical purposes for a long time, because high bile pigment levels in urine and faeces can be an indication for a clinical liver function (Watson, 1931 in Bloomer *et al.*, 1970). More than a century ago, faecal pigments were described and methods for their detection were developed (Jaffe, 1868, cited in Watson, 1969; Van Lair and Masius, 1871, cited in Watson, 1969; Schlesinger, 1903). In environmental science however, these pigments have been discovered to be useful as an indication for faecal pollution much later. In combination with high performance liquid chromatography, fluorescence detection of faecal pigments in a river was studied in Japan in the early 1990s (Miyabara *et al.*, 1994c). Since then there have been several studies on the subject of using faecal pigments as indicators of faecal pollution in water with different methods. Studies about the detection of faecal pigments in wastewater were done (Loganathan *et al.*, 2009; Miyabara *et al.*, 1994d), and as well about urobilin as an indicator of faecal pollution in the environment (Jones-Lepp, 2006; Piocos and La Cruz, 2000). However, the use of fluorescence spectroscopy to detect urobilin in water, without prior enrichment or help of other methods, has, at present, only been documented by Bixler *et al.* (2014), who used cavity-enhanced fluorescence spectroscopy.

In a previous Master thesis from Technologiezentrum Wasser (TZW - DVGW Technologiezentrum Wasser, Außenstelle Dresden), unknown fluorescence peaks were found in wastewater (Dang, 2016), which were suspected to be faecal pigments. Now it should be examined whether faecal pigments can be detected in wastewater by means of fluorescence spectroscopy and if fluorescence spectroscopy of faecal pigments could potentially serve as a stand-alone method for monitoring faecal pollution in water.

2 Aim

The aim of this work was to characterize not interpreted fluorescence signals, which were found in wastewater in a previous study at TZW, and to assess the applicability of using fluorescence detection of faecal pigments for the indication of faecal pollution.

For this purpose, some research questions were raised

- Is fluorescence spectroscopy suitable for the detection and quantification of faecal pigments in water?
- Can the pigments' fluorescence signal be enhanced for a more sensitive detection?
- Is faecal pigment fluorescence stable over time?
- Can faecal pigments be detected and quantified in wastewater by using fluorescence spectroscopy?
- Which fluorescent faecal pigments are present in wastewater?

In the process of answering these questions the following aspects were investigated

- Characterization of stercobilin, urobilin and urobilinogen standards by means of fluorescence spectroscopy, HPLC and MS
- Establishing a relationship between faecal pigment concentration and fluorescence intensity by the construction of calibration curves and determination of analytical limits for fluorescence detection of the pigments
- Examination of fluorescence enhancement by zinc addition to urobilin and stercobilin
- Assessment of faecal pigment stability in different media and under different storage conditions
- Determination of pH influence on faecal pigment fluorescence
- Screening of wastewater samples (WWTP inflow and outflow) from five different wastewater treatment plants for faecal pigments by fluorescence spectroscopy, HPLC, and MS
- Characterization and quantification of faecal pigments (urobilin and stercobilin) present in wastewater
- Development of a general model for identification and quantification of faecal pigments in water

3 Background

3.1 Faecal pigments

The term faecal pigments refers to pigments present in the intestine, which are excreted with faeces and urine, giving them their characteristic colour. As faecal matter is excreted from the body, its pigments are as well, together with countless microbes. Some of the pigments in faeces are fluorescent, which makes them detectable with optical methods and a promising alternative to microbial indicators of faecal pollution on food as well as in water (Bixler *et al.*, 2014). Additionally, faecal pigments have their excitation and emission maxima in a region, where not many other substances appear, making them relatively well distinguishable.

3.1.1 The heme metabolism

Faecal pigments have their origin in the heme metabolism. Heme is a porphyrin, a cyclic tetrapyrrole, with iron in its center. It is present as a part of hemoglobin in red blood cells, as well as in hemoproteins which can be found in all mammalian cells (Crawford *et al.*, 1988). The majority of faecal pigment comes from the degradation of red blood cells, which die off after a lifetime of approximately 120 days (Crawford *et al.*, 1988). During the degradation process of heme, its porphyrin structure is broken up, the iron is removed, and a linear tetrapyrrole is created (Figure 1, Boiadjiev and Lightner, 1999). The word "linear" refers to a cleavage of the porphyrin ring structure, rather than the actual shape of the molecule (Crawford *et al.*, 1988).

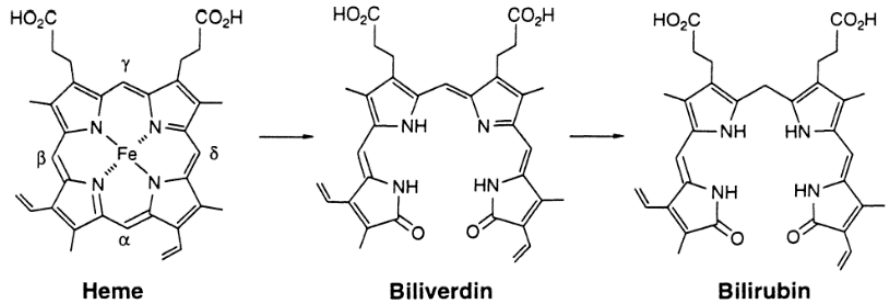


Figure 1. Conversion of the porphyrin (cyclic tetrapyrrole) heme to bilirubin, a linear tetrapyrrole (Boiadjiev and Lightner 1999, with permission).

Via several steps, heme is converted to bilirubin in the plasma and then transferred to the intestines (Figure 2). A series of reduction steps performed by intestinal microbes then transforms bilirubin to several so-called urobilinoids, which are, like bilirubin, linear tetrapyrroles. The "end products" excreted with faeces and urine are mostly urobilinogen and stercobilinogen, which are colourless, as well as their coloured oxidation products stercobilin and urobilin. Stercobilin is considered to be the dominant pigment in faeces (Orten, 1971). Apart from those mentioned above, the presence of more urobilinoids in faeces has been documented, some examples are half-stercobilinogen, half-stercobilin, and several urobilins with different molar weights (Watson, 1969). A part of urobilinogen is reabsorbed from the intestine and excreted in urine, becoming oxidized to urobilin. Most of the bile pigment is, however, excreted

via the faecal route (Crawford *et al.*, 1988). A daily excretion of 1-2 mg urobilin and 150-250 mg stercobilin is considered to be normal for an adult person (Orten, 1971). While stercobilin is stable to oxidation, urobilin can be further transformed via mesobiliviolin to glaucobilin (Watson, 1969). The transformations in the intestines are complex and, although urobilin and stercobilin were already detected in 1868 and 1871, respectively, there was an active discussion going on about structure and relationships of faecal pigments until the 1970s (Petryka *et al.*, 1975). Since then, the active discussion about the topic has decreased, but the reaction pathways of faecal pigments in the intestinal tract are still not fully understood (Vitek *et al.*, 2006).

Figure 2 shows a schematic view of the faecal pigment metabolism, which was strongly simplified to ensure clarity. For a full picture including all important urobilinoids the reader is referred to Watson (1969).

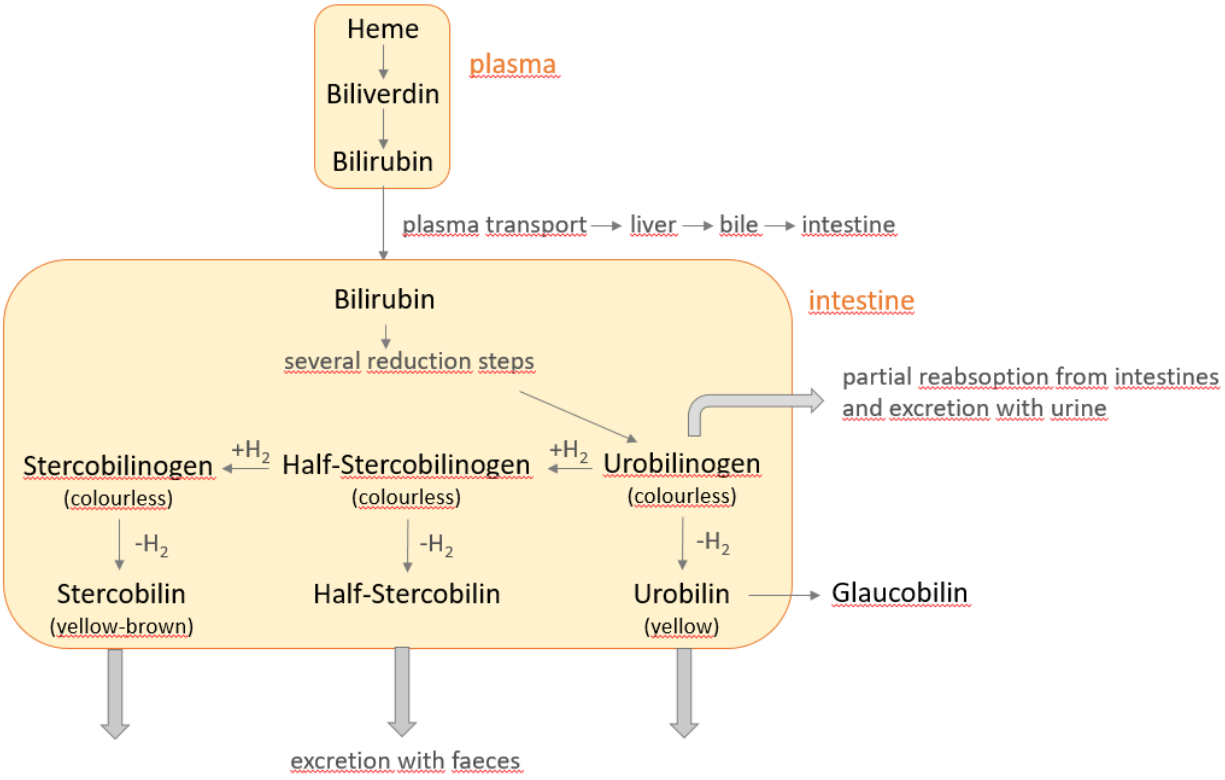


Figure 2. Simplified figure of the faecal pigment metabolism (modified after Watson, 1969 and Vitek et al., 2006).

It has to be mentioned that there are several different forms of the urobilinoids, which differ in their optical activity. In nature there is often one dominant form, which is laevorotatory (-)-stercobilin and dextrorotatory (+)-urobilin (Boiadjiev and Lightner, 1999). Synthetic urobilin and stercobilin can contain several different isomers and thus differ substantially from the naturally obtained pigments. To simplify, the different forms are not considered in this thesis. Stercobilin, urobilin, and urobilinogen are shown in Figure 3 (after Vitek *et al.*, 2006).

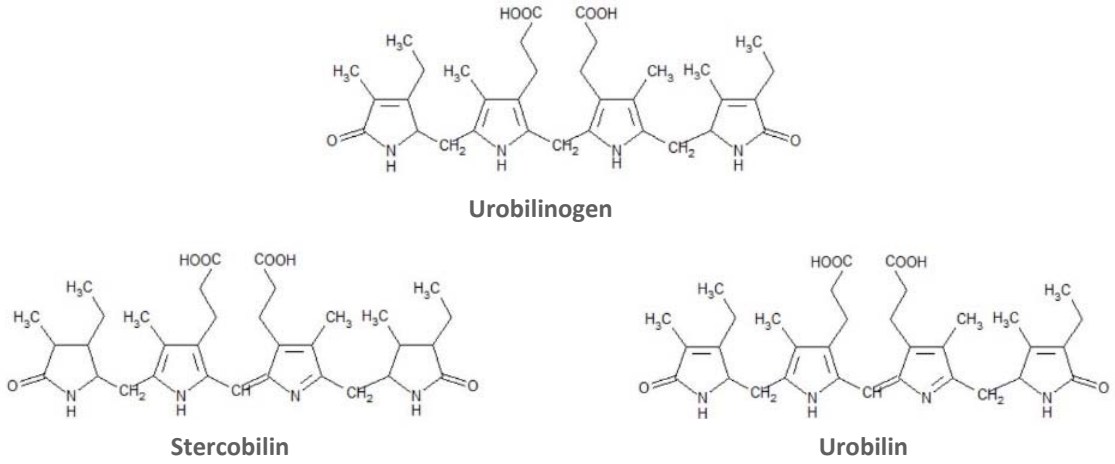


Figure 3. Structure of urobilinogen, stercobilin, and urobilin (Chemograph; after Vitek et al. 2006).

3.1.2 Indicator function

Usually, the contamination of water is assessed by the help of indicators for faecal pollution, instead of directly measuring pathogens (Cimenti *et al.*, 2009). Faeces, especially of human origin, are the primary source of many important waterborne pathogens in developing regions (Ashbolt, 2004). These include bacteria (e.g. *Salmonella typhi*, *Shigella spp*., *Vibrio cholera*), viruses (e.g. rotaviruses, enteroviruses), protozoa (e.g. *Entamoeba histolica*, *Chryptosporidium parvum*), and helminths (e.g. *Ascaris lumbricoides*) (Ashbolt, 2004). Indication of faecal pollution can therefore indicate an increased risk for the presence of pathogens. Typical indicators for faecal pollution are microbes present in faeces, but it can also be chemicals that fulfil certain requirements. The indicator should, according to Maier (as cited in Cimenti *et al.*, 2009)

- always be present in the case of faecal contamination,
- not be present in the environment without faecal contamination,
- be suitable for different environments (ground water, surface water, sea water),
- be present in at least the concentration of the pathogen or greater,
- be more persistent than the most persistent pathogen, and
- be detectable faster, more sensitive and less expensive than the pathogen.

Miyabara et al. (1994c) concluded that urobilin was a suitable indicator for faecal pollution in water and behaved, under laboratory conditions, similarly to total as well as faecal coliforms in water obtained from a polluted river in Japan. About the behaviour of faecal pigments in the environment under non-controlled conditions, however, not much is known.

3.1.3 Metal complex formation

In nature, many of the remarkable functions of the porphyrins, the parent molecules of faecal pigments, are connected to metal complexation. In hemoglobin, an Fe^{2+}-porphyrin complex is the heme part and in chlorophyll, the equivalent is a Mg^{2+}-porphyrin complex. The ability to complex with certain metals is still given when the porphyrin ring structure is broken up, like in faecal pigments. If such metals are present in a water matrix, for example Zn^{2+} or Mg^{2+}, they can bind to the pigments, changing their fluorescence properties. This property can be used as an advantage for the fluorescence detection of bile pigments. When zinc is added to a solution containing urobilin or stercobilin, the fluorescence of these pigments is enhanced and their emission spectrum shifted to longer wavelengths, also called a bathochromic or ”red” shift (Schlesinger, 1903; Miyabara *et al.*, 1992). The intensification makes the pigments much easier to detect in a solution containing low concentrations of pigment. This phenomenon was used by Schlesinger (1903), who developed a method for the fluorescence detection of urobilin in urine with the addition of zinc.

3.2 Fluorescence

Fluorescence is a form of luminescence, in which molecules that absorb light are excited to a higher energy level and subsequently emit light when they relax back to the ground state (Figure 4, after Lakowicz, 2010). Absorption of energy in the form of light is therefore a prerequisite for fluorescence. When a molecule absorbs a certain amount of light energy, called a quantum, it can be excited to a higher energy state (S1). Within this excited state there are various vibrational energy levels. The molecule relaxes to the lowest of vibrational levels in S1. From this level, it can relax back to the ground state via various ways, of which one is the emission of light. This light, emitted while relaxing from the lowest level of the first excited state to the ground state, is called fluorescence and lasts for approximately 10^{-9} (10^{-11}-10^{-7}) seconds (Coble, 2014). Several other relaxation processes compete with fluorescence, causing the molecule to lose energy without emitting light. How much light is emitted depends on the relation between fluorescence and other relaxation forms. The relation between emitted and absorbed light is called quantum yield (Coble, 2014).

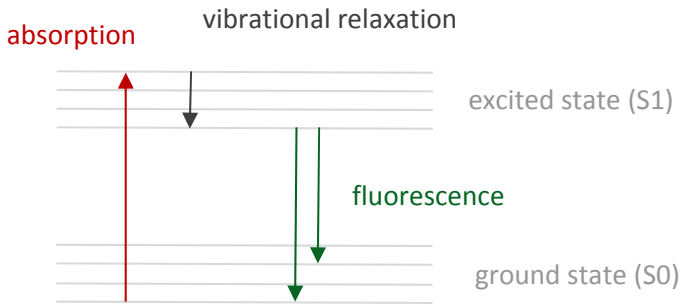


Figure 4. Jablonski diagram of energy levels (modified after Lakowicz, 2010).

The energy of light is proportional to its frequency and inversely proportional to its wavelength, which means that light with a short wavelength has a higher energy than light with a longer wavelength. As energy in the excited state is not entirely conserved, emission of light always takes place at longer wavelengths, i.e. lower energy, than the absorption of light (Coble, 2014). Which wavelength the emitted light has, depends largely on the molecular structure of the fluorophore, i.e. the fluorescent substance, and the possible electron transitions. The less energy lies between the lowest excited state and the ground state, the longer the wavelength of the emitted light (Coble, 2014).

While almost all molecules absorb light, only some molecules are able to exhibit fluorescence. This makes fluorescence spectroscopy a very specific method, which is, however, as the name implies, limited to fluorescent substances (Guilbault, 1990).

3.2.1 Fluorescence spectroscopy

When measuring the fluorescence of a sample with a fluorescence spectrometer, the result is a matrix consisting out of measured fluorescence intensities at certain excitation and emission wavelengths, called excitation emission matrix (EEM). The matrix can be taken apart into excitation spectra, showing the efficiency of a certain wavelength in exciting the sample, and emission spectra, showing at which wavelengths the sample emits light (Figure 5).

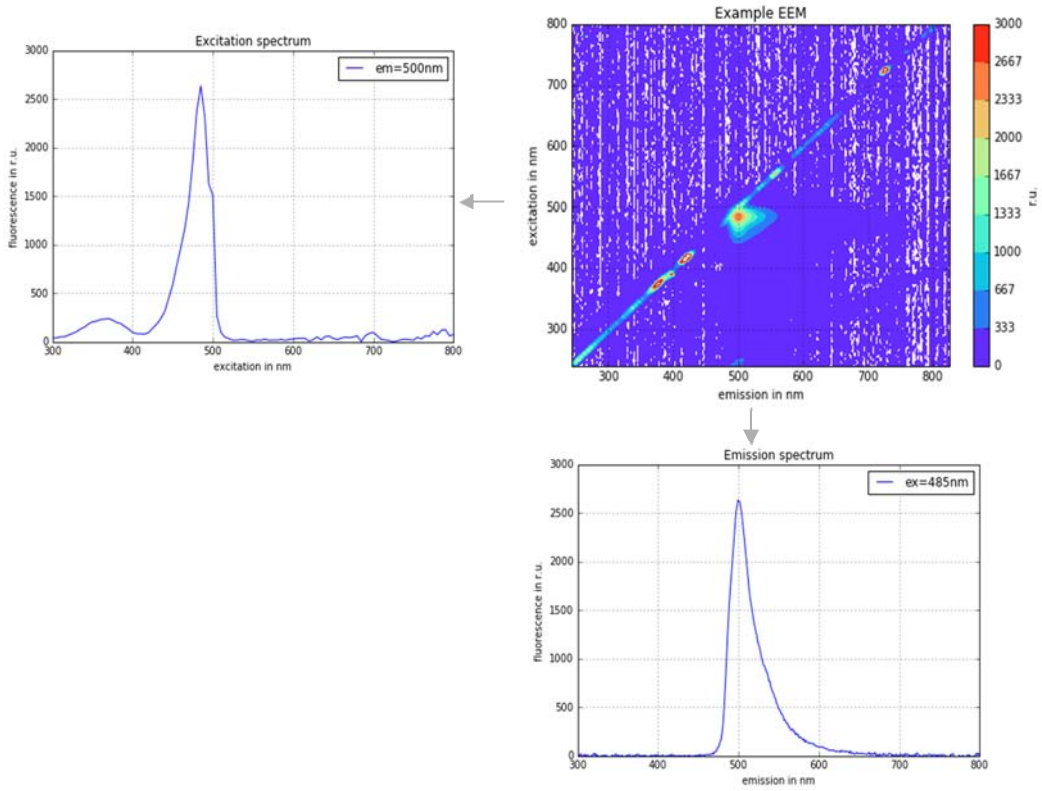


Figure 5. Composition of an EEM (top, right): Excitation (top, left) and emission (bottom, right) spectra.

Every fluorescent substance has a characteristic excitation and emission spectrum, which can be used for its identification. The emission spectrum of a substance (Figure 5, bottom) is thus independent of

the excitation wavelength and always has the same shape – only the intensity of the signal will vary depending on the excitation wavelength. If a substance is measured in different concentrations, its fluorescence intensity, i.e. the height of the fluorescence signal, will be different, but not the form of the spectra.

An EEM shows a composition of all fluorescence signals obtained when measuring a sample. If there are several fluorescent substances in a sample, they will all reflect in the matrix. However, signals can overlap and it is possible that different substances have the same excitation and emission spectra and appear as one peak instead of separated peaks. If this is the case, it is impossible to distinguish between substances by using fluorescence spectroscopy alone. On the other hand, if there are several fluorescence peaks differing in their excitation or emission spectra, there must be several substances present. As an example, Figure 5 shows an EEM with only one fluorescent substance, which has its main peak at an excitation wavelength of 485 nm and an emission wavelength of 500 nm ($\lambda_{ex/em}$ = 485/500 nm).

A part of the light used to excite the sample is scattered (Coble, 2014). There are several forms of scatter, with the ones most relevant for this study being Rayleigh and Raman scatter.

- Rayleigh scatter is caused by the scattering of light from the sample without loss of energy (slope=1), which shows as a diagonal band in an EEM (Figure 5).
- Raman scatter is caused by the solution, e.g. water molecules, scattering light, which leads to a loss of energy. The Raman scatter can be removed by measuring only the solvent and removing this as a blank value. Removal of the Raman scatter is done as a part of pre-processing of fluorescence data. Therefore, Raman scatter is not visible in the EEMs anymore. It is however important for the normalization of fluorescence data (3.2.2).

There is a 1st and 2nd order scatter. The first order appears at the wavelength the sample is excited with and the 2nd order scatter appears at double the excitation wavelength.

3.2.2 Fluorescence spectrometer design

EEMs are recorded with a fluorescence spectrometer. A sample is excited at certain wavelengths and simultaneously a detector detects the light, which is emitted by the sample (Figure 6). First, light from a bulb is directed to an excitation monochromator, where its spectrum is split up into its wavelengths and only a certain wavelength-fraction at a time is led to the sample. Part of the light goes to a reference detector, which monitors the intensity of the exciting light source, while the other part reaches the sample. When the sample emits light, the light is again directed to an emission monochromator and split up, so it can be detected which wavelengths are emitted in which intensity. Usually, one wavelength after another is registered by a detector. In the Horiba Aqualog fluorescence spectrometer, which was used in this study, a detector registers all emitted wavelengths simultaneously. Apart from the fluorescence, transmission, which is the light that passes the sample, is measured as well. From this, the absorbance of a sample can be calculated.

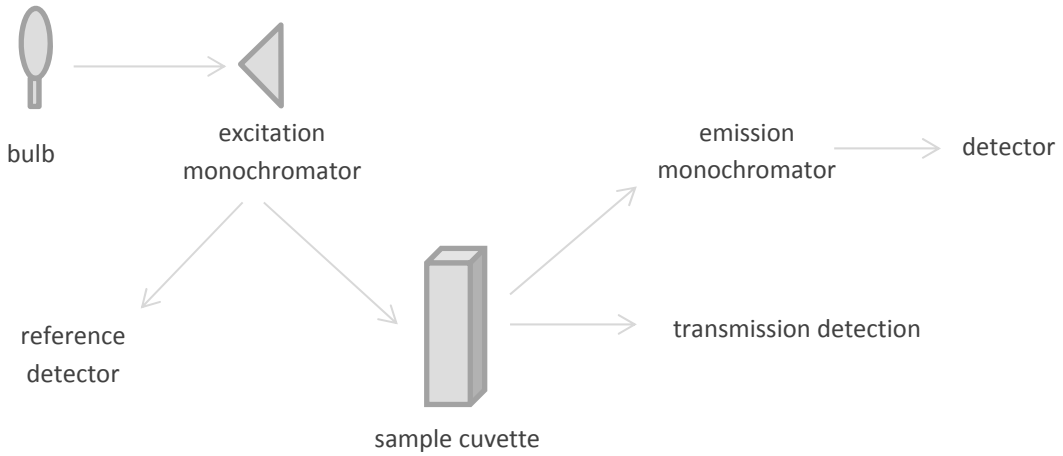


Figure 6. Schematic fluorescence spectrometer design.

There are problems comparing between results obtained with different spectrometers, because spectrometer properties can vary. Even when measurements are done with the same spectrometer, the measurements need to be normalized. Integration over the area of the Raman peak, i.e. the intensity of the Raman scatter, can be used for normalization of the intensities between different samples. For this purpose, a standard water cuvette filled with pure water should be measured at λ_{ex} 350 nm together with the samples to serve as reference. The intensity of such normalized samples is given in raman units (r.u.). If the samples are not normalized with the intensity of the Raman peak, the intensity can only be given in arbitrary units (a.u.).

3.2.3 Fluorescence influencing factors and quenching

The term quenching describes all processes that lead to an extinction or diminishing of fluorescence. Environmental factors, such as pH and temperature of the sample, will influence the result. High temperature causes molecules in the water to move faster, thereby increasing the probability of molecules colliding. If they collide, they are transferred back to the ground state without emitting light, which lowers the fluorescence intensity of the sample. Therefore, the fluorescence intensity will be higher if the samples are measured at a lower temperature (Guilbault, 1990). If the sample contains particles, the result has to be corrected for that. It is best, however, if the sample is filtrated first and all particles are removed (Guilbault, 1990). A change in pH can lead to protonation or deprotonation of functional groups in molecules. This can alter the fluorescence intensity by increasing or decreasing the relation between fluorescence and other relaxation processes without light emission. Spectra can also be shifted to shorter or longer wavelengths by a pH change (Coble, 2014). Quenching can take place both in the ground state (static quenching), or in the excited state (dynamic quenching). Temperature quenching (as described above), is an example of dynamic quenching, while the change in molecular structure leading to a loss of fluorescence, e.g. by a binding reaction, belongs to static quenching.

4 Materials and methods

4.1 Materials

4.1.1 Chemicals

Standards of urobilinogen (AppliChem, $C_{33}H_{42\text{-}50}N_4O_6$, MW 590.7-598.8 g/mol), urobilin hydrochloride (Frontier Scientific, $C_{33}H_{43}ClN_4O_6$, MW 627.2 g/mol), stercobilin hydrochloride (Chemos GmbH, $C_{33}H_{47}ClN_4O_6$, MW 631.2 g/mol), and uranin (Serva, $C_{20}H_{10}Na_2O_5$, MW 376.2 g/mol), a sodium salt of fluorescein, were used. Urobilin and stercobilin hydrochloride standards, which were in crystalline form, were dissolved in ultra-filtrated water (MilliQ) to stock solutions of 85 mg/l and 60 mg/l of pigment, respectively. Urobilinogen standard was present as a liquid solution of 40 g/l. All standards and solutions thereof were kept refrigerated at 4°C.

Apart from pigment standards, zinc acetate dihydrate (Merck, $Zn(CH_3COO)_2 \cdot 2H_2O$) was used for the fluorescence measurements, as well as potassium dihydrogen phosphate (Chemsolute, KH_2PO_4), and disodium hydrogen phosphate dihydrate (Merck, $Na_2HPO_4 \cdot 2\ H_20$), to create buffer solutions. For chemicals used during the reference methods (SPE, as well as HPLC and MS analysis), see Appendix A.

4.1.2 Real water samples

Wastewaters from five different German wastewater treatment plants (WWTPs) were used in this study. The samples were taken in confidence, so details to the location of the plant cannot be made public. Three of the treatment plants were relatively small (<40000 people), while two treatment plants were larger (>500000 people).

Drinking water before disinfection, after passing through active coal filtration (HOWI), for the analysis of the pigment standards was obtained from the drinking water plant Dresden-Hosterwitz. Real water samples served as a medium for measuring the pigment standards, and the wastewater samples in particular were examined for the presence of faecal pigments.

4.2 Analytical Methods

4.2.1 General characterization and preparation of the samples

Wastewaters were filtrated through a 0.45 µm GF/C glass fibre filter (Whatman) before further analysis to eliminate particles that could be disturbing during fluorescence measurements. If necessary, they were centrifuged for 15 min at 4700 rpm prior to the filtration. As the pH value can influence the fluorescence result, pH was measured with a pH meter (WTW pH 540 GLP). For a detailed element analysis, wastewater samples were sent to an external laboratory (M.U.T. Meißner Umwelttechnik GmbH, Ingenieurbüro für angewandten Umweltschutz).

The pH value of the pigment standard solutions was assumed to be the same as the pH of the water medium they were diluted in. As water media, real water samples with a pH from 6.8 to 8.4 were used as well as MilliQ water buffered with a phosphate buffer at pH 6.6 ("phosphate buffer"). The phosphate buffer stock solution with a pH of 6.6, containing 2.38 g/l KH_2PO_4 and 1.30 g/l $Na_2HPO_4 \cdot 2\ H_2O$, was prepared in MilliQ and diluted 1:20 with MilliQ for the measurements. A phosphate buffer solution with a pH of 8.5, to test the influence of a higher pH, was prepared from the same compounds, containing 0.34 g/l KH_2PO_4 as well as 11.43 g/l $Na_2HPO_4 \cdot 2\ H_2O$, which was then diluted 1:10 with MilliQ. If there is no further pH indication, the term "phosphate buffer" refers to the buffer with pH 6.6.

4.2.2 Spectral characterization

Standards as well as the pure wastewater samples were first characterized fluorometrically with a Horiba Aqualog fluorescence spectrometer ("Aqualog") to compare their fluorescence excitation emission matrices (EEMs). Approximately 3 ml of sample were measured in a quartz glass cuvette. In the fluorescence EEM measurements, the samples were excited from 800 nm to 240 nm in steps of 5 nm. The emission was recorded in steps of 1.16 nm between 244.2 and 825.7 nm. Detector sensitivity was high and the integration time was 1 second. As fluorescence measurements are temperature sensitive, the samples were, as far as possible, measured at a constant room temperature.

4.2.3 HPLC-DAD-FLD

High performance liquid chromatography (HPLC) separates a liquid sample in its constituents, depending on their solubility. A liquid sample is sent over a column to which the desired components adsorb. Afterwards, the adsorbed substances are gradually eluded with a solvent mixture of varying polarity. In reversed phase HPLC, which was used here, the more water soluble the adsorbed substances are, the earlier they are eluded from the column, while less polar compounds stay on the column longer. At the end of the column, a detector documents the time at which every substance arrives. There are several possibilities for detection. In this case, a UV-Vis absorption detector (diode array detector, DAD) and fluorescence detector (FLD) were used. For the comparison of two substances, their retention times are compared. Under constant conditions, the same substance should always appear at the same time.

After their fluorescence was recorded in the form of an EEM, pigment standards and wastewaters were chromatographically separated with HPLC to separate different substances within the sample from each other. Later, this was repeated with enriched wastewater samples (for enrichment, see 4.2.4). 25 µl of sample volume were injected. The sample first went through a self-filled C8 10 µm (20 mm x 2 mm) pre-column and then over the HPLC column, which was a C18 AB 5 µm from Macheray Nagel (250 mm x 3 mm). Column oven temperature was 30°C. For the separation, a solvent gradient between 90% MilliQ and 10% methanol up to 10% MilliQ and 90% methanol was used, each with 1mM ammonium acetate as a buffer. The flow rate was 0.4 ml/min, more details to the gradient can be found in Appendix A. A DAD and FLD sensor detected the absorption and fluorescence signal of the separated samples over time. For the absorption, a wavelength of 490 nm was observed (spectra from 240 nm to 590 nm in 2 nm steps were stored) and for the fluorescence detection, the signal at an excitation of 485 nm and an emission of 500 nm was used, as this is where faecal pigments were expected to emit the most light.

4.2.4 Solid phase extraction and enrichment

With solid phase extraction (SPE), components can be extracted from a liquid sample by adsorption to a solid phase. Whether a compound will be adsorbed or not, depends on the chemical and physical properties of both the compound and the solid phase. The solid phase is therefore a key point for the extraction and has to be adapted to the desired compound. SPE can also be used as a step in the enrichment of substances from a sample containing low concentrations of the compound of interest. After the desired compounds are adsorbed to the solid phase, they are then eluded with a suitable solvent, which is added in smaller amounts than the volume of the original sample, resulting in an enrichment.

In this study, SPE was used to enhance the fluorescence signal of several selected wastewaters, from which a pigment signal could be expected. For this, 400 ml of each selected wastewater were enriched with the help of 6 ml SDB cartridges (1g) from Phenomenex. Before the procedure, the pH of the filtrated wastewater samples was, if necessary, adjusted to 7±1 by adding hydrochloric acid or ammonium hydroxide and confirmed stable over 4 hours. The cartridges were successively conditioned with 4 ml of acetone, 8 ml methanol, and 8 ml MilliQ water. 200 ml of wastewater sample was loaded to one cartridge with a flow rate of 3-4 ml/min (pump). After the extraction, two washing steps were performed (4 ml MilliQ and 4 ml methanol 75%). Then the extract on the cartridge was, in succession, partially eluded with 4ml of

1. 50% methanol and 50% MilliQ (50% MeOH)
2. 75% methanol and 25% MilliQ (75% MeOH)
3. 100% methanol (100% MeOH).

Each eluate was collected separately. This lead to an enrichment of the factor 50 in the eluates. For fluorescence measurements, the eluates were then, to obtain a larger volume, diluted by a factor of 1:10, leading to an enrichment of the factor 5 compared to the original wastewater. For further HPLC and MS analysis, the eluates were evaporated with nitrogen gas at 50 °C until 0.7 ml of each eluate remained. The evaporated eluates were then diluted with 0.3 ml methanol (eluate 50% and 75% MeOH) or 0.3 ml MilliQ (eluate 100% MeOH).

The enriched wastewater sample fractions were, like the unaltered wastewater samples, analysed with the Aqualog, HPLC-DAD-FLD and additionally with MS.

4.2.5 Mass spectrometry (MS)

Mass spectrometry is used to identify the exact mass of compounds present in a sample. A small amount of vaporized sample is ionized and accelerated. While the ions are travelling through a tube, they are deflected by a magnetic field. The amount of deflection depends on the charge and mass of the ions, i.e. for ions of the same charge, the heavier they are, the less they are deflected. At the end of the tube is a detector. Only ions that directly hit the detector are registered by it. By varying the strength of the magnetic field and with it the deflection, it can be influenced which ions (ions with a certain mass to charge ratio) hit the detector. It is then possible to calculate back from the deflection and velocity to obtain the

mass of an ion. Not only single compounds can be identified, but substances can as well be fragmented and identified from their specific mass transfer.

Mass spectra of the standards and selected enriched wastewaters were obtained. The used system was an LC-MS/MS API2000 (Sciex). The mode was electrospray ionization (ESI) with positive ionization in Q1 (scan) and multiple reaction monitoring (MRM) mode.

HPLC and MS, in combination with SPE, served as reference methods for the results obtained with fluorescence spectroscopy. The reference methods were already established in the TZW laboratory and were performed by the responsible operator.

4.2.6 Calibration series

Figure 7. Calibration series preparation; different concentrations of urobilin in water (0 to 1 mg/l).

As it is not only wanted to qualitatively detect faecal pigments in water, but to also quantify them, one must be able to connect the measured fluorescence intensity to the pigment concentration. For this purpose, calibration curves of urobilin and stercobilin standards were established. Calibration series with known concentrations of urobilin and stercobilin standards were measured with the Aqualog in various media (phosphate buffer, HOWI, WWTP outflow and WWTP inflow) to relate a certain fluorescence intensity with the respective pigment concentration. Urobilin and stercobilin concentrations used for the calibration ranged from 0 to 1 mg/l. The addition of pigment standard to real waters leads to undesired dilution effects of the real water background fluorescence. To avoid this, the same amount of real water was used throughout one series (while the amount of pigment standard increased in the series) and the remaining volume of the volumetric flasks was filled up with MilliQ (80% real water, 20% standard and MilliQ in varying proportion). Thereby the real water concentration was the same, while the pigment concentration increased. Figure 7 shows a picture of a calibration series preparation of urobilin. The maximum fluorescence intensities caused by the pigments were determined by subtracting the blank value, which is the signal of the pure medium without added pigment, from the total signal of the measured calibration series and then using the fluorescence intensity at the maximum of each concentration for the calibration. A relationship between concentration and fluorescence intensity was established by fitting a straight line to the data points.

4.2.7 Zinc addition

Selected fluorescence measurements were repeated with an addition of 20 mg/l zinc as zinc acetate to both the standard solutions as well as the wastewater samples. An increase in fluorescence intensity was expected from this. Also, different zinc concentrations were tested and reaction kinetics at a given excitation wavelength were observed by doing continuous measurements of the samples after zinc addition for 30 minutes. During the kinetics measurement, an emission spectrum was recorded every 5 seconds. The excitation wavelength was 480 nm and the integration time 1 second. There was a waiting time of 1 minute between zinc addition to a sample and the start of the measurement.

Remark: A time-dependent behaviour of the fluorescence intensity after zinc addition was observed in the course of the experiments. Before this dependency was known, usually a time of one to two hours passed between the addition of zinc acetate to the sample and the measurement. After the time dependency had been observed, the method was changed to a waiting time of 60 seconds between zinc addition and start of the fluorescence measurement to assure comparability between the results. The data with longer or unknown time between zinc addition and the measurement thus cannot be evaluated quantitatively.

4.2.8 Stability

Concerning the suitability of faecal pigments as indicators of faecal pollution, their stability is an important factor. For all standards, stability tests under different storage conditions were done. For the stability measurements, pigment standards were diluted in different water media, i.e. phosphate buffer, HOWI, and WWTP inflow. In addition to the phosphate buffer, HOWI and wastewater were chosen to see whether components of a real water sample would have an influence on the pigments' fluorescence stability. The pigment concentration was chosen after doing some pre-tests because of the pigments' different fluorescence intensities. Stercobilin and urobilin standards showed a much higher intensity than the substances in the urobilinogen standard. The intensity should be well detectable, but not too intense for the measurement device. One group of standard solutions was then stored at room temperature under light influence ("warm"), while the other group was stored in the fridge at 4 °C in darkness ("cool"). These different storage conditions represent the influence of light and temperature on the stability of the pigments in water.

There were sixteen different solutions, each prepared in a 100 ml volumetric flask,

- 0.5 mg/l stercobilin
- 0.5 mg/l urobilin
- 5 mg/l urobilinogen (only in HOWI and phosphate buffer).

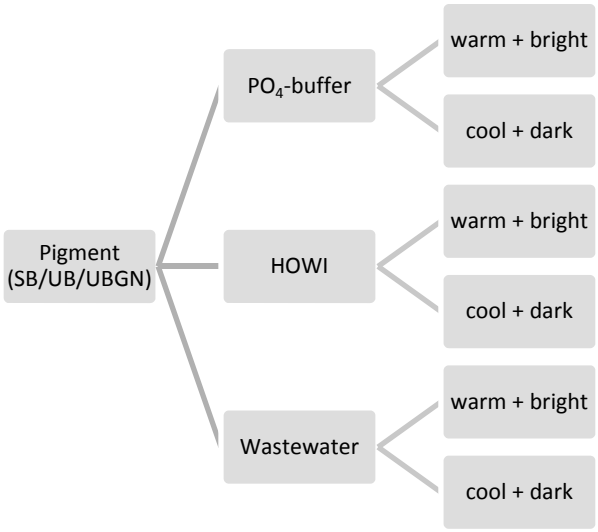


Figure 8. Schematic presentation of the stability test setup.

Fluorescence EEMs of the samples were measured regularly with the Aqualog to follow the development of the samples' fluorescence over time. The measurements were performed as described in 4.2.2. During the first working week (5 days), samples were measured daily with the Aqualog. Afterwards, samples were measured once per week for four weeks. Before the measurement, the flasks from the fridge were brought into the laboratory to adjust to room temperature.

4.2.9 Coupling of HPLC and Aqualog (+MS)

The HPLC was coupled with the Aqualog to get detailed pictures of the fluorescence of single fractions separated by HPLC. This was realized with a flow-through cuvette, coming from the HPLC, which lead the separated sample to the Aqualog. The sample was first separated with a solvent gradient by HPLC (Appendix A) and then reached the flow-through cuvette in the Aqualog, where the fluorescence intensity and emission wavelengths of the sample fractions were recorded continuously.

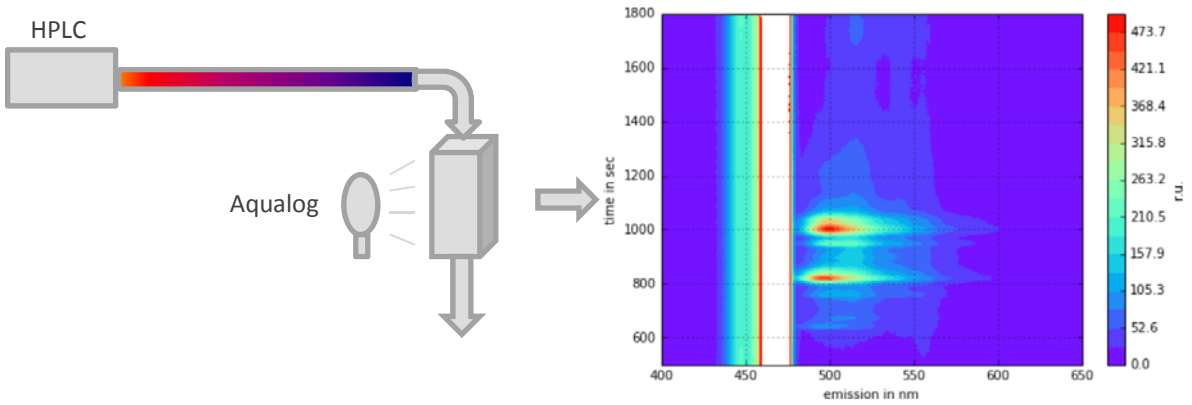


Figure 9. Coupling of HPLC and Aqualog: Flow-through design and resulting fluorescence matrix.

Instead of producing "normal" EEMs that show a matrix of fluorescence intensities at several excitation and emission wavelengths as a result, the sample was only excited at one certain excitation wavelength while the emission was recorded between 244 and 825 nm in 1.16 nm steps. This leads to a result that shows fluorescence intensity depending on the emission wavelength and time (instead of emission wavelength and excitation wavelength). The samples were, based on the peaks detected in the EEMs, measured at excitation wavelengths of 385, 465, and 480 nm, however only the excitation wavelength of 465 nm will be presented in the results.

The resulting matrix (Figure 9) shows fluorescence emission spectra of the separated sample over time. It is possible to decompose the matrix and look at the emission spectra of the separated sample at a certain time or at the chromatogram (at which time the emission occurs and with which intensity) at a certain wavelength (Figure 10).

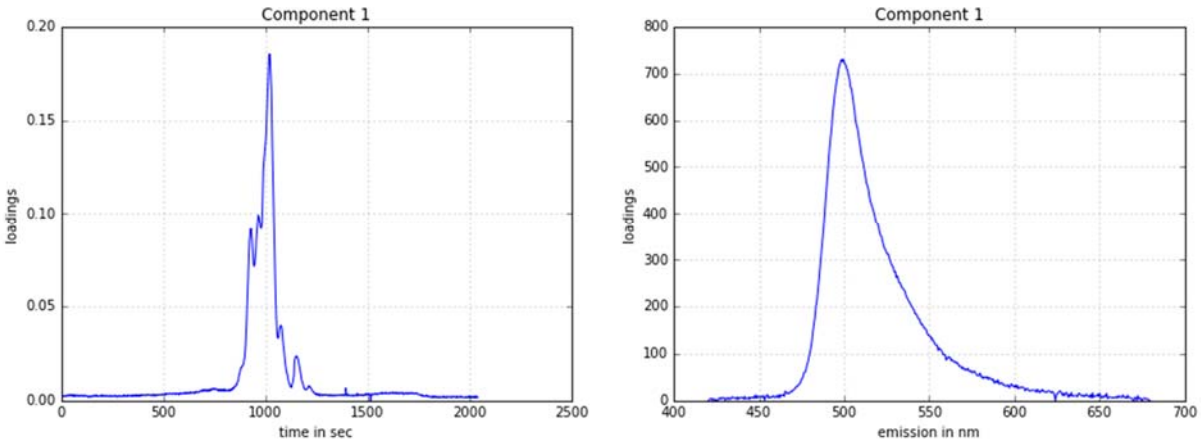


Figure 10. Result of HPLC-Aqualog coupling: Chromatogram (left) and emission spectrum (right) of a separated fluorescent matrix component.

In typical HPLC-FLD measurements, the fluorescence intensity at one single excitation-emission wavelength combination is recorded over time. On a "normal" fluorescence EEM, there is a whole matrix of excitation and emission spectra, but the sample is not separated and different fluorescence signals can overlap. Coupling both techniques has the advantage of being able to observe the whole emission spectra of the separated compounds in a mixture. The aim of this separation and fluorescence measurement combined was to better understand the single components constituting the fluorescence picture in the faecal pigment standard and wastewater EEMs. It can happen that some components overlap or have the same spectra, but it is very unlikely that different components appear at the same time and have the same emission spectrum. In this way, it can be determined whether compounds separated by HPLC have the same emission spectrum, and they can also be compared. If they appear at the same time and have the same emission spectrum it is very likely the same substance. Some changes in the molecular structure do not influence the fluorescence characteristics of a molecule, but they do often influence the polarity. Two substances can for example not be distinguishable in their fluorescence spectra, but they appear at different times in an HPLC chromatogram.

The problem with this method is, that one can compare different samples, but single substances still cannot be clearly identified. For this, an additional coupling of a mass spectrometer to the HPLC-Aqualog-coupling was done. However, the analysis of the combined data is very complex and only some results from the coupling of MS are presented in this work.

4.3 Data analysis

4.3.1 Analytical limits

Analytical limits are an important tool to know how sensitive a technique is, but also to assess how realistic obtained results are when applying calibration series to measured data.

- Limit of detection (LOD) is the minimum detectable concentration ($\alpha = 0.05$, $\beta = 0.5$).
- Limit of quantification (LOQ) is the minimum concentration necessary for quantification with a defined precision (k) of the result. In this case, k was set to 0.25. (Reichenbächer and Einax, 2011)

If a measured concentration of a substance is below the LOQ, it can only be qualitatively assessed but not quantified and if it is below the LOD, the presence of the substance is not certain enough (Reichenbächer and Einax, 2011).

The detection and quantitation limits can be estimated from the fluorescence intensity data of a linear calibration series with known concentrations. Analytical limits of the pigments were determined from fluorescence data (EEMs) of calibration series of the different standards in phosphate buffer, HOWI, WWTP outflow and WWTP inflow. From the calibration series, the detection and quantitation limits of stercobilin and urobilin were quantified by using the DIN 32645 test. The test is an internationally recognized German industrial norm method for determining analytical limits of substances from linear relationships. Outliers were identified by an outlier test (included in the DIN-procedure), and removed prior to performing the calculation. As the test is only valid for linear relationships, a test on linearity of the data (included in the DIN-procedure) was performed and only linear data was used.

4.3.2 Non-negative Matrix factorization (NMF)

Non-negative Matrix factorization (NMF) is a way of separating a matrix into its components. It is based on the assumption that every matrix can be represented by a set of smaller matrices, which, summarized, resemble the original matrix. In this study, NMF was used on the EEMs of the pigment standards in phosphate buffer, to decompose the standards into their fluorescent components. By this, it can be determined how many substances are present in the standards and what their fluorescence excitation and emission spectra look like.

4.3.3 Parallel factor analysis (PARAFAC)

Similar to NMF, PARAFAC can be used to identify single components in matrices. However, while NMF is restricted to one matrix, PARAFAC is used for the decomposition of multi-way data, which is for instance the case if there are several matrices (resulting in one more dimension). It uses the fact that a change in concentration only changes the fluorescence intensity, but not the emission and excitation spectra and can calculate back from the matrices to the spectra of single components. The matrices are arranged to a "data cube" and analyzed using alternating least squares, which means that the sum of the squares of the residuals is minimized (equation 1, Bro, 1997; Stedmon and Bro, 2008).

$$x_{ijk} = \sum_{f=1}^{F} a_{if} b_{jf} c_{kf} + \varepsilon_{ijk}$$

In the case of fluorescence EEMs, i refers to the sample number, j is the excitation wavelength and k is the emission wavelength. x_{ijk} is the measured fluorescence intensity of the i^{th} sample at the j^{th} excitation wavelength and k^{th} emission wavelength, while ε_{ijk} is the part of the signal, which is not explained by the model (residuals). The measured fluorescence signal is always the sum of all contributing fluorophore (F) signals. The result of PARAFAC is a choice of several models that give qualitative and quantitative information about the components modelled from the data. In the end, the concentration (a), emission spectra (b) and excitation spectra (c) of the components present in the matrices are given as a result (Stedmon and Bro, 2008). However, the right model first needs to be chosen.

Using PARAFAC, a general model of all used pigment standards and wastewaters was created. The goal of the model was to get a tool for the evaluation of unknown water samples regarding the presence of faecal pigments. The model should be able to recognize known compounds in water samples of unknown composition. By this, it would be able to detect and quantify for instance faecal pigments in a real water matrix based solely on an EEM.

4.3.4 Selection of appropriate NMF/PARAFAC models

PARAFAC and NMF modelling results in several models with a range of components that is decided upon by the person analyzing the data. First, the matrix is explained by the least number of components and one more component is added until the highest given number of components is reached. As an example, if 3 components are expected in a matrix, PARAFAC/NMF models with 1 to 5 components can be calculated. PARAFAC/NMF offers the models and gives out statistics on how well the data is explained by each model, but it needs to be decided which model is the right one. If a model with too few components is chosen, several sample components might be summarized to one model component. It should, however, also be avoided to choose a model with too many components, because it is an "overfit" – it will divide true sample components into several theoretical model components, which do not represent reality. Choosing the right number of model components is therefore vital to get a realistic model. To help in choosing the right model, lack of fit and core consistency can be used. The lack of fit is the percentage of a matrix or several matrices that cannot be explained by the model. A small lack of fit means that the model explains the data well. If there is a big improvement (decrease) in lack of fit

between a model with a certain number of components and the model with one component more, this indicates that that the additional component is likely a "real" water component and vital to explain the data. If the difference in lack of fit between two models is small, the additional component does not explain much more of the data and might not be necessary or even an overfit (Wagner, 2014). Core consistency describes the interdependence of model components with each other. If the core consistency is low (<50), different model components influence each other and such a model should not be chosen. However, core consistency is not necessarily the best tool to evaluate a model as it can be too strict for real-world data and can lead to an underestimation of components (Murphy *et al.*, 2013). In this work, core consistency is therefore excluded from the evaluation.

The NMF and PARAFAC calculations were done in Python (Python Software Foundation) with help of a library developed by Dr. Martin Wagner at TZW.

5 Results and Interpretation

5.1 Characterization of the pigment standards

5.1.1 Fluorescence

The fluorescence EEMs of all three examined faecal pigment standards showed a major peak at approximately $\lambda_{ex/em}$ 485/500 nm as well as a smaller peak with the same emission spectrum at $\lambda_{ex/em}$ 360/500 nm (Figure 11). These signals were of similar intensity in the urobilin and stercobilin standard, while they were weaker in the urobilinogen standard. The urobilin standard did not show any other fluorescence signals, while the stercobilin standard had one other peak at $\lambda_{ex/em}$ 540/560 nm. The urobilinogen standard showed many other fluorescence signals, e.g. a double peak at $\lambda_{ex/em}$ 385/620 and 385/680 while its strongest fluorescence signal appeared at $\lambda_{ex/em}$ 260/420 nm.

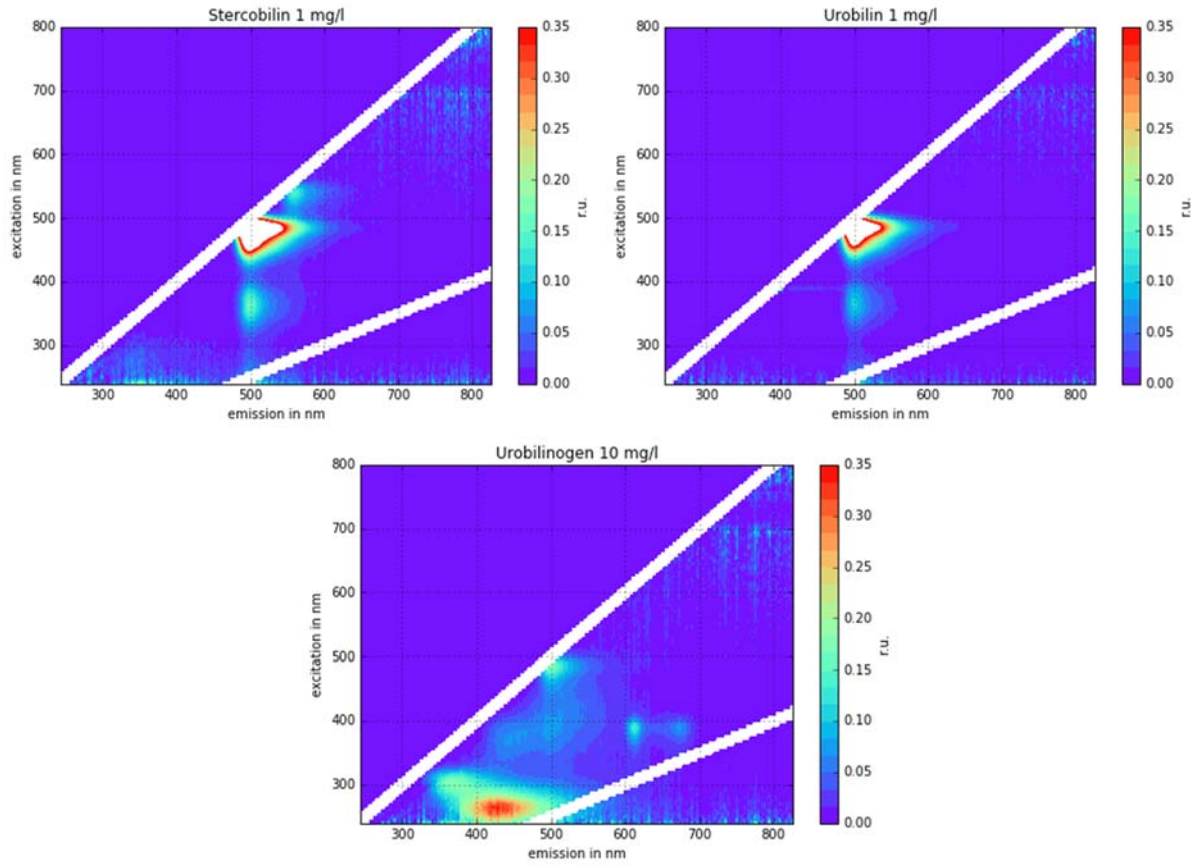


Figure 11. EEMs of the faecal pigment standards in r.u.. Upper left: stercobilin (1 mg/l), upper right: urobilin (1 mg/l), lower middle: urobilinogen (10 mg/l); Rayleigh scatter of 1st and 2nd order was removed.

According to NMF modelling, the stercobilin standard contained two real components with maxima at $\lambda_{ex/em}$ 485/500 nm and 540/560 nm, plus background noise (3 component model). The urobilin standard was explainable by one component only (1 component model). For the urobilinogen standard however,

4 components plus noise were necessary to explain the matrix (5 component model). Spectra of the modelled compounds can be found in Appendix B.

Urobilin and stercobilin could not be distinguished from each other in their fluorescence spectra. Figure 12 shows the spectra of urobilin and stercobilin, which were extracted from the respective standard's EEM with the help of an NMF analysis. The excitation as well as the emission spectra looked identical.

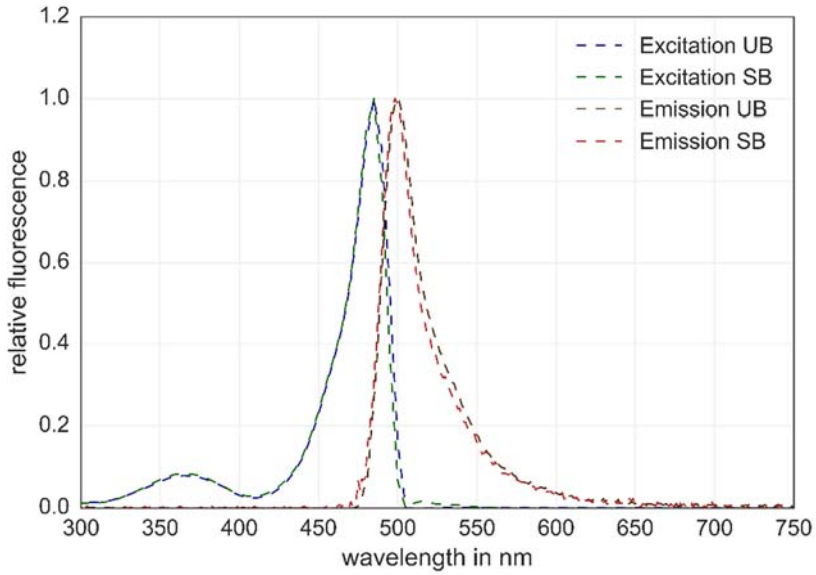


Figure 12. Excitation and emission spectra of urobilin (UB) and stercobilin (SB), extracted with an NMF model.

Every substance in a standard should be explainable by one emission spectrum, independent of the excitation wavelength. If this is not the case, more than one substance is present, which could be seen in the stercobilin standard and, in an extreme manner, in the urobilinogen standard (Figure 11). This can be a sign of pollution of the standard, or it can be metabolites of the main substance, which show a different fluorescence behaviour than the parent compound. The weak signal at $\lambda_{ex/em}$ 485/500 nm in the urobilinogen standard can be explained by the fact that urobilinogen itself is not fluorescent (Watson, 1969). If there was only urobilinogen in the standard, there would not be any fluorescence signal detectable. Every signal which can be seen in the urobilinogen standard's EEM must come from another substance. It is likely that the signal at $\lambda_{ex/em}$ 485/500 is urobilin, to which urobilinogen can be oxidized. Although different in structure, stercobilin and urobilin had identically looking fluorescence spectra, extracted by NMF from their respective standard's EEM. This implies that they cannot be distinguished from each other by fluorescence spectroscopy, which was to be expected from literature studies (Miyabara *et al.*, 1992).

5.1.2 HPLC-DAD-FLD and MS

In the previous section, the standards were characterized by fluorescence spectroscopy. To examine what comprises the standards' main fluorescence signal, standards were also separated and analysed by HPLC-DAD-FLD and MS. The focus was laid on fluorescence in the region around $\lambda_{ex/em}$ 485/500 nm, because this is the region where the main faecal pigment fluorescence appears.

Although the main faecal pigment fluorescence peak appeared at an excitation wavelength of 485 nm ($\lambda_{ex/em}$ 485/500), an excitation of 465 nm was chosen to evaluate the fluorescence. The reason for this is that the data presented in this section comes from coupling of HPLC, Aqualog and MS – if an excitation of 485 nm had been chosen, only parts of the emission spectra would be visible, as the Rayleigh scatter overlays a part of the emission spectrum. Through choosing a shorter wavelength, the whole spectra are visible, although at a lower fluorescence intensity. HPLC chromatograms showing the absorption of the separated standards can be found in Appendix C.

Stercobilin

The HPLC separated stercobilin standard showed 5 different fluorescence peaks between 1005 and 1145 seconds, when excited with light of 465 nm wavelength (Figure 13 a, c). The MS chromatogram showed 5 peaks with the mass to charge ratio (m/z) of 595, which corresponds to stercobilin (Quinn *et al.*, 2012), as well as, in smaller concentrations, m/z of 591 and 593, pointing to urobilin and urobilinogen being present in the stercobilin standard as well (Figure 13 b). Urobilinogen is however not fluorescent and can therefore not contribute to the fluorescence. Stercobilin was apparently present in the form of several stereoisomers. The different structure of the stereoisomers had no influence on the fluorescence spectra, as the fluorescence EEM in the region $\lambda_{ex/em}$ 485/500 nm only showed one main peak (Figure 11), while all different isomers contributed to the fluorescence (Figure 13 c). However, the hydrophilic properties differed between the isomers, so they appeared at slightly different times in the chromatogram. Coupling of HPLC and Aqualog showed that all the different peaks had identically shaped emission spectra with an emission maximum at approximately 500 nm, when excited at 465 nm (Figure 13 d).

Fluorescence peaks with $\lambda_{ex/em}$ 465/500 appeared at 1005, 1045, 1075, 1125, and 1145 seconds.

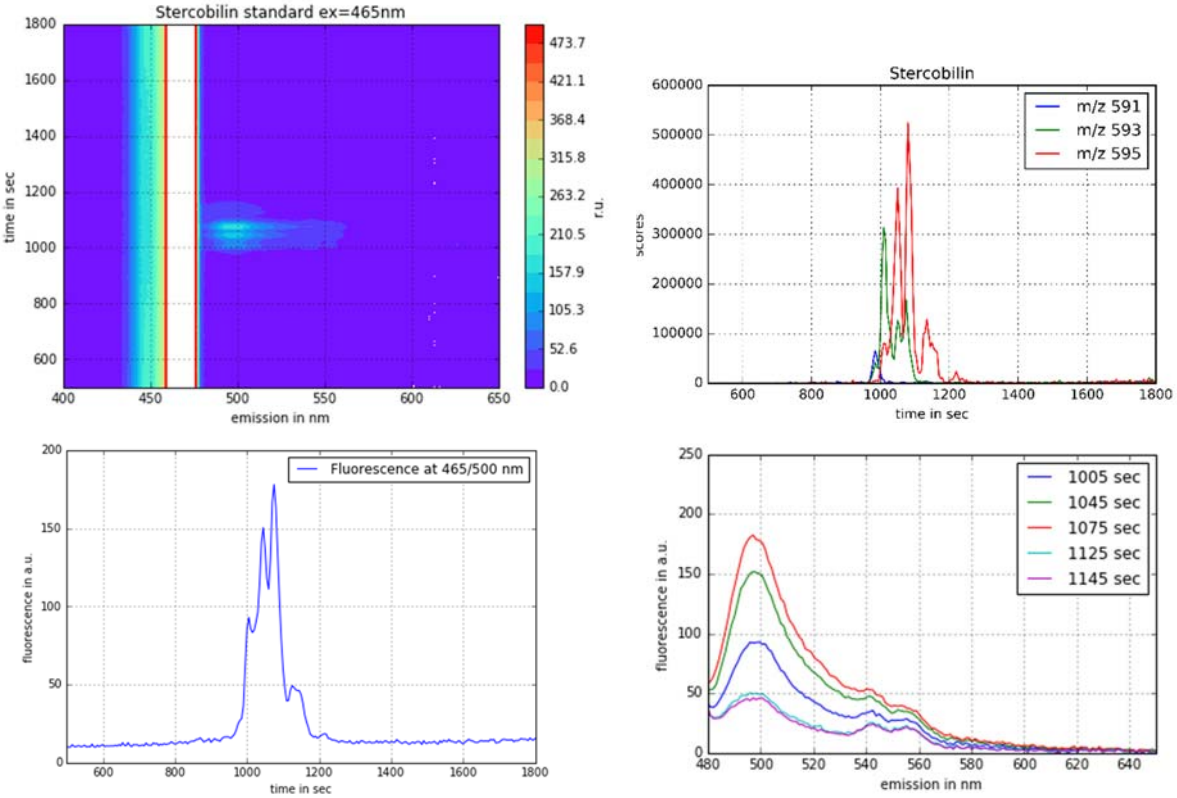


Figure 13. Results from HPLC-Aqualog-MS coupling of the stercobilin standard. Top left (a): Fluorescence matrix; top right (b): MS chromatogram; bottom left (c): Fluorescence chromatogram at $\lambda_{ex/em}$ 465/500 nm; bottom right (d): Emission spectra of the flourescence peaks in the chromatogram.

Urobilin

The separated urobilin standard showed two large fluorescence peaks and a smaller peak at a wavelength combination of $\lambda_{ex/em}$ 465/500 nm (Figure 14 a, c). The main peak at 1010 seconds had an m/z of 591 (Figure 14 b), which is urobilin (Quinn *et al.*, 2012), as should be expected in urobilin standard. It appeared earlier than the main peaks in the stercobilin standard. At the position of the second highest fluorescence and absorption peak at 1035 seconds, the MS chromatogram showed a peak with an m/z of 593. The fluorescence at this position can however not be caused by the non-fluorescent urobilinogen. There must be some other pigment there contributing to the fluorescence at 1035 seconds, possibly a faecal pigment metabolite. There was almost no stercobilin in the urobilin standard. The fluorescence peaks of the urobilin standard, like those of the stercobilin standard, all had the same emission spectra with a maximum at λ_{em} 500 nm, as shown in the coupling of HPLC and Aqualog (Figure 14 d). Unlike in the stercobilin standard, which showed several stercobilin isomers, there was only one urobilin-peak with m/z 591 in the urobilin standard (Figure 14 b).

Fluorescence peaks at $\lambda_{ex/em}$ 465/500 nm appeared at 1010, 1035, and 1100 seconds.

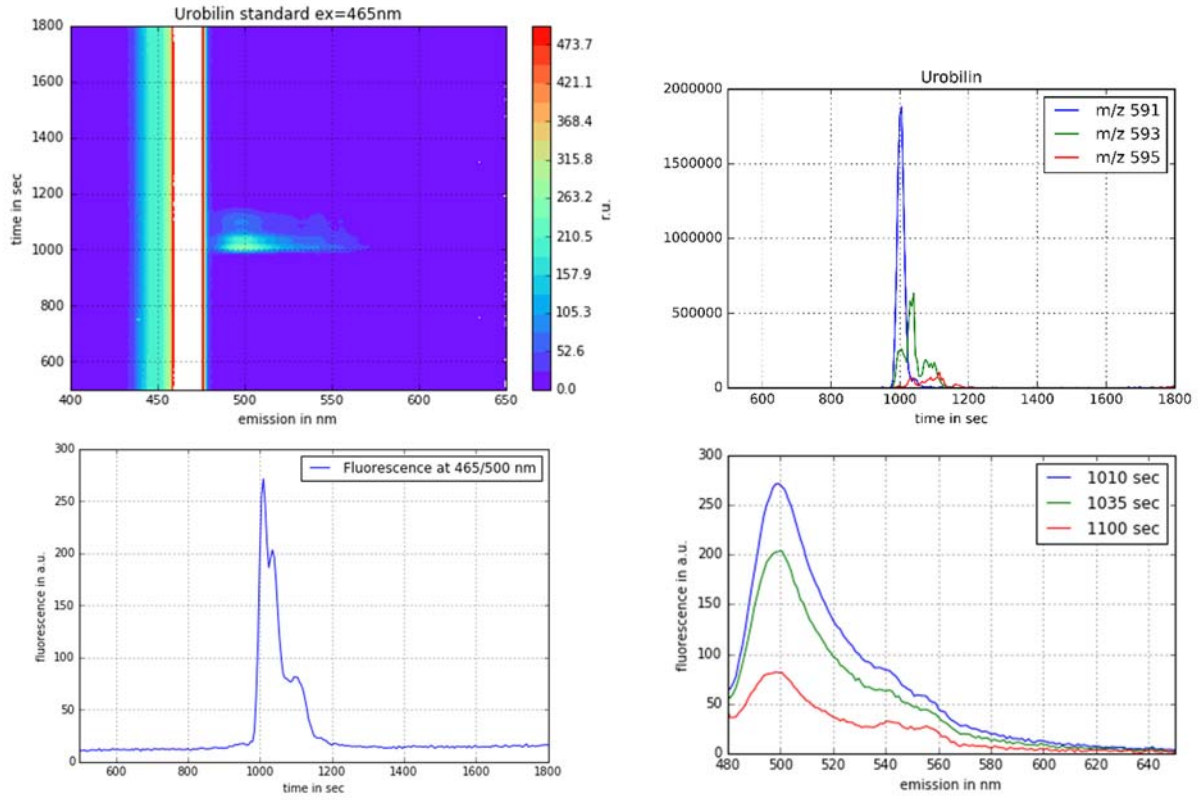


Figure 14. Results from HPLC-Aqualog-MS coupling of the urobilin standard. Top left (a): Fluorescence matrix; top right (b): MS chromatogram; bottom left (c): Fluorescence chromatogram at $\lambda_{ex/em}$ 465/500 nm; bottom right (d): Emission spectra of the flourescence peaks in the chromatogram.

Urobilinogen

As the fluorescence EEM already showed, the urobilinogen standard was far from being clean. It contained several substances fluorescing at λ_{ex} 465 nm (Figure 15). The largest fluorescent peaks appeared at 820 seconds, 950 seconds, and 1000 seconds (Figure 15 a, c). The emission spectra of these chromatogram peaks did not look entirely identical, the peak at 820 seconds (m/z 468) having its emission maximum at slightly lower wavelengths than the peak at 1000 seconds (Figure 15 d). A large part of the urobilinogen standard's fluorescence was caused by urobilin (m/z 591), the main peak appearing at 1000 seconds (Figure 15 b). Urobilinogen (m/z 593) appeared at approximately 1200 seconds (Figure 15 b) and did, as expected, not show any fluorescence (Figure 15 c).

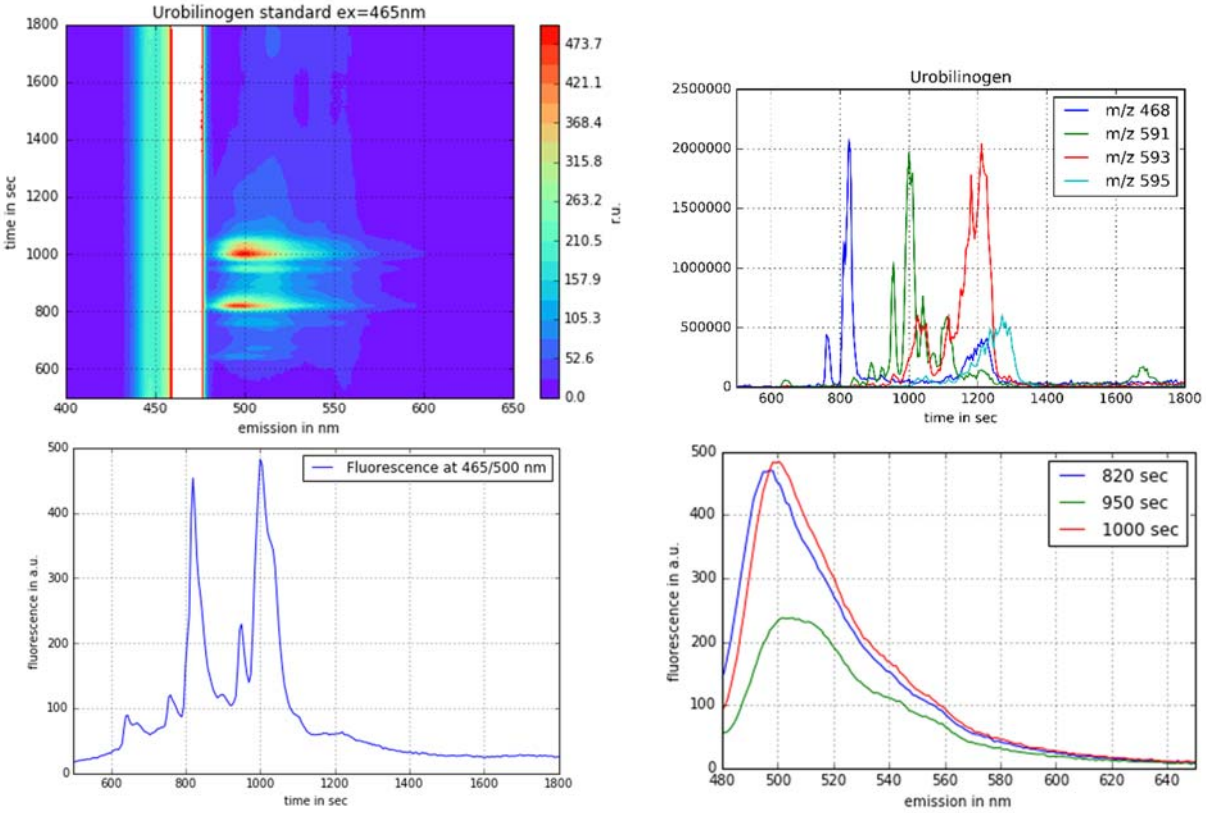


Figure 15. Results from HPLC-Aqualog-MS coupling of the urobilinogen standard. Top left (a): Fluorescence matrix; top right (b): MS chromatogram; bottom left (c): Fluorescence chromatogram at $\lambda_{ex/em}$ 465/500 nm; bottom right (d): Emission spectra of the flourescence peaks in the chromatogram.

The separation by HPLC showed that the main fluorescence signal of the pigment standards was composed by several different fluorescent compounds of different masses. It became clear that none of the standards only contained the substance it should contain, which gives a more realistic basis for interpretation of the results. Knowing the composition and the appearance of the different substances and isomers in a standard is furthermore a prerequisite for the comparison with real water samples later on.

While urobilin and stercobilin have similar fluorescence spectra, their HPLC chromatograms look vastly different. Not only are the structural differences between the urobilin and stercobilin shown, but

also the pigments' structural isomers, if present, are expressed in several peaks at different times in the chromatogram. HPLC was therefore a good tool to differentiate between the two pigments. HPLC of urobilin and stercobilin was first reported by Bull *et al.* (1981). In that study, stercobilin and urobilin (as well as half-stercobilin) could also be fully separated by HPLC.

The impurities in the standards are most likely not pollution from outside, but rather related forms of bile pigments which are metabolites of the examined pigments. Depending on the production of the standards (synthetic or "natural"), some impurities were expected. Assuming that there was only urobilinogen in the standard when it was manufactured, it seems to have oxidized to other urobilinoids, including urobilin as can be seen in the MS chromatogram (Figure 15 b). The fluorescent peak appearing at 820 seconds could be caused by a degradation product of a linear tetrapyrrole, where one pyrrole ring was split off. The compound with m/z 468 could be a linear oligopyrrole with three pyrrole rings, instead of four like in the faecal pigments (Quinn *et al.*, 2012).

5.2 Properties of the pigment standards

5.2.1 Calibration series and analytical limits

Urobilin and stercobilin standards exhibited different fluorescence intensities at the same concentration, which showed in the fact that their calibration curves differed in slope (Figure 16). Stercobilin standard showed, at the same concentrations, higher fluorescence intensities than the urobilin standard.

Both pigment standards differed in fluorescence intensity, depending on the medium they were measured in. Compared to phosphate buffer as a medium, the fluorescence intensity of stercobilin standard seemed to be quenched in HOWI, and, even stronger, in WWTP inflow. No quenching was observed for stercobilin standard in WWTP outflow (Figure 16, left). Urobilin standard was quenched in all real waters, compared to phosphate buffer. The calibration curves for the urobilin standard in HOWI, WWTP outflow, and WWTP inflow were very similar (Figure 16, right).

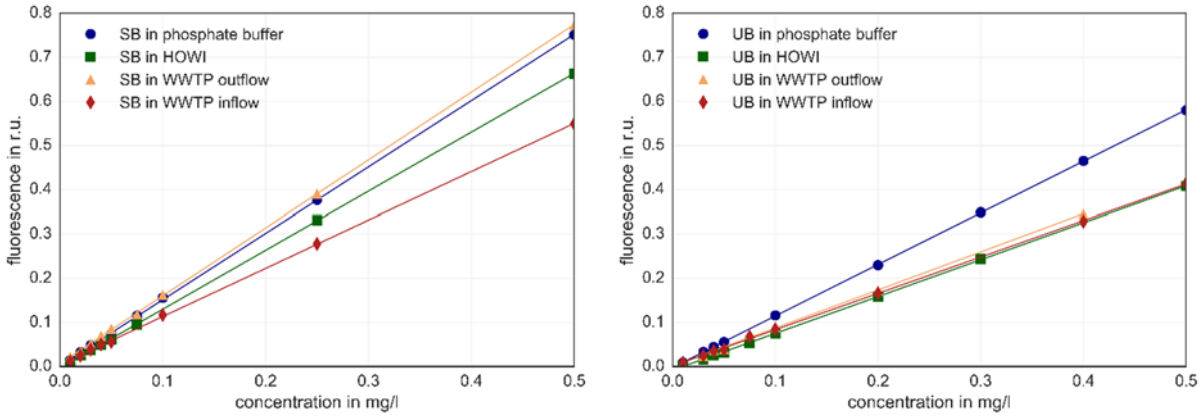


Figure 16. Calibration curves of stercobilin standard (left) and urobilin standard (right) in different media.

The relationship between fluorescence intensity and pigment concentration was linear in lower concentration ranges from 0.01 to 0.5 mg/l. Urobilin in WWTP outflow was an exception and only showed linear behaviour until 0.4 mg/l (Figure 16, right). If higher concentrations were included, a quadratic function fit the data better than a linear one. In this study, lower concentrations were more interesting for a practical application. Therefore, the calibration curves were limited to the concentration range up to 0.5 mg/l (0.4 mg/l for urobilin in WWTP outflow), in which the relationship was linear. This makes the calibration only valid for the range shown in Figure 16.

Analytical limits for the pigment standards, as well as properties of the calibration series are shown in Table 1 and Table 2. The urobilinogen standard is not shown here, as urobilinogen itself is non-fluorescent and can therefore not be quantified using fluorescence spectroscopy.

Table 1. Analytical limits (limit of detection (LOD) and limit of quantification (LOQ)) and calibration curve properties for the stercobilin standard.

Stercobilin	**Phosphate buffer**	**HOWI**	**WWTP outflow**	**WWTP inflow**
LOD [mg/l]	0.004	0.003	0.003	0.005
LOQ [mg/l]	0.020	0.014	0.015	0.027
Slope	1.50	1.34	1.54	1.09
R^2	0.9999	1.0000	1.0000	0.9999

Table 2. Analytical limits (limit of detection (LOD) and limit of quantification (LOQ)) and calibration curve properties for the urobilin standard.

Urobilin	**Phosphate buffer**	**HOWI**	**WWTP outflow**	**WWTP inflow**
LOD [mg/l]	0.002	0.003	0.010	0.007
LOQ [mg/l]	0.010	0.016	0.046	0.035
Slope	1.17	0.84	0.86	0.82
R^2	1.0000	1.0000	0.9995	0.9998

The results showed differences in fluorescence intensity between urobilin and stercobilin at the same concentration. Urobilin and stercobilin have a very similar molar weight (590 g/mol and 594 g/mol),

therefore this difference still exists when calculating with molar concentrations. The difference in fluorescence intensity between the two pigments could mean that they differ in their fluorescence properties (have a different quantum yield), or be a false impression caused by inaccuracy when preparing a stock solution from the standard, because there were very small amounts of substance dissolved in a lot of water. Additionally, none of the standards contained 100% urobilin or stercobilin, which made it difficult to know the actual concentration of the pigments in the calibration series. Unfortunately, there was only one standard of each substance, so no repetitions with a different standard could be done and it remains unclear, what caused this difference in fluorescence intensity at apparently the same concentration. In the case that the differences were attributable to the pigments themselves and not to any analytical inaccuracies, it would be impossible to connect one faecal pigment concentration to a certain fluorescence intensity. The reason is that the two pigments are indistinguishable by fluorescence spectroscopy, but the slope of their calibration curves is different, so they contribute differently to a fluorescence signal. For a practical application it is however necessary to conclude from a certain fluorescence intensity found in a sample to a faecal pigment concentration. One possibility to solve this is to calculate a range of possible concentrations, assuming that a fluorescence signal results from 100% urobilin or 100% stercobilin (5.6.3). The true concentration will then lie in the range between these two calculated values.

The observed quenching of urobilin and stercobilin in media other than phosphate buffer might be a pH effect, caused by a difference in ionic strength, or by metal ions present in the water. pH influence is illuminated in the next chapter.

The theoretical detection limits observed in this study were as low as 2 µg/l for urobilin and 3 µg/l for stercobilin, but depended on the medium (Table 1 and Table 2). To evaluate the detection limit from a practical point of view, a daily production of 150 mg faecal pigment for one person may be assumed (150-250 mg; Orten, 1971). For reaching the detection limit of 3 µg/l, the amount of faeces produced by one person in a day would need to be diluted in approximately 50000 litres of water, which is still a relatively high contamination, considering that the technique should be used for drinking water monitoring. However, LOD could probably be lower, if lower pigment concentrations for the calibration series were chosen. If calibration series are repeated with zinc addition, even lower limits of detection are to be expected. Miyabara *et al.,* (1992), got a detection limit of 0.2 µg/l after the addition of zinc (0.1% zinc acetate in 75 mM boric acid buffer at pH 6) and separation by HPLC. They were not able to detect any urobilin or stercobilin without zinc addition. Probably the technique was not sensitive enough. They used the area under the fluorescence peak (separated by HPLC) for the calibration and got a linear relationship between 1-1000 µg/l, which was a broader range than observed here. Bixler *et al.* (2014) calibrated the fluorescence intensity of urobilin in the presence of zinc by integrating the area of the emission curve after the blank value (ethanol) was removed. They observed detection limits of several ng/l, but only used pure alcohol and had no disturbances present in real water samples. Bixler *et al.* (2014) as well as Miyabara *et al.* (1992) both determined the fluorescence of the pigment zinc complex, determined with the Schlesinger method, and not of the pure pigment. Additionally, Miyabara *et al.* (1992) used HPLC and Bixler *et al.* (2014) used a cavity-enhanced form of fluorescence spectroscopy. Although these two studies are the most similar ones to the present study, the differences in methods make the results hardly comparable with the obtained detection limits in this study.

5.2.2 pH influence

The pH value had an effect on the faecal pigments' fluorescence intensity (Figure 17). The fluorescence intensity of both, urobilin and stercobilin, was lower at a pH of 8.5 compared to a pH of 6.6 (Figure 17). Fluorescence maxima were at approximately $\lambda_{ex/em}$ 485/500 nm, independent of the pH.

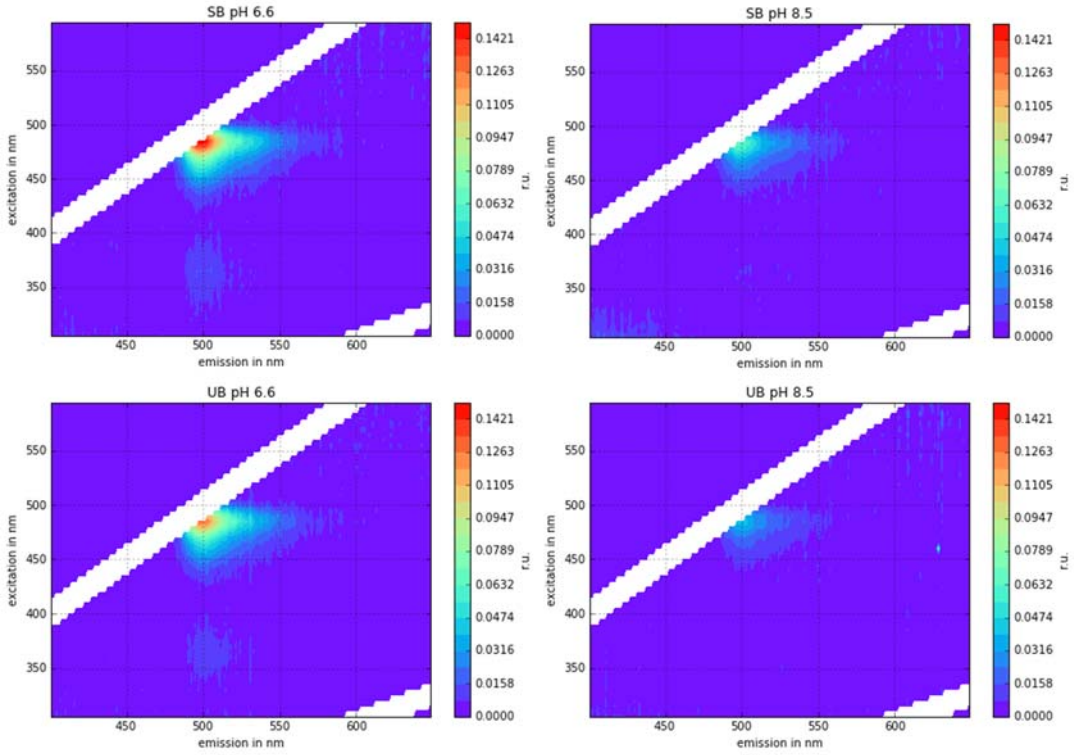


Figure 17. Fluorescence EEMs of stercobilin and urobilin (both 0.1 mg/l) in phosphate buffer. Left: pH 6.6, right: pH 8.5.

As the fluorescence intensity of urobilin and stercobilin in phosphate buffer was influenced by the pH value, the in 5.2.1 observed differences in the slope of the calibration line between different media might have been caused by pH. Unfortunately, the standards were opened at different times and therefore the calibration series of urobilin was measured in different water than the calibration series of stercobilin. pH was only measured initially after sampling and not for all media. For this reason, no direct relationship between the calibration series results and pH of the waters could be established. For the stercobilin calibrations, water from the screening of WWTP A was used (inflow pH 7.6, outflow pH 6.8), as well as freshly sampled HOWI (pH 7.9), but for the calibration series of urobilin, a second sample from WWTP A had to be taken and the pH of this sample is unknown. The HOWI used for this calibration could also not be freshly sampled but was three weeks old, which can have had an influence on the pH. The pH could explain why the calibration series look different depending on the medium, but this will have to be confirmed in future tests.

5.2.3 Stability

The fluorescence signal of urobilin and stercobilin standards, both diluted to a concentration of 0.5 mg/l, according to the concentration given by the manufacturer, was over the observed time relatively stable in phosphate buffer, with even an increase in fluorescence intensity in the beginning, while it decreased in real waters (Figure 18). In wastewater, the fluorescence intensity decreased the fastest (with urobilin in HOWI at room temperature being an exception), while the decrease in fluorescence intensity in HOWI under cool and dark conditions took more time. In phosphate buffer, both urobilin and stercobilin were stable (≥ 80% of the initial intensity) over the days studied. Light and temperature differences did not have a clear influence on the stercobilin fluorescence in phosphate buffer, while for urobilin in phosphate buffer the warm storage resulted in a weaker signal. However, although the fluorescence intensity of the sample under warm conditions in phosphate buffer remained lower than for the cool sample, they both had the same curve shape and overall behaviour (Figure 18). Under some conditions, there was an increase in intensity directly in the beginning of the stability experiments of urobilin and stercobilin, before the fluorescence intensity started to decrease.

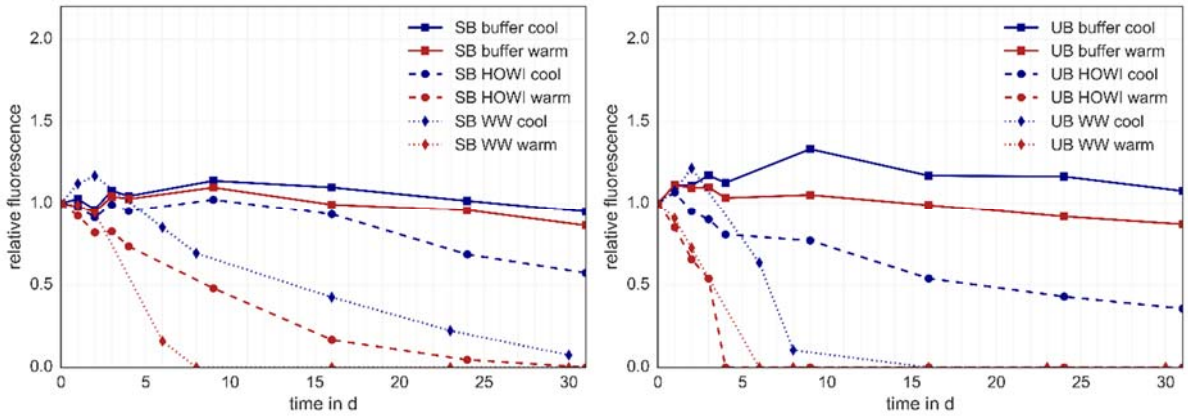


Figure 18. Relative fluorescence intensity of stercobilin (left) and urobilin (right), both 0.5 mg/l, over time in phosphate buffer, HOWI, and WWTP inflow under different storage conditions. For a better comparison, relative fluorescence is shown.

The urobilinogen standard showed an especially interesting behaviour (Figure 19). Its fluorescence in the faecal pigment region was barely detectable on day 0, but then increased to a multiple of the initial value. The time at which the highest intensity was reached strongly depended on the storage conditions, with the samples stored in warm conditions reaching the peak earlier than the samples stored in cool conditions. The peak intensity occurred between 4 and 24 days (HOWI warm vs. buffer cool). After reaching a peak, the fluorescence intensity declined again. No stability tests for the urobilinogen standard in wastewater were done.

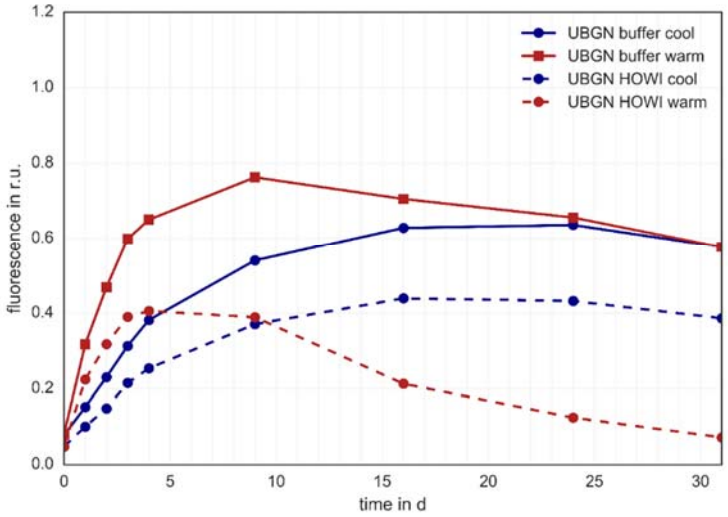


Figure 19. Stability of "urobilinogen" (5 mg/l) in phosphate buffer and HOWI.

The stability of the pigments' fluorescence intensity differed between media and storage conditions, whereas the water, in which the pigment was diluted, seemed to have a higher influence on the stability than temperature or light influence. It can therefore be assumed that there is not only a photochemical degradation, but also a microbial degradation. The influence of microbial degradation becomes clear when comparing between wastewater, HOWI, and phosphate buffer. The fluorescence intensity in phosphate buffer, which should have no or only little microbial pollution, was most stable, independent of the storage conditions, while the degradation was faster in HOWI and wastewater. Also, room temperature and/or light enhanced the loss in fluorescence, but only in HOWI and wastewater. This is a sign that microbial degradation was probably the main cause for loss of fluorescence. Both, photochemical and microbial degradation should be more pronounced in the samples stored at room temperature and under light influence. To find out whether photochemical degradation of a relevant degree takes place, tests with one group of samples in light and another group of samples in darkness, both at the same temperature, would need to be done. Degradation, in this case, refers to the diminishing of fluorescence intensity. It is not clear, whether only the fluorescence intensity diminishes over time, or whether the pigment molecules are really degraded.

The stability of faecal pigment fluorescence also has to be taken into account when evaluating the pigments as an indicator of faecal pollution. Miyabara *et al.* (1994c) found that 90% of urobilin in river water was lost within the first two days of storage in the laboratory. The findings in the present study showed a more stable behaviour of stercobilin as well as urobilin than in the study of Miyabara *et al.* (1994c). In the present study, there were still 50% of pigment left after three days, even in the samples in HOWI and wastewater at room temperature. These big differences in the results might be explainable by the different methods used. If Miyabara *et al.* (1994c) examined the zinc-complex fluorescence only, which was observed to be less stable than the fluorescence of the pigment alone (5.3.1), it is reasonable that they recorded a high loss within the first two days.

Stercobilin was, according to literature, expected to be more stable than urobilin, because stercobilin is considered to be a stable end product, while urobilin can be further oxidized to mesobiliviolin and glaucobilin (Bull *et al.*, 1981; Watson, 1969). However, the assumption of a more stable stercobilin could not be entirely confirmed in this study. Fluorescence intensity of urobilin and stercobilin behaved relatively similar in phosphate buffer. Urobilin fluorescence seemed to diminish faster in HOWI and wastewater, but more data is needed to compare the fluorescence intensity of stercobilin and urobilin over time.

Higher fluorescence intensity values in the cool samples compared to the samples stored at room temperature, e.g. for urobilin in phosphate buffer, can partly be a result from insufficient waiting time between taking the samples out of the fridge and the measurement, because cooler temperature enhances the fluorescence (3.2.3; Guilbault, 1990).

In the beginning of the experiment, there was an increase in the fluorescence intensity of several samples stored under cool as well as warm conditions, which cannot be explained by any temperature bias. This might be caused by the transformation of some urobilinogen and stercobilinogen in the standards to urobilin and stercobilin, causing an increase in fluorescence intensity. The fluorescence of the urobilinogen standard increased over the first days and reached a maximum of several times the starting value. Most likely, also here the non-fluorescent urobilinogen was gradually transformed to urobilin, which shows a fluorescence signal. However, compared to urobilin and stercobilin, the urobilinogen standard did not reach a high intensity and approximately 10% of urobilinogen were transformed to urobilin (given that 5mg/l urobilinogen were used, compared to only 0.5 mg/l urobilin and stercobilin).

In natural waters, the pigments would sooner or later be exposed to light. The temperature however depends strongly on the conditions (wastewater pipe, tropical lake, alpine river, etc.). Because of this, it cannot be judged which one of the storage conditions is the more realistic one. Given the experimental setup, it would be very unrealistic to assume that the stability in a glass bottle in the laboratory is equivalent to the stability in a natural water body. However, to know how to handle the pigments themselves, and water for pigment analysis after sampling, knowledge about the stability under such static conditions can be very useful.

Apart from the already mentioned possible temperature differences in the stability samples, the measurements presented here were done with samples of approximately the same temperature, which should not have any measureable influence on the fluorescence.

5.3 Reaction to zinc addition

When zinc acetate was added to faecal pigment standards diluted in a real water sample medium, the fluorescence intensity increased and the emission as well as excitation spectra shifted to longer wavelengths. The fluorescence intensity increase depended on the medium (5.3.2). The position of the fluorescence maximum shifted from $\lambda_{ex/em}$ 485/500 nm to $\lambda_{ex/em}$ 500/513 nm when zinc acetate was added, which was the same for both urobilin and stercobilin (Figure 20).

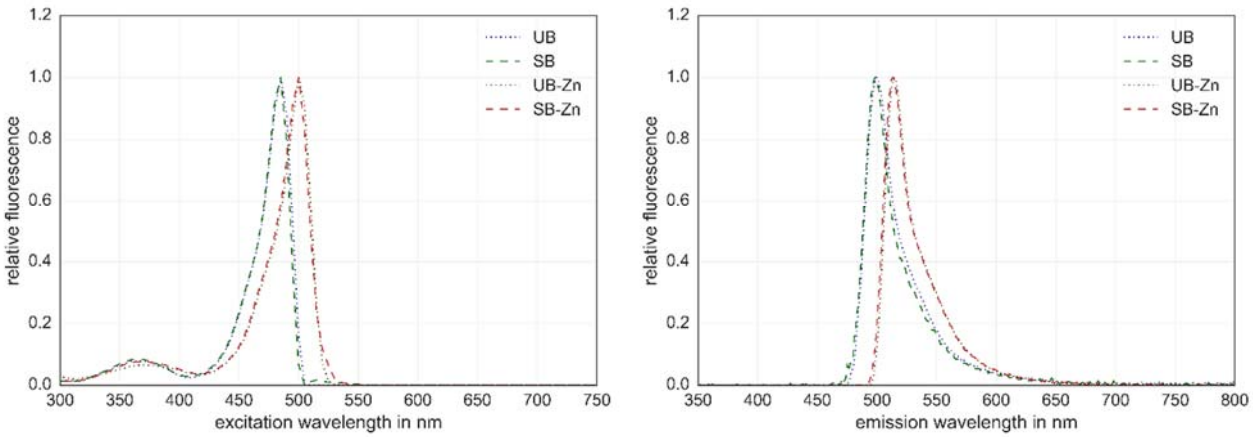


Figure 20. Normalized excitation (left) and emission (right) spectra of urobilin and stercobilin before and after zinc addition.

The intensification of fluorescence after zinc addition can be quantified by using a gain factor. The gain factor is calculated by dividing the peak fluorescence intensity after zinc addition by the peak intensity before zinc addition. It is important to take into account that the position of the peak shifts and that the slope of the peak is very steep. If this is not considered in the calculations, the peak maximum is missed, and it will come to unrealistically high or low gain factors.

$$\text{Gain factor} = \frac{F\ at\ 500/513\ nm}{F_0\ at\ 485/500\ nm}$$

As an example, for 0.1 mg/l stercobilin in HOWI, gain factors of 30-40 were observed at an addition of 20 mg/l zinc after a delay of 1 minute between zinc addition and measurement start.

5.3.1 Zinc kinetics

The gain in fluorescence intensity when adding zinc to a solution containing urobilin or stercobilin was time-dependent. Fluorescence intensity increased within seconds after zinc addition, but it was not stable and decreased rapidly, e.g. for 0.1 mg/l urobilin and stercobilin in HOWI with 20 mg/l zinc the intensity was nearly halved within 30 minutes (Figure 22). While the pigment signal became weaker with time, Rayleigh scatter increased, as can be seen in Figure 21 where Rayleigh scatter was not removed.

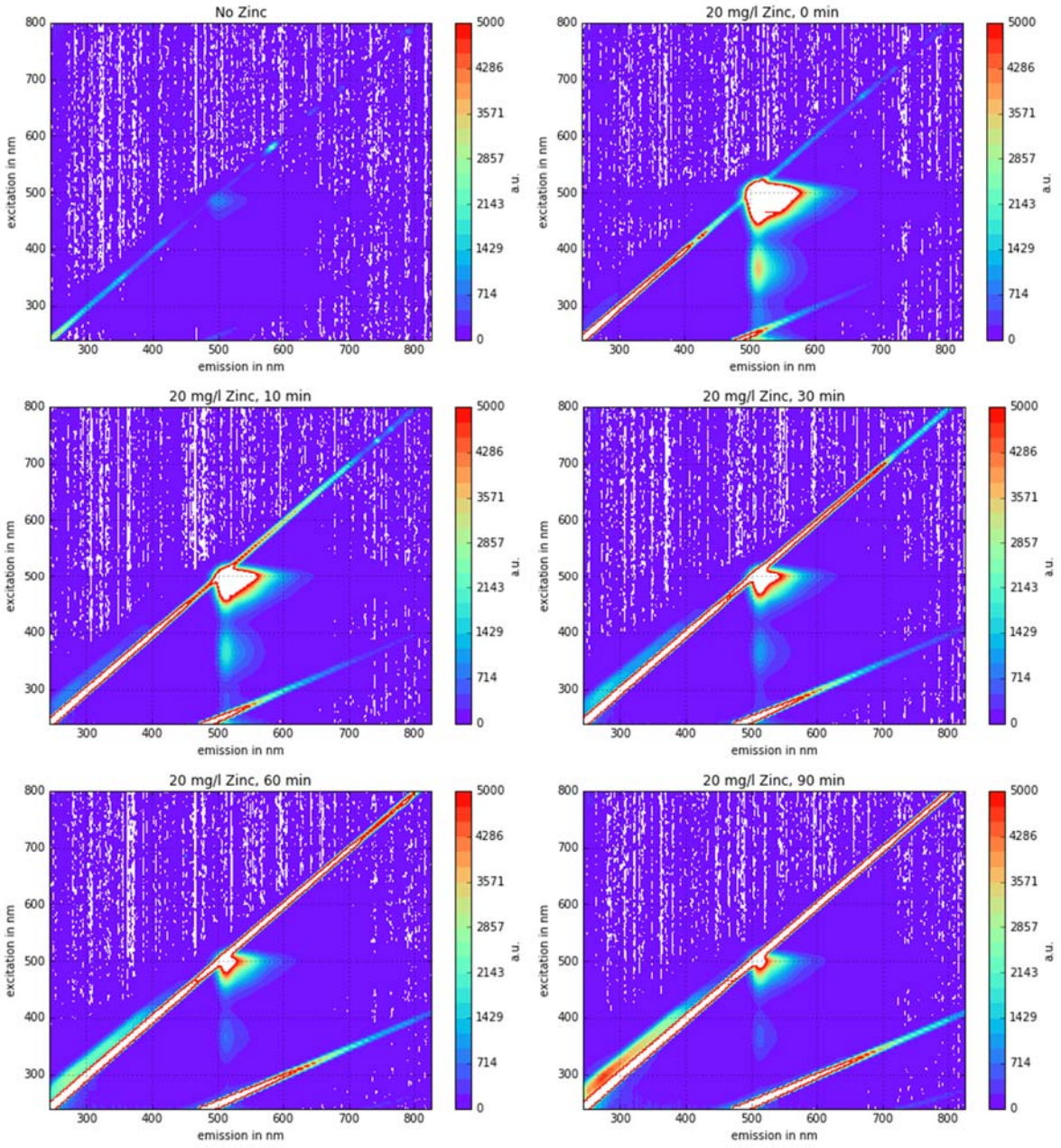


Figure 21. Fluorescence intensity of 0.5 mg/l stercobilin in HOWI over time after 20 mg/l zinc were added (a.u.). Rayleigh scatter not removed.

Within seconds after zinc addition, the colour of the urobilin and stercobilin solutions changed from yellow to a light pink. For higher zinc concentrations (>10 mg/l) flocculation could be observed with the bare eye.

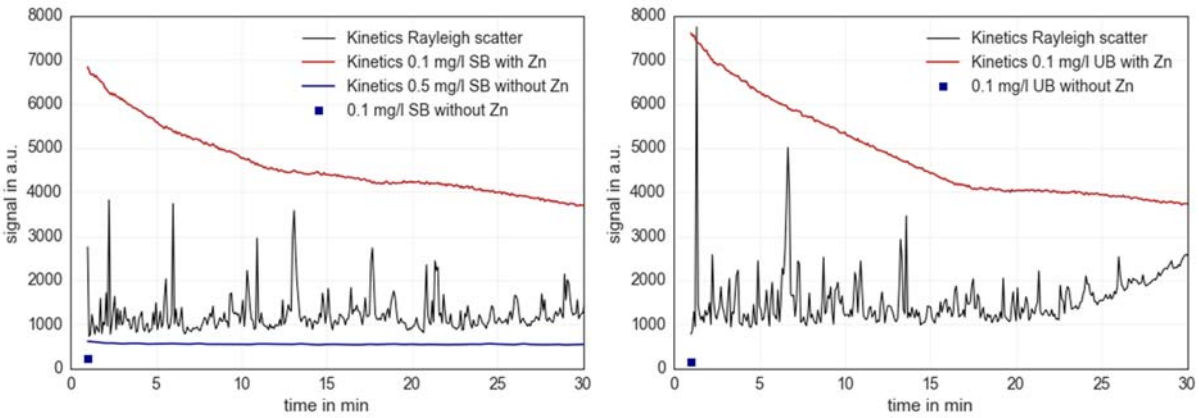


Figure 22. Time-dependent behaviour of Rayleigh scatter and faecal pigment fluorescence in HOWI after zinc addition (20 mg/l) over the course of 30 minutes. Measurements were started 1 minute after zinc addition. Left: stercobilin in HOWI, right: urobilin in HOWI.

Figure 22 shows the fluorescence of 0.1 mg/l stercobilin and urobilin in HOWI in the kinetics mode after addition of 20 mg/l zinc. From the start of the measurement, which was one minute after zinc addition to the pigment solutions, a continuous decrease in fluorescence intensity was observed (Figure 22, red line). Rayleigh scatter (Figure 22, black line) was very unstable during the observed time of 30 minutes. When no zinc was added, the fluorescence intensity of stercobilin in HOWI was stable over the time of 30 minutes (Figure 22, blue line), compared to stercobilin with zinc addition (Figure 22, red line). The same concentrations of pigment with and without zinc are not shown in the kinetics mode, as the signal would have been either too low for a stable measurement without zinc, or too high with zinc addition.

How fast the decrease in relative fluorescence intensity proceeded depended on the added zinc concentration. When 20 mg/l zinc were added to 0.1 mg/l stercobilin, the loss of fluorescence within the first 4 hours was over 80%, while it was only approximately 25% when only 1 mg/l zinc was added (Figure 23). While the fluorescence intensity of the zinc complexes decreased within three days to between 2% and 30% of the initial value, the red shift remained the same. This was observed for three days after zinc addition, then the observations were stopped.

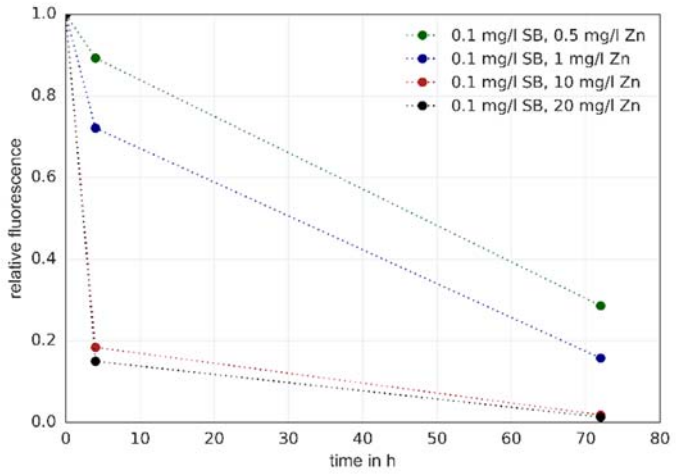


Figure 23. Relative fluorescence intensity of 0.1 mg/l stercobilin over time with different zinc concentrations.

The gain in fluorescence intensity when adding zinc to urobilin or stercobilin in water was time-dependent, which is a phenomenon that has not yet been described for these pigments in similar studies. Neither Miyabara *et al.* (1992) nor Bixler *et al.* (2014) observed a time-dependent reaction following zinc addition. Kinetics of metal ion binding to e.g. proteins is, however, a known phenomenon (Cavatorta *et al.*, 1994). Both Miyabara *et al.* (1992) and Bixler *et al.* (2014) used the Schlesinger method by dissolving urobilin (hydrochloride) in ethanol and adding zinc acetate. In the present study, a zinc acetate solution dissolved in MilliQ was added into the water sample without the use of ethanol, but the results concerning an enhanced fluorescence intensity and red shift were similar. It is not known whether a fluorescence decrease with time also existed in prior studies but was either not noticed or not described, or if it was not present. It is possible that the difference in method due to the absence of ethanol is responsible for the observed decrease of fluorescence over time.

When adding zinc acetate to faecal pigment samples, a red shift was visible within seconds after zinc addition, supporting that the reaction leading to a red shift and fluorescence intensity increase happened immediately after zinc addition. The fluorescence of the complexes was not stable, as well as the fate of the complexes unknown. It is at this point unclear, whether the pigment complexes stay complexed in a dissolved state, are degraded, or flocculate. It also has to be determined, if the rapid loss of fluorescence intensity is caused by a de-complexation or flocculation, or if there is an equilibrium in the solution to be reached between complexed and free zinc and pigments. The unstable Rayleigh scatter visible in the first 30 minutes of the zinc kinetics with urobilin and stercobilin, as well as a further increase in Rayleigh scatter, is a sign that some particles were starting to develop. A flocculate, which could be observed with the bare eye at high zinc concentrations, points to flocculation of some complexes – either stercobilin-zinc, or some other zinc complex, e.g. zinc phosphate. While the intensity decreased with time, the red shift remained the same, indicating that there was no de-complexation but rather an increase in radiationless processes competing with fluorescence. Then the absorbance should stay the same over time, while only the fluorescence decreases. A measurement of fluorescence, in combination with absorbance, in the kinetics mode could help to understand the process.

In a practical application, time has to be monitored when using zinc to enhance faecal pigment fluorescence. To make the measurements comparable and for practical reasons, the measurements of zinc solutions were started one minute after zinc addition to the sample, after there was probably already some decline in fluorescence intensity. The given gain factors are therefore probably relatively conservative estimations. The true gain factors might be much higher. For the zinc kinetics measurements, an excitation wavelength of 480 nm was chosen to avoid disturbances by Rayleigh-Scatter. However, as the peak of the zinc-complex at $\lambda_{ex/em}$ 500/513 is very steep, as well as red-shifted compared to the pure pigment, the peak can be missed, if an excitation wavelength so low is chosen. An excitation wavelength of 495 nm should give more accurate results. If the measurement is conducted as soon as possible after zinc addition, Rayleigh scatter should not disturb the fluorescence measurement. Using the whole area under the fluorescence peak can give a more robust result than taking only the value with the highest intensity (compare to Bixler *et al.*, 2014), because the fluorescence peaks are very steep and the peak intensity can be missed, causing a bias. Especially for the zinc kinetics, this would be a valuable method of calculating the fluorescence intensity, because the intensity decreases over time. Calibration series of pigment standards with zinc were done, before the time-dependency of the complex fluorescence was discovered. This means, that these calibration series were not, or only under limitations, evaluable and were not presented. For reasons of lacking time and standard material, these calibrations could not be repeated. Therefore no analytical limits are shown for the pigment-zinc complexes.

According to Miyabara *et al.* (1992) there are big differences between metals in their influence on faecal pigment fluorescence, with zinc causing the biggest gain in fluorescence intensity, compared to other metals. The fluorescence reaction of stercobilin and urobilin in the presence of other metals than zinc should be determined in future studies.

5.3.2 Influence of media and pH on the zinc reaction

In phosphate buffered MilliQ at pH 6.6, the pigments' fluorescence intensity behaved differently when zinc was added, than it did in drinking water or wastewater. While there was a strong increase of fluorescence intensity in drinking water and wastewater, as well as a red shift, there was only a smaller intensity increase and no red shift observed in phosphate buffer. In this section, the influence of pH and the water medium as possible causes were examined.

As was observed in chapter 5.2.2, when no zinc was added to urobilin and stercobilin in phosphate buffer, the fluorescence intensity was lower at a pH of 8.5 than at a pH of 6.6. When zinc was added, the fluorescence behaved the opposite way. With zinc, the samples with a pH of 8.5 showed a stronger intensity than the samples with a pH of 6.6. The gain factor of zinc addition was thus higher at a pH of 8.5 compared to a pH of 6.6 (Figure 24). Also, a red shift from $\lambda_{ex/em}$ 485/500 nm to 500/513 nm after zinc addition could be observed in the samples with pH 8.5. This was missing in the samples with pH 6.6.

Urobilin and stercobilin in HOWI (pH 7.9), had a much higher intensity after zinc addition, and thus higher gain factors, than they had in phosphate buffer at both lower (pH 6.6) and higher (pH 8.5) pH values (Figure 24). Similar intensifications like in HOWI were observed in wastewater (results not shown).

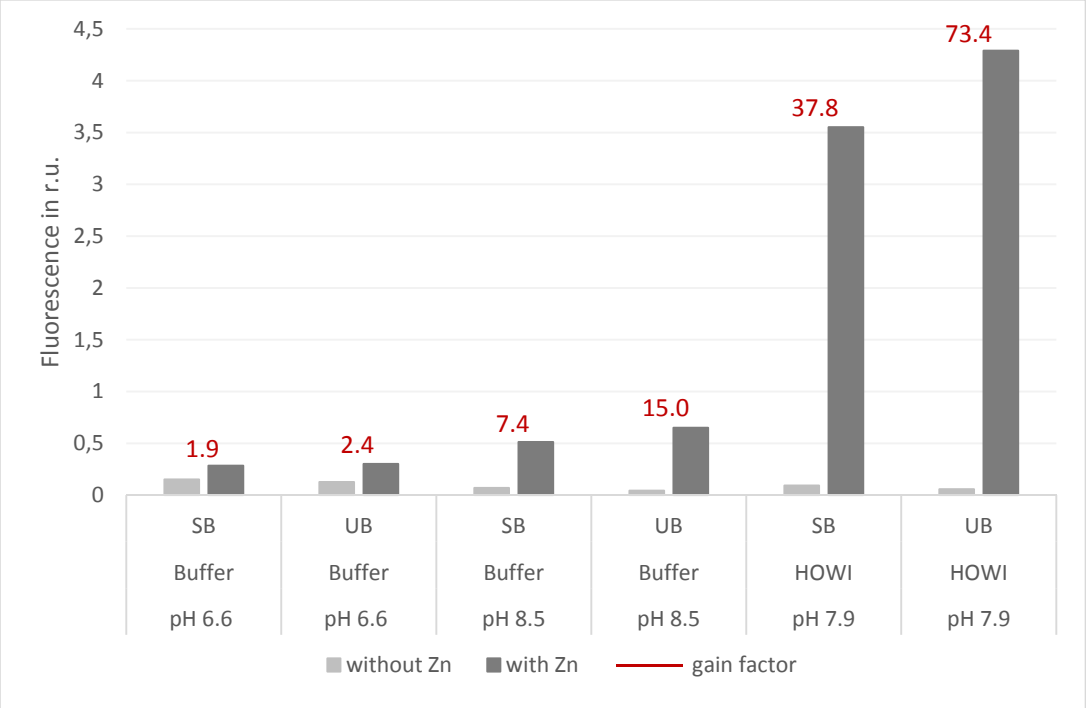


Figure 24. Fluorescence intensities and gain factors of 0.1 mg/l urobilin and stercobilin in phosphate buffer with pH value 6.6 and 8.5, as well as in HOWI before and after zinc addition in r.u.

The results showed that the pigments reacted differently on the addition of zinc in phosphate buffer and real water. As the phosphate buffer (pH 6.6) had a lower pH than the real waters (pH 6.8-8.4), this difference in pH could be suspected to cause this different reaction. The results imply that the pH has an influence on the reaction of zinc with the pigments. However, when the intensity after zinc addition is compared between faecal pigments in phosphate buffer of either pH and faecal pigments in HOWI, it becomes evident that there must be some additional influence other than the pH catalysing the zinc complexation and the following strong fluorescence intensity increase. The increase was much stronger in HOWI (pH 7.9) when zinc was added than in either of the phosphate buffers (pH 6.6 and pH 8.5). Thus, the pH value plays a role, but the pH as the only cause for the different reaction in phosphate buffer and real waters was excluded.

Other probable causes could be that the complexation is catalysed by some other ions present in natural waters, or that the ionic strength in general causes the difference. According to Schmidt and Scholtis (1964), a phosphate buffer disturbs the fluorescence measurement when zinc is added because of the precipitation of zinc phosphate. This could explain why there was little enhancement of the fluorescence when adding zinc acetate to the faecal pigments in phosphate buffer, compared to real waters. A large proportion of the zinc was probably bound to phosphate and was not available for complexation with the faecal pigments anymore.

The influence of pH on faecal pigment fluorescence was different, depending on whether zinc had been added to the water medium or not. It is unclear, why this was the case. Concerning a practical application, the influence of pH on the zinc complex fluorescence is especially interesting. Miyabara *et al.* (1992) got, with 0.1% zinc acetate in the solution, a maximum fluorescence response of urobilin and

stercobilin at a pH of approximately 6.5, decreasing towards pH 5 and pH 7. This is very different to the results that were found here. Schmidt and Scholtis (1964) found a pH of 7.5-8.5 to be optimal using the Schlesinger test (addition of zinc in alcohol) on several urine samples, which matches better with the results obtained here. Only two pH buffer solutions (pH 6.6 and pH 8.5) were used in the present study. To support the results from the literature, more tests on a broader range of pH values would be necessary. In the present study, pH and temperature could either be controlled or were at least known, but when measuring real waters on-line, they have to be taken into account. For all practical applications, with and without zinc addition, these results imply that the pH value has to be monitored when measuring fluorescence.

5.3.3 Dependency on the zinc concentration

The influence of different zinc concentrations on the fluorescence of faecal pigments was tested with stercobilin in HOWI. Urobilin was not tested, but is expected to behave similarly. The higher the zinc concentration, given a constant pigment concentration, the higher was the observed fluorescence signal (Figure 25). The increase showed a non-linear behaviour and the slope of the curve decreased at higher zinc concentrations, indicating that a saturation could be reached at higher zinc additions (Figure 25).

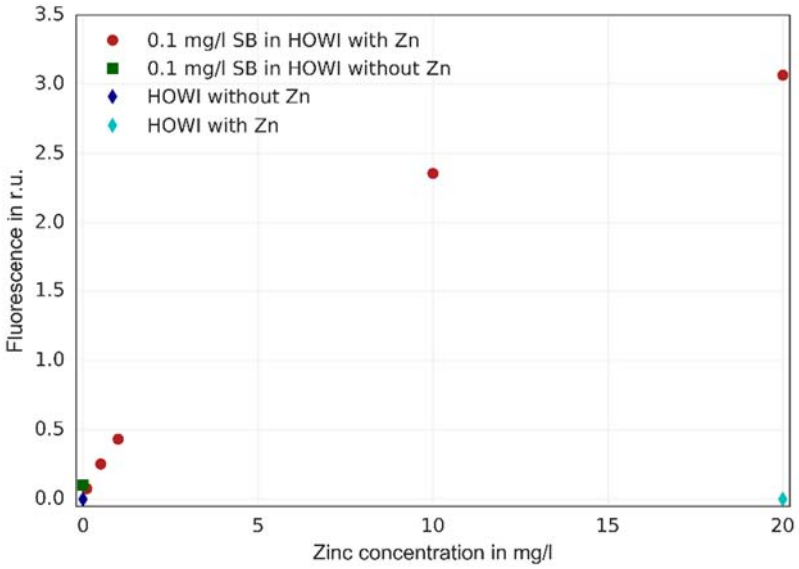


Figure 25. 0.1 mg/l SB in HOWI with varying zinc concentrations (1 min between zinc addition and measurement start). Molar relations between SB and zinc were approximately 0, 1:9, 1: 45, 1:90, 1:900, and 1:1800 from 0 to 20 mg/l zinc addition.

To ensure that it was a reaction of zinc with stercobilin, and not zinc alone or other zinc complexes causing the fluorescence increase, 20 mg/l zinc were added to HOWI without the addition of pigment. There was no fluorescence observed, confirming that it was the zinc-pigment-complex causing the increase in fluorescence intensity (Figure 25, cyan marker).

It was concluded that the addition of a high amount of zinc leads to a big fluorescence intensification, but also to a faster loss in relative fluorescence intensity, than when only small amounts of zinc are

added (Figure 23, Figure 25). Furthermore, the maximum fluorescence intensity was probably not reached at an addition of 20 mg/l zinc (Figure 25). It is surprising that an addition of 20 mg/l zinc in the form of zinc acetate did apparently not lead to a saturation with zinc, although the molar ratio between zinc and pigment was 1800:1 at a pigment concentration of 0.1 mg/l (0.17 µmol/l stercobilin; 0.31 mmol/l zinc). In theory, all available pigment molecules should have formed a complex. However, there were likely many other molecules, e.g. phosphate and dissolved organic matter, present in water, which can bind to zinc, making it unavailable for the complexation with stercobilin or urobilin. That a saturation did apparently not occur at a zinc addition of 20 mg/l, means that the experiments made with this zinc concentration, despite the excess in zinc, do not represent the highest possible gain factor.

5.4 Fluorescein

The uranine standard (fluorescein disodium salt) showed a very similar fluorescence signal to urobilin and stercobilin with its maximum at approximately $\lambda_{ex/em}$ = 485/510 nm. Its excitation and emission spectra are shown in Figure 26.

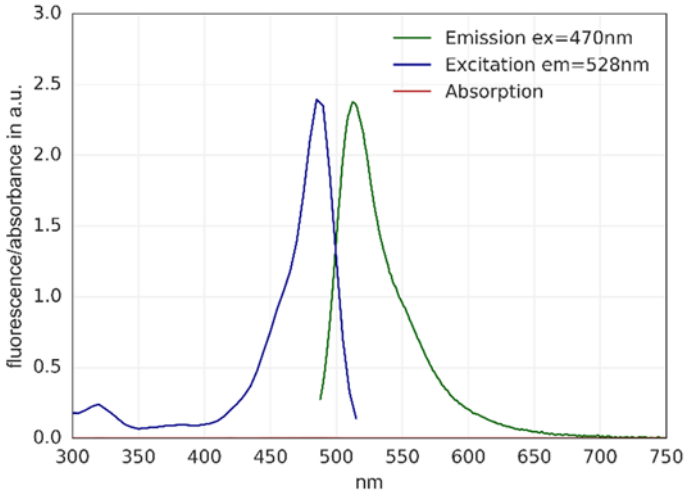


Figure 26. Absorption, excitation and emission spectra of uranine standard (10 µg/l fluorescein) in phosphate buffer (pH 6.6).

While its fluorescence signal was very strong at a concentration of only 10 µg/l, fluorescein showed almost no absorbance (Figure 26). The absorbance was, due to the low concentration, so small that it was hardly recognizable compared to the strong fluorescence (Figure 26, red line close to 0).

The measured fluorescence spectra of fluorescein imply a danger to confuse its fluorescence signal with that of urobilin or stercobilin and their zinc complexes. The excitation spectrum of fluorescein looks very similar to the faecal pigments' excitation spectra, apart from a small peak showing at 320 nm. Urobilin and stercobilin have such a small "pre-peak" at 360 nm excitation. Fluorescein's emission spectrum, on the other hand, looks similar to the emission spectra of the faecal pigments' zinc complexes.

5.5 Wastewater

This section will illuminate the question, whether the pigments can be found in wastewater samples taken from the WWTP screening as well as compare the different wastewater samples concerning faecal pigments. Samples of WWTP inflow and outflow from five different treatment plants were after reception and pH measurement first measured fluorometrically (EEM) and by HPLC-DAD-FLD. Some wastewaters, which showed a faecal-pigment-like fluorescence signal, were selected for enrichment. The enriched fractions were again measured with the Aqualog and also by coupling of HPLC-DAD, Aqualog and MS. The different wastewater treatment plants are referred to as WWTP A to E.

5.5.1 Wastewater treatment plant screening (not enriched)

The pH values of the sampled wastewaters are presented in Table 3. Measured pH values were between 6.8 and 8.4. The pH of WWTP E was however not measured on the sampling day, therefore it might have changed from the original values. Zinc concentration, obtained from a detailed element analysis, was between 0.023 and 0.097 mg/l. WWTP B's outflow had the lowest zinc concentration, while WWTP D's inflow had the highest zinc concentration.

Table 3. Overview over pH of the sampled wastewaters from the WWTP screening and approximate size of the treatment plants.

WWTP	pH inflow	pH outflow	zinc inflow (mg/l)	zinc outflow (mg/l)	size (people)
A	7.6	6.8	n.a.	n.a.	>500000
B	7.6	7.4	0.069	0.050	15000
C	7.7	7.6	0.065	0.023	5000
D	7.9	7.7	0.097	0.039	>500000
E	8.4*	8.1*	0.058	0.079	35000

***** *The pH of WWTP E was measured later and might not be representative for freshly sampled water.*

EEMs of all sampled wastewaters can be found in Appendix E. Example EEMs of a typical WWTP inflow and outflow are given in Figure 27. The maximum fluorescence intensity of the wastewaters was located below 300 nm excitation and between 300 and 400 nm emission, where protein-like fluorescence occurs (Coble, 1996). In the inflow, the maximum fluorescence intensity was much higher than in the outflow, which was to be expected as the outflow should be cleaner than the inflow.

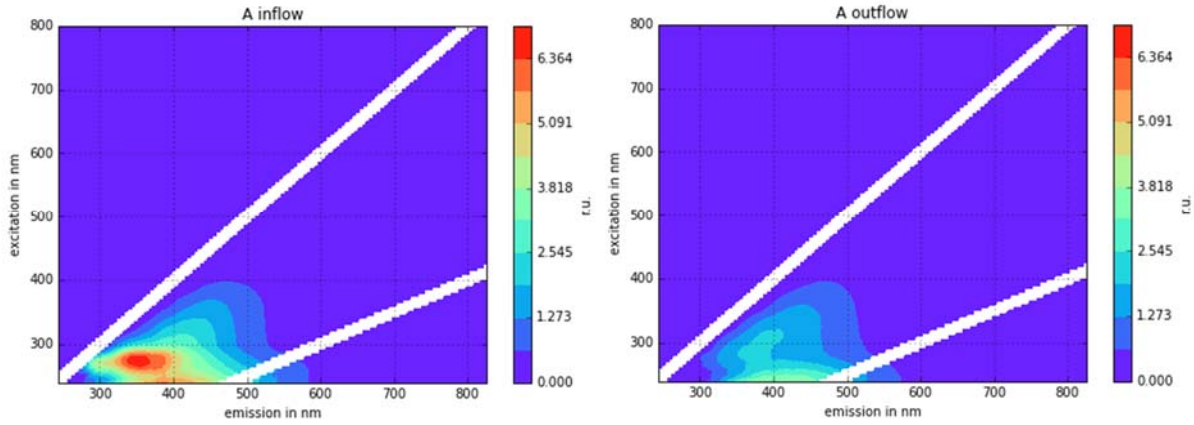


Figure 27. EEMs of inflow and outflow of WWTP A (r.u.).

The native wastewaters did, apart from WWTP D, not show any pigment-like fluorescence signal. The emission maxima were not at 500 nm as for faecal pigments, but at approximately 520 nm. Fluorescence at λ_{ex} 480 nm, which could be caused by faecal pigments or their complexes, seemed to be higher in the inflow of WWTPs A, C, and E than in the outflows, but the difference was not very pronounced and there was no clear peak, except from WWTP D (Figure 28). In the sample of WWTP B, the smallest of all plants, there was no visible difference between in- and outflow. WWTP D, however, was an exception as the in- and outflow both showed a much stronger signal in the faecal pigment region than the water of all other plants. It is as well remarkable that the signal of WWTP D in the faecal pigment region was higher in the outflow than it was in the inflow.

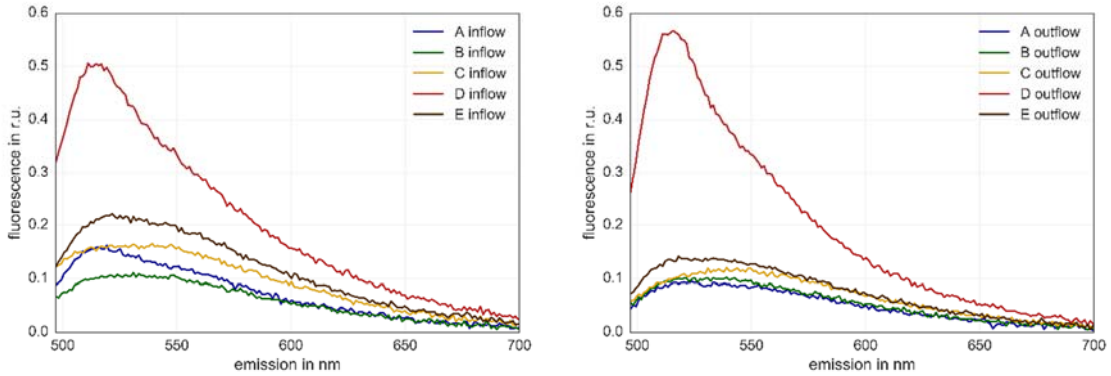


Figure 28. Emission spectra of WWTP inflows (left) and outflows (right) from the WWTP screening at λ_{ex} 480 nm.

WWTP D stood out from the other wastewaters as having a strong fluorescence signal at λ_{ex} 480 nm. The inflow of WWTP D also had the highest zinc concentration of the wastewaters, suggesting that a fluorescence intensification after zinc complexation might cause the strong fluorescence signal. However, WWTP D's outflow did not have a very high zinc concentration and still showed the strongest fluorescence signal. Therefore it is suspected that the zinc concentration was not the main reason for the difference in fluorescence intensity in the faecal pigment region between the wastewaters. The emission maxima were not at 500 nm, but at approximately 520 nm, which could be caused by a metal complex

with faecal pigments. Possibly, also a signal from other wastewater components might overlap with any faecal pigment signals.

The wastewaters after separation with HPLC are shown in Figure 29. Absorption at 490 nm and fluorescence at λ_{ex} 485 nm of the separated wastewater in- and outflows are shown. Stercobilin and urobilin are given as a comparison.

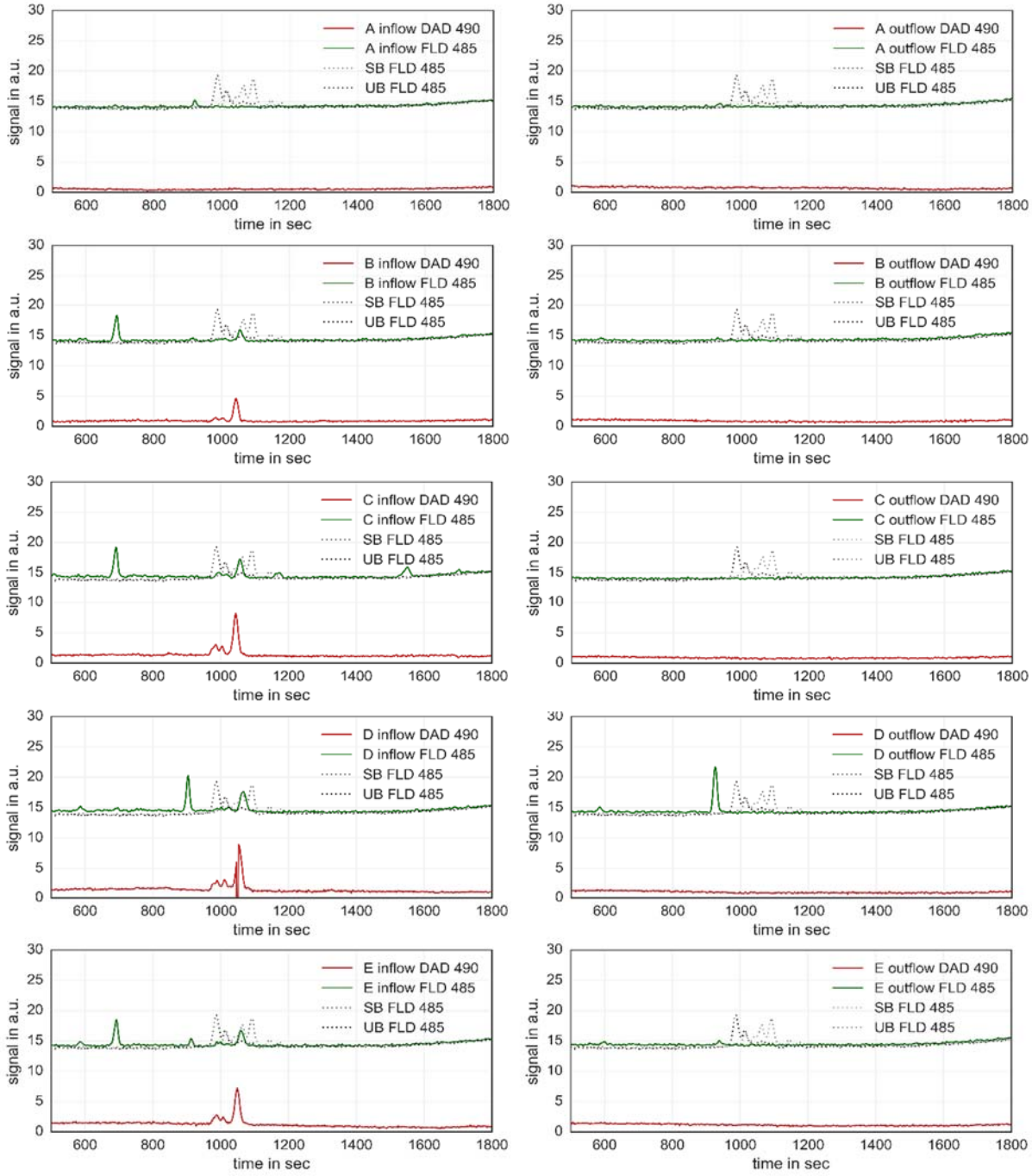


Figure 29. Chromatograms of WWTP inflows (left) and outflows (right), separated by HPLC (a.u.). UV-Vis absorption at 490 nm and fluorescence emission at $\lambda_{ex/em}$ 485/500 nm are given. For comparison, chromatograms of urobilin and stercobilin standards are shown.

Separation of the wastewaters with HPLC showed a more pronounced difference between WWTP in- and outflows than could be seen in their fluorescence spectra in Figure 28. Apart from the outflow of WWTP D, there was hardly any fluorescence or absorption signal at relevant wavelengths in the outflows. The outflow of WWTP D showed a strong fluorescence peak at slightly over 900 seconds, while there was no absorption peak. This peak could also be found in the inflow of WWTP D and – much smaller – in the inflows of WWTP A and E. Another fluorescence signal lacking an absorption signal appeared at 700 seconds in the inflows of WWTPs B, C, and E. WWTP C had a small fluorescence signal at over 1500 seconds. Apart from WWTP A, all WWTP inflows had three peaks, two very small peaks and one larger peak, between 980 and 1100 seconds, reflecting in the absorption as well as the fluorescence signal.

The substances causing the three fluorescence and absorption signals between 980 and 1100 seconds in WWTP inflows C, D, and E have a strong absorption, as well as fluorescence. Comparing the separated wastewaters with urobilin and stercobilin standards, separated with the same method, shows that these peaks might be caused by stercobilin and urobilin. This must, however, be confirmed. While the stercobilin standard, separated by HPLC, showed 5 different fluorescent isomers of stercobilin, there was only one peak resembling stercobilin in the wastewaters. It is suggested that this peak corresponds to the natural (-)-stercobilin. The separated urobilin standard showed only one main peak caused by urobilin.

The absence of any relevant fluorescence or absorption signal in the outflows suggested that no faecal pigments were present in detectable concentrations. Some fluorescence peaks were not reflected in the absorption measurement, which means that the substances causing them were probably present in very small concentrations only, but have a very high quantum yield. WWTP D's inflow and outflow had such a signal at approximately 900 seconds. The absence of an absorption signal and the fact that the fluorescence signal appeared at another time than stercobilin and urobilin suggests that the strong fluorescence in WWTP D's outflow, and also partly in the inflow, was not caused by urobilin or stercobilin.

5.5.2 Reaction of wastewater to zinc addition

There was an increase in fluorescence intensity and a red shift in all of the WWTP inflows when adding zinc. However, this strong increase was absent in the outflows. Even in the outflow of WWTP D, which exhibited a very high signal in the faecal pigment region, no intensity increase could be observed when zinc was added (Figure 30).

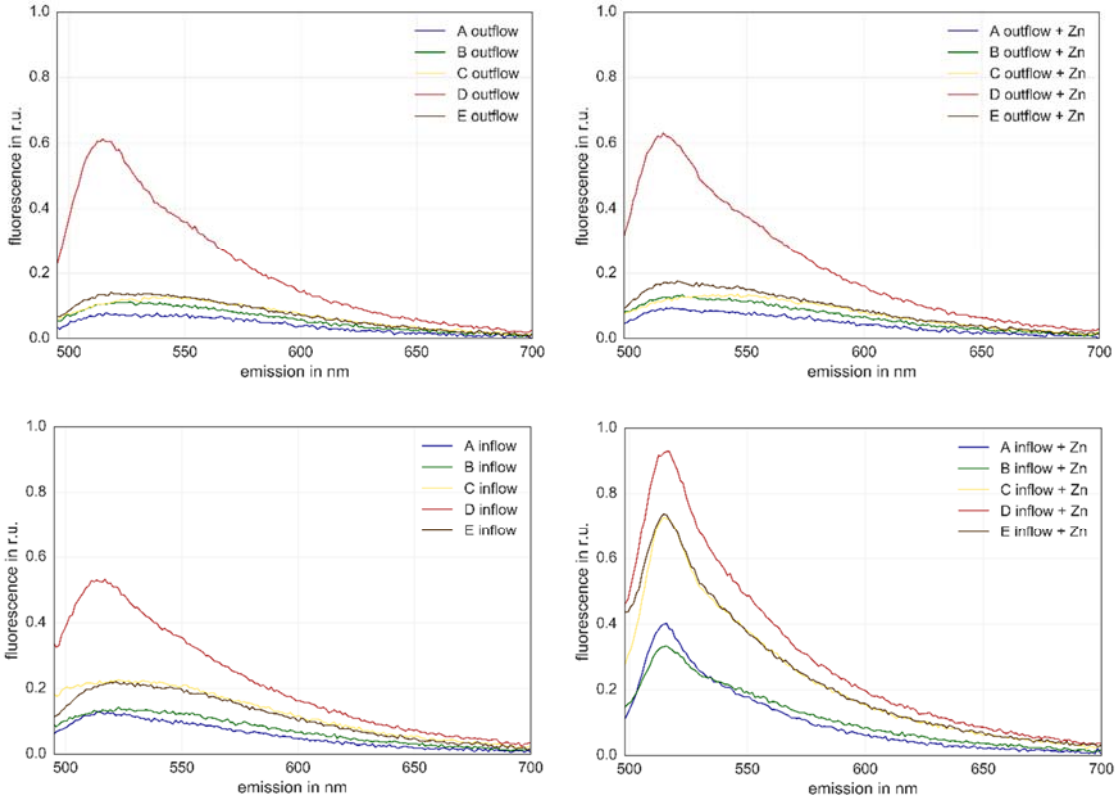


Figure 30. Fluorescence emission of the WWTP outflow (top) and inflow (bottom) in r.u., without (left) and with (right) addition of 20 mg/l zinc at λ_{ex} 480 nm.

At this point, together with the HPLC-results, it can already be concluded that the fluorescence signal in the outflow of WWTP D was very likely not caused by a faecal pigment, because it did not react to zinc addition. Although low concentrations of zinc had already been present in the native wastewaters, the wastewater fluorescence in the inflows responded to an addition of zinc at a high concentration. This increase in the WWTP inflows when adding zinc points strongly to the presence of urobilinoids. It is not known whether the gain in intensity would have been even stronger, if there had been no zinc in the water before the addition. As the time-dependency of the zinc reaction was not yet known at the time of the WWTP screening, the time between zinc addition and measurement was not recorded exactly and was approximately 1-2 hours in the case of the wastewater samples. Therefore, no quantitative evaluation of the fluorescence intensity increase in the WWTP inflows was possible. However, qualitatively, the increase in fluorescence intensity after zinc addition supports the presence of faecal pigments in the WWTP inflows.

5.5.3 Enriched wastewaters

Figure 31 shows EEMs of the eluates of enriched wastewater samples. There was an enrichment by a factor of 5 compared to the native wastewaters. The original eluates had an enrichment factor of 50, but for reasons of lacking sample amount they had to be diluted for the fluorescence measurements (4.2.4).

For a better overview, the figures are limited to the matrix section from 350 to 600 nm excitation and 400 to 700 nm emission.

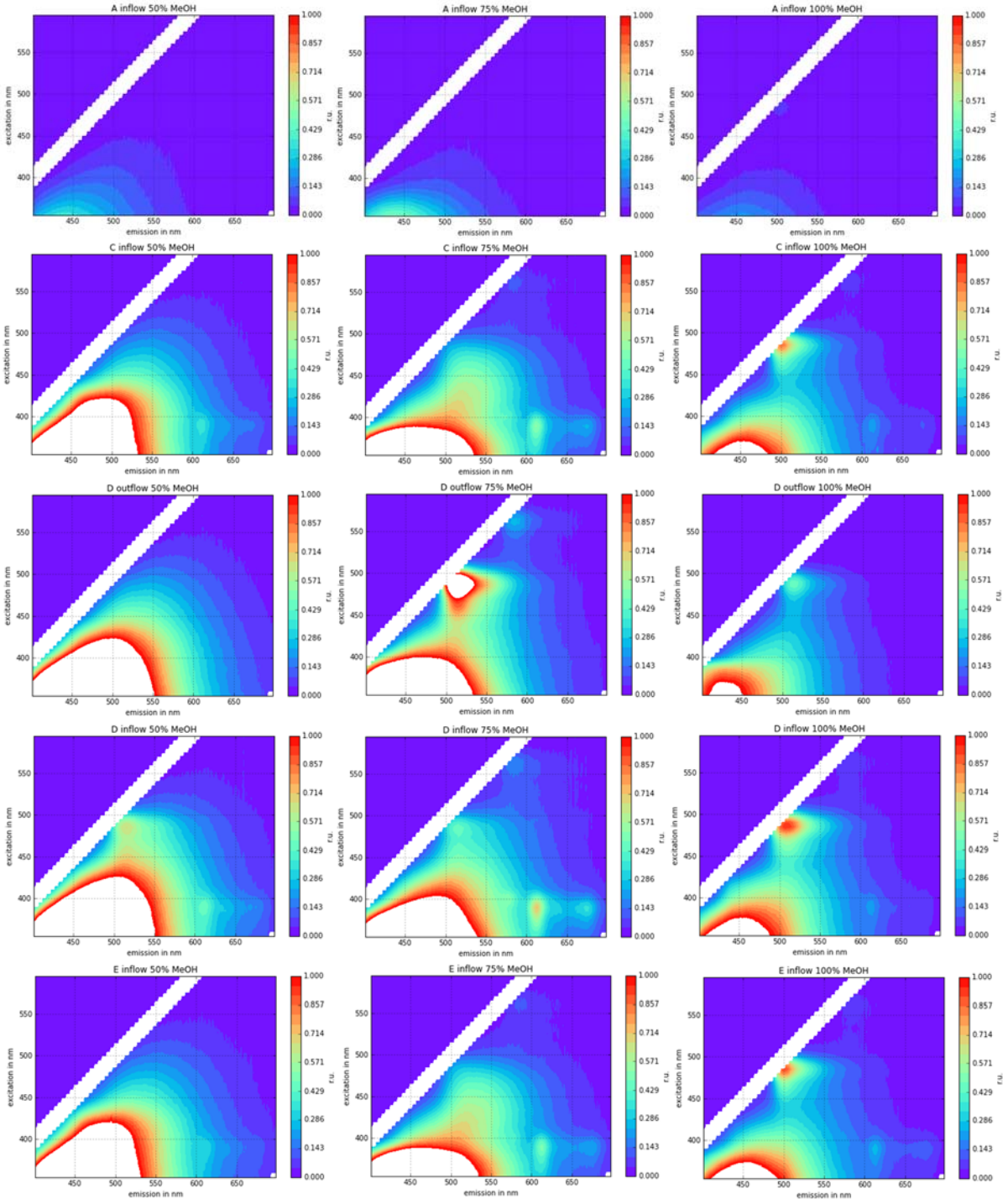


Figure 31. EEMs of selected enriched wastewater eluates from WWTPs A, C, D, and E (r.u.). From left to right: 50% MeOH, 75% MeOH, and 100% MeOH. Rayleigh scatter removed.

Compared to the other WWTPs, WWTP A showed a very weak signal in all fractions. The signal intensity in the region where faecal pigment fluorescence appears ($\lambda_{ex/em}$ 485/500 nm), seemed to increase from the 50% MeOH fraction to the 100% MeOH fraction in WWTPs A, C, and E, with the majority of faecal pigment-like fluorescence appearing in the 100% MeOH fraction. The 50% MeOH fraction (Figure 31, left column) contained least pigment-like signal, when comparing the different fractions, with the exemption of WWTP D's inflow that showed a very high signal. The 100% MeOH fraction of all WWTP inflows showed the highest pigment-like signal. WWTP D was, however, an exception. Its inflow showed a very high signal in the 50% MeOH fraction, while the outflow had a very high signal in the 75% MeOH fraction and only a smaller signal in the 100% MeOH fraction.

The 100% MeOH fraction of all WWTP inflows showed the highest pigment-like signal, which is an indication that faecal pigments were eluded in the 100% MeOH fraction. The exception of WWTP D indicates that the very high signal in the outflow of WWTP D, as well as parts of the signal in the inflow, are not caused by the same substance as the signal in the other WWTP inflows.

5.5.4 Coupling of HPLC-DAD, Aqualog, and MS

The EEMs of the enriched wastewaters showed relatively strong fluorescence in the faecal pigment region of the eluates of WWTP C inflow 100%, WWTP D outflow 75% and 100% MeOH, WWTP D inflow 50%, 75% and 100% MeOH, and WWTP E inflow 100% MeOH. In the following section, these enriched wastewater fractions are examined more closely by analysing the results from coupling of HPLC, Aqualog and MS. The evaluation process is shown exemplary for two eluates: WWTP C inflow 100% MeOH and WWTP D outflow 75% MeOH. Summarized results of all detailed wastewater analyses can be found in Table 6, while the reader is referred to Appendix F for a detailed analysis.

WWTP C inflow 100% MeOH

The fluorescence at an excitation wavelength of 465 nm in the 100% MeOH eluate of the inflow of WWTP C was composed mainly of stercobilin, appearing at 1045 seconds (m/z 595.4, λ_{em} max. at 500 nm; Figure 32; Table 4). The mass spectrum showed that stercobilin (m/z 595.4) was also the main component concentration-wise at 1045 seconds (Figure 33). Other contributors to the fluorescence at λ_{ex} 465 nm were urobilin (m/z 591.4, λ_{em} max. at 500 nm) at 1000 seconds, and another substance appearing at 905 seconds in the chromatogram with an emission maximum at 515 nm (Figure 32).

Table 4. Fluorescence of WWTP C's 100% MeOH eluate at λ_{ex} 465 nm.

	Time	Emission maximum	m/z
Peak 1 (highest)	1045 sec	500 nm	595.4
Peak 2	1000 sec	500 nm	591.4
Peak 3	905 sec	515 nm	unknown

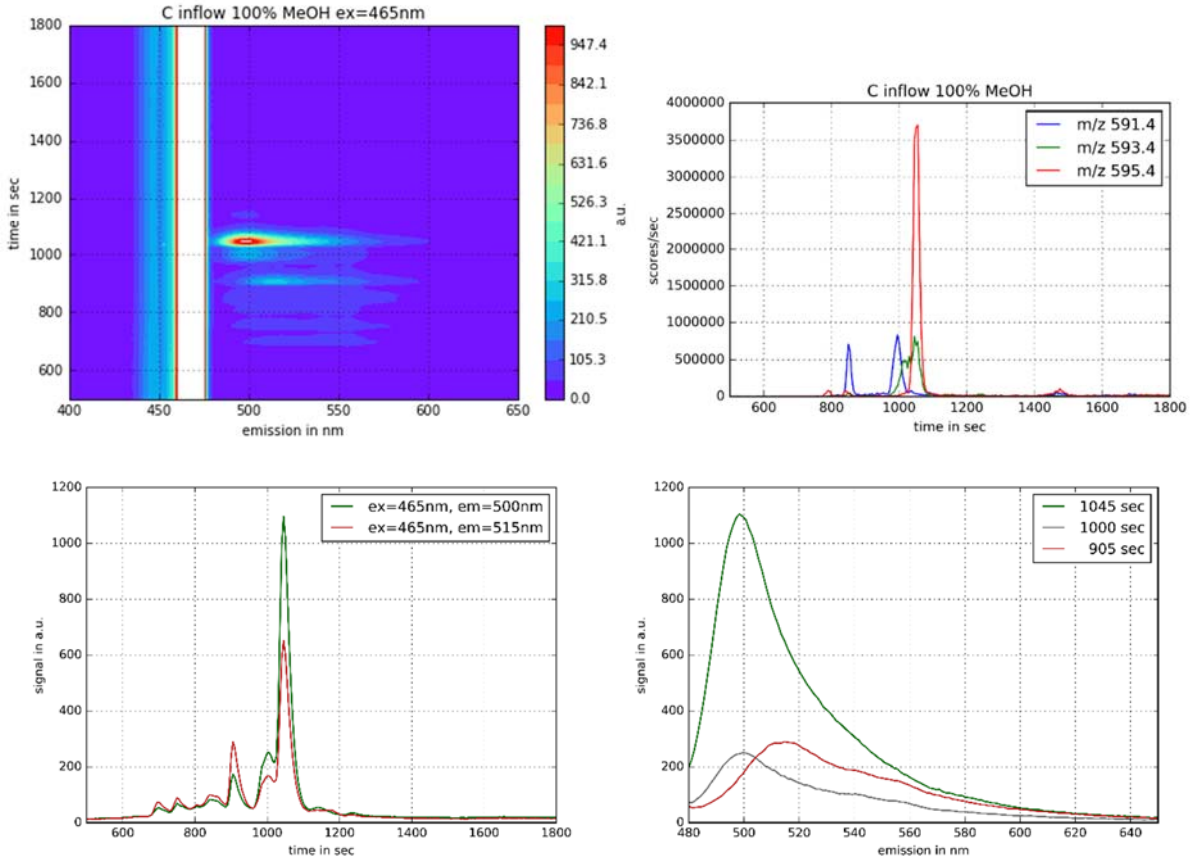


Figure 32. WWTP C eluate 100% MeOH; results from coupling of HPLC, Aqualog and MS. Top left (a): Matrix of fluorescence emission over time; top right (b): MS chromatogram; bottom left (c): fluorescence chromatogram; bottom right (d): fluorescence emission spectra at different times.

The fluorescence peak at 905 seconds was neither stercobilin nor urobilin, nor could it be identified as fluorescein by MS. It might however still be fluorescein in concentrations too low to be detected by MS. MS also showed a peak with m/z of approximately 700 at 905 seconds (Figure 33). It is not known whether this mass belongs to a fluorophore and it was not examined any further.

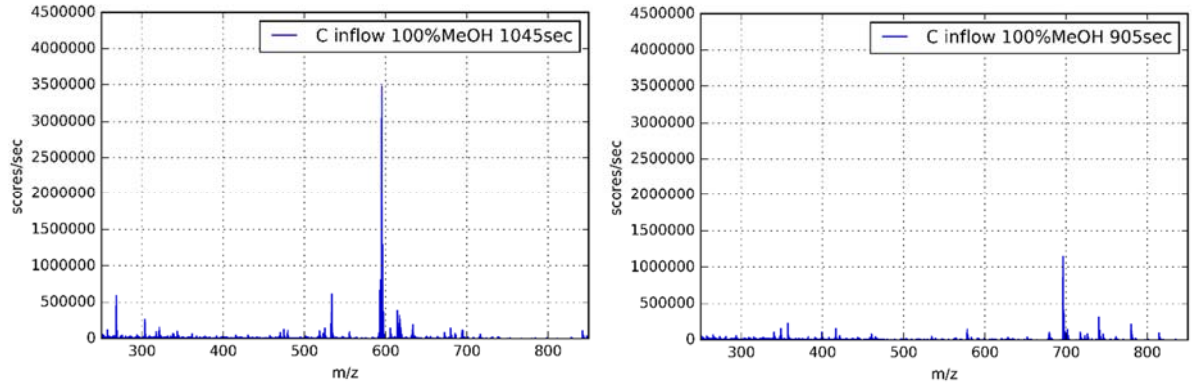


Figure 33. Mass spectrum of the eluate WWTP C inflow 100% MeOH at 1045 sec (left) and 905 sec (right).

WWTP D outflow 75% MeOH

In the 75% MeOH eluate of WWTP D's outflow, a fluorescence maximum at 880 seconds and 515 nm emission was observed at λ_{ex} 465 nm (Figure 34, Table 5).

Table 5. Fluorescence of WWTP D's 75% MeOH eluate at λ_{ex} 465 nm.

	Time	Emission maximum	m/z
Peak 1	880 sec	515 nm	333

It is very likely fluorescein (m/z 333) that caused this fluorescence peak. Fluorescein was only present in small concentrations (100000 counts, Figure 35), but can still cause a high fluorescence intensity due to its high quantum yield (Sjöback *et al.*, 1995). The eluate's mass spectrum at 880 seconds showed, apart from fluorescein, some unidentified peaks with an m/z between 400 and 600 (Figure 35).

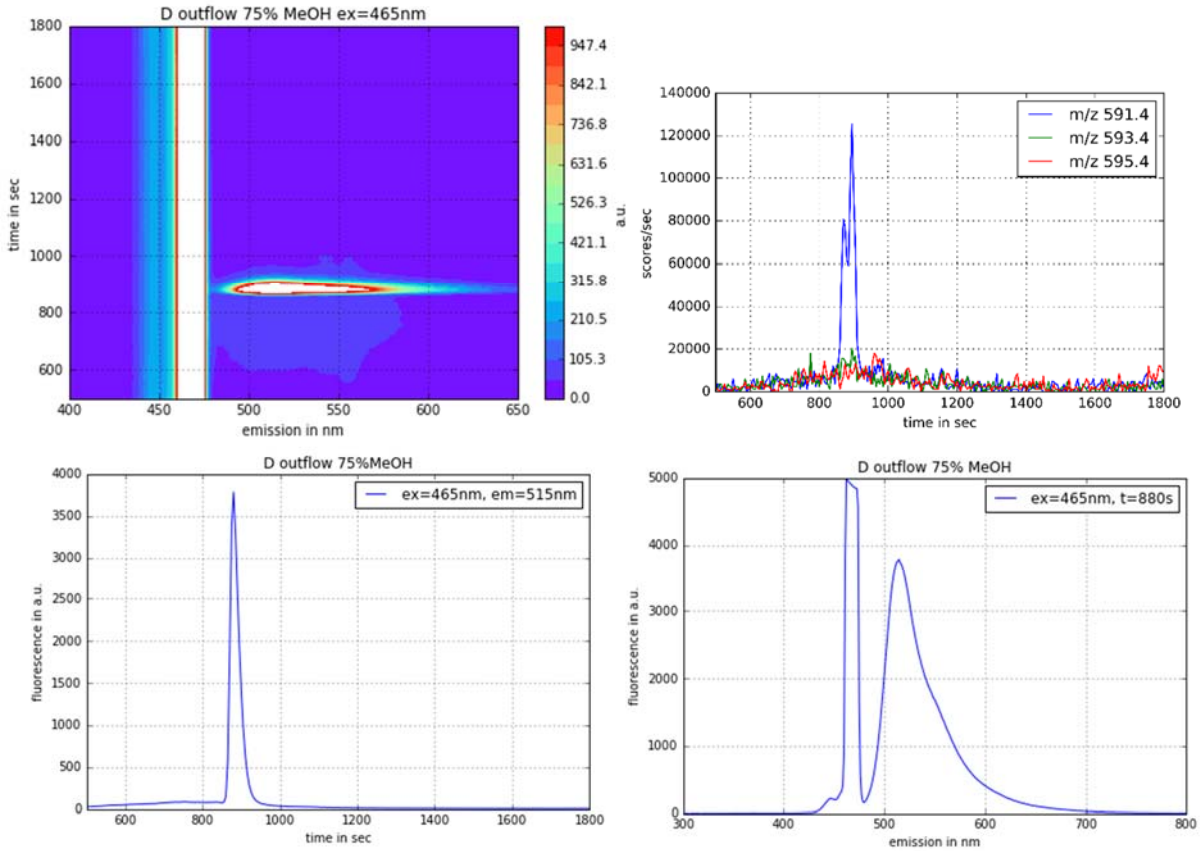


Figure 34. WWTP D eluate 75% MeOH; results from coupling of HPLC, Aqualog and MS. Top left (a): Matrix of fluorescence emission over time; top right (b): MS chromatogram; bottom left (c): fluorescence chromatogram; bottom right (d): fluorescence emission spectrum at 880 sec.

Faecal pigments were not observed in concentrations relevant for the fluorescence of this eluate (Figure 34 a, c). They would have appeared later in the chromatogram. Urobilin, stercobilin and urobilinogen were not detected by MS (Figure 34 b). A compound with m/z 591.4, the same as for urobilin, appeared at approximately 900 seconds, which was too early to be urobilin, but could be a substance structurally related to faecal pigments (Figure 34 b).

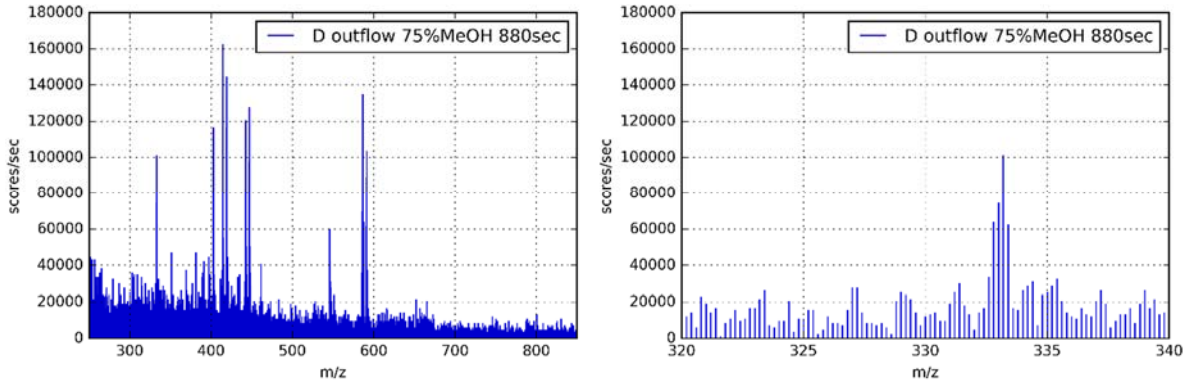


Figure 35. Mass spectrum of the eluate WWTP D outflow 75% MeOH at 880 sec. Left: whole spectrum from m/z 250 to m/z 850. Right: Section between m/z 320 and m/z 340.

In Table 6, the results of the coupling between HPLC, Aqualog and MS are summarized.

Table 6. Stercobilin, urobilin, fluorescein, and other relevant signals in the wastewaters, identified by coupling of HPLC, Aqualog and MS. Timing of peaks in HPLC and fluorescence emission maxima are shown.

	Stercobilin (m/z 595)	Urobilin (m/z 591)	Fluorescein (m/z 333)	Unidentified relevant fluorescence signals
C in 100	1045s/500nm	1000s/500nm		905s/515nm
D out 75			880s/515nm	
D out 100			880s/515nm	
D in 50				950s/513nm
D in 75				905s/515nm
D in 100	1050s/500nm	1000s/500nm		895s/515nm
E in 100	1040s/500nm	1000s/500nm		875s/515nm

The detailed analysis of the enriched wastewater fractions (Appendix F) showed that

- Urobilin and stercobilin were present in the examined WWTP inflows
- Faecal pigments were eluded in the 100% MeOH fraction.
- Wastewater fluorescence at λ_{ex} 465 nm was in most of the examined waters composed by several substances
- Stercobilin was, of the studied pigments, the dominant faecal pigment in WWTP inflow
- Fluorescein caused a large part of WWTP D's fluorescence in the faecal pigment region
- Fluorescein was difficult to detect by MS and not detectable with DAD in the UV absorption, because its concentration was so low.

In the examined wastewaters, the dominant detected pigment was stercobilin. Miyabara *et al.* (1994b) often found mainly urobilin in the environment, which is surprising, as much more stercobilin is excreted by the body compared to urobilin (Orten, 1971) and stercobilin is not considered to be less stable than urobilin. In the present study, the stability of both pigments seemed to be similar, with a trend of urobilin degrading faster in HOWI and wastewater (4.2.8), but this needs to be confirmed. According to Bull *et al.* (1981), urobilin should be expected to be less stable towards oxidation than stercobilin.

WWTP A showed a very weak fluorescence signal when separated with HPLC as well as in the enriched fractions, but it reacted to zinc addition. The water from WWTP A was three weeks old at the point of the HPLC analysis and enrichment, while the fluorescence EEM and zinc addition measurements were done when the water was freshly sampled. This time difference is probably the reason why there was hardly any fluorescence signal in the HPLC separated water as well as in the enriched fractions, while the faecal pigment fluorescence reacted strongly to zinc addition. Additionally, it was one of the first water samples received (Appendix D), and an earlier, not yet optimized enrichment technique, differing from the technique used for the other wastewaters, was used.

For all of the wastewater samples, the fluorescence measurements of the pure wastewaters were taken directly after the samples were obtained. HPLC-DAD measurements were done one week after that. The enrichment took some additional time, so the enriched samples were measured even later and it is possible that a part of the pigment in the samples was biologically degraded over that time (Appendix D). The potential degradation can be estimated from the stability results of urobilin and stercobilin observed in wastewater (5.2.3), where under cool and dark storage conditions, urobilin fluorescence was only approximately 50% of the initial value after one week, and stercobilin fluorescence was at 75% of the original value. Assuming that this fluorescence loss reflects the pigment degradation, only a part of the originally present faecal pigments were still present in the wastewaters at the time of HPLC and MS analysis. It is possible that the wastewaters initially contained a higher pigment concentration than the one detected by the reference methods, because some pigment might have been degraded in the time between sampling and analysis.

WWTP D stood out as different from the water of the other plants, as it had a high fluorescence signal in the faecal pigment area already in the pure water of inflow as well as outflow. It did, however, not react to zinc addition, indicating an absence or a very low concentration of faecal pigments. Already after separation of the pure wastewaters with HPLC and zinc addition to the samples, it became clear that the signal in WWTP D was caused by something different than what caused the signal in the other wastewaters. By the use of coupling Aqualog, HPLC and MS, an identification as fluorescein was possible. Fluorescein could also have been present in other wastewaters, but if it was, the concentration was too low for an identification with MS.

At the first glance, it does not seem logical that there is much more fluorescein in the outflow of WWTP D than in the inflow, resulting in the outflow having a stronger fluorescence signal. However, the samples of inflow and outflow were taken at the same time. It is possible that fluorescein was a one-time occurrence and had almost completely passed the treatment plant already in the treated water, while there was not so much in the untreated water anymore, if new water without or with less fluorescein entered the treatment plant. To confirm this, more samples from all plants need to be taken in regular time intervals and it has to be excluded that the plant uses fluorescein as a marker in the treatment process.

When comparing wastewaters from the WWTP screening before and after zinc addition, an excitation wavelength of 480 nm was chosen for both measurements, before and after zinc addition. With a higher excitation wavelength Rayleigh scatter was so strong that a part of the spectrum was lost. At the time of the WWTP screening, the time dependency had not yet been discovered and therefore also not the increase in scatter connected to the time after zinc addition. It should, with new samples, however be possible to repeat the experiment, measure 1 minute after zinc addition and get less scatter which should make the use of an optimal wavelength for the pigment-zinc-complex excitation (e.g. λ_{ex} 495 or 500 nm) possible. Both, an earlier measurement and a more optimal excitation wavelength, will likely result in a higher gain factor.

The WWTP inflows differed from the outflows in faecal pigment detectability. While faecal pigments could be detected by fluorescence spectroscopy after zinc addition, as well as with HPLC-DAD-FLD and MS in the inflows, no faecal pigments were detected in the outflows. As the lacking MS (only WWTP D) and HPLC-DAD signals of the WWTP outflows showed, not only the fluorescence properties of the pigments were altered, but the pigments were not present in detectable concentrations. This points to a loss of the pigments during the wastewater treatment. Detection limits for HPLC-DAD and MS were, however, not determined. If faecal pigments cannot be detected in WWTP outflow, pollution detected e.g. in a river is likely not caused by the treated wastewater discharge, but from either untreated WWTP overflow or direct faecal pollution. However, MS analysis was only done for the outflow of WWTP D, because it was the only outflow with a fluorescence signal in the faecal pigment region. It is possible that faecal pigments were present in the WWTP outflows as well, but in concentrations not recognizable by fluorescence detection and HPLC-DAD. There was hardly any reaction of the outflows to zinc addition (5.5.2), which leads to the conclusion that there was no or only a very small concentration of faecal pigment present in the outflows already when the wastewater was sampled. An additional degradation possibly took place in the time between the sampling and measurement with HPLC-DAD.

The loss of urobilin in wastewater treatment plants is supported by several studies (Miyabara *et al.*, 1994d; Loganathan *et al.*, 2009; Mowery and Loganathan, 2007). Loganathan *et al.* (2009) determined urobilin in wastewater in- and outflow in Kentucky, U.S., with HPLC-ES-MS and documented a urobilin removal efficiency of 99.9% during wastewater treatment and no detectable or very low concentrations (<37 ng/l) of urobilin in WWTP outflow. Different results came from Miyabara *et al.* (1994d), who detected on average 0.71 µg/l urobilin in outflows of WWTPs and septic tanks and 0.59 µg/l in river water in Japan with HPLC-FLD. They concluded that WWTP outflows were the major source of urobilin in aquatic environments. An explanation for the different results in literature might be a more effective wastewater treatment in modern plants with a higher removal efficiency. More studies are needed about faecal pigment concentrations in treated and untreated wastewater.

The results of the stability tests with urobilin and stercobilin in wastewater implied that the time needed for the water treatment, in combination with a very high microbial activity, will likely cause the degradation of faecal pigments during the treatment process. Unfortunately, the treatment plants did not provide details about their chemical water treatment, but apart from microbial degradation, an additional effect of flocculation agents on the pigments is also possible. As urobilin can bind to sediment, it is also possible that faecal pigments bind to particles and are removed with the sludge (Miyabara *et al.*, 1994a). Miyabara *et al.* (1994d) however suggested that urobilin adsorption to sludge was of minor importance, while microbial degradation during activated sludge process occurred. They documented the degradation of urobilin and stercobilin standards during different wastewater treatment steps as well as of the pigments in wastewater and suggested that the degradation occurred by aeration, bacterial degradation, or free chlorine treatment (Miyabara *et al.*, 1994d).

There are many studies, in which wastewater from only one WWTP is used, which is then generalized to "wastewater" in general. As it was shown with the different wastewaters from the WWTP screening, wastewaters can be quite different (even if they are all from one country or region) and generalization should be done with care. It would be good to have more wastewater data and compare wastewater between regions and maybe even countries to secure these results. This is especially necessary as the technique is supposed to be useable in development countries where wastewater and wastewater treatment might look very different from Central Europe.

5.6 General Model

Until this point, pigment standards, their zinc complexes and real waters were measured and characterized with fluorescence spectroscopy. With the help of HPLC and MS, the presence of faecal pigments in several wastewater samples could be verified. The goal was, however, to detect and quantify faecal pigments with fluorescence techniques only (e.g. from an Aqualog EEM), without the help of HPLC and MS and without the need for an enrichment. Therefore, a PARAFAC model was developed to evaluate the fluorescence data. The aim was to create a model, which would be applicable to any fluorescence data belonging to the different types of water used in this study, and therefore generalizable. It should be able to recognize and quantify faecal pigments in any given EEM from a real water sample. Apart from that, the model was intended to help in understanding the fluorescence components of the examined wastewaters.

5.6.1 Model fitting and evaluation

The model was fitted with Aqualog EEMs of stercobilin, urobilin, and urobilinogen standards in different media, as well as drinking- and wastewater samples. Fluorescein standard data was added as well. First, representative samples of all pigment standards as well as real waters were chosen. A list of the sample data used for modelling can be found in Appendix G. The EEMs of the samples were normalized over the Raman peak intensity to be comparable and prepared for modelling by removing Rayleigh scatter. Outliers and samples, which had a big influence on the model, but were described unsatisfyingly, were identified and excluded from the model. As the intensity of the wastewater fluorescence at lower wavelengths, probably caused by amino acids either free or bound in proteins or bacterial cell walls (Hudson *et al.*, 2007), was overwhelming and strongly influenced the model, all excitation wavelengths below 300 and above 600 nm, as well as all emission wavelengths below 450 and above 700 nm were removed. The resulting matrices thus had a stronger focus on the region where faecal pigment fluorescence appears. After the pre-processing, a PARAFAC analysis with 1 to 10 components was performed. During modelling, data was normalized to avoid higher influence of more intense matrices, and re-normalized afterwards. For choosing the right model, the different models were compared with each other, the lack of fit, as well as the sum of squared error (SSE) were evaluated and it was checked whether some samples or wavelengths still had a high influence on the model, while not being sufficiently explained by it. Core consistency was not taken into account, because it can be misleading when analysing real world data and result in an underestimation of model compounds (Murphy *et al.*, 2013). In this case, core consistency was negative for all models with more than 5 components (Figure 36), meaning that all models with more than 5 components should not be chosen because of an interdependency of the components.

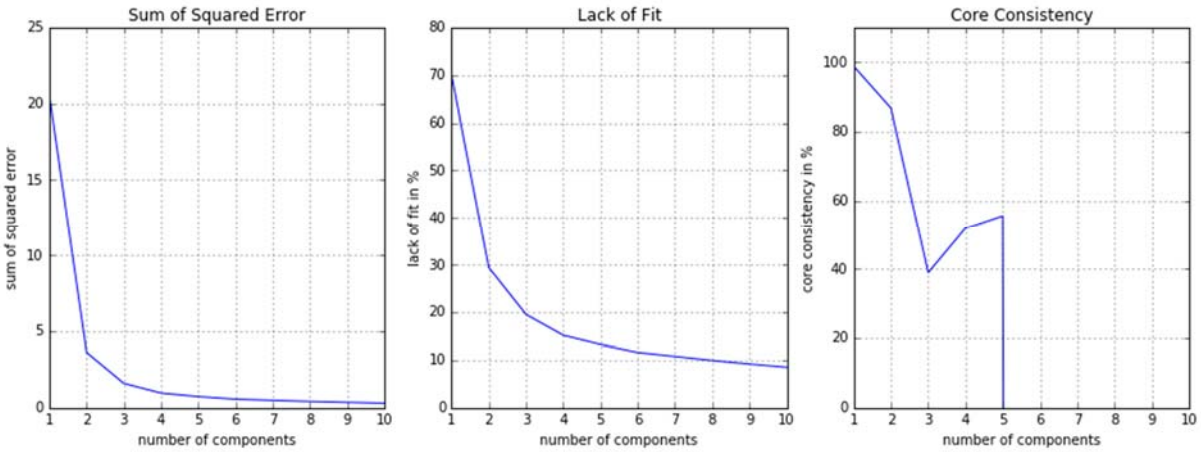


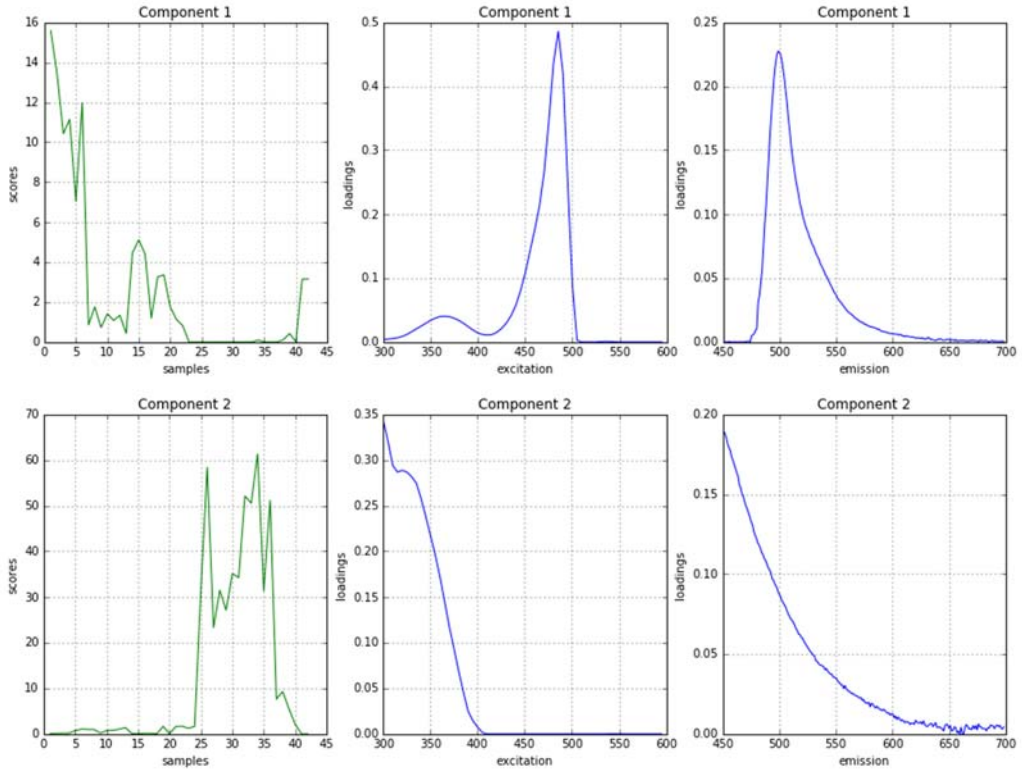
Figure 36. SSE, Lack of fit and Core consistency of different calculated PARAFAC models with 1 to 10 components.

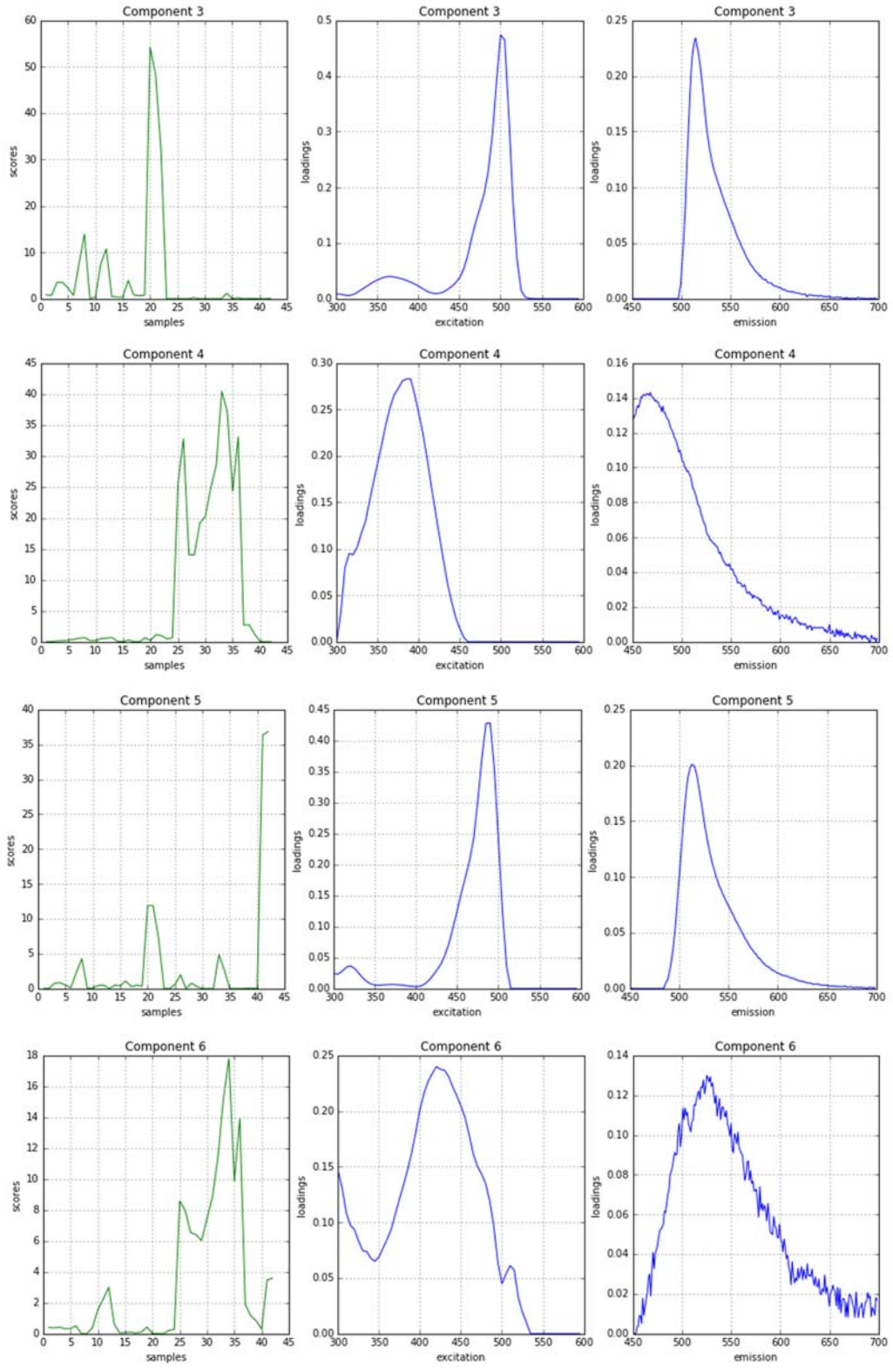
Lack of fit decreased substantially with every added component until component 4. A number of 4 components could explain approximately 85% of the data. After that, the addition of every new component only lead to a small improvement (Figure 36).

Core consistency was negative for all models with more than 5 components. Five components were, however, not considered to explain the data sufficiently well, because they did not represent all real components present in the examined waters. An addition of more model components lead to a better explanation of more real water constituents. As core consistency likely underestimated the real number of components and there was no substantial difference in the lack of fit between the models with a higher number of components, the final model was chosen by comparing the different models with each other and examining the plausibility of their components.

5.6.2 Components

Of all calculated models with between 1 to 10 components, a model with 8 components was found to describe the chosen matrices best. In Figure 37 the model components are shown. The figure in the middle shows the excitation spectrum, the emission spectrum is visible to the right, and on the left figure are the scores per sample, telling in which samples this model component was present and in what intensity. Components 2, 4, and 6 describe wastewater fluorescence. Stercobilin and urobilin could not be separated by the model, as the spectra looked identical. Component 1 therefore represents the faecal pigment fluorescence, without specification which of both pigments. Component 3 shows the pigment-zinc-complex, again for both pigments. Component 5 shows fluorescein, and component 8 depicts the double peak at $\lambda_{ex/em}$ 385/620 nm and 385/680 nm, which could be found in the urobilinogen standard, as well as in many wastewaters. Component 7 shows some noise, but could depict one component in the stercobilin standard, appearing at $\lambda_{ex/em}$ 540/560 nm.




Component 3
scores
samples
Component 3
loadings
excitation
Component 3
loadings
emission
Component 4
scores
samples
Component 4
loadings
excitation
Component 4
loadings
emission
Component 5
scores
samples
Component 5
loadings
excitation
Component 5
loadings
emission
Component 6
scores
samples
Component 6
loadings
excitation
Component 6
loadings
emission

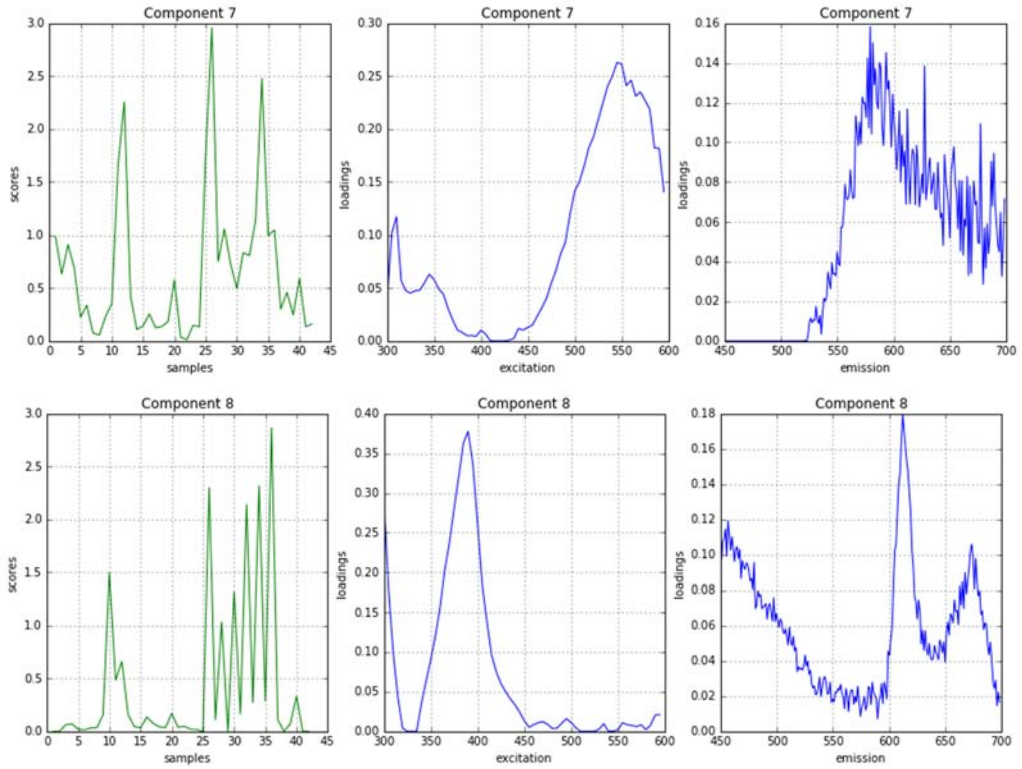


Figure 37. Spectra of the general model.

The model was able to distinguish between the pigment, the pigment complex and fluorescein. Especially the distinction between the pigment(-complex) and fluorescein is remarkable. It was probably possible due to the different minor peaks of the two substances appearing at different wavelengths. While the main peaks of fluorescein and the pigments as well as the zinc complexes are very close together, the pre-peak of fluorescein is at $\lambda_{ex/em}$ 320/500 nm while the pre-peak of the pigments is at about $\lambda_{ex/em}$ 360/500 nm and $\lambda_{ex/em}$ 360/513 nm. For a practical application this means that there is less risk that fluorescein traces in the water are misinterpreted as faecal pigments.

5.6.3 Application of the general model to the wastewater data

Figure 38 shows the application of the model to the data it was trained with as well as related data (EEMs of enriched wastewater eluates). The model was to a certain degree capable of detecting the pigment-zinc complex and fluorescein in the pure wastewater samples without prior enrichment (Figure 38, left). It was, however, not able to reliably detect the pure pigment, if its concentration was too low, as was the case in non-enriched wastewater (Figure 38, left, upper part). Then also pure pigment and zinc complexes could be confused. If the concentration was higher, like in the enriched wastewater samples, the model was able to recognize and distinguish between the pure pigment (urobilin/stercobilin), the pigment-zinc complex, and fluorescein (Figure 38, right). It recognized the pure pigment compound in the 100% MeOH eluates of WWTP C, D, and E inflow.

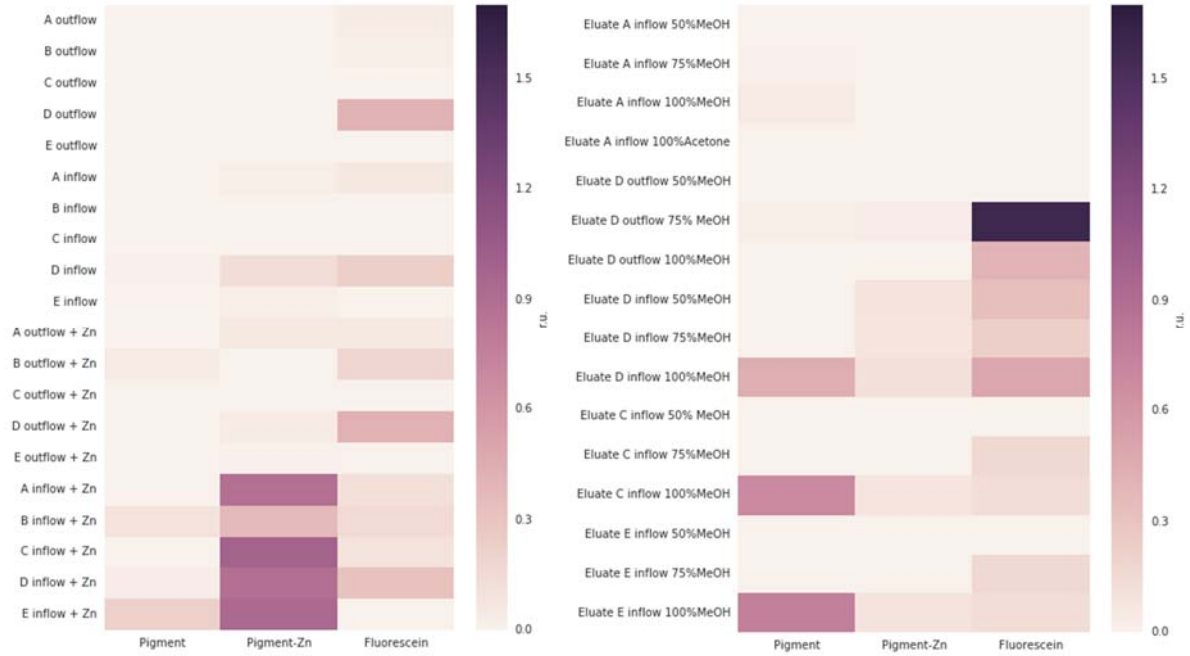


Figure 38. Modelled intensities of pigment, pigment-zinc complex and fluorescein in wastewaters in r.u. (without zinc, with zinc, and in eluates after enrichment).

The model reflected the differences in pigment concentration between WWTP inflow and outflow, if zinc was added (Figure 38, left) and for WWTP D after enrichment (Figure 38, right). Other outflows were not enriched, therefore only the eluate of WWTP D's outflow was available for comparison. Also, the model recognized the absence, or very low concentration, of faecal pigments in eluates with 50% MeOH and 75% MeOH. Fluorescein in WWTP D's outflow with 75% MeOH was described and, according to the model, the fluorescence in WWTP D's inflow 100% MeOH was partly comprised of both faecal pigment and fluorescein.

If calibration curves for the pigments and complexes are established, it is only a small step to calculate concentrations from the fluorescence intensity per component in a sample. The faecal pigment fluorescence intensities calculated by the model from the fluorescence EEMs of enriched wastewaters (eluate 100% MeOH) were transferred to concentrations. For comparison, concentrations of urobilin and stercobilin in the pure wastewaters were also determined by HPLC-DAD. The concentrations calculated by

the model were then compared with the HPLC data to evaluate the performance of the model (Table 7). For the transformation from fluorescence intensities to concentrations, calibration curves of urobilin and stercobilin standards in phosphate buffer were used. As the slope of the calibration curves was different for stercobilin and urobilin, the total pigment concentration is given as a range (Table 7). According to HPLC and MS, most of the pigment in the studied wastewaters was stercobilin. Using the stercobilin calibration equation would therefore be reasonable as well. However, if only fluorescence spectroscopy without HPLC is used, the pigment composition is not known and one can only get a range of possible concentrations.

Table 7. Comparative overview about pigment concentrations calculated (1) by HPLC and (2) by the fluorescence model in combination with a calibration in phosphate buffer.

	Urobilin (mg/l)	**Stercobilin** (mg/l)	**Total** (mg/l)
B inflow			
HPLC	<0.01	0.05	0.05-0.06
Model			-
C inflow			
HPLC	0.02	0.1	0.12
Model			0.09-0.12
D inflow			
HPLC	0.02	0.12	0.14
Model			0.06-0.08
E inflow			
HPLC	0.02	0.08	0.1
Model			0.1-0.13

In the inflow of WWTPs C and E, the estimation of the model agreed well with the HPLC measurement. The concentration of faecal pigments in WWTP D inflow was underestimated by the model (Table 7).

The evaluation shows that the general model works relatively well, but has some difficulties in distinguishing between the pure faecal pigments and their zinc complexes, if the pigment concentration, and with that the fluorescence signal, is not high enough. The reason for this is likely that the peaks and

the position of their maxima are not as well defined at low concentrations as they are at higher concentrations. For a practical application in wastewater, this means that either zinc should be added, or prior enrichment is necessary to reliably identify faecal pigments. If there is an enrichment, the pure pigment can be detected as well (Figure 38, right). The underestimation of the faecal pigment concentration in WWTP D's inflow shows that there might still be some difficulties in distinguishing exactly between fluorescein and faecal pigments, if both are present (Table 7).

Furthermore, the model needs to be tested and evaluated on more data. The model was trained with data from the WWTP screening. It has to be evaluated with more samples, which are unknown to the model but of which the actual composition is known. A problem is that the model tries to explain unknown fluorescence signals with known components. If a compound in a dataset does not fit exactly to the known compounds of the model, the model still tries to explain this fluorescence with known compounds, instead of giving the result that some parts of the data cannot be explained. This can lead to wrong concentration estimations, if there is a compound in the water which is similar to a compound described by the model, but not the same.

To get an even more precise separation of the compounds in the faecal pigment region, the excitation steps could be even smaller (1 nm instead of 5 nm) and the matrix recorded by the fluorescence spectrometer could be restricted to the range selected for creating the model (5.6.1) to avoid unnecessary computation effort. For an on-line sensor it would be good to have only one or two relevant wavelength positions to observe. However, in the case of faecal pigment quantification, the underlying wastewater or drinking water fluorescence signal has to be removed to isolate the signal caused by faecal pigments. This procedure avoids an overestimation of the faecal pigment concentration caused by other fluorescent substances in the water, which add up to the total fluorescence signal in the faecal pigment region. This separation can only be achieved if enough data is present for a model to recognize different water components. Also, the pigments, their zinc complexes, and fluorescein are very similar in their fluorescence signal and distinguishing between them would very likely not work with one or two wavelength combinations only. However, recording a whole EEM, like it was done here, requires much computational effort and time. It has to be tested, if a combination of the peaks at $\lambda_{ex/em}$ 485/500, 500/513, 360/500, and 360/513 nm (for faecal pigments and their zinc complexes), as well as 490/510 and 320/510 nm (fluorescein) would work as representative peaks to recognize faecal pigments or their zinc complexes and also avoid false positive errors by fluorescein. Otherwise, instead of recording the fluorescence intensity at some wavelength combinations only, whole emission spectra at certain excitation wavelengths can be recorded.

It might be rewarding to examine two other signals appearing in most of the wastewater matrices at $\lambda_{ex/em}$ 385/620 and 385/680 nm as well as in the urobilinogen standard. They are far away from other disturbing signals and are depicted by the model as a relevant component. These signals might be caused by compounds structurally related to urobilin and stercobilin, but could also result from something different. If they are caused by a faecal pigment, this signal could be chosen as a reference wavelength to create a more robust model.

6 Concluding Discussion

6.1 Summary

First, pigment standards were characterized by fluorescence (EEM), HPLC-DAD-FLD and coupling of HPLC, Aqualog and MS. They showed some pollution, which was suspected to be caused by degradation products of faecal pigments. Urobilin and stercobilin had alike-looking fluorescence spectra and could thus not be distinguished from each other by fluorescence spectroscopy. Urobilinogen itself is not fluorescent (Watson, 1969), however, its standard showed, due to low purity, several fluorescence signals.

With the pigment standards, stability tests were performed. Urobilin and stercobilin fluorescence was stable in phosphate buffer but not in real water. It was concluded that the stability was likely influenced by microbial degradation. Fluorescence in the faecal pigment region of the urobilinogen standard increased over time, indicating an oxidation of urobilinogen to urobilin.

Calibration series of urobilin and stercobilin standard were measured in phosphate buffer, drinking water, WWTP outflow, and inflow. Urobilin and stercobilin seemed to have different fluorescence intensities at the same concentration, but further studies need to confirm this. The media also influenced the fluorescence intensity, and with that, the slope of the calibration curve. Urobilin as well as stercobilin were quenched in real waters compared to phosphate buffer. Differences in pH value were suspected to be the cause. To confirm this, calibration series need to be repeated with waters of known pH value and pH needs to be measured before and after pigment standard addition. The limit of detection determined from the calibration was 3µg/l for stercobilin and 2 µg/l for urobilin.

The influence of pH on faecal pigment fluorescence was tested with urobilin and stercobilin in phosphate buffer solutions of pH 6.6 and pH 8.5, containing the same ingredients in different composition. The pigments showed a lower fluorescence at pH 8.5, compared to pH 6.6. Location of the excitation and emission maxima remained unchanged. In the case of zinc addition to the pigments, the reaction was the opposite way. Fluorescence of the zinc complexes was stronger at a pH of 8.5 compared to a pH of 6.6. Together with results from literature, it was concluded that pH has a substantial influence on the fluorescence intensity and has to be monitored when performing fluorescence measurements.

When zinc acetate was added to urobilin or stercobilin in water, an intensification of the fluorescence and a red shift of their fluorescence spectra from $\lambda_{ex/em}$ 485/500 to $\lambda_{ex/em}$ 500/513 nm was observed. The intensification happened immediately after zinc addition and fluorescence decreased afterwards. This time dependency was, according to what was found in literature, not observed before. To get a maximum intensification effect, samples should be measured as soon as possible after zinc addition. The gain factor upon zinc addition, as well as the velocity of the relative decrease afterwards, depended on the zinc concentration. A high concentration lead to a high fluorescence intensity, but also to a fast relative loss afterwards. Gain factors of >30 were observed, which can be useful in detecting low concentrations of faecal pigments.

An examination of the fluorescent marker fluorescein (present in the form of uranine, a fluorescein sodium salt) showed that its fluorescence spectra looked very similar to urobilin and stercobilin and those of their zinc complexes. There is a danger to confuse fluorescein, and potentially also other substances, with faecal pigments when using fluorescence spectroscopy, resulting in a false positive.

To find out whether faecal pigments could be detected in wastewater by fluorescence spectroscopy, as well as to characterize the wastewater fluorescence in the faecal pigment region, samples of 5 WWTPs were taken. They were, like the pigment standards, characterized by fluorescence spectroscopy (EEM), HPLC-DAD and MS as well as in coupling of these methods. Also, the wastewaters were added zinc.

EEMs of the native wastewaters did not show any clear signal in the faecal pigment region, apart from WWTP D where a strong signal was visible in both, the in- and outflow. Upon zinc addition, all WWTP inflows showed an increase in fluorescence, which could not be seen in the outflows, indicating the presence of faecal pigments in the inflows, but not in the outflows. The strong signal in the outflow of WWTP D did, like the other outflows, not either react to the zinc addition, which pointed against the presence of faecal pigments. Also, the outflows which were separated by HPLC did not show any sign of faecal pigment presence. However, the wastewater was not fresh anymore at the point of HPLC and MS analysis, so a degradation can have taken place and statements about the potential absence of faecal pigments in outflows need to be made with care.

Results from the coupling of Aqualog, HPLC and MS could show that the fluorescence in the WWTP inflows in the faecal pigment region was comprised of several substances. Stercobilin, urobilin, and, in wastewater from WWTP D, fluorescein could be found. Stercobilin was the dominant faecal pigment in the wastewater inflows, while there was only little urobilin present. Urobilin could have either degraded faster than stercobilin, or it was already present in the wastewater in lower amounts from the start because more faecal pigments are excreted in faeces in the form of stercobilin (Orten, 1971). It is, however, possible, that more urobilin as well as stercobilin were present at the time of the sampling but were degraded in the time before the analysis.

A PARAFAC model based on fluorescence data of faecal pigment standards in different media, fluorescein standard, HOWI, and wastewater samples was developed. Aim of the model was to be generally applicable, meaning that it can be applied for the interpretation of data from many different types of water and recognize different components typical for e.g. wastewater or faecal pigments. An application of the model to the data it was trained with as well as on related data showed that it was successful in distinguishing between faecal pigments (urobilin/stercobilin), pigment-zinc-complexes (urobilin/stercobilin), and fluorescein. This was however only the case if the concentration, and thus the fluorescence intensity, was high enough for the fluorescence peaks to be clearly separated. Either an enrichment or the addition of zinc to the wastewater samples was necessary to accomplish that.

6.2 Indicator function of faecal pigments

As faeces are the main source for many waterborne pathogens (Ashbolt, 2004) the indication of faecal pollution can indicate the presence of pathogens. However, it was in this work not possible to do any tests on the connection between the presence of pathogens or common indicators and the presence of faecal pigments in water.

Faecal pigments can only be of good indicator function where pathogen presence and concentration are related to the amount of faecal contamination. If pathogens do not have their origin in faecal pollution, a faecal pollution indicator is not useful. Sometimes, small concentrations of pathogens do not pose too high a risk, but problems arise when they multiply e.g. during storage and transport of drinking water. Faecal pigments have their limit when it comes to growth. While problematic pathogen populations can potentially grow from a small population (Vital *et al.*, 2008; Vital *et al.*, 2010), faecal pigment concentration will only indicate the initial pollution and not subsequent growth of pathogenic bacteria. On the contrary, the pigment will degrade, which was recorded in this study in real water media (5.2.3) as well as in literature (Miyabara *et al.*, 1994c).

It might also be a problem for the suitability as an indicator, if faecal pigments are not detectable in the outflow of WWTPs (5.5.4; Loganathan *et al.*, 2009). WWTP outflow can still contain large amounts of faecal bacteria (e.g. Ajonina *et al.*, 2015; Naidoo and Olaniran, 2013) and carries a risk of discharging pathogens into the environment. If faecal pigment concentration is diminished during the wastewater treatment more than the pathogen concentration is diminished, faecal pigments fail to indicate possible microbial risks from wastewater treatment plant effluents. However, faecal pigment fluorescence might be suitable to indicate direct faecal pollution and the presence of untreated wastewater.

Miyabara *et al.* (1994c) found a good correlation between the concentration of urobilin and faecal as well as total coliforms in river water, which points to urobilin being a suitable indicator for microbial pollution in water. However, a lot of research in this field is based on this study. When it comes to the suitability of urobilin as an indicator, always the same papers by Miyabara *et al.* (1994c; 1994b) are cited (e.g. by Lam *et al.*, 1998; Piocos and La Cruz, 2000; Jones-Lepp, 2006; Bixler *et al.*, 2014). More studies that connect faecal pigments with pathogens or at least other indicators of faecal pollution, like faecal coliforms or enterococci, under different conditions are needed. Both, urobilin and stercobilin, have to be tested for their suitability as indicators, in case they behave differently. Miyabara *et al.* (1994b) found e.g. more urobilin in sewage-contaminated rivers than stercobilin. Urobilin has already been examined as a potential indicator of faecal pollution (Miyabara *et al.*, 1994c; Jones-Lepp, 2006; Miyabara *et al.*, 1994b), but stercobilin would be the more interesting pigment, as it is the "faecal urobilin" and constitutes most of the excreted faecal pigments (Orten, 1971). In this study, stercobilin was the dominant faecal pigment in wastewater, suggesting that the potential of stercobilin as a faecal pollution indicator should not be neglected.

According to Ashbolt (2004), there is no universal indicator. There are different requirements for indicators, depending on whether the indicator should reflect the pollution history (sediment) or the current level of pollution (water) (Miyabara *et al.*, 1994c). If water is used for drinking water supply, indicators must reflect current pollution (Miyabara *et al.*, 1994c). Pathogens additionally show a diverse

behaviour in water, which needs to be accounted for: viruses can attach to particles and can therefore travel with the particles in the water, but they can also be inactivated and settle into the sediment with particles. Bacteria can also be transported and additionally they can grow or die off, depending on the circumstances (Jung *et al.*, 2014). To find out, under which conditions and for what kind of pathogens faecal pigments are suitable indicators, more studies about their behaviour in the environment need to be done. According to Miyabara et al. (1994c), urobilin in a river was distributed between water and sediment. While urobilin was degraded quickly in river water, Miyabara et al. (1994c) found urobilin in river sediment to be relatively stable. If urobilin and stercobilin attach to particles, it could however be a problem for fluorescence monitoring, because only dissolved pigment is measurable with fluorescence techniques and a filtration should be done before the measurement.

6.3 Practical application and limitations

Faecal pigments could not be identified in the native (non-enriched) wastewaters by fluorescence spectroscopy alone, without any zinc addition or enrichment. The help of reference techniques was needed to quantify faecal pigments in wastewater. An addition of zinc in combination with a PARAFAC model could make the use of reference techniques such as HPLC and an enrichment unnecessary at some point. The overall aim is to be able to detect very low concentrations of faecal pigments, which are relevant for the application in drinking or surface water monitoring. For this, the technique needs to be much more sensitive than it is at this point.

The theoretical detection limits observed in this study were 2 µg/l for urobilin and 3 µg/l for stercobilin. In the wastewaters, the faecal pigment concentration determined by HPLC and Aqualog in combination with PARAFAC was between 50 and 140 µg/l. As a comparison, 120 µg/l would correspond to 100 g of faeces dissolved in approximately 1000 l of water, assuming a daily excretion of 150 mg faecal pigments (Orten, 1971) and a median daily amount of 128 g of faeces (Rose *et al.*, 2015). According to the detection limit, the detection of faecal pigments should have been possible in wastewater without further enrichment, zinc addition, or other technique. This tells that the real detection limit using just fluorescence EEMs is much higher than 3 µg/l. LOD is just a theoretical value assuming ideal conditions, but the discrepancy between theoretical and practical detection limit observed here was very high. If calibration series are repeated with zinc addition, lower limits of detection are to be expected. However, time needs to be controlled, because the fluorescence intensity after zinc addition was not stable.

In case that a part of the pigments from urine and faeces is still present in the form of reduced urobilinogen and stercobilinogen, it would be best to first oxidize every sample to urobilin or stercobilin with e.g. iodine and then measure the fluorescence like it was done by Miyabara *et al.* (1992) in urine samples.

A PARAFAC model was able to distinguish between the compounds used here, if the concentration was high enough and with that it is possible to calculate back from the intensity of a compound to its concentration. However, if the concentration was not high enough, there was no clear peak (too much noise) and similar compounds could not be distinguished well. Either the addition of zinc in a high concentration or an enrichment is then needed to increase the fluorescence intensity. Enrichment takes

a lot of time, so the addition of zinc is a promising alternative to be used in an on-line measurement. It is therefore very important to find a way of measuring the zinc complex fluorescence, which is repeatable and robust. As the fluorescence intensity of the zinc complexes diminishes with time after zinc addition, already small changes in the measurement time can lead to great insecurities concerning the accuracy of the quantification. A standardized measurement technique, with a defined time between zinc addition and the measurement, has to be developed to get reproducible results.

Zinc addition leads to a high intensity increase, which enables the qualitative detection of low concentrations of faecal pigments by fluorescence techniques. Making use of the zinc-complexion reaction is therefore helpful, but also problems can arise. Instability of the fluorescence intensity in the presence of zinc, as well as its dependency on the zinc concentration complicates an exact quantification of the pigment concentration present in the water. For a measurement, it would be practical to add an overload of zinc leading to all pigments being present as zinc complexes. In theory this would result in the highest possible intensification and also avoid problems of the model confusing the pure pigment with the complex, as there is hardly any pure pigment left. However, one major problem is that the native metal concentration of a real water is usually not known before a detailed analysis, so it is unclear whether a part of the pigments in the water are already present in a complexed state. In the case that the pigments present in water are already present as a chelate, they are not available for complexation anymore. For example, pigments could form a complex with magnesium in the water and are then not available for complex forming with zinc. This can lead to a wrong estimation when using fluorescence spectroscopy to measure faecal pigment concentration. More tests need to be done, especially concerning the complex chemistry. This applies in particular for time dependency, dependency on the concentration of zinc as well as faecal pigments, other metal ions with complexation potential (e.g. Mg^{2+}), and the influence of pH in different waters. Important questions are:

- Can some metals be exchanged for others? How is the preference?
- Which metals complex with urobilin and stercobilin? Do they enhance or quench the fluorescence?
- Do they all cause the same red shift?
- How stable are the complexes?

For fluorescence monitoring, it would pose a problem if the pigment metal complexes stayed irreversibly complexed but lost fluorescence intensity with time. An addition of zinc would then not result in any intensity increase and this would lead to an underestimation of faecal pigment concentration (false negative result). The tests with different zinc concentrations showed, however, that the metal concentrations in water would have to be very high for all of the pigments being present in a complexed state. Also the wastewaters already contained zinc in small concentrations, but still reacted to zinc addition. Fluorescence detection could potentially serve as an early warning signal, which is fast and simple, after which other methods can serve for an exact quantification.

The pH value of the waters to be measured as well as time after zinc addition has to be monitored. The time between zinc addition and the measurement should be as short as possible to get a maximal intensification of the fluorescence signal.

Apart from the need for optimization of the measurement technique itself, there is always a risk for interferences by fluorescent industrial chemicals or other substances present in the wastewater, which are no body excretions (industrial dye, fluorescein, etc.). Fluorescein for example is, due to its high quantum yield (Sjöback *et al.*, 1995), already in small concentrations a disturbance when measuring faecal pigment fluorescence, while it has no influence on the absorption measurements. If the created PARAFAC model could not distinguish between fluorescein and faecal pigments, as well as their zinc complexes, this would pose a serious problem and a high risk for false positive alarms in drinking water monitoring for faecal pollution using fluorescence spectroscopy. A robust model combined with a suitable measurement technique is therefore necessary. For on-line monitoring, a sensor detecting only one or two wavelength combinations will most likely not be enough. An option to keep fluorescein apart from faecal pigments might be to record full emission spectra at several excitation wavelengths, e.g. 320 nm, 360 nm, and 485 nm. The pre-peak of faecal pigments at 360/500 can overlap with humic acids (Coble, 1996), which might also pose a difficulty, but model evaluation with more, independent data needs to show that. An improvement in the separation sensitivity could be achieved by exciting in 1 nm steps instead of 5 nm steps and using a smaller observation window. However, if fluorescein is interfering, there is the possibility that other unknown chemicals or similar organic compounds interfere as well. An unknown compound is a problem, if the model tries to explain unknown compounds with known components instead of signalling something unknown. Solving this in the modelling process would reduce the risk of erroneous results and false positive alarms substantially.

Faecal pollution of drinking water is not a common problem in Central Europe (e.g. Bain *et al.*, 2014), but will likely apply more in regions with lacking sanitation or insufficiently designed sanitation systems as well as after disasters. Therefore this technique might be more relevant for developing than industrialized countries in the case of drinking water monitoring. However, according to Sinton *et al.* (1998) illnesses are also often transferred by faecal pollution of recreational waters, which is relevant for every region.

6.4 Future Research

Future research needs include

- Evaluation of ideal conditions for fluorescence measurement of faecal pigments in water (metal concentration and timing, pH, temperature, etc.)
- Testing the behaviour of different metal ions for complexation with faecal pigments and their influence on the fluorescence properties (replacement of one metal by another; ranking of metals after binding preferences; fluorescence intensification; detection limits)

- Examination of the nature of the zinc-kinetic behaviour (absorption, flocculation, de-complexation, re-complexation, equilibrium, etc.)
- Tests on the behaviour of faecal pigments in the environment (adsorption, sedimentation, degradation)
- Studies on the correlation between faecal pigments and microbial indicators of faecal pollution, as well as relation to pathogen behaviour.

7 Conclusion

The main question was, whether fluorescence spectroscopy can be used as a stand-alone method to detect faecal pigments in real waters. It was in this study not possible to detect and quantify faecal pigments in wastewater by fluorescence spectroscopy alone. However, the use of an enrichment prior to fluorescence measurement, in combination with a PARAFAC model, made a quantification of faecal pigments in wastewater treatment plant inflow possible. To avoid the need for enrichment in the future, an addition of zinc is suggested to enhance the fluorescence intensity. For this purpose, metal complexes with faecal pigments in water need to be studied further, because the fluorescence of the complexes is not stable and depends on several factors (zinc concentration, pH, medium). The fate of the complexes is unknown: it is at this point unclear, whether the pigment complexes stay complexed in a dissolved state, de-complex, are degraded, or flocculate. The wastewater analysis showed that the unidentified fluorescence signal of wastewaters in the faecal pigment region was comprised of several substances. Stercobilin, urobilin, and fluorescein could be identified. Faecal pigments were responsible for a fluorescence signal in samples of WWTP inflow, but not in the outflow, indicating a degradation of faecal pigments during wastewater treatment. Fluorescein, as well as other interfering markers and dyes, can disturb faecal pigment assessment in water. However, fluorescein could be recognized by a PARAFAC model able to distinguish between faecal pigments, their zinc complex and fluorescein. A distinction between urobilin and stercobilin is not possible by fluorescence spectroscopy, because their spectra look alike. For a practical application in water, pH and temperature, as well as time in case of the pigment-zinc-complexes, have to be monitored.

On the basis of the present study it is concluded that 2D fluorescence detection, in combination with a PARAFAC model, is, with limitations, a promising tool for the detection of faecal pigments in water. There is, however, a great need for more research to obtain a more sensitive, reliable, and robust method which is suitable for the detection of faecal pigments in concentrations relevant for drinking and surface water applications.

Acknowledgements

I would like to thank all the people, who have accompanied me on my way here.

First of all, I owe my gratitude to my supervisors Martin Wagner and Annika Nordin, who put a lot of effort in me and in this work. Thank you for your energy and commitment.

I am very happy that I got the opportunity to perform my thesis project at TZW and would like to thank the TZW staff for the time there. Special thanks to Lutz Imhof, for technical advice but also for the welcoming atmosphere in the office and lab. It was great working with you.

This thesis would not have been possible without the help of SLU and its staff, especially Ulf Grandin, who made this thesis possible by creating a 45 credit Master thesis course in no time.

I would also like to thank all the other people, who have contributed to this thesis, like my examiner Björn Vinnerås, my opponent and classmate Jelena Rakovic, as well as my other classmates, for valuable discussions and comments.

And last, but not least, thanks to my friends, my parents, my godparents, my grandmother, and my partner, short, to all my families - in Germany and Sweden - for your encouragement and support.
I'm very grateful to have you.

References

Ajonina, C., Buzie, C., Rubiandini, R.H. and Otterpohl, R. (2015), "Microbial pathogens in wastewater treatment plants (WWTP) in Hamburg", *Journal of toxicology and environmental health. Part A*, Vol. 78 No. 6, pp. 381–387.

Ashbolt, N.J. (2004), "Microbial contamination of drinking water and disease outcomes in developing regions", *Toxicology*, Vol. 198 No. 1-3, pp. 229–238.

Bain, R., Cronk, R., Hossain, R., Bonjour, S., Onda, K., Wright, J., Yang, H., Slaymaker, T., Hunter, P., Pruss-Ustun, A. and Bartram, J. (2014), "Global assessment of exposure to faecal contamination through drinking water based on a systematic review", *Tropical medicine & international health TM & IH*, Vol. 19 No. 8, pp. 917–927.

Bixler, J.N., Cone, M.T., Hokr, B.H., Mason, J.D., Figueroa, E., Fry, E.S., Yakovlev, V.V. and Scully, M.O. (2014), "Ultrasensitive detection of waste products in water using fluorescence emission cavity-enhanced spectroscopy", *Proceedings of the National Academy of Sciences of the United States of America*, Vol. 111 No. 20, pp. 7208–7211.

Bloomer, J.R., Berk, P.D., Howe, R.B., Waggoner, J.G. and Berlin, N.I. (1970), "Comparison of fecal urobilinogen excretion with bilirubin production in normal volunteers and patients with increased bilirubin production", *Clinica Chimica Acta*, Vol. 29 No. 3, pp. 463–471.

Boiadjiev, S.E. and Lightner, D.A. (1999), "ChemInform Abstract. Optical Activity and Stereochemistry of Linear Oligopyrroles and Bile Pigments", *ChemInform*, Vol. 30 No. 33, no-no.

Bro, R. (1997), "PARAFAC. Tutorial and applications", *Chemometrics and Intelligent Laboratory Systems*, Vol. 38 No. 2, pp. 149–171.

Bull, R.V., Lim, C.K. and Gray, C.H. (1981), "High-performance liquid chromatography of bile pigments: separation and characterization of the urobilinoids", *Journal of chromatography*, Vol. 218, pp. 647–652.

Cavatorta, P., Giovanelli, S., Bobba, A., Riccio, P., Szabo, A.G. and Quagliariello, E. (1994), "Myelin basic protein interaction with zinc and phosphate. Fluorescence studies on the water-soluble form of the protein", *Biophysical Journal*, Vol. 66 No. 4, pp. 1174–1179.

Cimenti, M., Hubberstey, A., Bewtra, J.K. and Biswas, N. (2009), "Alternative methods in tracking sources of microbial contamination in waters", *Water SA*, Vol. 33 No. 2.

Coble, P.G. (1996), "Characterization of marine and terrestrial DOM in seawater using excitation-emission matrix spectroscopy", *Marine Chemistry*, Vol. 51 No. 4, pp. 325–346.

Coble, P.G. (Ed.) (2014), *Aquatic organic matter fluorescence, Cambridge environmental chemistry series*, Cambridge University Press, New York.

Crawford, J.M., Hauser, S.C. and Gollan, J.L. (1988), "Formation, hepatic metabolism, and transport of bile pigments: a status report", *Seminars in liver disease*, Vol. 8 No. 2, pp. 105–118.

Dang, C. (2016), "Characterization of waste water by using 2D-fluorescence spectroscopy", Master thesis, Faculty of environmental sciences, Technische Universität Dresden, Dresden, 2016.

Falk, H. (1989), *The Chemistry of Linear Oligopyrroles and Bile Pigments, Monatshefte für Chemie/Chemical Monthly Supplementa*, Vol. 1.

Guilbault, G.G. (Ed.) (1990), *Practical fluorescence, Modern monographs in analytical chemistry*, Vol. 3, 2. ed., rev. and expanded., Dekker, New York NY u.a.

Henderson, R.K., Baker, A., Murphy, K.R., Hambly, A., Stuetz, R.M. and Khan, S.J. (2009), "Fluorescence as a potential monitoring tool for recycled water systems: a review", *Water research*, Vol. 43 No. 4, pp. 863–881.

Hudson, N., Baker, A. and Reynolds, D. (2007), "Fluorescence analysis of dissolved organic matter in natural, waste and polluted waters—a review", *River Research and Applications*, Vol. 23 No. 6, pp. 631–649.

Jones-Lepp, T.L. (2006), "Chemical markers of human waste contamination: analysis of urobilin and pharmaceuticals in source waters", *Journal of environmental monitoring JEM*, Vol. 8 No. 4, pp. 472–478.

Jung, A.-V., Le Cann, P., Roig, B., Thomas, O., Baures, E. and Thomas, M.-F. (2014), "Microbial contamination detection in water resources: interest of current optical methods, trends and needs in the context of climate change", *International journal of environmental research and public health*, Vol. 11 No. 4, pp. 4292–4310.

Lakowicz, J.R. (2010), *Principles of fluorescence spectroscopy,* 3. ed., corr. 4. print, Springer, New York, NY.

Lam, C.W., Lai, C.K. and Chan, Y.W. (1998), "Simultaneous fluorescence detection of fecal urobilins and porphyrins by reversed-phase high-performance thin-layer chromatography", *Clinical chemistry*, Vol. 44 No. 2, pp. 345–346.

Loganathan, B., Phillips, M., Mowery, H. and Jones-Lepp, T.L. (2009), "Contamination profiles and mass loadings of macrolide antibiotics and illicit drugs from a small urban wastewater treatment plant", *Chemosphere*, Vol. 75 No. 1, pp. 70–77.

Miyabara, Y., Miyata, K., Suzuki, J. and Suzuki, S. (1994a), "Evaluation of fecal pollution of river sediment by detection of urobilin", *Environmental pollution (Barking, Essex 1987)*, Vol. 84 No. 2, pp. 111–115.

Miyabara, Y., Sakata, Y., Suzuki, J. and Suzuki, S. (1994b), "Estimation of faecal pollution based on the amounts of urobilins in urban rivers", *Environmental Pollution*, Vol. 84 No. 2, pp. 117–122.

Miyabara, Y., Sugaya, N., Suzuki, J. and Suzuki, S. (1994c), "Estimation of urobilin as a fecal pollution indicator in the aquatic environment", *Bulletin of Environmental Contamination and Toxicology*, Vol. 53 No. 1.

Miyabara, Y., Suzuki, J. and Suzuki, S. (1994d), "Degradations of urobilin and stercobilin during activated sludge treatment of night soil", *Environmental Pollution*, Vol. 84 No. 2, pp. 105–109.

Miyabara, Y., Tabata, M., Suzuki, J. and Suzuki, S. (1992), "Separation and sensitive determination of i-urobilin and l-stercobilin by high-performance liquid chromatography with fluorimetric detection", *Journal of chromatography*, Vol. 574 No. 2, pp. 261–265.

Mowery, H. and Loganathan, B. (2007), *Persistent organic compounds in wastewater: Azithromycin and urobilin concentrations in wastewater treatment plant samples from Murray, Kentucky, USA*.

Murphy, K.R., Stedmon, C.A., Graeber, D. and Bro, R. (2013), "Fluorescence spectroscopy and multi-way techniques. PARAFAC", *Analytical Methods*, Vol. 5 No. 23, p. 6557.

Naidoo, S. and Olaniran, A.O. (2013), "Treated wastewater effluent as a source of microbial pollution of surface water resources", *International journal of environmental research and public health*, Vol. 11 No. 1, pp. 249–270.

Orten, J.M. (1971), "Metabolism of hemoglobin and bile pigments", *Annals of clinical laboratory science*, Vol. 1 No. 2, pp. 113–124.

Petryka, Z.J., Weimer, M., Lightner, D.A., Chedekel, M., Bovey, F.A., Moscowitz, A. and Watsorr, C.J. (1975), "PRESENT STATUS OF THE d-UROBILINS", *Annals of the New York Academy of Sciences*, Vol. 244 No. 1 The Biologica, pp. 521–531.

Piocos, E.A. and La Cruz, A.A. de (2000), "SOLID PHASE EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH PHOTODIODE ARRAY DETECTION OF CHEMICAL INDICATORS OF HUMAN FECAL CONTAMINATION IN WATER", *Journal of Liquid Chromatography & Related Technologies*, Vol. 23 No. 8, pp. 1281–1291.

Quinn, K.D., Nguyen, N.Q.T., Wach, M.M. and Wood, T.D. (2012), "Tandem mass spectrometry of bilin tetrapyrroles by electrospray ionization and collision-induced dissociation", *Rapid communications in mass spectrometry RCM*, Vol. 26 No. 16, pp. 1767–1775.

Reichenbächer, M. and Einax, J.W. (2011), *Challenges in Analytical Quality Assurance*, Springer-Verlag Berlin Heidelberg, Berlin, Heidelberg.

Rose, C., Parker, A., Jefferson, B. and Cartmell, E. (2015), "The Characterization of Feces and Urine: A Review of the Literature to Inform Advanced Treatment Technology", *Critical reviews in environmental science and technology*, Vol. 45 No. 17, pp. 1827–1879.

Schlesinger, W. (1903), "Zum klinischen Nachweis von Urobilin", *Deutsche medicinische Wochenschrift*, XXIX No. 32, pp. 561–563.

Schmidt, N.A. and Scholtis, R. (1964), “Urobilin in urine”, *Clinica Chimica Acta*, Vol. 10 No. 6, pp. 574–576.

Sinton, L.W., Finlay, R.K. and Hannah, D.J. (1998), “Distinguishing human from animal faecal contamination in water. A review”, *New Zealand Journal of Marine and Freshwater Research*, Vol. 32 No. 2, pp. 323–348.

Sjöback, R., Nygren, J. and Kubista, M. (1995), “Absorption and fluorescence properties of fluorescein”, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, Vol. 51 No. 6, L7-L21.

Stedmon, C.A. and Bro, R. (2008), “Characterizing dissolved organic matter fluorescence with parallel factor analysis. A tutorial”, *Limnology and Oceanography: Methods*, Vol. 6 No. 11, pp. 572–579.

Vital, M., Hammes, F. and Egli, T. (2008), “Escherichia coli O157 can grow in natural freshwater at low carbon concentrations”, *Environmental microbiology*, Vol. 10 No. 9, pp. 2387–2396.

Vital, M., Stucki, D., Egli, T. and Hammes, F. (2010), “Evaluating the growth potential of pathogenic bacteria in water”, *Applied and environmental microbiology*, Vol. 76 No. 19, pp. 6477–6484.

Vitek, L., Majer, F., Muchova, L., Zelenka, J., Jiraskova, A., Branny, P., Malina, J. and Ubik, K. (2006), “Identification of bilirubin reduction products formed by Clostridium perfringens isolated from human neonatal fecal flora”, *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*, Vol. 833 No. 2, pp. 149–157.

Wagner, M. (2014), “DOC-Analytik mittels 2D-Fluoreszenzspektroskopie. Characterisierung und Quantifizierung natürlicher organischer Wasserinhaltsstoffe mittels Fluoreszenzspektroskopie”, Dissertation, Technische Universität Dresden, Dresden, 2014.

Watson, C.J. (1969), “Gold from Dross. The First Century of the Urobilinoids”, *Annals of Internal Medicine*, Vol. 70 No. 4, p. 839.

Appendix A

Standard operation procedure for SPE, HPLC and MS

Table 1: Chemicals. Not all CAS and article numbers are given.

Name	CAS	Company	Article Number
Urobilin hydrochloride	28925-89-5	Frontier Scientific	U590-9
Stercobilin hydrochloride	34217-90-8	Chemos GmbH	152095
Uranine	518-47-8	Serva	
Acetone			
Methanol			
MilliQ water			
Ammonium hydroxide			
Hydrochloric acid			
Ammonium acetate			

Table 2: Materials.

Name	Company	Article Number	Remarks
SPE cartridge	Phenomenex	8B-S014-JCH	1 g Strata SDB, 6 ml tube
GF/C glassfibre filter	Whatman	1822-047	47 mm
Column HPLC	Macheray Nagel	720936.30	Nucleosil 100-5 C18 AB

HPLC-DAD-FLD-MS/MS method

Table 3: Main parameters, materials and chemicals

Parameter	Description
Chemicals	MilliQ water
	Methanol (gradient grade)
	Ammonium acetate (LC-MS grade)
Precolumn	C8 10 µm self-filled column (20 mm x 2 mm)
HPLC Column	C18 AB 5 µm from Macheray Nagel (250 mm x 3 mm)
Eluent	A: 10% methanol, 90% MilliQ- water, 1 mM ammonium acetate
	B: 90% methanol, 10% MilliQ- water, 1 mM ammonium acetate
Column oven	30 °C
Injection Volume	25 µl
DAD	UV channel 490 nm, 230 nm, 270 nm, 385 nm
DAD Spectrum	Store all spectra from 240 nm to 590 nm, step 2 nm
FLD	Slit 10 – 20 nm (dependent of your device), Gain factor x100 (Waters) or 15-16 (Agilent), path length of measurement cell is 10 mm
	Wavelength: excitation: 490 nm, emission: 520 nm

Table 4: Gradient and Flow

Time in min	Eluent in % B	Flow in ml/min
0	15	0.4
2	15	0.4
10	50	0.4
20	60	0.4

Time in min	Eluent in % B	Flow in ml/min
25	80	0.4
27.5	80	0.4
27.6	50	0.4
28	15	0.4
40	15	0.4

MS/MS parameters

The used system was LC-MS/MS API2000 (Sciex). All mentioned parameters below regard this system.

Mode: ESI (electrospray ionization) with positive ionization in Q1 (scan) and MRM (multiple reaction monitoring) mode.

Table 5: Source dependent parameters for API2000 with electron spray interface (ESI)

Parameter	**Value**
Curtain gas (CUR)	25
Nebulizer gas (GS1)	30
Heater gas (GS2)	50
Collision gas (CAD)	3
Temperature heater gas (TEM)	350 °C
Ion spray voltage (IS)	+5000 V

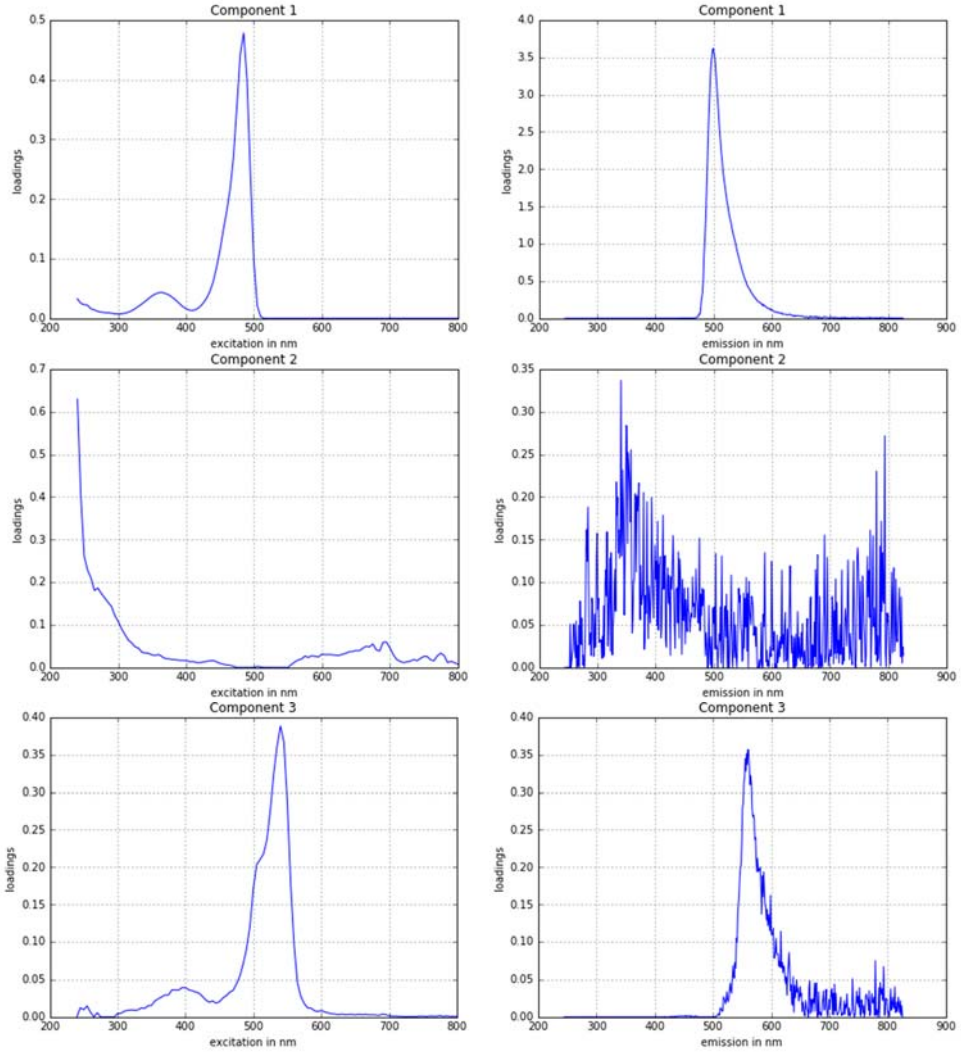
Table 6: Compound dependent LC-MS/MS parameters for API2000

Analyte	**Q1 mass** m/z	**Q3 mass** m/z	**Declustering potential** (DP) / V	**Collision energy** (CE) / V
Stercobilin	595.3	345.1	80	50
	595.3	166	80	70
	595.3	470.2	80	40
Urobilin	591.3	343.2	60	50
	591.3	164.1	60	70
	591.3	136.1	60	70
Urobilinogen	593.3	344.2	50	50
	593.3	180.1	50	55
Uranine	333.1	202	35	70
	333.1	189	35	85

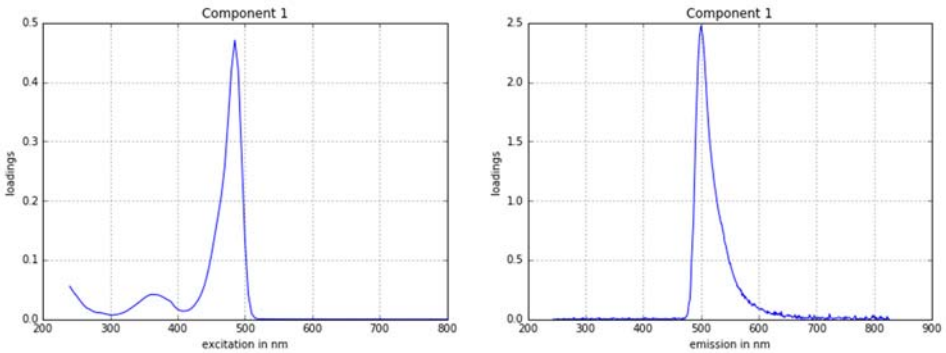
Appendix B

NMF-Models of the pigment standard fluorescence

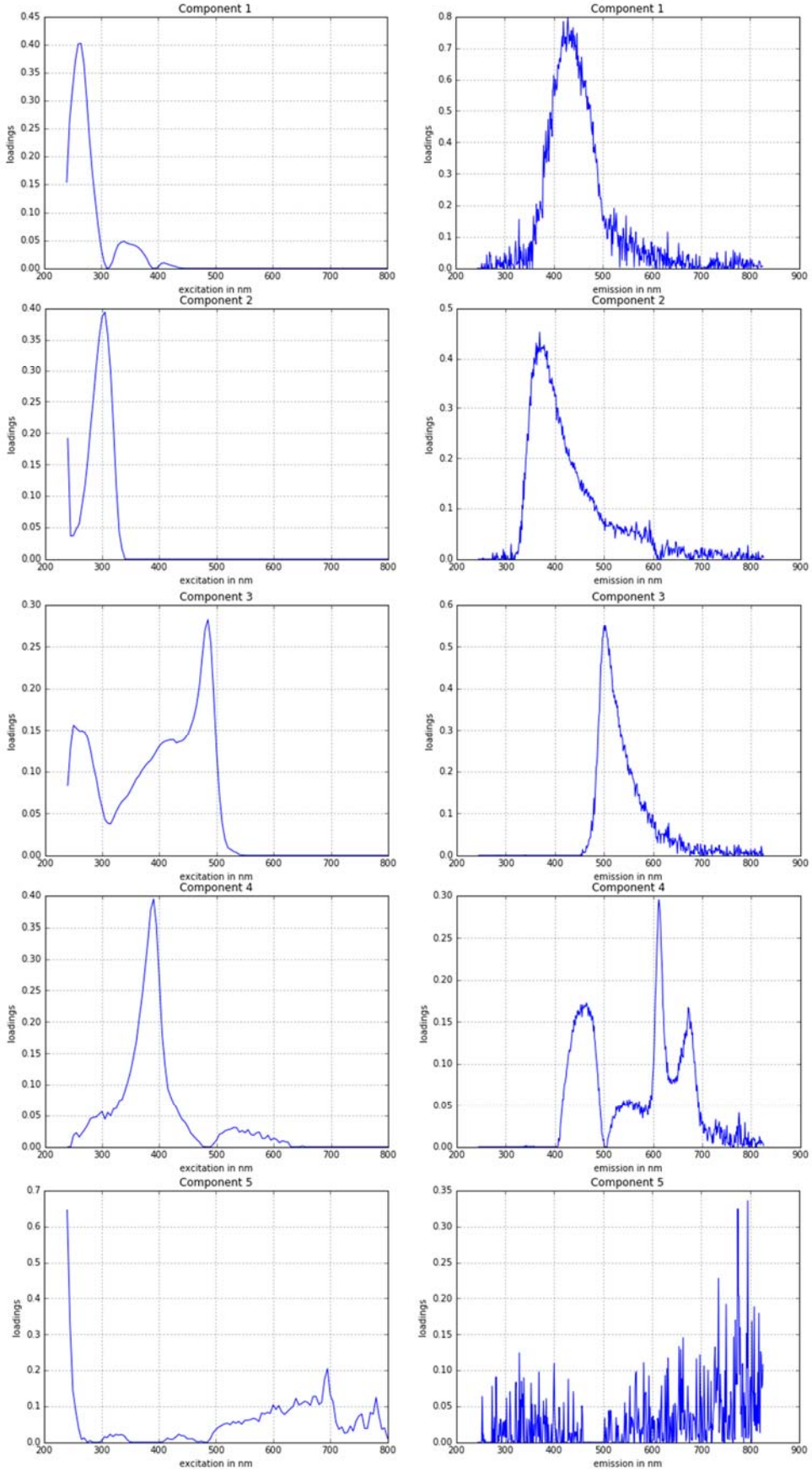
Stercobilin standard



Urobilin standard



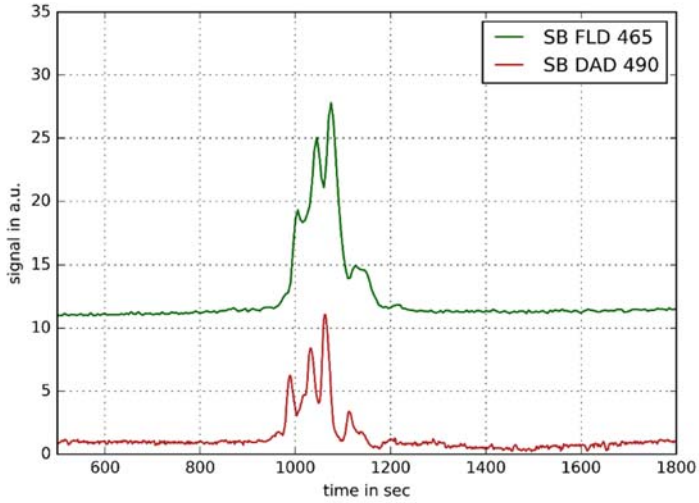
Urobilinogen standard



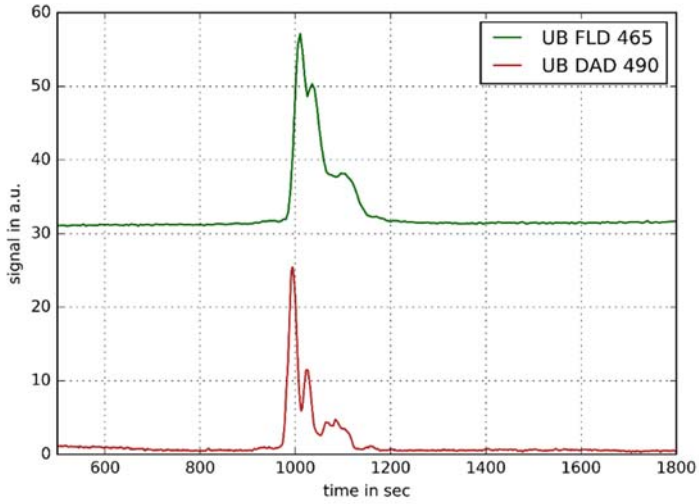
Appendix C

HPLC-DAD and FLD chromatograms of the pigment standards

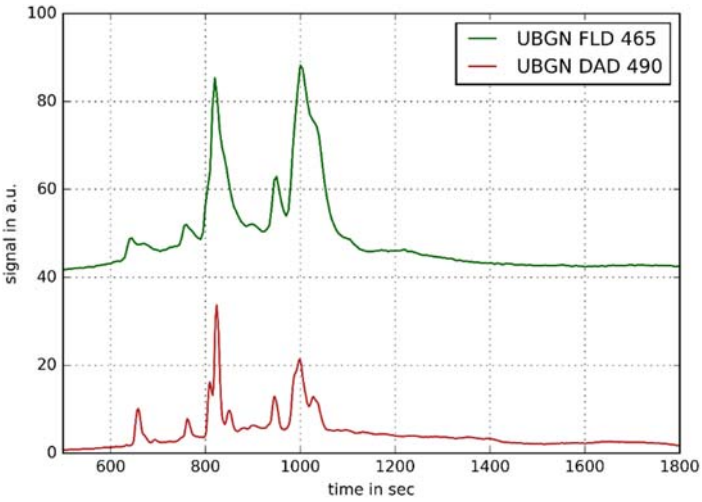
Stercobilin



Urobilin



Urobilinogen



Appendix D

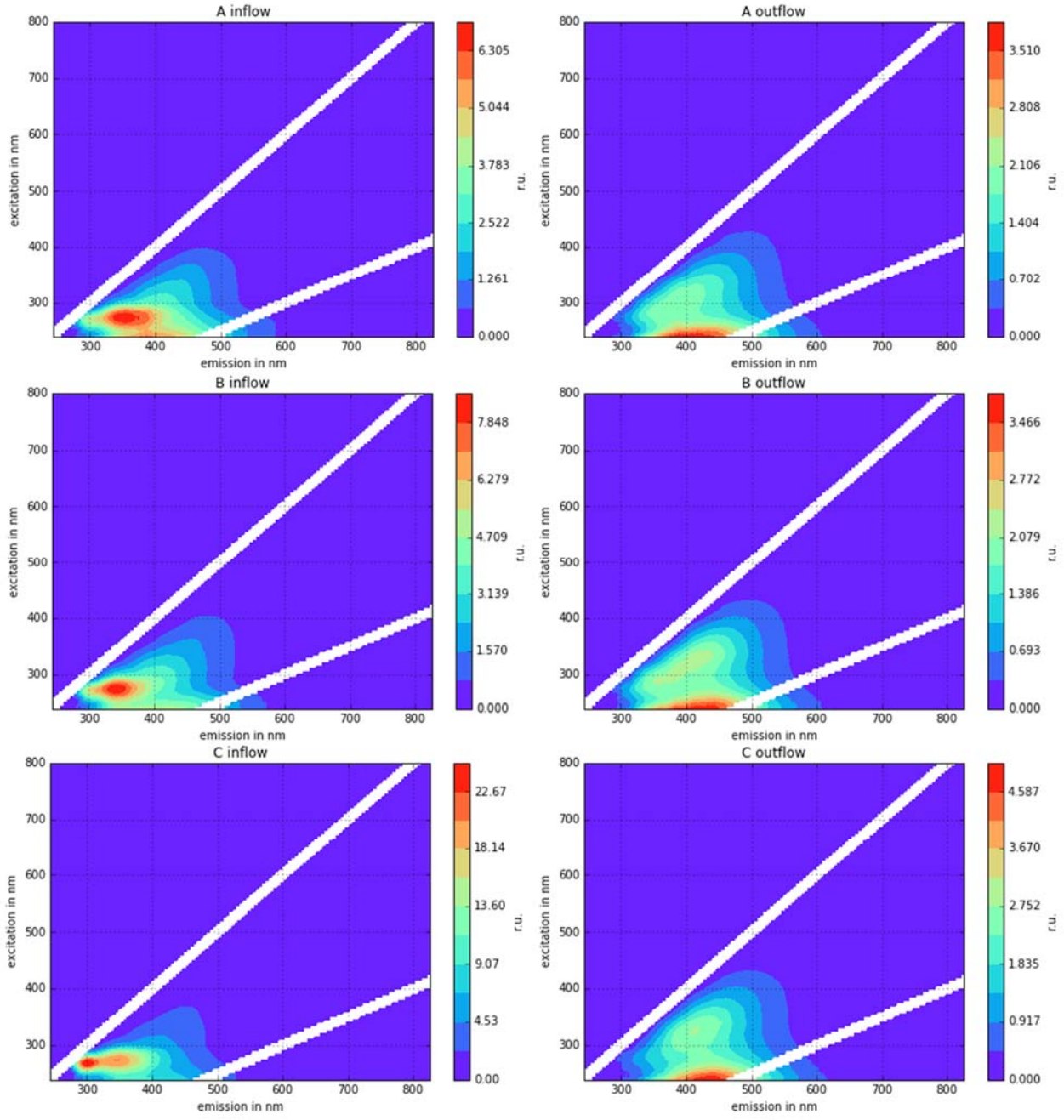
Time overview about wastewater and standard processing

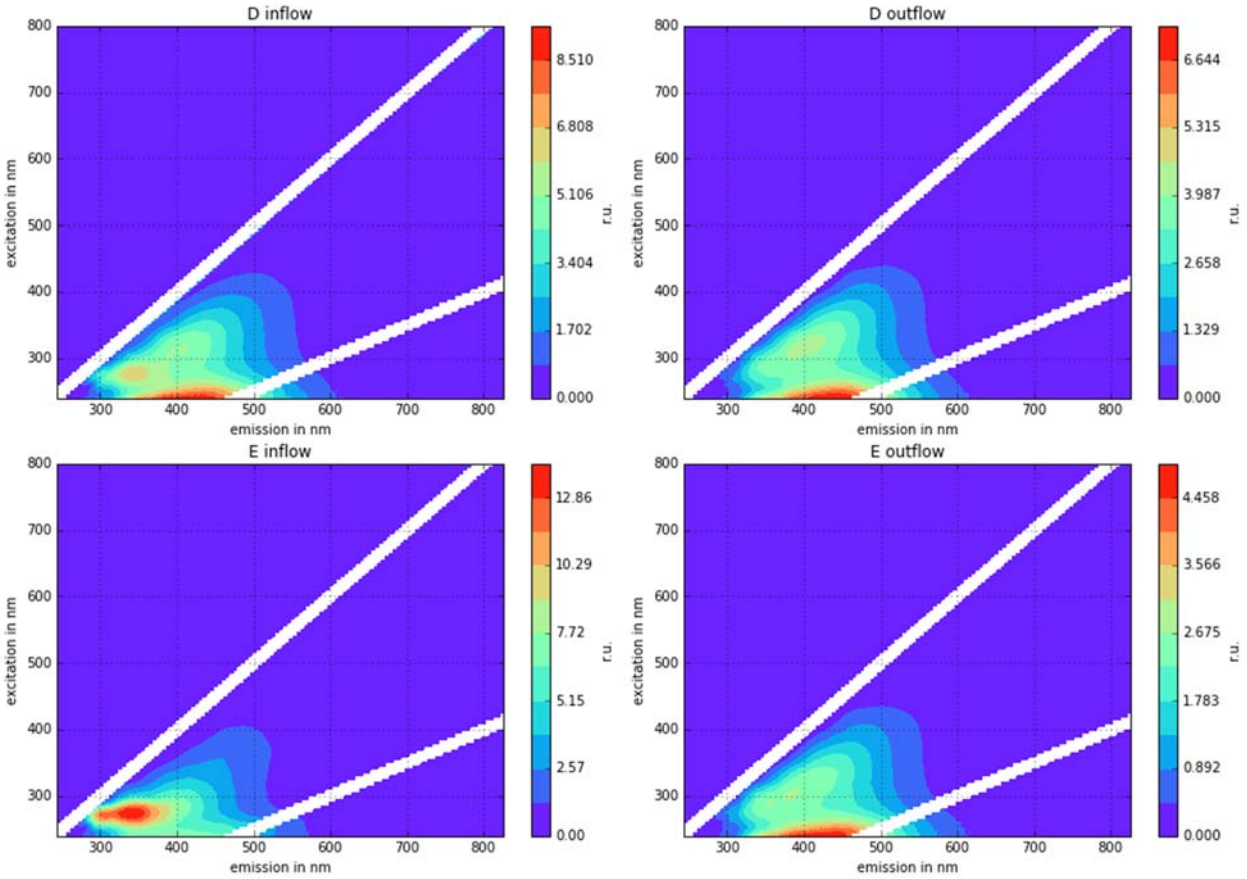
Name	Sampling	pH	Aqualog EEM	HPLC	SPE	HPLC-Aqualog-MS
A	14.06.2016	15.06.	15.06.	15.06./12.07.	--	--
B	06.07.2016	06.07.	06.07.	12.07.	--	--
C	06.07.2016	06.07.	06.07.	12.07.	20.07.	01.09.
D	06.07.2016	06.07.	06.07.	12.07.	20.07.	01.09.
E	12.07.2016	22.07.	12.07.	12.07.	20.07.	25.08.

Name	Standard opened	pH	Aqualog EEM	HPLC	SPE	HPLC-Aqualog-MS
UBGN	Several	-	several	12.07.	-	30.08.
SB	26.04.16	-	several	12.07.	-	30.08.
UB	11.07./20.09.	-	several	12.07.	-	30.08.

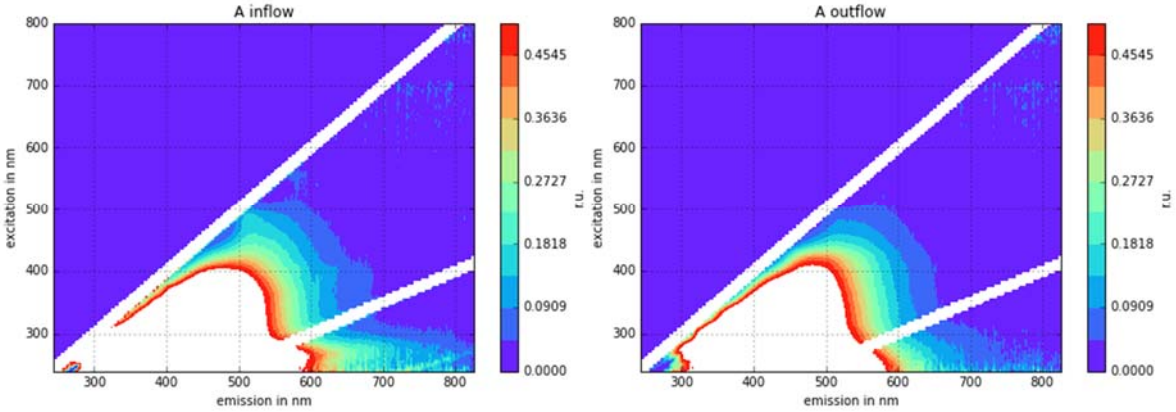
Appendix E

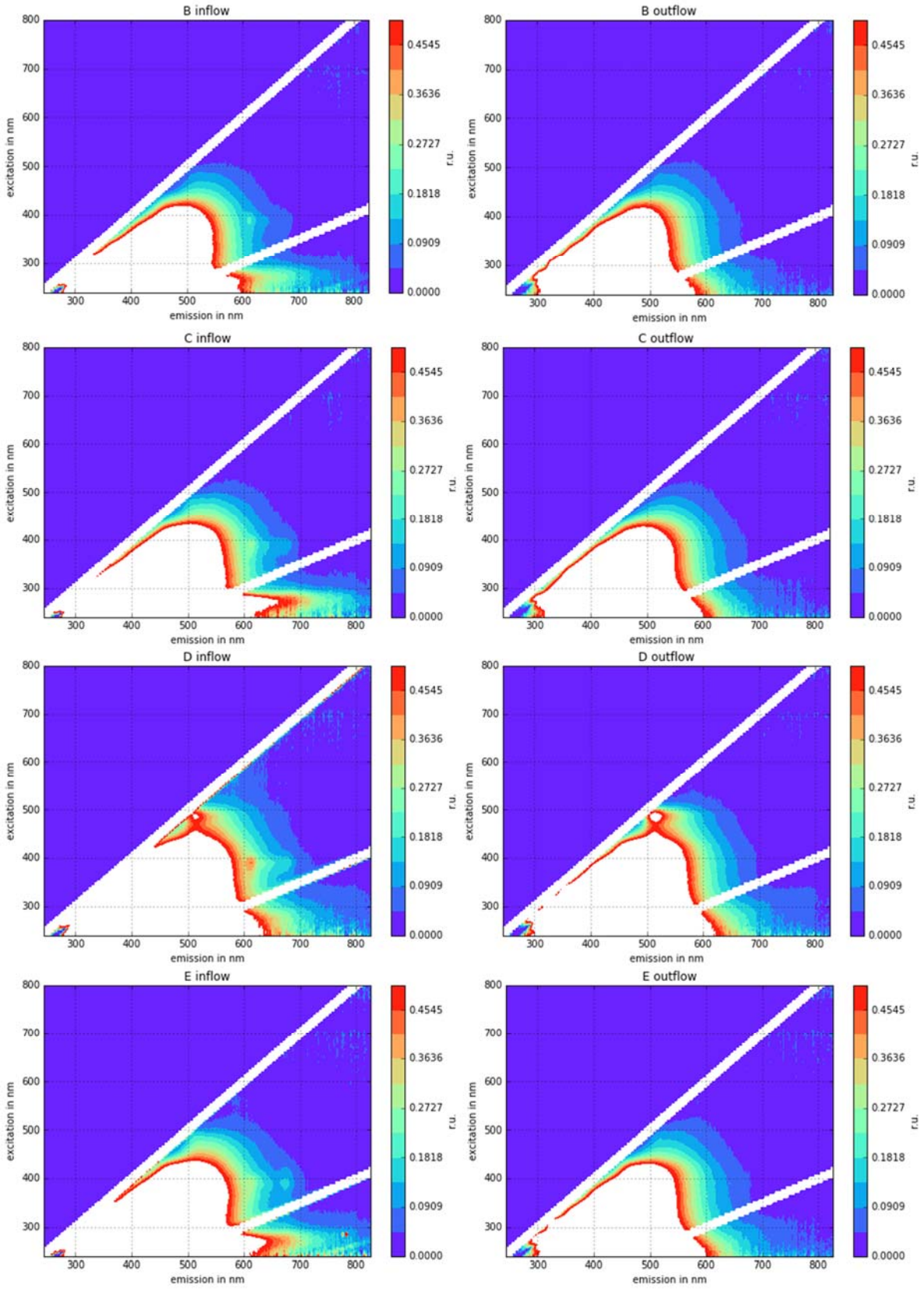
EEMs of the (not enriched) wastewaters from the WWTP screening





Wastewater EEMs scaled to a maximum intensity of 0.5 r.u.

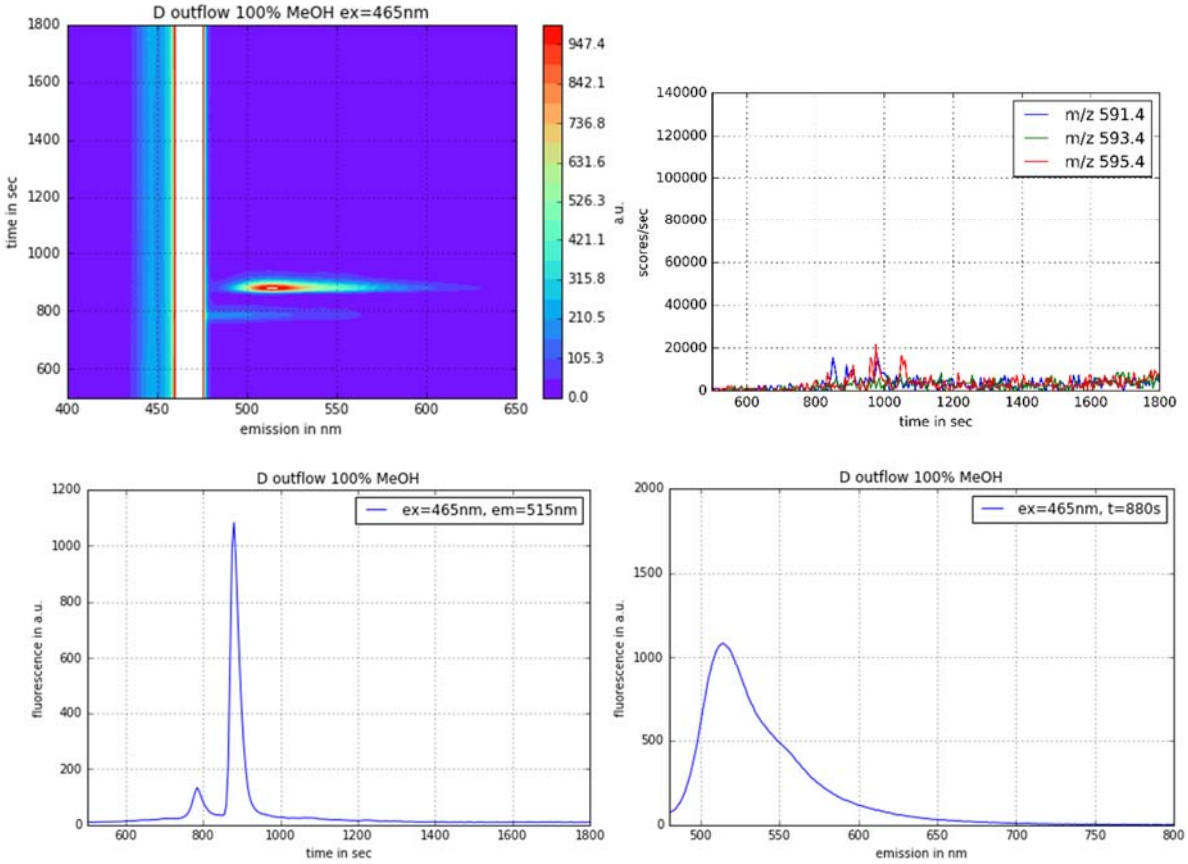



B inflow
B outflow
C inflow
C outflow
D inflow
D outflow
E inflow
E outflow
excitation in nm
emission in nm
r.u.
300
400
500
600
700
800
0.0000
0.0909
0.1818
0.2727
0.3636
0.4545

Appendix F

WWTP D outflow 100% MeOH

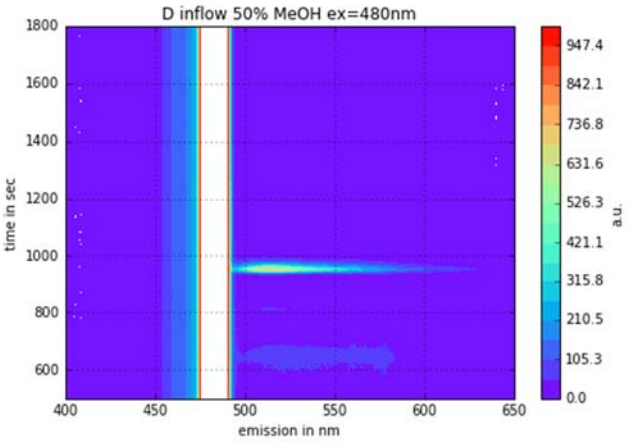
Fluorescence at λ_{ex} 465 nm	time	wavelength	m/z
Peak 1 (highest)	880 sec	515 nm	333

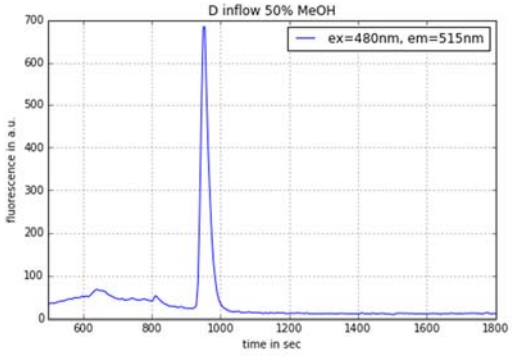


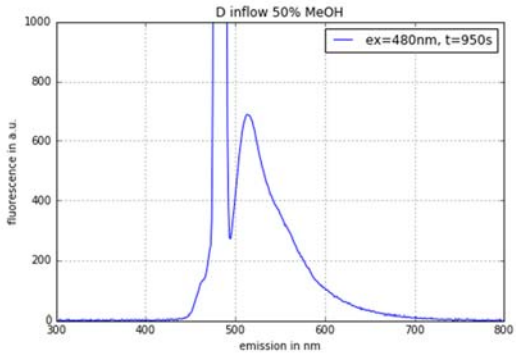
Faecal pigments were not clearly identified by MS. If they were present, the concentration was very low. Another small fluorescent peak at 785 seconds was not identified.

WWTP D inflow 50%MeOH

Fluorescence at λ_{ex} 480 nm	time	wavelength	m/z
Peak 1 (highest)	950 sec	515 nm	unknown

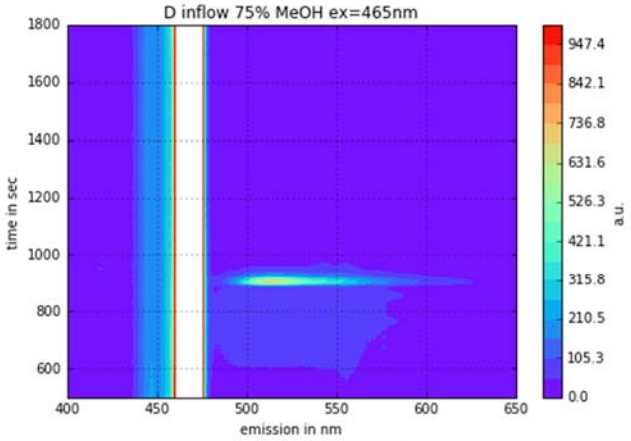


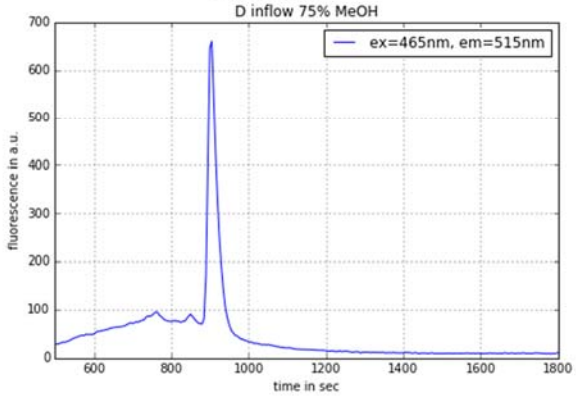


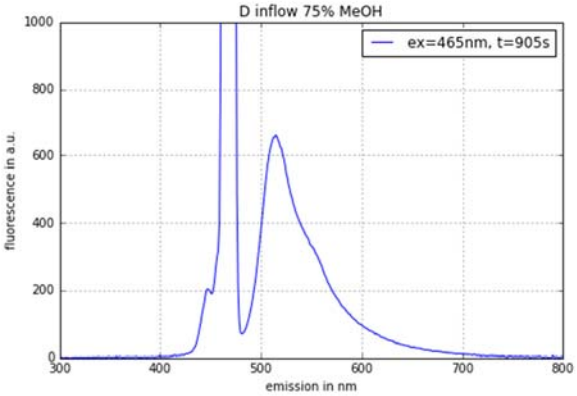


WWTP D inflow 75% MeOH

Fluorescence at λ_{ex} 465 nm	time	wavelength	m/z
Peak 1 (highest)	905 sec	515 nm	unknown

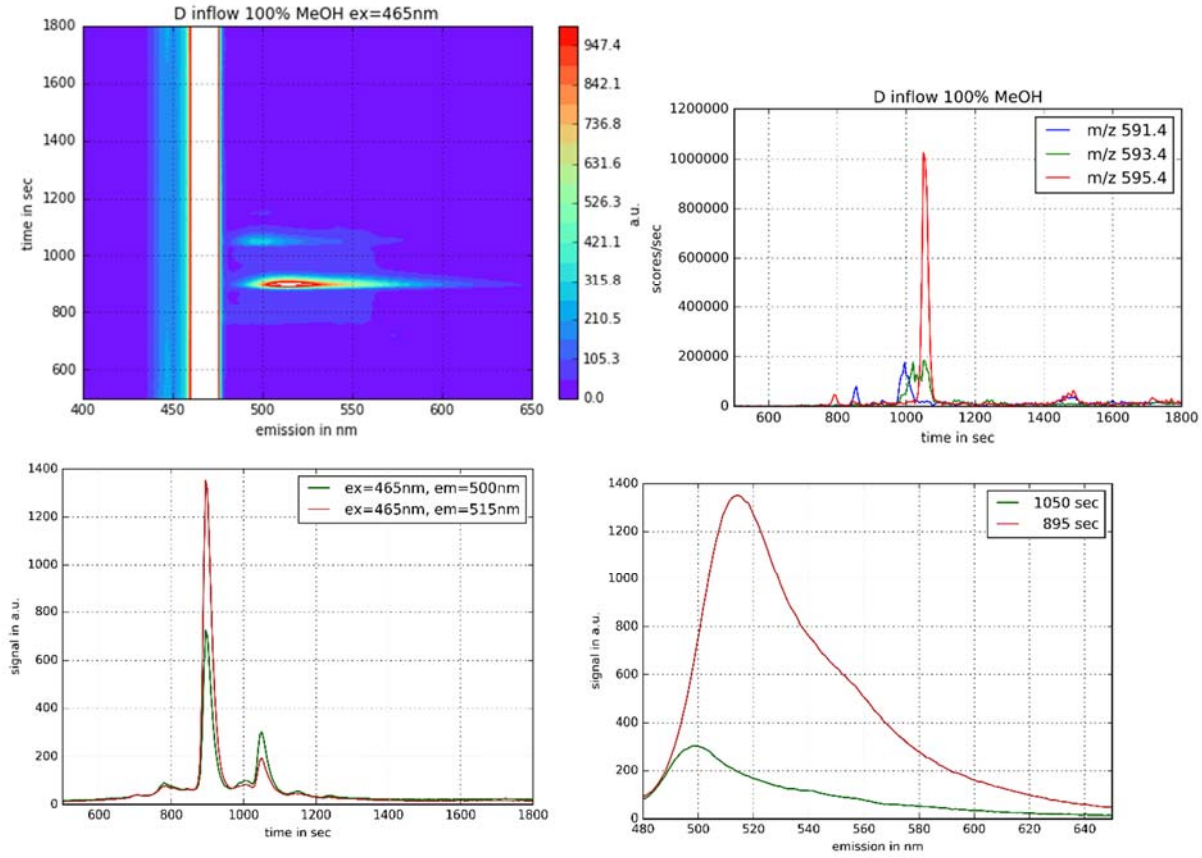






WWTP D inflow 100% MeOH

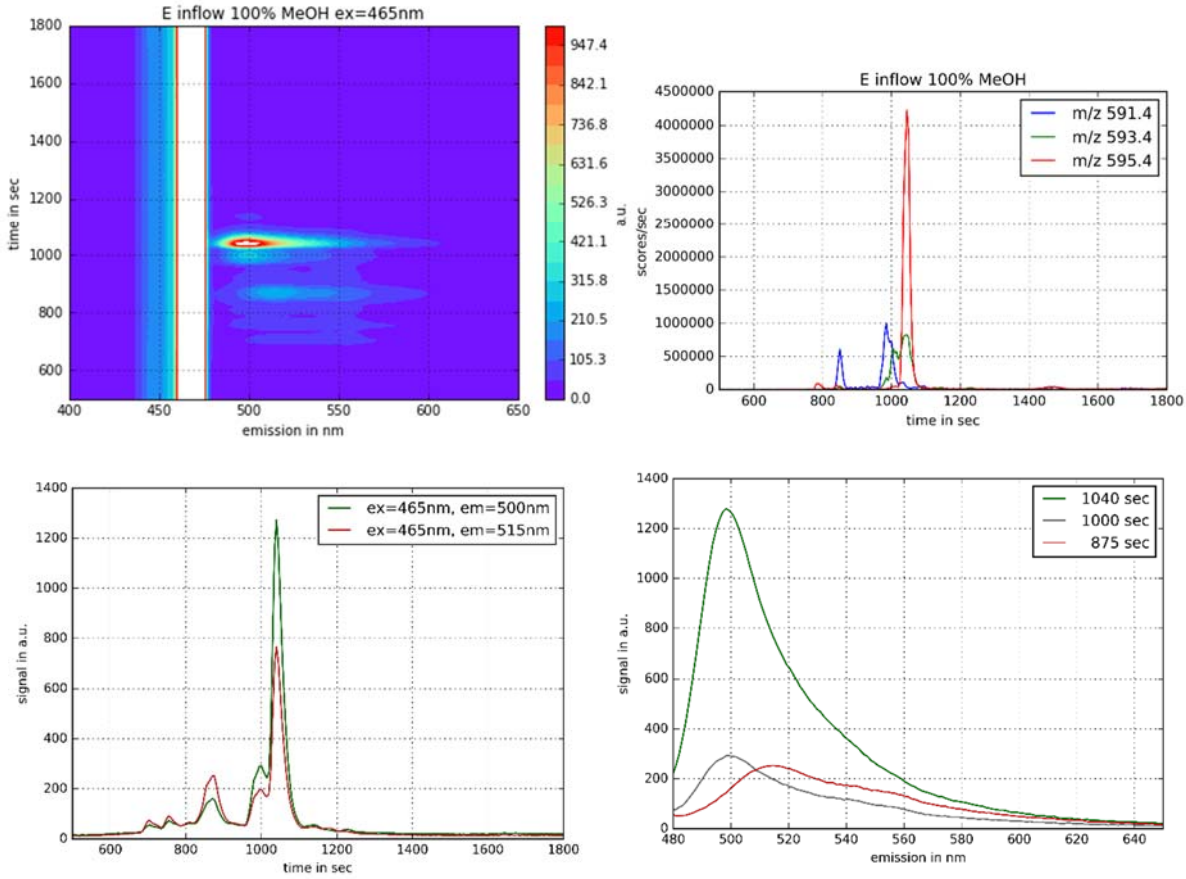
Fluorescence at λ_{ex} 465 nm	time	wavelength	m/z
Peak 1 (highest)	895 sec	515 nm	unknown
Peak 2	1050 sec	500 nm	595.4
Peak 3/4	1000 sec		591.4



The peak at 895 seconds could be caused by fluorescein in a concentration too small to be detected by MS.

WWTP E inflow 100% MeOH

Fluorescence at λ_{ex} 465 nm	time	wavelength	m/z
Peak 1 (highest)	1040 sec	500 nm	595.4
Peak 2	1000 sec	500 nm	591.4
Peak 3	875 sec	515 nm	unknown



The peak at 875 seconds could be caused by fluorescein in concentrations too small to be detected by MS.

Appendix G

Data used for fitting of the general model

Stercobilin in phosphate buffer

1: 1 mg/l stercobilin in phosphate buffer pH 6.6

2: 1 mg/l stercobilin in phosphate buffer pH 6.6

3: 1 mg/l stercobilin in phosphate buffer pH 6.6 + zinc

4: 1 mg/l stercobilin in phosphate buffer pH 6.6 + zinc

Stercobilin in HOWI

5: 1 mg/l in HOWI

6: 1 mg/l in HOWI

7: 1 mg/l in HOWI + zinc

8: 1 mg/l in HOWI + zinc

Urobilinogen in phosphate buffer

9: 5 mg/l in phosphate buffer

10: 10 mg/l in phosphate buffer

11: 7.5 mg/l in phosphate buffer + zinc

12: 10 mg/l in phosphate buffer + zinc

Urobilinogen in HOWI

13: 2 mg/l + zinc

Urobilin in phosphate buffer

14: 0.5 mg/l in phosphate buffer

15: 0.5 mg/l in phosphate buffer

16: 0.5 mg/l in phosphate buffer + zinc

17: 0.5 mg/l in phosphate buffer + zinc

Urobilin in HOWI

18: 0.5 mg/l in HOWI

19: 0.5 mg/l in HOWI

20: 0.5 mg/l in HOWI + zinc

21: 0.1 mg/l in HOWI + zinc

22: 0.1 mg/l in HOWI + zinc

HOWI

23: HOWI (not diluted, freshly sampled)

24: HOWI (not freshly sampled, stored)

Wastewater

25: WWTP A outflow (09.05.)

26: WWTP A inflow (09.05.)

27: WWTP A outflow (14.06.)

28: WWTP A inflow (14.06.)

29: WWTP B outflow

30: WWTP B inflow

31: WWTP C outflow

32: WWTP C inflow

33: WWTP D outflow

34: WWTP D inflow

35: WWTP E outflow

36: WWTP E inflow

WWTP A (14.06.) enriched

37: Eluate 50% MeOH

38: Eluate 75% MeOH

39: Eluate 100% MeOH

40: Eluate Acetone

Fluorescein

41: 10 µg/l in phosphate buffer

42: 10 µg/l in phosphate buffer + zinc

Sveriges Lantbruksuniversitet
Institutionen för energi och teknik
Box 7032
750 07 UPPSALA
http://www.slu.se/institutioner/energi-teknik/

Swedish University of Agricultural Sciences
Department of Energy and Technology
P. O. Box 7032
SE-750 07 UPPSALA
SWEDEN
www.slu.se/en/departments/energy-technology/