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Microbial, chemical and physical influences on uranine fluorescence measurements

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Abstract

Uranine, the sodium-salt of fluorescein, is a fluorescent pigment with the empirical formula $Na_2C_{20}H_{12}O_5$ and is used, among many other applications, as drill-water tracer in groundwater studies. Uranine is known to be pH sensitive, photochemically instable and heat sensitive. Concerns have been raised that uranine could be degraded by microorganisms and/or if fluorescent siderphores (e.g. pyoverdines) produced by microorganisms in groundwater could influence uranine measurements in groundwater investigations. Aerobic growth studies with three bacterial strains, *Shewanella putrefaciens*, *Pseudomonas stutzeri*, and *Pseudomonas fluorescens*, and 20 mg L^{-1}, 40, 100, and 200 µg L^{-1} uranine were performed for up to 60 days. Anaerobic growth studies under nitrate-reducing and sulphate-reducing conditions were also done with respectively *P. stutzeri* and *Desulfovibrio aespoeensis*. Fluorescence intensity was measured with fluorescent spectrophotometry and the results were confirmed with high-performance liquid chromatography (HPLC).

Fluorescence intensity increased rapidly in the aerobic bacterial growth cultures. The fluorescence increased also in some sterile controls, to a somewhat higher intensity in medium controls than in controls prepared with sterile water. In some controls the fluorescence intensity decreased. Pyoverdine production was followed in cultures of *P. fluorescens.* HPLC analyses of uranine in these cultures showed that part of the pyoverdins fluoresced with the settings for uranine measurements indicating that some of the fluorescence intensity measured in *P. fluorescens* cultures came from pyoverdins produced by this bacterium. *S. putrefaciens* and *P. stutzeri* did not produce any fluorescent substances during incubation.

There was no increase in fluorescence neither in the medium controls nor in the anaerobic cultures of the nitrate-reducing and the sulphate-reducing bacteria, during incubation for 135 days. Comparisons of uranine fluorescence in glass and plastic bottles with cultures of *P. fluorescens*, did not show any difference. Study of uranine in groundwater samples was done. In most samples the fluorescence remained stable for 21 days but in five of the 12 samples, the fluorescence had decreased on day 21.

The results from the different experiments in this study showed that influence from microorganisms but also storage in water solution can give values from the fluorescence analyses that overestimate the uranine concentration in samples. These results, together with the decrease found in two sterile controls, indicate that uranine fluorescence is not stable over time in solution. The actual cause of the increased fluorescence intensity was not identified in this study. One explanation could be that chemical interaction between uranine and compounds produced by microorganisms or present in the growth medium, decreased the energy loss in the emission process. This can be caused by the collision of analyte molecule with the molecules of the solvent. If the energy loss in the collision is less, the more energy has to be released in the emission and by that give higher fluorescence intensity. Another possibility could be that the uranine molecule is altered during the incubation and by that the fluorescence behaviour of the molecule is changed.

Sammanfattning

Uranin, natriumsaltet av fluorescein, är ett fluorescerande färgämne med den empiriska formeln $Na_2C_{20}H_{12}O_5$ och används bland annat som spårämne för rester av spolvatten i grundvattenundersökningar. Uranin är känt för att vara känsligt för pH, ljus- och värme. Farhågor har framförts att uranin även kan brytas ned av mikroorganismer men också att fluorescerande sideroforer av pyoverdintyp, som produceras av mikroorganismer i grundvattnet, kan påverka uraninmätningar i samband med grundvattenstudier. I det här projektet gjordes aeroba tillväxtförsök med tre bakteriestammar, *Shewanella putrefaciens* , *Pseudomonas stutzeri*, och *Pseudomonas fluorescens*, för att studera eventuell nedbrytning av uranin. Till odlingarna tillsattes 20 mg L^{-1} respektive 40, 100 och 200 µg L^{-1} uranin och försöken pågick i upp till 60 dagar. Även anaeroba odlingsförsök under nitrat- respektive sulfatreducerande förhållanden gjordes med bakterierna *P. stutzeri* och *Desulfovibrio aespoeensis*.

Fluorescensintensitet mättes med fluorescensspektrofotometri och resultaten verifierades med vätskekromatografi (HPLC) med fluorescensdetektor. Under försöken noterades att fluorescensintensitet ökade snabbt i de aeroba bakteriekulturer men även i vissa sterila kontroller och då något mer i kontroller gjorda med medium än i kontroller med sterilt vatten. I ett fåtal kontroller minskade dock fluorescensintensiteten. I odlingar med *P. fluorescens* följdes även produktionen av pyoverdiner under tillväxtförsöken. HPLC-analyser av dessa odlingar visade att pyoverdiner från *P. fluorescens* fluorescerade vid samma våglängd som uranin. Detta resulterade i att odlingar med *P. fluorescens* gav en högre fluorescensintensitet vid uraninmätning än i odlingar där bakterierna inte producerar pyoverdiner. I odlingarna med *S. putrefaciens* och *P. stutzeri* producerades det inga sådana fluorescerande ämnen.

Det blev ingen ökning av fluorescensintensiteten vare sig i kontroller med medium eller i anaeroba odlingar med nitrat- respektive sulfatreducerande bakterier. Det blev ingen skillnad i ökningen av fluorescensen mellan odlingar av *P. fluorescens* i glas- respektive plastflaskor, vilket visar att uranin inte påverkades av materialet i odlingskärlen.

I studier av uranin i grundvattenprover förblev fluorescensen stabil i de flesta proverna under 21 dagar, men i fem av 12 prov hade fluorescensen minskat efter 21 dagar. Resultaten från de olika experimenten i den här studien demonstrerar att fluorescensen hos uranin i vattenlösning, även utan mikrober, kan påverkas vid lagring under en längre tid. Den faktiska orsaken till den observerade ökningen i fluorescensintensiteten kunde inte fastställas. En förklaring kan vara att det uppstår en växelverkan mellan uranin och substanser som producerats av mikroberna eller som finns i odlingsmediet. Om sådana ämnen bidrar till en minskad energiförlust i emissionsprocessen till exempel vid kollision med den analyserade molekylen desto mer energi måste frigöras under emissionen, vilket därmed skulle ge högre fluorescens. En annan möjlighet skulle kunna vara att uraninmolekylen förändras och därmed också fluorescensen hos molekylen.

Contents

1 Introduction

Uranine has routinely been used as drill-water tracer in the investigations of groundwater and bedrock for a future repository for radioactive waste. Concerns have been raised that uranine could be degraded by microorganisms and by that indicate a lower drill-water content in a groundwater sample than what it actually is (Gutowski et al. 2015 and references therein). Gutowski and co-workers studied aerobic degradation of uranine and its transformation products from photo-degradation and they concluded that some biodegradation could not be ruled out. The degradation studies continued for 28 days and the degradation was measured indirectly as oxygen consumption. In addition, the opposite situation has been considered, that microorganisms produce fluorescent compounds that indicate too high drill-water content in the groundwater. These concerns initiated this study, which aimed to investigate the effect of pure cultures of microorganisms on the measured uranine fluorescence.

1.1 Uranine

Uranine is the common fluorescent dye also known as sodium-fluorescein (or D&C Yellow no.8). It has the empirical formula $Na_2[C_{20}H_{12}O_5]$ and the Chemical Abstracts Service (CAS) Registry Number 518-47-8 (Figure 1-1). Uranine is freely soluble in water and alcohol and after dissolution, it emits a bright yellowish-green fluorescence with reported excitation and emission maxima at 494 nm and 521 nm, respectively.

Uranine is a widely used dye for tracer experiments. It is known to be a nearly conservative tracer, and the material cost is comparatively low. Uranine is used extensively as a diagnostic tool in bio-chemical and medical research and health care applications and as stated above, it is also used in hydrology tests.

The disadvantages of uranine are reported to be its photochemical instability and pH sensitivity (Kola and Amataj 2006). Furthermore, it has been suggested that uranine solutions are unstable when heated. Uranine has a major pKa of 6.4 and the pH value has a major influence on the uranine signal over the range of 5 to 9 (Miller et al. 2004). Uranine have also pKa of 2.2 and 4.4, which refer to ketone and alcohol group, respectively. These pKa can be obtained at extreme conditions and are not considered at the present work (Sabnis 2007).

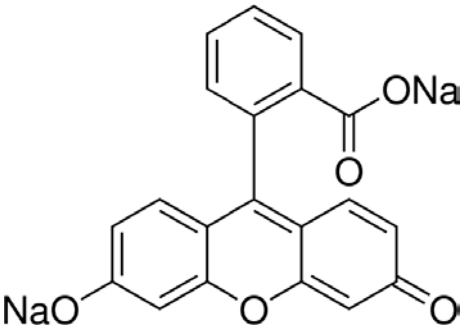


Figure 1-1. *The chemical structure of the fluorescein sodium salt from http://www.sigmaaldrich.com/catalog/product/sial/f6377?lang=en&region=SE, 2013*

1.2 Fluorescence

Fluorescence is a kind of luminescence, emission of light by a substance. Fluorescence occurs when an atom or molecule release surplus energy as emitted light after excitation by electromagnetic radiation. The phenomenon is associated with energy transfer between different fixed energy levels in the electron orbitals of an atom or molecule.

The phenomena can occur in both molecules and within atoms. Only molecules can absorb and emit light in the ultraviolet to visible range (Skoog and Leary 1992a, Larsson 2007).

When a molecule absorbs radiation, the electrons enter a higher energy level. The energy is absorbed and the molecule is now in a less stable electronic configuration and will try to relax to the lowest stable energy level. This relaxation happens through different pathways. Within the energy levels, it can occur by non-radiative vibrational relaxation but usually the relaxation between the major energy levels has to be accomplished by release of radiation. In the case of fluorescence, the electrons relax at first within the energy level and then fall back to the lowest level, which leads to the less energy of the emitted light and thus longer wavelength, than the absorbed one. If the same energy is applied, then the amount of energy detected at the longer wavelength is determined by the characteristics of the molecular orbitals in the molecule and the amount of molecules present. Different molecules have different properties and the ideal situation for analysis is a molecule that has high absorption of radiation and a large vibrational relaxation that causes the emitted light to be shifted to a considerably longer wavelength than the excitation wavelength (Skoog and Leary 1992a, Larsson 2007).

1.2.1 Fluorescent spectrometry

Fluorescence in a sample can be analysed by fluorescent spectrometry, sometime also called spectrofluorometry. It consists of a beam of light, usually ultraviolet light, that excites the electrons in molecules and make them emit light. At low concentrations, the fluorescence intensity will generally be proportional to the concentration of the fluorophore.

Fluorescence spectroscopy is used in, among others, biochemical, medical, and chemical research fields for analysing organic compounds which can provide luminescence. Spectrofluorometry has some certain advantages, it is a quite sensitive and fast method of analysis and it functions with small sample volumes (Skoog and Leary 1992b, Larsson 2007).

One problem with fluorescence spectrometry is that some molecules behave relatively similar when it comes to fluorescence. There is a theoretical difference between the emitted wavelengths but the difference can be too small to be differentiated with normal instrumentation.

In laboratory analysis, a pure substance in a clean solution is often used for calibration. However, real samples may contain all sorts of known and unknown substances apart from the fluorescent molecule of interest.

In an unfortunate situation, some of these molecules might cause a similar fluorescence to the one from the molecule of interest, upsetting the quantitative results. One solution for this problem is to add a separation step before the detection (Skoog and Leary 1992b Larsson 2007).

1.2.2 HPLC with fluorescence detection

High Performance Liquid Chromatography (HPLC) is a separation technique used to separate different molecules in a mixture. The technique originates from normal preparative chromatography. In HPLC, the liquid sample is pumped through a packed column filled with adsorptive material. A common variation is to use a packed column with a stationary phase coated with C18-chains. This phase will absorb and retain less polar organic compounds.

The C18-film is linked to the surface of silica particles and remains stationary in the packed column. To facilitate the separation, the particles are kept very small, giving a large surface area. The drawback is that a column filled with these very small particles gives a high backpressure to the liquid flowing through the column. In practice, a pump must be added to push the solvent through the column.

When a mixture of different molecules is pumped through the column, the molecules equilibrate between the stationary C18–phase and the solvent flushing through the column. The molecules are

retained on the surface of the particles. By pumping gradually higher concentrations of organic solvent like acetonitrile through the column, the adsorbed compounds become more and more mobile. The solvent competes with the stationary phase. Eventually the molecules elute from the column when they are more soluble in the solvent than in the stationary phase and are detected by the fluorescence detector, which is placed immediately after the separation column.

With HPLC, different molecules are separated before the fluorescence measurement. The key identification for a molecule is the time spent in the column, the retention time. However, the measured fluorescence cannot be used for exact identification but is suited for quantification. This is because an on-line detector together with very fast acquisition rate is used, and the drawback is less spectral specificity. (Skoog and Leary 1992b).

1.3 Microorganisms

Microorganisms exist almost everywhere; from low to high temperatures, from extremely acidic to highly alkaline environments. Some bacteria are heterotrophic using organic compounds as their carbon and energy source, others are autotrophic and synthesize organic substances from CO_2 and an inorganic energy source. Microbes can be characterized according to how well they tolerate atmospheric oxygen. For example, an obligate aerobe requires oxygen to accomplish cellular respiration, while an obligate anaerobe is the opposite and cannot use oxygen as their terminal electron acceptor. A facultative aerobe can utilize oxygen if it is present but can also survive without it (Madigan et al. 2012a, b). Many aerobic bacteria produce siderophores (iron chelators with extremely high affinity for ferric iron) in order to supply the cells with iron as iron has low solubility at aerobic conditions and neutral pH. The siderophores are divided into classes based upon their functional groups e.g. hydroxamates, catecholates and carboxylates. There are also mixed ligand siderophores such as the fluorescent pyoverdines (Figure 1-1) that are secreted by *P. fluorescens* among others under iron deficient conditions. They comprise a family of structurally related compounds and are non-ribosomal peptides containing a dihydroxyquinoline derivative which primarily differs in the amino acid composition and configuration. Over 40 structures have been described. The chromophore, (1S)-5-amino-2,3-dihydro-8,9-dihydroxy-1H-pyrimido [1,2-a] quinoline-1-carboxylic acid on the other hand, is the same with the exception of azobactin from *Azotobacter vinelandii*, which possesses an extra urea ring (Meyer 2000). The Fe (III)-binding ligands form relatively stable iron chelates with binding constants of approximately 10^{32} at alkaline pH (Cody and Gross 1987). The fluorescence of pyoverdines is pH dependent and has a reported fluorescence maximum at pH 7.0 (Xiao and Kisaalita 1995).

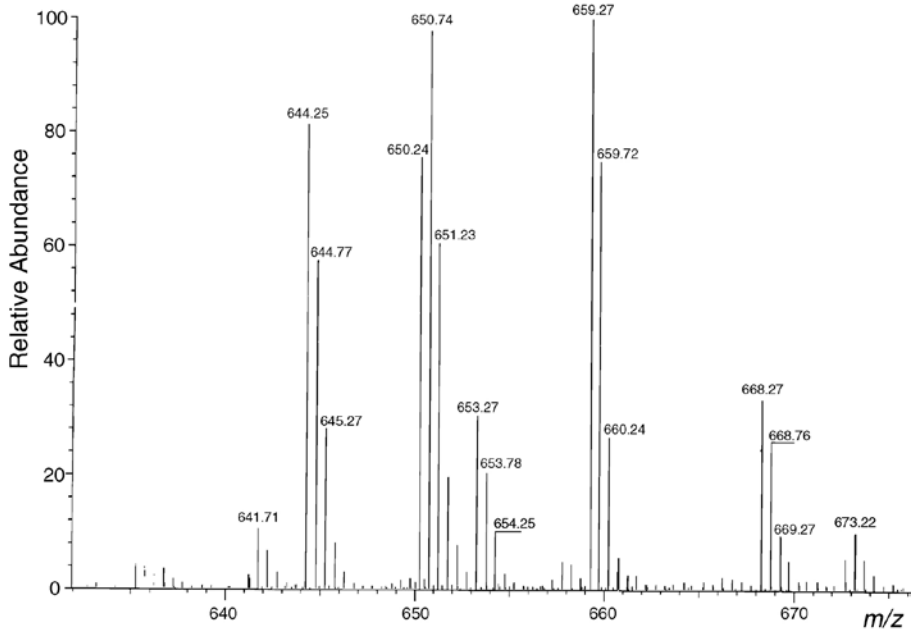
1.3.1 *Pseudomonas fluorescens*

Most strains of *Pseudomonas fluorescens* are obligate aerobes and use oxygen as terminal electron acceptor. However, some strains are known to be facultative anaerobes and can use nitrate as electron acceptor in the carbon metabolism in anaerobic environments. *P. fluorescens* is a gram-negative rod with polar flagellum and is found in soil and water (Madigan et al. 2012c). *P. fluorescens* synthesize different kinds of siderophores, of which the most typical group is the pyoverdines that fluoresces yellow/green detected around a wavelength of 460 nm (Meyer and Abdallah 1978). The bacterium has been detected in Fennoscandian groundwater and has been isolated from Äspö HRL groundwater.

1.3.2 *Pseudomonas stutzeri*

Pseudomonas stutzeri is gram-negative, a member of the genus *Pseudomonas* and has a typical rod shape phenotype with a single polar flagellum, and found in soil and groundwater. The bacterium has a wide strain specific temperature growth range between 4 °C to approximately 40 °C; the optimum temperature is approximately 35 °C. *P. stutzeri* does not tolerate acidic environments and fail to grow when pH is lower than 4.5. The bacterium grows well with oxygen and nitrate as terminal electron acceptor and is characterized as facultative anaerobic. Some strains of *P. stutzeri* can use chlorate and perchlorate as terminal electron acceptor. It is a strong denitrifier involved in the denitrification process when using nitrate as alternative electron acceptors. Some *P. stutzeri* strains produce siderophores but no fluorescent compounds (Lalucat et al. 2006).

A



B

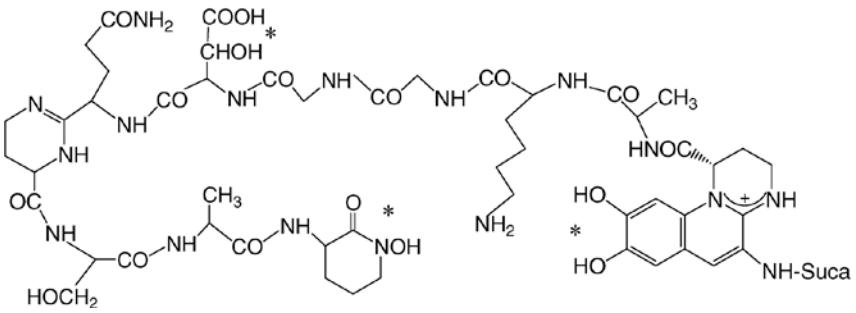


Figure 1-2. *(A) Molecular ion region ([M + 2H]2+) of the pyoverdine mixture produced from P. fluorescens (CCUG 32456). (B) Structure of the pyoverdine from P. fluorescens (CCUG 32456) with a succinamide (Suca) side chain (Suca-Chr-Ala-Lys-Gly-Gly-OHAsp-(Gln-Dab)-Ser-Ala-cOHOrn). Asterisks indicate the complexation sites. (from Moll et al. 2008).*

1.3.3 *Shewanella putrefaciens*

Shewanella putrefaciens is a gram-negative motile rod-shaped bacterium, characterized as a facultative anaerobe with oxygen as the major terminal electron acceptor. The bacterium can use many different electron acceptors for example, O_2, NO_3^-, NO_2^-, Fe(III), and Mn(IV) and is found in many different environments like marine and freshwater-sediments and groundwater (Cooper et al. 2003). *S. putrefaciens* produces siderophores of dihydroxamate-type (Ledyard and Butler 1997).

1.3.4 *Desulfovibrio aespoeensis*

Desulfovibrio aespoeensis is a sulphate-reducing bacterium isolated from groundwater in the Äspö HRL. It belongs to the genus *Desulfovibrio* and can grow with lactate as energy and carbon source and electron donor or with H_2-gas as energy source and electron donor and acetate as carbon source (Motamedi and Pedersen 1998).

2 Aim of study

This study aimed to investigate if microbial metabolism has any influence on the uranine concentration in a solution. The study consisted of two parts, 1) a laboratory study with pure cultures of microorganism performed by Microbial Analytics Sweden AB and 2) a study of uranine-amended groundwater from six borehole sections in six different boreholes in the tunnel (Table 3-2) at Äspö HRL, performed by the SKB Chemistry Laboratory at the Äspö HRL.

The microbial degradation study in the laboratory was divided in two experimental sub sets, one with aerobic microorganisms and one with anaerobic microorganisms.

The study included the following parts:

- Investigation of the optimal pH for uranine analyses.
- Create a standard curve to clarify optimal detection level of uranine.
- Clarify detection level and precision of the fluorescence measurements.
- Study possible degradation of uranine in groundwater over time
- Study the influence of microorganisms on the fluorescence of uranine.
- Study if pyoverdines produced by *Pseudomonas fluorescens* interfered with the uranine measurements.
- Analyse samples from growing cultures of microorganisms with uranine, on HPLC to detect any interfering substances produced by the microorganisms.

2.1 Experimental subsets

2.1.1 Laboratory study

Aerobic degradation

For the aerobic degradation study, uranine was added to cultures of *Pseudomonas fluorescens*, *Pseudomonas stutzeri*, and *Shewanella putrefaciens*. *P. fluorescens* produces a yellow/green pigment, pyoverdine that is similar to uranine and is detected around the same emission wavelength; 460 nm (e.g. Edberg et al. 2010). Pyoverdine production was studied to control if produced pyoverdines influenced the fluorescence measurement of uranine. In addition, an aerobic experiment with *P. fluorescens* was done in glass and plastic flasks to evaluate possible effects of adherence of uranine to the different materials.

Anaerobic degradation

The anaerobic degradation study followed the outline of the aerobic degradation study. Uranine-degradation by one nitrate-reducing bacterium (NRB), *P. stutzeri*, and by one sulphate-reducing bacterium (SRB) *D. aespoeensis* was investigated. Lactate, acetate, nitrate and sulphide concentrations were measured in the growth media and the fluorescence was monitored by fluorescence spectrophotometry and HPLC.

2.1.2 Groundwater study

The uranine degradation in groundwater was studied to check the stability of uranine at conditions similar to those that prevail in groundwater contaminated by flushing water from drilling. Each one of the six groundwater samples from six boreholes was divided into 13 subsamples of 100 mL with uranine added to a final concentration of around 200 µg L^{-1}. Twelve of the subsamples were stored in brown glass bottles and one sample in a brown plastic bottle. The uranine concentration in the samples was measured with a fluorescence spectrophotometer at six occasions, the last after 21 days. The numerous subsample analyses were performed to provide a statistical basis for the observations. Furthermore, the possible impact of two different materials as well as the time dependence was examined.

3 Materials and methods

3.1 Laboratory study

All pH measurements were made with a laboratory pH meter, CG 843P, Schott Instruments. Fluorescence was measured using a Hitachi fluorescent spectrophotometer F-7000 with Xenon light source. The uranine used was sodium-fluorescein, 46970-100G-F, Sigma Aldrich, Stockholm, Sweden. In some figures in this report, uranine is named fluorescein.

3.1.1 pH

Samples were prepared by dissolving uranine in sterile, anoxic Millipore analytical grade water, from now on called AGW, with the final concentration of 20 mg L^{-1}. pH was adjusted with 0.1 M NaOH and 0.1 M HCl within the range of 2-11. For measurements at different concentrations of uranine, the following series of uranine samples was prepared: 2, 0.2, 0.02, 0.002 mg L^{-1}. The λ_{ex} was 490 nm, slit = 5 nm and 400V was used for the fluorescence measurements.

3.1.2 Standard curve

Fluorescence was measured at λ_{ex} = 490 nm ($slit_{ex}$ = 5 nm, $slit_{em}$ = 5 nm) and at λ_{ex} = 475 nm ($slit_{ex}$ = 5 nm, $slit_{em}$ = 10 nm) for high and low concentrations respectively. All standards were prepared from a stock solution with 0.2 g L^{-1} uranine in 0.2 M Tris(hydroxymethyl)aminomethane-buffer with pH 9.0, to the final concentrations of 400, 350, 300, 250, 200, 150, 100, 80, 60, 50, 40, 20, 10, 5, 2, 1, 0.5, 0.2 µg L^{-1}.

3.1.3 HPLC

The HPLC analysis was performed on an Agilent 1100 system (Agilent Technologies, Palo Alto, USA). The system contained the following modules: Degasser G1322A, Binary pump G1312A, Autosampler G1313A, Column thermostat G1316A and Fluorescence detector G1321A and was controlled by Clarity 4.0.4 software (DataApex, Prague, Czech Republic). The analytical column was a SunShell C18 2.6 µm core-shell column with the dimensions 4.6 × 150 mm (ChromaNik Technologies Inc., Osaka, Japan). The gradient used was based on ammonium acetate (CAS 631-61-8, Sigma Aldrich, Stockholm, Sweden) adjusted to pH 8.4 with NaOH as aqueous buffer and acetonitrile (CAS 75-05-8, VWR HiPerSolv CHROMANORM, Leuven, Belgium) as organic solvent. The analytical column was not stable at pH higher than 9 and the analysis was done at pH 8.4 in 0.5 M ammonium acetate buffer. pH was adjusted to 8.4 by drop wise addition of 3 M NaOH-solution while monitoring the pH with an electrode and after that, the solution was degassed in an ultrasonic bath for 15 minutes.

With the gradient in Table 3-1, uranine was detected at approximately 9 min. retention time. The rinse with almost pure acetonitrile was added to wash less polar organic molecules from the column between analyses. such compounds can be present in natural samples.

The temperature of the analytical column was kept at 35 °C. For the detection, an excitation wavelength of 490 nm and an emission wavelength of 515 nm were used. Four calibration standard solutions where prepared with the following concentrations: 1.0 µg L^{-1}, 5.0 µg L^{-1}, 10.0 µg L^{-1} and 20.0 µg L^{-1}.

Table 3-1. The gradient used for HPLC analysis.

Time (min)	Vol% A (0.5M NH_4OAc pH 8.4)	Vol% B (ACN)	Flow (ml min^{-1})
0	90	10	0.80
1	90	10	0.80
10	85	15	0.80
11	1	99	0.80
15	1	99	1.50
17	90	10	1.30
18	90	10	0.80

The calibration curve was calculated by linear regression and not forced through zero. During the analysis, a calibration standard was re-analysed after every 8–10 samples to monitor instrument drift. The results were not corrected since the drift was negligible. A blank solution was measured to verify that no background signal could interfere with the uranine peak. The peaks were evaluated using peak area as integrated by the software. Estimated limit of detection was 0.1 µg L^{-1} and limit of analysis 0.3 µg L^{-1}. Estimated uncertainty of measurement in this kind of analysis is 10 % (with U=2, coverage factor of 95 %, determined according to Eurachem). Measurement uncertainty, limit of detection (LOD) and limit of quantification (LOQ) are not verified but based on visual evaluation of the peaks and experience from similar analysis on equivalent instrumentation.

Since the concentration in the samples was expected to be at least ten-fold higher than the calibration standards the analysed samples were diluted by mixing 100 µL of sample with 900 µL of AGW in the testing vials prior to analysis. The injected volume in the HPLC was small, 10 µL, so the pH of the buffer was expected to dominate over the pH of the sample. Because of this, the samples were diluted in water and not in buffer solution.

3.1.4 Bacterial strains

The bacterial strains used in the study were *Pseudomonas fluorescens* (CCUG 32456A, isolated 1993 from Hålö, Simpevarp, Sweden), *Pseudomonas stutzeri* (CCUG 36651, isolated 1993 from Äspö, Sweden), and *Shewanella putrefaciens* (CCUG 36599, isolated 1995 from LakeTranebärssjön, Sweden) and ordered for this project from The Culture Collection, University of Gothenburg, Sweden (HYPERLINK www.ccug.se). All of these three strains produce siderophores (Kalinowski et al. 2004).

Desulfovibrio aespoeensis (DSM 10631, isolated 1998 from Äspö, Sweden), was ordered for this project from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Germany (HYPERLINK https://www.dsmz.de/).

3.2 Aerobic subset

3.2.1 Aerobic growth with 20 mg L^{-1} uranine

The medium used for the experiment contained in g L^{-1}: NaCl, 7.0; $CaCl_2 \times 2H_2O$, 1.0; NH_4Cl, 1.5; KH_2PO_4, 0.2; $MgSO_4 \times 7H_2O$, 0.1; KNO_3, 3.05. After sterilization, Na-lactate (50 %), approximately 0.55 ml L^{-1} was added to the medium giving a final lactate concentration of 0.27 g L^{-1}. Uranine, 0.2 g L^{-1} dissolved in sterile AGW, was filtered through a sterile 0.2 µm 25 mm membrane filter and added to the medium to a final concentration of 20 mg L^{-1}, and pH adjusted to between 7.0 and 7.5 with NaOH (0.1 M and 1 M). The three bacterial strains were added with a sterile loop from pure cultures on agar slant tubes to approximately 100 ml medium in 250 or 300 ml Erlenmeyer flasks. Sterile controls were prepared with medium and uranine. The cultures were made in duplicates giving eight samples in total. Figure 3-1 shows the bright yellow/green colour of cultures and control with 20 mg L^{-1} uranine. Cultures were incubated on an orbital shaker at 160 rpm at room temperature (20 °C).

3.2.2 Aerobic growth with 200, 100, and 40 µg L^{-1} uranine, respectively

The medium used in the experiment was identical to the medium described in section 3.2.1. After sterilization, sodium-lactate (50 %), approximately 0.45 ml L^{-1} was added to the medium to a final lactate concentration of 0.23 g L^{-1}. Uranine, 0.2 g L^{-1}, dissolved in sterile AGW was filtered through a 0.2 µm 25 mm membrane filter and added to the medium to final concentrations of 200, 100 and 40 µg L^{-1} uranine. Four cultures with 75 ml medium in 250 or 300 ml Erlenmeyer flasks were prepared for each concentration. pH was adjusted to between 7.0 and 7.5. The inoculums were added with a sterile loop from a pure culture on slant agar tubes to the cultures. A control with medium and uranine was prepared for each concentration. An extra control was made with 200 µg L^{-1} uranine and sterile anoxic Millipore analytical grade water. Figure 3-2 shows cultures and control with 40 µg L^{-1} uranine. Figure 3-3 shows the cultures and control irradiated with UV-light, highlighting the strong fluorescent blue coloured pyoverdine produced by *Pseudomonas fluorescens*. The cultures were incubated at room temperature around 20 °C, on an orbital shaker at 160 rpm in the dark. At the end of the incubation, samples from 40 µg L^{-1} and 200 µg L^{-1} samples were divided into two portions of which one was filtered through a sterile 0.2 µM GTTP 13 mm isopore membrane filter.

Figure 3-1. *Growth cultures and control with 20 mg L^{-1} uranine. From left: the control, Shewanella putrefaciens, Pseudomonas stutzeri, and Pseudomonas fluorescens.*

Figure 3-2. *Growth cultures and control with 40 µg L^{-1} uranine. From left: Control, Shewanella putrefaciens, Pseudomonas stutzeri, and Pseudomonas fluorescens.*

Figure 3-3. *Growth cultures and control with 40 µg L^{-1} uranine irradiated with UV-light, demonstrating pyoverdine production. From left: Control, Shewanella putrefaciens, Pseudomonas stutzeri, and Pseudomonas fluorescens.*

To analyse if the bacteria produced substances that could interfere with the fluorescence measurements, control cultures without uranine were prepared. Four samples were prepared with 75 ml media in 250 or 300 ml Erlenmeyer flasks and pH adjusted to between 7.0 and 7.5. Bacteria were added from cultures on slanting agar tubes using a sterile loop. The cultures were incubated, on an orbital water shaker at 160 rpm at around 22 °C.

3.2.3 Aerobic growth with 100 µg L^{-1} uranine in plastic or glass flasks

Approximately 0.5 mL *P. fluorescens* was added from a pure-culture with > 10^6 cells mL^{-1}, to glass and plastic Erlenmeyer flasks containing growth medium. Uranine, 0.2 g L^{-1} dissolved in sterile water and filtered through a sterile 0.2 µm 25 mm membrane filter, was added to the glass and plastic flasks to a final concentration of approximately 100 µg L^{-1} uranine. Cultures were incubated at room temperature (20 °C), on an orbital shaker at 115 rpm. Bacterial samples and sterile controls with uranine were covered with aluminium foil. The experiment was repeated twice since the results from the first one were difficult to interpret. The second and third experiments followed the outline of the first.

3.3 Anaerobic subset

3.3.1 Anaerobic growth – nitrate reducing bacteria (NRB)

The basal medium used for anaerobic microbial degradation the study of uranine by NRB contained in g L^{-1}: NaCl, 7.0; $CaCl_2 \times 2H_2O$, 1.0; KCl, 0.1; NH_4Cl, 1.5; KH_2PO_4, 0.2; $MgCl_2 \times 6H_2O$, 0.1; $MgSO_4 \times 7H_2O$, 0.1; $Na_2MoO_4 \times 2H_2O$, 0.001; and $MnCl_2 \times 4H_2O$, 0.005. After sterilization, the medium was made anoxic by gassing with a mixture of nitrogen (80 %) and carbon dioxide (20 %).

After cooling, additions of heat sensitive solutions, such as KNO_3 and vitamin solutions, were added to the medium as described in Hallbeck and Pedersen (2008). As carbon and energy sources, sodium lactate (50 %), approximately 0.36 mL L^{-1} and sodium acetate, approximately 10 mL L^{-1} were added to the medium, receiving a final lactate and acetate concentration of 0.20 g L^{-1} and 0.72 g L^{-1}, respectively. The pH was adjusted with NaOH or HCl (1M) to between 6.5 and 7.5. Approximately 50 mL medium was added to 120 mL sterile anaerobic butyl rubber-stopped bottles with syringes.

3.3.2 Anaerobic growth – sulphate-reducing bacteria (SRB)

The basal medium for the anaerobic microbial degradation study of uranine by SRB contained in g L^{-1}: NaCl, 7.0; $CaCl_2 \times 2H_2O$, 1.0; KCl, 0.67; NH_4Cl, 1.0; KH_2PO_4, 0.15; $MgCl_2 \times 6H_2O$, 0.5; $MgSO_4 \times 7H_2O$, 3.0. The procedure after sterilization was identical to the medium described in section 3.3.1, except that no sodium-acetate was added.

3.3.3 Anaerobic growth – experimental set up

The cultures were prepared with growth medium, bacteria and uranine. The positive controls were growth medium with added bacteria, negative controls had growth medium with sterile-filtered bacterial cultures and the sterile controls were made with growth medium, uranine and sterile-filtered cultures.

P. stutzeri and *D. aespoeensis* were added from anaerobic pure cultures with > 10^6 cells mL^{-1}, approximately 0.5 mL from the culture to the anaerobic glass tubes with media for NRB or SRB (Sections 3.3.1and 3.3.2). Uranine, 0.2 g L^{-1} dissolved in sterile water and filtered through a sterile 0.2 µm 25 mm membrane filter was added to the anaerobic glass tubes to final concentrations of approximately 40, 100, and 200 µg L^{-1} uranine. Cultures were incubated at room temperature, around 20 °C. The bottles with uranine were covered with aluminium foil to be kept in the dark.

3.4 Analyses

3.4.1 Uranine

The samples with 20 mg L^{-1} uranine were centrifuged at 20 000 × g, the supernatant was diluted 10 times in AGW and pH was adjusted to 9.2–9.5 with NaOH (0.1 M and 1 M). The samples were measured in duplicates at room temperature in daylight, with λ_{ex} = 490 nm, slit = 5 nm, 250V in disposable cuvettes.

Samples with 200, 100, and 40 µg L^{-1} uranine were centrifuged at 20 000 × g, the supernatants were diluted 2, 5, or 10 times in 0.2 M Tris(hydroxymethyl) aminomethane-buffer (hereafter Tris-buffer) and pH was adjusted to pH 9.0 with 1 M HCl; to a final concentration of 20 µg L^{-1}. The samples were prepared at room temperature in light but kept dark until analysis. Duplicates were measured using disposable or quartz cuvettes with λ_{ex} = 490 nm, slit = 5 nm, 400V and emission was scanned from 400 to 700 nm.

3.4.2 Pyoverdine

Pyoverdines were measured simultaneously with uranine in the *P. fluorescens* cultures with 200, 100, and 40 µg L^{-1} uranine. However, pyoverdine was analysed at different settings compared to the uranine with λ_{ex} = 400 nm, slit = 5 nm, 400V; and emission scanned from 350 to 600 nm.

3.4.3 Total number of cells

The acridine orange direct count (AODC) method was used to measure the total number of cells (TNC). A portion of the sample was filtered onto a 0.2 µM 13 mm black Nuclepore filter at −20 KPa and stained for 5–7 minutes with 0.1 % acridine orange solution at pH 7.2. The number of cells was counted under blue light (390–490 nm) in an epifluorescence microscope (Pedersen and Ekendahl 1990).

3.4.4 Lactate

Lactate concentration was determined with an enzymatic UV absorbance method (Kit # 10139084035; Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany) and a Genesys 10 UV spectrophotometer (Thermo Fischer Scientific, Waltham, MA) following the instructions from the manufacturer.

3.4.5 Acetate

Acetate concentration in the NRB series was measured with an enzymatic UV absorbance method (Kit # 10148261035; Boehringer Mannheim/R-Biopharm AG, Darmstadt, Germany) and a Genesys 10 UV spectrophotometer (Thermo Fischer Scientific, Waltham, MA) following the instructions from the manufacturer.

3.4.6 Sulphide

Sulphide production in the SRB samples was analysed using $CuSO_4$ (5mM) and a Genesys 10 UV spectrophotometer as described by Widdel and Bak (1992).

3.4.7 Nitrate

Nitrate-reduction in the NRB samples was determined by the measurements of NO_3^- concentration with the chromotrophic acid method no. 10020 (Test'N Tube™ NitraVer® X Nitrate; HACH Lange AB, Sköndal, Sweden) and a HACH DR/2500 Odyssey spectrophotometer following the instruction from the manufacturer.

3.5 Groundwater study

The stability test was done with groundwater from six borehole sections in six different boreholes and the purpose was also to check if the groundwater composition could have an impact on possible degradation of uranine. The sampled borehole sections are listed in Table 3-2 together with flow rate, pH, temperature and electrical conductivity (EC) measured in the field.

Table 3-2. Sampled borehole sections and measured flow rate, pH, water temperature and electrical conductivity in the field.

Idcode:section no.	Section (m b l)**	Flow rate (L min^{-1})	pH-field*	Temperature-field* (°C)	EC-field* (mS/m)
KA2862A:1	0–15.98	1.06	7.88/7.89	13.6/13.6	3690/3690
KA2051A01:9	51–67	1.89	7.48/7.48	12.4/12.4	820/820
KA3385A:1	32.05–34.18	0.42	7.49/7.49	14.7/14.7	2010/2008
SA2600A:1	1.35–19.4	3.32	7.57/7.58	13.8/13.9	3120/3130
HD0025A:1	0–15	6.72	7.35/7.42	14.5/14.3	1361/1394
KA3105A:4	17.01–19.51	3.36	7.38/7.45	15.0/14.7	749***

* Two separate measurements.
** m b l = Metre borehole length.
***Only one measurement was available.

The groundwater samples were collected in 5 L cans. All cans and bottles were washed with acid (10 % super pure HNO_3), rinsed six times with reagent grade water, dried and sterilised in an autoclave prior to use. Three 100 mL portions of each sample were saved as blank samples (two portions in brown glass bottles and one in a brown plastic bottle). Subsequently, uranine was added to a theoretical concentration of approximately 200 µg L^{-1}. Possible earlier uranine addition, for example during drilling of the boreholes, was disregarded. Each groundwater sample was then divided into 12 100 mL portions in brown glass bottles and one portion in a brown plastic bottle. All the bottles were stored in the dark at room temperature prior to analyses.

The analyses included pH (ISO 10523), EC (ISO 27888) and uranine (Uraninbestämning med Spectrofluorimeter SKB MD 452.017, Internal controlling document). The blanks were analysed at start and the samples with uranine additions were analysed after 0, 2, 4, 7, 14 and 21 days.

The method used for the uranine analyses implies measuring the fluorescence of the samples at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. A RF-1501 Shimadzu Spectrofluorophotometer was used for the measurements and the instrument was configured to auto-shutter, which prevents unnecessary degradation. One droplet of 1 M NaOH solution was added to the samples prior to the analyses in order to assure high enough pH (≥ 9).

In addition to the uranine experiment, samples for regular groundwater monitoring were collected from the listed borehole sections, see Table 3-2, and relevant groundwater composition data are available for the groundwater in question.

4 Results

4.1 Laboratory study

4.1.1 pH

To investigate the influence of pH on uranine fluorescence, the measurements at different pH were carried out and results are shown at Figure 4-1. Highest fluorescence intensity was achieved when pH was above 9 and the highest emission intensity was found at around a wavelength of 516 nm, data not shown.

To investigate if the pH dependence was the same in lower concentrations, measurements of fluorescence at four different pH, 7, 8, 9 and 10, and with four different concentrations, 2, 0.2, 0.02 and 0.002 mg L^{-1}, were made and the results are shown at Figure 4-2. The pH range, where uranine had the highest fluorescence intensity was chosen based on the previously obtained results. It can be seen that the most pronounced difference in intensity was found with 2 mg L^{-1} uranine (Figure 4-2) and that the maximum emission wavelength varied between 514 and 516 nm depending on the concentration of uranine (Figure 4-2b).

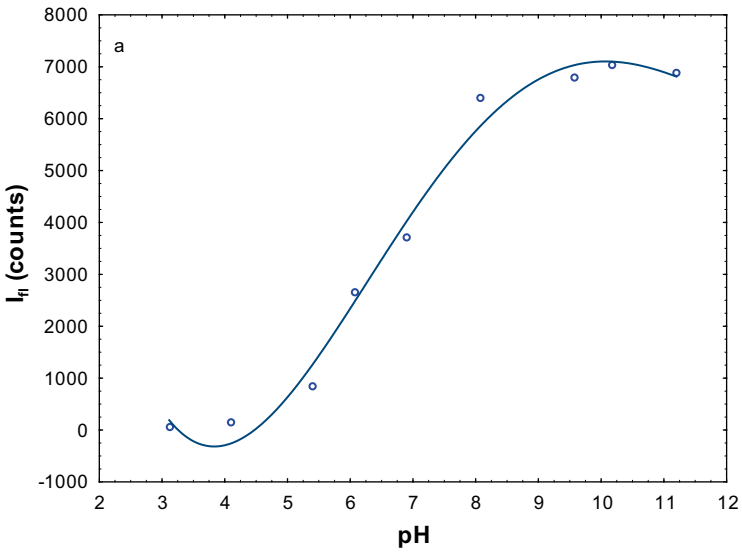


Figure 4-1. *The influence of pH on the emission intensity on uranine fluorescence. The solid line is the results of calculations using polynomial quartic fit model.*

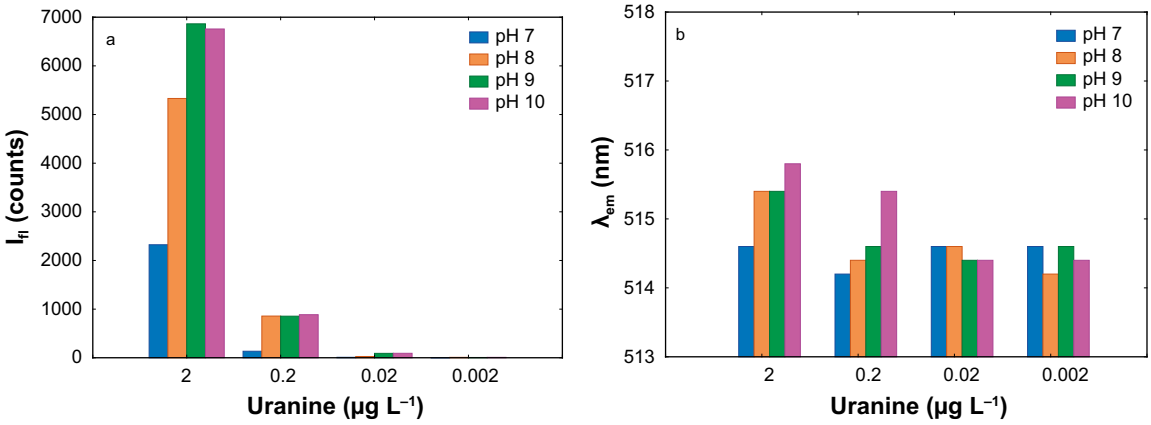


Figure 4-2. *Emission intensity (a) and maximum emission wavelengths (b) at different pH for different uranine concentrations.*

Fluorescence of pyoverdines was measured in the *P. fluorescence* cultures at different pH in the range from 3 to 12 and showed no significant relation to pH (no data shown).

Measurement uncertainty of the method was estimated to 3 % and the limit of detection was 0.1 µg L^{-1}. Two standard curves were made for uranine at different conditions. The first standard curve was prepared in Tris-buffer with uranine concentrations in the range of 20–400 µg L^{-1}. The fluorescence was measured using standard settings (λ_{ex} = 490 nm, λ_{em} = 514 nm, $slit_{ex}$ = 5 nm) and the standard curve is shown in Figure 4-3. It was quite difficult to reach satisfactory results for the low concentrations with the original settings (λ_{ex} = 490 nm, λ_{em} = 514 nm, $slit_{ex}$ = 5 nm) because of strong emission at 490 nm where system peak overlapped the peak of uranine. For that reason, measurements of sample with 1 µg L^{-1} uranine were done at different λ_{ex} and with different slit sizes and the optimal settings for fluorescent measurements at low concentrations were chosen to be λ_{ex} = 475 nm, λ_{em} = 514 nm, $slit_{ex}$ = 5 nm and $slit_{em}$ = 10 nm. A standard curve was after that prepared in the concentration range 0.2–50 µg L^{-1} (Figure 4-4).

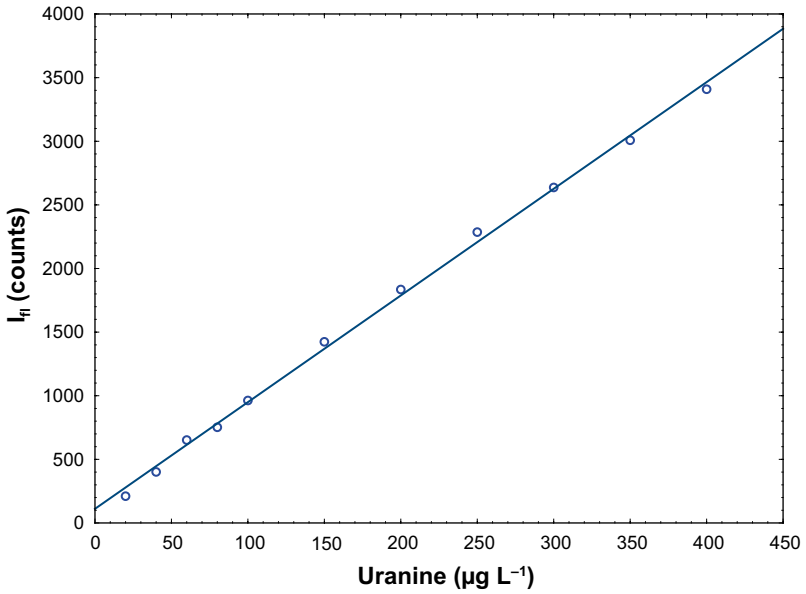


Figure 4-3. *Standard curve for uranine up to 400 µg L^{-1}.*

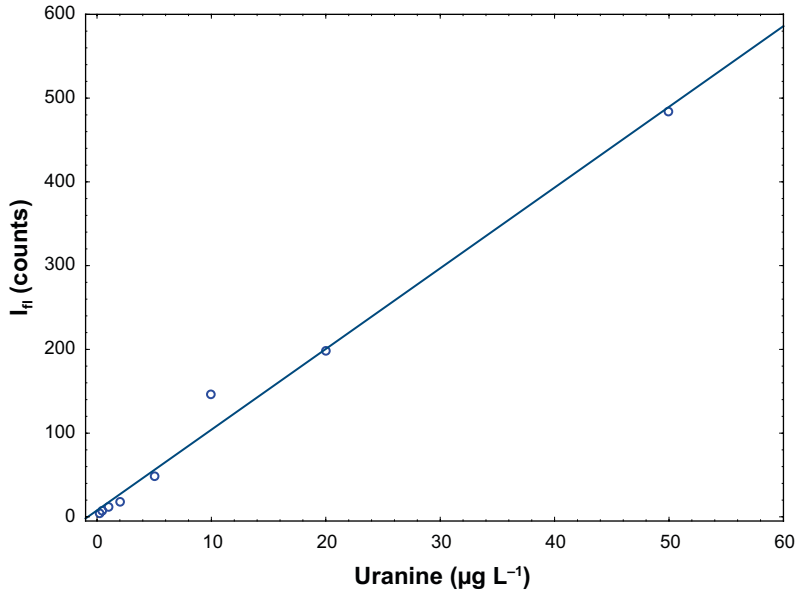


Figure 4-4. *Standard curve for uranine up to 50 µg L^{-1}.*

4.2 Aerobic growth

4.2.1 20 mg L^{-1} uranine

To study possible bacterial influence on the fluorescence measurements of uranine, two growth cultures of each bacterial strain were prepared. The concentration of uranine was 20 mg L^{-1} in growth medium with 0.27 mg L^{-1} lactate. As negative controls medium with 20 mg L^{-1} uranine but without bacteria were used. The emitted light was measured using a fluorescent spectrometer with excitation wavelength 490 nm; slit 5 nm, and voltage 250 V. The mean values of fluorescence intensity from duplicate samples are shown in Figure 4-5. Because of the lower voltage used in this experiment, compared to the 400 V used in the measurements of the following experiments and the standard curves, the emitted intensity is lower as can be seen in Figure 4-5.

The emitted light mean value in both cultures and control increased from day 21 to day 37. The control without bacteria, diluted in Millipore-water and pH adjusted to 9.2–9.5 with NaOH (0.1 M and 1 M) showed an increase with 544 counts as shown in Figure 4-5. Fluorescence intensity in the *S. putrefaciens* cultures increased with 516 counts, the *P. stutzeri* cultures with 484 counts and the fluorescence intensity in the *P. fluorescens* cultures increased 637 counts.

4.2.2 40, 100 and 200 µg L^{-1} uranine

To examine whether the fluorescence also increases at low uranine concentrations, sample series with each bacterial strain were prepared with 40 µg L^{-1}, 100 µg L^{-1} and 200 µg L^{-1} uranine in 0.23 g L^{-1} lactate medium. Negative controls with medium and uranine were made for each concentration. An extra control with 200 µg L^{-1} uranine in sterile water was also prepared, here called *Uranine*. All cultures and controls were made in duplicates and mean values were calculated. The emitted light was measured using fluorescence spectrometry with excitation wavelength 490 nm, slit 5 nm, and voltage 400 V. To study if the presence of cells in the samples could increase the fluorescence intensity, samples were filtered and fluorescence was measured from day 46. In addition, HPLC analyses were made from day 49 to investigate if the increase in fluorescence were caused by other substances than uranine. Due to the time needed for instrument setup and method development only samples collected after 49, 54 and 57 days could be analysed with HPLC.

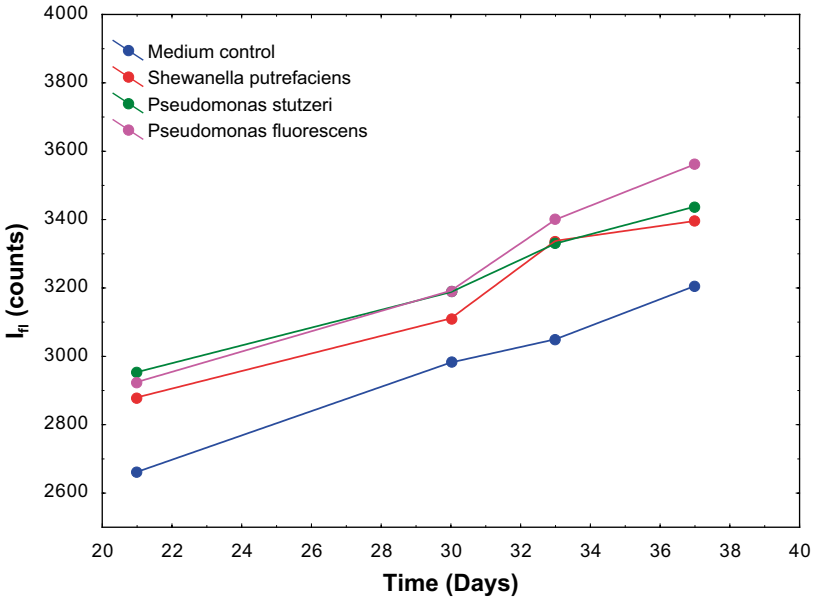


Figure 4-5. *Fluorescence intensity in growth experiment with 3 different bacterial strains and medium control with 20 mg L^{-1} uranine measured with a fluorescence spectrophotometer.*

The fluorescence measured in HPLC is not an absolute measurement since the result depends on, for example variations in atmospheric pressure, flow variation due to wearing of the pumps and temperature effects. The instrument was therefore calibrated before each analysis with standard solutions in the concentrations 20, 10, 5 and 1 µg L^{-1} uranine.

The fluorescence intensity measured with fluorescent spectrophotometer was recalculated to the unit µg L^{-1} uranine, shown in Figure 4-6 to Figure 4-8 using the standard curve presented in Figure 4-3. It is important to remember that it is the fluorescence intensity that is measured and not necessarily an actual increase in uranine concentration.

In the experiment with 40 µg L^{-1} uranine, all cultures with the three tested bacteria, showed an increase in fluorescence intensity corresponding to between 67 µg L^{-1} for *S. putrefaciens* to 92 and 93 µg L^{-1} for *P. stutzeri* and *P. fluorescens*, respectively. The increase started after day 30, see Figure 4-5.

The HPLC measurements showed that the *S. putrefaciens* cultures had a fluorescence intensity corresponding to 82 µg L^{-1} uranine on day 49 and 87 µg L^{-1} by day 57. The intensity corresponded in *P. stutzeri* samples to 157 µg L^{-1} on day 49 and 171 µg L^{-1} on day 54. *P. fluorescens* samples had an intensity corresponding to 106 µg L^{-1} day 49 and 136 µg L^{-1} by day 57. The filtered portion of *P. fluorescens*, measured on HPLC decreased from 58 on day 49 to 48 µg L^{-1} by day 57.

The negative control, measured on fluorescent spectrophotometer, showed a slight increased from 32 µg L^{-1} on day 1 to 35 µg L^{-1} on day 29. This sample was not measured on HPLC, since it unfortunately became contaminated after 30 days of incubation.

The results from the HPLC analysis verified the elevated fluorescence measured in the samples. The results are similar to the results from the fluorescent spectrophotometer. The differences might be due to the dilution steps needed and the fact that the samples were exposed for light during the analysis run that lasted up to ten hours. To elucidate if the presence of cells or other substances could increase the fluorescence intensity filtered samples also were analysed on HPLC.

The recalculated fluorescence data from the bacterial cultures with 100 µg L^{-1} uranine, measured on fluorescent spectrophotometer, is shown in Figure 4-7. The fluorescence intensity, recalculated to the corresponding amount of uranine, increased in the *S. putrefaciens* culture in total around 100 µg L^{-1} during the 57 days. The concentration *in P. stutzeri* cultures increased 80 µg L^{-1} and *P. fluorescens* cultures with 100 µg L^{-1} uranine, the concentration increased in total 95 µg L^{-1} by day 57.

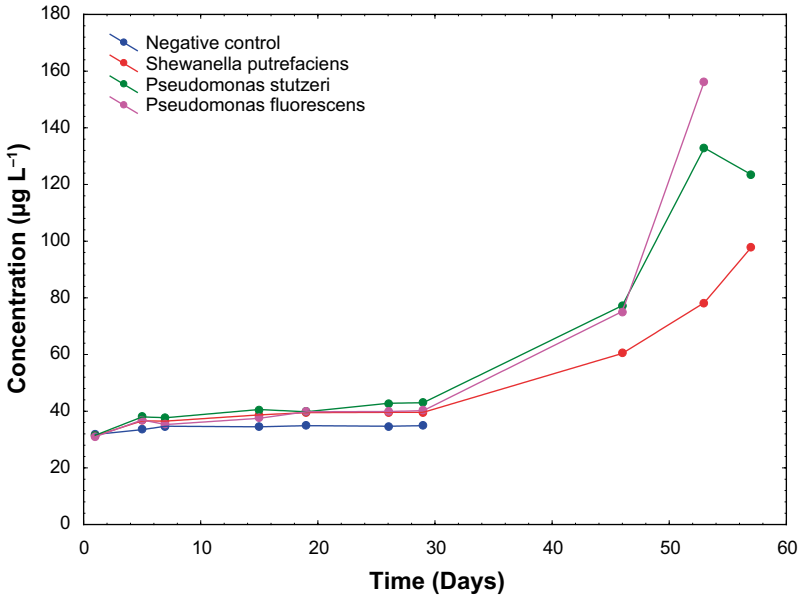


Figure 4-6. *The recalculated fluorescence results obtained in the growth experiment with 3 different bacterial strains and negative control with 40 µg L^{-1} uranine measured using a fluorescent spectrometer.*

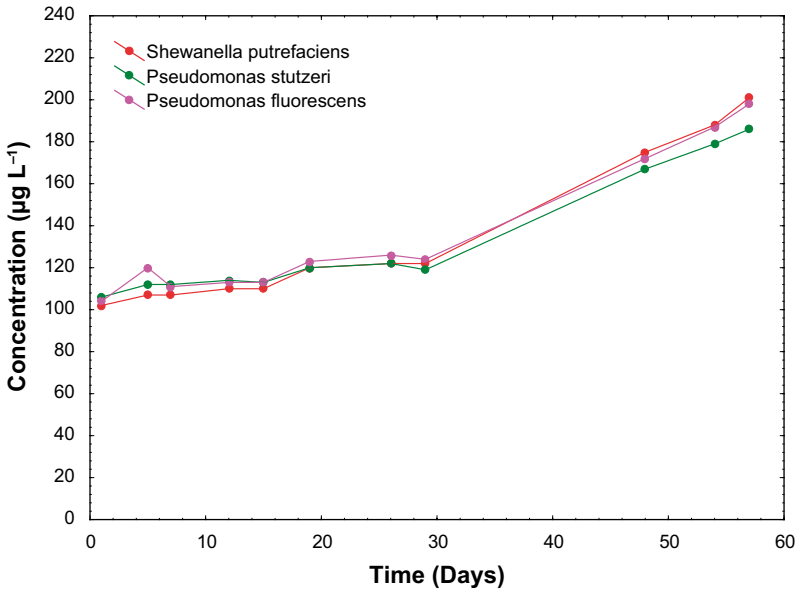


Figure 4-7. *The recalculated fluorescence results obtained in the growth experiment with three bacterial strains and 100 µg L^{-1} uranine measured with fluorescence spectrophotometer.*

The concentration in *S. putrefaciens* cultures, measured with HPLC increased from 150 µg L^{-1} on day 49 to 165 µg L^{-1} by day 57. The concentration in *P. stutzeri* cultures increased from 149 µg L^{-1} on day 49 to 155 µg L^{-1} by day 57and in *P. fluorescens* cultures the concentration decreased from 230 µg L^{-1} on day 1 to 160 by day 54, ending at 168 µg L^{-1} by day 57. No data are shown for the negative control with 100 µg L^{-1} uranine, due to contamination.

The *S. putrefaciens* sample with 200 µg L^{-1} uranine increased from 216 µg L^{-1} on day 1 to 483 µg L^{-1} by day 57, an increase of 267 µg L^{-1} as shown in Figure 4-8. The *P. stutzeri* sample, measured on fluorescent spectrophotometer increased 176 µg L^{-1} on the same time. The concentration in the *P. fluorescens* sample increased from 213 µg L^{-1} on day 1 to 461 µg L^{-1} by day 57, an increase of 248 µg L^{-1}. The concentration in the extra control sample, named Uranine, shown in Figure 4-8, measured on fluorescent spectrophotometer, increased from 220 µg L^{-1} on day 1 to 252 µg L^{-1} by day 57.

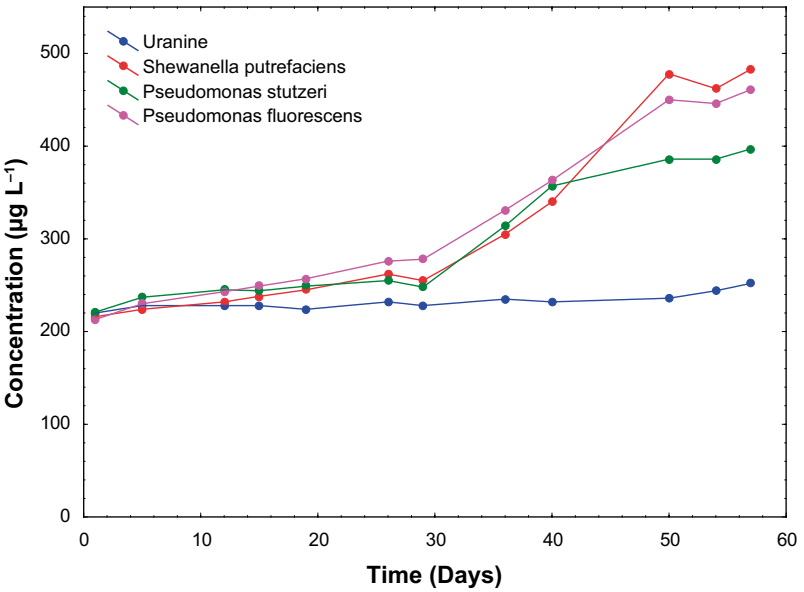


Figure 4-8. *The recalculated fluorescence results obtained in the growth experiment with 3 different bacterial strains and extra control, Uranine with 200 µg L^{-1} uranine measured using a fluorescence spectrophotometer.*

The uranine concentration in the Uranine control measured on HPLC, increased from 190 µg L^{-1} on day 49 to 203 µg L^{-1} by day 54, ending at 194 µg L^{-1} by day 57. The filtered portion of the uranine control increased from 137 µg L^{-1} on day 1 to 146 µg L^{-1} by day 54, ending at 142 µg L^{-1} by day 57. In *S. putrefaciens* cultures he concentration decreased from 511 µg L^{-1} on day 49 to 389 µg L^{-1} by day 57. The *P. stutzeri* sample decreased from 438 µg L^{-1} on day 49 to 320 µg L^{-1} by day 57. Concentration in *P. fluorescens* decreased from 473 µg L^{-1} on day 49 to 385 µg L^{-1} on day 57.

In all cultures, the concentration started to increase with a higher rate from day 28 compared to the much lower increase the first 28 days of incubation, see Figure 4-6, Figure 4-7 and Figure 4-8.

4.2.3 Aerobic growth without uranine

To investigate if also *S. putrefaciens and P. stutzeri* synthesized fluorescent substances that could interfere with the uranine measurements, cultures without uranine were prepared with medium and bacteria added from cultures on slanting agar tubes. Fluorescence in samples was measured at excitation wavelength 490 nm, at 700 V and at pH 7.

There was no fluorescens in these cultures that could interfere with uranine. Fluorescence intensity in the *S. putrefaciens* cultures changed from 70 on day 28 to 61 by day 37. The *P. stutzeri* fluorescence intensity changed from 67 on day 28 to 56 by day 37.

4.2.4 Pyoverdines

Pyoverdines produced by Pseudomonas fluorescens

In the cultures without uranine, which were prepared to study if the bacteria produced substances that could interfere with the uranine measurements, pyoverdines were measured in these *P. fluorescens* cultures. The measurements were done at pH 7 and at the settings for pyoverdine measurements (λ_{ex} = 400 nm, slit = 5 nm, 400V).

Pyoverdine fluorescence intensity in the *P. fluorescens* sample without uranine is shown in Figure 4-9. There was a slight decrease in intensity from day 1 to day 26 in the cultures. There was no large difference between non-centrifuged and centrifuged pyoverdine samples from day 23 to day 26.

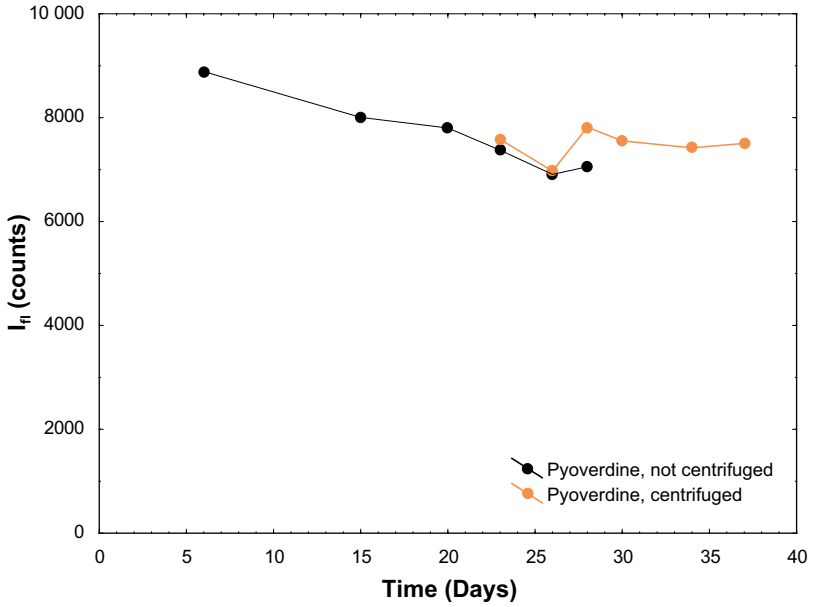


Figure 4-9. *Pyoverdine fluorescence intensity in Pseudomonas fluorescens culture without uranine.*

Pyoverdines produced by Pseudomonas fluorescens with 40, 100 and 200 µg L^{-1} uranine

In the *P. fluorescens* cultures with 40, 100, and 200 µg L^{-1} uranine, pyoverdines were measured simultaneously, diluted in Tris-buffer and at excitation wavelength 400 nm.

Pyoverdine fluorescence intensity in the *P. fluorescens* cultures with 40 µg L^{-1} uranine shown in Figure 4-10, increased from 6888 on day 5 to 7432 by day 26 when the fluorescence intensity rapidly increased to 17 902 by day 53. The emitted light of pyoverdines in *P. fluorescence* sample with 100 µg L^{-1} uranine increased from 8590 on day 5 to 16 227 by day 57. Pyoverdine intensity in the 200 µg L^{-1} uranine sample increased from 9225 on day 5 to 10 865 by day 26 when the intensity spiked to 20 503 by day 54, ending at 20 690 by day 57.

4.2.5 TNC

To control if fluorescence changes were related to cell growth, the total number of cells were counted in samples with 20 mg L^{-1}, 40, 100 and 200, µg L^{-1} uranine using the acridine orange direct count method. The results are presented in Appendix, Section A1.

4.2.6 Lactate

Lactate concentration was determined in cultures with 20 mg L^{-1}, 40, 100, and 200 µg L^{-1} uranine using an enzymatic UV absorbance method. The results are presented in Appendix, Section A2.

4.2.7 HPLC

Analysis of Pseudomonas fluorescens samples

In the HPLC analysis of uranine an excitation wavelength of 490 nm and an emission wavelength of 515 nm were used. It was clear from the analyses that other substances than uranine, with similar fluorescence characteristics were present in the cultures with *P. fluorescens*. Figure 4-11 and Figure 4-12 show a chromatogram from a *P. fluorescens* culture compared to a chromatogram from a uranine standard. In the beginning of the chromatogram from the *P. fluorescens* culture, there are several peaks originating from substances that are more water soluble than uranine. Since the area under the curve represents the total fluorescence from the sample at the selected wavelengths, it is clear that the detected substances interfere with measurements on a fluorescence spectrophotometer.

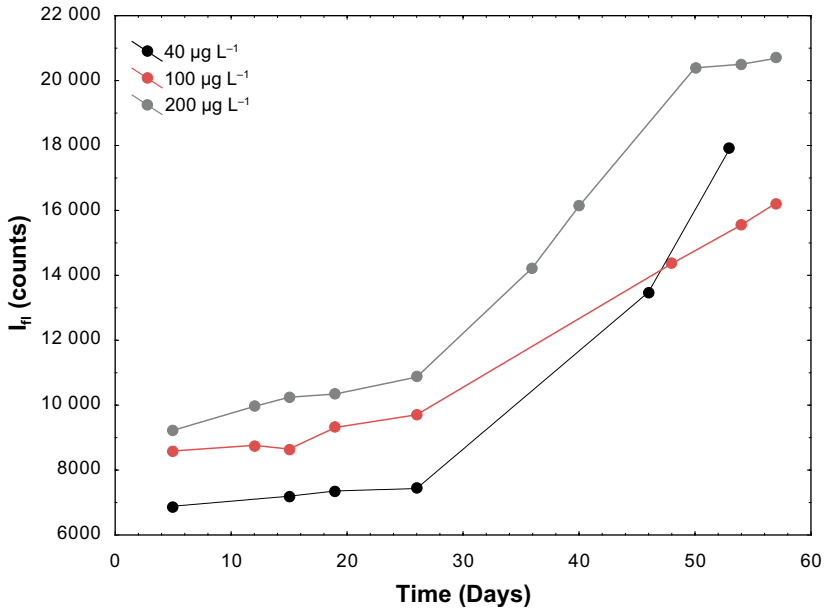


Figure 4-10. *Pyoverdine fluorescence intensity in Pseudomonas fluorescens cultures with 200, 100, and 40 µg L^{-1} uranine.*

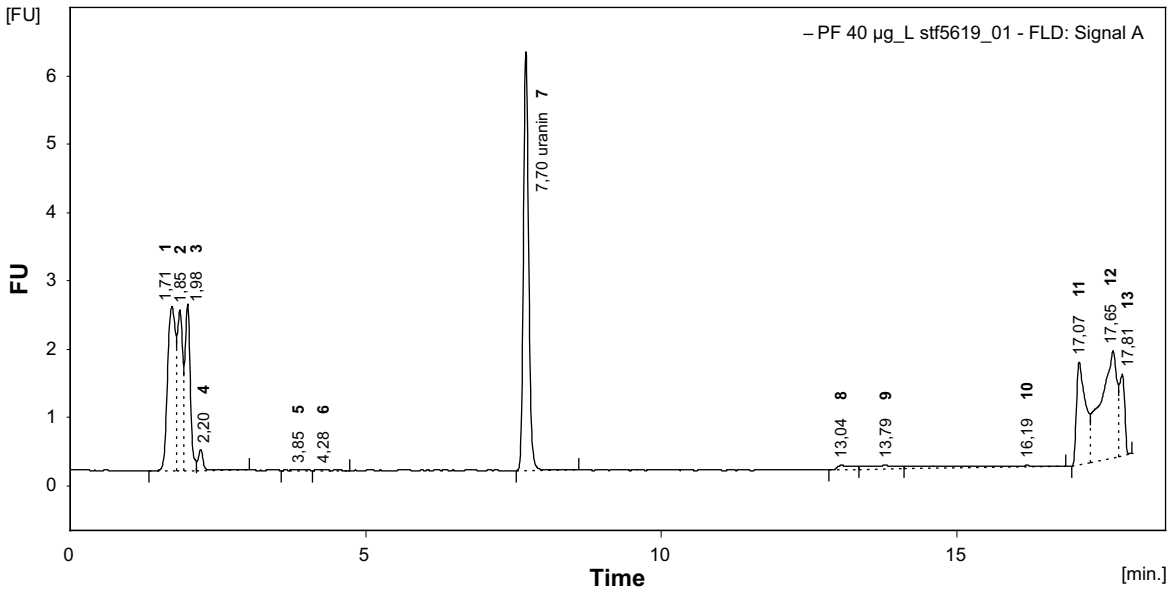


Figure 4-11. *Chromatogram from a Pseudomonas fluorescens culture with 40 µg L^{-1} uranine.*

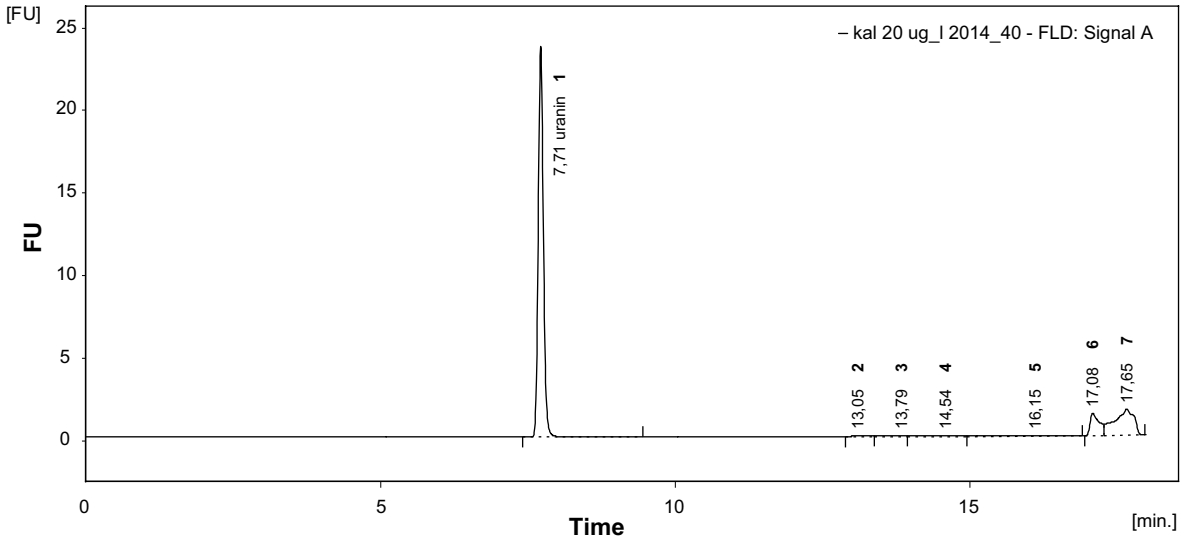


Figure 4-12. *Chromatogram from standard containing 20 µg L^{-1} uranine.*

Samples from cultures of *P. stutzeri* and *S. putrefaciens* did not show any extra peaks in the chromatograms and were very similar to the standard solution chromatogram.

It should be noted that the extra peaks in the end of the chromatogram probably are an artefact from the final flush with 99 % of organic solvent in each run. They were present also in analysis of pure MP water.

The gradient used for the analysis was optimized for the detection of uranine only. Peaks at the beginning and at the end of the chromatogram elute under steep gradients and were not resolved. It is possible to resolve these peaks as well but at the cost of a significantly longer analysis time.

Analysis of samples without uranine

In one of the three analyses on HPLC, centrifuged samples from cultures of the three bacterial strains were analysed to verify that no substances originating from the bacteria only, could interfere with the peak for uranine. In these chromatograms very small peaks could be detected at the retention time for uranine, but after quantification the obtained concentrations were less than 0.1 µg L^{-1}. The small peaks could be due to carry-over between injections on the HPLC.

AGW was analysed at the start of the measurement sequence and no peaks could be detected at the retention time for uranine.

Controls with known uranine concentration

Samples with 200 µg L^{-1} uranine were analysed in each sequence as external control of the analysis. These samples showed a good correlation with the expected concentration.

Effect of filtration

Filtrated samples generally contained less uranine than unfiltered samples of the same concentration. There might be a problem with uranine adsorbing to the filter or uranine might coordinate to larger complexes in the sample that are retained on the filter.

4.3 Anaerobic growth with 40, 100, and 200 µg L^{-1} uranine

4.3.1 Nitrate reducing bacteria – NRB

In order to study anaerobic microbial degradation of uranine a NRB, *P. stutzeri* was used. All cultures were made in duplicates and mean values of each culture, for example the positive controls, was calculated and presented in Figure 4-13 – Figure 4-15. The positive and negative controls showed no fluorescence. Bacterial cultures and sterile controls had 40, 100, or 200 µg L^{-1} uranine in each series and all sterile control were amended with sterile-filtered cultures. Uranine concentrations were determined by fluorescence measurements on a fluorescent spectrophotometer and with HPLC as described in Material and Methods.

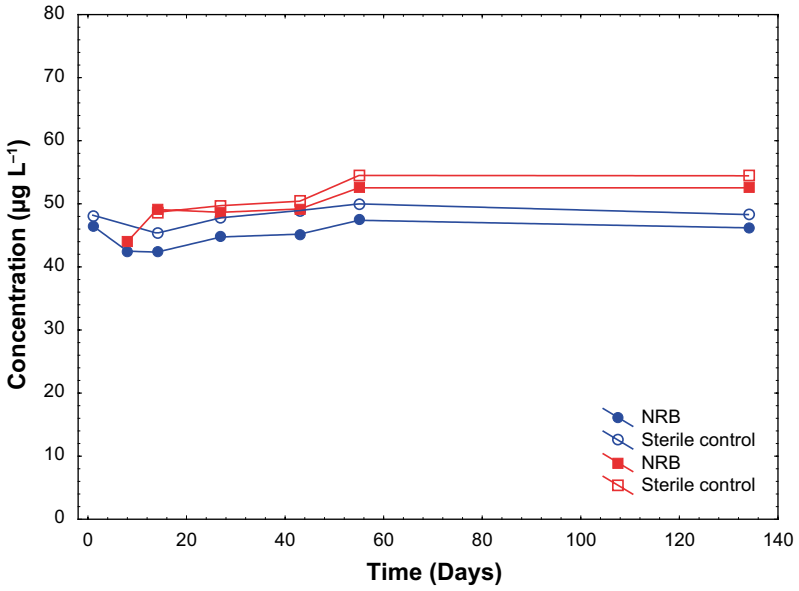


Figure 4-13. *Fluorescence measured on a fluorescence spectrophotometer (●; ○) and HPLC (■; □). The fluorescence intensity was recalculated amount of uranine with a standard curve and mean values of P. stutzeri cultures and sterile controls with 40 µg L^{-1} are shown.*

40 µg L^{-1} uranine

The results from this experiment are presented in Figure 4-13. The samples were measured both with fluorescence spectrophotometry and HPLC. The uranine concentration both in bacterial cultures with 40 µg L^{-1} uranine and controls, analysed by fluorescence spectrophotometry, stayed almost stable during the incubation of 134 days. In addition, the HPLC data showed stable data for this experiment, although somewhat higher than with fluorescence spectrophotometry.

100 µg L^{-1} uranine

The fluorescence in the bacterial cultures and controls with 100 µg L^{-1} uranine stayed also relatively stable during the incubation of 134 days, both when measured with fluorescent spectrophotometry and HPLC as seen in Figure 4-14.

200 µg L^{-1} uranine

Also the fluorescence in the bacterial cultures and controls with 200 µg L^{-1} uranine stayed also here stable during the incubation of 134 days, both when measured with fluorescence spectrophotometry and HPLC as seen in Figure 4-15.

4.3.2 NRB: Chemical analysis

Lactate

Cultivation medium was prepared with approximately 0.36 mL L^{-1} Na-lactate (50 %), giving a final concentration of 0.2 g L^{-1} lactate in the cultures. Lactate concentration was measured, as shown in Table 4-1, using an enzymatic UV absorbance method on day 1 and day 55 for each concentration.

The lactate concentrations in the positive controls and NRB cultures decreased from approximately 0.23 g L^{-1} on day 1 to 0.18 g L^{-1} by day 55. The negative controls and sterile controls showed no decrease in lactate concentration during the incubation. The mean lactate consumption in the NRB cultures was 0.048 g L^{-1}.

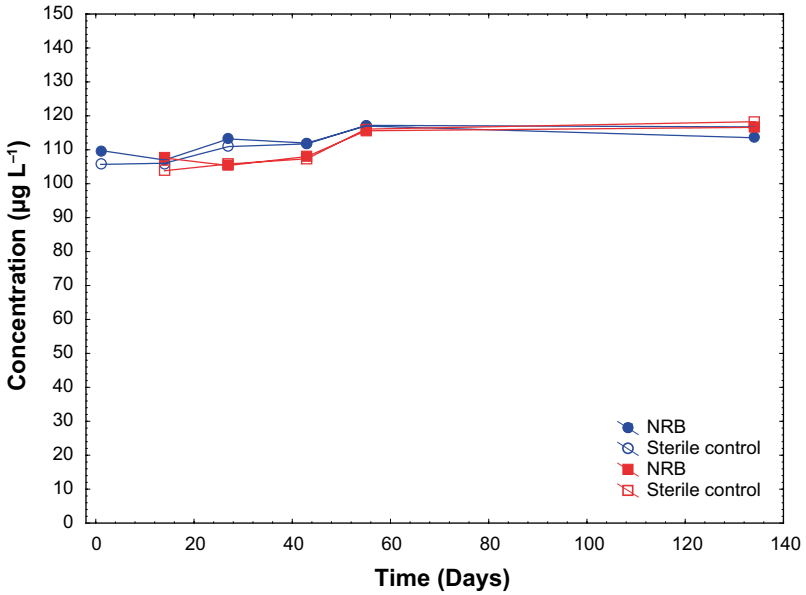


Figure 4-14. *Fluorescence measured on a fluorescence spectrophotometer (●; ○) and HPLC (■; □). The fluorescence intensity was recalculated amount of uranine with a standard curve and mean values of P. stutzeri cultures and sterile controls with 100 µg L^{-1} are shown.*

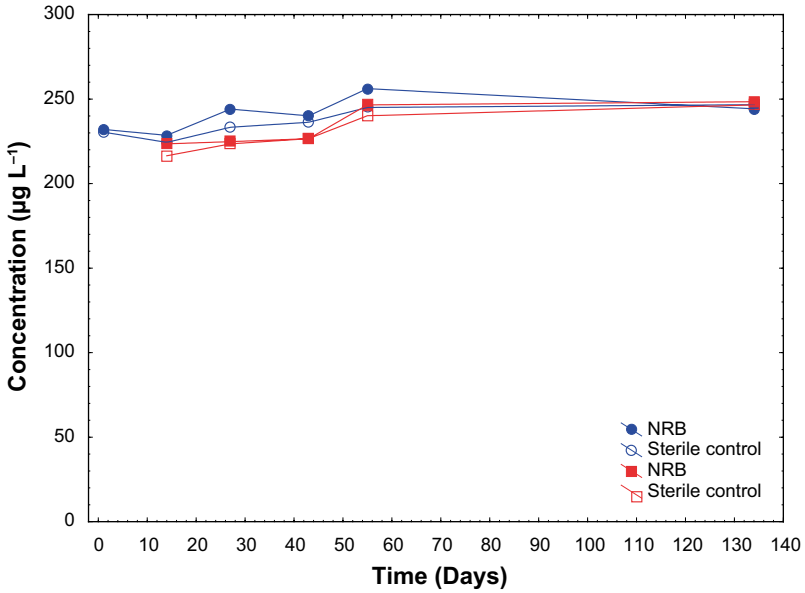


Figure 4-15. *Fluorescence measured on a fluorescence spectrophotometer (●; ○) and HPLC (■; □). The fluorescence intensity was recalculated amount of uranine with a standard curve and mean values of P. stutzeri cultures and sterile controls with 200 µg L^{-1} are shown.*

Table 4-1. Lactate concentration in NRB-cultures with 40, 100, and 200 µg L^{-1} uranine, analysed at day 1 and 55, using an enzymatic UV absorbance method.

Sample	Lactate g L^{-1} day 1	Lactate g L^{-1} day 55
Positive control: 1	0.22	0.18
Positive control: 2	0.24	0.18
Negative control: 1	0.23	0.22
Negative control: 2	0.23	0.22
Sterile control: 1 (40 µg L^{-1})	0.23	0.23
Sterile control: 2 (40 µg L^{-1})	0.23	0.23
Sterile control: 1 (100 µg L^{-1})	0.23	0.22
Sterile control: 2 (100 µg L^{-1})	0.22	0.22
Sterile control: 1 (200 µg L^{-1})	0.22	0.23
Sterile control: 2 (200 µg L^{-1})	0.23	0.23
NRB: 1 (40 µg L^{-1})	0.23	0.18
NRB: 2 (40 µg L^{-1})	0.23	0.18
NRB: 1 (100 µg L^{-1})	0.22	0.19
NRB: 2 (100 µg L^{-1})	0.23	0.18
NRB: 1 (200 µg L^{-1})	0.23	0.18
NRB: 2 (200 µg L^{-1})	0.23	0.18

Acetate

Approximately 10 mL L^{-1} Na-acetate was added to the growth medium, giving a final concentration of 0.72 g L^{-1} acetate. Acetate concentration was measured, as shown in Table 4-2, with an enzymatic UV absorbance method on day 1 and day 55 for each concentration.

The acetate concentrations in the positive controls and NRB cultures decreased from approximately 0.55 g L^{-1} on day 1 to 0.24 g L^{-1} by day 55 except bacterial sample 1 with 40 µg L^{-1}. The negative controls and sterile controls remained at a stable concentration from day 1 to day 55 with an acetate concentration at 0.57–0.60 g L^{-1}. The mean acetate consumption in NRB cultures and positive controls was 0.32 g L^{-1}.

Table 4-2. Acetate concentration in NRB-cultures with 40, 100, and 200 µg L^{-1} uranine, analysed at day 1 and 55, using an enzymatic UV absorbance method.

Sample	Acetate g L^{-1} day 1	Acetate g L^{-1} day 55
Positive control: 1	0.55	0.24
Positive control: 2	0.57	0.25
Negative control: 1	0.57	0.59
Negative control: 2	0.57	0.60
Sterile control: 1 (40 µg L^{-1})	0.57	0.59
Sterile control: 2 (40 µg L^{-1})	0.59	0.59
Sterile control: 1 (100 µg L^{-1})	0.56	0.59
Sterile control: 2 (100 µg L^{-1})	0.58	0.60
Sterile control: 1 (200 µg L^{-1})	0.56	0.60
Sterile control: 2 (200 µg L^{-1})	0.56	0.59
NRB 1: (40 µg L^{-1})	0.51	0.25
NRB 2: (40 µg L^{-1})	0.59	0.25
NRB 1: (100 µg L^{-1})	d.m.*	0.25
NRB 2: (100 µg L^{-1})	0.59	0.24
NRB 1: (200 µg L^{-1})	0.59	0.25
NRB 2: (200 µg L^{-1})	0.58	0.24

*data missing

Nitrate

Nitrate consumption was measured by determination of NO_3^- concentration in cultures and controls at day 1 and day 55 using the chromotrophic acid method. Approximately 10 mL L^{-1} KNO_3 (1M) was added to the cultures giving a final theoretical nitrate concentration of 0.62 g L^{-1}.

The NO_3^- concentration in positive controls and NRB cultures decreased from measured values of 0.66–0.83 g L^{-1} NO_3^- on day 1 to 0.07– 0.19 g L^{-1} on day 55. The mean total NO_3^- consumption was around 0.59 mg L^{-1}. NO_3^- concentration in the negative controls and sterile controls also differed from the theoretical concentration and the apparent decrease in NO_3^- concentration during the incubation may due to the uncertainty of the method.

Table 4-3. NO_3-concentration in NRB-cultures with 40, 100, and 200 µg L^{-1} uranine, analysed at day 1 and 55, using the chromotrophic acid method.

Sample	NO_3^- g L^{-1} day 1	NO_3^- g L^{-1} day 55
Positive control: 1	0.66	0.19
Positive control: 2	0.73	0.10
Negative control: 1	0.70	0.61
Negative control: 2	0.80	0.54
Sterile control: 1 (40 µg L^{-1})	0.74	0.58
Sterile control: 2 (40 µg L^{-1})	0.85	0.63
Sterile control: 1 (100 µg L^{-1})	0.75	0.67
Sterile control: 2 (100 µg L^{-1})	0.72	0.66
Sterile control: 1 (200 µg L^{-1})	0.73	0.58
Sterile control: 2 (200 µg L^{-1})	0.71	0.60
NRB: 1 (40 µg L^{-1})	0.68	0.07
NRB: 2 (40 µg L^{-1})	0.71	0.16
NRB: 1 (100 µg L^{-1})	0.72	0.14
NRB: 2 (100 µg L^{-1})	0.71	0.08
NRB: 1 (200 µg L^{-1})	0.69	0.11
NRB: 2 (200 µg L^{-1})	0.83	0.13

4.3.3 Sulphate reducing bacteria – SRB

The SRB, *D. aespoeensis* was used to study if SRB had any influence on uranine. All cultures were made in duplicates and mean values of the fluorescence intensity re-calculated to uranine concentration in each culture is presented in Figure 4-16 – Figure 4-18. Neither positive nor negative controls showed any fluorescence. SRB cultures and sterile controls were amended with 40, 100, or 200 µg L^{-1} uranine, respectively; and all sterile controls were amended with sterile filtered cultures. Fluorescence was measured on a fluorescent spectrophotometer and HPLC, and emission was measured with specific settings for uranine at excitation wavelength 490 nm. A second peak was identified in the chromatograms; most likely resazurin, a redox indicator added to the growth medium (data not shown).

40 µg L^{-1} uranine

The uranine concentration in the bacterial cultures with 40 µg L^{-1} uranine was analyzed on a fluorescence spectrophotometer, as shown in Figure 4-16. The concentration was relatively stable in both cultures and controls. The difference between measurements with fluorescence spectrophotometer and HPLC was minor.

100 µg L^{-1} uranine

There were no large differences in fluorescence intensity between the cultures and controls in the experiment with 100 µg L^{-1} uranine, neither measured on a fluorescence spectrophotometer or HPLC see Figure 4-17.

200 µg L^{-1} uranine

The growth experiment with SRB and 200 µg L^{-1} uranine gave similar results as in the experiments with 40 and 100 µg L^{-1}, respectively. There were some changes in concentration of uranine but only corresponding to 20 µg L^{-1}. The results are presented in Figure 4-18.

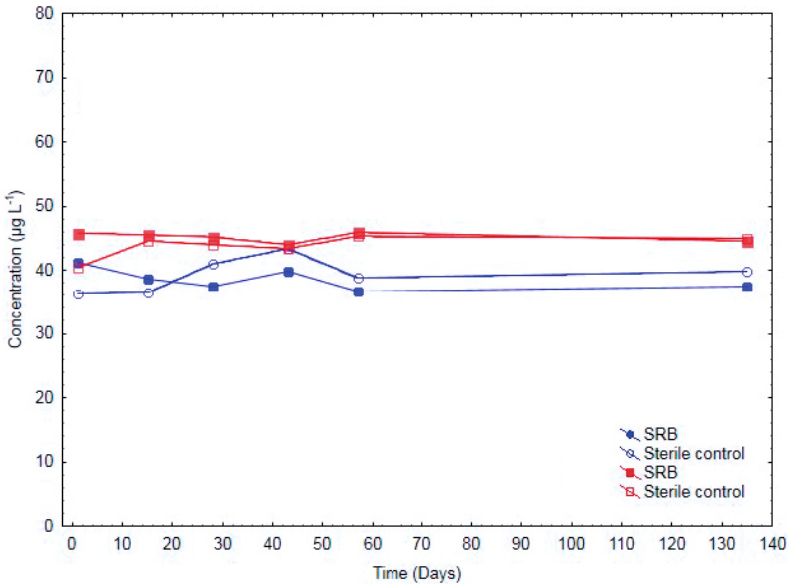


Figure 4-16. *Fluorescence measured on a fluorescent spectrophotometer (●; ○) and HPLC (■; □). The fluorescence intensity was recalculated amount of uranine with a standard curve and mean values of Desufovibrio aespoeensis cultures and sterile controls with 40 µg L^{-1} are shown.*

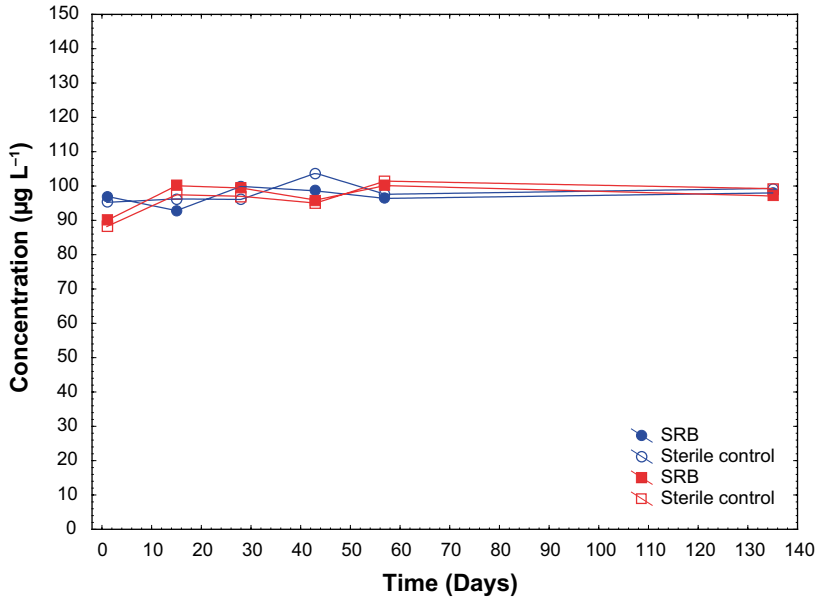


Figure 4-17. *Fluorescence measured on a fluorescent spectrophotometer (●; ○) and HPLC (■; □). The fluorescence intensity was recalculated amount of uranine with a standard curve and mean values of Desulfovibrio aespoeensis cultures and sterile controls with 100 µg L^{-1} are shown.*

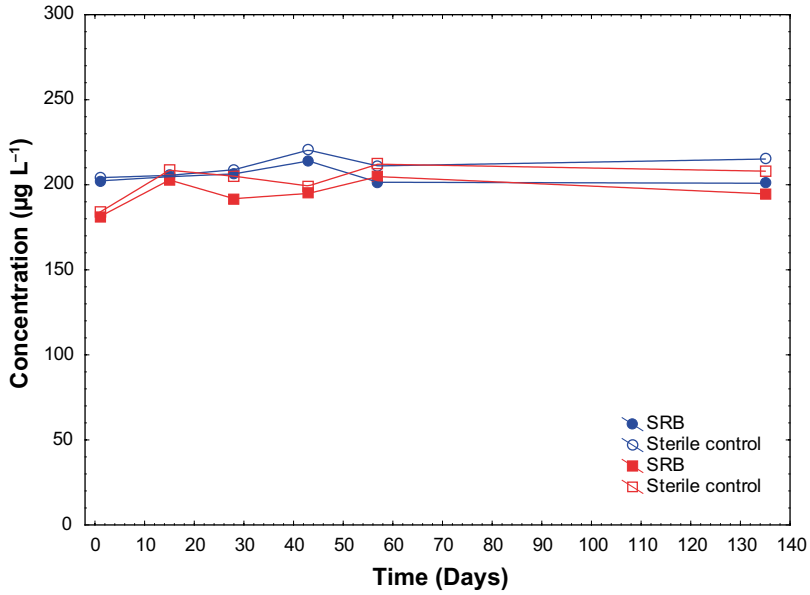


Figure 4-18. *Fluorescence measured on a fluorescent spectrophotometer (●; ○) and HPLC (■; □). The fluorescence intensity was recalculated amount of uranine with a standard curve and mean values of Desulfovibrio aespoeensis cultures and sterile controls with 200 µg L^{-1} are shown.*

4.3.4 SRB: Chemical analysis

Lactate concentration

Approximately 0.36 mL L^{-1} Na-lactate (50 %) was added to the SRB medium, giving a final concentration of. 0.2 g L^{-1} lactate in the cultures. The lactate concentration was measured using an enzymatic UV absorbance method on day 1 and day 57 for each culture and concentration.

The lactate concentrations in the positive controls and bacterial samples, shown in Table 4-4, were completely consumed by day 57. The negative controls and sterile controls remained a stable concentration from day 1 to day 57 with approximately 0.20 g L^{-1} lactate; except sterile control 2 with 40 µg L^{-1} uranine that had consumed all lactate indicating that the sterile control was not sterile.

Table 4-4. Lactate concentration in SRB-cultures with 40, 100, and 200 µg L^{-1} uranine, analysed at day 1 and 57, using an enzymatic UV absorbance method.

Sample	Lactate g L^{-1} day 1	Lactate g L^{-1} day 57
Positive control: 1	0.19	0
Positive control: 2	0.20	0
Negative control: 1	0.20	0.19
Negative control: 2	0.20	0.19
Sterile control: 1 (40 µg L^{-1})	0.20	0.20
Sterile control: 2 (40 µg L^{-1})	0.20	d.m.*
Sterile control: 1 (100 µg L^{-1})	0.20	0.19
Sterile control: 2 (100 µg L^{-1})	0.20	0.20
Sterile control: 1 (200 µg L^{-1})	0.20	0.20
Sterile control: 2 (200 µg L^{-1})	0.20	0.20
SRB 1: (40 µg L^{-1})	0.20	0
SRB 2: (40 µg L^{-1})	0.19	0
SRB 1: (100 µg L^{-1})	0.19	0
SRB 2: (100 µg L^{-1})	0.19	0
SRB 1: (200 µg L^{-1})	0.19	0
SRB 2: (200 µg L^{-1})	0.19	0

* Data missing

Sulphide concentration

The amount of sulphide was analysed using $CuSO_4$ (5mM) in the cultures at day 1 and 57. The sulphide concentrations in the positive controls and bacterial samples, increased from a range of 8–14 mg L^{-1} on day 1 to 34–63 mg L^{-1} by day 57. The negative controls and sterile controls remained at stable concentration from day 1 to day 57 at 9–15 mg L^{-1} sulphide, as shown in Table 4-5.

Table 4-5. Sulphide concentration in SRB-cultures with 40, 100, and 200 µg L^{-1} uranine, analysed at day 1 and 57, using $CuSO_4$.

Sample	S^{2-} mg L^{-1} day 1	S^{2-} mg L^{-1} day 57
Positive control: 1	11	43
Positive control: 2	9	39
Negative control: 1	10	12
Negative control: 2	11	10
Sterile control: 1 (40 µg L^{-1})	10	9
Sterile control: 2 (40 µg L^{-1})	13	15
Sterile control: 1 (100 µg L^{-1})	10	9
Sterile control: 2 (100 µg L^{-1})	9	7
Sterile control: 1 (200 µg L^{-1})	10	9
Sterile control: 2 (200 µg L^{-1})	9	10
SRB 1: (40 µg L^{-1})	10	52
SRB 2: (40 µg L^{-1})	14	63
SRB 1: (100 µg L^{-1})	9	51
SRB 2: (100 µg L^{-1})	10	43
SRB 1: (200 µg L^{-1})	9	46
SRB 2: (200 µg L^{-1})	8	34

4.4 Aerobic growth with 100 µg L^{-1} uranine in plastic and glass flasks

To investigate if uranine could absorb to bottle surfaces and by that affect the fluorescence measurements, growth cultures with *P. fluorescens* and 100 µg L^{-1} uranine in glass and plastic flasks were prepared. Positive controls with *P. fluorescens* and negative controls with filter sterilized inoculum in medium, without uranine, showed no fluorescence. Samples with 100 µg L^{-1} uranine were analysed with fluorescence spectrophotometry and HPLC. The uranine concentration was calculated from a standard curve from three parallel measurements and mean values were calculated. The experiment was repeated three times.

4.4.1 Fluorescence measurements of *P. fluorescens* in plastic growth flasks

Bacterial cultures in plastic flasks with 100 µg L^{-1} uranine were analysed with fluorescence spectrophotometry, and the data are shown in Figure 4-19. There was only a minor variation, around 10 µg L^{-1} in the measured concentration from day 1 to day 50, both in the cultures and the controls. The variation in concentration between the measurements with fluorescence spectrometry and HPLC was also in that size.

When the first experiment ended, it was found that the bacteria had not grown satisfactorily and therefore a second experiment was set up. The results from the second experiment were very similar to the first experiment.

The result from the third experiment is shown in Figure 4-20. There was an increase in fluorescence during the 34 days of the experiment for both the bacterial culture and the two different controls, one in sterile water and the other one in sterile medium. The increase corresponded to 35.0 ± 2.3 µg L^{-1} of uranine in the growth culture, 31.3 ± 7.6 µg L^{-1} the water control and 41.4 ± 4.5 µg L^{-1} in the medium control.

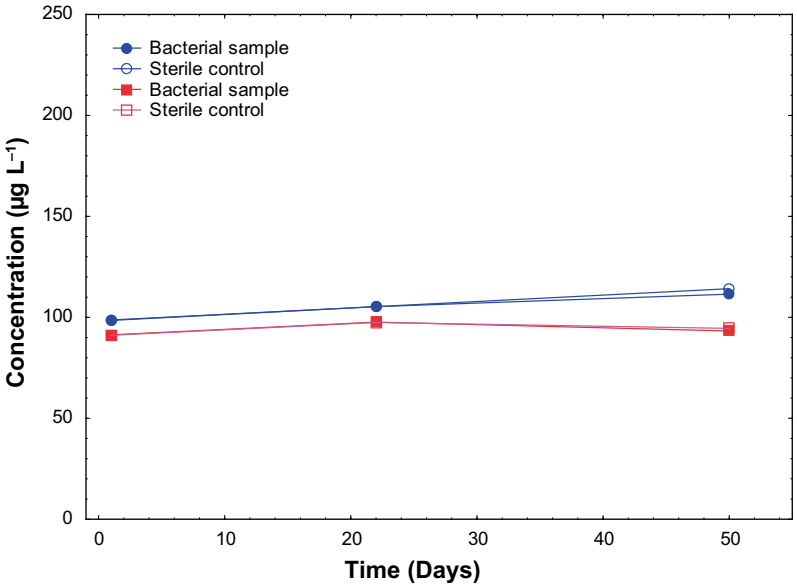


Figure 4-19. *Fluorescence measured with fluorescence spectrophotometry (●; ○) in plastic bottle in the first experiment and HPLC (■; □). The intensity was recalculated to uranine concentration with a standard curve produced in a plastic bottle. The values are mean values from duplicates with 100 µg L^{-1}.*

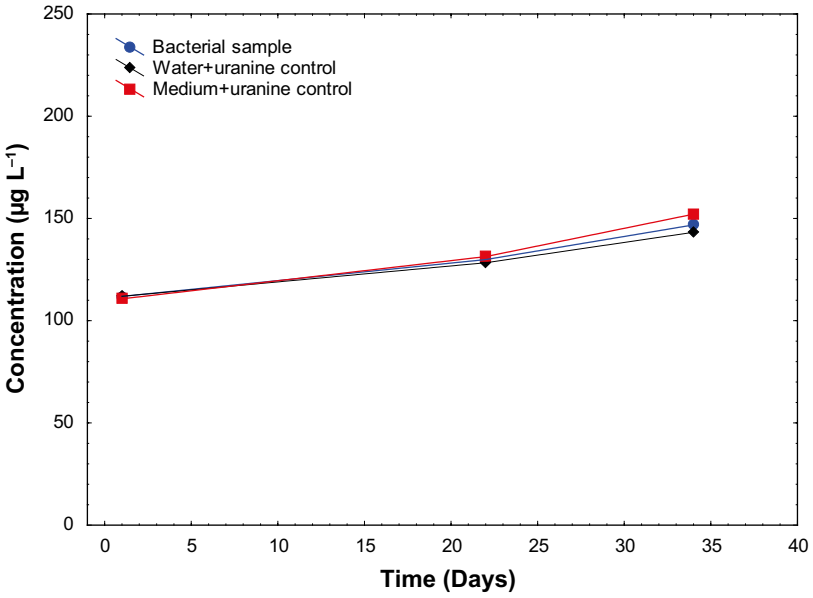


Figure 4-20. *Fluorescence in aerobic cultures from plastic flask in the third experiment measured on a fluorescence spectrophotometer. The intensity was recalculated to uranine concentration with a standard curve prepared in a plastic bottle. The values are mean values of the measurements and duplicates of the growth cultures and controls with 100 µg L^{-1} uranine.*

4.4.2 Fluorescence measurements with *P. fluorescens* in glass flasks

This test was done three times with different results. In the first experiment with *P. fluorescens* in glass flasks with of 100 µg L^{-1} uranine, the fluorescence recalculated to concentration increased 71 µg L^{-1} during the 50 days of incubation. The concentration in the sterile control increased 85 µg L^{-1}. The HPLC measurements showed an increased concentration of uranine for the bacterial growth cultures of 70 µg L^{-1}. The concentration in the sterile control increased 82 µg L^{-1} from day 1 to day 50.

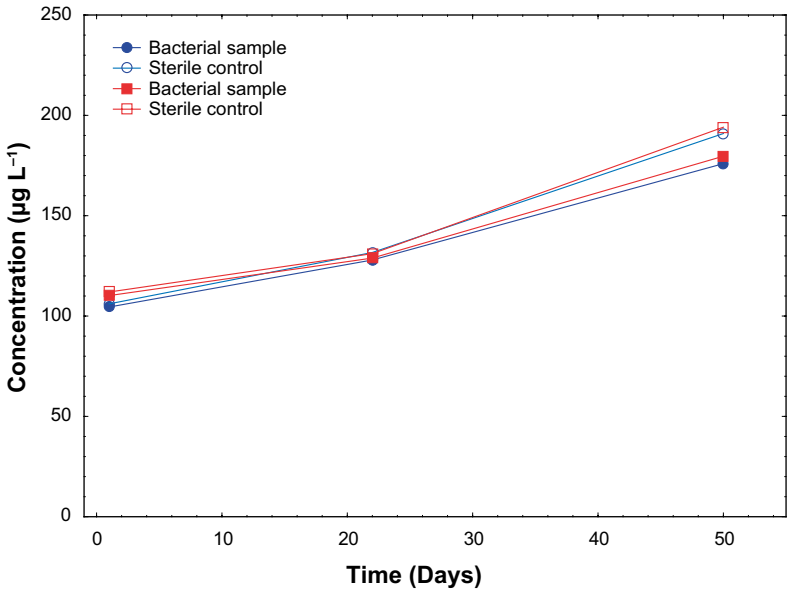


Figure 4-21. *Fluorescence measured with fluorescence spectrophotometry (●; ○) in the first experiment in glass flasks and with HPLC (■; □). The intensity was recalculated using a standard curve produced from a glass bottle and mean value of bacterial samples and sterile controls containing 100 µg L^{-1} uranine were estimated.*

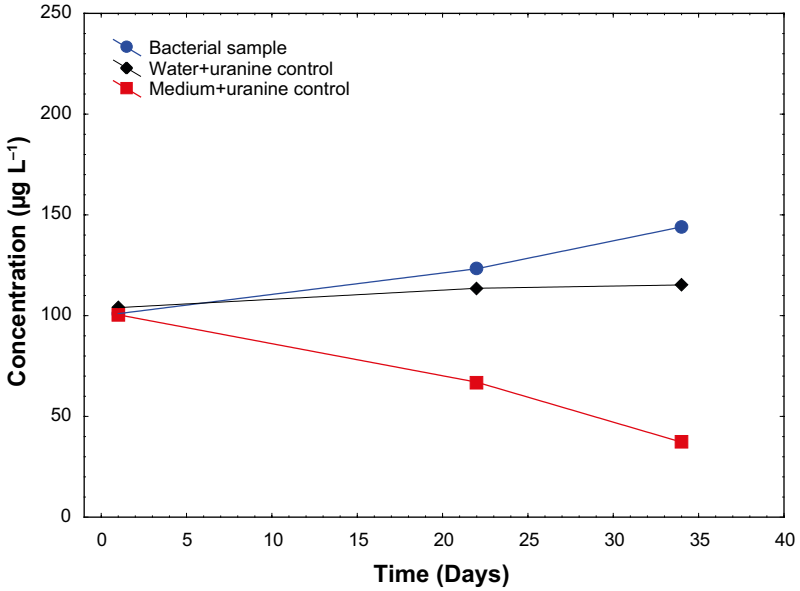


Figure 4-22. *Fluorescence measured with fluorescence spectrophotometry in the third experiment in glass flasks. The intensity was recalculated to uranine concentration with a standard curve prepared in a glass flasks. The values are mean values from the duplicates in the experiment with 100 µg L^{-1}.*

The second experiment with glass flasks was done at the same time as the one with plastic flasks. The controls used were two different, one with uranine and sterile water and one with uranine and sterile medium. The fluorescence increased in the bacterial sample, water + uranine control and medium + uranine control corresponded to 33, 25, and 27 µg L^{-1}, respectively.

In the third experiment with glass flasks, the growth cultures showed an increase in fluorescence during the 34 days of the experiment. The increase corresponded to 43.1 ± 3.3 µg L^{-1} of uranine. The fluorescence in the water control increased but less than in the growth cultures and it corresponded to 11.3 ± 7.8 µg L^{-1}. The fluorescence in the medium controls on the other hand showed a decrease and the mean value corresponded to a decrease of 63.2 µg L^{-1}. The results differed between the duplicates and the standard deviation of the samples were ± 30.4 µg L^{-1}.

4.5 Control experiment with uranine in glass flasks

Because of the varying results in fluorescence over time, in both growth cultures and sterile controls, one additional control experiment was prepared. Four controls with approximately 160 µg L^{-1} uranine + medium and four with 170 µg L^{-1} uranine but in sterile water, were prepared in Erlenmeyer flasks. Two of the four flasks from each series were kept on an orbital shaker at 150 rpm while the remaining two flasks from each series were kept still. Samples from the different flasks were analysed with fluorescence spectrophotometry at five occasions, day 1, 16, 30, 47, and 58, and uranine concentration was calculated from a standard curve.

Figure 4-23 shows the fluorescence intensity, as uranine concentration, in the uranine and medium controls. The fluorescence increased in the shaking uranine + medium controls corresponding to 83 and 91 µg L^{-1}, respectively. The fluorescence in one of the two still controls increased but less than the shaking controls, corresponding to 63 µg L^{-1}.The fluorescence in the second still control decreased, corresponding to 65 µg L^{-1} of uranine.

The fluorescence intensity in the uranine + water controls are presented in Figure 4-24. The fluorescence in the shaking controls increased corresponding to 112 and 41 µg L^{-1}, respectively. The intensity in the first control significantly increased after 30 days of incubation. A somewhat lower fluorescence intensity was found in the still samples, where the intensity increased 9 and 17 µg L^{-1}, respectively.

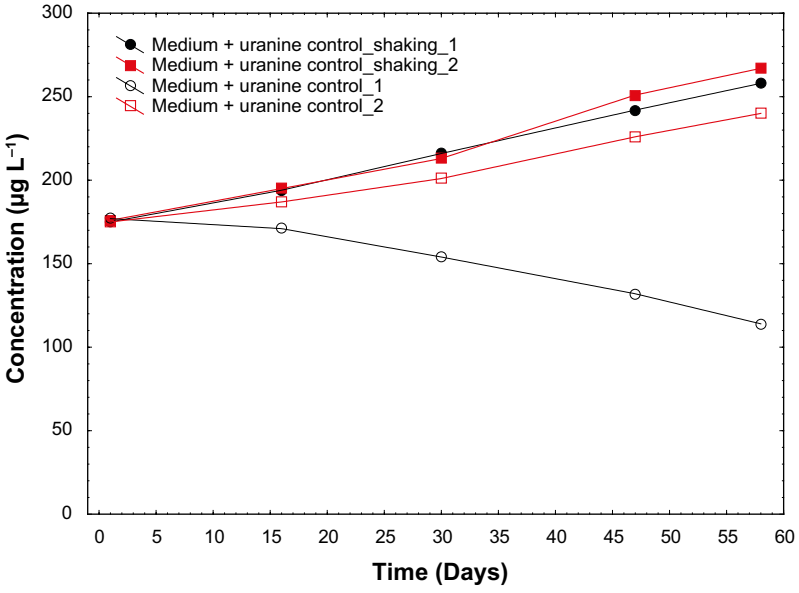


Figure 4-23. *Fluorescence measured with fluorescence spectrophotometry in medium + uranine controls that were shaking or still. The intensity was recalculated to uranine concentration from a standard curve prepared in glass flasks.*

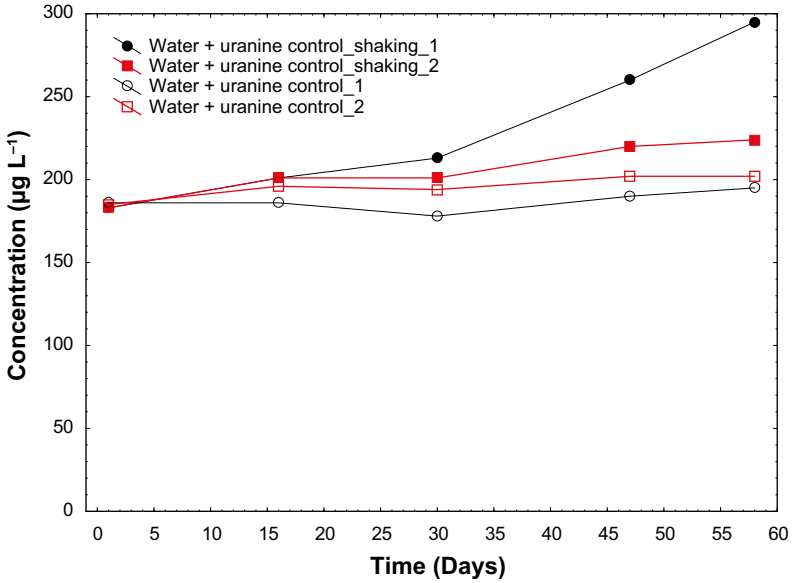


Figure 4-24. *Fluorescence measured with fluorescence spectrophotometry in water + uranine controls that were shaking or still. The intensity was recalculated to uranine concentration from a standard curve prepared in glass flasks.*

4.6 Laboratory studies with groundwater

The measured uranine concentrations for the samples and blanks are presented in Figure 4-25 and Table 4-6. The corresponding measured pH and EC values are plotted in Figure 4-26 and Figure 4-27, respectively. The results can be summarised as follows:

- The measured fluorescence in the blanks corresponds to uranine concentrations between 0.0782 and 0.661 µg L^{-1}. The blank concentrations are lower for the three most saline groundwaters (boreholes KA2862A, KA3385A and SA2600A). This is consistent with the lower DOC concentrations in these waters. Comparison between blanks in glass bottles and in plastic bottles does not reveal any clear relations or trends, and also the variation between true replicate analyses is equally large.
- Generally, the variation in measured uranine concentration within the time series for each borehole stays well within ± 5 %. For the three most saline groundwaters (boreholes KA2862A, KA3385A and SA2600A) there is a tendency of decreasing uranine concentrations after 21 days in the glass bottles. This is not observed in the corresponding samples in plastic bottles. However, the observations are few and the differences are not large and may still be due to analytical error.
- The pH variation in the different time series for each borehole is generally within the measurement uncertainty (± 0.1 pH units) and the few deviating values seem to be of a random nature. A possibly significant difference in pH can, however, be observed i.e. pH is generally lower (7.3–7.6) in the groundwater showing the decreasing uranine trend for glass bottles, compared to 7.6–7.9 in the rest of the bottles. This is probably due to that the samples represent different types of groundwater. The pH in the plastic bottle with groundwater from KA2862 shows a decrease after the 21 days which is difficult to explain and may be an error in the measurement.
- Furthermore, the electrical conductivity values agree in the different time series and are stable within the measurement uncertainty (± 5 %).

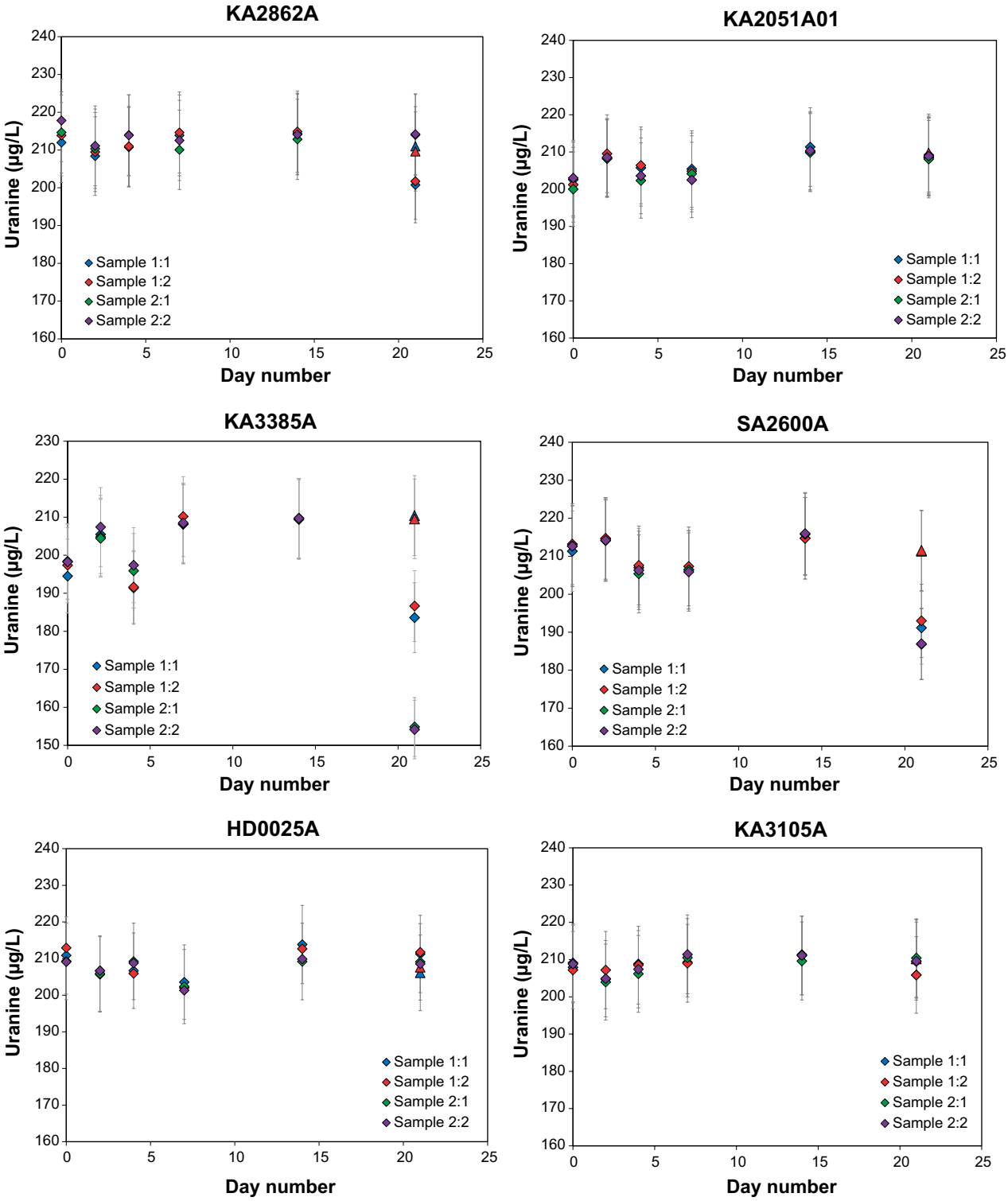


Figure 4-25. *Uranine concentration (two samples and replicate measurements) for each one of the six sampled borehole sections after 0, 2, 4, 7, 14 and 21 experimental days. Two of the samples from each borehole were kept in plastic bottles for 21 days and then analysed. The concentrations in these samples are given as triangles. The error bars correspond to ± 5 %.*

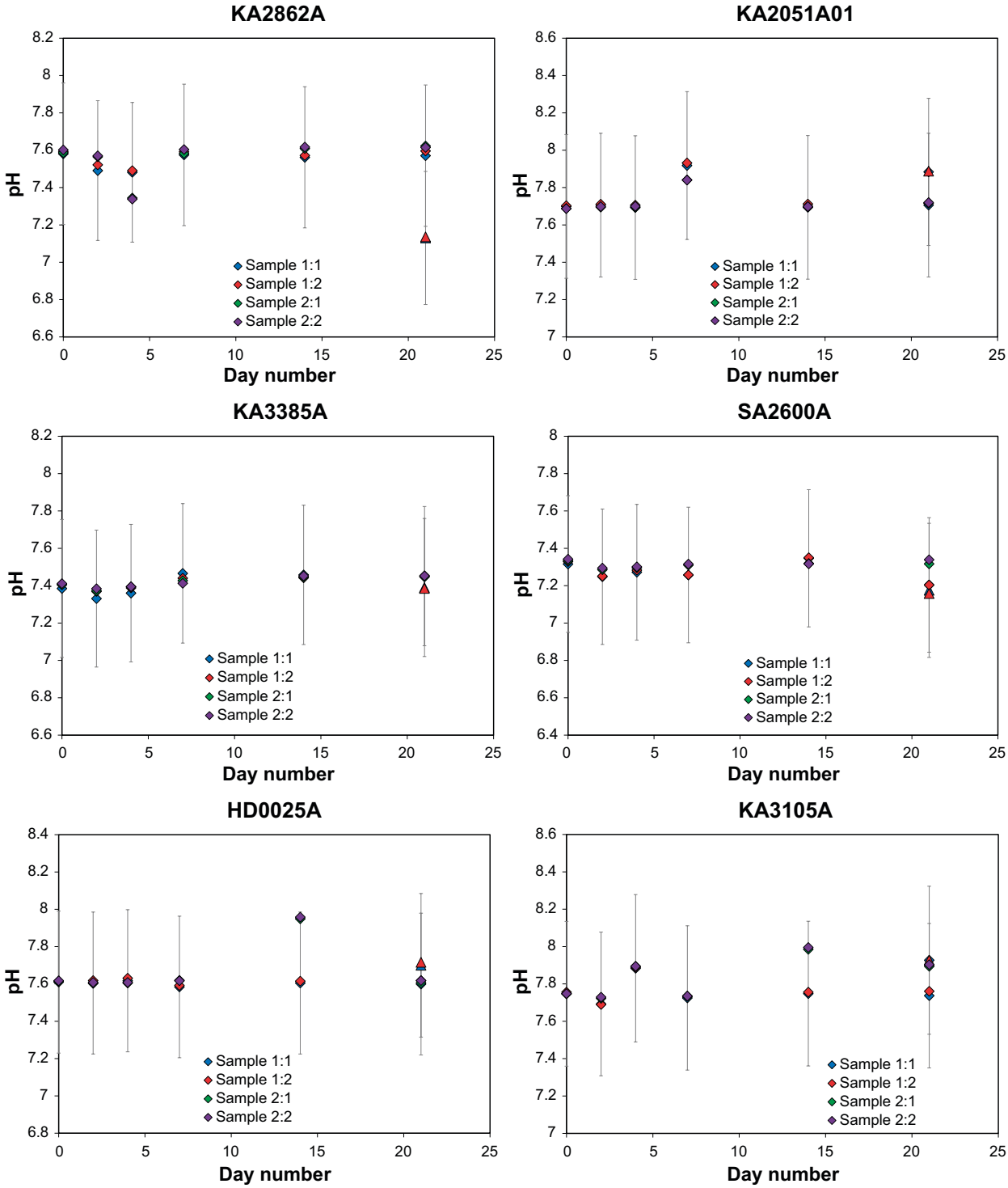


Figure 4-26. *Measured pH (two samples and replicate measurements) for each one of the six sampled borehole sections after 0, 2, 4, 7, 14 and 21 experimental days. Two of the samples from each borehole were kept in plastic bottles for 21 days and then analysed. The concentrations in these samples are given as triangles. The error bars correspond to ± 0.1 pH units.*

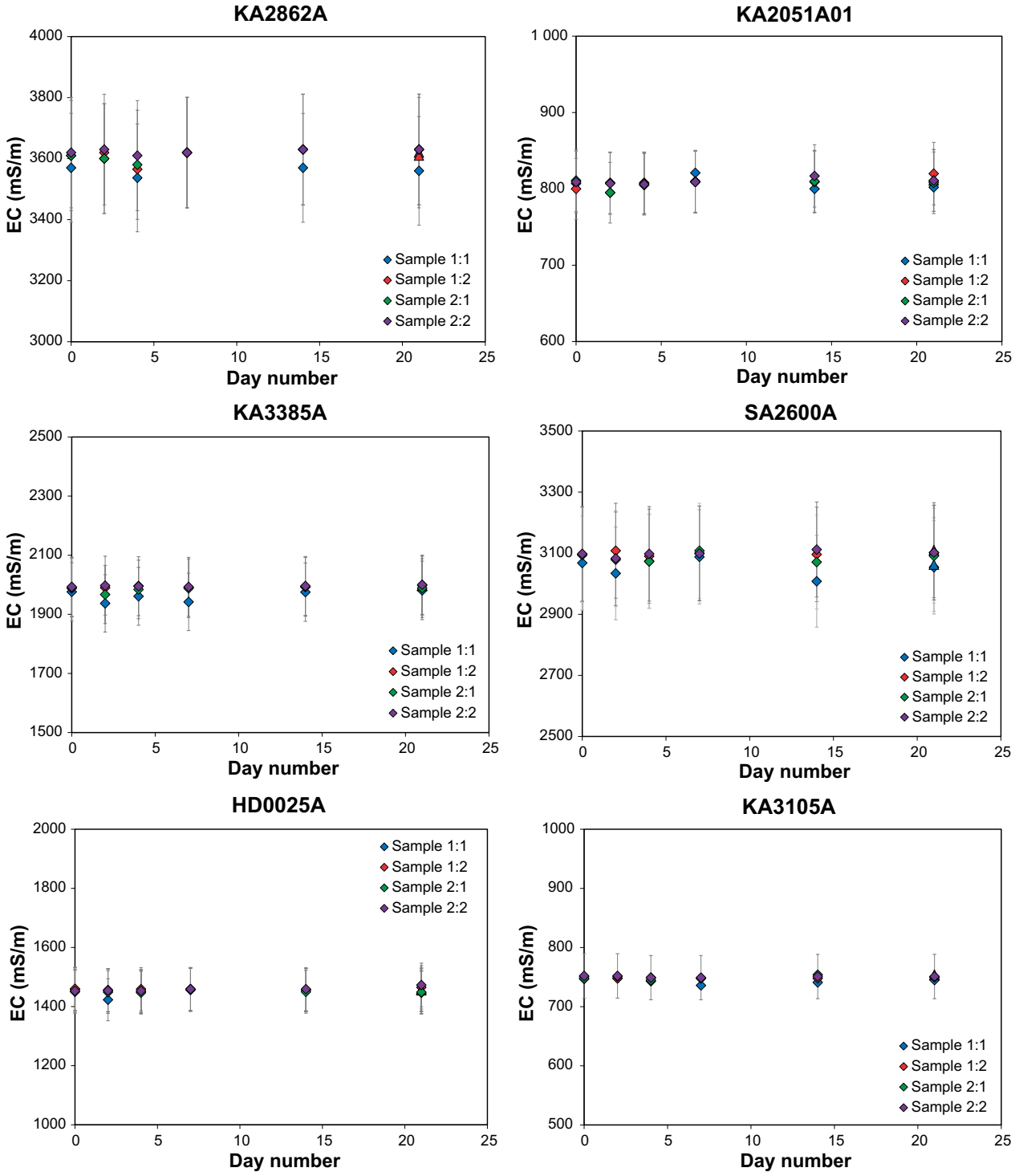


Figure 4-27. *Electrical conductivity (EC) (two samples and replicate measurements) for each one of the six sampled borehole sections after 0, 2, 4, 7, 14 and 21 experimental days. Two of the samples from each borehole were kept in plastic bottles for 21 days and then analysed. The concentrations in these samples are given as triangles. The error bars correspond to ± 5 %.*

Table 4-6. Uranine concentrations in samples with and without (blanks) uranine addition after 0, 2, 4, 7 and 21 days. Possible effects from plastic bottles were tested in the blank and in the sample stored for 21 days. The few values that indicate a decrease in the uranine concentration of a size amounting to about 3 Std or more, as calculated from the previous ten measured values are marked with grey shading.

	Uranine concentrations (µg L^{-1})											
Sample type	**KA2862A:1**		**KA2051A01:9**		**KA3385A:1**		**SA2600A:1**		**HD0025A:1**		**KA3105A:4**	
	Sample 1	**Sample 2**	**Sample 1**	**Sample 2**	**Sample 1**	**Sample 2**	**Sample 1**	**Sample 2**	**Sample 1**	**Sample 2**	**Sample 1**	**Sample 2**
Blank, glass bottle	0.151 0.165	0.116 0.0782	0.661 0.610	0.525 0.470	0.197 0.250	0.170 0.238	0.199 0.256	0.245 0.180	0.411 0.480	0.576 0.606	0.528 0.536	0.486 0.485
Blank, plastic bottle	0.226 0.354	N	0.410 0.422	N	0.147 0.248	N	0.180 0.295	N	0.193 0.310	N	0.499 0.482	N
0 days, glass bottle	212 214	215 218	203 201	200 203	195 197	198 198	211 213	213 213	211 213	209 209	208 207	209 209
2 days, glass bottle	208 209	210 211	208 210	208 209	205 205	204 207	215 215	214 214	206 207	206 207	205 207	204 205
4 days, glass bottle	211 211	214 214	206 206	202 204	191 192	196 197	207 208	205 206	207 206	209 209	209 208	206 207
7 days, glass bottle	214 215	210 213	205 205	204 202	208 210	208 209	206 207	206 206	204 202	202 201	209 209	210 211
14 days, glass bottle	214 215	213 214	211 210	210 210	208 210	209 210	215 215	216 216	214 213	209 210	211 211	210 211
21 days, glass bottle	201 202	214 214	209 209	208 209	184 187	155 154	191 193	187 187	211 212	209 209	206 206	210 210
21 days, plastic bottle	211 210	N	209 210	N	210 210	N	211 212	N	206 208	N	209 210	N

5 Discussion

This study was initiated because concerns had been raised that microorganisms could degrade uranine which then could give erroneous estimations of drill-water content in water samples from groundwater investigations. During the study it was found that cultures and sterile controls often showed increased fluorescence intensity instead of the expected decrease in fluorescence as a result of microbial degradation. This study therefore comprises several experiments that have been performed with the aims of obtaining consistent data and to evaluate the stability of uranine in bacterial cultures and sterile controls.

The initial studies on the effect of pH showed that uranine fluorescence was strongly dependent on pH from 5 to 10 with the maximum emission at pH 10. It was determined to keep pH at 9 with a buffer which decreased fluctuations in pH and thereby fluorescence. The buffer made the fluorescence measurement more stable when standard curves and samples from growth cultures were measured compared to if pure water and pH adjustment with NaOH was done. Fluorescence measurements are sensitive to external parameters such as changes in room temperature, atmospheric humidity and air pressure, which can change the intensity. Internal standards with defined intensity were, therefore, used as controls of the fluorescence spectrophotometer and influences of external parameters on the measurements.

The results from the aerobic growth experiments showed an increase in fluorescence during growth of the three bacterial strains used. After the first experiment with 20 mg L^{-1} (Figure 4-5) uranine were finalised, theories on why the fluorescence intensity increased instead of decreased were investigated. An increased fluorescence in samples diluted in AGW compared to Tris-buffer resulted in speculations regarding the influence of sodium ions, but the idea was rejected later on (no data shown). Since the result was the opposite to the expected decrease due to microbial degradation of uranine, additional analyses of number of cells and lactate concentration were later included and the results are presented in the Appendix. The cultures grew well and lactate was consumed as discussed in more detail below. In this experiment the fluorescence increased both in cultures and in the sterile control with medium and uranine for unknown reasons.

In the second aerobic growth experiment, the uranine concentrations were in the same range (200 µg L^{-1}) or lower than the concentration used in drill-water tracing (40 µg L^{-1} and 100 µg L^{-1}). During this experiment the fluorescence intensity increased all samples from the bacterial cultures, especially after 30 days. Unfortunately, in this experiment the sterile controls were contaminated and could not be used after 30 days. The control with 200 µg L^{-1} uranine in sterile water showed no increase in fluorescence. The controls that were made to elucidate if the presence of cells in the samples could increase the fluorescence, showed that this was not the case. Filtered samples showed a somewhat lower fluorescence both for samples and controls and that effect probably came from adsorption of uranine onto the filters. The filtered portion of growth cultures did not show an increase in fluorescence as the unfiltered cultures did. The conclusion from the tests of possible influence of cells on the fluorescence measurements is therefore that presence of cells does not enhance the measured fluorescence intensity. On the other hand, this growth experiment showed that the increase in fluorescence started after 30 days and occurred only in the growth cultures and not in the sterile control with AGW. Since the increase started after 30 days it was not connected to the growth phase of the bacteria. The increase must have been caused by some increase in fluorescence that occurred in one or several of the growth medium components or by a change in the uranine molecule.

There was no significant difference in fluorescence intensity between cultures of *P. fluorescens* in plastic and glass bottles or between respective controls in the first experiment. The repeated experiment with two bottle materials showed, on the other hand, the same increasing trend in fluorescence intensity as the aerobic experiments with the three bacterial strains. The medium controls in glass bottles in this experiment showed a decrease in fluorescence. Such decrease was also observed in one of the medium controls without shaking from the last control experiment (Figure 4-23) and in groundwater samples from the boreholes KA3385A and SA2600A, (Table 4-6). In the last measurements on day 21, the fluorescence in the uranine amended groundwater from these two boreholes had decreased to intensities corresponding to a decrease in concentrations between 11 and 30 µg L^{-1}.

The anaerobic growth experiment with one nitrate-reducing and one sulphate-reducing bacterium and uranine did not show any large increase in fluorescence over time neither in cultures nor in sterile controls. The bacterial cultures grow well and the carbon and energy sources added were consumed and the electron acceptors were reduced.

The tests with uranine additions to groundwater samples did not show any changes in intensity except for the decrease discussed above. It has to be noticed that the samples were incubated for 21 days to be compared with the growth experiments that lasted for at least 58 days. In the growth experiments the increase started after between 20 to 30 days.

Results from the growth experiments with bacteria in aerobic conditions all showed an increase in fluorescence intensity in the l cultures, when the bacteria grew satisfactorily. The controls showed varying results in these experiments. There might have been a more pronounced increase in fluorescence in controls with medium than in controls with water only and the last control experiment showed that shaking resulted in more fluorescence than without shaking. The increase occurred after around 30 days, which is well after the growth cultures had reached the stationary growth phase.

There was no or insignificant fluorescence at the wavelengths used for uranine measurements in cultures with *P. stutzeri* and *S. putrefaciens* without uranine at pH 9. In cultures of *P. fluorescens* without fluorescein, pyoverdines were detected at pH 7, and showed highest fluorescence values shortly after start, then the intensity decreased slightly over time when the cultures reached stationary growth phase because of nutrient limitations.

In *P. fluorescens* cultures with uranine additions, fluorescence from pyoverdines was measured at pH 9 at 400 nm, and the fluorescence increased over time for cultures with the three uranine concentrations, 40, 100 and 200 µg L^{-1}, in opposite to the cultures without uranine additions as described above. HPLC analyses showed four peaks from pyoverdine detected also at the excitation wavelength 490 nm during the analyses of uranine (Figure 4-11). The presence of pyoverdine in cultures of *P. fluorescens,* therefore, gave higher fluorescence when measured by fluorescence spectrophotometry and may, consequently mistakenly be considered as uranine in a groundwater sample with *P. fluorescens*. The analyses of pyoverdines showed that there was no significant difference in fluorescence intensity from pyoverdines when measured at pH 7 or 9. In *P. stutzeri* and *S. putrefaciens* cultures with uranine additions, fluorescence also increased over time for all three uranine concentrations which cannot be caused by pyoverdines, but there may be other siderophores that could influence the fluorescence intensity. Both *P. stutzeri* and *S. putrefaciens* produce siderophores but with other chemical characteristics than pyoverdines. Since these compounds are non-fluorescent, they were not detected with the analytical methods used in this study but they could indirectly influence the fluorescence of uranine. The retention time for uranine on the HPLC changed somewhat for the bacterial cultures compared to the standard and this difference could be due to interactions between uranine and such compounds. On the other hand, in the majority of the sterile medium controls and in some AGW controls, the fluorescence increased as well.

The actual cause of the increased fluorescence intensity in growth cultures and controls could not be identified in this project. From the obtained results at least two possible explanations can be discussed. One explanation could be that it was a substance in the medium that interacted with the uranine molecule, possibly in combination with aeration, since increases were absent in the anaerobic cultures. The anaerobic medium had approximately the same composition as the aerobic medium had. An interfering substance could cause a chemical interaction with the uranine molecules that will decrease the energy loss in the collision between the analysed molecule and solvent molecules, during emission. If the energy loss in the collision is less with than without the solvent molecules, more energy has to be released in the emission thus giving higher fluorescence intensity. Another possible explanation can be that the uranine molecule changed structure during the incubation and by that changed the fluorescence intensity. Such possible changes in the uranine molecule structure can be analysed with liquid chromatography combined with mass spectroscopy (LC MS/MS).

The results from the experiments showed that fluorescence measurements indicated up to the double concentration of uranine in the laboratory experiments after to 60 days of incubation compared to what was added. The three bacterial stains used in the study were isolated from groundwater and are indigenous in groundwater of the Fennoscandian Shield. They produce and release siderophores that may interact with uranine in groundwater samples. The test with groundwater and uranine did not show any increase during the period the bottles were incubated but as commented above, the incubation period was shorter than for the growth experiments. Therefore, a test with exposure times equivalent to what was used for the growth cultures is recommended to confirm the absence of an increase in groundwater. In such tests the stability of uranine could be studied with LC MS/MS to elucidate if a change in fluorescence intensity depends on changes in the structure of the uranine molecule.

Measuring uranine concentration with fluorescence seems to be sensitive to several components and it could be a good idea to consider another tracer for drill-water measurements in the future.

6 Conclusions

- The three bacterial strains used in this study increased the uranine fluorescence during growth but also some sterile controls showed an increase during incubation.
- *Pseudomonas fluorescens* produced pyoverdines that fluoresce at pH 9 and at the same wavelength as uranine and due to this, the measured uranine concentration in a sample will be overestimated compared to the actual concentration.
- Because of the influence on the fluorescence intensity by microbial activity or changes in the uranine molecule, degradation of uranine could not be verified in this study.
- The effect of microbial metabolism, compounds in growth media or changes in the uranine molecule, on the fluorescence could indicate up to twice the amount of uranine in the laboratory experiments. Measurements of uranine in groundwater can possibly also give overestimated concentration values, however, this has not been verified since the test periods were probably too short.

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Appendix

A1 Determining the total number of cells

The acridine orange direct count method was used to determine the number of cells to investigate if fluorescence changes were due to cell growth (Pedersen and Ekendahl 1990).

A1.1 Microbial growth with 20 mg L^{-1} uranine

Table A-1. Number of cells determined in samples with 20 mg L^{-1}.

Sample	Cells mL^{-1}	Mean (cells mL^{-1})
Control:1	0.0	0.0
Control:2	0.0	
Shewanella putrefaciens:1	7.6×10^7	9.3×10^7
Shewanella putrefaciens:2	1.1×10^8	
Pseudomonas fluorescens:1	2.6×10^8	2.2×10^8
Pseudomonas fluorescens:2	1.8×10^8	
Pseudomonas stutzeri:1	1.4×10^8	1.5×10^8
Pseudomonas stutzeri:2	1.6×10^8	

A1.2 Microbial growth with 200, 100, and 40 µg L^{-1} uranine

Table A-2. Number of cells determined in samples consisting 200, 100, and 40µg L^{-1} uranine, analysed at days 14–15 and 26–27.

Sample	Cells mL^{-1} at days 14–15	Cells mL^{-1} at days 26–27
Control 40 µg L^{-1}	0.00	
Uranine in water 200 µg L^{-1}	0.00	
Shewanella putrefaciens 200 µg L^{-1}	3.2×10^7	7.9×10^7
Shewanella putrefaciens 100 µg L^{-1}	3.7×10^7	5.1×10^7
Shewanella putrefaciens 40 µg L^{-1}	1.8×10^8	5.6×10^7
Pseudomonas stutzeri 200 µg L^{-1}	3.3×10^8	2.2×10^8
Pseudomonas stutzeri 100 µg L^{-1}	3.1×10^8	3.8×10^8
Pseudomonas stutzeri 40 µg L^{-1}	1.8×10^9	4.7×10^8
Pseudomonas fluorescens 200 µg L^{-1}	3.7×10^8	2.9×10^8
Pseudomonas fluorescens 100 µg L^{-1}	3.4×10^8	2.4×10^8
Pseudomonas fluorescens 40 µg L^{-1}	3.4×10^8	2.9×10^8

A2 Lactate analysis

An enzymatic UV absorbance method was used to study that the added lactate were consumed by the bacteria in samples consisting 20 mg L^{-1}, 200, 100, and 40 µg L^{-1} uranine.

A2.1 Microbial growth with 20 mg L^{-1} uranine

Media was prepared and approximately 0.55 ml L^{-1} sodium – lactate (50 %) was added after sterilization, and the samples lactate concentration were measured on day 35.

Table A-3. Lactate concentration in samples consisting 20 mg L^{-1} uranine, analysed at day 35.

Sample	Lactate mg L^{-1} day 35
Control:1	247
Shewanella putrefaciens:1	0.0
Pseudomonas fluorescens:1	0.3
Pseudomonas stutzeri:1	0.0

A2.2 Microbial growth with 200, 100, and 40 µg L^{-1} uranine

Media was prepared and approximately 0.45 ml L^{-1} sodium – lactate (50 %) was added after sterilization, and the samples lactate concentration were measured on day 6, 12, and 20.

Table A-4. Lactate concentration in samples consisting 200, 100, and 40 µg L^{-1} uranine, analysed at days 6, 12 and 20.

Sample	Lactate mg L^{-1} day 6	Lactate mg L^{-1} day 12	Lactate mg L^{-1} day 20
Control 100 µg L^{-1}	257	–	–
Control 40µg L^{-1}	259	–	–
Uranine in water 200µg L^{-1}	0.00	–	–
Shewanella putrefaciens 200 µg L^{-1}	252	246	195
Shewanella putrefaciens 100 µg L^{-1}	256	257	207
Shewanella putrefaciens 40 µg L^{-1}	180	25	–
Pseudomonas stutzeri 200 µg L^{-1}	0.96	–	–
Pseudomonas stutzeri 100 µg L^{-1}	0.96	–	–
Pseudomonas stutzeri 40 µg L^{-1}	50	–	–
Pseudomonas fluorescens 200 µg L^{-1}	0.96	–	–
Pseudomonas fluorescens 100 µg L^{-1}	1.28	–	–
Pseudomonas fluorescens 40 µg L^{-1}	0.96	–	–

A3 Internal standard

Internal standards consisting 200 µg L^{-1} uranine in sterile Millipore-water, or medium were prepared to investigate the standard deviation and external disturbances influence on the fluorescence measurements.

Internal standards with AGW had a standard deviation of approximately 4 percent, while the internal standard with medium had a standard deviation of approximately 3 percent.

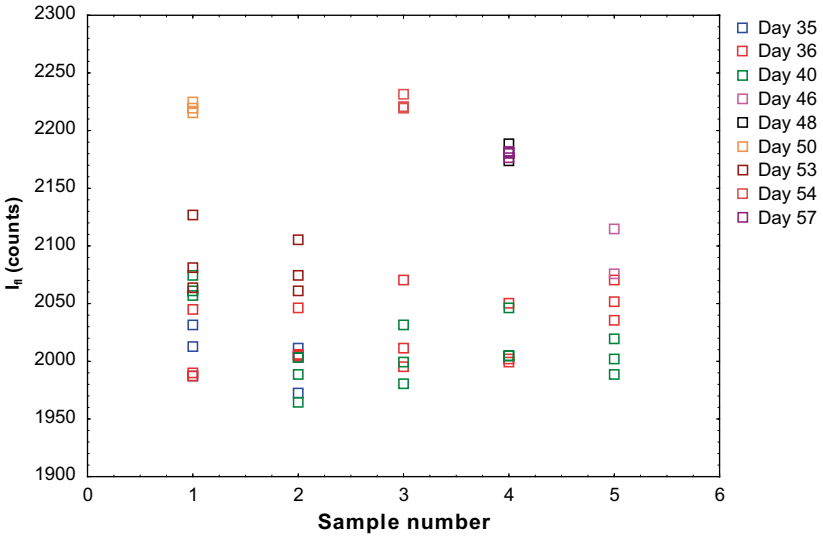

Figure A-1. *Internal standard consisting 200 µg L^{-1} uranine and sterile AGW. Mean value = 2067; standard deviation = ± 80.*

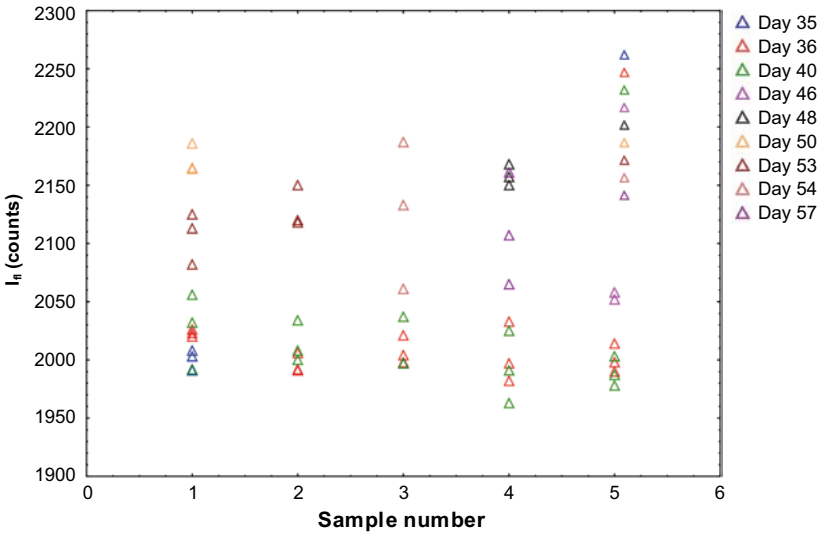

Figure A-2. *Internal standard consisting 200 µg L^{-1} uranine and medium. Mean value = 2051; standard deviation = ± 66.*

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