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A New Approach to Quantify Na-Fluorescein (Uranine) in Acid Mine Waters

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Abstract Sodium fluorescein (uranine) is one of the most popular fluorescent dyes for tracer experiments due to its chemical properties, low detection limits and low costs. As a limiting factor, it is generally assumed that Na-fluorescein cannot be properly detected under acid conditions because of weak fluorescence intensities at the standard excitation wavelength (490 nm). This laboratory study introduces a method to quantify Na-fluorescein in low pH waters without having to raise pH to alkaline conditions, which spares the time- and cost-intensive filtration of hydroxide precipitates prior to analysis. It was applied for recovery tests in water samples from five mining sites in Germany. These were buffered to pH 1.5 where Na-fluorescein shows a second fluorescence intensity maximum at an excitation wavelength of 438 nm. The method had satisfying recovery rates although, as expected, a higher detection limit compared to standard wavelength and pH. High Fe contents in the waters are a limiting factor—the impact of increasing Fe concentrations at a constant dye spike was evaluated and yielded a distinct negative trend between Fe and detected Na-fluorescein, probably due to Fe-fluorescein complexation. However, good recovery can be expected in mine waters with up to ≈ 100 mg L^{-1} Fe. The method of standard addition offers a potential calculative solution for higher Fe concentrations, resulting in significantly better Na-fluorescein recovery rates, compared to direct measurements. The method introduced here represents a promising approach for improving Na-fluorescein applicability in acid environments.

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Introduction

Sodium fluorescein ($C_{20}H_{10}Na_2O_5$), also known as uranine, is one of the most widely applied fluorescent dyes for tracer experiments due to its conservative behaviour, low detection limits, and low cost. Therefore, it is tempting to use it in all environments. One challenge until now is the application of Na-fluorescein in low pH mine water.

Usually, an excitation wavelength of 490 nm with a corresponding emission wavelength of 511 nm is used for measuring Na-fluorescein concentrations in a spectrofluorimeter because at this wavelength, fluorescence intensity is maximized in alkaline conditions (Fig. 1). Under acidic conditions, however, the fluorescent anion transforms into a cation with weak fluorescence intensities at this excitation wavelength. At pH 2, the intensity is less than 5% of the maximum intensity at pH 9 (Käss 2004). Furthermore, simply increasing the pH to the optimum conditions with suitable buffer solutions is not recommended because mine waters often contain metal cations, especially Fe, which precipitate as hydroxides at higher pH values. These can interfere with the measurements, negatively influencing Na-fluorescein recovery rates due to dye adsorption on the hydroxide surfaces and subsequent loss by filtration (Aldous and Smart 1988), or—if samples are not filtered—causing an intensity overestimation due to scattering effects caused by the increased turbidity in the sample. For these reasons, it is generally assumed that Na-fluorescein is not a suitable option for tracer experiments in low pH mine waters and so other tracers, like salt or drift tracers (e.g. lycopodium spores or microspheres), are preferably used
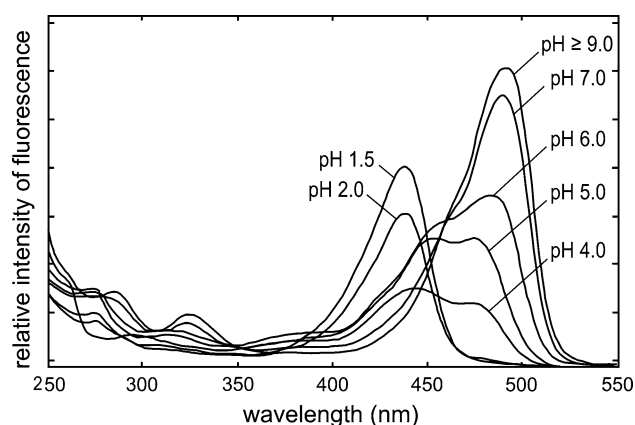
Fig. 1 Relative fluorescence intensity of Na-fluorescein at different excitation wavelengths and different pH values (modified after Behrens 1988, cited in Käss 2004)

(Aldous and Smart 1988; Davis 1994; Mather et al. 1969; Wolkersdorfer 2002, 2008; Wolkersdorfer et al. 2002).

On the other hand, to the authors' knowledge, it was not considered until now that the Na-fluorescein cation has a maximum fluorescence intensity as well, albeit at a shifted excitation wavelength of 438 nm (emission wavelength: 517 nm) that can be used in strongly acidic environments (Fig. 1). Thus, it should be possible to measure Na-fluorescein concentrations under acidic conditions at this shifted wavelength. To quantify this, we performed a laboratory study using water samples from five German mining locations as matrices.

We compared Na-fluorescein concentrations, measured under optimum conditions at pH 9, with measurements under acid conditions at pH 1.5, the cation's fluorescence intensity maximum (Fig. 1). Different buffer solutions were used to achieve the respective pH values. During the experiments, it became clear that the fluorescence intensity is influenced not only by the pH value but also by the Fe concentration of the samples. Therefore, we conducted further tests to quantify the influence of Fe on the fluorescence. Furthermore, the method of standard addition is introduced as a possible approach to minimize the effects of Fe on fluorescence intensity.

Materials and Methods

In total, eight mine water samples were obtained from five different locations across Germany (Fig. 2). The sites at Hambach and Groitzsch are open cast lignite mines. Anthracite is mined in the subsurface at Ibbenbüren. Samples were also collected at the Braubach site, an abandoned sulphidic Pb-Ag mine, and at Marsberg, an abandoned sulphidic Cu mine. All samples were collected

Fig. 2 Sampled mining locations in Germany (*B* Belgium, *DK* Denmark, *L* Luxembourg)

in new polyethylene bottles and stored in the dark at 4°C until analysis.

To characterize the different matrices, certain physico-chemical parameters, electrical conductivity (eC), dissolved oxygen (DO), pH, and Eh were determined on site. In addition, organoleptic parameters (colour, clouding, precipitates) were documented. Total Fe and Mn concentrations were determined colorimetrically using a spectrophotometer (Dr. Lange Casas 100) (Table 1). All samples were filtered with Whatman 589/1 filter papers (retention 12–25 μm) before the measurements to minimize disturbance due to clouding or precipitates.

To prepare the samples for fluorescence measurements at the two intensity peaks (Fig. 1), buffer solutions for pH 1.5 and pH 9 were prepared. For the former, a solution of 25 mL KCl (0.2 mol L^{-1}) and 20.7 mL HCl (0.2 mol L^{-1}) was used (Küster and Thiel 2008). Tests with an alternative buffer solution for pH 1.5, consisting of 2.3325 g citric acid ($C_6H_8O_7 \cdot H_2O$), 22.2 mL NaOH (1 mol L^{-1}), and 38.9 mL HCl (1 mol L^{-1}), filled up to 500 mL with deionized water (Küster and Thiel 2008), led to yellowish colouration in Fe-rich samples (probably Fe citrate precipitation). It was therefore not used in the subsequent experiments. For pH 9, a solution of 4.797 g sodium borate, 40.25 mL NaOH (1 mol L^{-1}), and 9.75 mL HCl (1 mol L^{-1}), filled up to 500 mL with deionized water, was used (Küster and Thiel 2008). The addition of this

Table 1 List of samples with parameters measured on site and Fe and Mn concentrations (mg L^{-1}); n.a. = not analyzed, DO: dissolved oxygen (mg L^{-1}), eC: electrical conductivity (μS cm^{-1}), Eh (mV)

Sample	eC	DO	pH	Eh	Colour	Clouding	Precipitate	Fe	Mn
1	1,363	7.57	5.9	410	Pale yellow	Precipitate	Red-brown	n.a.	n.a.
2	5,550	4.96	3.6	570	Brown	Brown	–	206	17.8
3	3,290	5.65	4.8	432	Brown	Brown	–	16.4	9.54
4	1,393	9.67	5.3	355	Brown	Brown	–	0.024	1.04
5	4,220	10.10	3.6	616	Pale red-brown	Pale red-brown	–	9.69	201
6	49,700	7.65	7.1	414	–	–	–	<0.005	1.81
7	4,460	5.12	6.7	417	–	–	–	<0.005	0.825
8	3,600	10.4	2.8	636	Yellow	Precipitate	Orange-brown	203	33.3
9	n.a.	n.a.	1.5	n.a.	–	–	–	1,000	n.a.
10	n.a.	n.a.	1.5	n.a.	–	–	–	200	1.81

buffer caused a yellow-orange precipitation in the Fe-rich samples 2 and 8 (Table 1), obviously Fe hydroxides. Apart from that, no further changes due to the buffering were observed. After adding the alkaline buffer to the Fe-rich samples 2 and 8, they were filtered to minimize the influence of the hydroxide precipitate.

A Varian Cary Eclipse fluorescence spectrophotometer was used to measure the Na-fluorescein concentrations. Calibration was done in deionized water using Na-fluorescein (CI 45350, producer: BASF) for six calibration ranges to maximize the possible span of the measurements. In the lowest range, concentrations between 0.001 μg L^{-1} and 0.01 μg L^{-1} can be measured. The highest range reaches from 100 to 1,000 μg L^{-1}. The other calibration ranges cover the four decades in between. Every standard solution was spiked with the previously prepared buffer solutions in the same ratio as the samples, which resulted in one calibration series for each of the two buffers. The detection limit at pH 9 is cdl(pH 9) = 0.003 μg L^{-1} and at pH 1.5 it is cdl(pH 1.5) = 0.015 μg L^{-1}. This higher detection limit is due to the fact that analysis under acidic conditions is performed on a "side peak" with lower relative fluorescence intensity, compared to the "main peak" under alkaline conditions (Fig. 1). The detection limits were calculated according to DIN 32645 of the German Institute for Standardization (2008).

To quantify the feasibility of measuring Na-fluorescein under acidic conditions, we conducted several recovery tests. First, the buffered samples without the Na-fluorescein spike were measured to determine possible background values. For the recovery tests, the samples were additionally spiked with Na-fluorescein concentrations in a dilution series from 0.005 to 50 μg L^{-1}. The same dilution was used for all tests, dilution accuracy is ±0.04 mL for the used 50 mL volumetric flasks and deviation of the Eppendorf pipette is ±0.0075 mL. Every fluorimeter measurement was repeated five times and the arithmetic mean was then used to determine one single concentration.
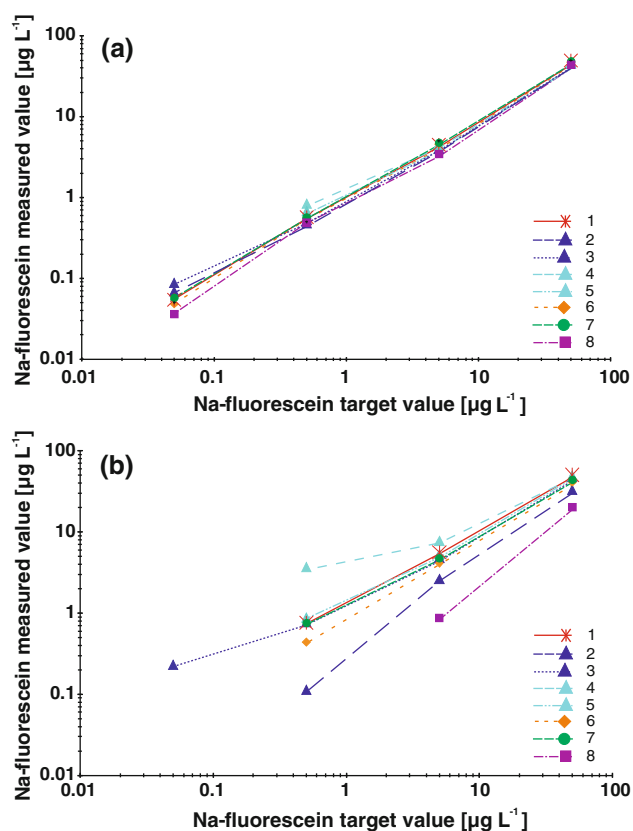
Results and Discussion

Sample characterization is shown in Table 1. Only samples No. 2, 5, and 8 showed both a low pH value and a high concentration of metal ions. Samples 2 and 8 yielded high Fe contents above 200 mg L^{-1}, whereas sample 5 had a high Mn concentration in the same range. The pH value varied between 2.8 and 3.6. The other samples were higher in pH, between 4.8 and 7.1, and had significantly lower metal concentrations. Very high eC values in sample 6 ($\approx$ 50 mS cm^{-1}) at neutral pH may be explainable by abundant saline waters in that mining area (Grobe and Machel 2002). Samples 9 and 10 were artificially prepared (see text).

Figure 3a shows the results of the first recovery test under optimum 'standard' conditions for Na-fluorescein quantification (pH 9). As mentioned above, samples 2 and 8 were filtered before the measurement. The average recovery rate over all concentration ranges was close to 100%, with larger scattering in the lowest concentration range.

This shows that it is possible to measure Na-fluorescein in acid mine waters with satisfying results by buffering the samples to the desired pH value of 9 if Fe-rich samples are filtered prior to tracer analysis. The filtration of the precipitate in samples 2 and 8 obviously had no effect on the Na-fluorescein concentration. It is, however, possible that this may not be the case with other mine waters that were not part of this study.

Figure 3b illustrates the results of the second recovery test performed at pH 1.5. Only sample 3 could be measured in the lowest concentration range between 0.01 and

Fig. 3 **a** Recovery tests for Na-fluorescein at pH 9 (Fe-rich samples were filtered prior to the measurement); **b** recovery tests for Na-fluorescein at pH 1.5 (filtration unnecessary). The Na-fluorescein input concentrations were 0.05, 0.5, 5, and 50 μg L^{-1}

intensity 'side peak' at an excitation wavelength of 438 nm and an emission wavelength of 517 nm, though with higher detection limits. This would, in consequence, lead to redundancy of the time- and cost-intensive filtration step in routine tracer laboratory work. Buffering the samples to pH 1.5 is not an additional effort since a buffering step (preparation of samples for Na-fluorescein measurement at pH 9) would have to be implemented anyway.

However, the measurements seem to be strongly influenced by the presence of elevated concentrations of Fe. Another experiment was conducted to further examine this phenomenon. We prepared 20 solutions with different Fe concentrations between 0 and 500 mg L^{-1} by diluting a 1,000 mg L^{-1} Fe standard solution ($Fe(NO_3)_3$ in 0.5 M HNO_3) with deionized water. Each solution was then spiked with three different Na-fluorescein concentrations (0.5, 5, and 50 μg L^{-1}) and buffered to pH 1.5. Additionally, 10 of the solutions with 5 μg L^{-1} Na-fluorescein were buffered to pH 9. Na-fluorescein recovery tests were conducted with these 70 samples (Fig. 4).

The results clearly show that increasing Fe concentrations led to reduced detectability of Na-fluorescein. While higher Na-fluorescein concentrations (5 and 50 μg L^{-1}) produced good recovery rates and a sub-parallel decrease with high Fe concentrations (Fig. 4b–d), the less concentrated Na-fluorescein spike showed increased scattering (Fig. 4a, d). Recovery started to deteriorate at Fe concentrations of 2.5 mg L^{-1} for the 5 μg L^{-1} Na-fluorescein spike at pH 1.5 (dropping to 84%) and pH 9 (dropping to 85%), and at 5 mg L^{-1} Fe for the 50 μg L^{-1} spike (dropping to 81%), suggesting a signal loss even at relatively low Fe concentrations (Fig. 4d). In concentrations above 100 mg L^{-1} Fe, measurable Na-fluorescein intensity diminished rapidly; the recovery at 500 mg L^{-1} Fe was below 5% of the input concentration for all three spikes at pH 1.5. The development at pH 9 was even more drastic, with a sudden drop down to only 1% recovery already at 50 mg L^{-1} Fe (Fig. 4b, d).

As an approach to overcome this problem, we applied the 'method of standard addition'. This method is often used in analytical chemistry and is described in norm DIN 32633 of the German Institute for Standardization (1998). The basic principle of this method is to add spikes with increasing Na-fluorescein concentrations to the sample with an unknown Na-fluorescein concentration and then measure all the solutions. Consequently, the Fe content should now reduce the signal intensity of the added spike in the same proportion as in the original sample. The interpretation of the measurement is carried out graphically by plotting the measured intensity of fluorescence in arbitrary units [a.u.] versus the spike concentrations; a regression line is fitted to the values. The interception point of this line with the x-axis then gives the real Na-fluorescein

0.1 μg L^{-1}. In the other samples, the background Na-fluorescein values exceeded this concentration range, so it is impossible to recover the Na-fluorescein spike of 0.05 μg L^{-1}. In most of the samples, the input concentration was recovered almost completely. The general trend was similar to measurements at pH 9, in that the divergence from the ideal values increased with decreasing concentrations. The overestimated values of sample 4 at the Na-fluorescein target values 0.5 and 5 μg L^{-1} reflect the high background signal in this sample, potentially caused by other organic components. Only the Fe-rich samples 2 and 8 showed an underestimation, with Na-fluorescein recovery rates between 18 and 66%. The instrumentally-computed relative standard deviations obtained for the recovery tests in these two samples are in the same range as the relative standard deviations for the low Fe samples (0.1–2.0%), suggesting an actual decrease of the Na-fluorescein signal rather than instrumental inaccuracy. The input concentrations of the Mn-rich sample 5 could be recovered without any significant deviation.

From these results, it is clear that it is not the pH of the mine waters that is problematic. It is certainly possible to measure Na-fluorescein concentrations correctly on the
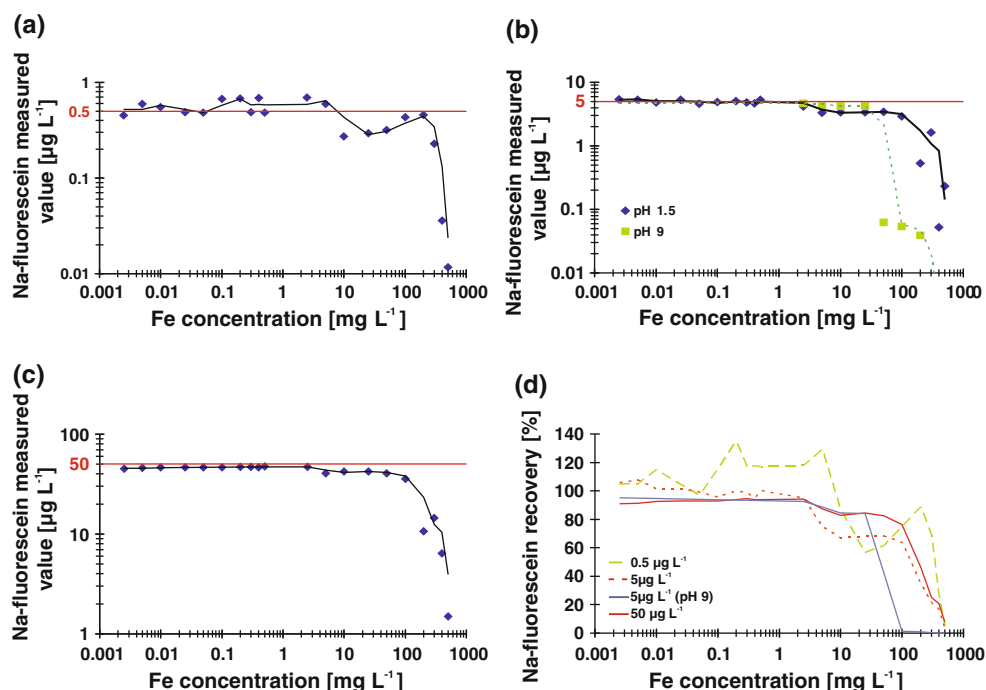
Fig. 4 Recovery tests for Na-fluorescein with increasing Fe concentrations at Na-fluorescein input concentrations of **a** 0.5 µg L^{-1}, **b** 5 µg L^{-1}, and **c** 50 µg L^{-1}, and development of recovery rates (**d**). All measurements were conducted at pH 1.5, except where pH 9 is indicated

concentration in the sample. It is important to perform all analysis in the same calibration range because otherwise the linear trend between the intensity and the concentration would not apply.

To test the applicability of this method, we selected sample 2 as a natural acid mine water with high Fe content and additionally created two artificial samples that are listed as samples 9 and 10 in Table 1. As a basis for artificial sample 10, we used sample 6, which has a neutral pH, low metal content, and could be measured without problems in the previously conducted recovery tests. We then added an Fe standard solution (Fe(NO$_3$)$_3$ in 0.5 M HNO$_3$) and a few drops of 65% HNO$_3$ solution to accomplish an Fe content of 200 mg L^{-1} and thus simulate a composition similar to natural acid mine water. Artificial sample 9 consists of pure Fe standard solution (Fe(NO$_3$)$_3$ in 0.5 M HNO$_3$) with an Fe content of 1,000 mg L^{-1}. These three samples were prepared with a Na-fluorescein concentration of 5 µg L^{-1}. Subsequently, we added Na-fluorescein spikes of 2.5 and 5 µg L^{-1} to gain three points for the regression line and then measured the samples with the fluorescence spectrophotometer at pH 1.5. The results are shown in Fig. 5.

The method of standard addition only partially provided the desired results. For sample 9, containing 1 g L^{-1} Fe, only 3.4 µg L^{-1} Na-fluorescein were determined, which is 68% of the tracer concentration in the sample. With a determined concentration of 5.5 µg L^{-1}, the Na-fluorescein in sample 10 was recovered completely with this method (with a slight overestimation). For sample 2, however, a Na-fluorescein concentration of 8 µg L^{-1} was
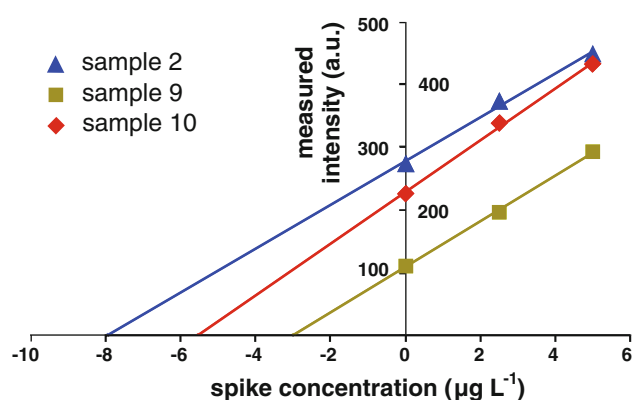
Fig. 5 Method of standard addition for three samples with pH 1.5 and a Fe content of 200 mg L^{-1} for samples 2 and 10 and a Fe content of 1,000 mg L^{-1} for sample 9

detected, which is 159% of the sample concentration and therefore an overestimation. The slope of all regression lines is similar but the measured intensities without the spike vary. It looks like the main problem is that, contrary to our assumption, the added Na-fluorescein spike is affected by Fe presence in a different way than the initial Na-fluorescein in the sample.

Nevertheless, it is interesting to compare the concentrations determined with the method of standard addition, with a direct measurement at pH 1.5. The latter only detected a Na-fluorescein concentration of 1.4 µg L^{-1} (28% recovery) for sample 2 and 1.1 µg L^{-1} (22%) for sample 10. The Na-fluorescein in sample 9 could not be detected at all, due to the high Fe concentration of 1 g L^{-1}

in this sample. In using the standard addition method, we thus recovered significantly more Na-fluorescein for all samples than by direct determination.

Due to the low number of data points in the applied standard addition method, statistically reliable statements on the confidence of the regression lines cannot be made. Therefore, it is possible that the correct concentration falls within the confidence range on the x-axis. Additional analysis is required to support the method.

Conclusions

This study showed that contrary to general assumptions, it is possible to quantify Na-fluorescein in acid mine waters with satisfactory results by buffering the samples to pH 1.5 and then measuring the intensity on the 'side peak'. This eliminates the time- and cost-intensive filtration of samples necessary when the classical approach of Na-fluorescein analysis at pH 9 is used. High Fe concentrations are a limiting factor for the introduced approach. Good Na-fluorescein applicability can be expected for mine waters with up to ≈ 100 mg L^{-1} Fe, but significant intensity losses were shown to occur at higher Fe concentrations. We assume that the generation of Fe-fluorescein-complexes is responsible for this phenomenon. The method of standard addition is shown to be a potential alternative to determine Na-fluorescein at even higher Fe concentrations which, however, will have to be enhanced in further studies. The approach introduced here represents a promising tool for improving Na-fluorescein applicability in acid environments and thus granting the mining and remediation sectors access to this easy to use and cost-effective dye.

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