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Diploma thesis

Optimisation of fluorescence tracers for flooded underground mines

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Munich, May 2008

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AFFIDAVIT

I hereby certify that I have prepared this thesis without the unauthorised assistance of third parties and without the use of aids other than those indicated; the ideas taken directly or indirectly from external sources are identified as such.

Munich, 27 May 2008Michael Kratzer

THANKS

First of all, I would like to thank Prof. Dr. Christian Wolkersdorfer for his many suggestions and advice over the past months. Especially helpful were the countless suggestions for corrections concerning the layout and the linguistic design of the work.

Then I would like to thank the members of the Peißenberg Miners' Association, especially Mr Franz Fischer, for their kind support in taking mine water from the deep gallery "Heilstollen" of the former Peißenberg mine. Furthermore, I would like to thank the laboratory staff in the hydrogeological laboratory of the Hydro- and Environmental Geology Working Group, Ms. Koci and Ms. Paschert, who helped me with my work whenever possible.

In addition, I would like to express my deep gratitude to my entire family, especially my parents, who have supported me unreservedly throughout this time, who have always been there for me with advice and support whenever necessary, and without whom this work would certainly not have been possible in this form.

Summary

The choice of tracers for conducting tracer tests in underground mines is very limited due to the often special requirements. In recent years, fluorescent microspheres have been used several times in such tests and have proven to be a suitable tracer. They are artificially produced and chemically and physically very variable polystyrene spheres with diameters from 0.02 to 100 µm. For use in conventional fields such as surface and ground water, several methods have been developed for this type of tracer, which is counted among the artificial tracers, that enable rapid and highly detectable evaluation. However, except for the time-consuming counting of the microspheres under a fluorescence microscope, these methods are not suitable for use in underground mines. Therefore, within the framework of this diploma thesis, a procedure that has been used as standard in physiology and medicine for years was to be adapted to the special requirements of mine water. In this method, the number of microspheres is not determined by counting, but directly via the fluorescence intensity of the fluorescent colours enclosed in the microspheres. For this purpose, the fluorescent dyes are dissolved from the polystyrene shells of the microspheres with the help of an organic solvent (2-ethoxyethyl acetate) and their intensity is measured in a spectral fluorimeter. The main focus was on the development of a method for sample preparation and the
-evaluation, with which the samples collected during the tracer experiments should be able to be evaluated easily and quickly.

Different microspheres were used in the tests carried out as part of the work: three colours from the Dye-Trak VII+ series and the entire series of Dye-Trak 'F' microspheres, consisting of four different colours. Both microsphere series are manufactured by Triton Technology. In addition, the tests were carried out with two different mine waters from the Straßberg underground mine and the former Peißenberg mine.

An important part of the work was to test whether it is possible to simultaneously measure different microspheres stained with different fluorescent colours. Fluorescent microspheres have the great advantage that they can be stained with different fluorescent colours. This makes it easy to distinguish between different tracer points at which one of the selected microsphere colours is added. In this context, it has been possible to distinguish between up to six different microsphere colours during only one measuring procedure.

Furthermore, it turned out that the measurements carried out with the spectrofluorophotometer are comparable to those of the counting method and that with a considerably lower amount of work. Furthermore, a method could be developed with which the microspheres could be detached from the filter nets used in the tracer tests and their number determined. In the tests carried out, differences were found between the two microsphere series used, Dye-Trak VII+ and 'F', and the two mine waters Straßberg and Peißenberg. At least some of the tests delivered reliable and accurate results. Finally, the detection limit, which was previously estimated at about 1000 microspheres, was lowered considerably. In this context, it was possible to detect numbers in the range of 50 microspheres.

Abstract

The assortment of tracers for tracer tests in mines is often highly limited because of special requirements. In the last few years fluorescent microspheres were used in multiple ways for such tests and they turned out as convenient tracers. Microspheres are synthetic and physically and chemically very flexible polystyrene beads with diameter from 0.02 to 100 µm. For the application at conventional fields like surface and ground water several methods for this tracer have been developed, which allow a fast and easy analysis. But they are, except for the time-consuming counting of the microspheres under a fluorescence microscope, not practical for an application in underground mines. For that reason in this work a method should be adopted to the special requirements of mine water, which is, by default, used in physiology and medicine for years now. Within this method the number of microspheres is not deter- mined by counting, but directly from the fluorescent intensity of the dyes in the microsphere beads. For that purpose the fluorescent dyes have to be eluted out of the polystyrene beads with the help of an organic solvent (2-ethoxyethyl acetate) and their intensity has to be measured in a spectral fluorimeter. The main focus of the proceeding lies on the development of a method for sample preparation and analysis, which should allow to analyse the samples collected during the tracer test in a simple and fast way.

Within the context of the work different microspheres were used for the performed experiments: three dyes of the Dye-Trak VII+ series and all dyes of the Dye-Trak 'F' microsphere series, including four different colours. All the microspheres are manufactured by Triton Technology. Furthermore two different mine waters from the abandoned underground mine Straßberg and the abandoned mine Peißenberg were used.

An important part of the work was to verify if a simultaneous measurement of different microspheres - marked with various dyes - is possible. Fluorescent microspheres have the advantage, they can be marked with various dyes. For this reason different injection points, where at a time one of the chosen microsphere dye gets injected, can be easily distinguished.

Furthermore there it was pointed out, that the measaurements which were made by spectral fluorimeter are comparable with those of the counting method and can be performed with a significant lower amount of work. Furthermore a method could be developed which allows to release the microspheres from the filters that are used at the tracer tests and to count their quantity. Within the passed experiments differences between the two used microsphere series Dye-Trak VII+ and 'F' and both mine waters from Straßberg and Peißenberg became evident. Thereby at least a part of the experiments delivered reliable and precise results. Finally the detection limit, which was mostly estimated at around one thousand microspheres before, should be reduced significantly. In this context it was possible to prove numbers around fifty microspheres.

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1 In tion

Coloured and fluorescent microspheres, 0.02 - 100 µm polystyrene beads, were originally developed for applications in medicine and microbiology. There they have been the subject of intensive research for more than two decades and are now used as standard, especially for determining blood flow in organisms (Raab 2003). Their potential as particle tracers in hydrological and hydrogeological systems, especially that of fluorescent microspheres, was first exploited in the mid-1980s. Since then, numerous field and laboratory experiments have been conducted and a wide variety of methods have been developed for the detection and analysis of microspheres for hydrogeological applications. The oldest and most common is the time-consuming and tedious counting of microspheres under a fluorescence microscope. Another was developed by Niehren (1999). This is a field-ready microspheres counter for groundwater applications that detects fluorescence using a single photon counting avalanche diode. However, this fast and highly detectable method only allows the detection of a single microsphere colour and is essentially designed for groundwater applications.

The choice of tracers for tracer tests in flooded underground mines is often very limited due to the special requirements that mine waters often place on possible tracer substances. Therefore, new, suitable tracer substances are constantly being sought in this field of application. For this reason, fluorescent microspheres were used for the first time in tracer tests in flooded underground mines a few years ago. Since then, they have proven to be suitable tracers on several occasions. Among other things, they have been used to clarify pollutant-related transport processes in mine water. In addition, these artificial particle tracers can also be used to obtain information on the flow direction and velocity, as well as on hydraulic connections, as part of a tracer experiment. The conditions in a mine often place high demands not only on the tracer substance, but also on the sampling and analysis procedures.
-evaluation in a tracer experiment. For this reason, it is also important to look for ways to improve and further develop the existing methods in this respect. All tracer tests carried out so far in underground mines have been evaluated under the fluorescence microscope using the counting method already mentioned above. Alternatives, such as the microsphere counter developed by Niehren, are not suitable for the evaluation of tracer tests in underground mines due to the sometimes high particle content in mine water (Käss 2004).

For this reason, a standard method used in medicine, the evaluation with the help of a spectral fluorimeter, was to be adapted to the special requirements of mine water within the framework of this diploma thesis. For this purpose, the fluorescent dye is first dissolved out of the microspheres using an organic solvent (2-ethoxyethyl acetate) and the intensity of the fluorescent dye is then measured in a spectral fluorimeter.

2 Material and Methods

2.1 Material

2.1.1 Equipment and chemicals

The spectrofluorophotometer RF-5001PC from Shimadzu was used to measure the fluorescence intensity. Its exact mode of operation is described in detail in chapter 2.3.2.

Cuvettes made of special optical glass from Hellma (Hellma GmbH & Co. KG, 79371 Müllheim, Germany) were used for the measurements, as cheaper disposable cuvettes made of polystyrene are not resistant to the solvent. The organic solvent (2-ethoxyethyl) acetate from Merck (Merck KGaA, 64293 Darmstadt, Germany) was used to release the fluorescent colours from the microspheres.
is used. This is the solvent recommended by the manufacturer of the microspheres used (Triton Technology Inc., San Diego CA 92109) to trigger the fluorescent dyes.

Nylon filter nets with a mesh size of 15 µm (NY 15 HC) and 41 µm (NY 41 HC) from the companies Heberle (Renate Heberle Netzfabrikation, 87452 Altusried, Germany) and Hydro-Bios (Hydro-Bios GmbH, 24161 Kiel-Holtenau, Germany) were used as filter material. The filter nets were cleaned with oxalic acid (0.05 mol/L), made from oxalic acid dihydrate (Merck KGaA, 64293 Darmstadt, Germany) and distilled water.

The KS 250basic horizontal shaker (IKA Labortechnik GmbH & Co. KG, 79219 Staufen, Germany) and the Rotofix 32 tabletop centrifuge (Andreas Hettich GmbH & Co KG, 78532 Tuttlingen, Germany) were used to accelerate the triggering process. In both cases, 50 mL centrifuge tubes made of polypropylene were used.

2.1.2 Microspheres

For the microspheres, Lemon VII+, Tangerine VII+ and Berry VII+, three of the seven microspheres of the Dye-Trak VII+ series, were used initially. These were later expanded to include the entire series of Dye-Trak 'F' microspheres, consisting of four fluorescent colours (Lemon 'F', Yellow 'F', Orange 'F' and Persimmon 'F') and one control colour (Navy 'F'). The colour Navy 'F' serves exclusively as an internal control to validate the sample preparation process. The microspheres, which all have a diameter of 15 µm, are manufactured by Triton (Triton Technology Inc., San Diego CA 92109). They are stored in concentrations of three (Dye-Trak VII+) or one (Dye-Trak 'F') million microspheres per mL in a 2 or 10 mL NaCl solution, mixed with 0.05 % Tween 80 and 0.01 % Thimerosal. Although the Dye-Trak VII+ series are not designated fluorescent microspheres, according to the manufacturer they are also stained with fluorescent dyes. Consequently, they could be used just as well in the tests as the microspheres of the Dye-Trak 'F' series. In addition, when selecting the three Dye-Trak VII+ microsphere colours from a total of seven available, special care was taken to ensure that their individual excitation maxima were far apart from each other. This measure was intended to exclude a possible spill-over effect (see chapter 2.4.1).

With the Dye-Trak 'F' microsphere series, as Tab. 2 shows, simultaneous use of all four Dye-Trak 'F' colours could be realised without any significant spectral overlap and mathematical correction. The respective excitation and emission maxima of the different Dye-Trak VII+ and Dye-Trak 'F' microspheres are listed in Tab. 1.

An important prerequisite that generally applies to labelling agents is that they are harmless to humans and ecotoxicologically. According to studies by the Working Group for the Human and Ecotoxicological Evaluation of Marking Agents in Waters (1997), this is demonstrably true of fluorescent microspheres. In view of their use as labelling agents in physiology, this result seems self-evident. Besides, in contrast to many other colour tracers, they usually do not cause any visible discolouration of the water even when larger amounts of tracer are added.

Colour	Excitation (nm)	Emission (nm)
Lemon (Dye-Trak 'F')	390	445
Yellow (Dye-Trak 'F')	440	485
Orange (Dye-Trak 'F')	495	525
Persimmon (Dye-Trak 'F')	545	575
Navy (Dye-Trak 'F')	640	670
Lemon (Dye-Trak VII+)	390	---
Tangerine (Dye-Trak VII+)	525	---
Berry (Dye-Trak VII+)	590	---

Tab. 1 Listed are the respective excitation and emission maxima of the different Dye-Trak VII+ and Dye-Trak 'F' microspheres, with Navy serving exclusively as the control colour. The release of the respective fluorescent dyes was carried out with the solvent (2-ethoxyethyl) acetate.

In addition to the diameters of 15 µm used, microspheres with diameters of 0.02 - 100 µm are available, depending on the problem. According to the manufacturer (Triton Technology), the fluorescent microspheres have a density of 1.06 g/cm³. Since the fluorescent dye is already introduced during the polymerisation process, the dye is distributed evenly over the entire sphere volume and does not remain on the surface. This enables a high stability of the fluorescent dye against all external influences. With appropriate storage, the shelf life of the microspheres is at least one year. The loss of microspheres after 6 months in dark storage is below 1 % (An et al. 1999). When storing the microspheres, only a few things need to be taken care of in comparison to the fluorescent dyes that are dissolved out later. Earlier experiments have shown that the microspheres can be stored both in the refrigerator (5 °C) and at room temperature (approx. 25 °C) without significant loss of fluorescence intensity (Raab 2003). According to the manufacturer's information, they are even stable in a temperature range of 0 - 60 °C. However, care should be taken that the microspheres are stored in the dark (An et al. 1999).

Another major advantage of fluorescent microspheres is their highly variable chemical and physical properties. During the manufacturing process, functional substances can be added to the surface of the otherwise neutrally charged polystyrene spheres.

groups (e.g. carboxyl and sulphate groups) can be attached. This allows both positively and negatively charged microspheres to be created. In addition, depending on the functional group, they have hydrophilic or hydrophobic properties.

2.2. Marking tests in underground mines

2.2.1 History

Although there are many different reasons for carrying out a tracer test in or around a mine, only a few tests have been carried out and described in the literature. In comparison, tracer tests have long been a proven tool in groundwater investigations. They provide information on hydraulic parameters, transport processes and possible connections between different groundwater bodies. In addition to these specifications, numerous other questions arise in connection with mines. On this basis, Wolkers- dorfer (2002) divides the tracer tests carried out so far into the following groups according to their objectives:

- Optimisation of the mining strategy
- Assessment of the risks or reasons for mine water intrusion/flooding
- Development of rehabilitation strategies for mines
- Assessment of the suitability for underground disposal of nuclear waste
- Assessment of the feasibility or effects of the storage of non-radioactive waste on the subsurface
- and settlement studies

It should be noted that most tracer experiments to date have not been aimed at marking the mine water in the mine itself. Instead, they were concerned with issues that involved a mine. Examples of this are the assessment of the suitability of an abandoned mine for the final underground storage of nuclear waste or the potential contamination of a groundwater body or surface water by escaping mine water. The latter was the subject of the historically first tracer tests in connection with mines. The sole purpose was to determine possible connections between groundwater or surface water and the mine. In contrast, the question about the hydraulic parameters of a water body, which is common in groundwater investigations, is usually much more difficult to answer in underground mines. This is mainly due to the fact that a tracer input far below the water surface at a predefined water depth is necessary without contaminating the mine water above the input point. In most of the tracer tests carried out so far, however, the tracer was either added directly at the surface, where it then entered the mine water via fissures, or it was added to the mine water via boreholes. Therefore, there are hardly any tracer tests described in the literature that contain statements about the hydraulic parameters. One of the first tracer experiments aimed at investigating the complex hydrodynamics of an underground mine was carried out in 1995 (Wolkersdorfer 1996). Lydia (**Lycopodium task probe**) was used, a device that had been specially developed in advance for the precise tracer input required for this purpose.

2.2.2 Objectives

As described in the previous chapter, tracer tests that deal exclusively with the mine water in an underground mine itself are a rarity. Tracer tests carried out in advance of a mine rehabilitation can help to work out a suitable or improved rehabilitation strategy. With this comparatively inexpensive method, knowledge can be gained about the hydrodynamics of a flooded underground mine. This can help to optimally adapt the often very cost-intensive remediation measures to the remediation object.

For such a case, there are the following basic objectives:

- Testing the effectiveness of sheet piling or dams
- Exploring the hydrodynamic conditions
- Determination of connections between mine and surface
- Clarification of the causes of mine water intrusion/flooding
- Exploration of the mass flow/flow rate
- Determination of the decrease or increase of pollutants

2.2.3 Tracer requirements and selection

When using tracers in underground mines, the following difficulty arises: many of the substances that are otherwise used in hydrogeological and hydrological investigations cannot be used in underground mines. This is often due to the physical and chemical parameters of many mine waters, which can contain extremely low pH values, high solute loads and metal concentrations. Due to these conditions, many of the tracers described by Käss (2004) are no longer available. Therefore, only a very limited number of tracers are available for use in underground mines. Since mostly only results of successful tracer tests are published, little can be said about the number of unsuitable tracers. Therefore, it is not possible to make statements about the entire selection of tracers that have been used in underground mines in the past.

Mine waters differ greatly from one another in their chemical and physical properties (e.g. pH, solubility, trace element concentrations). Therefore, it is essential to carry out laboratory tests with all potential tracer substances and the mine water with the expected composition prior to a tracer test. It is particularly important that the potential tracer remains stable in relation to the mine water over the entire duration of the tracer test. With their highly variable chemical and physical properties, fluorescent microspheres have good prerequisites for use in mine water. Especially in the respect that the number of different tracers is limited in a multitracer test. Using the same tracer but different microsphere colours, up to seven (thirteen) different input points can be distinguished from each other. In addition, eight other tracers have been described in the literature that have been successfully used in underground mines. These include Na- fluorescein, *lycopodium* spores (*Lycopodium clavatum*) and NaCl (Wolkersdorfer 2006).

2.2.4 Use of filter networks

In order to obtain reliable results from a tracer test, continuous sampling should always be aimed for. At least with water-soluble tracers such as salt and dye, it is often sufficient to simply take water samples at regular intervals and then examine them in the laboratory. With other tracers, however, the requirement of continuous sampling often poses a greater problem for various reasons. In the case of particle tracers such as lycopod spores or microspheres, for example, this procedure is only possible if either very high tracer concentrations are expected or water quantities greater than one litre can be filled. An analysis of smaller sample quantities would not lead to any usable result due to concentrations that are difficult to detect because they are too low. In order to nevertheless enable continuous sampling and thus a good quantitative evaluation, Niehren (1999) developed a microsphere counter suitable for the field. At its heart is a specially designed flow cytometer. According to Käss (2004), however, this device is only a sensible alternative to the otherwise used counting method for tracer experiments in low-freight groundwater and surface water. In this method, the number of microspheres is determined by time-consuming counting under the fluorescence microscope. An application in underground mines is out of the question because of the associated special requirements and conditions. These often include high flow rates, cramped sampling conditions and heavily polluted mine water. For this reason, only filter nets were used for sampling in the tracer tests with microspheres in underground mines. These are made of stable nylon and have different mesh sizes of 15 and 41 µm. They ensure that the tracer accumulates on the surface of the filter nets over a certain period of time and is thus concentrated. In the course of the tracer experiment, the filter nets are then removed at regular intervals and replaced by new ones. The period of time after which the filter nets must be removed is determined in advance of the tracer test. The following consideration plays a decisive role here. The longer the period, the more the tracer is concentrated. However, the poorer the temporal resolution. Which of these is more important depends on the circumstances and the question. What is certain is that with this sampling technique, semi-quantitative results can still be achieved during the evaluation. Originally, this type of sampling was developed for another particle tracer, stained lycopod spores. Both tracers are very similar in many properties. Thus, the original methodology did not need to be strongly adapted to use microspheres instead of Lycopodium spores. A major difference between the two tracer substances is the differing mesh sizes. These are 30 µm for lycopod spores and 15 µm for microspheres. A detailed description of the use of lycopod spores and their sampling with filter nets can be found in Käss (2004).

Until now, a major disadvantage of this sampling method has been the time-consuming evaluation of the samples using a fluorescence microscope. With the intended use of a spectrofluorophotometer, the aim is now to address precisely this point and enable rapid sample preparation and evaluation. The differences in evaluation between spectrofluorophotometer and fluorescence microscope will be discussed in detail in chapter 2.5.

As will be read in more detail in the historical analysis in chapter 2.3.1.2, the use of microspheres with a diameter of 15 µm, outside of an underground mine, has only been described once in the literature (Petrich 1998). The decisive factor here is the criteria for selecting the diameter of the microspheres.

As a rule, the size of the particle whose transport behaviour is to be simulated and the average pore diameter of the aquifer to be investigated are of decisive importance. In this context, Käss (2004) comes to the following conclusion: microspheres with a diameter of about 1 µm are best suited for simulating the transport behaviour of microorganisms, such as bacteria, in pore aquifers. In other aquifer types, too, only microspheres with diameters of up to 6 µm have been used so far. This is because with larger diameters there is a risk that the microspheres will not move permanently in the water body due to the associated increase in weight. Instead, they sediment before reaching the sampling point and thus no longer allow a useful evaluation of the tracer experiment. Due to the usually high sediment load in mine waters and the planned use of close-meshed filter nets, the following problem arose: because of the sometimes high sediment load, the mesh size could not be chosen too narrow. Therefore, the use of microspheres with small diameters of up to 5 µm was out of the question. Numerous laboratory tests were carried out on this aspect between 1994 and 2006 by the "Tracer Tests" working group (Dr. Ch. Wolkersdorfer) at the TU Clausthal and TU Bergakademie Freiberg. It turned out that filter nets with mesh sizes below 10 µm were not suitable for sampling. After conducting further tests, this time with wider-meshed filter nets, it was finally decided to use a diameter of 15 µm.

2.2.5 Hydrodynamics

The hydrodynamic properties of a water body are often part of the question in tracer tests. In this respect, flooded underground mines behave essentially like karst aquifers (Sammarco 1995; Burbey et al. 2000). For this reason, the principles that apply to karst aquifers, which Käss (2004) discusses in detail, can be used as a strong guide for appropriate experiments in an underground mine. Both in karst and in mines, the effective velocities are high, the storage capacity is low and there is usually a non-linear or turbulent flow. Despite these many similarities, Wolkersdorfer (2008) lists the following main differences between a karst aquifer and a flooded mine:

- Roadways in modern mines are mostly not curved and meet each other at right angles
- normally, the diameter of the track does not change on longer routes
- Changes in the water level are relatively minor
- Cavities in the mine are not subject to solution processes

In addition to the large cavities, such as shafts and galleries, which in simplified form can be equated with karst cavities, there are often also extensive fissure systems in the vicinity of most underground mines. These can either have existed before or be caused by mining operations. Even if the fracture systems play a minor role compared to the large cavities, they can cause an increased flow rate in special cases. This is the case, for example, during heavy rainfall events. During the multitracer test in the abandoned Königshütte mine, it was possible to prove that during such a heavy rainfall event, the flow rate was increased.

In the event of a rainfall event, the fracture systems above the water surface are hydraulically connected to the mine water below (Wolkersdorfer et al. 2002). Finally, there are areas of backfill material in most mines. These can be regarded as pore aquifers in addition to the normal rock matrix of the surrounding rock. Therefore, if you look at it more closely, a flooded underground mine usually does not just have the characteristics of a karst aquifer. Instead, it has characteristics of all three typical aquifers: pore, fracture and karst aquifers. These each have a greater or lesser share in the level of water flow.

In addition, when carrying out a tracer test in an underground mine, the following advice should be taken into account: one should always be prepared for anything and especially not disregard the fact that surprising developments can occur at any time, which one has not taken into consideration beforehand. An example of this is the presence of shafts or tunnels that are not recorded in this form in the mine plan, but which can have a considerable influence on the hydrodynamics of an underground mine.

2.3 Use of microspheres

2.3.1 History

2.3.1.1 Physiology

The use of microspheres has a comparatively long tradition in physiology. In 1967, microspheres were used for the first time for blood flow measurements (Rudolph and Heyman 1967). These first microspheres consisted of carbon and oxygen instead of polystyrene. In addition, they contained radioactive isotopes whose activity could be determined by means of a gamma counter. Since then, the properties, not only of radioactive microspheres, have been continuously developed. Nowadays, microspheres with diameters of 0.02 - 100 µm and different chemical and physical properties are available on the market. In addition, the integration of functional groups offers great flexibility. This makes it possible to create microspheres with carboxyl- and sulphate-modified surfaces that can be either positively or negatively charged and behave either hydrophilically or hydrophobically. In the case of radioactive microspheres, numerous different nuclides are available (including cadmium-109, indium-114, ruthenium-103). Each of them has a specific energy spectrum and can thus be determined without doubt by gamma spectrometry even in the presence of other isotopes. Even though radioactive microspheres are still used in a wide variety of applications, their use entails numerous disadvantages. For this reason, alternative substances for labelling the microspheres were sought only a few years after their first use. One disadvantage of radioactive microspheres is their low stability over time. On the one hand, this results from the short half-life of the available radioactive isotopes, which ranges from 27.7 to 462.6 days (Steinhagen 2004). On the other hand, a small percentage of the radioactive isotopes contained in the microspheres escapes from the polystyrene shell during longer experiments (Van Oosterhout 1998). For these two reasons, radioactive microspheres are not suitable for experiments that exceed a period of 24 hours (Van Oosterhout 1998; Glenny 1993). In addition, there are high disposal costs, a licensing requirement for any kind of experiments with radioactive microspheres and high safety requirements for handling the radioactive material.

Subsequently, non-radioactive microspheres were developed that were labelled with different dyes instead of radioactive isotopes. Hale (1988) used these for the first time in 1988 to determine blood flow. The preparation of these microspheres was as follows: The microspheres were separated from the dissolved tissue samples by centrifugation and then measured in the haemocytometer. The dyes stored in the microspheres can be distinguished from each other due to a specific wavelength (absorption wavelength) with which they absorb a maximum of the light. Another advantage over radioactive microspheres is the greater stability of the dyes. They can therefore also be used for longer experiments. In addition to these advantages, coloured microspheres also have some disadvantages. These include the strong overlapping of the spectral maxima that occurs between the different colours. For this reason, at the beginning, only three different colours could be used at the same time without a mathematical correction of the individual spectra and in contrast to radioactive isotopes (Hodeige 1999). In addition, there was a comparatively complex sample preparation at the beginning. In order to be able to measure the colour intensity, the microspheres first had to be extracted from the tissue in an elaborate way. Even though the sample preparation and thus the reliability of the method could be greatly simplified by a different processing protocol (Kowallik et al. 1991), an alternative to the coloured microspheres was still sought. This was found in the use of fluorescent dyes. In contrast to the simple dyes, these have a narrower absorption and emission maximum and are therefore easier to distinguish from one another. For the measurement of the colour intensity, a spectral fluorimeter is used instead of an absorption photometer, as still used for the coloured microspheres. This allows a significant increase in sensitivity to be achieved.

Fluorescent microspheres were used for the first time in the early 1980s. In these first tests, a fluorescence microscope was used to count the individual microspheres, as is still common today in groundwater investigations. A spectral fluorimeter to determine the intensity, which can then be used to calculate the number of microspheres in a sample, was used for the first time in 1993 (Glenny 1993). The prerequisite for this is that the exact number of microspheres can be determined via the intensity. Initially, the fact that the fluorescence intensity cannot be measured directly in the samples, as is the case when using radioactive microspheres, posed a serious problem. When the microspheres were released from the tissue samples, there was often a loss of microspheres. In addition to this, there was also the risk of losing the fluorescent dye that had been released. Both of these factors lead to a change in the dye concentration and thus to a more or less large measurement error. For this reason, the method of extracting the microspheres from the tissue was modified and improved many times in the following years (Glenny 1993; Van Oosterhout 1995 and 1998; Raab 2003).

In addition, in numerous experiments, either different fluorescent microspheres were compared with each other or fluorescent microspheres were compared with radioactive microspheres. In both cases, very good agreement was found. For the coefficient of determination (R), values of R^2= 0.98 - 0.99 were obtained when comparing fluorescent with radioactive microspheres and R^2= 0.99 for different fluorescent microspheres among each other (Glenny 1993).

One finding that can be drawn from many studies carried out over the last few years is the following: The use of fluorescent microspheres offers many advantages compared to radioactive microspheres. For this reason, fluorescent microspheres can

compared to radioactive microspheres are considered either equivalent or even superior (Raab 2003).

2.3.1.2 Ground and surface waters

Microspheres have been used in hydrogeology for about 20 years. Almost exclusively fluorescent microspheres of different sizes and surface modifications have been used. Usually, microspheres with diameters of 0.2 to 5 µm and a neutral or carboxyl-modified surface are used. Carboxyl-modified microspheres are negatively charged and behave hydrophilically. With these microspheres, not only because of their similar size, the transport behaviour of small particles, e.g. bacteria and viruses, in the subsurface can be simulated in a suitable way. With their electroneutral surface, the polystyrene spheres show similar transport behaviour to bacteria (Harvey 1989). In addition, they have clear advantages over the use of bacteria, in this case as a tracer substance, in many respects (Käss 2004). The areas of application of microspheres can be divided into two large groups. On the one hand, there are column experiments and small-scale experiments and, on the other hand, field experiments. The first use of microspheres in a labelling experiment in groundwater took place in the mid-1980s in the Chalk River experimental area, Ontario, Canada (Käss 2004). In the following years, the properties of microspheres and their suitability in tests in groundwater and surface water were researched in detail. Until the end of the 1990s, microspheres with diameters of 0.2 to 2.0 µm and carboxyl-modified surfaces were widely used (Harvey 1989, 1993, 1995; Becker 1999; Reimus 1999; Niehren 1999). Initially, great difficulties were encountered. Harvey (1989) came to the following conclusion after his first experiments: none of the microspheres used were suitable for simulating bacterial transport in groundwater. Subsequently, however, numerous independent experiments in pore, karst and fissure aquifers provided revealing insights into the transport properties of microorganisms.

Although they have been widely used, Käss (1992 and 2004) considers carboxyl-modified microspheres unsuitable for groundwater marking due to their active surface. This assessment cannot be confirmed, however, if one considers the results of a large number of tests carried out to date. On the other hand, it is clear that in recent years microspheres with larger diameters above 1 µm have been increasingly used (Ward 1997; Petrich 1998; Bäumle 2001; Göppert 2005, 2006, 2007). With diameters of 1 to 15 µm, these are suitable for simulating both small particles such as bacteria and larger organisms. An example of this are the experiments of Petrich (1998), who successfully used microspheres with diameters of 2, 5 and 15 µm in 1995 on a site of the University of Idaho, approx. 3.2 km east of the city of Moscow, Idaho. The subject of the investigation was the transport behaviour of encapsulated cells ("encapsulated cell microbeads") in a heterogeneous aquifer.

In addition to the field tests, numerous column tests and small-scale experiments were also carried out (Harvey 1993; Vilks 1996; Ward 1997; Becker 1999; Cumbie 1999; Niehren 1999; Gamerdinger 2001; Knappett 2006). These mostly served to simulate the transport properties of particles on a small scale. Subsequently, an attempt was made to transfer the results obtained to the conditions of areas where field tests were carried out. An important difference between the column tests and the field tests is that the analytical methods used in the column tests are often different.

methods were used. With a few exceptions (Ward 1997; Becker 1999), a fluorescence microscope or various types of particle counters were used in the field tests. In the column experiments, on the other hand, spectral fluorimeters were often used. The reason for this is the higher detection limit of this method. Göppert (2005) gives this as approx. 1000 microspheres per mL sample quantity.

2.3.1.3 Underground mines

In underground mines, coloured and fluorescent microspheres have only been used in marking tests for a few years. The number of tracer tests carried out so far is easy to survey. The first well-documented multitracer test, in which microspheres were also used, took place in 2000 in the underground mine Straßberg in the Harz Mountains (Wolkersdorfer & Hasche 2001). This was followed by three more multitracer tests in 2001, 2003 and 2004 in Brixlegg, Königshütte and Ehrenfriedersdorf. In the flooded Tyrolean mine Georgi-Unterbau near Brixlegg/Tyrol, four different microsphere colours (orange, red, green and blue; Triton Technology) with a diameter of 15 µm were used in the first of two tracer tests in 2001 (Wolkersdorfer et al. 2002). The same applies to the test in Straßberg the year before. Instead of the colour blue, however, microspheres of the colour yellow-green were used in this case. In the third tracer experiment in the former mining area of Ahrendfeld near Königshütte/Harz, six different microsphere colours (red, orange and green: FluoSpheres; white, orange and tangerine: Dye-Trak VII+) were used (Wolkersdorfer & Hasche 2003). The last multitracer test so far took place in 2004 in the Sauberger mining district of the abandoned Ehrenfriedersdorf tin ore mine in the Ore Mountains. Three microsphere colours were used: white, tangerine (Dye-Trak VII+, Triton Technology) and red (FluoSpheres, Molecular Probes, Eugene, Oregon, USA).

2.4 Determination of the fluorescence intensity

2.4.1 Physical basics of fluorescence

Fluorescence is a physical phenomenon in which light of a certain wavelength (excitation or excitation wavelength) is absorbed by a fluorescent substance and then immediately emitted again as light with a longer wavelength (emission wavelength). During this process, photons initially hit some electrons of an atom and raise them to a higher energy level. Within approx. 10^{-9} s, the electrons then release their energy again in the form of light. This causes them to fall back to their more stable, lower initial energy level. In the process, the electrons emit another photon, but this one is lower in energy and thus has a longer wavelength than the excitation photon. This so-called emission wavelength is specific to each fluorescent dye. There is a difference between the excitation wavelength and the emission wavelength, which is called the Stokes shift.

The fluorescent dyes in the different fluorescent microspheres were selected and optimised in such a way that they are excited exclusively within a narrow colour spectrum and in turn also emit the light again within a narrow spectrum. This allows up to 7, and with the help of a mathematical correction even up to 13 fluorescent colours within the visible colour spectrum to be measured simultaneously. This is done without the individual spectral curves overlapping each other in a disturbing way.

This overlapping effect, which must be avoided, is also called spill-over effect. It only plays a major role with a few colours, such as green (FluoSpheres, Molecular Probes). In order to eliminate this spill-over effect and thus enable the simultaneous use of up to 13 different fluorescent colours, there are two possibilities. One is based on a mathematical correction. For this purpose, the proportion of the overlapping colour at the neighbouring curve maximum is determined by numerous comparative measurements. This proportion is then subtracted from the determined apparent maximum for all subsequent measurements. In the other method, the excitation of overlapping spectra takes place only after a slight shift of the excitation wavelength towards the blue end of the colour spectrum. This reduces the danger of exciting a colour at the red end of the colour scale, which is now further away.

Colour	Lemon	Yellow	Orange	Persimmon	Red	Crimson
Ex / Em wavelength	390 / 445	440 / 485	495 / 525	540 / 560	578 / 598	610 / 630
Lemon	100 %	0 %	0 %	0 %	0 %	0 %
Yellow	0 %	100 %	0 %	0 %	0 %	0 %
Orange	0 %	3 %	100 %	0 %	0 %	0 %
Persimmon	0 %	2 %	0 %	100 %	3 %	0 %
Red	0 %	0 %	0 %	0 %	100 %	2 %
Crimson	0 %	-1 %	0 %	0 %	2 %	100 %

Tab. 2 Spectral overlaps (in per cent) of equal concentrations of the four different "Dye-Trak 'F'" and two "FluoSpheres" microspheres.

In this study, Lemon 'F', Yellow 'F', Orange 'F' and Persimmon 'F' (Dye-Trak 'F'; Triton Technology) were used, four colours for which the spill-over effect has been shown to play little to no role. Tab. 2 shows the spectral overlaps (in percent) of the four different microsphere colours. If required, it is also possible to expand the existing colour spectrum by two additional colours, red and crimson (FluoSpheres; Molecular Probes), from four to six microspheres.

2.4.2 Fluorescence quenching (Quenching)

Basically, all processes that lead to a reduction in the intensity of the fluorescence are referred to as quenching. The effect usually occurs at very high dye concentrations, which leads to a reduction in the intensity of the fluorescence signal compared to the dye concentration. Two causes for this are complex formation and energy transfer to other molecules. However, a decrease in intensity can also be caused by contamination in the sample to be measured, an increase in temperature or a lower viscosity of the solvent.

2.4.3 Construction and function of the spectrofluorophotometer

A spectrofluorophotometer model RF-5001PC from Shimadzu (Shimadzu Europe GmbH, 47269 Duisburg, Germany) was used to measure the fluorescence intensity,

Germany) is used. The schematic structure of the optical system used in such a device is sketched in Fig. 1.

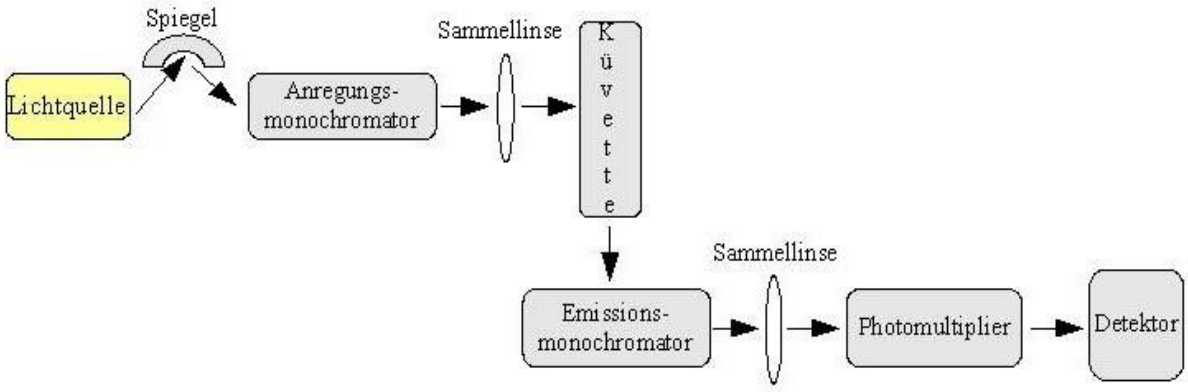


Fig. 1 Optical system of the spectrofluorophotometer RF-5001PC

A 150 Watt xenon lamp serves as the light source. The light spot of the xenon lamp is first expanded by an arrangement of various elliptical and concave mirrors, bundled and finally guided through the entrance slit of the excitation monochromator. The monochromator makes it possible to filter out a specific (excitation) wavelength from the continuous light spectrum of 200 to 1200 nm produced by the xenon lamp. The light beam is then focused again by a converging lens before it next hits the cuvette with the sample liquid inside. The light emitted by the sample then passes through the emission beam path, which is at a 90° angle to the excitation light. This arrangement is intended to prevent incident excitation light from entering the emission beam path and thus falsifying the measured values. In the following, the emitted light is again passed through a lens and then through a slit before it hits the emission monochromator. Before the detector, the intensity of the light beam is amplified by a photomultiplier. In the detector, the colour intensity is finally determined and converted into AU (arbitrary units).

An important component to compensate for errors caused, for example, by device or mains fluctuations, is the internal division of the device into two beam paths. A distinction is made between a sample beam path and a reference beam path. With this method, only 90 % of the light is directed into the sample chamber, while the remaining 10 % hits a reference photomultiplier and is measured there as a reference value.

Depending on the dye concentration, the intensity of the signal to be measured can extend over several orders of magnitude. Therefore, it is often of decisive importance to be able to adjust the spectral resolution, which corresponds to the sensitivity of the spectrofluorimeter. This can be achieved by adjusting the width of both the absorption and emission slits. The following slit widths can be selected for the RF-5001PC: 1.5, 3, 5, 10, 15 and 20 nm. In the present case, slit widths of 3 - 10 nm have proven to be favourable for measurements of the fluorescence intensity of 15 to 2000 microspheres. Due to the direct correlation between slit width and sensitivity, the sensitivity can be significantly increased by using a larger slit width.

increase. However, it should not be forgotten that a larger slit width also results in an increase in the background value of the source. This in turn leads to an increase in the signal-to-noise ratio. Both aspects, sensitivity and signal-to-noise ratio, should therefore be taken into account when selecting the slit width. In the measurements carried out to determine the standard curves, however, no significant background value could be detected for any of the three selected slit widths. Finally, the following should be pointed out: When measuring on two fluorescence measuring devices of the same type, slight differences in the measured fluorescence intensity may occur due to slight variability in the light intensity of the source. Therefore, it is essential to create a standard curve for each microsphere colour as described in chapter 2.4.4 before each series of experiments.

2.4.4 Standard curves

2.4.4.1 Dye-Trak VII+

As explained in the previous chapter, the calculation of standard curves is an important preparatory step for the subsequent measurements. With the help of a standard curve, the fluorescence intensity measured in the spectrofluorophotometer can be inferred from the number of microspheres in the sample. A decisive prerequisite is the direct proportionality between the number of fluorescent microspheres and the colour concentration. To determine the fluorescence intensity per microsphere of each microsphere colour, dilution series were prepared. These comprised six dilution levels each with 10, 50, 100, 250, 500 and 1000 microspheres. Due to a five percent solubility of distilled water in the solvent used (2-ethoxyethyl) acetate, the dilution could be made with distilled water. Only from a percentage of more than 5 % does a formation of two phases occur (An et al. 1999). From the diluted microsphere solution prepared in this way with a concentration of 10 microspheres per µL, 1 to 100 µL were then pipetted off accordingly.

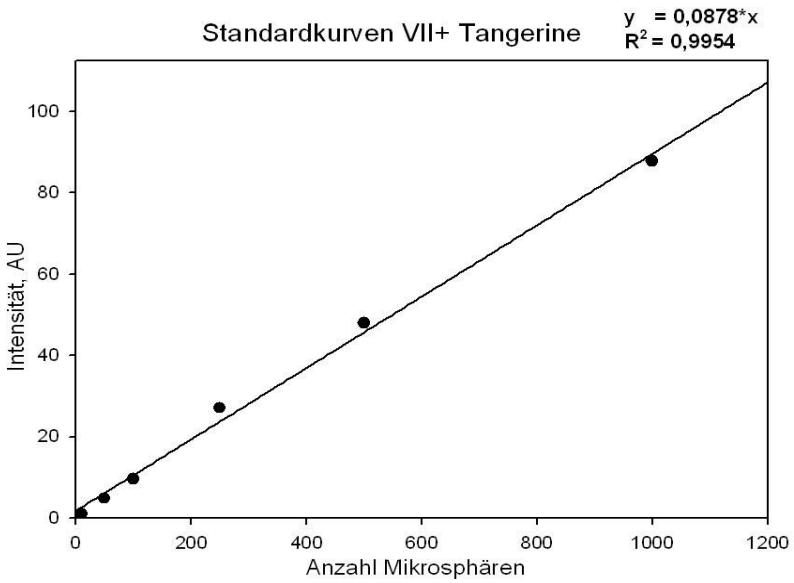


Fig. 2 Standard curve of the colour Tangerine (Dye-Trak VII+)

Subsequently, the fluorescent dye was dissolved out in a glass cuvette with 2 mL solvent and the fluorescence intensity was measured in the spectrofluorophotometer RF-5001 PC. Finally, a standard curve was determined for each colour from the values obtained (see Fig. 2).

2.4.4.2 Dye-Trak 'F

For the fluorescent microspheres, six dilution steps were also used to determine the standard curves. From the initial concentration of 1000 microspheres/µL, a microsphere solution with a concentration of 10 microspheres/µL was prepared again by dilution with distilled water.

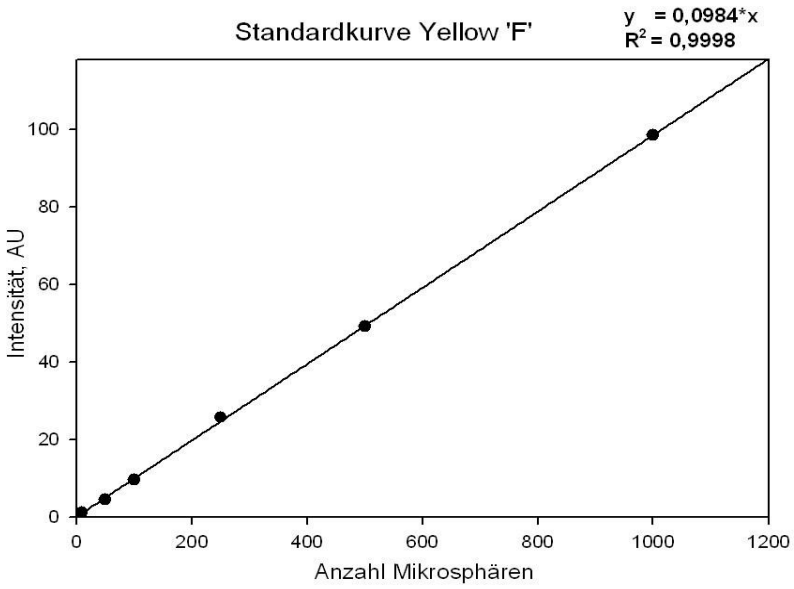


Fig. 3 Standard curve of the colour Yellow 'F' (Dye-Trak 'F')

To obtain the required microsphere concentrations of 10, 50, 100, 250, 500 and 1000 microspheres, 1 - 100 µL of microsphere solution was pipetted into a glass cuvette as with the Dye-Trak VII+ microspheres. To this, 2 mL of solvent (2-ethoxyethyl acetate) was added to trigger the dyes. In addition, 4 µL (concentration 500 microspheres/µL) of the control colour Navy was added to each microspheres solution. The addition of the control colour made it possible to detect possible contamination of individual samples and thus prevent falsification of the measured values. Fig. 3 shows the standard curve of the colour Yellow 'F' representing all four colours of the Dye-Trak 'F' series.

2.5 Spectrofluorophotometer vs. Fluorescence microscope

Despite some possible alternatives, including the microsphere counter developed by Niehren (1999), the evaluation of tracer experiments is still mostly carried out using the time-consuming and laborious method of counting under the fluorescence microscope. This is largely due to the fact that this method has so far proven to be reliable and applicable everywhere.

Both an evaluation with the spectrofluorophotometer and counting under the fluorescence microscope have their advantages and disadvantages. In both cases, several microsphere colours can be measured or counted simultaneously. The detection limit with the fluorescence microscope is, at least so far, still significantly lower than that of the spectrofluorophotometer. While individual microspheres can already be detected on the filter plates under the microscope, (Göppert 2005) gives a detection limit of approx. 1000 microspheres/mL for the spectrofluorophotometer. This is usually not sufficient to evaluate tracer experiments with low concentrations. For this reason, the spectrofluorophotometer has so far only been used to evaluate column experiments in current studies in groundwater (Göppert 2005, 2006 and 2007).

When preparing the samples, the fluorescence microscope reveals clear disadvantages. Before counting, a complex cleaning of the filter net is necessary in the laboratory. This is the only way to remove possibly disturbing foreign material on the filter paper. It has been shown that impurities can make counting the individual filters considerably more difficult. For example, chlorophyll bodies fluoresce red, textile fibres brightly light blue and calcite crystals also light blue (Käss 1992 and 2004). In addition, contamination of the filters by foreign microspheres can easily occur during sample preparation. These can either originate from other filter nets or they are introduced by contaminated working material (e.g. laboratory gloves, spatulas and glass containers). Finally, the final counting process proves to be extremely time-consuming and exhausting. In this context, the human component, the person in charge of counting, represents a major potential source of error (pers. comm. Wolkersdorfer). After a few hours, microscopy already shows strong signs of fatigue, which can quickly lead to erroneous results. As a result, only about 4 - 6 filter preparations can be counted per hour (Käss 2004).

In the spectrofluorophotometer, on the other hand, a large number of samples can be measured one after the other without increasing the measurement error. As comparable studies in medicine (e.g. Raab 2003) show, the measurement in the spectrofluorophotometer is also preceded by a more or less complex sample preparation. A possible source of error is the high sensitivity of the fluorescent colours to changes in pH and temperature. However, this can be minimised by selecting a suitable solvent, in this case (2-ethoxyethyl) acetate, and by maintaining constant temperatures during the measurement process.

Ward (1997) made use of the numerous advantages of an evaluation with the spectrofluorophotometer as early as the mid-1990s. He developed a method with which both water and soil samples could be analysed. In the column experiments and field experiments with 1, 6 and 10 µm microspheres, he used acetone as a solvent instead of the solvents (2-ethoxyethyl acetate (Glenny 1993) and 2-2-ethoxy ethoxy ethyl acetate (Van Oosterhout 1995)), which were already common in physiology at the time. In the experiments, a detection limit of less than 1 µg/L was achieved, which should correspond approximately to a microsphere number of 1000 microsphere/L. Despite the rather high detection limit, these early experiments already show that an evaluation of field experiments with the help of a spectrofluorophotometer can be quite practicable.

A disadvantage of the fluorescence microscope compared to the spectrofluorophotometer that has received little attention so far is that only microspheres with a minimum diameter of 100 nm can be counted under the microscope (Käss 2004). However, since such small microspheres have not yet been used in any tracer experiments in hydrology, this limitation has not yet come into play. However, it seems quite conceivable that in future

also use microspheres with the diameter of viruses, and thus with sizes smaller than 100 nm, in tracer tests. However, due to the lack of investigations, it is not possible at this time to make statements about an effective and highly detectable analysis of such microspheres with the spectrofluorophotometer.

3 V request

3.1 Devices

3.1.1 Filter unit

The filter unit used for the tests is part of the automatic sample collector MeFiSTo (Multi Filter Storage Tool; Fig. 4) developed in recent years by Wolkersdorfer and Berger especially for sampling in underground mines. In the tests carried out, not the entire sample collector was used, but only a single filter system unit. This consists of three PVC pipes of different lengths (KG pipes DIN 150) and two filter nets (20 x 20 cm; nylon) with different mesh sizes (41 and 15 µm). These can be clamped between two pipe sections with the help of two quick-action plastic connectors (Fig. 4 left).

Fig. 4 left: the different components of a single filter unit; right: Multiple Filter Storage Tool (MeFiSTo; from Wolkersdorfer 2006).

3.1.2 Filter nets

In the case of the filter nets, it was important to ensure that the material was resistant to the mine water, but above all to the solvent used. The filter nets have already been successfully used in various tracer tests as part of the automatic sample collector MeFiSTo (Multiple Filter Storage Tool) developed by Wolkersdorfer and Berger in recent years. For this reason, the only question that remained to be answered in this work was: Is the filter material also suitable for the newly designed sample preparation? As Raab (2003) found out by comparing different potential filter materials, polyamide (nylon) is very suitable as a filter material due to its mechanical and chemical properties. The filter nets proved to be permanently resistant to the solvent and showed hardly any traces of defects in the filter material even after repeated use. However, since contamination of the filter material by introduced microspheres is a potential source of error, the tests were not repeated. The filter nets were therefore only used once each.

3.2 Mine water

In order to be able to test the newly developed method for sample preparation and evaluation under different conditions, the tests were carried out with two different mine waters. For this purpose, mine water from the overflow 539 of the abandoned underground mine Straßberg in the Harz region (SBG-1203-Ü539) and mine water from the deep gallery "Heilstollen" of the former mine Peißenberg (TSP-1701-TSH) were used. A comparison of the two mine waters can be found in Tab 3.

Locality	Underground mine Straßberg/Harz Brachmannsberg, shaft no. 539	Peißenberg mine Deep gallery "Heilstollen
Designation	**SBG-1203-Ü539**	**TSP-2403-TSH**
Date	12.03.2007	24.03.2007
Temp. (°C)	10,4	16,5
Electr. conductivity (µS/cm)	542	3590
pH	6,52	6,9
Redox potential (mV)	137,4	na
O2 content (mg/L)	0,18	na
Fe, total (mg/L)	13,71 ± 1,86	0,13
Mn (mg/L)	0,987 ± 0,20	0,023
Zn (mg/L)	0,05 ± 0,03	< 0,014
Al (mg/L)	0,6	< 0,33
Ba (mg/L)	na	< 0,25
K (mg/L)	2,168 ± 0,35	19,36
Mg (mg/L)	18,2 ± 1,58	101
Cl (mg/L)	32,32 ± 1,42	16,03
NO3 (mg/L)	< 0,74	27,55
SO4 (mg/L)	146,61 ± 2,12	1561,4
F (mg/L)	2,21 ± 0,17	1,66
Ca (mg/L)	41,5 ± 3,94	275,2
Na (mg/L)	22,66 ± 2,55	410,4
Cu (µg/L)	7,62 ± 0,051	3,54
Ni (µg/L)	11,936 ± 0,036	23,25
Pb (µg/L)	1,684 ± 0,006	1,80
Cd (µg/L)	0,349 ± 0,016	na
As (µg/L)	131,895 ± 0,608	6,53
Sr (mg/L)	na	7,90
Cr (µg/L)	na	0,98
KB (mmol/L)	11,84 ± 0,12	na
KS (mmol/L)	1,15 ± 0,01	na
HCO3 (mg/L)	70,17 ± 0,70	30.59
TIC (mg/L)	na	96,2
TOC (mg/L)	na	6,33

Tab. 3 Water analyses of the mine waters used from the Straßberg underground mine and the former Peißenberg mine; na = value not determined

3.3 Comparison of counting method with Spectrofluorophotometer measurements

3.3.1 Evaluation of the Ehrenfriedersdorf tracer test

The central point of this experiment is the following question: Can the measurements on the spectrofluorophotometer be compared with the results of the counting method? To answer this question, the microspheres were detached directly from the cellulose acetate filter plates used in all previous tracer experiments (black with 8 µm mesh size; diameter 47 mm, Sartorius, 37075 Göttingen, Germany). Subsequently, the fluorescent dyes of the respective microsphere colours (red, tangerine and white) were dissolved out of the polystyrene shells with the help of the solvent (2-ethoxyethyl) acetate and their intensity was measured in the spectrofluorophotometer.

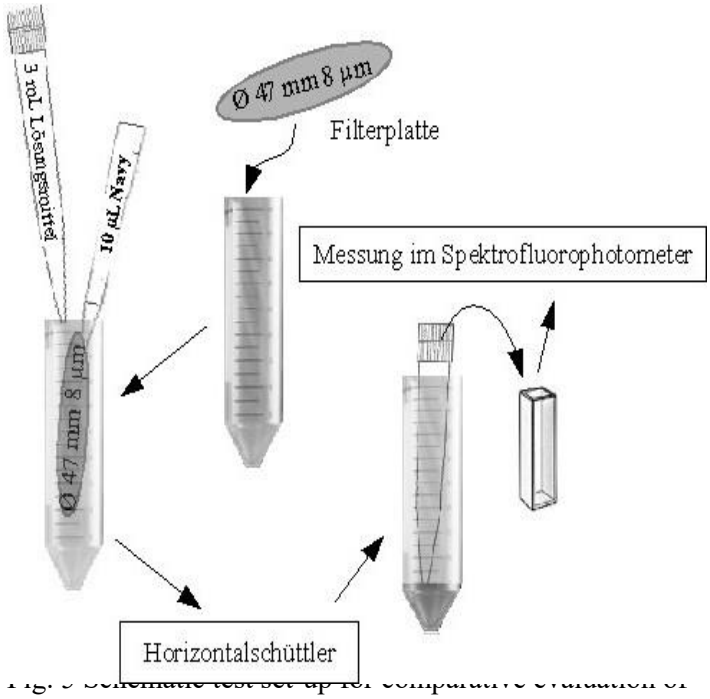
In order to be able to make a comparison between the two different methods, filter plates from the Ehrenfriedersdorf tracer experiment (Wolkersdorfer & Hasche 2004b) that had already been counted under the fluorescence microscope were used in the experiment. The results obtained at that time (Tab. 4) were then compared with those of the newly performed measurements. The work steps necessary for the experiment, as also shown in Fig. 5, are now described again in detail below.

Sample number	Red (FluoSpheres)	Tangerine (Dye-Trak VII+)	White (Original Dye-Trak)
EFD-0906-12	0	7533	2
EFD-0906-15	0	313	2
EFD-0906-23	0	33	2
EFD-0906-24	0	31	0
EFD-0906-25	0	26	2
EFD-0906-31	0	32	2
EFD-1606-14	0	16	1
EFD-1606-15	0	3	0
EFD-1606-21	0	7	2
EFD-1606-22	0	11	3
EFD-1606-23	0	3	1
EFD-1606-24	0	2	0
EFD-1606-25	0	3	0
EFD-1606-31	0	6	1
EFD-1606-32	0	4	1
EFD-1606-33	0	6	4
EFD-1606-35	0	0	1
EFD-2306-11	0	3	2
EFD-2306-13	0	9	1
EFD-2306-22	0	3	0

Tab. 4 Aliquot results of microspheres from the measuring site Tiefer Sauberger Stollen, Gesenk 2. Sohle (E-TSG12; modified from Wolkersdorfer & Hasche 2004b)

To remove the microspheres from the filter plates, they were carefully detached from the underlying glass plate with the help of tweezers and placed in a 50 mL centrifuge tube. Then 10 µL (concentration 200 microspheres/µL) of the control colour Navy 'F' was pipetted onto the filter already in the centrifuge tube. The addition of the control colour served the purpose of detecting contamination of the sample or a possible loss of microspheres during the further workflow. Then 3 mL of the solvent (2-ethoxyethyl)-acetate was added to the filter material. After adding the solvent, the polystyrene shells of the microspheres dissolved and the different fluorescent dyes were released.

In the run-up to the investigations, the test arrangement was tried out on empty cellulose acetate filters. This revealed the following: Not only the polystyrene sleeves, but also the the filter material began to dissolve after only a short contact with the organic solvent. Therefore, a way had to be found to dissolve the colours from the microspheres without also dissolving parts of the filter. This was the only way to prevent contamination of the solvent by dissolved filter material. Exactly this objective was pursued in a series of experiments in which the centrifuge tubes with the filters inside were run for different periods of time (1, 3 and 5 minutes) at two different speeds, 200 and 5 minutes respectively.
250 revolutions per minute into a horizontal shaker (KS 250 basic; IKA Labortechnik GmbH) were used (Tab. 5).



the Ehrenfriedersdorf tracer test

5 min / 250 revolutions per minute	2 min / 250 revolutions per minute	3 min / 200 revolutions per minute	2 min / 200 revolutions per minute	1 min / 200 revolutions per minute
EFD-1606-14	EFD-1606-33	EFD-1606-23	EFD-0906-12	EFD-1606-32
EFD-1606-15	EFD-2306-13	EFD-1606-24	EFD-0906-15	EFD-1606-35
EFD-1606-21		EFD-1606-25	EFD-0906-23	EFD-2306-11
EFD-1606-22		EFD-1606-31	EFD-0906-24	EFD-2306-22
			EFD-0906-25	
			EFD-0906-31	

Tab. 5 Different test set-ups to determine a suitable method for triggering and later evaluating microspheres deposited on cellulose acetate filters

The shaking was intended to accelerate the release process of the fluorescent dye from the microspheres and thus avoid prolonged contact with the filter material. Finally, 2 mL of the solvent with the dye dissolved in it was pipetted from the centrifuge tube into a glass cuvette. Finally, the colour intensity was determined in the spectrofluorophotometer.

3.3.2 Recovery of the microspheres from the filter networks

A second experiment dealt with the question of whether microspheres remain on the filter net during the cleaning process carried out as preparation for the counting method. Thus, microspheres would already be lost during the sample preparation process. For the experiment, already cleaned filter nets from the Ehrenfriedersdorf tracer experiment were used and checked for microsphere residues. The filter nets have been stored in 250 mL amber glass bottles since the end of the tracer experiment in mid-2004.

In this test, a total of 15 filter nets (Tab. 7) were examined for possible microsphere residues. In the first step, the filter nets, some of which were still wet, were carefully removed from the 250 mL amber glass bottles and dried in the drying cabinet at max. 50 °C. They were then put back into the amber glass bottles and 4 mL of solvent was pipetted into the amber glass bottle for each filter net. The amber glass bottles were then tightly closed and placed in an overhead shaker at 10 rpm for approx. 1 hour. From the amber glass bottles, 2 mL of solvent was then pipetted into glass cuvettes and then measured in the spectrofluorophotometer.

3.4 Development of a new method for sample preparation and evaluation

3.4.1 Experimental setup

The development of a new method for sample preparation and evaluation focused on the following objective. The aim was to replace counting the microspheres in the fluorescence microscope by determining the intensity in the spectrofluorophotometer, which can then be converted into the number of microspheres accordingly. For this purpose, a method was to be devised with which the microspheres could be detached from the filter nets (15 and 41 µm) as simply and quickly as possible and the intensity of the released fluorescent dye could be measured in the spectrofluorophotometer.

The chosen experimental set-up made it possible to simulate the sampling in the mine. The different initial parameters, such as the concentration of the microspheres in the mine water, the amount of water or the pH value, could be changed depending on the objective of the experiment. The set-up and the individual work steps, as also shown in Fig. 6, are described below.

First, the two filter nets (NY 15 and 41) with a size of 20 x 20 cm are clamped between two pipe sections using the quick-release fasteners. The 41 µm filter net is mounted above the 15 µm filter net. Its sole purpose is to filter out larger particles and suspended matter in the mine water in order to prevent clogging of the finer-meshed filter below. Since the microspheres with their diameter of only 15 µm should basically fit entirely through the meshes of the upper filter net, the 41 µm filter net no longer plays a decisive role in the later evaluation. However, as shown in Fig. 6, there is the possibility of using both filter meshes on microspheres.

examine. This makes it possible to determine whether a certain proportion of the microspheres already remains on the coarser filter mesh. Furthermore, in the case of mine waters with a low particle content, after prior testing, the use of the additional 41 µm filter net can be dispensed with altogether.

Afterwards, the previously determined number of microspheres, for example 300 microspheres of one or more fluorescent colours, are pipetted together into a Teflon cup. The investigation of how many or which microsphere colours can be determined next to each other in only one measuring procedure formed a large part of the work. In most cases, several microsphere colours were used simultaneously. Based on the manufacturer's specifications (Tab. 2) and the experience gained in determining the standard curves, there was no reason to fear mutual interference between microspheres of the same series (Dye-Trak VII+ or Dye-Trak 'F'). Therefore, all colours of a microsphere series were used at the same time and with the same concentrations.

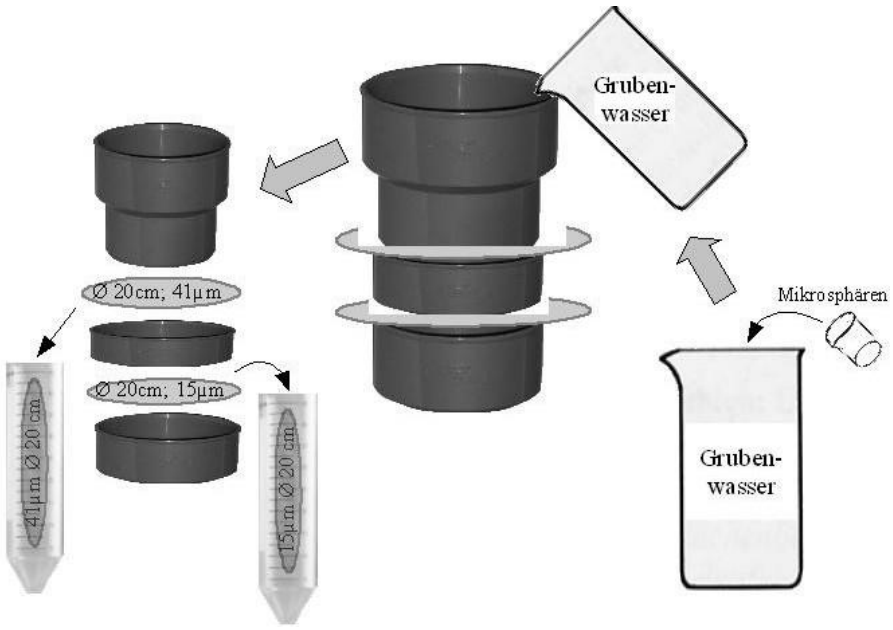


Fig. 6 Schematic sketch of the experimental set-up for the development of a novel method for sample preparation and evaluation

An approximate amount of 2 mL of distilled water is then added to the microspheres in the Teflon cup. The additional water in the Teflon cup serves the sole purpose of facilitating the subsequent addition of the microspheres to the sample water. This is because, despite the dilution that has already taken place, quantities of only 2 to 200 µL were usually used.

Then the microspheres are mixed with the water by gently shaking the Teflon beaker with the lid closed. Next, a certain amount of sample water, usually 1 to 2 litres, is measured in a large glass measuring cup. Two different mine waters were used as sample water: on the one hand, mine water from spillway 539 of the abandoned Straßberg underground mine in the Harz Mountains (SBG-1203-Ü539) and, on the other hand, mine water from the deep gallery "Heilstollen" of the former Peißenberg mine (TSP-1701-TSH). In the next step, the contents of the Teflon cup are carefully poured into the sample water. Afterwards the

Teflon beaker rinsed several times with distilled water. The water must also only be poured into the sample water. This is to prevent small amounts of microspheres from remaining in the Teflon cup and thus causing a loss of microspheres.

Afterwards, all the sample water with the microspheres in it is poured into the top of the filter unit. Here, too, the measuring cup should be rinsed several times to prevent the loss of microspheres, and the additional water should be poured into the filter unit. Afterwards, it is important to wait until all of the sample water has completely passed through the two filter nets. This can take up to an hour, depending on the pit water and the amount of sample. At this point, approx. 100 - 500 mL of a 0.05 mol/L oxalic acid was added to the filter net during the tests with the mine water from the "Heilstollen" Peißenberg. The reason for this was to avoid subsequent cleaning of the filter net after it had been removed from the filter unit. This would have meant additional work and would also have been difficult to implement. The exact reasons for this are explained in detail in section 3.4.1.1. Subsequently, possible residues of oxalic acid on the filter net are removed by adding tap water to the filter unit.

Since, as already mentioned in chapter 2.4.4, water is only miscible in small quantities with the organic solvent (2-ethoxyethyl) acetate, it was necessary to dry the two filters completely in the next step. This can be done either at room temperature or, as in this case, in a drying oven at a maximum of 50 °C. It seems sensible to dry the filter nets at room temperature. It seems sensible to leave the filter nets in the filter unit during the drying process to prevent contamination. In the next step, the two filter nets are removed from the filter unit and folded together with extreme care and using latex gloves, again to prevent contamination. The folded filter nets are then placed in 50 mL polypropylene centrifuge tubes. The filter nets can be safely stored in these tubes for a longer period of time. Observe the necessary storage conditions.

As described above, the drying process can take some time. It therefore seems impractical to carry out the drying directly in the mine during an ongoing tracer test. Alternatively, the filter nets can be folded up while still wet and placed in appropriately sized amber glass bottles for storage and transport. For this purpose, 250 or 500 mL vessels are suitable. The filter nets are then dried in a drying cabinet in the laboratory, as described in the previous section.

3.4.1.1 Cleaning the filter networks from impurities

Initial tests have shown that mineral-rich deposits on the filter networks can influence the further treatment process. Therefore, possibilities were sought to remove them as simply and completely as possible. The choice fell on strongly diluted oxalic acid (0.05 mol/L). This had already been used successfully for this purpose in earlier tracer experiments (Wolkersdorfer 2002b). By adding a certain amount of oxalic acid, iron hydrates and carbonates that have previously been deposited on the filter mesh can be dissolved and thus removed from the surface of the filter mesh. This step is done at a time when all sample water has already flowed through the filter net.

When using oxalic acid, the following had to be taken into account: The fluorescent colours embedded in the microspheres react very strongly to even small deviations from the neutral pH value as soon as they are released from the polystyrene shells of the microspheres. This property is explained by the way the microspheres are manufactured. At the beginning of the manufacturing process, the microspheres have a sponge-like structure that absorbs the fluorescent dye. The outer pores only close when the microspheres are placed in a hydrophilic medium after the dyeing process. In this way, the fluorescent dye, which is actually highly sensitive to pH, is shielded from the outside and stable against changes in pH until it is released again from the polystyrene shells of the microspheres.

However, if there are still residues of oxalic acid on the filter mesh during the triggering process, part of the fluorescent dye can be destroyed and thus the strength of the fluorescence intensity measured later can be reduced. There are several ways to prevent this. On the one hand, the filter net can be cleaned of the oxalic acid residues by rinsing the filter net with a large amount of water after the cleaning process, thus removing as many oxalic acid residues as possible. On the other hand, the low pH value produced by the oxalic acid can be buffered with the help of a phosphate buffer until a neutral pH value is reached on the surface of the filter net. Comparative tests did not reveal any significant differences between the two above-mentioned procedures. Since the solvent used (2- ethoxyethyl) acetate also has a pH value of 4 - 5, the use of a phosphate buffer was dispensed with.

3.4.2 Sample preparation

3.4.2.1 Work steps

For the actual sample preparation, 4 mL of the organic solvent (2- ethoxyethyl) acetate is pipetted into each of the two centrifuge tubes with the filter nets inside. The centrifuge tubes are then tightly closed and centrifuged at 20 rpm for 10 minutes. During this centrifugation process, the solvent dissolves the polystyrene shells of the microspheres, releasing the respective fluorescent dyes. After the 10 minutes, the centrifuge tubes are gently shaken in an upright position. This causes the solvent, with the fluorescent dyes dissolved in it, to settle at the bottom of the centrifuge tube. The filter net is then carefully removed from the centrifuge tube with tweezers. It should be noted that the solvent is a toxic chemical that should not come into contact with the eyes or skin. Therefore, any handling of the solvent should be done under the given precautions (fume cupboard, protective gloves, safety goggles). Finally, pipette 2 mL of the remaining liquid from the centrifuge tube into a glass cuvette. This is followed by the measurement in the spectrofluorophotometer, where the fluorescence intensity of the individual fluorescence colours is determined via the respective spectral maxima. From this, the number of microspheres on the filter network can be calculated.

The reason why 4 mL of solvent have to be used instead of 2 mL as in the determination of the standard curves is as follows: It was not possible to extract the entire amount of solvent from the filter mesh after the triggering process. However, in order to be able to extract 2 mL of sample liquid, solvent with the fluorescent dyes dissolved in it, in the

spectrofluorophotometer, the initial amount of solvent had to be increased to 4 mL at the beginning.

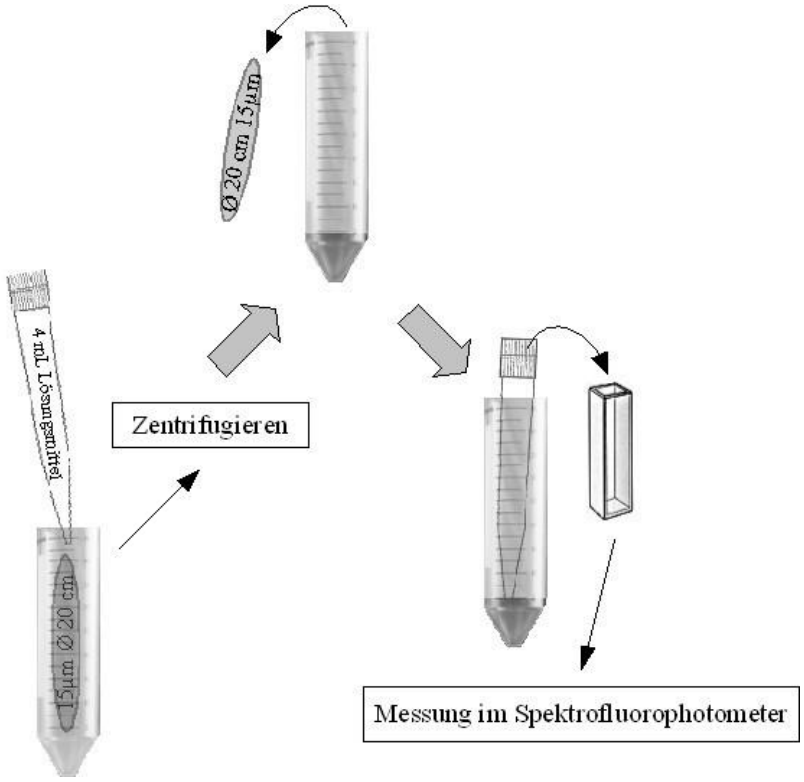


Fig. 7 Schematic representation of the individual work steps of a newly designed method of sample preparation and evaluation; in each case for the 15 and 41 µm filter network

3.4.2.2 Influence chemicals and materials

In order to exclude a falsification of the measured values, all materials and chemicals used were examined for their influence on the measurement signal prior to the measurements. In this context, an empty glass cuvette, 2 mL of the solvent (2- ethoxyethyl) acetate, 2 mL of solvent, which was previously centrifuged for 10 min at 20 rpm in a centrifuge tube, and 2 mL of a 0.2 mol/L oxalic acid were measured for their intensity signal in the spectrofluorophotometer. The same measurement settings were used as for all other measurements.

In addition, three tests were carried out with the aim of determining or quantifying the effects of oxalic acid purification on the measured value. Essentially, the steps listed in chapter 3.4.1 were followed. However, the procedure after adding the mine water to the filter unit was slightly varied in each case. In experiment 1, the filter network was not cleaned at all. In contrast, cleaning with 0.05 molar oxalic acid was carried out in experiment 2. In experiment 3, cleaning the filter net with oxalic acid was followed by washing the filter net with a larger amount of water. Finally, the influence of the oxalic acid was to be determined by comparing the three experiments. For all three tests, microspheres of the Dye-Trak 'F' series were used, each with an initial quantity of 100 microspheres. Mine water from the deep tunnel was used as sample water.
"Heilstollen" of the former Peißenberg mine.

3.4.2.3 Dye-Trak VII+ and Dye-Trak 'F' Compatibility

The question of the compatibility of the two microsphere series Dye-Trak VII+ and 'F' was not of great importance. However, with regard to a possible complementation of the respective colour palettes, an interesting side aspect emerged. It was investigated how many of the eight or seven peaks could be distinguished simultaneously during a measuring process. For this purpose, equal concentrations of each colour were pipetted into a glass cuvette and the fluorescent dyes were then released using 2 mL (2- ethoxyethyl) acetate. The colour Lemon VII+ was not used for the following reason: The colours Lemon VII+ and Lemon 'F' have their respective emission maximum at the same position (see Table 1), which would inevitably lead to an overlapping of the two peaks. Thus, the colours Tangerine VII +, Berry VII+, Lemon 'F', Yellow 'F', Orange 'F', Persimmon 'F' and Navy 'F' were examined.

3.4.3 Trials with the filter unit

3.4.3.1 Dye-Trak VII+

Just as with the different dilution levels for determining the standard curves, an initial concentration of 10 microspheres/µL was assumed for the experiments carried out. Of this, 1 to 200 µL of the three microsphere colours Lemon VII+, Tangerine VII+ and Berry VII + were pipetted into a Teflon cup according to the procedure described in chapter 3.4.1 and then added to the pit water.

In the first series of experiments, 2, 10, 20, 50, 100 and 200 µL were added twice each to the pit water of the Straßberg underground mine (SBG-1203-Ü539). The resulting numbers of microspheres of 20, 100, 200, 500, 1000 and 2000 microspheres for the respective experiment thus correspond exactly to a doubling of the dilution levels used in the determination of the standard curves. This doubling of the initial quantities compared to the standard curves compensates for a later dilution of the sample. This results from the use of twice the amount of solvent. In the later measurements in the spectrofluorophotometer, concentrations of 5 to 500 microspheres per mL of solvent could thus be expected.

In a second series of tests, mine water from the deep gallery "Heilstollen" of the former Peißenberg mine (TSP-1701-TSH) was used as sample water. The initial quantities used in the first series of tests were otherwise left unchanged. While the first tests were carried out without cleaning the filter network with 0.05 molar oxalic acid, the test procedure was supplemented with cleaning with oxalic acid after unpromising results (for more details see chapter 3.4.1.1). Subsequently, the entire test series was carried out again with this slightly modified test procedure.

3.4.3.2 Dye-Trak 'F

The series of tests with the Dye-Trak 'F' microspheres, Lemon 'F', Yellow 'F', Orange 'F' and Persimmon 'F' were carried out in the same way as the tests with the Dye-Trak VII+ series described in the previous chapter. This was to ensure a direct comparison between the two microsphere series (Dye-Trak VII+ and Dye-Trak 'F'). Again, six different starting quantities were chosen and two series of tests were carried out. One of them with the mine water from the Straßberg underground mine and the other with that from Peißenberg. Based on the findings from the previous

tests, oxalic acid was used from the beginning to clean the filter networks in the tests with the mine water from Peißenberg. Furthermore, there was a small difference to the series of tests with the Dye-Trak VII+ microspheres. In the test series with the Straßberg mine water, a different quantity of 250 microspheres was used instead of the usual starting quantity of 200 microspheres.

3.4.3.3 Influence pH value of the mine water

As described in chapter 3.4.1.1, the fluorescent dyes used are protected from pH influences by their polystyrene shell before the release process. Nevertheless, the following question should be clarified by means of a simple experiment: Does the pH value of a mine water influence the later measurement result in any way? A similar question had already been raised when oxalic acid was used to clean the filter networks. To investigate this question, a certain amount of mine water from Straßberg was brought to a pH value of 4, 7 or 10. Originally, this had an approximately neutral pH value (see Tab. 3). Apart from this, the experiments were carried out in the same way as described in chapter 3.4.1. Microspheres of the Dye-Trak VII+ series with an initial quantity of 240 microspheres each were used.

3.4.3.4 additional use of the 41 µm filter mesh

Since hardly any particles with diameters larger than 41 µm were found in the two mine waters used, Straßberg and Peißenberg, the 41 µm filter mesh was not used as a prefilter in most of the tests. However, the tracer tests carried out so far have shown that the use of such a pre-filter can be quite useful or even necessary. Therefore, it should also be investigated what influence the use of an additional, coarser filter net might have. In this context, the following question had to be answered:

a certain proportion of the added microspheres already remains on the coarser filter mesh. Thus, a corresponding examination for microspheres, as described in chapter 3.4.2.1, would also be necessary for the 41 µm filter mesh. Although no differences were to be expected at least between the different microspheres series (Dye-Trak VII+ and Dye-Trak 'F'), tests were carried out with both series and also both mine waters, Straßberg and Peißenberg.

3.5 Measurement accuracy

As far as measurement accuracy is concerned, there were several aspects that were investigated in more detail. First of all, it was to be clarified whether the spectrofluorophotometer used would produce a measurement error due to slight fluctuations of the light source (for more details see chapter 2.4.3). For this purpose, one and the same sample was measured several times within short time intervals and the mean value and the mean error were determined.

In addition, great attention was paid to the topic of measurement accuracy in connection with the determination of the standard curves. This is because in physiology, as well as in column tests in connection with groundwater investigations, up to now

exclusively high microsphere concentrations were measured in the spectrofluorimeter. Therefore, in addition to the question of the detection limit, it was also necessary to clarify how large the measurement error would be at correspondingly low microsphere concentrations. For this purpose, the dilution levels of the respective microsphere colours were prepared and measured several times.

In the tests with the filter unit, great importance was attached to the assessment of the respective recovery rates of the individual tests with regard to measurement accuracy. The reproducibility of the individual test results, on the other hand, played a rather subordinate role. This can be explained by the fact that for reasons of time and material expenditure, in contrast to the dilution steps in the determination of the standard curves, the test series could not be repeated as often as desired.

4 E rresults

4.1 Comparison of counting method with Spectrofluorophotometer measurements

4.1.1 Evaluation of the Ehrenfriedersdorf tracer test

In the first two test arrangements (samples EFD-1606-14 to EFD-2306-13), parts of the cellulose acetate filter were dissolved in each case, which resulted in contamination of the solvent. Therefore, peak maxima for the colour tangerine could only be determined for half of the measurements. For the remaining three samples (EFD-1606-14, EFD-1606-22, EFD-1606-33) the measured results deviate strongly from those of the counting method.

Sample number	Results Counting	Results Spectrofluorophotometer	Deviation in %	Test parameters
EFD-1606-14	16	88	450	
EFD-1606-15	3	na	na	5 min / 250 rev. per minute
EFD-1606-21	7	na	na	
EFD-1606-22	11	22	100	
EFD-1606-33	6	55	817	2 min / 250 rev. per minute
EFD-2306-13	9	na	na	
EFD-1606-23	3	6	100	
EFD-1606-24	2	17	750	3 min / 200 rev. per minute
EFD-1606-25	3	26	767	
EFD-1606-31	5	13	160	
EFD-0906-12	7533	7406	2	
EFD-0906-15	313	241	23	
EFD-0906-23	33	31	10	2 min / 200 rev. per minute
EFD-0906-24	31	34	15	
EFD-0906-25	26	22	3	
EFD-0906-31	32	31	6	
EFD-1606-32	4	8	100	
EFD-1606-35	0	0	0	1 min / 200 rev. per minute
EFD-2306-11	3	0	na	
EFD-2306-22	3	3	0	

Tab. 6 Comparison of the results obtained with the counting method with those of the spectrofluorophotometer for the colour tangerine (Dye-Trak VII+); na = peak not detectable

In the third test set-up, the dissolution phenomena were no longer so strong. Thus, at least one peak maximum could be determined for all four samples. However, these measured values still deviate greatly from the microsphere numbers determined by the counting method.

In the last two test arrangements, peak maxima could be determined for all ten samples, if present. There were no visible signs of dissolution of the cellulose acetate filter. Only the background value determined was lower for all samples.

twenty measurements is significantly higher than that of the reference measurement. As it turned out, however, the quality of the measurement results is hardly influenced by this, if at all.

4.1.2 Recovery of the microspheres from the filter networks

A total of 15 of the 46 filter nets were checked for microspheres that remained on the cleaned filter nets after the treatment process. Significant peak maxima were measured in seven filter nets, from which conclusions could be drawn about the number of microspheres still present (Tab. 7). For three (values in brackets) of the seven measured values, the peak maxima were shifted towards the lower wavelength range and each had their peak maximum at a wavelength of 506 nm. Because of this, these three peaks cannot be unequivocally assigned to the microsphere colour tangerine VII+, whose emission maximum is at about 525 nm (see Tab. 1). The possibilities of explaining such a peak shift will be discussed in detail in section 5.3.2 in connection with the interpretation of the measurement results obtained. In any case, it is clear that these three peak maxima, converted into microsphere numbers, lie in areas far above the expected result.

Sample number	Results Count (Tangerine VII+)	Results Spectrofluorophotometer (Tangerine VII+)
EFD-1606-14	716	268
EFD-1606-15	313	80
EFD-1606-24	31	(8213)
EFD-1606-31	32	0
EFD-0906-32	32	(116)
EFD-0906-33	42	21
EFD-1606-21	7	0
EFD-1606-34	1	0
EFD-2306-11	3	0
EFD-2306-12	91	0
EFD-2306-13	9	0
EFD-2306-15	6	105
EFD-2306-23	2	0
EFD-2306-25	3	(2835)
EFD-2506-11	0	0

Tab. 7 Comparison of the numbers of microspheres of the colour Tangerine VII+ determined by counting under the fluorescence microscope with the numbers of residual microspheres determined in the spectrofluorophotometer on the cleaned filter nets of the Ehrenfriedersdorf tracer experiment (left column modified from Wolkersdorfer & Hasche 2004); values in brackets = peak maxima at a wavelength of 506 nm.

For the remaining four samples, the peak maxima were at a wavelength of about 525 nm, as expected. This suggests that they belong to the microsphere colour Tangerine VII+.

are to be assigned. Without going into more detail about the results obtained, it can already be definitively said that microspheres have remained on the cleaned filter nets.

4.2 Development of a new method for sample preparation and evaluation

4.2.1 Influence chemicals and materials

In view of the problem with the easy solubility of the cellulose acetate filter plates, the following question arose before the start of the tests with the filter unit: Does prolonged contact between solvent and the materials used also lead to an influence on the measured value? According to the experiments by Raab (2003), however, such a clear and serious influence on the measured value was not to be expected.

Chemical/Material	Wavelength peak, nm	Intensity, AU
empty glass cuvette	271	3,583
Oxalic acid 0.2 mol/L	275	3,813
Solvent 2-(Ethoxyethyl) acetate	284	64,145
	307	15,074
	573	**3,060**
Solvent (centrifuged)	283	64,410
	572	**2,948**

Tab. 8 Measurement results of several tests to clarify the influence of the chemicals and materials used in the sample preparation process; highlighted are the peaks at 573 and 572 nm, which are important for determining the intensity of the colour Berry VII+.

The results of the tests carried out in this context are listed in Tab. 8. It can be seen that only one of the peaks (573 or 572 nm) is important in determining the intensity of the individual relevant peaks. All other peaks are outside the wavelength range of about 390 - 630 nm in which the peaks of the different microsphere colours are located. They therefore have no influence on the measured intensities. The peak in the range of 570 - 575 nm is generated by the solvent (2-ethoxyethyl)-acetate recommended for these microsphere series and thus increases the peak of the colour Berry by an amount of 3.7 AU on average. This applies at least to the measurement settings chosen for the spectrofluorophoto- meter in this work. However, the peak height of the colour Berry VII+ can be corrected by this value in a simple way (see also chapter 4.3.3.2). The prerequisite for this is that the value remains approximately constant, regardless of the other concentration of the fluorescent colours.

4.2.1.1 Influence oxalic acid

As far as the influence of chemicals on the measured value is concerned, oxalic acid plays a significant role. In addition to the measurements listed in Tab. 8, three further experiments were therefore carried out. With the help of the first two experiments it was to be investigated what the effects of a purification with 0.05 mol/L

and to what extent the measurement result can be changed or improved. In addition, the following had to be clarified for experiment 2. Firstly, to what extent a negative change in the measurement result due to possible oxalic acid residues (see chapter 3.4.1.1) on the filter nets seems conceivable. Secondly, does a subsequent cleaning with a larger amount of water prevent such a negative effect? A comparison of experiments 2 and 3 should provide information on the latter.

Output quantity (microspheres)	Microsphere colour	Experiment 1 (without oxalic acid)	Experiment 2 (with oxalic acid)	Experiment 3 (with oxalic acid and H_2O)
100	Lemon 'F	204	195	112
100	Yellow 'F	134	118	114
100	Orange 'F	92	117	116
100	Persimmon 'F	97	147	109

Tab. 9 Results of the experiments on the influence of oxalic acid on the subsequent measurement result; converted into the number of recovered microspheres

The results of these three experiments, which are summarised in Tab. 9 and additionally shown graphically in Fig. 8, allow several conclusions to be drawn. From the results of experiment 1, the effects of overlapping the two front peaks (Lemon 'F' and Yellow 'F') can be clearly seen, as can also be seen in Fig. 9 below (red curve). This results in a considerable overestimation of the two peak maxima mentioned above. In the case of the colour Lemon 'F', this is reflected in an approximate doubling of the value. A similar effect can be observed in experiment 2. However, this effect is mitigated by the use of oxalic acid. A negative effect on the measured intensity signal, possibly due to oxalic acid residues, may become apparent in the peak of the colour persimmon 'F'. At least in this context there is a considerable influence on the measured value. The results of experiment 3 then show quite clearly how much an additional cleaning of the filter network contributes to the correctness of the measurement result that is finally determined.

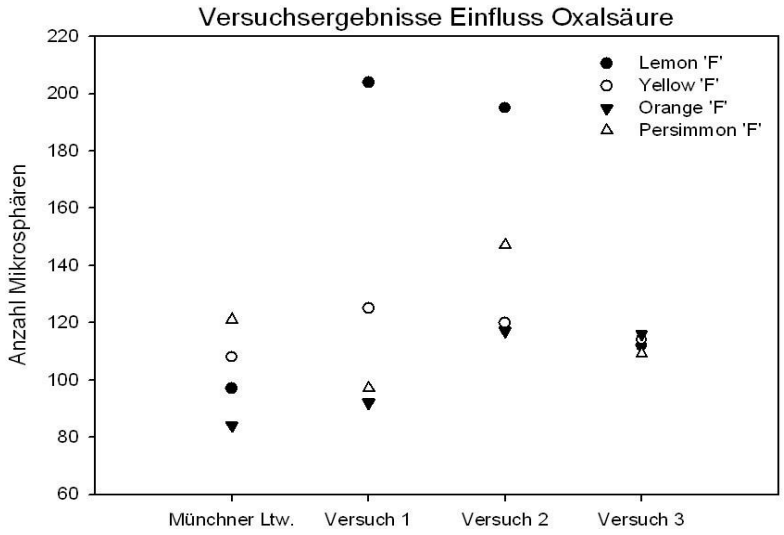


Fig. 8 Graphical representation of the test results on the influence of oxalic acid on the measurement result; compare this with the results of a test with Munich tap water.

Although there is still an overlapping of the two front peaks, this can be eliminated by a correction carried out afterwards. Thus, experiment 3 ultimately delivered a meaningful result. The values of test 3, which are consistently slightly above the initial quantity, can be attributed on the one hand to the usual measurement error and on the other hand to the slightly increased background value.

Fig. 8 shows a comparison of the results of the three tests carried out with Munich tap water instead of mine water. This shows that cleaning with oxalic acid and subsequent rinsing of the filter net yields results that are comparable to those of "clean" water. The following can also be concluded from the results with tap water: Deviations of up to about 20 % between the individual microsphere colours are probably not due to the properties of the mine water. Instead, such variations are probably due exclusively to the way the tests were carried out and evaluated. However, the effects that the knowledge gained from the experiments is likely to have on the final measurement result and the associated measurement error will be discussed in more detail in the following chapters.

4.2.2 Dye-Trak VII+ and Dye-Trak 'F' Compatibility

As the test result shows (Tab. 10), it is possible to distinguish the peaks of five of the seven possible and available microsphere colours from each other during one measurement process. Only the emission peak of the colour Persimmon 'F', which is approximately at 545 nm, is covered by the adjacent and more pronounced peak of the colour Tangerine VII+ to such an extent that, at least at the same microsphere concentration, a differentiation of the two peaks is not possible with the method presented here.

Output quantity (microspheres)	Microsphere paint	Wavelength peak, nm	Intensity, AU	Number of microspheres
100	Lemon 'F	402	5,742	80
100	Yellow 'F	448	8,648	86
100	Orange 'F	491	24,291	124
100	Persimmon 'F	na	na	na
2000	Navy 'F	626	1,177	2056
120	Tangerine VII+	524	33.143	357
120	Berry VII+	574	8,433	214

Tab. 10 Test result for investigating the compatibility of the two microsphere series Dye-Trak VII+ and Dye-Trak 'F'; na = peak not determinable

4.2.3 Trials with the filter unit

4.2.3.1 Introduction

In the experiments carried out with the filter unit, there are several partial aspects that are of interest. The question of whether the concentration of the different microsphere colours of a microsphere series can be measured simultaneously in a sample could basically already be answered positively when determining the standard curves. The results of the tests carried out with the Dye-Trak VII+ and 'F' listed in Tab. 9 and Tab. 10 further confirm this assumption. In response to the questions about the measurement accuracy and the

The detection limits achieved in the tests are described in detail for each microsphere series in chapters 4.3.4.1 and 4.3.4.2 and 4.4.2.2 and 4.4.2.3.

A fundamental and probably generally valid finding from the experiments carried out is the following observation: In all experiments, the measured background signal is higher than the signal measured to determine the standard curves. However, this effect is of little importance for the following reason. The influence of the peak height by the mostly only slightly increased background signal is usually below the measurement error.

In contrast, the deposits on the filter networks caused by the mine water have a considerably greater influence on the measured signal. The results of the first tests with the "Heilstollen" Peißenberg mine water show this clearly. This is because such deposits have a considerable influence on the solvent added at a later point in time. Thus, they can also lead to a far-reaching change in the measurement result. As the red curve in Fig. 9 below makes sufficiently clear, large parts of the wavelength range in which the expected peaks of the various fluorescence colours lie are overlapped to a considerable extent. This leads to a situation where a correct determination of the peak height is made extremely difficult or impossible. As a result, all experiments with the Peißenberg mine water, without purification with oxalic acid, yielded results that could only be evaluated to a limited extent or not at all. Two of these tests are listed in Tab. 9 for comparison purposes.

In the tests with the Straßberg mine water and the filter networks cleaned with oxalic acid, the influence of the overlays caused by the deposits was significantly lower. As long as the individual peaks were not completely overlaid, but only influenced to a small extent, the share of the overlay in the total height of the peak could mostly be successfully determined. This was done by comparing the background value and the measured peak height. By making a correction in this way, it was finally possible to determine the original height of the intensity signal generated by the fluorescent dye.

4.2.3.2 Dye-Trak VII+

As the results in Tab. 11 show, a subsequent correction of the measured value leads to comparatively good results. As described in the previous chapter, the correction is made by comparing the background value and the measured peak height. The magnitude of the influence of such an overlay on the measured peak becomes particularly clear with low microsphere numbers. There, an overestimation of the Lemon peak often occurs, since this is most affected by the superposition due to the low wavelength (Fig. 9 below).

As Fig. 10, in which the results from Tab. 11 are graphically plotted side by side, shows, the results of the test series with the Straßberg and Peißenberg mine water do not deviate greatly from each other. In both cases, up to an initial quantity of 100 microspheres, all microspheres colours could be found and their concentration determined. The quality of the results and the associated measurement errors are discussed in detail in section 4.3.4.1. In addition to the comparison of the two mine waters, the results obtained also allow an equally interesting comparison of the two microsphere series used (section 4.2.3.4).

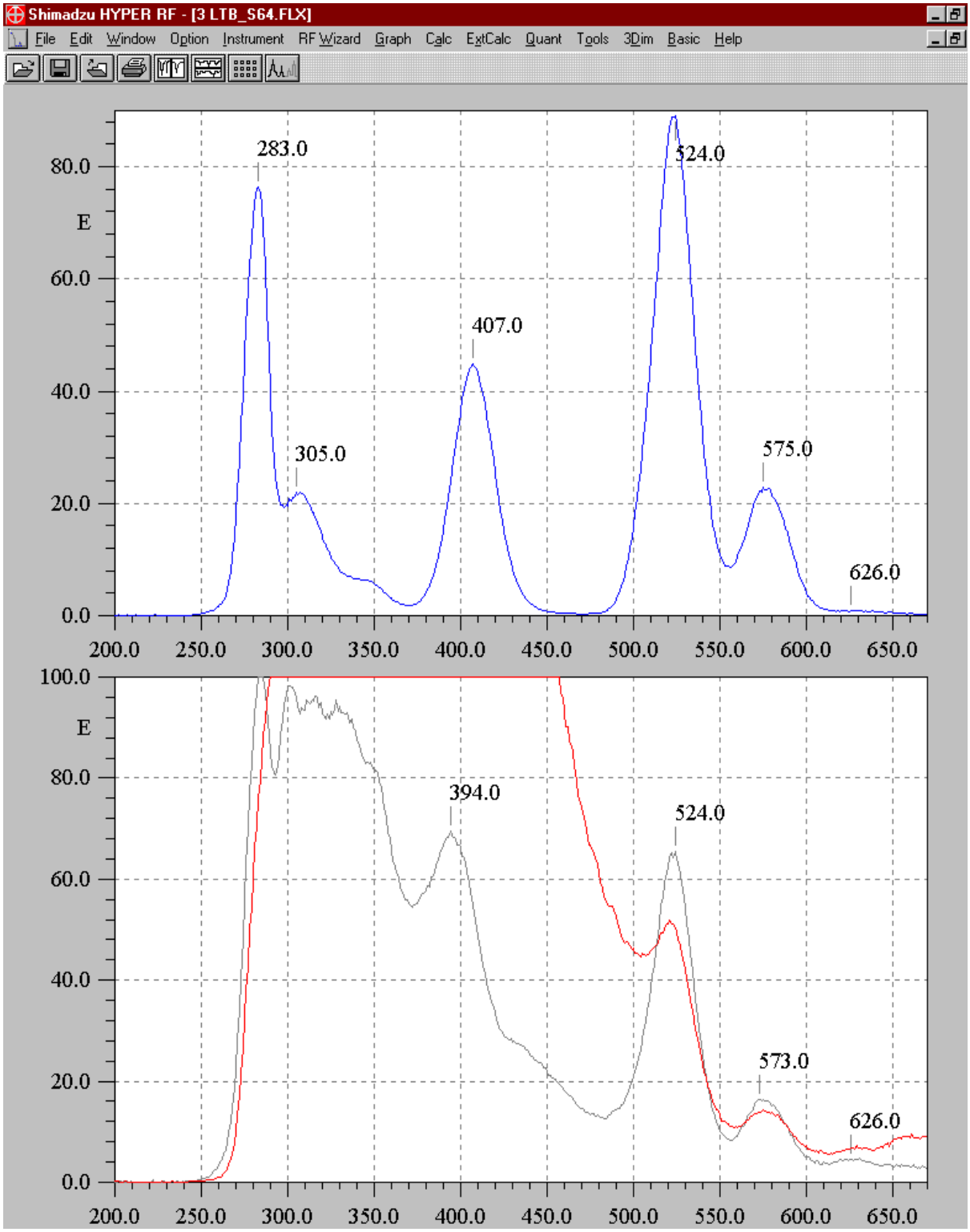


Fig. 9 top: the curve of a measurement to determine the intensity of the Dye-Trak VII+ peaks at a concentration of 500 microspheres/mL (blue curve); bottom: Comparison of two curves of experiments with (black) and without (red curve) oxalic acid purification; a part of the red curve is partially above the measuring range.

Number Microspheres	Microsphere-colour	Straßberg (SBG-Ü539-1203)	Peißenberg (TSP-1701-TSH)	Peißenberg (purified with oxalic acid)
2000	Lemon VII+	1781		2134
2000	Tangerine VII+	1859		2178
2000	Berry VII+	1495		2342
1000	Lemon VII+	1106	na	818
1000	Tangerine VII+	1047	1238	1179
1000	Berry VII+	960	682	964
500	Lemon VII+	480	na	339
500	Tangerine VII+	529	na	426
500	Berry VII+	523	548	357
200	Lemon VII+	164		378
200	Tangerine VII+	100		106
200	Berry VII+	119		86
100	Lemon VII+	170		132
100	Tangerine VII+	119		62
100	Berry VII+	107		150
20	Lemon VII+	53		na
20	Tangerine VII+	53		na
20	Berry VII+	56		38

Tab. 11 Results of the two test series with microspheres of the Dye-Trak VII+ series (LTB) and the Straßberg (SBG) and Peißenberg (TSP) mine waters; additionally two results of tests with the Peißenberg mine water without subsequent oxalic acid purification; na = peak not determinable

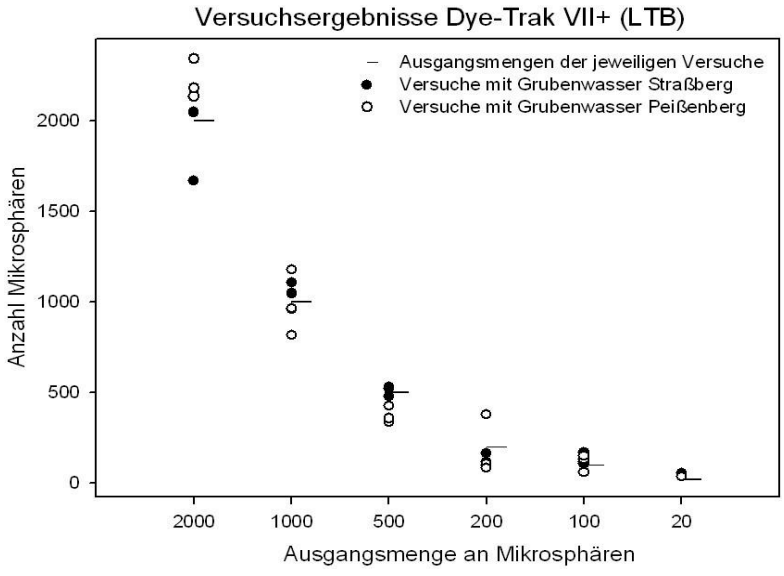


Fig. 10 Graphical representation of the results of the two test series with microspheres of the Dye-Trak VII+ series (LTB) and the mine waters Straßberg (SBG) and Peißenberg (TSP).

4.2.3.3 Dye-Trak 'F

In contrast to the comparatively good test results with the microspheres of the Dye-Trak VII+ series, the results of the tests with the Dye-Trak 'F' microspheres listed in Tab. 12 give a completely different picture. In the tests with high initial quantities and the Straßberg mine water, there is an enormous discrepancy between the determined microsphere numbers of the colour Lemon 'F' on the one hand and the values of the remaining three colours Yellow 'F', Orange 'F' and Persimmon 'F' on the other. In addition, the high numbers of the colour Lemon 'F' can most probably not be attributed to overlapping effects. How this strong discrepancy ultimately comes about could not be clarified either in the course of the trials or afterwards.

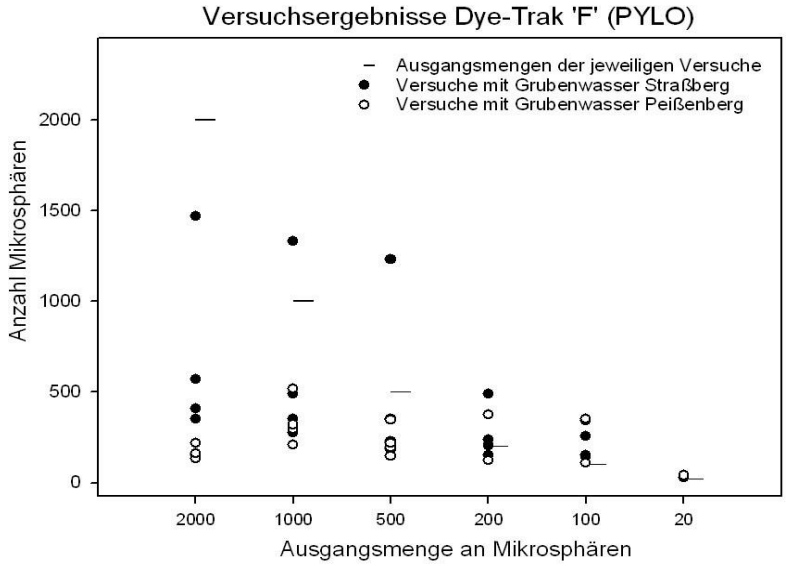


Fig. 11 Graphical representation of the results of the two test series with microspheres of the Dye-Trak 'F' series (PYLO) and the mine waters Straßberg (SBG) and Peißenberg (TSP).

Regardless of this, the results of the first three tests with initial quantities of 2000, 1000 and 500 microspheres for both mine waters are considerably below the expected number of microspheres. With initial quantities below 500 microspheres, the picture is completely different. Apart from the mostly quite low recovery rates, the following trend can at least be read from the calculated results: As the initial quantity decreases, the number of recovered microspheres also decreases, although in many cases only slightly. An exception to this is the test with the Peißenberg mine water and an initial quantity of 2000 microspheres. It should be noted that from the results of the three experiments with initial quantities of 200, 100 and 20 microspheres and the Peißenberg mine water, insofar as a peak could be determined at all, further conclusions should only be drawn with certain reservations.

A difference between the results of the test series with the two mine waters, which has already been mentioned, is the following fact: In the tests conducted with the Straßberg mine water, all but one of the microsphere colours could be recovered. In the tests with the Peißenberg mine water, on the other hand, only individual peaks could be found with initial quantities of 200 microspheres and below.

can be measured and the respective microsphere numbers determined from this. This has a corresponding effect on the detection limit determined for the Dye-Trak 'F' microspheres. However, this will be discussed in more detail in chapter 4.4.2.3.

Number of microspheres	Microsphere colour	Straßberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
2000	Lemon 'F	1469	153
2000	Yellow 'F	571	131
2000	Orange 'F	353	163
2000	Persimmon 'F	408	220
1000	Lemon 'F	1332	516
1000	Yellow 'F	490	297
1000	Orange 'F	277	209
1000	Persimmon 'F	351	320
500	Lemon 'F	1232	346
500	Yellow 'F	350	194
500	Orange 'F	187	147
500	Persimmon 'F	229	219
250/200	Lemon 'F	489	374
250/200	Yellow 'F	237	na
250/200	Orange 'F	153	123
250/200	Persimmon 'F	206	na
100	Lemon 'F	343	353
100	Yellow 'F	256	na
100	Orange 'F	145	108
100	Persimmon 'F	153	na
20	Lemon 'F	30	na
20	Yellow 'F	30	na
20	Orange 'F	29	41
20	Persimmon 'F	na	na

Tab. 12 Results of the two test series with microspheres of the Dye-Trak 'F' series (PYLO) and the mine waters Straßberg (SBG) and Peißenberg (TSP); na = peak not determinable

4.2.3.4 Comparison Dye-Trak VII+ with Dye-Trak 'F'

The differences in the results within the respective microsphere series have already been discussed (Figs. 10 and 11). What follows now are the results of the individual test series. They are shown side by side on the next pages in a total of six figures (Figs. 12 to 17) according to their initial quantities. In this way, the differences between the four series of experiments are made clear. The accompanying texts deal separately with the results shown in the respective figures and explain their differences.

One of the biggest differences between the respective tests with Dye-Trak VII+ and Dye-Trak 'F' microspheres on the one hand and the mine waters from the Straßberg underground mine and the former Peißenberg mine on the other hand can already be seen in Fig. 12. There are considerable differences between the tests with the Dye-Trak VII+ (SBG LTB and TSP LTB) and Dye-Trak 'F' (SBG PYLO and TSP PYLO) microspheres. While the determined microsphere numbers of the two trials SBG LTB and TSP LTB are in the range of the initial quantity of 2000 microspheres, the recovery rate of the two other trials is considerably lower with 35 and 8 %, respectively. The results of the respective test series with the same microsphere series show comparatively more similarities. The deviations of individual microsphere colours are also considerable in some cases, but for the most part the differences do not exceed 20 %. A major exception in this context is again the discrepancy between the value of the colour lemon 'F' and those of the other three colours in the test of the test series SBG PYLO, which was already mentioned in chapter 4.2.3.3.

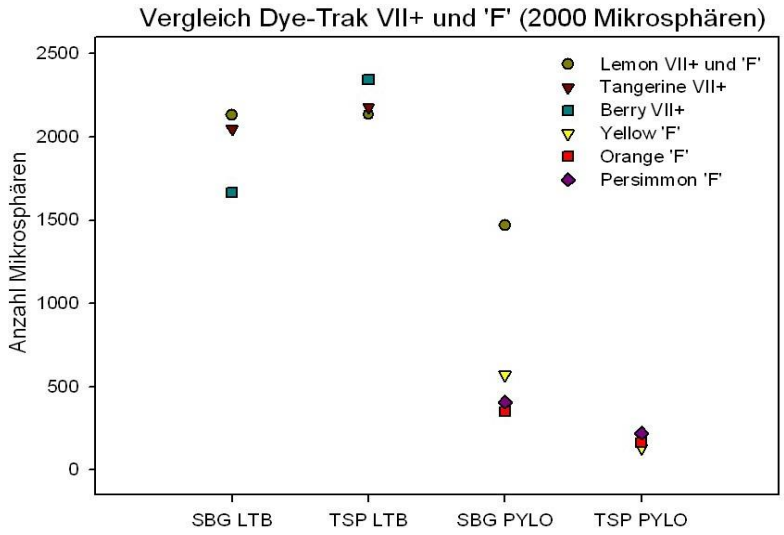


Fig. 12 Graphical representation of the test results of the four test series with microspheres of the Dye-Trak VII+ (LTB) and 'F' (PYLO) series, each with an initial quantity of 2000 microspheres and the mine waters Straßberg (SBG) and Peißenberg (TSP).

This discrepancy is also clearly evident in Fig. 13. In addition, the clear difference between the results of the SBG LTB and TSP LTB tests on the one hand and the SBG PYLO and TSP PYLO tests on the other hand is noticeable in the tests with initial quantities of 1000 microspheres. Even though this is comparatively smaller. This is also reflected in the different recovery rates of the individual trials with 104 and 99 % (SBG LTB and TSP LTB) and 61 and 34 % (SBG PYLO and TSP PYLO). The difference between the two tests with the Dye-Trak 'F' microspheres is almost exclusively due to the considerable differences in the two values of the colour Lemon 'F' (1132 and 516 microspheres respectively). In addition, the results of the respective test series with the same microsphere series are again relatively close to each other. Just as in the tests with an initial quantity of 2000 microspheres, the differences between the tests with different mine waters are below 20 %. The variance in the values of the different microsphere colours within a test is also in this range.

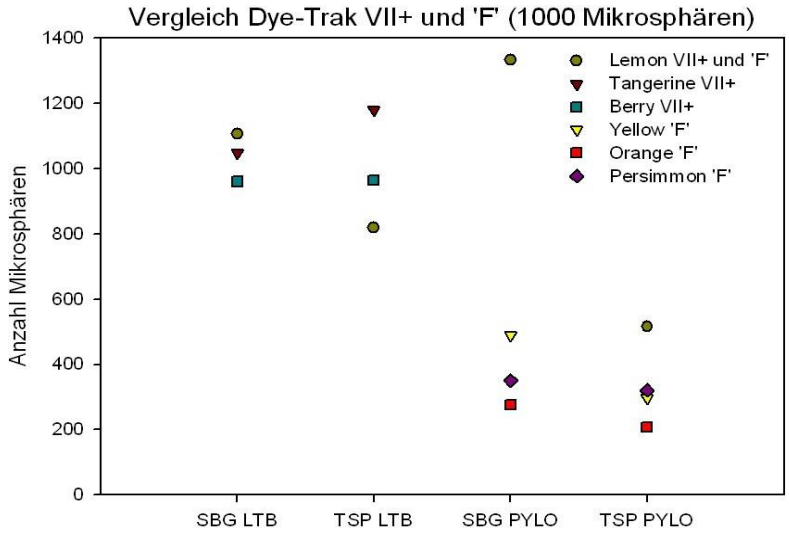


Fig. 13 Graphical representation of the test results of the four test series with microspheres of the Dye-Trak VII+ (LTB) and 'F' (PYLO) series, each with an initial quantity of 1000 microspheres and the mine waters Straßberg (SBG) and Peißenberg (TSP).

The results of the four different experiments with an initial quantity of 500 microspheres (Fig. 14) do not bring anything surprisingly new. Therefore, essentially the same findings can be drawn from them as from the trials with an initial quantity of 1000 microspheres. Again, the value of the colour lemon 'F' in test SBG PYLO deviates strongly from the other three values. This time, too, they are clearly below the initial quantity. In this case, the three values of the respective microsphere colours of the trials SBG LTB and TSP LTB show an even smaller variance than in the two previous trials. However, they differ from each other by an average of 27 %. However, this is probably still within the range of the expected measurement error.

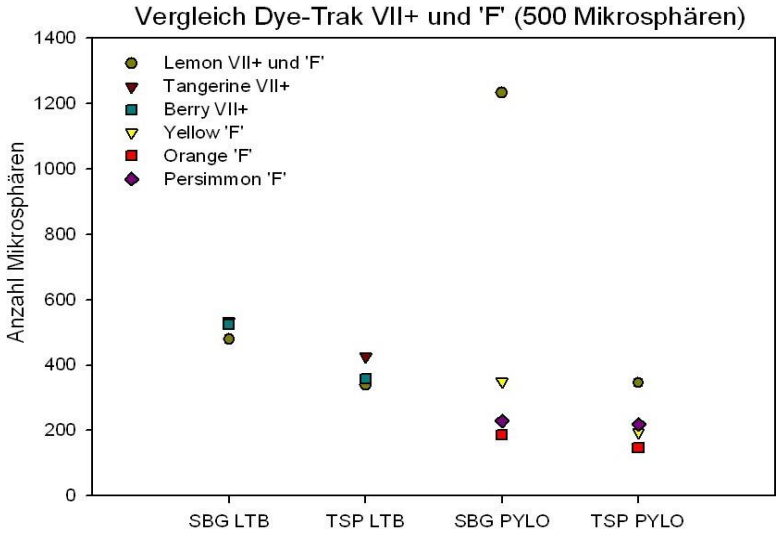


Fig. 14 Graphical representation of the test results of the four test series with microspheres of the Dye-Trak VII+ (LTB) and 'F' (PYLO) series, each with an initial quantity of 500 microspheres and the mine waters Straßberg (SBG) and Peißenberg (TSP).

Fig. 15 then shows a completely different picture compared to the previous figures. On the one hand, the values of the SBG LTB and TSP LTB tests are for the first time overall lower than those of the other two tests. On the other hand, with an initial quantity of 200 microspheres, for the first time not all microspheres added to the sample water could be found. In the TSP PYLO experiment, detectable peaks could only be measured for the two colours lemon 'F' and orange 'F'. In addition, the overestimation of the peak of the colour lemon, which has already been mentioned several times, does not only occur in test SBG PYLO. This time, the two tests with mine water from Peißenberg (TSP LTB and TSP PYLO) are also affected. If one disregards the strongly overestimated value of the Lemon VII+ colour, the recovery rates of the two Dye-Trak VII+ trials, at 64 and 48 % respectively, are for the first time significantly below the initial quantity of 200 microspheres.

It should also be noted in this figure that 250 microspheres were used in the SBG PYLO experiment instead of the 200 microspheres that would otherwise have been used. Therefore, the recovery rate for this experiment is not 100 % as suggested by the figure, but "only" 80 %. Once again, the highly overrated value of the colour lemon 'F' is not taken into account.

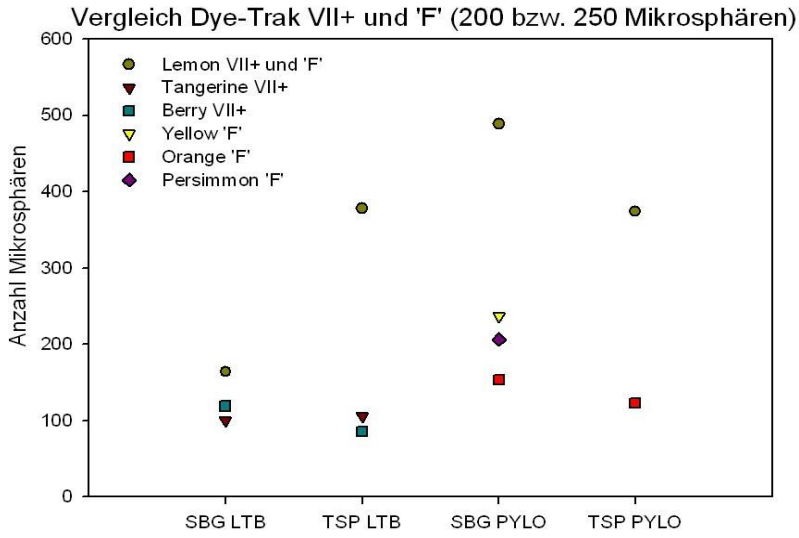


Fig. 15 Graphical representation of the test results of the four test series with microspheres of the Dye-Trak VII+ (LTB) and 'F' (PYLO) series, each with an initial quantity of 250 and 200 microspheres, respectively, and the Straßberg (SBG) and Peißenberg (TSP) mine waters; the deviating initial quantity of 250 microspheres refers exclusively to the SBG PYLO test.

Fig. 16 shows a picture that is difficult to interpret at first glance, with relatively widely scattered measurement results. A large variance within the different microsphere colours can be observed in all four tests. In this case, too, there is a clear overestimation of the lemon 'F' peak in the SBG PYLO and TSP PYLO tests. The same probably applies to the SBG LTB trial, albeit to a considerably lesser extent. Taking this into account, the results of this experiment are otherwise relatively close to the initial quantity of 100 microspheres. In contrast, the scatter of the values in the TSP LTB test is in the range of about 40 %. As with an initial quantity of 200 microspheres, only two of the four microsphere colours, lemon 'F' and orange 'F', can be detected in the TSP PYLO test.

can be found. Of these, at least the value of the colour orange 'F' is within the range of the initial quantity of 100 microspheres. The results of the SBG PYLO experiment indicate that, in addition to the value of the colour lemon 'F', the other three values are also clearly overestimated. The calculated number of microspheres of the colour Yellow 'F', which is noticeably increased compared to the two colours Orange 'F' and Persimmon 'F', strongly reinforces this impression. Furthermore, the peak maximum of the colour yellow 'F' lies in a range in which an increased background value has a comparatively stronger effect on the measured peak height. This can possibly be explained by contamination.

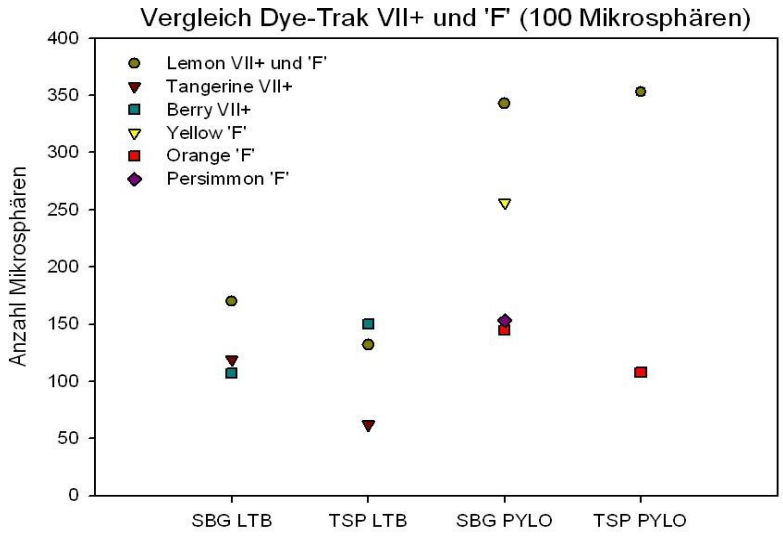


Fig. 16 Graphical representation of the test results of the four test series with microspheres of the Dye-Trak VII+ (LTB) and 'F' (PYLO) series, each with an initial quantity of 100 microspheres and the mine waters Straßberg (SBG) and Peißenberg (TSP).

When looking at the measurement results shown in Fig. 17, there is one aspect that should be the main focus of attention. After all, these four experiments provide important information about the detection limit to be expected. With regard to the results obtained with the standard curves, the following question arises: Can the detection limit of 10 microspheres on average obtained with the standard curves also be transferred to the experiments carried out? Although this topic will be dealt with in detail in chapter 4.4.2, the following should be said at this point. In the four experiments carried out with a respective initial quantity of 20 microspheres, only 8 of the maximum 14 peaks could be measured. Especially the two tests with the mine water from Peißenberg, where only one microsphere colour could be detected with Berry VII+ (TSP LTB) and Orange 'F' (TSP PYLO). All other peaks were either absent or below the background value. This is probably also the reason for the high intensities of the other peaks in relation to the initial quantity of only 20 microspheres. The intensity values of the eight measured peaks are on average 185 % above the expected values. In this context, the very low variance among the different microsphere colours of the SBG LTB and SBG PYLO tests is remarkable and also a little surprising. It amounts to just 2.5 and 1.5 % respectively.

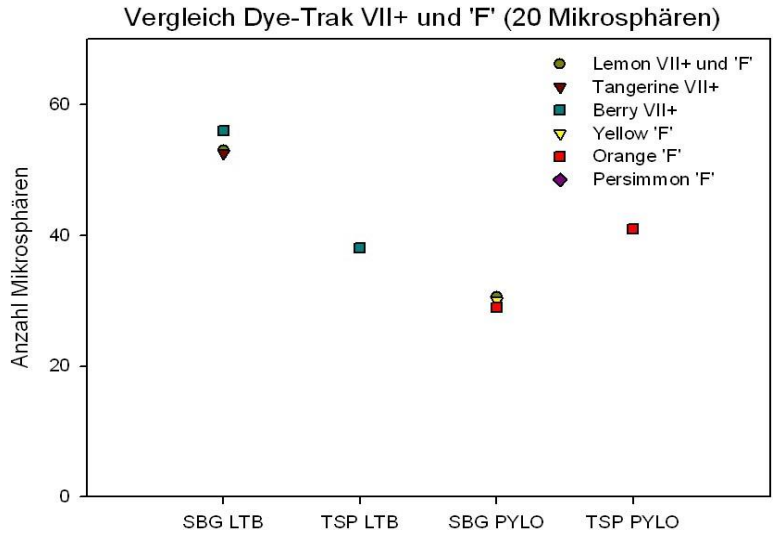


Fig. 17 Graphical representation of the test results of the four test series with microspheres of the Dye-Trak VII+ (LTB) and 'F' (PYLO) series, each with an initial quantity of 20 microspheres and the mine waters Straßberg (SBG) and Peißenberg (TSP).

4.2.3.5 Influence pH value of the mine water

What is striking about the results obtained (Fig. 18), irrespective of the pH value of the respective sample water, is that the recovery rate of the different Dye-Trak VII+ colours differs greatly in some cases. While the recovery rate for the colour Tangerine VII+ is above 80 %, only 54 % (Lemon VII+) and 34 % (Berry VII+) of the original 240 microspheres could be recovered for the other two colours.

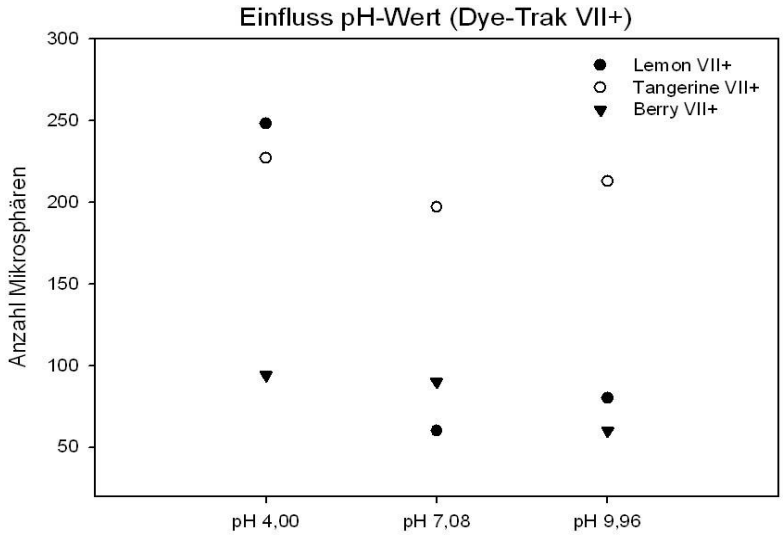


Fig. 18 Results of the tests with microspheres of the Dye-Trak VII+ (LTB) series, an initial quantity of 240 microspheres each and sample waters with different pH values

In contrast, the measured differences between the individual experiments are not so great. If one considers the low microsphere concentrations described in Chap. 4.3.4.1.

calculated measurement error of 36.2 %, at least the deviations in the measured values of the two colours Tangerine VII+ (5 %) and Berry VII+ (18 %) are within the expected measurement error. Only the strong deviation in the measured value of the colour Lemon VII+ at a pH value of 4.00 is striking. With 248 microspheres, however, the value is still within the expected range. A correlation between the pH value of the sample water and the measured value can therefore not be proven.

Instead, two scenarios are conceivable. On the one hand, the recovery rate for the colour Lemon VII+ may have been significantly higher during the experiment for reasons that cannot be determined. Secondly, residues of the mine water could have changed the pH value on the surface of the filter net to such an extent that it influenced the fluorescent dye released in the further course.

4.2.3.6 additional use of the 41 µm filter mesh

In the tests carried out, the main aim was to determine whether a certain proportion of the microspheres already remained on the filter mesh with a mesh size of 41 µm and what percentage this proportion represents of the total number. Therefore, no assessment of the recovery rates or measurement errors achieved during the tests will be made in the following. Accordingly, Table 13 does not show the respective test results in absolute numbers. Instead, the results were converted into the respective share of the two filter nets (15 and 41 µm) in the overall result and plotted next to each other.

The results of the first test with microspheres of the Dye-Trak 'F' series and an initial quantity of 100 microspheres, using the mine water from Peißenberg, provide hardly any usable information with regard to the question. Instead, this only confirms the result that was already achieved without the additional use of the 41 µm filter mesh (see Table 12).

Output quantity (microspheres)	Microsphere series	Mine water	Filter mesh 15 µm	Filter mesh 41 µm
100	Lemon 'F	Peißenberg	0,0	0,0
100	Yellow 'F	Peißenberg	0,0	0,0
100	Orange 'F	Peißenberg	100,0	0,0
100	Persimmon 'F	Peißenberg	100,0	0,0
300	Lemon VII+	Straßberg	82,3	17,6
300	Berry VII+	Straßberg	100,0	0,0
1000	Lemon VII+	Peißenberg	46,1	53,9
1000	Tangerine VII+	Peißenberg	100,0	0,0
1000	Berry VII+	Peißenberg	98,7	1,3
5000	Lemon VII+	Peißenberg	71,4	28,6
5000	Tangerine VII+	Peißenberg	79,8	20,2
5000	Berry VII+	Peißenberg	69,5	30,5

Tab. 13 Results of the four tests carried out converted into the respective share of the two filter meshes (15 and 41 µm) in the total result in %.

In the other three tests, however, microspheres were also found on the 41 µm filter meshes. Consequently, some of the microspheres remain on the coarser 41 µm filter mesh despite the diameter of 15 µm. If one looks at the individual test results after this very general consideration, the following can be determined: In some cases, a considerable percentage of the microspheres remain on the upper filter mesh. The maximum value is around 54 %. However, this high value is limited exclusively to the colour Lemon VII+. Furthermore, it can be determined, if a conclusion can be drawn at all from the few tests, that the percentage of microspheres remaining on the 41 µm filter mesh increases with increasing output quantity. This may be explained as follows, especially with low output quantities: Small numbers on the 41 µm filter net can no longer be detected for the sole reason that they are below the detection limit.

In summary, one important finding can be drawn from the tests carried out: As soon as the 41 µm filter net is used as a pre-filter in a tracer test, it should be examined for microspheres just like the 15 µm. This is based on the knowledge that at least a small proportion of the microspheres in the sample water are already deposited on the 41 µm filter mesh.

4.3 Measurement accuracy

4.3.1 General aspects

The following chapter deals with a few general sources of error that either occurred in the course of the experiments or were already known as such in advance. The next section then deals with the measurement errors resulting from the determination of the standard curves and the test series. The different areas are each dealt with separately in a separate chapter. In addition, the measurement error of the spectrofluorophotometer already mentioned in chapter 3.5 should be mentioned here. As expected, this error is low at less than one percent.

4.3.2 Sources of error

With regard to sources of error, particular attention had to be paid to a possible loss of microspheres during the sample preparation process. In the past, there has been evidence of a loss of microspheres during the cleaning of the filter nets in the course of the preparation process. This is shown by the results of the tests on the recovery of microspheres from the filter nets in chapter 4.1.2.

One means of preventing a loss of microspheres is to develop a method with as few and standardised steps as possible. One requirement, similar to that in medicine, was to leave the filter net in a sample vessel from the time it is removed from the filter unit until the microspheres are released by means of the solvent (2-ethoxyethyl) acetate, and to carry out all the steps necessary for sample preparation inside. In this case, 250 and 500 mL amber glass bottles were used first, which had already been used in earlier tracer experiments (see section 3.3). These were then replaced by 50 mL centrifuge tubes during the development process, as the use of a centrifuge accelerated the triggering process and made it easier to pipette the solvent from this sample vessel.

Another possible source of error is contamination of the samples either by entrained microspheres or other fluorescent substances. In the analysis with the fluorescence microscope, the occurrence of very isolated microspheres in some samples can often be attributed to carry-over (Wolkersdorfer & Hasche 2003 and 2004b). In contrast, the introduction of individual microspheres during measurements in the spectrofluorophotometer does not pose such a great danger, also due to the lower detection strength. The intensities measured in the experiments were often above the expected values. However, this can probably be explained solely by fluctuations in the measuring device or the level of measurement error. Contamination in the form of introduced microspheres is probably not an explanation in this context.

The result of one of the experiments carried out with the Dye-Trak 'F' microspheres shows that conscientious cleaning of the sample and measuring vessels used in previous experiments is necessary to prevent possible contamination. The occurrence of peaks that were not to be expected was most likely due to inadequate cleaning of the centrifuge tube or glass cuvette used. In addition to the four expected peaks of the colours lemon 'F', yellow 'F', orange 'F' and navy 'F', two additional peaks occurred. These could be clearly assigned to the two Dye-Trak VII+ colours Tangerine and Berry. A positive side effect, however, is the fact that the following could be determined in this unintentional way: The six microsphere colours listed above can be detected next to each other during only one measurement process. This in turn confirms the results obtained in chapter 4.2.2.

The sensitivity of the fluorescent colours already dissolved from the microspheres to strong changes in temperature, pH and strong incidence of light can be another source of error. Therefore, care should be taken during sample preparation: keep the temperature conditions constant and store the centrifuge tubes as far as possible in the dark from the time the solvent is added until the measurement in the spectrofluorophotometer. The influence of mine waters with strongly differing pH values on the measurement result was investigated within the scope of this work (section 3.4.3.3**).** It was found that the differences in intensity are within the range of the usual measurement error and therefore no negative influence on the measurement result is to be expected.

4.3.3 Standard curves

4.3.3.1 Dye-Trak 'F

Since the first measurements with both the Dye-Trak VII+ and the Dye-Trak 'F' microspheres already showed large fluctuations between the individual measurements, the measurements of the six dilution levels were carried out several times. The respective mean errors were then determined from these. In order to detect possible contamination, which could have led to a falsification of the measurement results, 4 µL (concentration 500 microspheres/µL) of the control colour Navy was added for each measurement. In the following, the respective mean errors in % of the individual microsphere colours of the Dye Trak 'F' series are listed (Tab. 14), which resulted from the measurements to determine the standard curves.

Number of microspheres	Lemon 'F	Yellow 'F	Orange 'F	Persimmon 'F	Navy 'F
10	6,39	12,79	17,81	14,19	2,51
50	6,40	8,35	2,92	17,51	4,88
100	6,51	7,27	1,88	7,64	1,76
250	2,78	5,65	1,30	4,01	0,35
500	3,14	4,88	1,88	2,82	3,10
1000	3,40	5,65	2,65	1,50	4,54

Tab. 14 Mean error in % of the respective dilution levels for the determination of the standard curves of each microsphere ink of the Dye-Trak 'F' series

If the corresponding percentage errors are converted into absolute numbers of microspheres (Tab. 14), the following becomes clear: The high error rates of up to 18 % for the strong dilutions, expressed in absolute numbers, hardly have a noteworthy influence on the subsequent measurement result. It can therefore be assumed that the size of the measurement error decreases with an increasing number of microspheres. However, no clear correlation could be identified in this respect from the measurements carried out.

Number of microspheres	Lemon 'F	Yellow 'F	Orange 'F	Persimmon 'F
10	1	1	2	1
50	3	4	1	9
100	7	7	2	8
250	7	14	3	10
500	16	24	9	14
1000	34	57	27	15

Tab. 15 Mean error in microsphere numbers of the respective dilution levels for determining the standard curves of each microsphere colour of the Dye-Trak 'F' series

Also, all four regression lines show a near zero crossing (-1.4 - 1.5 AU). Furthermore, the comparison of the intensities of the control colour Navy suggests that with increasing microsphere numbers of the other four microsphere colours, a slight weakening of the intensity accompanies. One reason for this could be fluorescence extinction caused by the increasing intensities. A conceivable approach to calculating the proportion of extinction in the total value is listed in Tab. 16. For this purpose, the percentage changes are calculated from the determined intensity values of the colour navy. Then a simple correction factor is formed from these. This correction factor can then be used to correct the values determined for the other four fluorescent colours by the amount of attenuation.

Number of microspheres	Intensity, AU	Change in %	Correction factor
10	1,226		1,000
50	1,192	2,77	1,028
100	1,149	6,26	1,063
250	1,092	10,93	1,109
500	1,007	17,83	1,178
1000	0,904	26,25	1,263

Tab. 16 Results of the measurements of the control colour Navy (conc. 2000 microspheres) in connection with the determination of the standard curves of the microspheres series Dye-Trak 'F'.

If one calculates standard curves for the four fluorescent colours from the values corrected in this way, one finds that these also show a very good correlation between the number of microspheres and the level of intensity (Fig. 19).

	Lemon 'F	Yellow 'F	Orange 'F	Persimmon 'F
Measured values	0,9986	0,9998	0,9994	0,9996
Corrected values	0,9993	0,9988	0,9968	0,9971

Tab. 17 Comparison of the microsphere colour specific values of the coefficient of determination of measured and corrected values of the Dye-Trak 'F' series

The comparison of the coefficient of determination (R^2) of measured and corrected values (Tab. 17) reveals only minor deviations between the two values. Except for the colour lemon 'F', the correlation of the values worsens slightly due to the correction. Consequently, a possible reduction of the intensities due to an assumed fluorescence extinction most likely does not lead to a significant impairment of the measurement result.

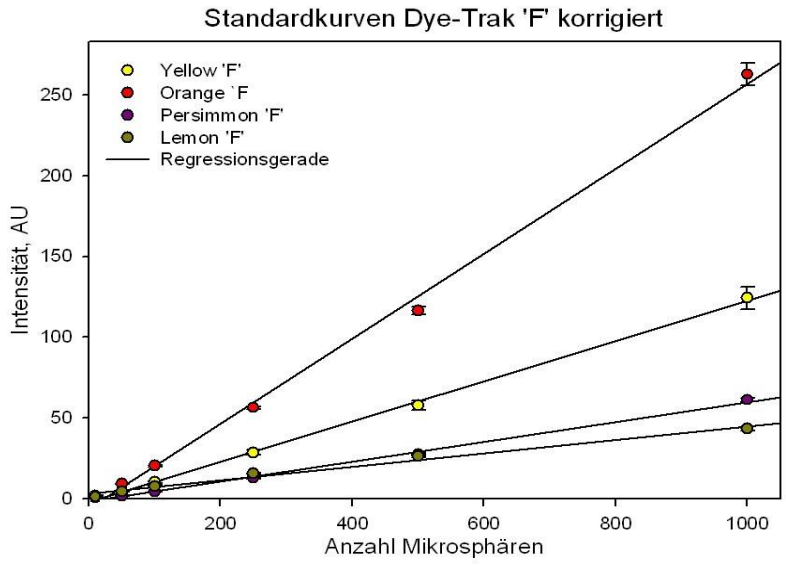


Fig. 19 Standard curves of the four different microsphere colours of the Dye-Trak 'F' series (corrected values); regression coefficients in Tab. 17

For this reason, it does not seem necessary to make such a correction. A possible explanation why a clear trend is recognisable in the colour navy, but not reflected in the same way in the other four fluorescent colours, could be the following assumption: The fluorescence extinction may not be equally pronounced in all colours, but varies slightly from colour to colour.

4.3.3.2 Dye-Trak VII+

The three Dye-Trak VII+ microsphere inks also showed considerable differences when the respective dilution levels were measured several times. The error was in the range of 0.9 - 28.2 %, converted into microspheres 1 - 36 microspheres. As with the Dye-Trak 'F' microspheres, the size of the error tends to decrease with increasing numbers of microspheres. From the results of the control colour Navy, which was also measured in this case in concentrations of 2000 microspheres, no clear trend can be read this time. The values range between 0.954 (±0.026) and 1.235 (±0.105), which corresponds to a maximum deviation of 29.5 %. However, since the intensity of the colour navy in this case does not decrease again with increasing microsphere concentration of the other colours, no correction factor can be determined with this. Furthermore, as already mentioned in the previous chapter, this result confirms the assumption that with an increasing number of microspheres up to at least 1000 microspheres, there is no or only a negligibly small fluorescence extinction.

A special case arises with the colour berry, since its intensity maximum in the wavelength range of 570 nm overlaps with the signal of the solvent (see chapter 4.2.1). However, since the intensity of this signal lies at an average value of 3.773(±0.114) AU, independent of the other microsphere concentration, the respective intensity maximum of the colour berry can be corrected by this amount. A regression line with a zero crossing of 0.72 AU can then be calculated from the corrected values. In comparison, the two other regression lines have a comparable zero crossing of 0.83 (lemon) and 1.76 (tangerine) AU.

4.3.4 Trials with the filter unit

4.3.4.1 Dye-Trak VII+

In the previous chapters, the determination of the various standard curves was dealt with in great detail. Now the question arises to what extent these results can also be transferred to the different test series with the filter unit. In Tab. 18, the respective deviation in % from the original initial value is plotted for each measured value obtained with the Dye-Trak VII+ microsphere series. This list is intended more to give an overview of the variance of the measured values and less to pick out individual values and discuss their correctness. The values summarised in Tab. 19 are much better suited for this purpose. In this case, all values of an experiment were combined and their average deviation from the initial value was determined.

On the one hand, this makes the differences between the tests with high compared to those with low initial quantities clear. On the other hand, it allows a good comparison of the two mine waters used from Straßberg and Peißenberg.

Number Microspheres	Microsphere paint	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
2000	Lemon VII+	6,5	6,7
2000	Tangerine VII+	2,3	8,9
2000	Berry VII+	16,7	17,1
1000	Lemon VII+	10,6	18,2
1000	Tangerine VII+	4,7	17,9
1000	Berry VII+	4,0	3,6
500	Lemon VII+	4,0	32,2
500	Tangerine VII+	5,8	14,8
500	Berry VII+	4,6	28,6
200	Lemon VII+	18,0	89,0
200	Tangerine VII+	50,0	47,0
200	Berry VII+	40,5	57,0
100	Lemon VII+	70,0	32,0
100	Tangerine VII+	19,0	38,0
100	Berry VII+	7,0	50,0
20	Lemon VII+	165,0	na
20	Tangerine VII+	165,0	na
20	Berry VII+	180,0	90,0

Tab. 18 Deviation from the initial value in % of the test results of both test series with microspheres of the Dye-Trak VII+ series (LTB) and the mine waters Straßberg (SBG) and Peißenberg (TSP); na = peak not determinable

As far as the results of the individual tests in relation to their initial quantity are concerned, the pure measured values have already been dealt with in detail in chapter 4.2.3. In Tab. 19, in which all values of the respective microsphere colours of a test were combined to an average value, the following trend can be seen overall: As the initial quantity decreases, the value of the percentage deviation from the initial value increases in both test series, with the corresponding pit water in each case, and thus the measurement error increases at the same time. In addition, further conspicuous features can be read from the values. First of all, the considerable jump between the values with an initial quantity of 500 and those with an initial quantity of 200 microspheres should be mentioned. This is quite obvious and can be seen in both test series, even if not necessarily with the same clarity (Peißenberg). A second jump can be seen between the initial quantities of 100 and 20 microspheres. Here, the discrepancy between the two values can be clearly seen. Especially in those of the experiments with the Straßberg mine water. The fact that the latter value of 170 % differs so clearly from the other five values will play a decisive role again in the following chapter. The fact that the corresponding value for the Peißenberg mine water is not so high can be explained as follows: The value is composed of only one and not three values, since only one peak could be measured at all in this test with the colour Berry VII+. When comparing the two pit waters, the following becomes clear: In all tests, the percentage deviations in the pit water are higher than in the other tests.

water Straßberg is lower than that of the corresponding tests with the mine water Peißenberg. The only exception is the last test mentioned above.

Quantity Microspheres	Microsphere series	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
2000	Dye-Trak VII+	8,5	10,9
1000	Dye-Trak VII+	6,4	13,2
500	Dye-Trak VII+	4,8	25,2
200	Dye-Trak VII+	36,2	64,3
100	Dye-Trak VII+	32,0	40,0
20	Dye-Trak VII+	170,0	90,0

Tab. 19 Average deviation from the initial value in % of all colours of the microsphere series Dye-Trak VII+ (LTB) for both test series with the mine waters Straßberg (SBG) and Peißenberg (TSP)

Finally, a comparison should be made between the measurement results of the two test series with those of the standard curves. A clear judgement can be made without having to compare the values individually. The measurement errors of the tests as a whole are clearly higher than those of the standard curves. However, what this ultimately says about the quality of the results obtained will not be discussed conclusively at this point (see Chap. 5.4.3.2).

4.3.4.2 Dye-Trak 'F

Just as for the two test series of the Dye-Trak VII+ microsphere series, the respective deviations in % from the original initial value were also plotted in a table (Tab. 20) for each measured value obtained in the two remaining test series with the Dye-Trak 'F' microsphere series. Once again, the list represents more of an overview of the variance of the measured values and served less to single out individual values and discuss their correctness. For this purpose, as was already the case for the Dye-Trak VII+ trials, all values of a trial were additionally summarised and their average deviation from the initial value was determined (tab. 21).

The consistently very high values are striking. These express large percentage deviations from the initial value and thus inevitably correspondingly high measurement errors. If one then compares the individual values with each other, it is much more difficult to identify a clear trend than in Tab. 20. In this case, an increase in the measurement error with decreasing initial quantity cannot be recognised. Even if one also takes into account that with initial quantities of 250 or 200 microspheres and below, in many cases only some of the microspheres colours could be detected. Consequently, the respective values are no longer necessarily based on an average of the results of all four microsphere colours lemon 'F', yellow 'F', orange 'F' and persimmon 'F', but only on that of one or two colours.

Number Microspheres	Microsphere paint	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
2000	Lemon 'F	26,6	92,4
2000	Yellow 'F	71,5	93,5
2000	Orange 'F	82,4	91,9
2000	Persimmon 'F	79,6	89,0
1000	Lemon 'F	33,2	48,4
1000	Yellow 'F	51,0	70,3
1000	Orange 'F	72,3	79,1
1000	Persimmon 'F	64,9	68,0
500	Lemon 'F	146,4	30,8
500	Yellow 'F	30,0	61,2
500	Orange 'F	62,6	70,6
500	Persimmon 'F	54,2	56,2
250/200	Lemon 'F	95,6	87,0
250/200	Yellow 'F	5,2	na
250/200	Orange 'F	38.8	38,5
250/200	Persimmon 'F	17,6	na
100	Lemon 'F	243,0	253,0
100	Yellow 'F	156,0	na
100	Orange 'F	45,0	8,0
100	Persimmon 'F	53,0	na
20	Lemon 'F	50,0	na
20	Yellow 'F	50,0	na
20	Orange 'F	45,0	105,0
20	Persimmon 'F	na	na

Tab. 20 Deviation from the initial value in % of the test results of both test series with microspheres of the Dye-Trak 'F' series (PYLO) and the mine waters Straßberg (SBG) and Peißenberg (TSP); na = peak not determinable

However, a comparison of the two mine waters shows that, with one exception, the test series with the Peißenberg mine water has a correspondingly higher measurement error than the comparable tests with the Straßberg mine water.

Quantity Microspheres	Microsphere series	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
2000	Dye-Trak 'F	65,0	91,7
1000	Dye-Trak 'F	55,0	66,5
500	Dye-Trak 'F	73,0	54,7
250/200	Dye-Trak 'F	39,0	62,8
100	Dye-Trak 'F	124,0	130,5
20	Dye-Trak 'F	48,0	105,0

Tab. 21 Average deviation from the initial value in % of all colours of the microsphere series Dye-Trak 'F' (PYLO) for both test series with the mine waters Straßberg (SBG) and Peißenberg (TSP)

4.3.4.3 Comparison Dye-Trak VII+ with Dye-Trak 'F'

With regard to the results shown in Tab. 22, the following should be pointed out first: The three percentages of the test series SGB LTB listed in the table are not based on the error values of all tests. This has the following background: The results of the tests with an initial quantity of 20 microspheres show, as already mentioned in chapter 4.3.4.1, disproportionately high deviations from the initial quantity (170 %) compared to all other tests carried out in this test series. Therefore, this test is not taken into account in the calculation of the average deviation listed in Tab. 22. In order to make it clear what difference, expressed in figures, this measure has made, the corresponding values, taking into account all available values in the above table, are given in brackets.

Microsphere paint	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
Lemon VII+	21,8 (45,7)	35,6
Tangerine VII+	16,4 (41,1)	25,3
Berry VII+	14,6 (42,1)	41,1
Lemon 'F	99,1	102,3
Yellow 'F	60,6	75,0
Orange 'F	57,7	65,5
Persimmon 'F	53,9	71,1

Tab. 22 Average deviation from the initial value in % of all test results of a microsphere colour of the series Dye-Trak VII+ (LTB) and 'F' (PYLO) with the mine waters Straßberg (SBG) and Peißenberg (TSP)

Tab. 22 shows the average percentage deviations from the initial value for each of the four test series carried out. This representation is intended to facilitate a comparison of the microsphere colours used. Such a comparison can be made both for each test series individually and for all tests together. To illustrate the respective differences, the values from Tab. 22 are shown again graphically in Fig. 20.

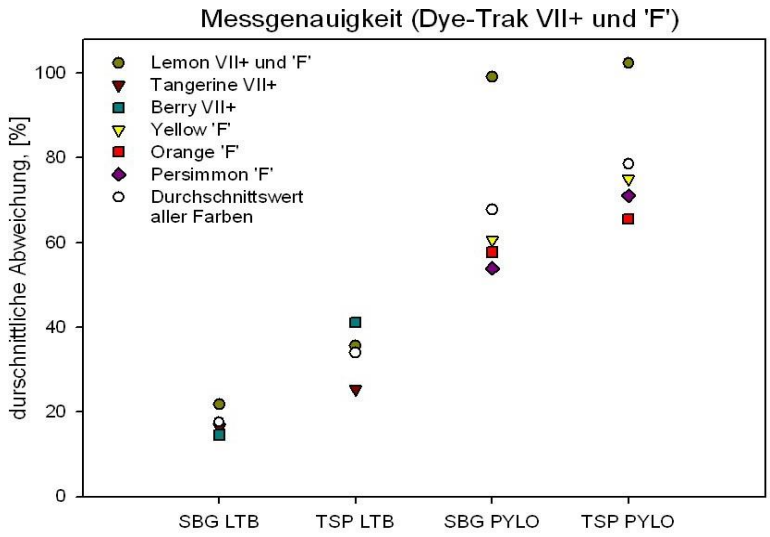


Fig. 20 Graphical comparison of the average deviations of each microsphere colour of the Dye-Trak VII+ (LTB) and 'F' (PYLO) series from the initial value in
% of the test results with the mine waters Straßberg (LTB) and Peißenberg (TSP)

In the following, we will discuss the overvaluation of the Lemon Peak, which was already discussed in Chap.
3.4.3.3 was discussed in detail. The overestimation of the lemon peak is presumably caused by an often quite pronounced overlapping of this peak. A comparison of the four different values with the average percentage deviation of the measurement results of the colour lemon VII+ or 'F' with the other values leads to the following result: In three of the four cases, the value for the colour lemon is the highest.

It should be noted that the test series in which the value for the colour Berry VII+ is above that of the colour Lemon VII+ (TSP LTB) is subject to similar conditions as the test series SBG LTB, where the overall result depends significantly on the one result of the test with an initial quantity of 20 microspheres. If this test, in which only the colour Berry VII+ could be detected, is not taken into account, the value changes from 41.1 to 31.3 %. This would also be below the value for Lemon VII+. In contrast, the values of the two or three other microsphere colours are mostly close to each other in their measurement errors. Consequently, the superimposition effect should play only a minor role or no role at all for these colours.

Otherwise, it can be determined for all values that the respective measurement errors of the test series with the Straßberg mine water are in part noticeably below those of the two other test series with the Peißenberg mine water. In addition, the results shown in Fig. 20 demonstrate even more clearly the differences in the measurement errors determined between the two different microsphere series Dye-Trak VII+ and Dye-Trak 'F'. The former are considerably smaller. However, this does not prejudge the quality of the respective measurement results.

Finally, on the subject of measurement accuracy in the tests with the filter unit, the average deviation from the initial value in % of all test results is listed. However, only a subdivision into the respective four test series was made (Tab. 23). In other words, the respective values represent an average value of all tests carried out within the framework of a test series.

Microsphere series	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
Dye-Trak VII+	17,6 (43,0)	34,0
Dye-Trak 'F	67,8	78,5

Tab. 23 Average deviation from the initial value in % of all test results of the microsphere series Dye-Trak VII+ (LTB) and 'F' (PYLO) with the mine waters Straßberg (SBG) and Peißenberg (TSP)

As in Tab. 22, the value given in Tab. 23 for the test series with the Dye-Trak VII+ microsphere series and the Straßberg mine water again does not take into account the test with an initial quantity of 20 microspheres. In this case, too, the value that would have resulted if all values had been taken into account is given in brackets for comparison purposes.

The four values listed in the table confirm the assumptions already concluded from the other tables and figures. On the one hand, the measurement error in the tests carried out with the Dye-Trak VII+ microspheres is comparatively much lower. Also, the test series with the mine water from Straßberg again show the lower measurement error.

4.4 Detection limit

4.4.1 Standard curves

One of the main goals of the laboratory tests was to lower the detection limit as much as possible. Even in the first tests, small numbers of only a few microspheres could be reliably detected. After the standard curves of both the Dye-Trak VII+ and the Dye-Trak 'F' microspheres reliably detected concentrations of 10 microspheres dissolved in 2 mL solvent, a further reduction of the detection limit was to be tested in further experiments.

When the number of microspheres was reduced to 5, only the colour orange 'F' could be detected in all three measurements of the Dye-Trak 'F' microspheres. The error of 9% determined from the three measurements was even lower than the average value of 11.5% for this colour. In addition, the colours Yellow 'F' could be detected twice, Lemon 'F' once and Persimmon 'F' not once. In the case of the Dye-Trak VII+ microspheres, the colour Lemon VII+ was detected in all measurements and the colours Tangerine VII+ and Berry VII+ in half of all measurements.

The following conclusion can be drawn from the results obtained: With the measurement settings chosen for the spectrofluorophotometer, detection limits of 5±1 microspheres per 2 mL solvent can be achieved for the colours Lemon VII+ and Orange 'F' and 10±2 microspheres per 2 mL solvent for the colours Tangerine VII+, Berry VII+, Lemon 'F', Yellow 'F' and Persimmon 'F'.

4.4.2 Trials with the filter unit

4.4.2.1 Introduction

Compared to the measurements to determine the standard curves, the situation is slightly different in both experiments with the filter unit. As described in detail in chapter 3.4.2.1, 4 mL of solvent is required during the preparation process to release the microspheres from the filter nets, of which only 2 mL are measured in the spectrofluorophotometer. Thus, for example, the intensity measured in the spectrofluorophotometer with an initial quantity of 100 microspheres corresponds to a number of only 50 microspheres. However, since the value can easily be corrected for the degree of dilution, this fact only plays a role in the case of low microsphere concentrations. For example, if only 10 microspheres of the colour yellow 'F' have accumulated on the filter net, the intensity measured in the device corresponds to only 5 microspheres. Consequently, no peak can be measured due to the previous dilution, although the detection limit for this colour is 10±2 microspheres.

Accordingly, no initial quantities below 20 microspheres were used in the experiments carried out. Which in turn means that in the best case only a detection limit of 20 microspheres could be achieved.

4.4.2.2 Dye-Trak VII+

Looking at the results in Tab. 11, the following can be determined, at least for the tests with the Dye-Trak VII+: In the tests with the Straßberg mine water, all microsphere colours could be found at least in attenuated form. From this it can be concluded that the detection limit in this case is 20 microspheres or even lower.

In the corresponding test with the Peißenberg mine water, however, only one of the three microsphere colours could be detected (Berry VII+). Consequently, the detection limit for the remaining two colours (Lemon VII+ and Tangerine VII+) is higher than 20 microspheres. With an initial quantity of 100 microspheres, all three microsphere colours could be detected. Consequently, the detection limit when using the Peißenberg mine water is in the range of 20 to 100 microspheres.

4.4.2.3 Dye-Trak 'F

The results of the tests with the Dye-Trak 'F' microspheres, on the other hand, show that the detection limit can depend primarily on the initial quantity, but also strongly on the colour used. In the tests in which the Straßberg mine water was used, all four microspheres of the Dye-Trak 'F' series could be successfully detected with an initial quantity of 100 microspheres. Only with an initial quantity of 20 microspheres was it no longer possible to determine an independent peak for the colour Persimmon 'F'. Consequently, the detection limits for the colours Lemon 'F', Yellow 'F' and Orange 'F' are about 20 microspheres and those for the colour Persimmon 'F' are in the range of 20 to 100 microspheres.

If there are no major differences between the respective microsphere colours up to this point, the series of tests with the Peißenberg mine water and the dye water show that there are no major differences.

Trak 'F' microspheres (TSP PYLO) that there can be significant differences between colours as mentioned above. In this case, only with an initial amount of 500 microspheres and above could all microsphere colours be successfully recovered. With an initial quantity of 200 microspheres and below, the colours Yellow 'F' and Persimmon 'F' could no longer be detected in each case. Microspheres of the colour Lemon 'F' could still be recovered up to an initial quantity of 100 microspheres. With an initial quantity of 20 microspheres, only microspheres of the colour orange 'F' could be found. This results in detection limits of approximately 500 microspheres for the colours yellow 'F' and persimmon 'F', 100 microspheres for the colour lemon 'F' and 20 microspheres for the colour orange 'F'.

5 D iscussion

5.1 Task

Up to now, the detection and analysis of microspheres in hydrogeological investigations has usually been carried out by means of a time-consuming and tedious counting procedure under the fluorescence microscope. The microsphere counter developed by Niehren (1999) is also not suitable for the evaluation of tracer tests in underground mines due to the sometimes high particle content in mine waters (Käss 2004). For this reason, a standard method used in medicine, the use of a spectral fluorimeter for analytical evaluation, was to be adapted to the special requirements of mine water within the scope of this work. Just as already developed for medical applications, it should be possible to separate the different fluorescent dyes from the microspheres and analyse them in as few standardised steps as possible. Since even the detection of a few microspheres can determine the success of a tracer experiment, the aim was also to achieve the highest possible sensitivity of a few microspheres per sample or filter network. In addition, it should be investigated how many and which microsphere colours can be measured simultaneously during a measurement process.

5.2 Standard curves

5.2.1 Introduction

While initially only the three microsphere colours of the Dye-Trak VII+ series were used in the laboratory tests, the spectrum was expanded in the course of the work to include further microsphere colours of the Dye-Trak 'F' series. In contrast to many other studies, in this case the determination of the standard curves was more than a simple preparatory step in the run-up to the work carried out.

First, a uniform measurement setting had to be found. In order to be able to achieve comparable values later, this setting could not be changed when determining the respective microsphere colours of the two series Dye-Trak VII+ and 'F'. In this context, it was also necessary to ensure that the entire range of different initial concentrations 5 to 2000 microspheres could be determined within one measuring range. Otherwise, the comparability of the values would have been lost by changing the slit width on the spectrofluorophoto-meter (see chapter 2.4.3). A large number of measurements were therefore necessary to find a suitable measurement setting. In the process, considerable differences in the peak maxima of the respective fluorescent dyes at the same concentration or number of microspheres became apparent between the two microspheres series, Dye-Trak VII+ and Dye-Trak 'F'. However, these are probably not related to the type of preparation. Instead, they can probably only be explained by the differences in the height of the respective excitation and emission maxima.

Then the individual measurements had to be carried out several times to determine the standard curves. This became more important when the first measurements already showed significant fluctuations between the individual measurements (see chapter 4.3.2). Otherwise it would not have been possible to obtain reliable results. Several reasons are conceivable for the sometimes quite high measurement errors. On the one hand, the rather small initial quantities of 1 to 100 µL may play a role. On the other hand, they are possibly due to slight deviations in the size and colouring of the

microspheres used. In the following two chapters, differences in microsphere colours within and between the Dye-Trak VII+ and Dye-Trak 'F' microsphere series are also listed.

5.2.2 Dye-Trak 'F

Simultaneous measurement of the four different fluorescent dyes of the Dye-Trak 'F' series previously extracted from the microspheres resulted in the standard curves shown in Fig. 21. The considerable differences in the peak maxima of the respective fluorescent dyes at the same concentration or number of microspheres are immediately apparent. These have already been pointed out in the previous chapter. In the comparison of all colours, the colour orange 'F' shows the highest intensities, which can also be seen in connection with the respective detection limit of each colour (see chapter 4.4.1). Otherwise, despite the measurement error already mentioned, a good correlation between the number of measured microspheres and the fluorescence intensity was found for all four colours (section 4.3.3). Unexpectedly, the following surprising observation was made in this context: In contrast to the other microspherical colours, there is no linear relationship between the number of microspheres and the intensity of the lemon 'F' colour. Instead, the course follows an exponential increase with a maximum value to which the curve approaches.

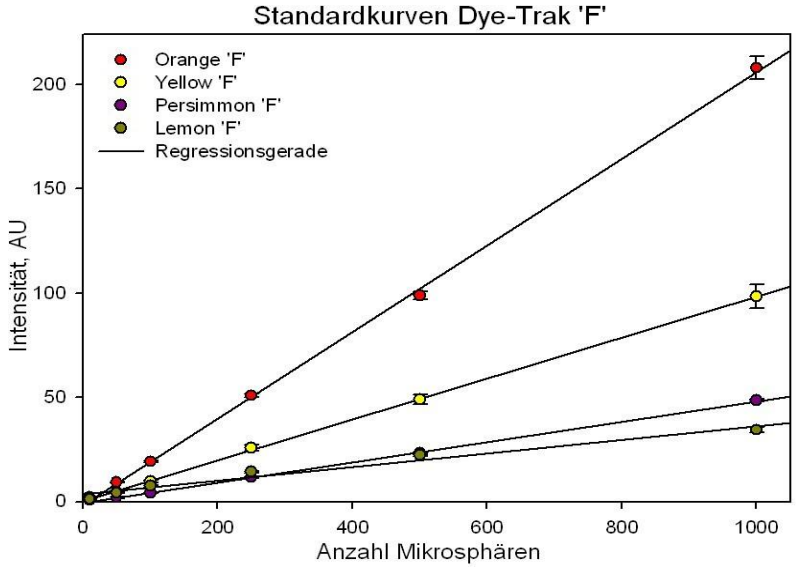


Fig. 21 Standard curves of the four different microsphere colours of the Dye-Trak 'F' series

This suggests that the measurements of the colour lemon 'F' tend to give better results at low microsphere concentrations and worse results at high microsphere concentrations. This is because the differences in the intensity maxima become smaller and smaller as the number of microspheres increases. In this context, Fig. 22 shows a comparison of the two standard curves of the Dye-Trak 'F' colours Lemon 'F' and Persimmon 'F'. This figure clearly shows the difference between the two different regression lines. It can be clearly seen that the values of the colour Lemon 'F' are comparatively higher up to the point of intersection between the exponential and the linear slope line. The point of intersection is almost exactly 450 microspheres.

As the results of the measurements to determine the respective detection limits (chapter 4.4.1) show, the difference between the colour Lemon 'F' and the other three colours of the Dye-Trak 'F' series is negligible. For this reason, it must be assumed that the above-mentioned special feature of the microsphere colour Lemon 'F' is most likely only significant at very high expected microsphere numbers. Consequently, it probably only influences the subsequent measurement result in ranges above 1000 microspheres. This in turn is based on the observation that at low microsphere concentrations the measurement error is so large that the advantage of the comparatively higher intensities of the colour Lemon 'F' is nullified.

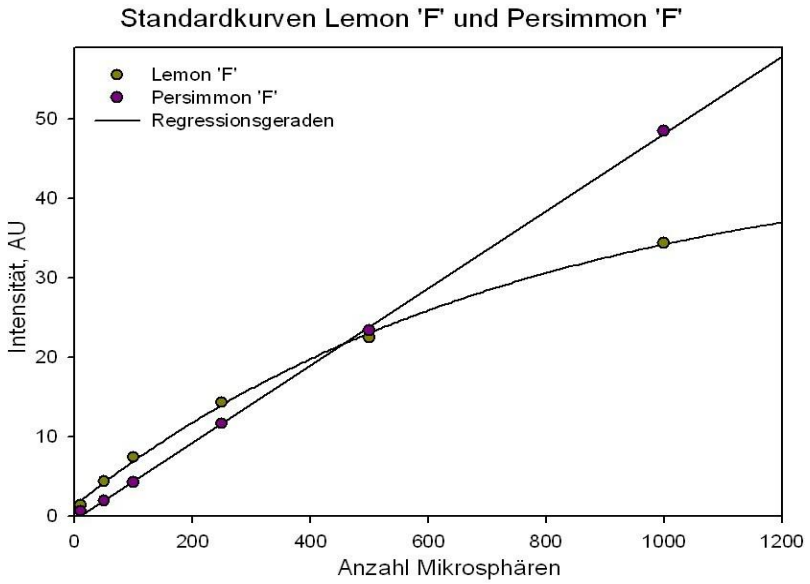


Fig. 22 Comparison of the two standard curves of the Dye-Trak 'F' colours Lemon 'F' and Persimmon 'F

5.2.3 Comparison Dye-Trak VII+ with Dye-Trak 'F'

In addition to the number of available microsphere colours, three and four respectively, further differences between the two microsphere series have emerged in the course of the work. These will now be discussed in more detail in this chapter. As already explained in chapter 2.1.2, the microspheres of the Dye-Trak VII+ series are not designated fluorescent microspheres. However, since these are also stained with fluorescent dyes, the intensity of the excitation or emission maxima can be measured not only in an absorption photometer, which is usually used, but just as well in a spectral fluorimeter. Since the sensitivity of an absorption photometer is comparatively much lower, the microspheres of the Dye-Trak VII+ series are particularly strongly coloured. Exactly this difference can be demonstrated in a direct comparison of the colours Lemon VII+ and Lemon 'F'. This is because the strength of the colouration is ultimately noticeable in a difference in the intensity maxima (Fig. 23).

The difference in the intensity maxima determined from the two curves is 40 % on average. This fact appears to be advantageous especially with low microsphere concentrations. Nevertheless, at least when determining the standard curves, no significant advantage could be drawn from this. This is probably mainly due to the fact that the difference in the intensity maxima is not large enough to allow a detection limit below 10 microspheres, as was the case when determining the standard curves for the

colour Lemon 'F' was determined. The same applies to the other two Dye-Trak VII+ colours Tangerine VII+ and Berry VII+.

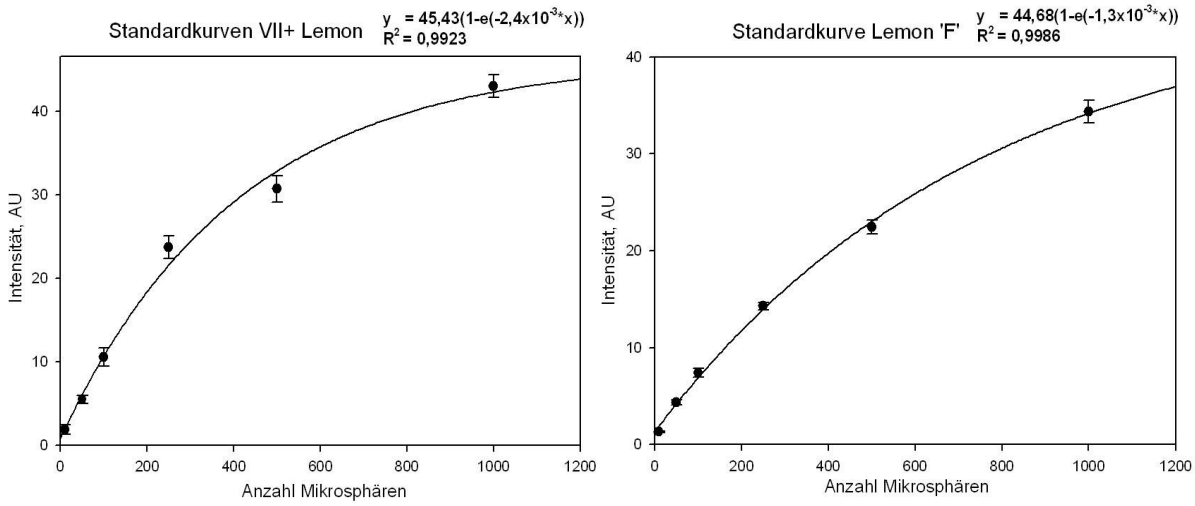


Fig. 23 Comparison of the course of the standard curves of the two colours Lemon VII+ (Dye-Trak VII+) and Lemon 'F' (Dye-Trak 'F')

5.3 Comparison of counting method with Spectrofluorophotometer measurements

5.3.1 Evaluation of the Ehrenfriedersdorf tracer test

The tests carried out have shown that reliable results can be achieved with the newly developed sample preparation. At least in the last two test setups, the results of the counting method could be confirmed throughout. The slightly lower average values can be explained by a slight loss of fluorescence in the microspheres over the long storage period. Even though the manufacturer of the microspheres (Triton Technology) only states a fluorescence loss of less than 1 % after 6 months in dark storage, a loss of several percent over a period of more than 3 years (the tracer test was carried out in 2004) seems quite conceivable. Furthermore, the average measurement error for the colour tangerine calculated from the standard curves is between 1 and 20 %. Based on these two prerequisites, a maximum deviation of 23 % (EFD-0906-15) from the results obtained by counting can be explained quite plausibly.

However, as the first two test setups clearly show, even a slight contamination of the solvent by dissolved components of the cellulose acetate filter considerably disturbs the measured result. If a peak maximum could be determined at all, it is so strongly overlaid that the measured peak height is up to 800 % higher than the expected value. Therefore, care must be taken to keep the time of contact between the filter material and the solvent as short as possible. In addition, the centrifuge tube should only be shaken lightly (low speed), as too high a speed favours the dissolving process of the filter, resulting in contamination by dissolved filter material after only two minutes. This becomes clear from the results of the second test arrangement (2 min at 250 rpm).

Furthermore, the following can be concluded from the results: Numbers of less than 5 microspheres can only be determined via the colour intensity partially and with a large

determine the accuracy of the measurements. This has already been shown in the determination of the standard curves.

5.3.2 Recovery of the microspheres from the filter networks

The tests carried out show that in almost half of the filter nets, significant numbers of microspheres remain on the filter nets despite extensive cleaning. As already mentioned in chapter 4.1.2, the following question arises for three of the seven measured intensity maxima (EFD-0906-24; EFD-0906-32 and EFD-2306-25): Can a connection be drawn between the measured peak maximum at a wavelength of 506 nm and the colour tangerine, whose emission maximum lies at about 525 nm? The conversion of the intensity values of these three samples resulted in microspheres of up to 8200 microspheres. Consequently, the question arises whether such a result can still be related to the numbers obtained by counting. Due to the enormous discrepancy between the two values (31 and 8213 microspheres), this seems unlikely. At least for sample EFD-0906-24 a possible explanation could be found in this context. The amber glass bottle in which the filter net was stored broke during transport. A resulting contamination of the filter net may explain such a high peak at 506 nm. However, this cannot be regarded as a doubtless explanation. For the other two samples, such contamination of the filter network could be ruled out. Nevertheless, they show equally high peak maxima at a wavelength of 506 nm. Ultimately, the question of the causes for the height of the three measured peak maxima remains unanswered. Presumably, however, the cause lies in a type of contamination that cannot be traced.

On the other hand, the other results of this experiment clearly suggest that a certain, small proportion of microspheres remain on the filter nets despite the complex cleaning process. Furthermore, it turned out that the microsphere numbers determined via the fluorescence intensity are mostly below the values obtained during counting. How serious the influence of the loss of microspheres and the associated change in the counting result is on the informative value of a tracer experiment cannot, however, be clearly stated with regard to the results obtained. It cannot be concluded with certainty from the results that the number of microspheres left behind is directly related to the total number of microspheres on the filter net. This is due to the small number of samples tested. To be able to clearly clarify this, a considerably larger number of filter nets to be examined would certainly be required. If such a direct correlation could be proven, it would mean the following: There is only a reduction in the number of microspheres counted, without any influence on the course of the tracer passage curve.

5.4 Development of a new method for sample preparation and evaluation

5.4.1 Influence chemicals and materials

As already discussed in detail in chapter 4.2.1, the chemicals and materials used in the treatment process partly influence the subsequent measurement result. The tests, especially with the oxalic acid used to clean the filter nets, led to the following conclusion: An influence of external influences on the measurement result can be excluded.

This can be successfully dealt with, on the one hand, by conscientious cleaning of the filter network and, on the other hand, by subsequent correction of the measured value. Consequently, the chemicals and materials used are all suitable for sample preparation and evaluation.

5.4.2 Compatibility Dye-Trak VII+ and Dye-Trak 'F'

The measurement result described in chapter 4.2.2 is not surprising, since the manufacturer, Triton Technologie, as shown in table 2, does not recommend one of the Dye-Trak VII+ colours to complement the Dye-Trak 'F' series, but the two colours Red and Crimson of the FluoSpheres series (Molecular Probes). The extent to which this recommendation also applies to the measurement settings chosen in this work could not be verified.

On the other hand, the question arose whether the three selected Dye-Trak VII+ colours could be extended to the total of seven possible colours of the series. These are the colours Yellow VII+, Orange VII+, Persimmon VII+ and Navy. Once again, the colour Navy is mainly used as a control colour. It can and should only be used as such. According to the manufacturer, they have the same emission maxima as the corresponding Dye-Trak 'F' colours. This suggests that, just as in the tests carried out, the peaks of the two colours Tangerine VII+ and Persimmon VII+ overlap. A reduction of the colour palette from seven to six colours would be the consequence. However, in order to be able to clarify this question conclusively, further experiments are needed that are coordinated with this question.

5.4.3 Trials with the filter unit

5.4.3.1 Introduction

If one looks at the results achieved in the experiments and wants to assess their significance or quality, the following parameters play a decisive role: On the one hand, the initial quantity and the associated recovery rate. On the other hand, the determined measurement error, which can be seen from the percentage deviation from the initial value. In contrast to the previous approach, in this case the measurement accuracy of the corresponding test series will not be dealt with separately in a separate chapter as before, but its significance will be considered in the overall context.

5.4.3.2 Dye-Trak VII+

If one looks at the test results of the Dye-Trak VII+ series divided according to their initial quantity (Tab. 19), then one finds the following for the tests with the mine waters from Straßberg and Peißenberg and an initial quantity of 500 and more microspheres: On the whole, they are comparable with the results of the standard curves in their recovery rate on the one hand and in their measurement error on the other. In the experiments with initial quantities of less than 500 microspheres, the individual microspheres colours (Lemon VII+, Tangerine VII + and Berry VII+) can be clearly detected in this context, but measurement errors of 35 - 65 % prevail. It can be concluded from this that the various fluorescent dyes can be reliably released from the microspheres, but the measured values are presumably strongly influenced by external influences, so that no reliable statements can be made about the exact number of microspheres.

Number of microspheres	Microsphere colour	Mine water	Peak height, AU uncorrected	Peak height, AU corrected	Deviation in %
2000	Lemon VII+	Straßberg	53,81	42,74	20,6
1000	Lemon VII+	Straßberg	44,20	24,35	44,9
500	Lemon VII+	Straßberg	18,96	15,75	16,9
200	Lemon VII+	Straßberg	29,51	9,89	66,5
100	Lemon VII+	Straßberg	30,30	8,70	71,3
20	Lemon VII+	Straßberg	9,79	3,64	62,8
2000	Lemon VII+	Peißenberg	114,75	42,75	62,7
1000	Lemon VII+	Peißenberg	69,43	29,25	57,9
500	Lemon VII+	Peißenberg	33,70	16,02	52,5
200	Lemon VII+	Peißenberg	60,26	17,42	71,1
100	Lemon VII+	Peißenberg	86,21	7,51	91,3
20	Lemon VII+	Peißenberg	na	na	na

Tab. 24 Comparison of the peak heights of the microsphere colour Lemon VII+ measured in connection with the two test series of the Dye-Trak VII+ (LTB) series (uncorrected/corrected); na = peak not determinable

The microsphere colour Lemon VII+ is particularly affected by external influences and especially by overlay effects, which are presumably caused by deposits on the filter nets. The figures in Tab. 24 clearly show that the peak of the colour Lemon VII+ was overlaid to a greater or lesser extent by a neighbouring peak in each measurement, irrespective of the initial quantity and the mine water used. On the one hand, the findings from many experiments indicate that such overlapping, which usually results in an overestimation of the lemon peak, can be corrected by suitable means. On the other hand, it can also be concluded from the figures in Tab. 22 that the correction carried out by no means leads to a usable result in every case. For this reason, it must be assumed that in the comparison of the three microsphere inks of the Dye-Trak VII+ series, the Lemon VII+ ink delivers the poorer quality results. Presumably, this is solely due to the way the sample was prepared or the selected measurement settings. This means that such an assessment should by no means be interpreted in such a way that the colour Lemon 'F' should no longer be used in future tracer tests. Instead, it seems advisable to carry out specially designed laboratory tests before each tracer experiment, on the basis of which it can subsequently be decided which microsphere colours are suitable in the respective case.

If we next look at an overview of the recovery rates of the respective tests, many of which have already been mentioned in Chapter 4.2.3.4 (Table 25), we can see the following: In the majority of the trials, the percentages are in the range of 75 to 100 %. It should be noted that all numbers above 100 % are not equally significant with a real recovery rate of over 100 %. Instead, they are probably the result of an overestimation of the measured value caused by a correspondingly large measurement error. The two relatively low values of 64 and 63 % can be explained on the one hand by a high measurement error.

explain. On the other hand, the latter value can also be seen in the background that only a part of the microsphere colours could be recovered.

Quantity Microspheres	Microsphere series	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
2000	Dye-Trak VII+	97	111
1000	Dye-Trak VII+	104	99
500	Dye-Trak VII+	102	75
200	Dye-Trak VII+	64	95
100	Dye-Trak VII+	132	115
20	Dye-Trak VII+	270	63

Tab. 25 Recovery rates in relation to the initial quantity in % of all colours of the Dye-Trak VII+ (LTB) microspheres series for both test series with the Straßberg (SBG) and Peißenberg (TSP) mine waters

If we finally look at the measurement errors of the two test series as a whole, we see the following: The measurement error of the test series with the Straßberg mine water (SBG LTB) and Peißenberg (TSP LTB) is not exactly low at 17.6 and 30.7 %. In this case, as explained in detail in chapter 4.3.4.3, the respective tests with an initial quantity of 20 microspheres are not taken into account. However, despite this, relatively reliable results can still be achieved, which are all the more accurate and reliable the higher the number of microspheres on the filter nets.

Finally, the following conclusion can be drawn: On the one hand, not only satisfactory results could be achieved in the experiments. On the other hand, at least interesting and usable findings could be drawn from most of the test results.

5.4.3.3 Dye-Trak 'F

The test results of the two test series with the microspheres of the Dye-Trak 'F' series paint a rather sobering picture. First of all, the colour Lemon 'F', as well as the colour Lemon VII+, delivers comparatively poorer quality results than the other three colours. This can again be clearly seen from the values in Tab. 22, which reflect the average deviations from the initial value of each microsphere colour of the Dye-Trak 'F' series. This is also indicated by the fact that the two values representing the two test series with the different pit waters for the colour Lemon VII+ are consistently more than 25 % higher than those of the other three microsphere colours.

If we next look at the respective recovery rates, we can see the following: On the one hand, the variance of the values is extremely high at 8 - 100 % (Tab. 26). At this point, however, it should again be pointed out that all percentages above 100 % are due to a very high individual measurement error and do not correspond to a real existing value. On the other hand, the recovery rates of the test series with Peißenberg mine water are low compared to the tests with Straßberg mine water. However, since the differences do not otherwise show a clear trend

and are also rather small in relation to the fluctuations between the individual values, no further conclusions can be drawn from this observation either.

In comparison, the following observation is of greater importance: In almost all tests with microspheres of the Dye-Trak 'F' series and an initial quantity of more than 250 microspheres, the recovery rates are very low. This is particularly important in view of the fact that this trend does not also appear in the experiments with initial quantities of 250 microspheres and below. This raises some questions for which no clear answers can be found at first glance. This becomes particularly clear if one compares the results with the microspheres of the Dye-Trak VII+ series. However, this comparison and the associated conclusions will be discussed in more detail in the following chapter.

If one also looks in this context at the measurement errors determined for the respective tests, there is a correlation between the clear differences in the recovery rates of the tests with initial quantities of more than 250 microspheres compared to those with 250 microspheres and below, and the respective measurement errors. If one assumes, as in the case of the Dye-Trak VII+ microspheres, that from initial quantities of 250 microspheres and below, external influences affect the ultimately measured peak maximum to such an extent that reliable measurement results can only be achieved to a limited extent, this would mean in this case that the corresponding peaks of the respective microsphere colours are overlaid and thus overestimated to such an extent that an actually low recovery rate is covered up and thus appears higher than is actually the case.

Quantity Microspheres	Microsphere series	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
2000	Dye-Trak 'F	35	8
1000	Dye-Trak 'F	61	34
500	Dye-Trak 'F	100	45
250/200	Dye-Trak 'F	109	62
100	Dye-Trak 'F	224	115
20	Dye-Trak 'F	111	51

Tab. 26 Recovery rates in relation to the initial quantity in % of all colours of the Dye-Trak 'F' (PYLO) microspheres series for both test series with the Straßberg (SBG) and Peißenberg (TSP) mine waters

If we look at the measurement errors of all microsphere colours of the Dye-Trak 'F' series for themselves and not in connection with the recovery rate, they are very high, both for each test individually (Tab. 21) and as a whole (Tab. 23), with 39.0 - 130.5 % and 67.8 and 78.5 % respectively. As can be seen from the last two values, the measurement error of the tests with Straßberg mine water is on average slightly below that of the tests with Peißenberg mine water. Ultimately, however, it must be consternated that even the lowest value of 39.0 % is still so high that hardly any reliable results can be expected.

The following conclusion can therefore be drawn: For reasons that cannot be understood, the results of the two test series with the microspheres of the Dye-Trak 'F' series provide only limited meaningful and, for the most part, hardly usable findings.

5.4.3.4 Comparison Dye-Trak VII+ with Dye-Trak 'F'

While the determination of the standard curves did not show any significant difference between the two microsphere series used, the tests with the filter unit, on the other hand, showed considerable differences in some cases. As already mentioned in the previous chapter, there are significant differences in the recovery rates, especially between the two microsphere series. This primarily affects the tests with output quantities above 250 microspheres. The considerably lower recovery rates in the tests with microspheres of the Dye-Trak 'F' series are surprising for the following reason: The microspheres of the Dye-Trak 'F' series have essentially the same properties as the microspheres of the Dye-Trak VII+ series. Consequently, they should actually only differ in the fluorescent colours used and the strength of the colouring. Such low recovery rates were therefore not to be expected in the run-up to the experiments, which is why it is also difficult to find a plausible explanation for them. One conceivable explanation is as follows: The microspheres of the Dye- Trak 'F' series possibly have a slightly smaller diameter on average than the 15 µm specified by the manufacturer and therefore partially fit through the meshes of the 15 µm filter net. According to the manufacturer, the microspheres of the Dye-Trak VII+ series have a diameter of 15.5±0.42 µm. This should also apply to the microspheres of the Dye-Trak 'F' series. Comparable investigations have shown that slightly larger deviations from the specified value can occur (Raab 2003). A check of the diameters of the respective microspheres would presumably have led to a clarification of this question, but could not be carried out within the framework of this work. Ultimately, however, an explanation of the low recovery rates by possible deviations in the diameter of the microspheres seems unlikely.

The observed differences between the two microsphere series also raise questions that are difficult to answer. This mainly concerns the measurement error and the recovery rate. Only the colours Lemon VII+ and Lemon 'F' are very similar in their properties. In addition, there is also a certain commonality in the fact that both microsphere series show the lower measurement errors in the tests with Straßberg mine water compared to those with Peißenberg mine water.

Finally, the following conclusion can be drawn from the comparison of the two microsphere series: Even if no obvious reasons can be found, the microspheres of the Dye-Trak VII+ series produce comparatively better and more reliable results than those of the Dye-Trak 'F' series. For this reason, it seems advisable to use only microspheres of the Dye-Trak VII+ series in tracer experiments for which the newly developed method for sample preparation and analysis is used.
-evaluation is used.

5.5 Detection limit

5.5.1 Introduction

One of the main reasons why fluorescence spectrometric analysis of microspheres in hydrogeology has so far only been used in column experiments and small-scale experiments was the previously assumed high detection limit of about 1000 microspheres/mL sample volume (Göppert 2005). One of the main aims of the laboratory experiments was therefore to reduce this detection limit as far as possible. The great advantage of the counting method using a fluorescence microscope is the detection of

only one microsphere. Comparably good detectability should also be achieved in determining the number of microspheres by measuring the colour intensity. Initial tests already suggested that it might be possible to achieve the set goal. With a slit width of 5 nm, intensities of around 2.5 AU could still be achieved in the measurements of 15 microspheres.

5.5.2 Standard curves

At least in the determination of the standard curves, it could be sustainably demonstrated that small numbers of microspheres can also be detected by measuring the fluorescence intensity, and this with only small deviations of one or two microspheres. It is true that it was not possible to detect only one single microsphere of a colour, as is the case with the counting method. Nevertheless, the detection limits of 5±1 microspheres for the colours Lemon VII+ and Orange 'F' and 10±2 microspheres for the colours Tangerine VII+, Berry VII+, Lemon 'F', Yellow 'F' and Persimmon 'F' proposed in chapter 4.4.1 can be described as quite detectable using 2 mL solvent.

5.5.3 Trials with the filter unit

Compared to the standard curves, the question of the respective detection limit cannot be answered so easily in the experiments with the filter unit. Above a certain initial quantity, not all microsphere colours could be detected. Consequently, it is not possible to speak of a uniform detection limit of about 20±4 microspheres for all the microspheres used in the Dye-Trak VII+ and 'F' series. Furthermore, there are considerably greater differences between the individual microsphere colours than assumed at the beginning.

Microsphere paint	Strasberg (SBG-Ü539-1203)	Peißenberg (purified with oxalic acid)
Lemon VII+	20	100
Tangerine VII+	20	100
Berry VII+	20	20
Lemon 'F	20	100
Yellow 'F	20	500
Orange 'F	20	20
Persimmon 'F	100	500

Tab. 27 Detection limits (in microsphere numbers) of the respective microsphere series Dye-Trak VII+ (LTB) and 'F' (PYLO) derived from the test results of the four test series carried out; divided into the respective microsphere colours and test series

Looking at the microsphere numbers listed in Tab. 27, the following conclusion can be drawn: At least for the two test series in which the mine water from Straßberg was used, relatively precise and undisputed detection limits are available. However, if one then additionally considers the determined measurement error of the test results relevant to the determination of the detection limits, then one finds the following: Example

In the case of the SBG LTB test series (Dye-Trak VII+ and Grubenwasser Straßberg), for example, the evaluation of the test with an initial quantity of 20 microspheres resulted in an average measurement error of 170 % (see Table 19). Furthermore, although the respective peaks could be determined without doubt, it should also be taken into account when finally setting the detection limits that reliable results are no longer possible with a very high measurement error. Consequently, qualitative evidence can be provided, but no statement can be made about the exact number of microspheres.

Furthermore, only a relatively small number of tests were carried out within the scope of the work. With such a high measurement error, as occurred in some of the tests, this has far-reaching consequences. It is hardly possible to make reliable statements about how reproducible the achieved results really are. This can be illustrated quite clearly by the results of a large number of tests with the same test parameters, which were carried out alongside the respective test series. The results shown in Fig. 24 show how reliable or to what fluctuations individual measurements with small initial quantities are exposed.

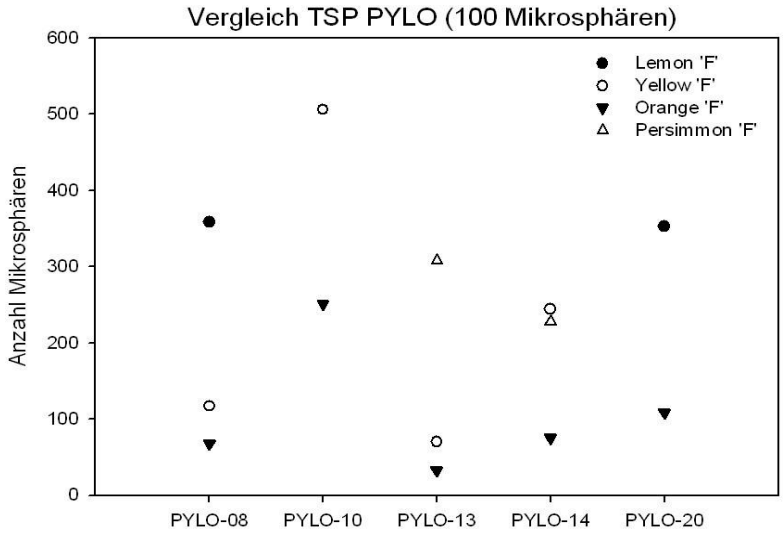


Fig. 24 Comparison of the test results of five different tests, each with the same test parameters; microspheres of the Dye-Trak 'F' series (PYLO) with an initial quantity of 100 microspheres each and Peißenberg mine water (TSP) were used.

For each test, microspheres of the colour Dye-Trak 'F' with an initial quantity of 100 microspheres each and mine water from Peißenberg were used. Furthermore, the filter nets were always cleaned afterwards with oxalic acid and then washed off with water. A total of eight identical tests were carried out, but microspheres could only be detected in five of the eight tests. The results of these five tests are shown graphically in Fig. 24. They show how large the differences between tests with the same test parameters can be.

Consequently, it seems more realistic to assume a detection limit in the range of 50 microspheres. Table 27 also clearly shows that the expected detection limit is not only dependent on the microsphere colour used, as already mentioned in chapter 4.4.2.2. The comparatively higher detection limits for the two test series with the Peißenberg mine water can be explained as follows

probably due to superposition effects and an associated increased background value. The same probably applies to the considerable measurement error (Tab. 18).

The quite clear difference between the two microsphere series in this respect is difficult to explain with the knowledge gained up to this point. To what extent there is a connection between the detection limit and the stronger colouring of the microspheres of the Dye-Trak VII+ series, which has already been discussed in detail in chapter 5.2.3, cannot be said with certainty. However, the example of the colour orange 'F' at least shows that the relationship between the number of microspheres and the height of the peak can play a significant role with regard to the detection limit. The peak of the colour orange 'F' can be reliably detected even with initial quantities of 20 microspheres, regardless of the mine water used. At the same time, it has the highest intensity maximum of all the microsphere colours used with the same number of microspheres.

In summary, it can be said that detection limits of 5 or 10 microspheres, as achieved with the standard curves, cannot be transferred to the tests with the filter unit. Depending on the pit water used and the related level of the background value and the microsphere colour used, an average detection limit of 50 - 100 microspheres can be assumed for the sample preparation and evaluation method developed in this work. In the best or worst case, detection limits of 20 or 500 microspheres can be expected.

5.6 Conclusions

Within the scope of the diploma thesis, numerous aspects were examined more closely via the evaluation of tracer experiments with microspheres. One of them is the comparison with the counting method under the fluorescence microscope, which has been used as standard so far. The question was whether the number of microspheres on the cellulose acetate filters used in the counting procedure could also be determined in a different way than under a fluorescence microscope. The experiments with samples from the tracer test carried out in 2004 in the abandoned Ehrenfriedersdorf tin ore mine (Wolkersdorfer&Hasche 2004b), which have already been discussed in detail in chapter 5.3.1, showed the following: A reliable determination of the number of microspheres is also possible by measuring the fluorescence intensity in a spectral fluorimeter.

Due to the error-proneness of this determination, which is based on contamination of the solvent (2-ethoxyethyl) acetate with the easily soluble filter material (cellulose acetate filter), the development of a new method for sample preparation and filtering was a challenge.
-evaluation represents the central part of the work carried out. In addition to numerous tests dedicated to a specific aspect, four larger test series were carried out with a filter unit of the automatic sample collector MeFiSTo (Multiple Filter Storage Tool). It turned out that there are partly considerable differences between the two used microsphere series Dye-Trak VII+ and Dye-Trak 'F' and also that the quality of the achieved measurement results depends on the properties of the mine water. A comparison of the two mine waters Straßberg and Peißenberg (Tab. 3) carried out during the tests showed that the measurement error occurring during the measurement of the triggered fluorescence colours of the respective microsphere colours and also the detection limit to be achieved can partly depend on the mine water. It should therefore be investigated in advance of a tracer test in any case using the respective mine water whether a reliable

It is not possible to determine the number of microspheres at all and how high the measurement error and the detection limit to be expected are. Based on the findings from the tests carried out, it is not possible to say with certainty whether the method developed for sample preparation and evaluation provides a reliable measurement result in every case and for every mine water.

It was also found that the tests carried out with the Dye-Trak 'F' series microspheres did not produce reliable measurement results. This applies to both series of tests carried out with the Straßberg and Peißenberg mine waters. However, since no reasons could be found that fundamentally speak against the use of this microsphere series, it seems sensible to examine its possible suitability more closely in further tests.

Probably the most valuable finding from all the tests carried out, however, is that the tests with the three Dye-Trak VII+ microspheres and the Straßberg and Peißenberg mine water provided reliable results. It can thus be said, albeit with certain limitations, that the newly developed method for sample preparation and
-evaluation has proven to have the potential to be a reliable alternative to the previous evaluation with the counting method in the future.

Finally, it should be noted that the method for sample preparation and evaluation developed in the context of this diploma thesis still has a susceptibility to errors that is not easy to control and therefore certainly still requires some checks and improvements.

What is certain, however, is that the time required for sample preparation and
-evaluation could be considerably reduced compared to the counting method. The preparation and evaluation of the samples alone takes about 15 - 30 minutes per sample. However, since several samples can always be centrifuged at the same time and a single measuring process in the spectrofluorophotometer only takes about one minute, this time is only an approximate guide value. Depending on the number of samples to be analysed, about 2 - 10 samples can be prepared and evaluated per hour.

5.7 Outlook

Even though it has been shown that the newly developed method for sample preparation and evaluation can deliver reliable results, there are, as already mentioned, still some aspects of the sample preparation process that can be improved. On the one hand, the cleaning with oxalic acid, which would still have to be carried out on site, i.e. in the mine, poses a certain problem in a tracer test. Therefore, the aim of further investigations should be to change the sample preparation process so that cleaning the filter network with oxalic acid is possible even after it has been removed. Such a measure could presumably also achieve complete cleaning of the filter networks from disturbing deposits, which has not always been possible up to now. This would presumably reduce the measurement error, which has been quite high in some cases, considerably. Whether this would also lead to a further reduction in the detection limit, on the other hand, is not so easy to predict. However, the lower limit, as achieved in the determination of the standard curves, should lie at a number of about 5 - 10 microspheres.

The extent to which the sample processing unit (SPU) developed by Raab (2003) for medical purposes is suitable for the improvements discussed in the previous paragraph could not be investigated in the context of this diploma thesis, but it is still possible to use the SPU.

this could also be the subject of further investigations. In addition to improving sample preparation, it could also be investigated to what extent other microsphere colours can be used alternatively or additionally. With the Fluo- Spheres (Molecular Probes) series, a range of up to 13 fluorescent colours is available, which means that up to 13 different tracer input points could potentially be distinguished from each other in a multitracer test using fluorescent microspheres alone.

Furthermore, an application in field tests in normal aquifers is also conceivable, if an on-line measurement with the particle counter developed by Niehren (1999) does not seem possible or advisable. It can be seen, therefore, that the investigations carried out do not so much represent the limits of an evaluation of tracer experiments with microspheres. Instead, they can hopefully contribute to the fact that the potential of an evaluation with the spectral fluorimeter, which has only been used to a small extent so far, will be exploited to a greater extent in the future and thus be used in an improved form in the future.

6 Lit erature

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