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A simple and inexpensive technique for assessing contamination during drilling operations

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Abstract

Subsurface exploration relies on drilling. Normally drilling requires a drilling fluid that will infiltrate into the drill core. Drilling fluid contains non-indigenous materials and microbes from the surface, so its presence renders a sample unsuitable for microbiological and many other analyses. Because infiltration cannot be avoided, it is of paramount importance to assess the degree of contamination to identify uncontaminated samples for geomicrobiological investigations. To do this, usually a tracer is mixed into the drilling fluid. In past drilling operations a variety of tracers have been used, each has specific strengths and weaknesses. For microspheres the main problem was the high price, which limited their use to spot checks or drilling operations that require only small amounts of drilling fluid. Here, we present a modified microsphere tracer approach that uses an aqueous fluorescent pigment dispersion with a similar concentration of fluorescent particles as previously used microsphere tracers. However, it costs four orders of magnitude less, allowing for a more liberal use even in large operations. Its applicability for deep drilling campaigns was successfully tested during two drilling campaigns of the International Continental Drilling Program (ICDP) at Lake Towuti, Sulawesi, Indonesia, and Lake Chalco, Mexico. Quantification of the tracer requires only a fluorescence microscope or a flow cytometer. The latter allowing for high-resolution data to be obtained directly on-site within minutes and with minimal effort, decreasing sample processing times substantially relative to traditional tracer methods. This approach offers an inexpensive, rapid, but powerful alternative technique for contamination assessment during drilling campaigns.

Exploration of deep subsurface environments relies on drilling, which requires the use of drilling fluid for cooling the bit and transporting cuttings out of the borehole (Kallmeyer et al. 2006; Kallmeyer 2011). This process inevitably causes infiltration of drilling fluid into the core, either by advection through the pore space of coarser and looser sediments or by being pushed through small fissures that can be created by drilling more fine-grained and more cohesive or brittle sediments (Lever et al. 2006). While drill fluid contamination is problematic for many analyses, it poses particular challenges for geomicrobiological studies. Microbial cell abundances in the subsurface are several orders of magnitude lower than those of surface environments (e.g., Kallmeyer et al. 2012; D'Hondt et al. 2015). Thus, even the slightest infiltration of drilling fluid into the sediment (nanoliters per

cm³ sediment) renders the sample unsuitable for microbiological and also certain geochemical investigations (Yanagawa et al. 2013). Therefore, it is essential to trace contamination of the sediment core to identify uncontaminated samples for geomicrobiological investigations.

To attribute the detected tracer to the infiltration of drilling fluid into the sample it is necessary that tracers (1) have no natural source, (2) are easy to detect even at extremely low concentrations, (3) are chemically inert.

Several techniques have been used in past drilling operations to assess microbial contamination, including fluorescent dyes (Phelps et al. 1989; Russell et al. 1992; Pellizzari et al. 2013), Perfluorocarbon tracers (PFT) (Senum and Dietz 1991; Colwell et al. 1992; Russell et al. 1992; Smith et al. 2000*b*; House et al. 2003; Lever et al. 2006) and microsphere tracers (Colwell et al. 1992; Smith et al. 2000*b*; Kallmeyer et al. 2006; Yanagawa et al. 2013). Fluorescent dyes like fluorescein or rhodamine are inexpensive, easy to handle on-site and allow sensitive detection of contamination (Russell et al. 1992). However, they are sensitive to light degradation (Diehl and Horchak-Morris 1987), pH and water chemistry

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(Smart and Laidlaw 1977; Krause et al. 2005). Furthermore, significant sorption onto clays, which are a common component of drilling fluids, and the presence of humic substances, can decrease the fluorescence signal (Magal et al. 2008; Hafuka et al. 2015). These features limit the applicability of fluorescent dyes in deep drilling campaigns. Moreover, fluorescent dyes will stain the entire drilling fluid in a bright color, which might cause problems for disposal of the mud after drilling.

PFT are chemically inert hydrophobic compounds that can be detected with high sensitivity (picogram per gram) via gas chromatography (Smith et al. 2000*a*). Detection of PFTs thus requires a gas chromatograph on-site to identify uncontaminated samples for immediate biogeochemical and geomicrobiological analyses that have to be carried out on fresh sample material. While this is a minor issue on large oceanographic vessels (PFT is currently the standard contamination control method during IODP drilling expeditions), it poses challenges for operations at remote locations or on smaller drilling platforms. Also, samples have to be taken within a few hours after core retrieval as the volatile tracer diffuses into the interior of the core making it impossible to distinguish between drilling induced contamination and contamination by diffusion (Smith et al. 2000*b*; Lever et al. 2006). Also, care has to be taken during sampling of core material. The tracer may volatilize from the outermost part of the core resulting in significant background concentrations in the air of the laboratory. If core material is sampled for contamination control in an environment with high PFT concentrations in the air, measurements in the low concentration range can no longer be trusted.

Microsphere tracers are small (0.2–0.5 μm diameter) fluorescent particles and have been used in both terrestrial and ocean drilling campaigns (Colwell et al. 1992; Smith et al. 2000*b*; Kallmeyer et al. 2006; Yanagawa et al. 2013). They are either mixed directly into the drilling fluid, or filled into a small plastic bag that is taped on the lower end of the core barrel. The bag bursts open when the core barrel hits the sediment and the released microspheres are supposed to mix locally with the drill mud and surround the drill core while entering the core barrel. It has been shown, however, that the distribution of tracer derived from these bags is rather uneven (Biddle et al. 2014) so the results have to be interpreted with caution. For analysis the microspheres are either extracted from the sediment by density centrifugation (Kallmeyer et al. 2006) or the sample slurry is counted directly, similar to Acridine Orange Direct Counting (AODC) for microbial cells (Fry 1988; Cragg et al. 1990). Irrespective of preparation, the sample is transferred onto a filter membrane and counted by fluorescence microscopy, with which a detection limit of 10^3 particles g^{-1} of sediment can be achieved (Colwell et al. 1992). However, microsphere tracers are exceptionally expensive, costing tens of dollars per milliliter, which renders them unsuitable for drilling campaigns that use large amounts of drilling fluid and therefore would require liters to tens of liters of microsphere solution. Also, microsphere quantification by fluorescence microscopy is rather time consuming.

Here, we present an inexpensive contamination control approach based upon fluorescent pigments that are normally used for paints or plastics. Their physical properties are similar to those of microsphere tracers but their price is nearly four orders of magnitude lower. Quantification of contamination can be achieved by fluorescence microscopy or by flow cytometry, the latter allowing for more rapid analysis. This study thus presents an inexpensive and simple contamination control approach that only requires a minimum of equipment and that offers a powerful technique to assess microbial contamination during terrestrial and marine drilling campaigns.

Materials and procedures

Tracer properties

As a tracer we used SPL-N fine grind fluorescent pigment dispersion (DayGlo, Cleveland, OH) with a pigment content of ca. 45%. The pigments are available in several colors and normally used for coatings, plastics and water-based inks. According to the data sheet provided by the supplier, the dispersion contains no organic solvents and is free from heavy metals, its specific gravity is 1–1.1 g/mL and viscosity ranges between 50 and 300 cP at 25°C. In contrast to normal microspheres, which have a very narrow size range and usually come in very precisely controlled concentrations, the size of fluorescent pigment particles ranges between 0.25 and 0.45 μm and the concentration may also vary by several percent. Given the normal size range of deep subsurface microbes (Kallmeyer et al. 2012), the pigments cover the natural range quite well. The few percent variability in tracer concentration is within the usual level of accuracy of our detection techniques. In Europe these pigment suspensions are supplied through Radiant Color NV, Houthalen, Belgium, and labeled RADGLO AFN. The pigment concentration is about 1×10^{15} particles per liter in undiluted form, which is in the same range as common microsphere tracers.

Drilling

The applicability of the pigment tracer for large drilling campaigns was tested during two International Continental Drilling Program (ICDP) Deep Drilling campaigns at Lake Towuti and Lake Chalco.

Lake Towuti: In June/July 2015, a 114 m long sediment core was retrieved for geomicrobiological investigations from Lake Towuti, located in Sulawesi, Indonesia (Russell et al. 2016). DOSECC Exploration Services carried out drilling operations using the ICDP Deep Lakes Drilling System (DLDS 2010). The borehole was drilled with specialized lake drilling tools. Cores were collected in standard IODP-style butyrate liners (66 mm core diameter) in 3 m intervals using P size
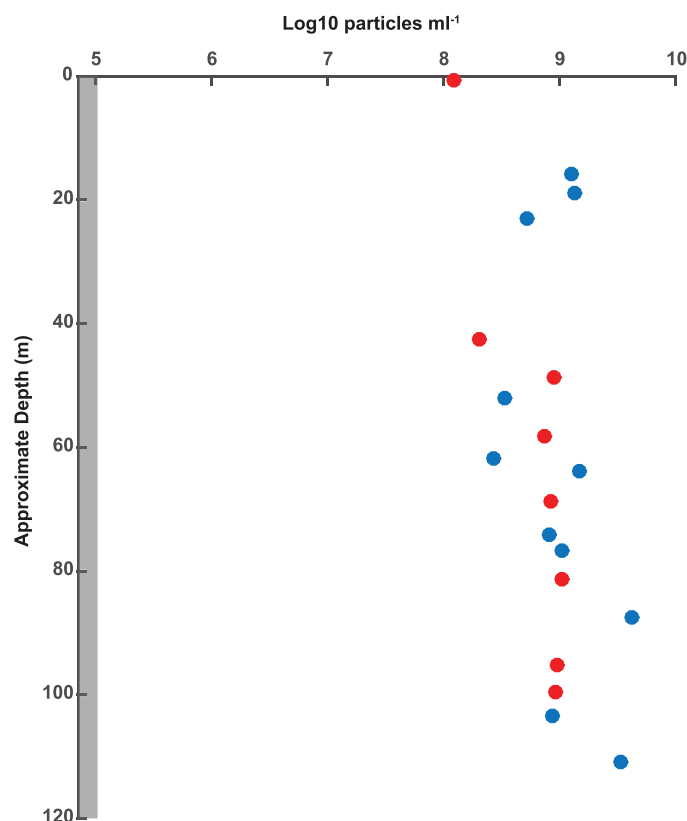
Fig. 1. Pigment particle concentrations per milliliter of liner fluid (blue circles) and drill fluid samples (red circles) from the Lake Towuti drilling. Concentrations are within one order of magnitude. The limit of detection (LOD) is indicated in dark grey.

Fig. 2. Tracer concentration in the drilling fluid over the course of the ICDP Lake Chalco drilling. The samples were taken directly from the gap between core and liner, i.e., as so-called liner fluid. Despite daily addition of tracer the concentration decreased over the course of the drilling operations.

drill rods (122.6 mm borehole diameter) (Russell et al. 2016). Hydraulic piston coring (HPC) was applied for soft unconsolidated sediment, and the rotating Alien corer (ALN) was used for more resistant layers such as lithified tephras. Surface Lake Towuti water was used as drilling fluid without any additives. We used a blue tracer with a green fluorescence under blue excitation (DayGlo SPL-19N; RADGLO AFN-29), The drilling fluid was stored in a 4000 L holding tank in which the pigment tracer was diluted 1 : 1000 to a final concentration of approximately 1×10^9 particles per mL drilling fluid, which colored the drilling fluid bright blue. The holding tank had an internal mixing system to ensure homogenous dispersion of any drill fluid additives. The flow rate of the drilling fluid was highly variable, ranging from tens to hundreds of liters per minute depending on the lithology. Drilling fluid was not recirculated and flowed out of the borehole on the lake floor into the lake. Drill fluid samples from the holding tank were collected and analyzed in regular intervals to ensure homogenous particle concentrations over the course of drilling. Also, a fluid sample from the gap between the drill core and the liner, i.e., the so-called liner fluid, was collected and the particles quantified
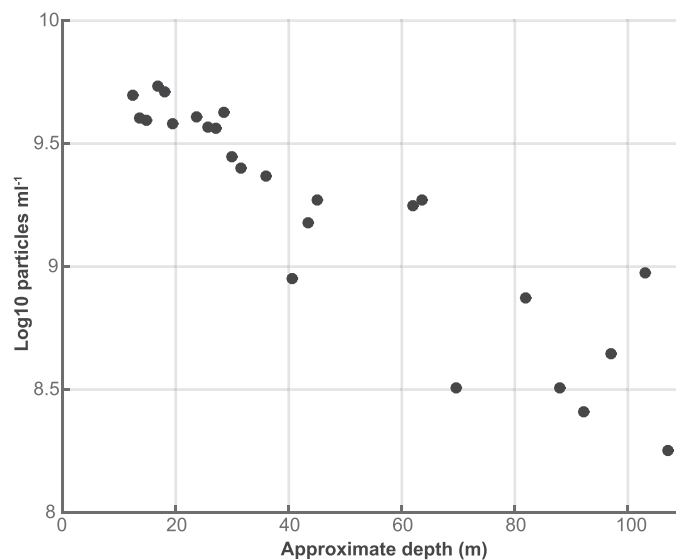
(Fig. 1). Particle concentrations of these samples were about 10^9 particles per milliliter and fluctuated within one order of magnitude.

Additionally, small plastic bags with undiluted tracer were attached to the bottom of the core to increase the tracer concentration right at the bottom of the drill hole. However, several of the bags did not burst and were pushed through the liner by the entering sediment. Only in two instances the liner fluid showed significantly higher (>1 order of magnitude) particle concentrations than the drill mud (87 and 111 m, Fig. 1). We interpret this as a general failure of the tracer bags at the bottom of the core. Only in two instances did this technique lead to a significantly higher particle concentration around the core.

Lake Chalco: Drilling of the ICDP Mexidrill drilling project at Lake Chalco was carried out with a track mounted diamond wireline drill rig. The cores were collected in plastic liners in 1.5 m intervals using H size (~ 96.3 mm diameter hole, 61.1 mm diameter core) drilling tools. The drilling fluid was recycled and topped up when necessary. The core reached a maximum depth of 500 m but only the upper 100 m were investigated for geomicrobiological and biogeochemical purposes. In the upper 100 m the drillers were using minimal rotation in an effort to maximize recovery of the relatively soft sediments. Field observations indicated good recovery of undisturbed sediment. However, upon subsequent splitting and initial description of cores, significant disturbances—interpreted as drilling artifacts—were frequently observed in core sections from this upper sequence.

A whitish tracer with light blue fluorescence under UV excitation (DayGlo SPL-594N; RADGLO AFN-09) was mixed into the mud holding system. Due to the agricultural/urban setting of the drill site, this tracer was chosen because it did not add any coloration to the drilling fluid. It would not have been possible to use and later dispose brightly colored drilling fluid, despite being non-toxic and environmentally harmless. After the initial amendment of the drill mud with tracer (dilution ca. 1 : 1000, ca. 1×10^9 particles per mL drilling fluid), about half of the initial amount of fresh tracer was added daily to make up for the loss of particles due to continual separation and removal of cuttings from the drill mud or to loss of drilling fluid into permeable sedimentary layers. As no fluorescence microscope or flow cytometer was available on site, this addition was based on the experiences of Kallmeyer et al. (2006). Upon later analysis, we found that the tracer addition was not completely sufficient as the tracer concentration dropped by over one order of magnitude over the course of the drilling (Fig. 2).

Sample collection and particle quantification

Immediately after core retrieval liner fluid was collected either from a fresh cut between sections or from the bottom of the core. At Lake Towuti the sediment core was cut into two subsections of 1.5 m length whereas at Lake Chalco the core was recovered in 1.5 m intervals. Unfortunately at Lake Towuti it was only possible to obtain 11 liner fluid samples from a total number of 61 core sections, due to gas expansion in the sediment caused by pressure loss during retrieval, which pushed out any remaining fluid and completely filled out the liner. Additionally, samples of the drilling fluid were collected in regular intervals from the holding tank. At Lake Chalco liner fluid could be collected from every core.

Samples for contamination control were either taken from whole round cores (WRC, Lake Towuti) or from the inside of the core just above the break between the shoe and the liner (Lake Chalco). In either case a few mm of the cut core surface was scraped off with a sterile scalpel and sediment samples of 1 and 2 cm^3 for the Towuti and Chalco cores, respectively, were retrieved with a sterile cutoff syringe from the rim, the center and an intermediate position of the core. The syringes were inserted by hand into the sediment perpendicular to the cut surface and the subsamples were transferred into a 15 mL Falcon tube containing 9 and 8 mL of MilliQ water for Lake Towuti and Lake Chalco, respectively, followed by homogenization.

For initial analysis by fluorescence microscopy, 100 μL of the sediment slurry from the Towuti drilling were diluted 1 : 100 with MilliQ water and filtered through a white 0.2 μm polycarbonate track-etched membrane filter (Ø 25 mm, Cyclopore, Whatman International Ltd., Maidstone, England). For the Chalco samples it was necessary to disperse pigment-particle aggregates prior to analysis. Therefore, 30 μL of the sediment slurry were mixed with 2670 μL MilliQ water and 150 μL each of methanol and Detergent Mix (36.8 g L^{-1} Na$_2$EDTA $\times$ 2 H$_2$O, 22.3 g L^{-1} Na-pyrophosphate $\times$ 10 H$_2$O and 5 mL TWEEN 80). This suspension was sonicated 1×10 s and 1×5 s with a 30 s cooling pause in between at 90% amplitude with a Bandelin MS73 ultrasonic probe (Bandelin electronic, Berlin, Germany). 500μL of this mixture was filtered through a white 0.2 μm polycarbonate track-etched membrane filter. Filter towers were cleaned with MilliQ after each filtration to avoid cross contamination between samples. Filters were embedded in glycerol, diluted 1 : 2 with MilliQ water, with 10 μL of this solution on both the microscope slide and cover slip. On each filter a total of 200 fields of view were counted on a Leica DM2000 fluorescence microscope using non-fluorescent immersion oil (Leica type F oil, $n_e^{23} = 1.518$, $v_e = 46$) and 63$\times$ or 100$\times$ objectives (Leica Plan Apo), depending on the amount of minerals on the filter. For enumeration of the tracer used at Lake Towuti, we employed a blue filter set (Leica Filter Cube F1/RH, excitation 490/15; 560/25 nm, dichromatic mirror 500; 580 nm, suppression 525/20; 605/30 nm). For the Lake Chalco tracer a UV filter set (Leica Filter Cube A, excitation BP340-380 nm, dichromatic mirror 400 nm, suppression LP425 nm) was used.

Quantification via flow cytometry

To expedite sample processing, we combined the microscopy approach with flow cytometry. For Lake Towuti samples we used a portable cytometer (BD Accuri C6, BD Biosciences, San Jose, CA), which is small and rugged enough to be taken into the field, enabling automatic and rapid determination of contamination directly on site. For each analysis 100 μL of sediment slurry or drilling fluid was diluted 1 : 100 in MilliQ water and the slurry fed into the cytometer by a peristaltic pump at a constant flow rate of 35 μL/min. MilliQ water was used as sheath fluid. A blue 488 nm laser and fluorescence detectors with optical filters at 533/30 nm and 585/40 nm were used. For the 533/30 nm filter a threshold was set to 900 to remove background noise events that are not associated with sediment or tracer particles.

For the Lake Chalco samples we used a FACS Aria III (BD Biosciences, San Jose, CA) instead of the Accuri C6 because the excitation wavelength of the tracer is in the UV range and therefore outside the range of the red and blue lasers of the Accuri. For analysis, 1 mL of the remaining slurries from the microscopy filter preparation was transferred into round bottom tubes through a 40 μm cell strainer cap to remove larger particles which may clog the flow cell. The sample was fed to the flow cell by air pressure at a flow rate of ca. 20 μL/min. The exact flow rates for each sample were determined from the mass difference of the sample tubes before and after each measurement. BD facs flow (BD Biosciences, San Jose, CA) was used as sheath fluid. A UV 375 nm laser and fluorescence detectors with optical filters at 510/50 nm and
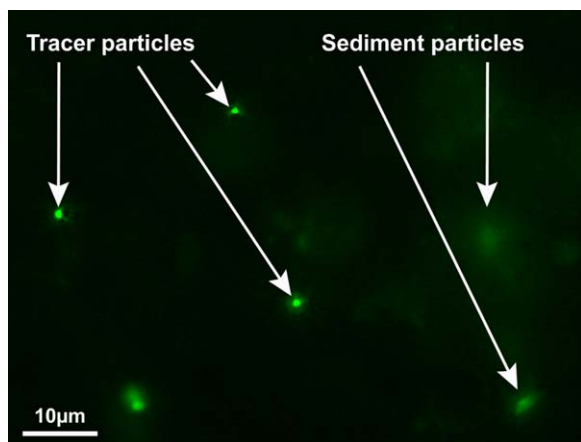
Fig. 3. Microscopic image of pigment particles (DayGlo SPL-19N) in a contaminated sediment slurry containing ca. 1×10^8 pigment particles per cm^3 sediment.

450/40 nm were used. For the 510/50 nm filter a threshold was set to 300. These parameters allowed the instrument to ignore background noise events.

Assessment

Before applying the approach in the field, several tests were conducted to assess fluorescence stability of the pigment tracer under a wide range of parameters. First, we tested whether tracer particles (DayGlo SPL-19N) remain detectable when they are mixed into a sediment slurry. A 1 : 10 diluted sediment slurry containing 1 cm^3 sediment from Lake Towuti was spiked with a tracer particle concentration of ca. 1×10^8 particles per milliliter and 10 μL of this mixture was diluted in 1 mL MilliQ water and transferred onto a white 0.2 μm polycarbonate track-etched membrane filter.

Figure 3 shows a microscopic image of this filter and illustrates that the tracer particles do not attach to sediment particles and can easily be quantified. This is also important for analysis with flow cytometers, where aggregates of sediment and tracer particles might not be clearly identifiable. Particle quantification by fluorescence microscopy could reproduce the known particle concentration.

Occasionally, scientific drilling operations are carried out in acidic, alkaline or saline environments. To determine the applicability of the pigment tracer in these environments we tested the tracer stability under extreme pH and salinity conditions. We prepared three solutions:

1. A 0.1 M HCl solution with a pH of 1.
2. A 0.1 M NaOH solution with a pH of 13.
3. A saturated NaCl solution.

Each of the three solutions was spiked with a tracer particle concentration of 10^6 particles per milliliter. After two hours, the particle concentration was quantified. Additionally, we
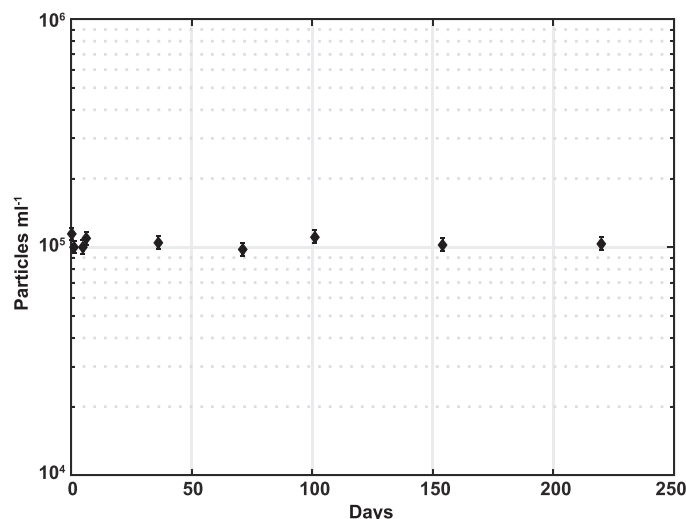

Fig. 4. Particle concentrations of a pigment tracer solution with a known concentration of 1×10^5 particles per mL over a time span of 220 days. The solution was stored under ambient light conditions over the whole period.

repeated the pH experiments with a tracer-contaminated sediment slurry to examine changes in adsorption behavior of tracer particles on sediment particles. Slurries were brought to pH 1 and 13, respectively, stored for twenty hours and particles were quantified. In all experiments the fluorescence intensity of the tracer particles remained unchanged and microscopic counts could reproduce the known particle concentration showing that the tracer is applicable over a wide range of pH or salinity conditions.

Photodegradation of particles

Considering the common applications of the pigment particles, we expected them to withstand even excessive exposure to sunlight. To verify this, we performed a long-term experiment by preparing an aqueous suspension of DayGlo SPL-19N with a final concentration of $\sim 10^5$ particles per mL. The suspension was stored in a plastic vial on a window sill and exposed to natural sunlight as well as fluorescent lighting from the room lights. Particles were repeatedly quantified over a period of 220 days. Figure 4 shows that the particle concentration did not decrease over this time period. Hence, this tracer remains usable even several months after initial core retrieval, which is a major advantage over fluorescent dyes and PFT.

Thermal degradation of pigment particles

If drilling operations are carried out in areas of hydrothermal or volcanic activity the drilling fluid can heat up extensively during drilling operations. Yanagawa et al. (2013) showed that microsphere particles are thermally degraded under those high-temperature conditions. To address this issue, we prepared an aqueous suspension of DayGlo SPL-19N containing 1×10^6 particles per mL and autoclaved it
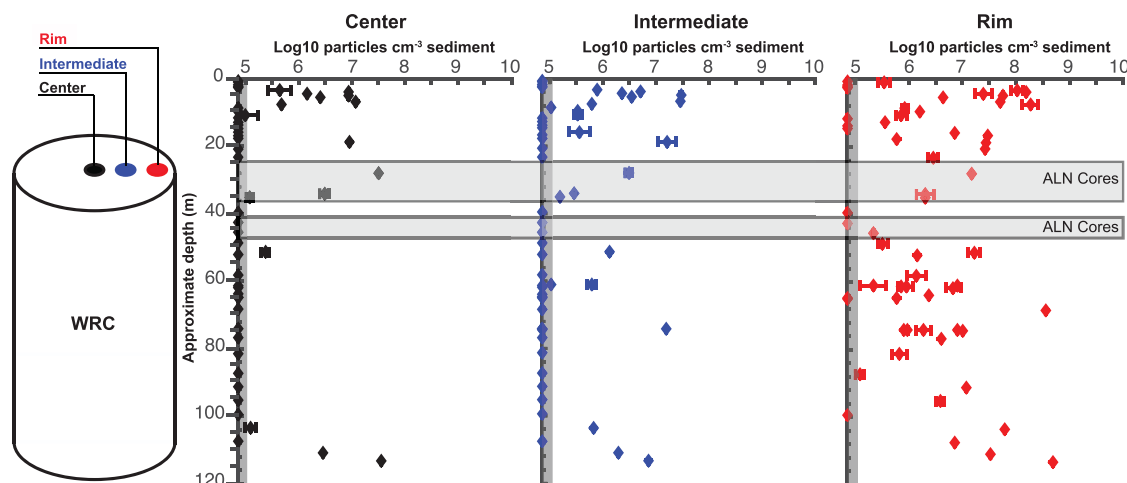
Fig. 5. Particle concentrations per cm^3 of sediment samples from the rim, the center and the intermediate position of whole round cores (WRC) of the Towuti drilling (DayGlo SPL-19N). Counts are based on fluorescence microscopy and were carried out in duplicates. Error bars represent one standard deviation. Cores retrieved using the ALN tool as well as the limit of detection (LOD) of the particle enumeration method, are indicated in light and dark grey, respectively.

for 25 min at 125°C and 3 bar (Certoclav Essential 12l, Traun, Austria). No fluorescence could be observed by microscopic analysis, so autoclaving led to a complete degradation of all particles. Therefore, like regular microsphere tracers, our pigment tracer is not applicable for high-temperature drilling operations. As we did not perform a thermal gradient experiment we cannot provide any data on the maximum temperature to which the tracer can be used.

Applicability for drilling campaigns

To test the applicability of the approach we determined particle concentrations at the rim, at an intermediate position and at the center of each WRC from the Lake Towuti drilling. Almost all samples from the rim contained particles as they were in direct contact with the drilling fluid that flows through the gap between the sediment core and the liner (Fig. 5). The number of particles and therefore the infiltration of drilling fluid decreased significantly toward the center of the core, which is in agreement with previous studies (Smith et al. 2000*b*; Lever et al. 2006).

In two WRC (at 28 and 34 m depth, Fig. 5) particle concentrations from the interior of the core exceeded those of the rim. The cores from which these WRC were retrieved were drilled with the ALN tool, where a rotating drill bit cuts the core. This tool is well known to cause disturbances in soft and/or semi-consolidated sediment due to excessive shearing (e.g., Glombitza et al. 2013). Particle concentrations in these samples thus indicate that the sediment was homogenized and reconstituted in a mixture of drilling fluid and sediment, rendering those samples unsuitable for geomicrobiological investigations. It should be noted that the sediment still appeared solid and unaffected upon first inspection. Only

during careful sectioning it became obvious that the original sedimentary structure was completely destroyed (Fig. 6).

Also, the upper 20 m of the core appear to be generally more contaminated than the lower parts, despite the use of the HPC tool. We presume that the softer sediment in this depth interval is more susceptible to infiltration than the deeper layers.

According to Kallmeyer et al. (2008) the limit of detection (LOD) can be determined using the following equation Eq. 1:

$$n = -\frac{T_{fov}}{C_{fov}} \ln(1-p) \qquad (1)$$

where n is the number of particles, C_{fov} is the number of fields counted, T_{fov} is the total number of fields on the filter and p is the confidence level to detect at least one particle. When using a 63× objective the T_{fov} of a 25 mm filter is 7854. When counting 200 fields of view n is 117, meaning that 117 particles are required on the filter to detect at least one particle with a 95% probability. For a sample containing 1×10^{-3} cm^3 sediment the corresponding detection limit is $\sim 10^5$ particles per cm^3 of sediment.

Infiltration of drilling fluid

It is possible to calculate the amount of liner fluid infiltrating into a sample if the particle concentration in the corresponding drilling fluid is known. The LOD can be calculated by Eq. 2:

$$LOD = \frac{n}{V_{sed} * C_{LF}} \qquad (2)$$

where *n* is the number of particles required in a sample to be able to detect at least one particle with a probability of 95%
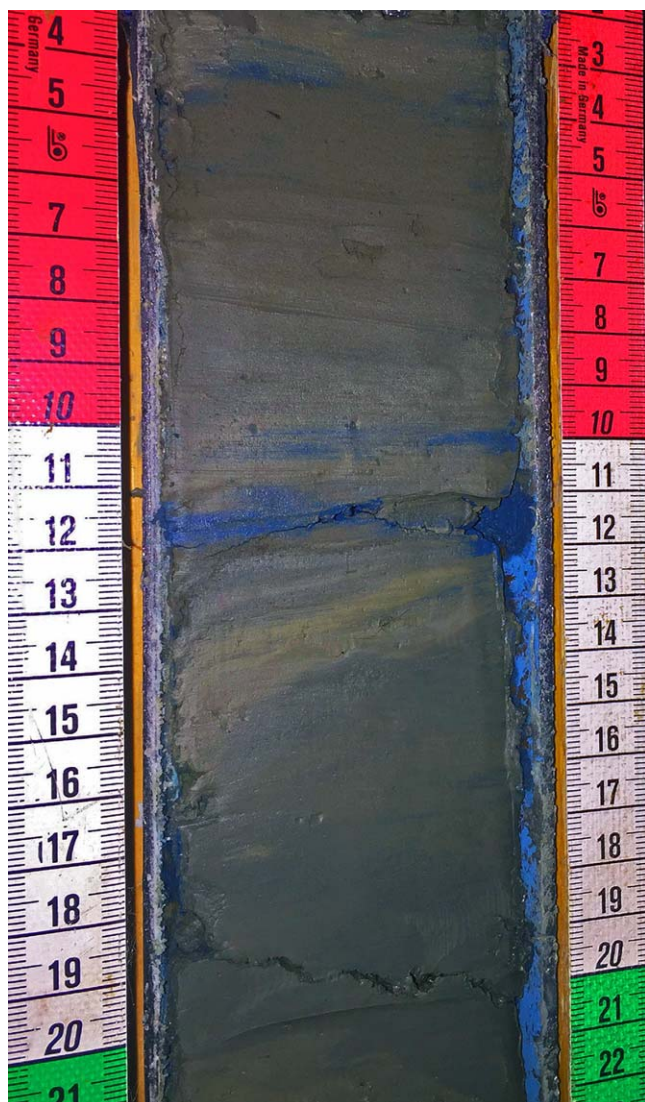
Fig. 6. Highly contaminated ALN core. The sediment structure was completely destroyed in the process of drilling, producing cracks and fissures and allowing drilling fluid to infiltrate deeply into the sediment core. Pressure during coring solidified the mixed up material again, producing sedimentary structures that at first glance appear to be original features.

(see Eq. 1), V_{sed} is the volume of sediment on the filter (cm^3) and C_{LF} is the particle concentration in the drilling fluid at the time the respective core was retrieved. Assuming that the average particle concentration was 1×10^9 particles per mL of drilling fluid (Fig. 1), the calculated LOD results in ca. 117 nL drilling fluid per cm^3 sediment (Fig. 7) if one particle was found in 200 fields of view. This is in the range of already established techniques like PFTs or microsphere tracers (Smith et al. 2000*a*; Kallmeyer et al. 2006). For the Lake Towuti drilling, surface lake water with a cell concentration of ca. 10^6 cells per mL was used as the drilling fluid, resulting in a LOD of ca. 117 nonindigenous cells per cm^3 sediment (Fig. 7).

For Lake Chalco particle quantification by fluorescence microscopy revealed severe contamination of the samples taken from the upper $\sim$ 100 m of the drill core (Fig. 8). Almost all samples from the center of the drilled core contained high tracer concentrations of up to 10^9 particles per cm^3 sediment, which in some cases exceeded particle concentrations of the rim or intermediate samples (Fig. 8). Contamination control of the Lake Chalco drill core thus revealed that the majority of samples are not suitable for geomicrobiological analysis. We note that all samples were taken from ends of core sections, which are more susceptible to contamination of drilling fluids than material within core sections. However sampling such material was not possible as there was not a dedicated hole for geomicrobiological sampling.

Quantification of pigment particles by flow cytometry

To determine the applicability of the flow cytometry approach we prepared three samples for an initial test:

1. A sediment-free tracer solution containing 1×10^6 pigment particles per mL.
2. A tracer-free blank containing 1×10^{-3} cm^3 sediment per mL of MilliQ water.
3. A contaminated sediment slurry (1×10^{-3} cm^3 sediment per mL of slurry) with a particle concentration of ca. 1.5×10^7 and 8×10^7 particles per cm^3 for the blue tracer (SPL-19N) and UV tracer (SPL-594N), respectively.

After analyzing the sediment-free tracer solution of the blue tracer (SPL-19N) it was possible to define the tracer particle populations (Fig. 9A). Analysis of the tracer-free sediment suspension of Lake Towuti sediment showed that the sediment particles plot as a distinctly different population outside this group (Fig. 9B). The contaminated slurry shows good separation of sediment and tracer particles (Fig. 9C). We then quantified the events within the tracer group.

For the UV tracer (SPL-594N) the same experiment was performed with sediment samples from Lake Chalco. Here, the sediment particles were plotting in a much wider distribution (Fig. 10B), most probably due to the fact that some minerals are fluorescent under UV light. Nevertheless, the dotplot of the contaminated slurry shows that the tracer particle population is separated from the sediment particle population (Fig. 10C).

To test if the gates were set correctly and the populations only contain sediment or tracer particles, respectively, we performed a particle sorting of the contaminated slurries using a BD FACSAria III cell sorter (BD Biosciences, San Jose, CA). For the SPL-19N tracer the optical parameters were very similar to those used on the Accuri (excitation laser wavelength 488 nm, optical filters 616/23 nm and 530/30 nm, threshold 200 for 530/30 nm), for the SPL-594N tracer the parameters are given in the Materials and Procedures section.

The dotplots of the Towuti samples were very similar to the ones from the Accuri and the gates were set accordingly. Two
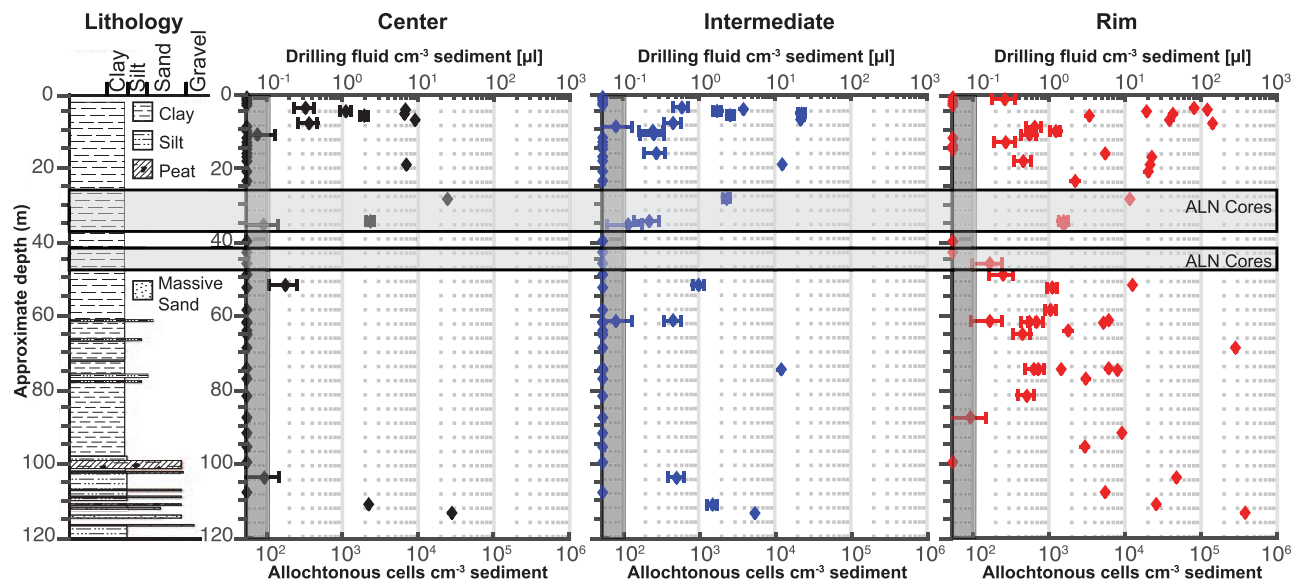
Fig. 7. Simplified stratigraphy of the drilled Lake Towuti core (Russell et al. 2016) and amount of drilling fluid (μL) that has infiltrated into one cm^3 of sediment during drilling. Intervals in which the Alien tool (ALN) was used are marked in light grey. The limit of detection (LOD), based on a particle concentration of 1×10^9 particles per mL of drilling fluid, is marked in dark grey.
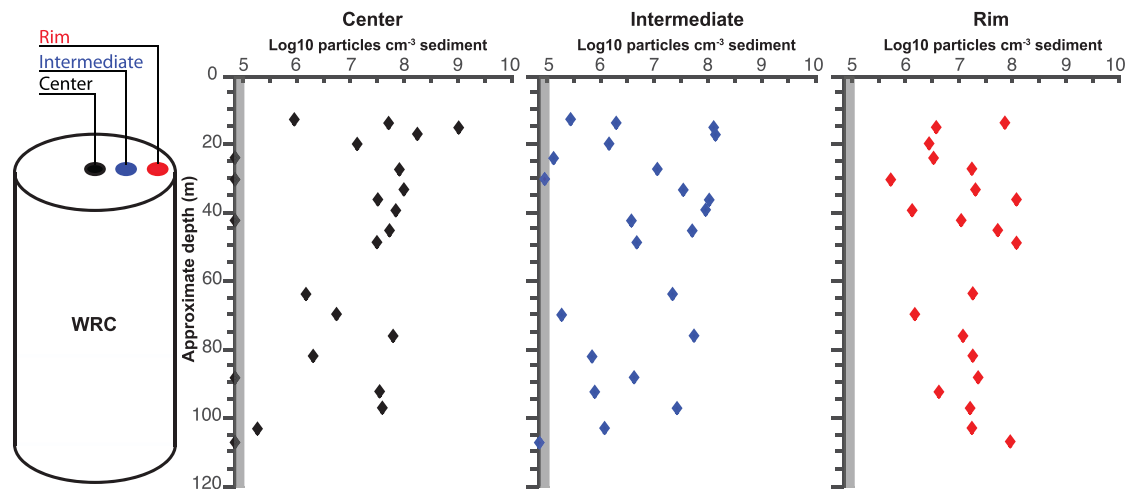

Fig. 8. Particle concentrations per cm^3 sediment of drill core samples from Lake Chalco (DayGlo SPL-594N). The limit of detection is indicated in dark grey.

distinct populations were sorted into separate vials preloaded with 5 mL MilliQ water. After sorting, the suspensions were vortexed and filtered onto white polycarbonate membranes, and the particles quantified via fluorescence microscopy using the protocol described above. The fraction of sorted tracer contained only tracer particles (Fig. 11A), whereas the sorted sediment fraction contained mostly sediment and very few fluorescent particles (Fig. 11B). The same observation could be made after sorting the Chalco samples (not shown).

To test whether the values obtained by fluorescence microscopy can be reproduced by flow cytometry over the entire range of particle concentrations found in these samples, we analyzed samples from both Lake Towuti and Lake Chalco.

We chose 23 samples from Lake Towuti and twenty samples from Lake Chalco from different depths to cover the widest possible range of particle concentrations. The results were in good agreement with the values obtained by fluorescence microscopy (Fig. 12). Thus, it is possible to detect and accurately quantify tracer particles by flow cytometry, which raises the possibility for accurate and rapid contamination control in sediment samples, potentially even directly on site.
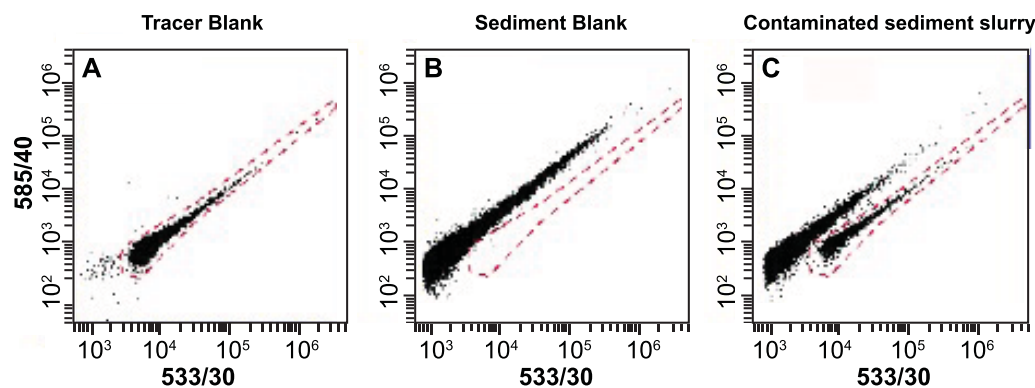
Fig. 9. Cytometer dotplots of (**A**) a sediment-free tracer solution (blue tracer, SPL-19N), (**B**) a tracer-free sediment suspension blank, and (**C**) a contaminated sediment slurry containing $\sim 1.5 \times 10^7$ tracer particles per cm^3 sediment.
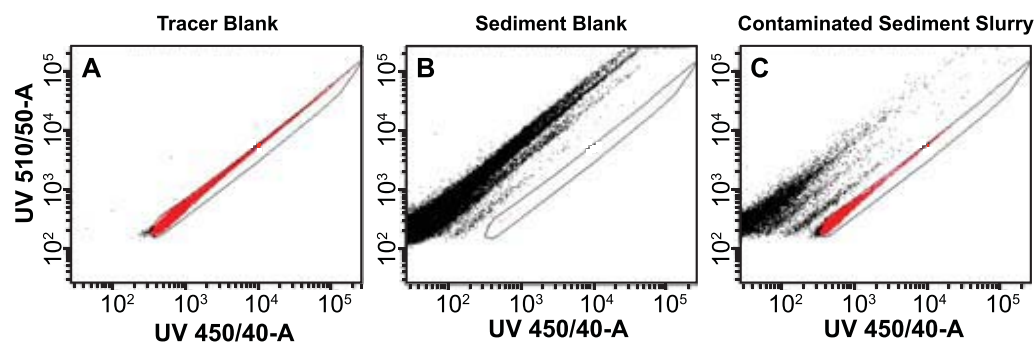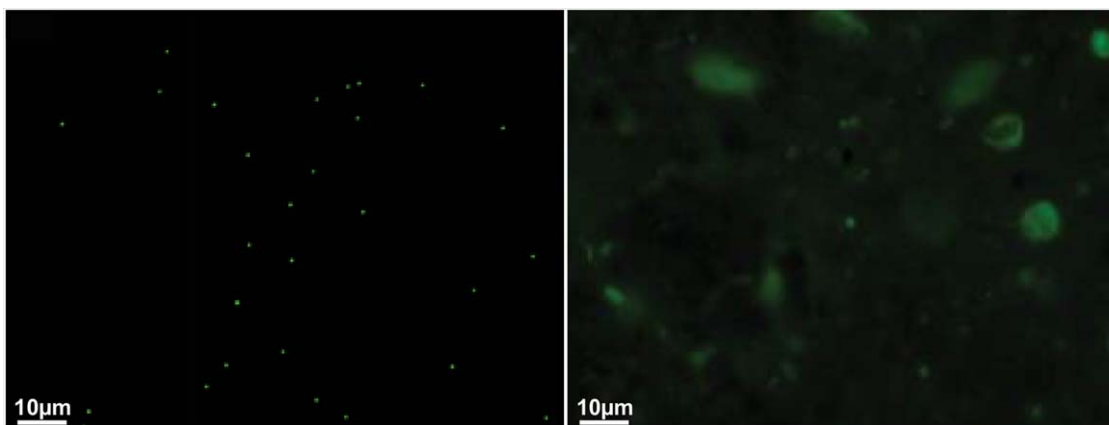

Fig. 10. Fig.10. Cytometer dotplots of (**A**) a sediment-free tracer solution (UV tracer, SPL-594N), (**B**) a tracer-free sediment suspension blank, and (**C**) a contaminated sediment slurry containing $\sim 8 \times 10^7$ tracer particles per cm^3 sediment.

Fig. 11. Microscopic image of FACS-sorted (**A**) tracer particles (SPL-19N) and (**B**) Towuti sediment particles.

Detection limit of the flow cytometry approach

The sensitivity of the flow cytometry approach depends on the number of non-tracer particles that fall within the population of the tracer, which in turn depends on the volume of sample that was analyzed. After analyzing each 1 mL of five tracer-free sediment blanks containing 1×10^{-3} cm^3 sediment from Lake Towuti the average number of particles found within the tracer population was 53 ± 29. Based on the approach used by Kallmeyer et al. (2008) and Morono and Kallmeyer (2014) we defined the LOD as the blank plus
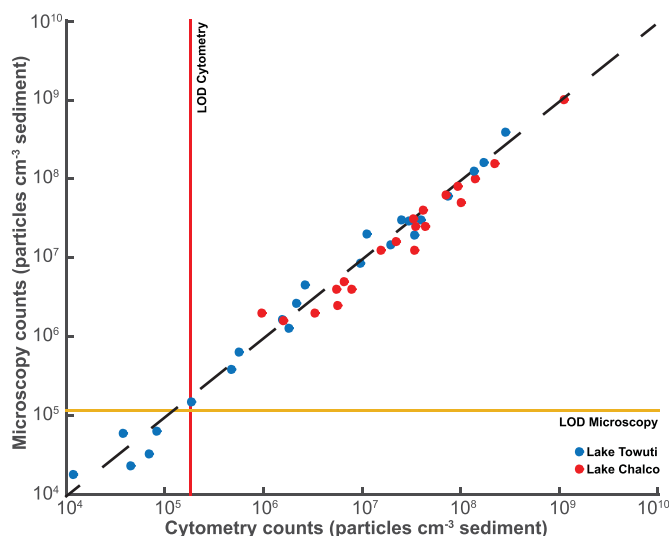
Fig. 12. Comparison of tracer particle counts obtained by fluorescence microscopy and flow cytometry for Lake Towuti (blue circles) and Lake Chalco (red circles) samples. The limit of detection of both fluorescence microscopy and flow cytometry is indicated in orange and red, respectively. The dashed line indicates the 1 : 1 ratio between the two techniques.

three times its standard deviation. The LOD thus accounts for 1.4×10^5 particles per cm^3 sediment (Fig. 12). Assuming a cell concentration of 10^6 cells cm^{-3} and a particle concentration of 1×10^9 pigment particles cm^{-3} in the drilling fluid this translates into 140 allochthonous cells per cm^3 of sample. For Lake Chalco four tracer-free sediment blanks containing 1×10^{-3} cm^3 sediment were analyzed. The average number of particles within the tracer gate was 87 ± 24 corresponding to a similar LOD of 1.5×10^5 particles (150 allochtonous cells per cm^3 of sediment).

Discussion and recommendations

Here we present a new tracer method for scientific drilling campaigns that uses an aqueous fluorescent pigment dispersion. Tests confirmed the applicability of our new tracer method for large drilling campaigns with volumes of drilling fluid in the order of tens to hundreds of thousands of liters. The method requires only a minimum of equipment and offers rapid and easy on-site analysis. The sensitivity of the new method is similar to the established microsphere tracer approach but it offers two major improvements. First, the price of the tracer is four orders of magnitude lower and among the lowest of all contamination control tracers. Second, by combining our approach with flow cytometry we decreased processing time per sample by at least a factor of four. It takes about 15 min to prepare a set of six filters, and at least 10 min per filter to count them (3 s per field of view), whereas difficult filters with many mineral particles and/or high background fluorescence might take up to 30

min each. For flow cytometry, the sample only needs to be diluted and analysis takes about three minutes per sample. Depending on the type of flow cytometer it is possible to use an autosampler, allowing for fully automated rapid on-site analysis. This opens the possibility to select samples of highest quality with respect to contamination and to decide whether changes in drilling techniques are required to optimize core quality.

Like regular microsphere tracers, the pigment tracer is stable to light degradation (Fig. 4), which allows for contamination assessment on archived core material even several months after initial core retrieval. This is a major advantage over PFT that, due to its volatility, will not reflect the initial drilling fluid contamination over this time interval and thus will not allow a later contamination assessment.

In contrast, autoclaving tests showed that our tracer particles experience a complete thermal degradation under high temperatures, which is similar to other microsphere particles (Yanagawa et al. 2013). For high-temperature drilling campaigns it is therefore recommended to use other tracers such as PFT, which has a boiling point of 76°C at atmospheric pressure (Yanagawa et al. 2013) or naphthalene sulfonic acids (NSA) that have been used as a thermally stable tracer for drilling in hydrothermal systems (Gunderson et al. 2002). However, the latter has its limitations concerning handling and environmental regulations. Also, a 16S rRNA gene tracer approach was used successfully for contamination control in hydrothermal drilling operations (Yanagawa et al. 2013).

Chemical tracers (PFTs, fluorescent dyes, etc.) and particulate tracers (microspheres, pigment tracer) differ in their diffusive properties, with chemical compounds diffusing much faster than particulate tracers, which behave more like microbial cells in that their diffusion is extremely slow, being limited to Brownian motion. Particulate tracers thus represent the most suitable, non-biological tracer to trace infiltration of non-indigenous microorganisms in subsurface materials even though their surface characteristics differ substantially from that of microorganisms (Harvey et al. 1989; Colwell et al. 1992). However, particulate tracers have limitations in systems where pore water chemistry is of interest, as drilling fluid may infiltrate into pores that exclude tracer particles. Here, a chemical tracer would be the option, as it would provide more realistic results. To achieve the most accurate contamination assessment with respect to microbial and chemical contamination a combination of particulate and chemical tracers would be recommended.

We consider the sensitivity of the presented approach to be sufficient for contamination assessment in low biomass environments. However, to further increase the sensitivity of the method, a plastic bag filled with undiluted tracer was attached to the core catcher of a HPC prior to deployment (Smith et al. 2000a; Colwell et al. 1992). In principle this would increase the amount of particles in the liner fluid

right at the core, leading to a better LOD of the amount of drilling fluid that has infiltrated into a sample. Using a 3 m long liner with a diameter of 66 mm and assuming a 2.5 mm gap between the sediment and the liner this would correspond to a volume of $\sim$ 1.5 L of drilling fluid that enters the liner during drilling. To reach a particle concentration of $\sim 1 \times 10^{10}$ mL^{-1} (corresponding to a LOD of $\sim$ 14 nL liner fluid infiltration and thus $\sim$ 14 allochtonous cells per cm^3 sediment for the flow cytometry approach) we used a plastic bag filled with 20 mL undiluted particle tracer containing in total $\sim 2 \times 10^{13}$ particles.

However, the results from the particle counts of liner and drill fluid show that only in two instances the liner fluid had particle concentrations in the 1×10^{10} mL^{-1} range (Fig. 1). All other liner fluids had concentrations that were similar to the drilling fluid. We can only speculate why the delivery of additional tracer from the bags did not work out as planned, but we assume that either the bags fell off during tripping of the core through the drill pipe, or the high flow rate of drill fluid flushed the additional tracer out of the liner. However, as the tracer concentration in the drill fluid was in the 1×10^9 mL^{-1} range and therefore sufficiently high, we do not think that the malfunctioning of the bags greatly impeded our results.

The presented approach proved to be successful in two different drilling campaigns using different tracers and different drilling techniques. At Lake Chalco it could be shown that tracer amendment into the drilling fluid is unaffected by a suite of polymer additives that are often added to the drilling fluid to stabilize the borehole and to suspend and carry cuttings out of the hole. Also, due to its non-toxic composition the tracer can be used irrespective whether the drilling fluid is discharged or recycled over the course of the drilling. We believe that the approach is likewise applicable for ocean and igneous rock drilling as the similar microsphere tracer approach has already been successfully applied in such operations (Smith et al. 2000*b*). Our new approach thus offers an inexpensive and rapid alternative approach for assessing contamination in future scientific drilling operations.

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Conflict of Interest

None declared.