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Science for Clean Energy

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Competitive low-carbon energy

D3.5
Report on the biodegradability and bioaccumulation of DNA-based particle tracers

WP 3 – Instruments and tools – Development and deployment

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History of the changes

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1.0	07-01-19	Julian Koch	First draft circulated internally at UCL
1.1	25-02-19	Alberto Striolo	Second version circulated to the consortium for comments and approval
1.2	26-02-19	Julian Koch	Second version after the reception of consortium's comments and feedback
1.3	28-02-19	Natalie Nestorowicz	Final version submitted to the EC

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Key word list

Ecotoxicity, DNA tracer, silica

Definitions and acronyms

Acronyms	Definitions
S4CE	Science for clean energy
DNA	Deoxyribonucleic acid
ROS	Reactive oxygen species
EC50	Concentration of a tested material that gives half-maximal response
EC20	Concentration of a tested material that gives 20% of maximal response
ENP	Engineered nanoparticles

1. Introduction

1.1 General context

Geo-energy operations like geothermal energy, carbon sequestration and enhanced gas recovery are considered essential for a green energy mix in Europe. The S4CE is concerned with implementing new technologies and mitigating the risks in these promising fields. Part of this consortium is specifically working on a new generation of environmental tracer based on DNA. We at ETH Zürich are evaluating its environmental impact, in the framework of S4CE as "Deliverable 3.5".

In geo-energy operations, understanding deep and shallow aquifer and groundwater reservoir connectivity is essential to improve the effectivity and decrease the environmental impact of sub-surface operations. Compared to analyzing surface connectivity, sub-surface systems require a multitude of analytical approaches in order to map the full sub-surface network, approaches that can be divided into modelling and experimental ones. The data for modelling of sub-surface connectivity comes from experimental work on a few test sites, thoroughly tested. However this gives rise to risk of oversimplification for an observed geological setup by assuming that the dataset is representative for other sites[1]. Experimental work varies from electrical resistivity imaging[2] to environmental tracer studies. Common environmental tracers include salts (NaCl or LiBr), fluorescent dyes (Fluorescein, uranine, rhodamine etc.) or labeled water (high tritium content or other isotope). Microbial[3] or DNA based tracers[4, 5] are currently being developed to provide new opportunities for multitracing applications. Tracer data, combined with modelling can yield particularly useful information about subsurface connectivity.

The fact that silca is widely present in surface and underground water make it a suitable tracer matrix for DNA tracing from an ecotoxicological viewpoint. Studies on oral toxicity and ecotoxicity of bulk silica found that this material is non toxic[6, 7] and also tested the nanostructured silica material, finding that it may induce the release of inflammatory response such as endosomal substances or reactive oxygen species. None of the tested nanomaterial however was shown to bioacumulate[7].

1.2 Deliverable objectives

Haelixa AG is developing a new generation of DNA tracers for sub-surface applications, with the DNA encapsulated in silica nanoparticles. The silica coating increases the stability and robustness of the DNA, making it a suitable tracer for conditions where free DNA strands would degrade. The particulate character of the tracer allows us to study sedimentation transport and aggregation dynamics. As silica nanoparticles have not been used yet in large scale underground tracer experiments, an ecotoxicological assessment is conducted in the framework of S4CE. The goal of this work is therefore to characterize the DNA tracers and

perform ecotoxicological tests with 3 different relevant organisms (Daphnia Magna, Ceriodaphnia and Algae) to be able to estimate the impact of large scale tracer use.

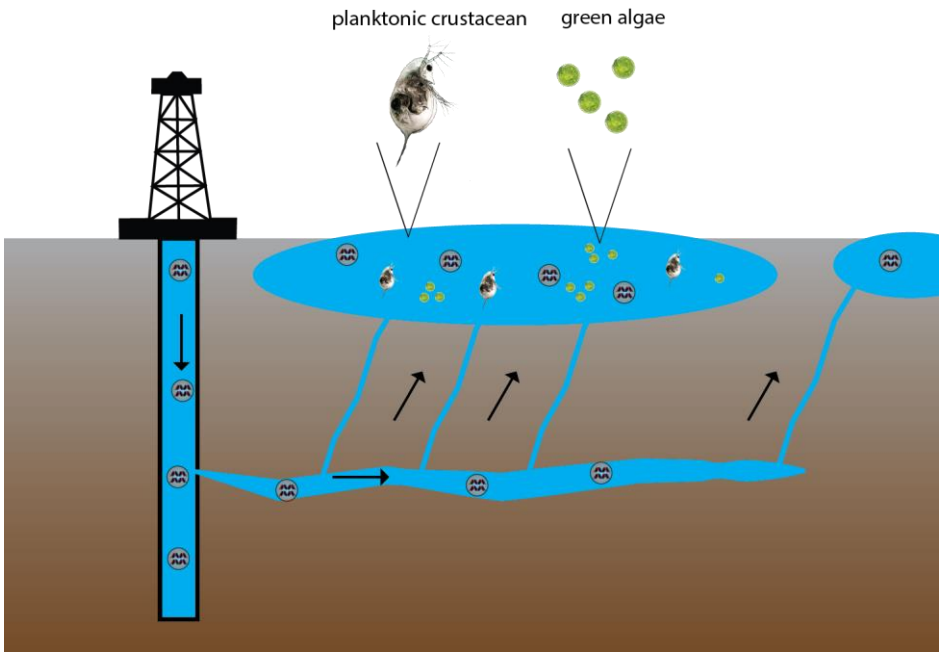

Figure 1: Illustration of potential application of DNA based tracer material and its impact on an ecosystem.

2. Methodological approach

Particle synthesis

The particle synthesis was based on the methods described by Paunescu et al.[8]. 50 mg silica nanoparticles (microparticles GmbH) in 1 mL ethanol were surface functionalized with 10 µL N-trimethoxysilylpropyl-N,N,N - trimethylammonium chloride (TMAPS, 50% MeOH, ABCR GmbH) for 12 h. To produce 1 batch, 2 mL of particles (50 mg/mL) were added to 400 µL of double stranded DNA (1mg/mL) in 30 mL of ultrapure water (Merck Millipore). The amount of unbound DNA was determined by NanoDrop 2000c Spectrophotometer. On top of the DNA layer, silica was grown through Stöber synthesis. 30 mL of ultrapure water, 5 µL of TMAPS and 5 µL of tetraethyl orthosilicate (TEOS, ≥90%, Aldrich) were added before shaking for 5 h. Then, 240 µL TEOS was added and the batch was shaken for 4 days. To control the size, the particles were milled from 2 to 8 min with 1 g per mg of particles of 0.1 mm zirconium beads (Nikkato Corp.) in a planetary micro mill (Fritsch GmbH).

Particle characterization

The particle size in suspension was measured with a dispersion analyser (LUMiSizer®, LUM) at a particle concentration of 1 mg/mL. The surface potential was measured by a zeta potential analyzer (Zetasizer Nano, Malvern) at a concentration of 0.1 mg/mL. The DNA loading was measured by adding buffered oxide etch to a known particle concentration and measuring the DNA concentration by Qubit assay. For SEM imaging, the particles were dispersed in isopropanol (>99.7%, VWR Chemicals) and deposited on a grapheme TEM grid. For chemical stability of encapsulated DNA, the particles were exposed to reactive oxygen species (ROS).

DNA stability assay

Encapsulated and non-encapsulated DNA were subjected to radical treatment stability assay[8], where a combination of ascorbic acid, H_2O_2 and $CuCl_2$ produce reactive oxidative species and test the protective properties of SPED towards DNA oxidation[8]. 5 µL of DNA/encapsulated DNA were added to 2.5 µL L-ascorbic acid (20 mM), 12.5 µL H2O2 (20 mM) and 17.5 µL CuCl2 (500 µM). After 10 minutes, the reaction was quenched by adding 17.5 µL of 100 mM EDTA and 20 µL of BOE. To measure the total amount of DNA, 50 µL of water and 20 µL BOE were added to 5 µL of DNA/encapsulated DNA. The DNA concentration of treated and untreated samples was measured by QUBIT assay. All reported values are averaged over quintuplets.

The DNA stability was also evaluated by subjecting it to household bleach. A bleach stock was prepared with 8.57 mL NaClO (14% activity), 1.164 mL NaOH 10 M and 30.266 mL H_2O. 143 µL of 100-fold diluted bleach stock was added to 143 µL of DNA (10 ng/mL) or SPED suspension (0.1 µg/mL) and incubated for 10 min. The reaction is quenched by adding 5 µL of thiosulfate (1.46 M). The unprotected DNA is directly measured by qPCR whereas to the SPED samples, 10 µL of BOE were added and then purified by QIAGEN QIAquick PCR Purification Kit with 50 µL elution buffer. The samples were quantified by qPCR (10 µL master mix (Roche, Lightcycler 480 SYBR Green I Master Mix), 8 µL of MilliQ grade water, 1 µL of primer mix (primers F0 and R0 (Microsynth AG), each at 10 µM), 1 µL diluted DNA pool, Cycling parameters were 95°C for 15s, 54°C for 30s, 72°C for 30s for 30 cycles). All values reported in this deliverable are averaged over triplicates.

Ecotoxicological tests

Three standardized tests, described in Table 1, have been used to establish the ecotoxicological profile of DNA-silica tracers. The *Daphnia magna* immobilization test is conventionally used to assess the short-term acute effects of chemicals and effluents whereas the algal growth test and the Ceriodaphnia dubnia test are standard protocols to evaluate chronic toxicity. All three tests were carried out according to their respective standard protocols certified by ISO. In each test, the organism was exposed to a concentration range of the tracer, which allowed to establish an EC_X value. These values are reported with a 95% confidence interval limits and calculated by logistic regression with Hill model (REGTOX v. 7.0.5 software, Vindimian, 2001). For the algal growth test, the tracer solutions were filtered with a 0.45 µm filter prior to preparing the different dilutions.

Table 1: Test parameters for the ecotoxicity tests used in this work

Test	*Daphnia magna* immobilization test	Algal growth inhibition test	*Ceriodaphnia dubnia* chronic toxicity test
Organism	*Daphnia magna* Straus	*R. subcapitata* (*S. capricornutum*)	*Ceriodaphnia dubnia*
Test method	ISO 6341	ISO 8692	ISO 20665
Endpoint	Mobility	Growth	Reproduction
Type of effect	Acute	Chronic	Chronic
Temperature	20 ± 2°C	24.3 ± 0.5 °C	25 ±1 °C
Lighting	Darkness	Continuous lighting during incubation	300 to 500 lux, with 16h:8h light:dark photoperiod
Test duration	48 hours	72 hours	7 days
Measurement	Immobility by eye at 24 and 48 hours	Cell counting with Coulter counter® at 72 hours	Organism and offspring counting at 7 days
Control and dilution medium	Synthetic medium (ISO 6341)	Synthetic medium (ISO 8692)	Synthetic medium (ISO 20665)
Agitation	None	Continuous (125 rpm, orbital shaker)	None
Test design	5 indivium per replicate, 4 replicates per concentration step, 4x5 individum as control	3 replicates per concentration, 4 controls	12 replicates per concentration, 24 control replicates
Test vessel	Glass tubes (15 mL)	Polystyrene microplate (2.2 mL)	Polypropylene beakers (25 mL)
Number of organisms	5 per replicate	Initial density:1000 cell/mL	12 per concentration
Expression of results	EC_{50} 48 hours	EC_{50} 72 hours	EC_{50} 7 days

3. Summary of activities and research findings

The first part of the project was concerned with identifying a realistic tracer exposure scenario. The tracer injection is a point source with a release of a total of 1 L tracer medium and 100 ppm tracer concentration (Figure 1) which is similar to the amount of tracer used in a recent DNA tracing study from Mikutis et al.[5]. In addition to dilution through diffusion and convection perpendicular to the flowing motion, most of the injected tracers will not arrive at the measurement spot downstream but be absorbed in a rock matrix, degrade due to the sun or air, or deviate from the main stream into smaller side streams. These effects lead to a decrease in tracer concentration to which organisms living in the concerned ecosystem will be exposed. As a worst-case scenario for acute exposure of an organism to the tracer over 48 h, the initial concentration of 100 ppm at the tracer injection point is chosen. For chronic exposure over the course of 7 days, based on actual tracing scenarios[5, 9-11], where tracer dilution varied from $x10^2$ to $x10^4$ from start to endpoint, a worst-case scenario of 1 ppm is envisioned.

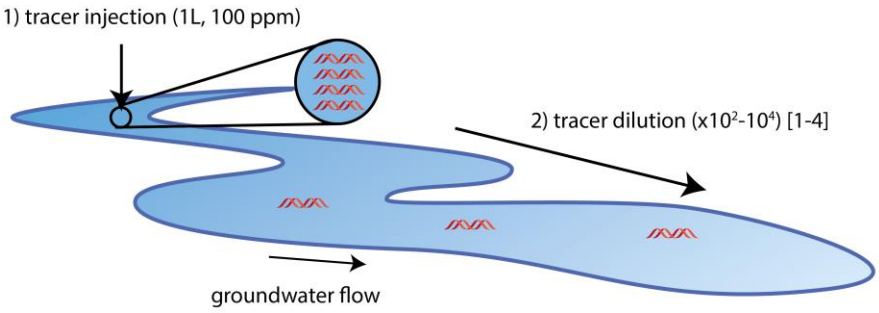

Figure 2: Tracer dilution and exposure scenario

The tracer synthesis was based on Paunescu et al.[8], an established synthesis protocol for DNA-silica encapsulated particles used in studies for environmental tracing[4, 5, 12] and other studies for food tagging[13] or bacteria particle uptake[14]. In short, negatively charged DNA is adsorbed onto positively charged SiO_2 nanospheres because of electrostatic interactions. The exposed DNA layer is covered by an additional layer of SiO_2 through Stöber synthesis, which is enough to protect the DNA from external factors such as high temperature[15] and chemical stress[8], as shown by exposing the particles to ROS (Table 2). For this study, we produced two different sized particle batches (Figure 2, Table 2). The surface potential of the particle tracer was measured at -24.2± 4.2 mV and -24.5 ± 4.5 mV, indicating that the particle suspension was stable. Indeed, sedimentation experiments showed that over the course of 24 h and 72 h, the concentration of particles was stable in the supernatant (Figure 3b).

Table 2: Particle characteristics from two batches used in this study. *Unprotected DNA had 95% DNA loss in ROS assay.

Particle batch	**Surface potential** (mV)	**DNA loading** (%wt)	**ROS test***	**Particle diameter (nm)**
1	-24.2 ± 4.2	0.35	8% DNA loss	147.8 ± 35.8
2	-24.5 ± 4.5	2	50% DNA loss	220.3 ± 91.5

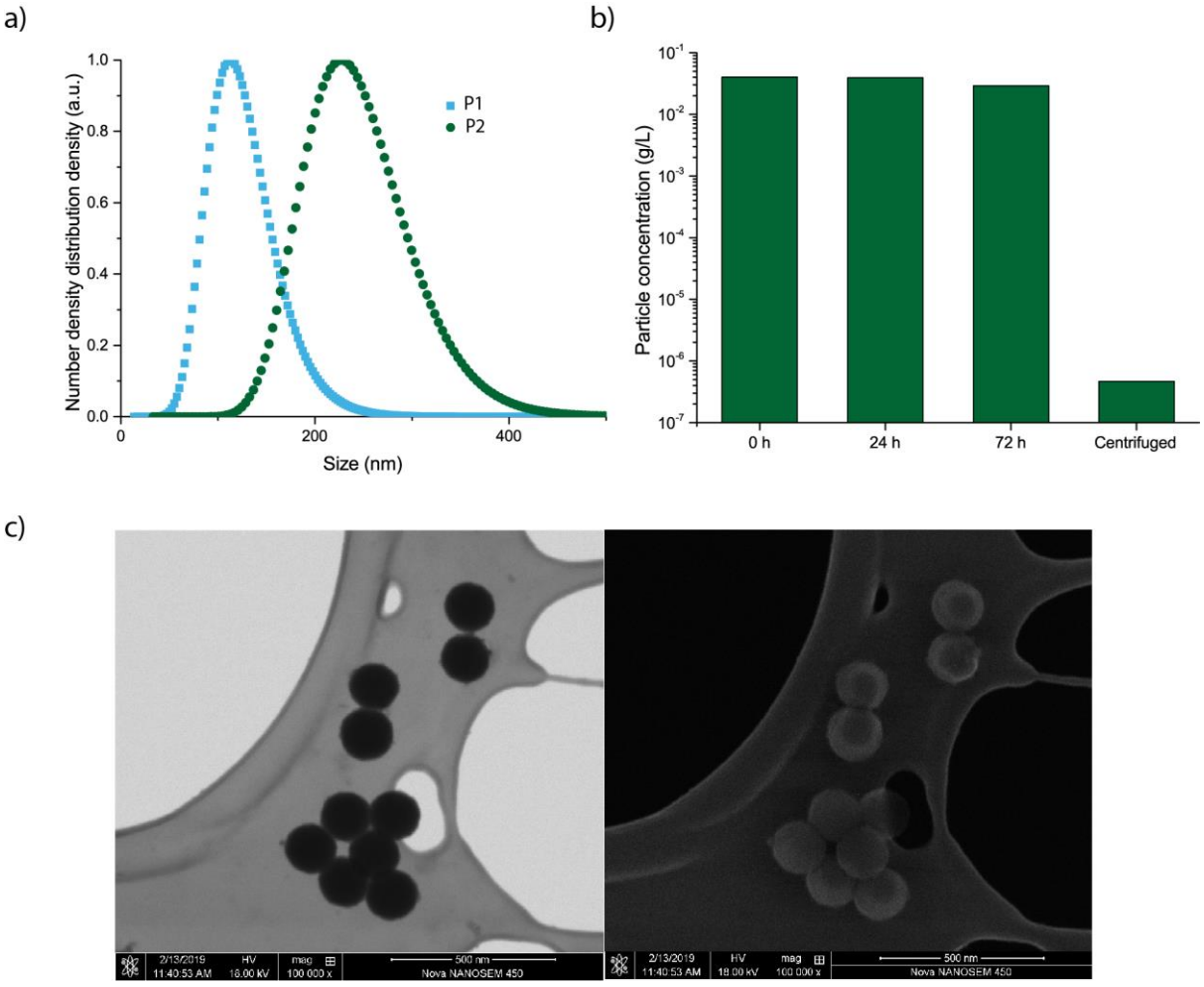

Figure 3: a) Particle size distribution measured by LUMiSizer® b) Supernatant particle concentration measured by qPCR. After 72 h, the suspension was centrifuged and the DNA concentration in the supernatant was measured c) SEM and STEM images from silica particles with encapsulated DNA

In collaboration with the laboratory SOLUVAL SANTIAGO, three bioassays were conducted to assess the ecotoxicological impact of silica particles with encapsulated DNA.

The freshwater organism *Daphnia Magna*, a standard assay in ecotoxicology, was used to test the acute toxicity of the tracer material after 24 h and 48 h. In this assay, the number of daphnids, which were immobilized, are counted by eye in each treatment group and control. At tracer concentrations up to 20 ppm, no impact on the organism's mobility was detected for both types after 24 h and 48 h (Figure 4a). At very high tracer concentrations (>300 ppm), inhibition can be observed after 48 h. This effect has been seen with other types of nanoparticles (1-100 nm) and fine particles (>100 nm) and is associated with increasing collision frequency and therefore higher resistance in concentrated particle dispersions[16, 17]. Previous reports on acute toxicity of SiO_2 particles for *Daphnia magna* measured an EC50 of 150 ppm for particles with a diameter from 10-20 nm[18]. Lee et al. studied the genotoxicity of ENPs like SiO_2, TiO_2 and CeO_2 on DNA integrity of Daphnia magna. For SiO_2 and TiO_2, they did not find any effects on DNA integrity[19]. The slightly higher effect of P1 on the mobility compared to P2 at the same concentrations could be linked to the higher surface area per mass of tracer[20] resulting from the smaller particle size distribution, therefore resulting in higher collision frequency[16, 20].

A second bioassay with green algae (*Pseudokirchneriella subcapitata*) was implemented to test the reproductive impact of the tracer during 72 h. The cell density (number of cells per mL) is measured with a Coulter® counter in each treatment group and control. As with the previous test, up to 20 ppm tracer concentration, no effect on population growth was observed for both types (Figure 4b). For high particle concentrations greater than 50 ppm, growth inhibition of algae was observed for both particle batches. This effect has been reported for different types of nanoparticles at high concentrations and is associated with overloading of particles in the test organism. This can then lead to physical effects such as shading the algae[21-23]. EC20 after 72 h of 318 ppm with a diameter of 10-40 nm[24] have been reported and in a different study an EC20 after 72h of 20 and 28 ppm with particle sizes of 12.5 and 27.0 nm respectively[20]. Again, the difference in size of P1 and P2 could explain the more important effect on algae due to the smaller size and therefore higher surface area.
Finally, to evaluate the chronic toxicity on crustaceans, two standard tests exist, one with *Daphnia Magna*, which takes 21 days and the other with *Ceriodaphnia dubia* for 7 days. For this work, only the assay with *Ceriodaphnia dubia* was implemented. *Ceriodaphnia dubia* is about 4 times smaller than *Daphnia Magna*[25] (Figure 4d) and has a shorter reproductive life-cycle[26], allowing for shorter meaningful assessment of chronic toxicity effects. It has been suggested that chronic toxicity data of *Ceriodaphnia* is more sensitive but similar to the data obtained with *Daphnia Magna*[26], making it an appropriate surrogate test. To measure the population growth inhibition of *Ceriodaphnia dubia*, the organisms are transferred once each day to a new vessel with renewed medium, at which point the survival of mothers and offsprings in each vessel are counted. As in the previous assays, P1 has more effect on the population growth compared to P2 (Figure 4c). The effect of nanoparticles on reproduction could come from uptake of particles and therefore deplacement of nutrients, which in turn increases the filtering activity and therefore results in higher energy usage[16]. Also the need for more nutrients leads to increased mobility in order to move to regions with higher nutrient

availability, again increasing energy usage, resulting in less energy available for reproduction[16].

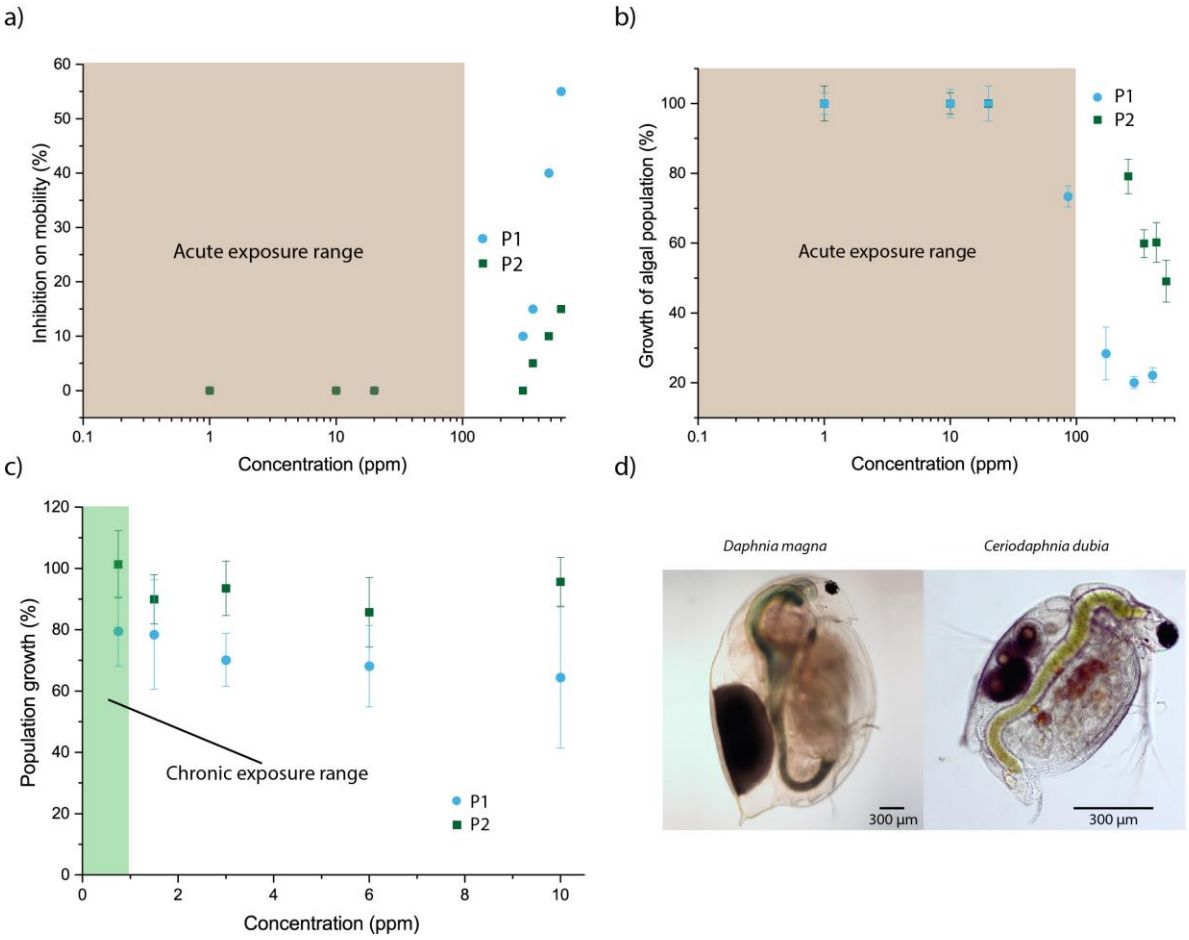

Figure 4: a) Inhibition of mobility of Daphnia magna in function of tracer concentration, measured at 24 h and 48 h. No apparent inhibition was measured at 24 h, only results at 48 h are displayed d) Images taken from zooplankton key Copyright © 2003-2013 Center for Freshwater Biology, Department of Biologcal Sciences, University of New Hampshire, Durham, NH 03824 USA

4. Conclusions and future steps

In this work, the tracer material was characterized and the ecotoxicity of SiO_2 particles with encapsulated DNA was assessed by means of 3 standard bioassays. The acute toxicity was tested with the crustacean *Daphnia Magna* during a time period of 24 h by observing the mobility. In the concentration range from the exposure scenario, the organism shows no decrease of mobility. The reproduction of green algae seems to be affected only by very high concentrations (>50 ppm) of tracer which indicates effects of shading which affect the reproducibility. Finally, over the course of 7 days, the crustacean organism *Ceriodaphnia dubia* is subjected to the tracer material for 7 day. A slight decrease of reproduction is observed, especially for the P2 particles, which is probably due to the smaller size distribution and therefore higher surface area. In general, the experimental results suggest that the tracer material can be used in the confinements of the exposure scenario without impacting the ecosystem. Additional examination for the difference of impact on the organism would be required to better understand the size related effect of the tracer material to the organism. The results obtained in this work, however indicate that a higher particle size would reduce the ecological impact on algae and crustacean species.

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