Ecotoxicity assessment using ciliate cells in millifluidic droplets

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Precise analysis of the aquatic cells and their responses to the toxic chemicals, i.e., water disinfective agents, is of crucial importance due to their role in the ecosystem. We demonstrate the application of the droplets based millifluidic tool for isolating and longtime monitoring of single *Paramecium tetraurelia* cells using a large number of water-in-oil emulsion droplets. Due to the automated monitoring of the fluorescence signal, the droplets containing cells are distinguished from the empty reservoirs. A viability indicator is used to follow the metabolic dynamic of the cells in every single droplet. Finally, we perform ecotoxicity tests in droplets, exposing the encapsulated paramecia cells to silver nitrate for determination of EC50 levels, and compare the output with the conventional microtiter plate assay.

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I. INTRODUCTION

Aquatic microorganisms play an important role for the ecosystem, since they participate in a food chain at multiple levels, both as *producers* that capture the sun's energy through photosynthesis, and as *consumers* engulfing photosynthetic algae cells, colonized bacteria, or waste materials.¹ Nature regulates the functions of aqueous habitants, such that they maintain their life environment clean in a self-consistent manner. For instance, well investigated paramecia from the group of ciliates can act as a cleaning agent of water environment, by uptaking freely dispersed and potentially harmful bacteria.² However, human activities significantly influence the natural processes, including those happening at the level of participants of the aquatic ecosystem.³ For example, increase of the environmental concentrations of substances, e.g., silver nitrate, commonly used for water disinfection,⁴ can reach toxic levels that impact the natural water purification efficiency. Furthermore, the use of the nanomaterials, i.e., nanoparticles of copper oxide (CuO), zinc oxide (ZnO), and especially nanosilver (nAg) to fight the undesirable growth of bacteria, fungi, and algae in industrially relevant environment^{5,32–34} and households, also might result in adverse effects on activated sludge organisms.⁶ Thus, the release of disinfection agents to wastewater treatment plants by sewage water takes place to a high degree, and coincidentally the concentration of toxic species in sewer systems and in effluent water is steadily increasing. At the moment, potential adverse effects for the wastewater treatment process and consequences for the aquatic ecosystem are under investigation. For environmental risk assessment, it is necessary to characterize the potential hazard of the toxins, applying test organisms, which are relevant in the investigated compartment (e.g., wastewater treatment plants).

In these regards, paramecia represent a well investigated model system and a representative of filter feeders in activated sludge, for establishing the ecotoxicity assays *in vivo*. In particular,

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this test organism is one of the most relevant genera of ciliates and is broadly used in previous works for risk assessment in European activated sludge. In particular, Burkart *et al.*⁷ presented a new nano-ecotoxicity test system for activated sludge, which employed *Paramecium tetraurelia* as relevant test organism using microtitre plates (96 wells). However, this method is restricted in terms of replicate numbers and numbers of tested concentrations due to the sample handling effort.

Generally speaking, the number of microorganisms, e.g., *Tetrahymena sp.*, has previously been used in ecotoxicological testing. However, in Europe, this genus is less abundant than *Paramecium*. Thus, in order to choose a relevant organism for activated sludge in Europe, and because of its availability in axenic culture, *P. tetraurelia* was used in the toxicity tests presented here.^{8,9}

Here, we present the *millifluidic* tool for isolating and further monitoring the single ciliates from exposure samples using a large number of water in oil (w/o) emulsion droplets. Due to the automated continuous monitoring of the fluorescence signal, the droplets containing the cells are efficiently distinguished from the background empty reservoirs. We record the viability curve for every single paramecia cell, using the metabolism driven resazurin to resorufin conversion.¹⁰ Finally, we perform the ecotoxicity tests in droplets, exposing the different concentrations of silver nitrate to the encapsulated ciliates, and determine its critical levels in the medium. Silver nitrate is used here as a model example, as it represents an easy to handle compound (compared to, e.g., nAg) for proof of principle of the method. The developed tool is similar to so-called droplet based *microfluidics* concept, operating the large amount of same size isolated reservoirs in parallel (linear dimensions of each droplet are in *micrometer* range, and volumes of reservoir can be scaled down to *femtoliters*).^{11–13} Nowadays, this technology makes an important contribution to the classical detection and characterization laboratory assays, substituting reaction tubes, flasks, and incubation chambers for, e.g., culturing microorganisms,^{14,15} cells¹⁶ and drug screening,¹⁷ protein analysis,¹⁸ nucleic acid test, and polymerase chain reactions.¹⁹

However, currently discussed *millifluidic* technique,^{20,21} operating *nanoliter* reservoirs with high efficiency (linear dimensions of each droplet are about a *millimeter*), in this particular case would outperform the *microfluidic* approach in terms of better suitability for quick screening of *larger volume* water samples (up to ~l) for quantitative analysis of its biological and chemical content. Furthermore, we believe that the presented concept is beneficial for the fast collection of aquatic unicellular organisms from environmental samples, whose dimensions reach few hundred micrometers. Finally, the ecotoxicity assessments, addressing the effect of the nanomaterials or disinfection agents on the participants of the water ecosystem, can be efficiently realized on single cell level.

II. MATERIALS AND METHODS

A. Fluidic circuit

In order to collect paramecia from *exposure samples* and to perform long term monitoring of every single cell, the automated fluidic machine, controlled by a developed LabView software, is designed (see Fig. 1(a)). The machine consists of two basic parts, carrying the specific functions, namely, (a) generation and storage of a large number of water in oil (w/o) emulsion droplets (up to 10^3) inside of the fluorinated ethylene propylene (FEP) capillary tube (Dolomite Microfluidics), and (b) a droplets' detection unit (see Figs. 1(b) and 1(c)). The fluids, e.g., oils and the nutrition medium for ciliates, are injected using syringe pumps (NEMESYS, Cetoni). The entire fluidic setup utilizes FEP tubing with a length up to 3.5 m and an inner diameter ID of about 0.5 mm, which is wrapped up into two loops of the same size for the compact storage of droplets. The detection area (fluorescence readout unit) is located in between the storage loops. For continuous detection, the generated droplet chain is pushed from the first storage loop into the second one, passing the readout unit, as demonstrated in Fig. 1(c). The measurements of the fluorescence in each droplet are performed repeatedly (up to 30 h) by pulling the droplets between the storage loops back and forth, similar to the fluidic circuit, presented in

FIG. 1. Schematic illustration of the entire setup enabling the long term monitoring nanoliter droplets. (a) The photo displays the experimental setup, including all building blocks, i.e., spectrometer, injection pump, connected to the droplet analysis box. Optical signal is detected using spectrometer and recorded using LabView software. (b) The main parts of the droplet analyser are shown here: The readout unit together with the laser diode and the optical detector and T-junction as part of the droplet forming unit are shown with two yellow boxes. Two loops for droplet storage and the third one is to support droplet generation. (c) In the droplet forming unit, a droplet chain is generated containing alternating reservoirs of mineral oil (red droplets) and culture medium with paramecia cells and viability dye (cyan droplets). The droplet train is pushed (blue arrows) by the HFE oil towards the readout unit. After the fluorescence of the entire train is been measured, it is located in the second loop. A reverse of the HFE oil flow is refilling the HFE oil syringe and is pulling (red arrows) the droplets backwards through the readout unit again. After the second measurement, one measuring cycle is complete.

Ref. 21. The implemented electric valves V1 and V2 realize control of the fluid flow in the circuit (the LEE Company) (see Figs. 1(b) and 1(c)). The flow outlets are opened to the atmosphere and controlled with basic valves, to assure either the detection of the droplets with encapsulated ciliates (V1—closed and V2—opened).

B. Droplet generation

Encapsulation of the paramecia into the droplets and the further viability assessments require three different fluids to be injected into the tubing. First, the hydrofluoroether oil (HFE, 3M) with 0.01% of Pico-Surf dispersed surfactant (Pico-Surf 1; 2% in Novec 7500, Dolomite Microfluidics) is used as a biocompatible solution for the continuous phase and in order to decrease the wetting of the FEP tubing by water droplets. It has been demonstrated that the lower concentration of surfactant is able to reduce the leakage kinetics of the dye into the continuous phase.³⁵

The analyte solution contains the paramecia dispersed in an aqueous axenic nutrient (axenic broth for ciliates—ABC; according to the protocol for axenic medium of Soldo and Van Wagtenonk,³⁰ modified after Kaneshiro *et al.*²⁹) and the metabolic indicator resazurin (Fluorometric Cell Viability Kit, Promo Cell GmbH). Finally, the mineral oil (M5904, Sigma Aldrich) is injected into the FEP tubing in the co-flow mode to serve as the spacers between the neighbouring aqueous reservoirs (see Fig. 2(a)). Similar technology is used to maintain the stability of the droplets' chain for the microbiological assays.²¹ Additionally, a long mineral oil plug with the length of about 4 cm is formed at the beginning and at the end of the droplets' package to support its stability and integrity throughout the duration of exposure and readout. Furthermore, the mineral oil at the edges helps to equilibrate the aerobic conditions for all

FIG. 2. (a) A sequence of mineral oil and medium droplets inside of FEP tubing. For a better visualization, a dye was added to the medium. (b) A single ciliate confined in water droplet. For a clear view, the droplet does not contain any dye or resazurin. (c) An example of the measurements: at the initial stage of the experiment ($T = 0$ h), the signal of the empty and filled droplets is all the same because no dye was converted yet. The fluorescence signal of a droplet containing a living organism has increased after 6 h due to the conversion of the metabolic dye.

droplet bio-reactors participating in the measurements. The generation of the droplets is realized by infusing the mineral oil and ciliate medium using two T-junction elements with inner diameter of 0.5 mm (Dolomite Microfluidics), plugged into the FEP tubing one by one, as previously demonstrated in Fig. 1(c). Before starting the sampling experiment, all tubing was flushed with ethanol and filled with the HFE oil. The initial mineral oil plug is generated and pushed towards the detection part. In this configuration, V2 is opened and V1 is closed. Afterwards, both valves are switched to their opposite state (V1—opened and V2—closed) to assure the formation of the droplets. Since the first droplets were always bad, they were washed out through V1. When the droplet forming unit was containing just good droplets, the valves were switched again (V1—closed and V2—opened) to inject the good droplets into the detection area. The valves were switched again (V1—opened and V2—closed) to wash out the remaining droplet and to push just mineral oil to the V1. Another switching accrued (V1—closed and V2—opened) to attach final a mineral oil plug. All components (HFE, mineral oil, and medium) of the emulsion are pushed with the flow rate of 5 ml/h each.

Resulting water based droplets had a length of approximately 0.8 mm and a volume of about 160 nl. This droplet volume was found to be optimal for isolation of actively swimming single *Paramecium*, having typical dimensions of about $180\ \mu\text{m}$ in length and about $50\ \mu\text{m}$ in width (Fig. 2(b)).

C. Automatic detection of droplets

Once the droplets are formed, the valve configuration is set to push the emulsion towards the detector part (see Fig. 1(c)). The detection unit consists of a green laser diode ($W = 5\text{mW}$, $\lambda = 532\text{ nm}$, Thorlabs) used for the excitation of fluorescence inside of every droplet. The focus of the laser was adjusted to the size of the tubing inner diameter ($500\ \mu\text{m}$) to illuminate entire droplet width. Active cells inside of a droplet did not have a significant effect to the fluorescent signal. Only the metabolism of the cells is changing the fluorescence. An optical fiber coupled UV-Vis spectrometer (QE 65000 Scientific-grade spectrometer, Ocean Optics) has been used as a detector. The developed LabView program utilizes a virtual filter which is set to block every wavelength shorter than 550 nm. This feature helps to eliminate the detection of, e.g., laser signal occurring due to its scattering in the FEP tube. Long time monitoring of the ciliates inside

FIG. 3. (a) The graph is showing the filling rate of the droplet with the ciliates (red bars), compared with the theoretical values of the Poisson distribution (green bars). The empirical data are in a good agreement with the Poisson distribution. (b) The graph is displaying the process kinetics of fluorescence intensity increase of multiple individual droplets (empty and filled). Filled droplets contain living cells which convert the resazurin, over many hours, to the highly fluorescent resorufin. (c) Example plot of four droplets, one without any living organism (black curve), two droplets very likely containing one paramecia cell (red curves), and one droplet probably containing two cells (green curve).

of droplets is realized by automating the measurements' procedure, similar as was presented by us elsewhere.²¹ However, in the current configuration, multiple detection runs are realized via alternating change of operation modes of the HFE syringe pump from pushing to refilling. This configuration helps to avoid the switching events of the integrated electric valves during the measurements' procedure that potentially can disturb the droplets' flow. To reduce the amount of data to be stored, the developed LabView program only records the *integrated* fluorescence intensity of every channel (above 550 nm) of the spectrometer. Furthermore, it was possible to perform the videomicroscopy observation of the emulsion samples in parallel with the fluidic machine, to monitor the content of every droplet (see Fig. 2(b)). In particular, the viability of the encapsulated ciliates can be straightforwardly checked by visualizing the motion pattern of every single cell (see supplementary video S1).³⁶ In contrast, the dead cells (exposed to toxic concentrations of silver nitrate) are observable as sedimented motionless objects inside of the droplet.

III. RESULTS AND DISCUSSION

A. Metabolic dynamics in droplets

The initial concentration of cells in the solution determines the filling factor of the droplet following Poisson statistics.¹⁶ Detecting the evolution of the fluorescence inside of every single droplet with time, as well as cross reference videomicroscopy measurements enabled the mapping of the Poisson distribution for the single ciliates, entrapped into the nanoliter droplets (see supplementary material, Fig. 2S).³⁶ To do this, metabolic dye resazurin was added to the cell culture immediately at a volume ratio of 1:11 before droplets' formation process. Whereas the fluorescence of the occupied droplets increases with the time due to the metabolic conversion of low fluorescent resazurin to high fluorescent resorufin, the signal of empty droplets stays practically unchanged (see Fig. 2(c), top and bottom panels). Fig. 3(a) summarizes a histogram of the experimentally observed cell distribution in the droplets for 1000 cells/ml, together with the data derived from Poisson distribution. Obtained results indicate that the one and two cells' encapsulation is obtained for only about 15% and 1% of droplets, respectively. Small deviation between theoretical Poisson prediction and empirical results is caused by a number of reasons, i.e., eventually not sufficient amount of droplets filled with cells for precise analysis. Additional factor, influencing the cells' encapsulation might be related to the inhomogeneous distribution of living cells inside the syringe, occurred due to the active natural motion of the ciliates. Although the potential negative effect of the active motion on the cell partition, the swimming of paramecia still helped to identify the survival of the cells in the droplets, to validate the test system throughout the duration of the experiment (20 h). This analysis helped to optimize the encapsulation conditions to vary the number of entrapped cells, with the aim to achieve an ultimate single cell/droplet case (see Fig. 2S in the supplementary material).³⁶

FIG. 4. Family of metabolic curves measured in 10 different droplets, eventually containing single ciliate cell.

For trapping single ciliate cells and monitoring their metabolic dynamics, the medium solutions with the cell densities of 1000 cells/ml were used in the following. The evolution of the fluorescence intensity over the time inside of every single droplet (see Fig. 3) was determined as a measure of cell viability. In particular, Fig. 3(b) represents the metabolic dynamics of the *Paramecia* cells, entrapped inside of the 434 droplets. It displays a series of metabolic curves reflecting the activity of the ciliates inside the droplets. Note that the signals recorded for a large fraction of the curves remained at the background level, indicating empty droplets. Approximately 50 droplets represented a positive signal increase over the time. The corresponding curves can be visually divided into groups, with respect to the measured rate of metabolic resazurin conversion: (i) single encapsulated *paramecia* with different metabolic activities (supplementary video S1);³⁶ (ii) two encapsulated ciliates (supplementary video S2);³⁶ and (iii) cell division event inside of the droplet (supplementary video S3).³⁶ The videomicroscopy observation of every filled droplet in the chain assisted to verify the status of the ciliates in droplets. Next, Fig. 3(c) demonstrates the corresponding metabolic curves recorded for single cells and two cells, respectively. Conversion rates were estimated via linear fitting of the obtained families of the curves. Naturally, a droplet housing two cells reveals about 30% higher resazurin conversion rate than a droplet containing just one cell (for comparison, $F_{2C}(x) = 0.29x$ versus $F_{1C}(x) = 0.2x$, respectively) and is characterized by a higher final fluorescence signal reached (see Figs. 3(b) and 3(c)). Furthermore, the metabolic curves, obtained for the single encapsulated ciliates reveal deviations in the conversion rate, as well as in the final reached signal, which could indicate the potential cell-to-cell heterogeneity within isogenic cell population, observed and actively investigated in bacterial cultures,^{22–24} in particular using droplets based techniques.²⁰ However, since data points taken from multiple droplets housing single cells in Fig. 4 obviously do not reveal the splitting into different subfamilies, and the observation time is too short for slowly dividing *paramecia*, we rather attribute these deviations to the effects of the different cell cycles.

B. Ecotoxicity assays

As a proof of principle for the application of the millifluidic analyzer in ecotoxicological testing, we characterize the toxicity of silver nitrate on the viability of *paramecia* cells and determine the so-called EC_{50} concentration, at which 50% of the introduced test organisms exhibit inhibition effects after a specified exposure duration (20 h). Toxicity analysis is performed using both millifluidic analyzer and conventional fluorescence based microplate reader technique, as a reference. For these purposes, the test organisms, *P. tetraurelia*, are cultured in ABC at 22 °C in the dark. Once a week, 0.5 ml of cell culture are transferred to 4.5 ml of fresh ABC under sterile conditions. The test system is previously described in detail elsewhere.⁷ In brief, the cell density of an axenic *P. tetraurelia* culture after incubation was determined, as described in the supplementary material (Section titled “Adjustment of cell density”).³⁶ For toxicity measurements, silver nitrate ($\geq 99.9\%$, p.a., Carl Roth GmbH) is used as a source of silver

FIG. 5. Summary of the ecotoxicity analysis, performed using a millifluidic machine. (a) The graph demonstrates the increase of fluorescence intensity in individual droplets, containing single ciliate cell, exposed to different concentrations of silver nitrate (0 mg/l—blue datapoints, 1 mg/l—green datapoints, and 1.5 mg/l—brown datapoints). (b) Histogram, summarising the viability of ciliates inside of droplets in percents (with respect to the total number of droplets), exposed to different concentrations of silver nitrate. (c) Concentration response curves drawn from probit functions, for determination of EC_{50} values. The black and red curves represent the toxicity assays realized in microtitre plate and using millifluidic device, respectively.

ions (Ag^+) in the solution. Silver nitrate stock solutions are prepared from high performance liquid chromatography (HPLC)-grade water (Chromanorm, VWR) at a concentration of 1 g/l. Silver nitrate pre-suspensions (diluted stocks) consisting of ABC medium and silver nitrate stock solution are prepared at concentrations representing the 11 fold target exposure concentrations. *P. tetraurelia* cell culture density is adjusted to 500 cells/ml by mixing respective amounts of cell culture and filtered ABC. The previously prepared $AgNO_3$ pre-solutions are mixed with the *P. tetraurelia* culture at a ratio of 1:10 to yield silver nitrate concentrations of 0, 1, 2, 4, 8, and 16 mg/l. In parallel, the blank samples are prepared with filtrated ABC medium. After 4 h of incubation, resazurin is added to the exposure solutions and blanks in a ratio of one part viability kit solution to eleven parts cell culture with $AgNO_3$. These exposure solutions are dispersed into the millifluidic system with droplet volumes of about 160 nl and 430 droplets per assay, as described above. Ten replicates of each exposure solution are filled in 96-well plates (pure grade S, transparent F-bottom, Brand GmbH & CO KG) at a volume of 110 μ l. Additionally, aliquots of the respective blanks are dispersed into six wells of the 96-well plate. Both assays are incubated for 16 h at room temperature (22 °C) in the dark. The fluorescence intensity for the 96-well plate is detected using a microplate reader (Tecan Polarion, Tecan Deutschland) with excitation wavelength of 560 nm and emission wavelength of 590 nm. Finally, the mean blank value of fluorescence intensity is subtracted from fluorescence intensities of the ten replicates for each exposure concentration.

Analogously to the microplate experiments, the exposure solutions, containing paramecia with the six different concentrations of silver nitrate in the range 0–16 mg/l, were sampled using the millifluidic machine. Note that the gradient of silver nitrate is not applied in these experiments, due to the low number of the droplets filled by paramecia and thus not sufficient statistical output of the assay. During the measurements, the data are sorted (empty/filled) according to the fluorescence spectrogram peaks (see Fig. 2(c)); only droplets filled with ciliates are used for further analysis. The mean fluorescence intensity of peaks in blank solution is subtracted from the measured peak maximum values. The number of peaks and blanked maximum fluorescence intensities are used for calculation of concentration response curves and respective half maximum effective concentrations (EC_{50}) values (see below). The data from both toxicity assays (microplate and fluidic) are analysed regarding concentration response curves and EC_{50} values using ToxRat® Standard Version 2.10 software. The viability of the cells versus silver nitrate concentration is fitted with probit function typically employed for toxicity analysis.

First, we visualize the activity of ciliates exposed to different concentrations of silver nitrate (from 1 mg/l to 16 mg/l) by recording their metabolic curves during 10–20 h. Furthermore, the percentage of droplets exhibiting positive growth are quantified at each concentration. Fig. 5 summarizes the ecotoxicity tests performed in 430 droplets, exposing the cells with density of about 500 cells/ml to different silver nitrate concentrations (see Section II). The curves, shown

in Fig. 5(a), correspond to the metabolic activity of the cells at 0 mg/l (blue curves), 1 mg/l (green curves), and 1.5 mg/l (brown curves), respectively. Naturally, the increase of the toxicant concentration leads to a decrease of cell activity, reflected in the slowdown of their metabolism, which corresponds to lower resazurin conversion rate (green and brown curves in Fig. 5(a)). After 20 h of measurements, we quantified the fraction of droplets still revealing fluorescent signal and summarized the analysis in Fig. 5(b). The histogram suggests that the substantial inhibition of the ciliate activity is achieved already at 2 mg/l of silver nitrate in the solution. These results allowed us to derive EC_{50} values, typically used for analysis of toxicity assays. For comparison, the experiments were carried out using conventionally applied microplate assay and the millifluidic device in parallel. The final fluorescence signal levels, averaged for each measurement, are drawn as a probit function with the log silver nitrate concentration (Fig. 5(c)). For the standard test assay, the determined EC_{50} was 3.4 mg/l within a 95% confidence interval of 2.6 to 4.4 mg/l. Goodness of fit was given with a $p(\text{Chi}^2)$ 0.9971 and $p(F)$ 0.004. For the millifluidic test assay (whole dataset analysis), the determined EC_{50} was 1.7 mg/l with a 95% confidence interval of 1.6 to 1.8 mg/l. Goodness of fit was given with $p(\text{Chi}^2)$ 1.000 and $p(F)$ 0.003. Compared to the standard assay, the concentration response curve of the millifluidic assay was steeper with only two determined values between zero and 100% inhibition. Statistical difference between the two EC_{50} values can be tested using Zajdlik' ad hoc method No. 1³¹ according to the equation

$$z = \frac{\log EC_{50_1} - \log EC_{50_2}}{\sqrt{(\sigma^2(\log EC_{50_1}) + \sigma^2(\log EC_{50_2}))}},$$

where σ is the standard error. If $|z|$ is larger than the critical value 1.96, the EC_{50} values are significantly different. Based on this equation, the nominal EC_{50} values of silver nitrate tests were compared for the two methods and proven to be not statistically different ($z = 0.785$) with $p(F) < \alpha$ ($\alpha = 0.05$). Still the small difference in the EC_{50} values could be caused by so-called inoculum effect, since the same cell densities were sampled into the reservoirs with different volumes (100 nl versus 110 μ l) and the resulting cell number per reservoir is distinct. Finally, an effect of unequal distribution of liquid in the millifluidic system regarding different exposure solutions and the resulting variance in peak numbers could also enhance the bias between the assays. For probit analysis, the number of peaks needs to be constant. The unequal peak numbers were adjusted filling data sheets with zero values representing the dead cells in droplets or empty and missing droplets. For the control samples, this leads to an apparently reduced number of surviving cells. In the highest exposure concentrations, probably many droplets were empty because the few surviving cells might easily escape the encapsulation by swimming actions. During encapsulation, a vertical striation was observed in the syringes filled with exposure solutions. Living cells tended to accumulate at the top of the syringes apparently by negatively geotactic movement. This problem could be overcome in the future by moving the syringes on orbital shakers during droplets' formation or by putting a small magnetic stirrer inside the syringes.

These findings indicate that the millifluidic system can generally outperform the available technology for toxicity testing in terms of statistical output, as well as pipetting work and thus handling time. One of the major benefits of this technique is the availability of data on a single cell level and thus opening possibility to address the cell heterogeneity issues. Compared to other available single cell techniques such as flow cytometry, millifluidics enable a high sample and replicate throughput at a large number of different exposure concentrations. For reason of comparability of the results obtained from the two techniques applied in this work, previously prepared exposure mixtures were used to feed the millifluidic device and the microtitre plates. The preparation of exposure mixtures shall be undertaken "online" with the dosage system within the millifluidic device in future experiments, in order to make use of its benefits mentioned above.

IV. CONCLUSIONS

Here, we present the *millifluidic* tool for isolating and further monitoring single ciliates from the water samples (or eventually cell culture) using a large number of water in oil (w/o) emulsion droplets. We present the viability curves recorded for every single paramecia cell, using the metabolism driven resazurin to resorufin conversion. Finally, we present the ecotoxicity tests in droplets, exposing the encapsulated paramecia cells to different concentrations of silver nitrate, and determine the critical levels of silver nitrate in the medium as EC_{50} . The ecotoxicity analysis obtained using millifluidic data is compared with the assay, conventionally performed in microtitre plate. While the millifluidic assay reveals the EC_{50} value to be about 1.7 mg/l, the standard test assay determines EC_{50} at 3.4 mg/l.

The further technological upgrades of this approach could enable the separation of the ciliates from the environmental water probes, via implementing droplet sorter for the qualitative and quantitative nanomaterials' detection, which to our knowledge is not realized at the moment. To realize it, the ciliates from the water sample have to be collected and investigated one by one to analyze the extracted content, which therefore reflects the concentration of the polluting materials in the matrix, as well as pollutants itself. It is therefore essential to develop and establish fast and efficient methods for high throughput *extraction* and *analysis* of ciliates from larger volume complex environmental matrices, e.g., activated sludge. The environmental concentrations of nanomaterials by now can only be approximated with exposure models,^{25–28} which are based on production volumes of the respective material. As registration of such materials was not obligatory for producers during the last decades, many assumptions had to be made for exposure assessment, which probably led to over- or underestimation of the environmental concentrations.

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