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Rapid serial diluting biomicrofluidic provides EC50 in minutes

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\section*{1. Introduction}

Water toxicity monitoring relies on manual sampling and lengthy protocols \cite{1}. Automated real-time toxicity monitoring would be an ideal alternative, as it could allow continuous water monitoring against bioterrorism, enforcing discharge regulations, and dynamic feedback in wastewater treatment plants. Inexpensive reagents such as resazurin, a green fluorescent dye that is metabolically reduced to pink fluorescent protein \cite{6}, are available to assess microorganism toxicity on the minute timescale. To rapidly evaluate unknown aqueous effluents, a segmented-flow microfluidic device is refined for real-time gross toxicity detection and quantification. A microfluidic chip integrating a magnetic stirrer for serial dilution is demonstrated to rapidly determine EC50 values of known and unknown toxicants, where 100 nL samples are suspended into a continuous oil phase with no interfering surfactants. A five logarithmic dilution sequence is evaluated in < 5 min, enabling feedback for dynamic processes e.g. dilution, ion exchange, or absorbant treatments. The resazurin-based assay is refined from traditional laboratory procedures to reduce sample volume and response time, with the advantages of short toxicant exposure (< 30 s), high density bacteria (10\textsuperscript{7} cell/mL), and continuous mixing in an oxygen-free medium. Simple scaling of the stirred chamber volume from 1 to 30 μL adjusts the number of samples from 100 to 2500 dilution droplets, respectively. A detailed analysis of the resazurin kinetics suggests the presence of two or more enzymes with distinct Michaelis constants. Enzyme kinetics and the resazurin reduction rate is dependent on growth phase and EC50 inhibition by mercury > zinc > copper > nickel cations. Resazurin concentration of 10 μM is optimal for \textit{Enterococcus faecalis} at 0.1 optical density. The adaptable method is transferable to other microorganisms, such as common baker's yeast, \textit{Saccharomyces cerevisiae}, where the resazurin reduction rate is 30% of \textit{Enterococcus faecalis} (4 nM/s vs. 13 nM/s per 100 nL droplet). Zinc and nickel cations are observed to increase the base resazurin reduction rate of baker's yeast by 25%, whereas copper is found to be more cytotoxic than mercury cations.

Complex water effluents pose a toxicity risk to biological wastewater treatments and environmental discharge. Dynamic sampling and risk assessment of effluents would mitigate downstream hazards, but few methods are available to determine organic toxicity on the minute timescale. To rapidly evaluate unknown aqueous effluents, a segmented-flow microfluidic device is refined for real-time gross toxicity detection and quantification. A microfluidic chip integrating a magnetic stirrer for serial dilution is demonstrated to rapidly determine EC50 values of known and unknown toxicants, where 100 nL samples are suspended into a continuous oil phase with no interfering surfactants. A five logarithmic dilution sequence is evaluated in < 5 min, enabling feedback for dynamic processes e.g. dilution, ion exchange, or absorbant treatments. The resazurin-based assay is refined from traditional laboratory procedures to reduce sample volume and response time, with the advantages of short toxicant exposure (< 30 s), high density bacteria (10\textsuperscript{7} cell/mL), and continuous mixing in an oxygen-free medium. Simple scaling of the stirred chamber volume from 1 to 30 μL adjusts the number of samples from 100 to 2500 dilution droplets, respectively. A detailed analysis of the resazurin kinetics suggests the presence of two or more enzymes with distinct Michaelis constants. Enzyme kinetics and the resazurin reduction rate is dependent on growth phase and EC50 inhibition by mercury > zinc > copper > nickel cations. Resazurin concentration of 10 μM is optimal for \textit{Enterococcus faecalis} at 0.1 optical density. The adaptable method is transferable to other microorganisms, such as common baker's yeast, \textit{Saccharomyces cerevisiae}, where the resazurin reduction rate is 30% of \textit{Enterococcus faecalis} (4 nM/s vs. 13 nM/s per 100 nL droplet). Zinc and nickel cations are observed to increase the base resazurin reduction rate of baker's yeast by 25%, whereas copper is found to be more cytotoxic than mercury cations.

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microplates, where the droplets act as distinct microreactors when separated by inert oil phases [9–13]. This design has the advantage of milliseconds delays from sample generation (e.g. water sample + bacteria + viability reporter) to fluorescent measurement, enabling faster kinetic assessments than manual protocols. Our previous publication solved a considerable hurdle of this microfluidic setup—incorporating a functional hydrophobic surface modification to remove all assay-interfering surfactants [14,15]. The novel disposable biomicrofluidic allows real-time monitoring of up to 100 droplet ‘microreactors’ independently, with the additional benefits of anaerobic conditions (not possible in PDMS) [13].

Here the platform is further expanded to generate logarithmic concentration gradients in < 3 min, designed with components for future portability (i.e. pressure-driven, magnetic stirring). These advantages are leveraged to explore the specificities of the resazurin reduction assay when performed in microfluidic format under continuous operation. The logarithmic serial dilution allows rapid evaluation of resazurin kinetics, Michaelis-Menten parameters, and toxicant(s) concentration. Five metals frequently found in wastewater were selected as model toxicants, from potent and non-essential (Hg2+) to essential (Cu2+, Co2+, Ni2+, Zn2+) and (D+) glucose were purchased from VWR, Singapore. Deionized water was purchased from Sigma Aldrich, Singapore. Yeast extract, peptone (7-Hydroxy-3H-phenoazin-3-one 10-oxide), resorufin (7-Hydroxy-3H-phenoazin-3-one sodium salt), CuSO4·5H2O, NiCl2·6H2O, HgCl2, CoCl2·6H2O, and brain heart infusion (BHI) broth were purchased from Sigma Aldrich, Singapore. Yeast extract, peptone and (D+) glucose were purchased from VWR, Singapore. Deionized water was used in all experiments. PMMA sheets 3 mm thick were obtained from Marga Cipta, Indonesia.

2. Experimental section

2.1. Materials and chemicals

Hexadecane, 99% and n-Dodecyltrichlorosilane (DTS), 96% were purchased from Alfa Aesar, USA. Colloidal silica Ludox TM-S0, resazurin (7-hydroxy-3H-phenoazin-3-one 10-oxide), resorufin (7-Hydroxy-3H-phenoazin-3-one sodium salt), CuSO4·5H2O, NiCl2·6H2O, HgCl2, CoCl2·6H2O and brain heart infusion (BHI) broth were purchased from Sigma Aldrich, Singapore. Yeast extract, peptone and (D+) glucose were purchased from VWR, Singapore. Deionized water was used in all experiments. PMMA sheets 3 mm thick were obtained from Marga Cipta, Indonesia.

2.2. Fabrication of PMMA microfluidic chip with integrated stirrer

Microfluidic chips were made by milling 3 mm thick PMMA sheets followed by solvent vapor adhesion, which is one of many methods available for bonding [16–19]. The design shown in Fig. 1 was created in Autodesk Fusion 360 and milled 100 μm deep on a desktop CNC mill (STEPCRAFT-2/300, Stepcraft). The main channel and inlets were 700 μm and 300 μm wide, respectively. A 1 μl or 30 μl chamber was also milled and a magnetic stirrer inserted (Fig. S1) before bonding as detailed in supporting information. Finally, the channels were coated with SiNPs and silanized with DTS, as previously reported [13], except the stirred chamber was blocked with paraffin wax prior to silanization to keep it hydrophilic. The magnetic stirrer was actuated at 800 rpm by an external magnetic field (UC151, Stuart).

2.3. Microfluidic device operation

Liquids were delivered from pressurized 15 mL centrifuge tubes as depicted in Fig. 1. On-chip serial dilution was achieved by running a diluent stream through the stirred chamber that was pre-loaded with a sample using a syringe pump (Mitos Duo XS-Pump, Dolomite). The outgoing stream with an exponentially decaying concentration was immediately segmented into droplets by a continuous stream of hexadecane, and up to two additional reagents (aqueous inlets 3 and 4) were injected continuously. For clarification, the inlet solutions are defined in the figure captions for each experiment, e.g. aq1 corresponds to aqueous inlet 1. Liquids were delivered from pressurized reservoirs, and their flow was turned on or off by manual rotary valves (P-732, Upchurch scientific) except for the diluent stream that was automated by computer controlled valve from the second channel of the pump (valve only, the syringe was removed). Flow rates ratio between inlets were set by fixed lengths of FEP tubings acting as hydraulic resistors, as detailed in the supporting information (Fig. S2A). The absolute flow rate was varied by adjusting the driving pressure generated by a peristaltic pump (KCS-N14-SB6A, KSP1302, Kamoer) and pressure sensor (24PCCFA6D, Honeywell), both controlled by a digital I/O card (Analog discovery, Digilent). During operation the pressure was adjusted to maintain a fixed incubation time through dynamic feedback of CCD camera-calculated droplet velocity as illustrated in Fig. S2B. Droplets tracking and fluorescence intensity recording was carried out as previously reported [13]. Briefly, the PMMA chip was placed in a custom holder (Fig. S3) and Fluorescence excitation was achieved by total internal reflection from 56 LEDs at 530 nm (151033GS0300, Würth Elektronik) surrounding the chip which were operated at a current of 5 mA. Images were acquired using a digital camera (acA1300-60gc, Basler) with a 12 mm focal length lens (M1214-MP2, Computar). C++ software was developed in-house using the OpenCV library [20] to simultaneously track and record fluorescence intensities of all droplets present in the chip.

Fig. 1. Microfluidic design for serial dilution with an integrated magnetic stirrer in 1.1 mm diameter chamber. Reservoirs are pressurized at the same pressure and the ratios between flow rates are pre-determined by the resistance of the different tubes. Logarithmic concentration gradients of a sample (aq1) diluted with a diluent (aq2) followed by two sequential reagents injection (aq3 and aq4) can be generated with simple shutoff valves.
2.4. Concentration gradient validation

The concentration gradient in droplets was calibrated via fluorescence measurements of resorufin dilutions. Resorufin (10 μM in PBS) was loaded as the sample (aqueous 1), while PBS was used as the diluent (aqueous 2) and additional reagents were injected as aqueous 3 and 4 (Fig. 1). A calibration curve for the serial dilution was obtained from the average intensity of the droplets in the incubation channel. The dilution sequence was repeated at least 3 times to determine the standard deviation of this method. No photobleaching was observed under the given parameters (Fig. S4).

2.5. Resazurin reduction kinetics by bacteria and yeast and toxicant inhibition curves

A series of experiments was carried out where the logarithmic gradients of resazurin, microorganisms, or toxicant were independently generated (aqueous 1 and 2) while the other two were injected at static concentrations at different inlets (aqueous 3 and 4). E. faecalis OG1RF (ATCC® 47077™) grown to stationary phase (period after log phase, where cell remains the same) in BHI broth was used as the sacrificial reporter. Unless otherwise indicated, 10 μM resazurin and sacrificial reporter (bacteria) were diluted to generate droplets with OD600 = 0.1 (optical density or absorbance reading at 600 nm), and the reservoir was kept on ice to prevent growth while the chip was maintained at 37°C. OD600 = 0.1 was estimated at 3.7 10^6 cell mL^−1 by the acridine orange direct count method [21]. Toxicant inhibition curves were also measured for Saccharomyces cerevisiae (commercial Baker yeast) regrown on yeast extract, peptone, dextrose (YPD) medium and an OD600 = 0.5 (OD 1 = 3E107 cells mL^−1) [22]. A magnetic stirrer was added to the yeast reservoir to prevent aggregation and sedimentation. Optical density of cell suspension in reservoirs was measured with 1 mm optical path (nanodrop 1000, Thermo Fisher) and normalized to 1 cm. Density in droplets was estimated from the dilution factor after all reagents were injected.

2.6. Determination of initial resazurin reduction rates and inhibition curves fitting

Fluorescence time course measurements of each droplet were acquired with the camera, and initial resazurin conversion rates were obtained from a linear fit of the fluorescence signals from 5 s after mixing the cells and 10% resazurin conversion [23,24] or the maximum incubation time was reached, as illustrated in Fig. S5A. Inhibition curves were obtained by fitting the conversion rates as a function of toxicant concentration with a 4 parameter logistic function (4PL), as shown in Fig. S5B. When data points in the lower plateau (i.e. complete inhibition) were not covered by the tested concentration range, the average rate in the negative control (without microorganisms) was used as a constraint for fitting. Reported standard deviations correspond to at least 3 independent dilution series (i.e. 3 samples loadings in the stirred chamber).

3. Results

3.1. Serial dilution profile is determined by the mixing chamber to droplet volumetric ratio

The microfluidic design incorporates many features for flexibility in fluorescent cytotoxicity analysis (see Fig. 1). Video algorithms calculate droplet velocity for feedback to the pressure-driven flow—permitting variable droplet incubation from 1 to 10 min in a 1-m long channel. Continuous droplet generation with sequential injection of 2 additional reagents is video monitored, providing temporal fluorescence data independently for all droplets. The PMMA microfluidic chip integrates a magnetic stirrer as an active mixing element to create a continuous logarithmic dilution of a pre-loaded sample. The aqueous stream exiting the stirred chamber is immediately split into aqueous droplets by a continuous stream of oil, removing time delays without Taylor-Aris dispersion. Assuming an ideal mixing chamber, the concentration is a function of time C(t):

\[ C(t) = C_0 e^{-Q t} \]

where \( C_0 \) is the initial concentration at \( t = 0 \), \( Q \) is the volumetric flow rate and \( V_c \) is the volume of the mixing chamber. Given the number of droplets generated \( x = \frac{Q t}{V_d} \) where \( V_d \) is the droplet volume, the concentration becomes:

\[ C(x) = C_0 e^{-\frac{x V_d}{Q}} \]

when \( V_d \gg V_c \) (i.e. when concentration in the chamber does not change significantly while a droplet is formed), it approximates the concentration of discrete droplets. The exponential decay lifetime \( V_d/V_c \) corresponds to the number of droplets required to reach ~37% of the initial concentration. Microfluidic chips are made with 1 and 30 μL chambers and the droplet volume is approximately 100 nL, as estimated from the channel cross-section and length. The gradient is characterized by dilution of fluorescent resorufin with PBS as a diluent. As shown in Fig. 2, when the stirrer is turned on, droplet fluorescence intensity follows an exponential decay, as predicted by Eq. 2. To analyse the effects of the mixing chamber, the stirrer is switched off and the data compared. Without mixing, the sample in the centre of the chamber is pushed out by advection resulting in the first few droplets at maximum concentration; as time progresses, the solute remaining in the chamber is eventually cleared by diffusion [25]. Despite the reproducibility, this regime is undesirable as the profile depends on the diffusion coefficient of the solute, which is unknown for most samples. The resolution of the gradient is determined by the chamber to droplet volumetric ratio; for example, high resolution provides better accuracy for the determination of EC50 from the dose response curves. EC50 refers to the toxic concentration that affects a 50% resazurin metabolism kinetics. This is beneficial for sharp sigmoid transitions where the region of interest is only covered by 1 or 2 points with traditional methods. However, higher resolution (i.e. more points) requires more time, and in the setup in Fig. 1, droplet generation rate is restricted by the incubation time. Time course curves are typically recorded for up to 3 min, corresponding to a droplet generation frequency of 0.5 Hz. Under these conditions, a single dilution sequence in Fig. 2A and B takes 3 min and 75 min, respectively. For continuous toxicity monitoring, this time corresponds to the sampling interval, thus a 1 μL chamber was selected in the remaining experiments as a compromise between assay time and resolution.

3.2. Continuous mixing in moving droplets increases the resazurin reduction rate

The microfluidic design aims to allow for the real-time recording of metabolic conversion of moving droplets independently, and hence under continuous mixing [13]. Mixing effects are further explored by measuring the resazurin reduction rate as a function of droplet velocity (i.e. mixing efficiency and incubation time). Resazurin is incubated with E. faecalis containing droplets with a final concentration of 10 μM and OD600 = 0.1, respectively. As shown in Fig. 3, the resazurin reduction rate first increased rapidly with increasing droplet velocity up to 3 mm s^−1 before stabilizing to about 3.0 μM min^−1. In order to maximize the fluorescent signal at the end of the incubation channel, a velocity of 4 mm s^−1, corresponding to an incubation of 3 min, is standardized in the following investigations.

3.3. Apparent toxicity increases with decreasing bacterial density

To test the effect of bacterial density on the sensitivity of the assay
towards a toxicant, a fixed concentration of copper ions known to cause partial inhibition from preliminary experiments is incubated with varying bacterial densities. Droplets containing serial dilutions of *E. faecalis* harvested in the stationary phase, 0 or 6 mg L$^{-1}$ Cu$^{2+}$ and 10 $\mu$M resazurin are generated to assess the effect of bacterial density on the apparent toxicity of copper. As shown in Fig. 4A, the resazurin conversion rate varied linearly with bacterial density in the range ~0.01–0.50, estimated as a bacterial density from 10$^6$ to 10$^9$ cell mL$^{-1}$.

For a given copper concentration, the apparent toxicity increases with lower bacterial densities down to 0.01 OD$_{600}$, observed in Fig. 4B. Increase in apparent toxicity is expected at lower bacteria densities because of a higher toxicant to biomass ratio. However, this is limited by the low signal to noise ratio when approaching the background signal, and the short 3 min incubation time in the microfluidic device. An optical density of 0.1 is chosen as a compromise between response linearity and the limit of detection.

### 3.4. Kinetics analysis suggests multi-enzyme reduction of resazurin to resoruvin

Droplets containing a serial dilution of resazurin, and a fixed density of *E. faecalis*, are generated to optimise the initial resazurin concentration. Initial reduction rates are calculated from fluorescence time course curves to measure the apparent Michaelis constant ($K_m$). As displayed in Fig. 5A, the enzymatic velocity (i.e. resazurin reduction rate) increased sharply at low substrate concentrations, and then appears linear at higher substrate concentrations, deviating from the Michaelis-Menten (MM) model. Departure from linearity is evident in the Lineweaver–Burk plot, as seen in Fig. 5B, and indicates the presence of multiple enzymes/substrate affinities [26]. The total velocity $v$ of two enzymes converting the same substrate in parallel is given by the summation of two MM processes:

$$v = \frac{[S]V_{max1}}{K_{m1} + [S]} + \frac{[S]V_{max2}}{K_{m2} + [S]}$$  \hspace{1cm} (3)
where \([S]\) is the substrate concentration, \(V_{\text{max}1}\) and \(V_{\text{max}2}\) are the maximum velocities and \(K_{\text{m}1}\) and \(K_{\text{m}2}\) are Michaelis constants that should differ by at least a factor 20 for the deviation to be apparent \([27]\). Experimental velocity profiles are compatible with this 2 enzyme model, suggesting the presence of two (or more) enzymes that are responsible for resazurin reduction to resorufin. It should be noted that estimated \(K_{\text{m}2} = 140 \mu M\) was extrapolated because higher substrate concentrations are not possible due to the limited solubility of the resorufin product (~10 \(\mu M\) in PBS).

### 3.5. Ratio between enzymes reducing resazurin rapidly changes during the late log phase

Standard assay protocols recommend exploiting bacteria in the exponential stage of growth \([28]\). However, this poses a problem for continuous toxicity monitoring because the long term maintenance of a cell in the log phase (period of exponential growth) requires a chemostat, thus introducing additional reagents, wastes and overall complexity. The impact of growth phase on the resazurin reduction kinetics is assessed by repeatedly measuring the resazurin kinetics during bacterial growth. Instead of culturing bacteria beforehand, they are used immediately after inoculation and the reservoir is maintained at 37 °C. Continuous monitoring resulted in a new dilution sequence of resazurin every ~7.5 min. Optical density is also measured by manual sampling every 30 min to help distinguish between contributions to the overall reduction rate from the number of bacteria, and from variations in metabolic activity. Overall the resazurin reduction rate increased as the bacteria grew, but the shape of the substrate dependent kinetics changed from quasi-linear to hyperbolic and back towards linear, as shown in Fig. 6A. Curves were globally fitted with Eq. 3 for 2 enzyme kinetics with shared \(K_{\text{m}1}, K_{\text{m}2}\). Note that this assumes that the enzyme concentration related to \(V_{\text{max}1}, V_{\text{max}2}\) changes, but not their Michaelis constants. This assumption is necessary because fitting individual curves with 4 parameters results in overparameterization for more
linear curves. Maximum velocities are plotted as function of culture time in Fig. 6B. According to this model, the concentration of both enzymes increased with bacterial growth, but their ratio varied substantially. The enzyme with the lower Michaelis constant $K_m \approx 3 \mu M$ increased during the early log phase before suddenly decreasing at the 4-h mark. The other enzyme, in contrast, increased monotonically following the bacterial density trend, albeit not proportionally, because of a non-linearity at high bacterial density, as shown in Fig. 4.

3.6. Copper and mercury inhibit the resazurin reduction by different pathways

The same kinetic study is repeated with bacteria at an OD$_{600}$ of 0.1, but with the addition of a fixed amount of mercury or copper at partially inhibitory concentrations. As shown in Fig. 6C, apparent inhibition by copper increases along with substrate concentration, while inhibition by mercury was predominant at low substrate concentrations. As a consequence, the choice of resazurin concentration and growth phase can dramatically change the apparent toxicity when assessed by the resazurin assay. For instance, with a low resazurin concentration (10 $\mu M$), copper appears more toxic in the stationary phase than during logarithmic growth, as detailed in supporting information (Fig. S7). A reverse trend is expected for mercury or at higher resazurin concentrations.

3.7. The resazurin assay with *E. faecalis* in droplet format is more sensitive towards copper and zinc compared to reported cytotoxicity

Once the bacterial growth phase, density and resazurin concentration are optimized, inhibition curves are measured independently for 5 heavy metals; zinc, cobalt, copper, mercury and nickel. Droplets containing a serial dilution of the metals (500 mg L$^{-1}$ stock) were generated followed by sequential injections of *E. faecalis* and resazurin. Some of the metals precipitated when injected, likely because of a change in solubility induced by the buffer [29]. At higher cobalt concentrations, interference with resazurin or resorufin impurities is also observed in negative controls (no bacteria). Therefore, this portion of the data is excluded. Precipitates are observed with Hg$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ resulting in droplet pinning and cross-contamination between droplets. Stock concentrations are adjusted accordingly: Cu$^{2+}$ 100 mg L$^{-1}$, Zn$^{2+}$ 50 mg L$^{-1}$ and Hg$^{2+}$ 25 mg L$^{-1}$. The apparent toxicity ranking based on their EC$_{50}$ is Hg$^{2+}$ > Zn$^{2+}$ > Cu$^{2+}$ > Ni$^{2+}$ > Co$^{2+}$, as shown in Fig. 7. In the case of Hg$^{2+}$ and Zn$^{2+}$, a plateau is reached before complete inhibition in which case the apparent EC$_{50}$ is reported. This
may be attributed to a few causes; 1) less toxic precipitates than their cation counterparts, or 2) inhibition of only one of the enzymes reducing resazurin. The EC80 values obtained from the resazurin assay in the microfluidic format are compared to MICs (minimum inhibitory concentration) previously reported, as listed in Table 1. Values calculated herein are comparable for Hg2+, whereas the Co2+ and Ni2+ EC80 values are substantially higher, bearing in mind that these are only extrapolations because complete inhibition was not reached in the concentration range tested. The EC80s for Cu2+ and Zn2+ are one to two orders of magnitude lower than their reported MICs.

3.8. Resazurin reduction in yeast is inhibited by copper but increased by zinc and nickel

Commercially available Baker’s yeast is a universal bioreporter that requires minimal preparation with the advantage of a long shelf life. Typical effluent cations (Hg2+, Cu2+, Co2+, Ni2+, Zn2+) are evaluated with Saccharomyces cerevisiae obtained from a local supermarket (active
Table 1
Comparison of heavy metal toxicity to E. faecalis from resazurin reduction EC80 in the microfluidic droplet format (3 min), and literature reported MICs based on growth inhibition on agar plates (14-24 h). Concentrations are in (mg L⁻¹).

| Resazurin EC80 (this work) | Hg²⁺ | Zn²⁺ | Cu²⁺ | Ni²⁺ | Co²⁺ |
|---------------------------|------|------|------|------|------|
| MIC                       | 2.6  | 5.1  | 28.6 | 1428 | 9000 |

Table 2, the reported EC50s vary by orders of magnitude among laboratories, even when similar methods are compared. A possible reason for these discrepancies is a difference in the bioavailable form of the cations (e.g. free cation, chelated cation, or protein bound cation), which depends on the culture medium [29]. The closest comparison to the technique developed herein is with a microplate toxicity screen for fungi/cides based on Saccharomyces cerevisiae and E. faecalis previously developed by Fai et al. [12]. Similar EC50 for Cu²⁺ are observed. The advantage of this microfluidic droplet assay vs. Fai’s microplate is the incubation time of 3 vs. 70 min. Further increases in sensitivity are possible with yeast as demonstrated for E. faecalis above.

4. Discussion

The resazurin assay is a robust and reliable method for cytotoxicity, and antibiotic screening, and bacterial contamination evaluation [28]. Our laboratory has previously demonstrated the method as an early warning assay for anaerobic digestion, where resazurin to resorufin (fluorescent) metabolism is only observed intracellularly [9-11,30,31]. However, as a laboratory based assay it is time and labour intensive, requires oxygen-free hoods, and does not present a suitable alternative for continuous cytotoxicity monitoring. To automate the process, development in hydrophobic surface modification of non-PDMS materials needed to be advanced to incorporate it in a microfluidic platform—allowing surfactant and low-oxygen conditions [13]. PDMS-based microfluidics are oxygen permeable, interfering with anaerobes and anaerobic respiration of facultative bacteria [32]. For the first time, a disposable PMMA microfluidic method is demonstrated that generates five logarithmic concentration gradients and simultaneous droplet spectrofluorometry with < 5 μL of sample. This achieves a sensitive EC50 value within 3 min (with a 1 μL mixing chamber), allowing feedback to downstream industrial processes e.g. remediation by dilution, ion exchange, or additives (nanoabsorbants) to mediate heavy metal cytotoxicity [33-35].

Concentration gradients in microfluidic devices can be obtained by adjusting the relative flow rates between reagents [36], but this requires complex pumps, and is practically limited to 2 logarithm dilution because flow rates must vary within the same dynamic range. Concentration gradients obtained by segmentation of a Taylor-Aris dispersion profile from a sample plug into droplets has been reported [37]. Nevertheless, for mixed or unknown samples a method independent of the diffusion coefficient simplifies reliability. Previously reported droplet traps in microfluidic serial dilutions suffer from incomplete mixing of the droplet content between 2 merge and split operations [38], or required excessive amounts oil/water ratios between droplets to passively stir the trap [39]. Gien et al. created gradients by generating droplets from an open micro well continuously mixed with a stirrer bar, which can be contaminated by the external environment [40]. To advance these designs, herein a custom magnetic stirrer was directly integrated into the chip as an active mixing element. Pressure driven flow is coupled to CCD-camera droplet velocity, which achieves a tunable incubation time from 1 to 10 min with inexpensive hardware components. Introduction of active stirring elements has the advantage of allowing a wide range of flow rates, and simple scaling of the stirred chamber volume from 1 to 30 μL adjusts the number of samples from 100 to 2500 dilution droplets (Fig. 2).

The gradient can be used to create dose response curves (Fig. S5B), but more generally reproduce assays comprised of a serial dilution followed by sequential addition of 2 other reagents in droplet format. Given the wide variations in reported protocols, and differences inherent in the microfluidic format, extensive validation was necessary. A major difference from a microplate format is the continuous mixing in moving droplets and rapid spectrofluorometric measurements after toxicant addition. Complex recirculating streamlines are formed in moving droplets, thus enhancing mixing and mimicking shear rates typically found in laminar flows [41]. Complete mixing requires the droplets to move 10–30 times their length, as shown experimentally [42] and by simulations [43]. Since resazurin reduction only occurs inside the cells [11], continuous mixing can mitigate or prevent local depletion of the substrate due to bulk phase diffusion by reducing the laminar boundary layer around the cell. The metabolic increase with droplet velocity and saturation at 4 mm s⁻¹ of the resazurin reduction rate is consistent with a transition from a bulk diffusion limited regime to a kinetic limited regime (Fig. 3). Consequently, the assay performed above this critical velocity reduces the detection time, but also increases the sensitivity towards toxic inhibition as bulk phase diffusion is no longer a limiting factor.

The assay time can also be optimized by bacterial density and resazurin concentration, in droplet format. Sample generation and fluorescence measurements are carried out with 100 ms resolution. Therefore, bacterial densities with complete conversion within minutes is possible within this biomicrofluidic, whereas microplate measurements would result in complete reduction before the first timepoint is completed. There is, however, a trade-off as a lower toxicant to biomass ratio makes the assay herein less sensitive (Fig. 4).

The incubation time cannot be increased by changing the length of the incubation channel because of the limited space available, and decreasing the flow rate/droplet velocity would compromise the mixing efficiency and raise probability of droplet pinning (< 1 mm s⁻¹). Therefore, a bacterial density of 3.7 10⁷ cell mL⁻¹ at 4 mm s⁻¹ was optimal for E. faecalis. Incubation time could be extended by modifying the channel dimensions greater than the 6 cm × 6 cm demonstrated, but this requires further optimization in terms of channel width, channel spacing, peripheral LED placement (see Fig. S3A), and CCD camera resolution. At this time priority was given to rapid production by milling to demonstrate a readily available method of fabrication, but more elegant methods are available to increase the channel width and length (e.g. photolithography).

The initial resazurin concentration can influence the apparent toxicity, but there is no consensus in the literature of an optimal amount. Concentrations ranging from 6 μM to 3 mM have been reported [44,45], while the manufacturer’s instructions for Alamar blue (and similar commercial kits) result in samples with 44 μM resazurin [46]. Justifications, when given, are usually maximization of the fluorescence signal, or linearity with respect to cell density. Inner filter effects from concentrated resazurin solutions (e.g. above 34 μM for 1 cm optical path [47]) are also widely overlooked. These impediments are avoided through optimizing resazurin concentration based on reduction kinetics. In order to detect potential competitive, non-competitive and uncompetitive inhibitors, the substrate (i.e. resazurin) should be equal.

dry yeast). Droplets containing a serial dilution of the metals are generated followed by sequential injections of yeast (~1000 cells per 100 nL droplet) in aq3 followed by resazurin in aq4. The apparent toxicity ranking based on EC50 is Cu²⁺ > Hg²⁺ > Co²⁺, as shown in Fig. B. Cation Cu²⁺ is five times more inhibitory to yeast than E. faecalis, while the other cations have less metabolic effect. Cation Zn²⁺ (8–30 mg L⁻¹) and Ni²⁺ (10–100 mg L⁻¹) had the effect of increasing resazurin reduction rates up to 25%.

Saccharomyces cerevisiae’s ubiquitous status in bioassays allows comparisons across cytotoxicity methods. However, as presented in Table 2, the reported EC50s vary by orders of magnitude among laboratories, even when similar methods are compared. A possible reason for these discrepancies is a difference in the bioavailable form of the cations (e.g. free cation, chelated cation, or protein bound cation), which depends on the culture medium [29]. The closest comparison to the technique developed herein is with a microplate toxicity screen for fungi/cides based on Saccharomyces cerevisiae and resazurin previously developed by Fai et al. [12]. Similar EC50 for Cu²⁺ are observed. The advantage of this microfluidic droplet assay vs. Fai’s microplate is the incubation time of 3 vs. 70 min. Further increases in sensitivity are possible with yeast as demonstrated for E. faecalis above.
to the Michaelis constant $K_m$ of the enzyme involved [48]. However, the situation is complicated by a deviation from Michaelis-Menten kinetics, indicating the presence of multiple enzymes reducing resazurin (Fig. 5). At least two enzymes, dihydrolipoamine dehydrogenase and NADH dehydrogenase, have separately been shown to reduce resazurin in vitro, but exactly which enzymes are primarily responsible for resazurin reduction in-vivo is still up for debate [46]. Another common deviation from the Michaelis-Menten model for in-vivo kinetics is due to diffusional limitations across the cell membrane [49], and around membrane-bound enzymes [50]. However membrane diffusion limitations with a single enzyme should result in a more linear curve compared to the Michaelis-Menten model, including in the low substrate region. A more likely explanation is that a first enzymatic reaction is rapidly saturated with increasing substrate concentration, while a second enzymatic reaction is limited by membrane diffusion, thus resulting in the linear increase in reduction rate at higher substrate concentration.

Since the assay can be performed in < 5 min, this enables a time-
Table 2

Comparison of heavy metals toxicity to Saccharomyces cerevisiae using the re-
sazurin reduction EC50 in a microfluidic droplet format (3 min), and literature
reported data. Concentrations are in (mg L$^{-1}$) [12].

|            | Cu$^{2+}$ | Hg$^{2+}$ | Co$^{2+}$ | Ni$^{2+}$ | Zn$^{2+}$ |
|------------|-----------|-----------|-----------|-----------|-----------|
| Resazurin (this work) | 2.0 | 33 | > 40 | > 300, rate variation increased | > 30, rate increased |
| Tetrazolium [73] | 79 | 101 | 615 | 20 | 19 |
| Tetrazolium [74] | 5.6 | 0.8 | 23 | 117 | 164 |
| Optical density | | | | | |
| [75] | | | | 11.4 | 75 |
| Optical density | | | | | 1.5 |
| Resazurin [12] | | | | | 604 |
| Optical density [12] | | | | | |

dependent analysis to be carried out which revealed that the ratio be-
tween the enzymes changes with different stages of bacterial growth (Fig. 6B).
Therefore, bacteria in the stationary phase are preferable for continuous monitoring because they introduce less variability with re-
spect to harvesting time, and are easier to maintain. It is generally as-
sumed that the inhibition of resazurin reduction indicates general cy-
toxicity or a reduction in cell metabolism. However, the different inhibition profiles from mercury and copper ions (Fig. 6C) suggest a more complex mechanism, with inhibition of specific enzymes reduc-
ing resazurin, or at least inhibition of distinct metabolic pathways, rather than indicating an overall decrease in cell viability. Other compound such as dicumarol can specifically inhibit enzymes reducing resazurin without being cytotoxic [51]. As a consequence the choice of resazurin concentration and growth phase may shift the overall sensitivity, but also the relative sensitivity towards different toxicants. For these rea-
sons, 10 μM resazurin and E. faecalis in the stationary phase were chosen for toxicity experiments, but future work will discern if specific resazurin reduction profiles may predict the specific metabolic toxins or dominant bacterial species.

The dose dependent inhibition curves with optimized assay parameters for five heavy metals (Hg$^{2+}$-Cu$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Zn$^{2+}$ – Fig. 7) do not necessarily correspond to cytotoxicity. In particular, the EC20 for Cu$^{2+}$ (28.6 mg L$^{-1}$) and Zn$^{2+}$ (5.1 mg L$^{-1}$) are far below reported MICs from growth inhibition assays [52,53]. This behaviour can be anticipated from the detailed kinetic studies above, and can be ex-
plained by an important reorganization of metabolic pathways that occurs at these concentrations which will be reflected by the rate of resazurin reduction [54-56]. This non-ideal behaviour of the resazurin assay is often overlooked; furthermore, the short exposure time in the droplet format (30 s) might accentuate the effect of metabolic activity on resazurin reduction without necessarily reflecting cytotoxicity be-
cause resistance mechanisms such as efflux pumps require longer timescale [57,58]. Deviation from an ideal viability indicator was also observed with yeast instead of bacteria (Fig. 8), where the reduction rate increased with addition of zinc and nickel. This could have been due to a variety of reasons, including activation of metal dependent enzymes [59,60], impaired mitochondrial oxidation [61], or an in-
crease in cellular activity as part of a toxicity resistance mechanism [62,63]. Stimulation is plausible, at least for zinc at tens of mg L$^{-1}$ [64,65], in which case the assay might be exploited for heavy metal stimulations [10]. Complementary assays with longer exposure times would, however, be needed to verify if the increased rate is sustained or temporary as a result of toxicity. Regardless of the mechanism, for the purpose of detecting toxicants in water, microorganisms can act as a “canary in a coal mine”. Therefore, their fate is not essential as long as the response is repeatable and the dynamic range of responses matches the application needs. For instance, E. faecalis would be a poor indicator for drinking water safety, but has considerable potential as an early warning indicator for wastewater bioreactors. For instance, Chen et al. previously reported that the addition of Ni$^{2+}$ or Co$^{2+}$ to anaerobic sludge at 5 mg L$^{-1}$ can stimulate the methane production, while 250 mg L$^{-1}$ are inhibitory [10], which is matched by the detection range from E. faecalis. One drawback of this approach would be that an extensive validation is required, including different types and combina-
tions of toxicants being needed to ensure overall correlation between both.

Overall, these results demonstrate that caution is warranted inter-
preting resazurin assay data, and inhibition of the reductase enzymes responsible for resazurin metabolism does not always reflect cell cyto-
toxicity. Although the resazurin assay is most often referred to as a viability assay, following the distinction made by Kwolek-Mirek et al., it would be best classified as a vitality assay [66]. The microfluidic ver-
sion of the resazurin assay is automated and works in continuous mode, which makes it a potential alternative to genetically engineered bior-
reposers for water toxicity monitoring [67]. It does not give the option to target specific compounds, but for gross toxicity it presents several advantages: 1) the response time is < 5 min compared to 0.5 to 2 h for engineered bioreporters [68], 2) whole effluent toxicity is readily quantifiable by on-chip serial dilution [69], 3) it can be adapted to a variety of microorganisms without genetic modifications, and 4) like in traditional bioassays, fresh samples are independent of previous ex-
posure to toxicants and matrix interferences are detected in control samples. Continuous reagents consumption can be a drawback, but resazurin is inexpensive and many alternatives are available for passive delivery [70,71], while the continuous oil phase is easily recycled. However, some limitations of the biomicrofluidic need to be addressed by future work. The main limitation is its restriction to hydrophilic toxicants and fluorescent metabolites—those that are hydrophobic or with a logP > 2 will partition into the oil phase and report an unusually high EC50. This can be addressed with fluorocarbon or silicone solvents as the oil phase. Droplet velocity is limited between 1 and 10 mm.s$^{-1}$, as slower rates raise the possibility of droplet pinning. Faster rates in-
crease the complexity of chamber mixing and inlet reagent injection.

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Conflicts of interest

There are no conflicts of interest to declare.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://
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