

Heat-Transfer Method (HTM): A Thermal Analysis Technique for the Real-Time Monitoring of *Staphylococcus aureus* Growth in Buffered Solutions and Digestate Samples

Kai Betlem¹, Amanpreet Kaur¹, Alexander D. Hudson¹, Robert D. Crapnell¹, George Hurst², Pankaj Singla⁴, Mikhajlo Zubko³, Silvia Tedesco², Craig E. Banks³, Kathryn Whitehead³, and Marloes Peeters^{5*}

1. Division of Chemistry and Environmental Science, Faculty of Science and Engineering, Manchester Metropolitan University, John Dalton Building, M15GD, Manchester, UK

2. Division of Engineering, Faculty of Science and Engineering, Manchester Metropolitan University, John Dalton Building, M15GD, Manchester, UK

3. Division of Biomedical Science, Faculty of Healthcare Science, Manchester Metropolitan University, John Dalton Building, M15GD, Manchester, UK

4. Department of Chemistry, UGC-centre for Advanced Studies-I, Guru Nanak Dev University, Amritsar 143005, India.

5. Newcastle University, School of Engineering, Merz Court, Newcastle Upon Tyne, NE1 7RU, United Kingdom.

* Correspondence: marloes.peeters@newcastle.ac.uk, Phone: +44 (0)191 208 8281

Abstract: The identification and quantification of microorganisms in water samples is crucial to improve processes in organic waste treatment facilities. Most of the currently available tests are either labour-intensive or costly, and they do not allow determination of the dynamics within microbial communities in digestate samples. This study is the first report on the use of thermal analysis, specifically the Heat Transfer Method (HTM), to monitor microbial load in aqueous systems and digestate samples. *Staphylococcus aureus* was used as a model organism and different concentrations in water were measured by HTM. It was demonstrated that there was a positive correlation between the thermal resistance and concentration of the bacterial cells. Subsequently, the influence of temperature on growth rates was studied and confirmed by plating experiments and Scanning Electron Microscopy (SEM). These results showed the possibility to monitor the temperature dependent growth of *S. aureus* using HTM. To determine if this technique can be applied for studying complex matrices, digestate samples were collected from a number of sources and plated on nutrient agar plates. The bacterial cultures derived from

single colonies were characterised and identified by sequencing of DNA regions for 16S ribosomal RNA. HTM measurements were performed in diluted or centrifuged digestate samples that were enriched with *S. aureus*. The results indicated that it is possible to evaluate microbial load even in samples containing other organic material. The thermal analysis method has the potential to provide a low-cost monitoring option, which is simple to use and provides *real-time* analysis, thus improving the existing monitoring procedures in organic waste treatment facilities.

Keywords: Microbial growth, Heat transfer method (HTM), Water Quality, *S. aureus*, Organic waste treatment

Corresponding author: Dr. Marloes Peeters, phone: +44 (0)191 208 8281

e-mail: marloes.peeters@newcastle.ac.uk

1. Introduction

The introduction of molecular techniques in wastewater microbiology made it possible to determine the species composition of microbial communities in these complicated and dynamic systems [1]. Knowledge about the microbial community structure is crucial to understand biodegradation pathways of organic pollutants within organic waste treatment facilities [2]. Organic pollutants can be resistant to degradation by microorganisms and therefore persist in wastewater, which compromises water quality [3]. Bioaugmentation (the addition of microorganisms to wastewater) is a low-cost and environmental friendly biodegradation method as compared to physiochemical approaches for removal of organic pollutants [4]. While this approach has been used for decades, there are numerous reports on bioaugmentation failure due to difficulty in monitoring interactions between the inoculated organisms and the host ecosystem [5]. In addition, membrane bioreactors that are used for filtration are prone to biofouling, and this can hamper their efficacy to degrade organic pollutants [6].

Besides issues related to biodegradation processes, there are concerns about the incidence of bacteria resistant to antimicrobials in organic waste treatment facilities [7,8]. These microorganisms are potential hot spots for spreading of antibiotic resistance since antibiotic residues and other substances with potential selective pressure can be present in high quantities within sewage water [9]. Conditions during the wastewater treatment process, such as the presence of chemicals and changes in temperature, can favor horizontal gene transfer [10].

Therefore, it is of a great interest to monitor bacteria in effluent streams in *real-time* to gain insight in changes of their structural and biological properties.

The harsh conditions in wastewater, including pH levels, contaminants, and solid fraction, complicate the development of biosensors for measuring microbial load in digestate samples. Fluorescent *in-situ* hybridization technique (FISH) allows characterisation of bacterial populations in complex ecosystems [11]. Culturing assays, such as the Total and Fecal Coliform Assay, rely on the use of indicator organisms to estimate the number of pathogens in wastewater samples [12]. The cultivation and analysis of these indicator microorganisms is a labour-intensive and time-consuming process [13], which can be overcome by the use of nucleic acid-based methods. Real-time PCR is a fast method and can be combined with pre-treatment steps that involve the addition of intercalating dyes, which allow to only monitor detection of DNA from viable microorganisms. It is possible to discriminate between deceased and living bacteria with this method [14,15]. However, this method would not allow evaluation of the dynamics in bacterial communities and influence of stochastic factors [16]. Table 1 provides an overview of these techniques and provides further information on the detection time, the range of detection and the ability to detect viability of the sample along with an indication of the setup and operational costs.

Table 1. Comparison between conventional methods for the determination of bacterial load [17,18].

Method	Time to detection	Limit of detection (CFU/mL)	Relative standard deviation	Equipment needed
Plate counting	Days	$\leq 10^4$	>10%	Plate reader (optional)
Quantitative polymerase chain reaction	Hours to days	$\leq 10^4$	$\leq 10\%$	PCR
Fluorescence microscopy	Hours	$\leq 10^4$	>10%	Microscope
Enzyme-linked immunosorbent assay	Hours	$\leq 10^4$	$\leq 10\%$	ELISA

Fluorescence-based microplate readers	Minutes	$\leq 10^6$	$\leq 10\%$	Fluorescence plate reader
Flow cytometry	Minutes	$\leq 10^4$	$\leq 5\%$	FACS

To the authors' knowledge, this work is the first report on the use of the Heat-Transfer Method (HTM) for the monitoring of bacterial growth in buffered solutions and complex wastewater samples. The principle of this thermal measurement technique is based on the monitoring of heat-flow at the solid-liquid interface and was first applied to studying DNA mutations and cell concentrations using Surface Imprinted Polymers as recognition elements [19,20]. Advantages of this method include low-cost of the electrode chips and equipment used, ease of measurement procedure, ability to operate under harsh conditions, and portable design of the device [19]. Recently, Betlem *et al.*, [22] demonstrated that it is possible to study growth of yeast in *real-time* with the use of gold electrodes. In order to facilitate longitudinal experiments, it was necessary to adapt the flow cell design.

In this study, the same design was employed to monitor growth of *S. aureus* in solution by using thermal analysis. Bacteria can be motile in liquid media, and choice of electrode material as well as nutrients are key in designing the appropriate experimental factors [23]. *S. aureus* is a non-motile microorganism and, although it is a common member of the microbiota in the body, it is an opportunistic pathogen that is the frequent cause for skin and respiratory infections [24]. It is one of the most common causes for hospital-acquired infections and recognized as a worldwide problem in clinical medicine due to the emergence of antibiotic-resistant strains including methicillin-resistant *S. aureus* (MRSA) [25]. The aim of this work was to demonstrate that thermal analysis could be used for determining bacterial load. Therefore, the influence of temperature and the presence of other microorganisms as well as organic material on the growth rate of *S. aureus* was evaluated. Complex digestate samples contain sediments, which required modifications in sample handling and pre-treatment, were further studied to demonstrate proof-of-application of the developed sensor platform. It is possible that this experimental set up could be used for determining water quality and studying the influence of contaminants, such as micropollutants, on growth and structural properties of bacteria.

2. Experimental

2.1 Media

Yeast extract, peptone bacteriological, nutrient agar (NA), nutrient broth, agar bacteriological (agar NO.1), D (+)-glucose, glycerol were purchased from Fisher Scientific, United Kingdom. Adenine sulfate was purchased from Alfa Aesar, United Kingdom. Yeast extract peptone dextrose (YEPD) and nutrient broth were standard liquid growth media (for yeasts and bacteria respectively). YEPD included 1% yeast extract, 2% peptone bacteriological, 2% D (+)-glucose and 0.03% adenine sulfate.

2.2. Electrode preparations

Silicon chips (1 cm × 1 cm, Agar Scientific Ltd., Essex, UK) with a thickness of 450 μm in (100) crystalline orientation were modified with a doped gold layer. Silicon substrates were initially covered by 20 nm adhesive layer of chromium which was followed by deposition of a 100 nm layer of gold, as previously described in ref 20. Gold doping was carried out using physical vapor deposition at 5×10^{-5} Pa. Glass microscopy slides (thickness 1-1.2 mm) were cut to 1 cm x 1 cm with a diamond scribe and cleaned with a standard Radio Corporation of America (RCA) cleaning protocol, involving washing with water, ammonia, hydrogen peroxide, and subsequent drying. Each electrode was freshly cleaned prior to analysis.

2.3 Cultivation of *S. aureus*

The strain of *Staphylococcus aureus* (ATCC 9144) was obtained from the microbiological laboratory of Faculty of Healthcare Science at Manchester Metropolitan University. Twenty millilitres of nutrient broth was inoculated with a single colony of *S. aureus* for the overnight growth at 37 °C while shaken at 150 rpm. Cells were harvested by centrifugation (3000 rpm for 10 minutes) and the pellet was washed with 20 mL of sterile (autoclaved) water. Finally, the cells were re-suspended in sterile water until an OD of 1.0 ± 0.1 ($\lambda = 600$ nm) was obtained, which corresponded to a concentration of 1.0×10^7 colony forming units per mL (CFU/mL), as determined by colony counting. Samples were diluted to the density 1.0×10^2 CFU/mL and plated out on NA.

To determine the attachment of *S. aureus* to the gold surface, SEM measurements were recorded on a Supra 40VP Field Emission from Carl Zeiss Ltd (Cambridge, UK) with an average chamber vacuum of 1.3×10^{-5} mbar and average gun vacuum of 1×10^{-9} mbar, after running growth experiments and after exposure to elevated temperatures. Prior to any SEM experiments

involving bacteria, samples were subjected to UV treatment to kill any bacteria present, whilst allowing them to maintain their size and shape. Bacteria samples were placed inside a TL-2000 Ultraviolet Translinker (UVP, CA, USA) and subjected to 100 mJ/cm² of UV light (252 nm). Samples for SEM were mounted onto aluminium SEM pin stubs (12 mm diameter, Agar Scientific, Essex, UK) using carbon tabs (12 mm diameter, Agar Scientific, Essex, UK). To enhance the contrast of these images, a thin layer of Au/Pd ((8 V, 30 s) was sputtered onto the electrodes with a SCP7640 from Polaron (Hertfordshire, UK). SEM measurements were conducted before and after a growth experiment to determine the concentration of bacterial cells increased over time in the presence of nutrients. For the temperature-dependent experiments, suspensions of *S. aureus* cells in water (1.0×10^6 CFU/mL) were incubated at 37°C, 50°C and 90°C for 30 min to determine the influence of temperature on morphology. Subsequently, these suspensions were dropcast onto the gold substrates and the morphology of the cells was determined with SEM. The size of the cells was determined with ImageJ software and four different areas of the electrodes were studied, with at least 90 cells considered to determine the error bar.

This experiment was carried out since it has been demonstrated that mutations occur in *S. aureus* genes at induced pressure, including growth at temperatures >42 °C [26].

2.4 Analysis of the digestate samples and sequencing with 16S ribosomal RNA gene fragments

Samples (numbered 1 and 2) were collected from a municipal solid waste (MSW) recycling centre. Sample 3 consisted of brewery and distillery effluents. All digestate samples were collected from anaerobic bioreactors, near completion of hydraulic retention time for organic waste treatment in Greater Manchester, United Kingdom.

The efficiency of removing larger sedimentations from the wastewater samples while retaining the bacteria was investigated in two ways: i) using dilution series and ii) removing sediments by centrifugation. In the first approach, serial dilutions from 10^{-1} up to 10^{-5} were prepared. In the second approach, 25 mL of the bacterial suspension was centrifuged three times at 4200 rpm for 30 min. After removal of supernatant, the pellet was re-suspended in 25 mL of nutrient broth. From each of those samples, 100 µL was plated and incubated for one day at either ambient atmosphere or in a 5.0 ± 0.1 % CO₂ environment using a LEEC CO₂ incubator at 37 ± 0.1 °C. For digestate sample 3 (originating from a brewery), additional platings were performed on YEPD plates due to the high presence of yeast in these samples as part of the beer brewing

process. The incubation period was extended to two days due to slower microbial growth of the bacteria from this sample, which is line with samples analysed previously containing yeast [22]. All colonies grown on plates were examined visually and by using a 4x magnification SR microscope (Zeiss, Germany). Any colony showing a unique morphology was streaked out onto a new plate to obtain single colonies. The single colonies were subjected to Gram staining and identification by using 16S rRNA sequencing. Gram staining was performed according to the protocol by Clause *et al.* [27], and the results were recorded with 1000 times magnification using a Leica DM 500 with a ICC50HD camera.

Subsequently, 16S ribosomal RNA gene sequencing was performed. For this, single colonies were re-suspended in 300 μ L of sterile water. The cells of each isolate were lysed by freezing at $-80\text{ }^{\circ}\text{C}$ for 15 min followed by heating at $95\text{ }^{\circ}\text{C}$ for 15 min. The obtained lysate was mixed and centrifuged at low speed (300 rpm for 3 min) to precipitate cell debris. The supernatant was used a DNA template source for PCR. The PCR mixture was prepared with the reagents described in Table 2.

Table 2. Reagents used for PCR

Reagent	Volume (μ L)
Biomix (Bioline)	7.5
16s 27F primer	0.5
16s 518R primer	0.5
DNA sample	1
PCR grade water	5.5
Total volume for the reaction	15

This mixture contained forward 27F-UNI primer (5'- AG AGT TTG ATC MTG GCT CAG-3') and reverse 518R primer (5'-CGT ATT ACC GCG GCT GCT GG-3') to amplify a hypervariable region of approximately 400 bp of the 16S rRNA gene. The PCR conditions included heating the mixture in a PCR thermocycler (QcyclerII, Quantabiotech, UK) to $94\text{ }^{\circ}\text{C}$ for 2 min. This was followed by 5 cycles of $94\text{ }^{\circ}\text{C}$ for 30 s and $40\text{ }^{\circ}\text{C}$ for 1 min, and then 30 cycles of $94\text{ }^{\circ}\text{C}$ for 30 s, $50\text{ }^{\circ}\text{C}$ for 1 min and $72\text{ }^{\circ}\text{C}$ for 3 min.

To visualise PCR products of the expected length, gel electrophoresis was performed in 1xTAE buffer with 1.5% agarose containing midori green (Geneflow, UK). From each PCR reaction, 5 μ L of the PCR products were loaded on the gel, electrophoresed at 90 V for 20 min, and visualised on the geneflash bio imager (Syngene, UK).

To clean the PCR samples for sequencing, 5 μL of the remaining PCR product was mixed with 2 μL of ExoSAPIT and incubated at 37 $^{\circ}\text{C}$ for 15 min. The ExoSAPIT reagent was inactivated by incubating the sample at 85 $^{\circ}\text{C}$ for 15 min. After that, 3 μL of the cleaned PCR product was added to a mixture containing 6 μL of PCR grade water and 1 μL of 27F-UNI primer. These samples were then sent for Sanger sequencing by the Genomic Technologies Core Facility, based within the Faculty of Biology at the University of Manchester.

To identify bacterial species, the obtained sequences were matched to those available on the BLAST database.

2.4 Heat Transfer Method (HTM) measurements

The detection of bacteria was based on monitoring the heat flow through electrode surfaces using a device developed in-house [19]. The principle of this thermal technique is shown (Figure 1), where the temperature in the flow cell was measured at the copper heat sink (T_1) and in the liquid (T_2). The flow cells used in this study had an internal volume of 100 μL .

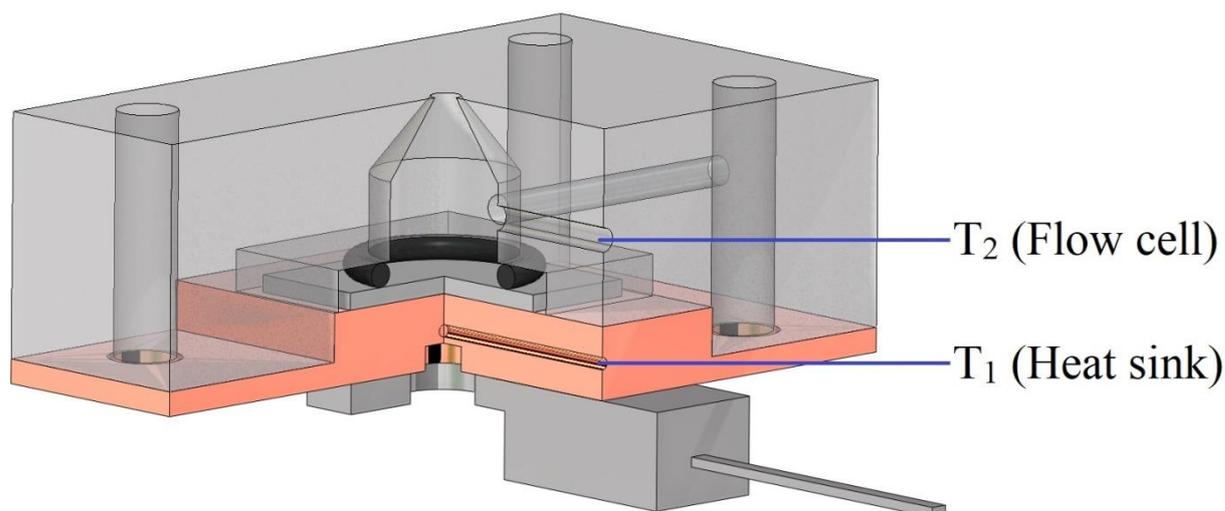


Figure 1. Flow cell developed to accommodate samples containing microorganisms.

The heat-transfer resistance, which was denoted as R_{th} , was dependent upon the temperature difference of the heat sink (T_1) and the temperature of the solution at 1.7 mm above the surface of electrode (T_2). T_1 was controlled with a Proportional-Integral-Derivative (PID) controller. For these measurements, the optimal PID value with the lowest noise on the signal consisted of settings $P = 1$, $I = 8$, $D = 0.1$ or settings $P = 1$, $I = 14$, $D = 0.3$, depending on the dimensions of the heat sink used. The noise on the signal was affected by the settings of the PID feedback loop and therefore impacted on the sensitivity of the sensor platform; hence, it was crucial to optimize the PID settings prior to conducting the measurements [28].

The R_{th} value was calculated by dividing the temperature gradient over the power required to maintain heat sink T_1 at the set temperature. The thermal resistance was measured over time, with the thermocouple (type K, Cole-Palmer, UK) recording a measurement every second.

$$R_{th} = \frac{(T_1 - T_2)}{P} \quad \text{Eq. 1}$$

Gold-coated electrodes were mounted in the flow cell and stabilized in either water or in nutrient broth. Bacterial suspensions were manually injected in the flow cell to ensure adherence to the gold substrate and prevent contamination of the tubing. Initially, experiments were conducted in water (pH = 7.4) to determine whether it was possible to discriminate between different concentrations of *S. aureus* in water. Five suspensions with *S. aureus* concentrations between 10^3 and 10^7 CFU/mL were created via serial dilution and used for the injections. Water was used in this experiment as it did not contain nutrients and thereby slowed the growth of *S. aureus*, thus making it possible to accurately determine the bacterial load.

In the next stage of this work, growth experiments were conducted with the temperature of the copper, T_1 , fixed at 37 ± 0.02 °C except for a temperature dependent growth measurement and one measurement in which bacteria were exposed to elevated temperatures (90 °C).

In order for the microorganisms to grow, the exchange of nutrients and waste products was ensured using a continuous flow of nutrient broth at a flow rate of 1 mL/h with an automated NE500 from ProSense (Oosterhout, the Netherlands). An initial experiment was conducted with *S. aureus* suspended in nutrient broth at a concentration of 1.0×10^4 CFU/mL. In subsequent measurements, suspensions with *S. aureus* concentrations of 1.0×10^2 CFU/mL were used. Lower concentrations were used due to the longer time of the experiment and to prevent saturation from occurring.

After inoculation of the flow chamber with cells, a continuous flow of nutrient broth (at a flow rate of 1 mL/h) was provided allowing for microbial growth. To demonstrate that the observed increase in the signal corresponds to the increase in microbial growth an experiment was performed during which the growing culture was thermally eliminated, thereby preventing a further increase of the signal. Therefore the mixture was heated and maintained at 90 °C for 10 min 8h into a growth measurement. This temperature ensured that the growth of *S. aureus* was fully inhibited. After the short exposure of the cells to 90 °C for 10 min, the heat sink was cooled down to 37 °C and again continuously supplied with a fresh broth solution by an

automated syringe pump (flow rate = 1 mL/h). The absence of a signal increases hereafter demonstrates that the change in thermal resistance was due to microbial growth. In further experiments, the temperature dependency of the growth was quantified. To this end, the temperature was varied from 35 – 55 °C with keeping the temperature fixed for 2 h at intervals of 5 °C. The slope of the graph at a given temperature was correlated to the growth of *S. aureus*. Analysis of variance (ANOVA) [29] was used to investigate the effect on cell growth (°C/Wh) of Temperature T_1 , using Excel and Design Expert (v.11).

In particular, a one-way ANOVA in Excel (level of confidence of 0.05) analysis. This was followed by a Least Significance Difference (LSD)-test as Post Hoc comparison method to assess which temperature regime has a major influence on cell growth.

Next, to see whether it was possible to determine microorganism growth in the complex digestate samples were used. Digestate sample 1 was centrifuged to remove all sediments and subsequently washed with NB three times. After manual injection of this suspension to the flow cell, the heat sink was kept at 37°C and the thermal resistance was monitored over 30 h. This experimental procedure was then conducted with digestate sample 2, except this sample was 1000x diluted and filtered to remove all sediments and added to a *S. aureus* suspension of 10^2 CFU/mL in NB in a 1:1 ratio.

3. Results and Discussion

3.1. Evaluating the concentration of *S. aureus* cells in samples

The thermal response of glass and gold-coated electrodes to aqueous solutions (pH= 7.4, $T = 37$ °C) with a range of increasing concentrations of *S. aureus* cells was studied. After incubating the gold-coated electrodes with water for 15 min, the R_{th} value stabilized at 2.3 ± 0.1 °C/W (Figure S1). Under identical conditions, R_{th} values of 2.8 ± 0.1 °C/W were obtained with the glass electrodes. This difference in thermal resistance can be explained by the lower thermal conductivity of glass compared to gold and the increased thickness of the glass substrates (1.2 mm for glass vs ~0.5 mm for gold electrodes). After exposure of the electrodes to increasing amount of cells, a significant increase was observed for both electrodes. The thermal resistance of a gold-coated electrode exposed to a *S. aureus* concentration of 1.0×10^7 CFU/mL in water increased to 7 ± 1 % compared to a gold-coated electrode stabilised in water. For the glass electrodes, a maximum increase of 6 ± 1 % was recorded at the same bacterial concentration. The normalized R_{th} values, defined as the thermal resistance at a certain concentration divided that over the baseline level, are shown in Figure 2.

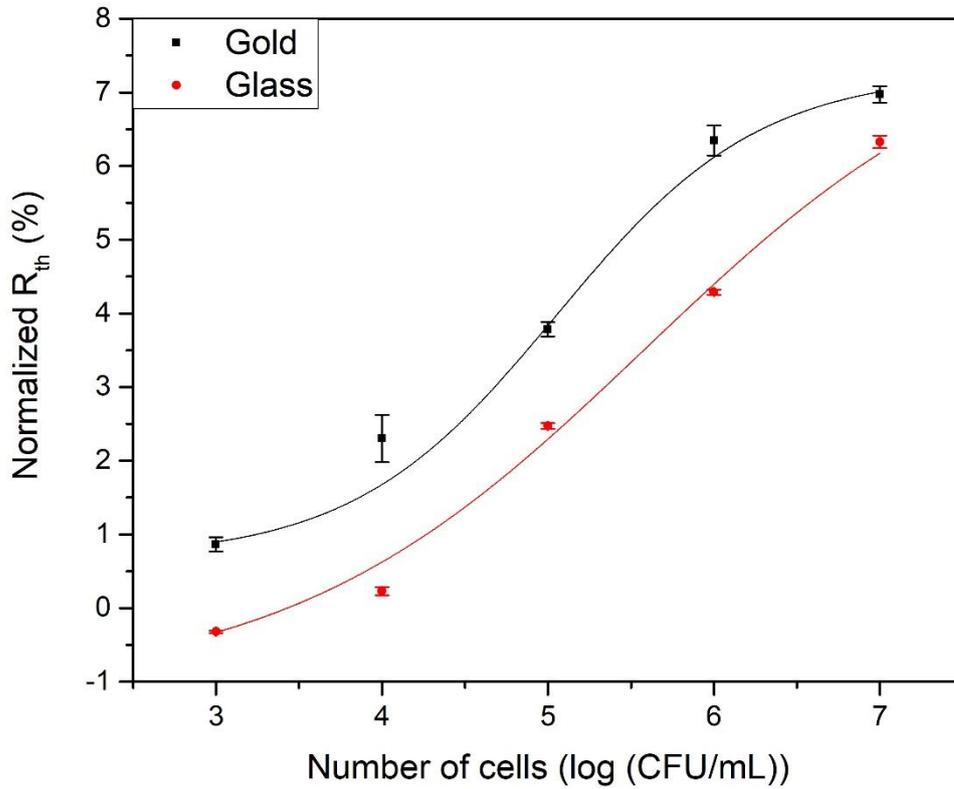


Figure 2. The normalized thermal resistance when gold-coated (black line) and glass (red line) electrodes were exposed to different concentrations of *S. aureus* in water ($T_1 = 37.00$ °C). The curve was fitted with a standard dose-response fit ($R^2 = 0.98$). Standard deviations were determined by taking the average of at least 600 points.

The syringe pump with a flow rate of $250 \mu\text{L}/\text{min}$ was used to administer *S. aureus* suspensions in water to the flow cell. Injection at a high flow rate was necessary to prevent adhesion of the cells to the gold-coated electrodes and ensure there is no build-up of microorganism in the cell, which could lead to inaccuracy in the measurements. Prior to the measurement, the CFU/mL were determined by standard UV-vis methods.

The dynamic range of the gold-coated and glass electrodes was between 1.0×10^4 and 1.0×10^7 CFU/mL. Previous work by Betlem *et al.*, [22] demonstrated the capability of gold-coated electrodes combined with thermal analysis to monitor suspensions of yeast in water in the range of $1.0 \times 10^4 - 1.0 \times 10^7$ CFU/mL, similar to results obtained in this study. To the authors' knowledge, this, is the first report on the use of gold electrodes and HTM to monitor bacterial concentrations.

The limit of detection of the developed sensor platform was estimated by taking the concentration at which the signal is equal to three times the standard deviation on the baseline signal (according to conventional three sigma method). For gold-coated electrodes this was equal to approximately 0.5×10^2 CFU/mL, while for glass electrodes 1.0×10^4 CFU/mL was attained. Due to the enhanced sensitivity of the gold-coated electrodes compared to glass, they were used in further experiments on studying the growth of *S. aureus* under different conditions.

3.2 Evaluation of *S. aureus* growth in the nutrient broth

The effect of a continuous flow of nutrients (flow rate = 1 mL/h) on the thermal response of the gold-coated electrodes over a prolonged time was evaluated. This flow rate based on results from previous work [22] ensured minimal disturbance in the thermal resistance signal while still providing sufficient nutrients for the microorganisms to grow. There was no significant effect on the thermal resistance and a clear baseline was established (Figure S2).

The determination of the impact of microorganisms on the thermal resistance at the solid-liquid interface of the gold-coated electrodes was then established. *S. aureus* has a generation time of approximately 30 min in aqueous media at physiological temperature [24]. Species of staphylococci are able to grow over at a wide temperature range but their optimal range is between 30-37 °C [30, 31]. Therefore, experiments were performed by maintaining T_1 of the heat sink (copper block) at 37 °C.

An experiment to determine bacterial growth was conducted with a starting concentration of *S. aureus* at 1.0×10^4 CFU/mL. The cells were maintained at 37 °C and supplied with a continuous flow of fresh nutrients with a syringe pump that administered fresh nutrient broth at a rate of 1 mL/h. After an initial lag phase (45 min) that was omitted from the graph, a continuous increase of 0.03 °C/W per hour was observed (Figure S3). SEM images confirmed that *S. aureus* cells adhered to the gold electrode, and the concentration of bacteria retained on the surface was dependent on the concentration in the medium (Figure S4).

Plating experiments were performed to determine the impact of temperature on the microorganism growth rate by exposing the bacteria to elevated temperatures for 30 min. The exposure of the bacteria to 50 °C slowed down their growth rate, which is to be expected since the viability of the cells is affected at this temperature.

Full inhibition of growth was observed after incubating the bacteria at 90 °C, which implies they are no longer viable. Previous reports indicate that disintegration of the bacterial cell wall can occur at this temperature.

SEM images (Figure S4) also revealed that the bacterial cell morphology was affected by exposure to elevated temperatures. An average size of 717 nm \pm 71 nm at 37 °C was found, which is under the optimal growth conditions of *S. aureus*. On the contrary, at 50 °C there was a mixture of bacteria present with an average of 195 nm \pm 83 nm (some cells intact and some shrunk) and at 90 °C all cells were significantly smaller or defragmented with an average size of 126 nm \pm 38 nm.

Figure 3 shows the thermal resistance in time after exposure of the cells to 90 °C for 10 min, which is known to impact on their viability and cell morphology.

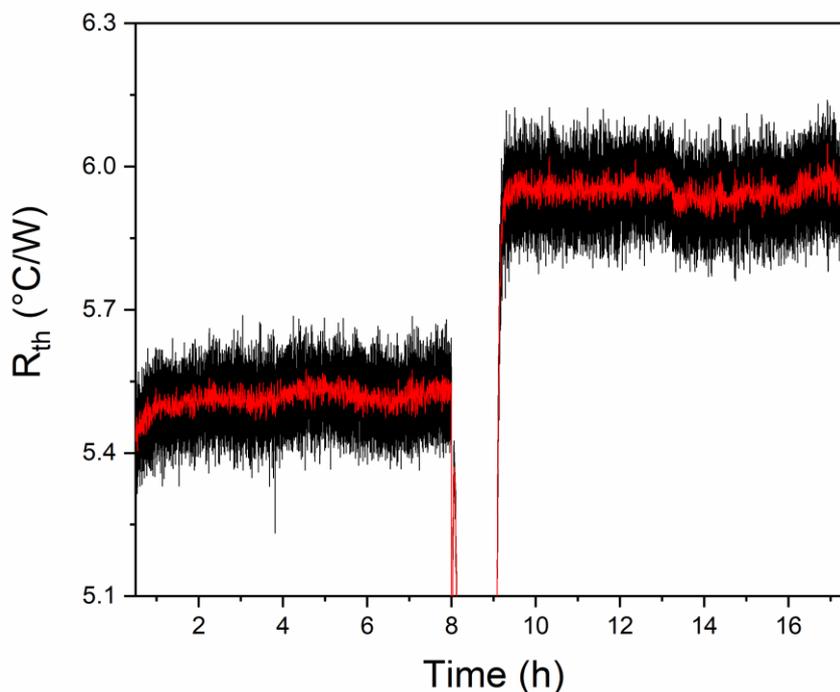


Figure 3. Thermal elimination of a suspension of *S. aureus* in water at 90.0 °C. The measurement was started with $\sim 10^2$ CFU/mL of *S. aureus* cells that were grown at 37.00 °C for 8 h. Hereafter, the temperature of T_1 was increased (in 10 min) to and kept at 90.0 °C for 10 min before returning back to 37.00 °C in 20 min. The red line corresponds to a gentle median filter (50 points) applied to the raw thermal resistance data (black line).

During replication of *S. aureus* at 37.0 °C, an increase in the thermal resistance (~ 0.025 °C/W per hour) was observed. After exposure of the bacteria to elevated temperatures, an increase of ~ 0.5 °C/W was observed that remained stable over time. The increase in signal can potentially be attributed to the disintegration of the dead bacteria that sink to the bottom of the electrode, thereby creating a dense layer that blocks heat-flow through the surface. This effect has previously been observed after boiling of yeast cells [22]. The fact that no more increase in the thermal resistance was observed after there were no viable cells left, confirmed that the increase in thermal resistance was caused by bacterial growth.

Furthermore, the impact of temperature on the growth of *S. aureus* was determined. To this end, *S. aureus* was added to the flow cell, and a continuous flow of nutrient broth was applied with a flow rate of 800 $\mu\text{L/h}$ (Figure 4).

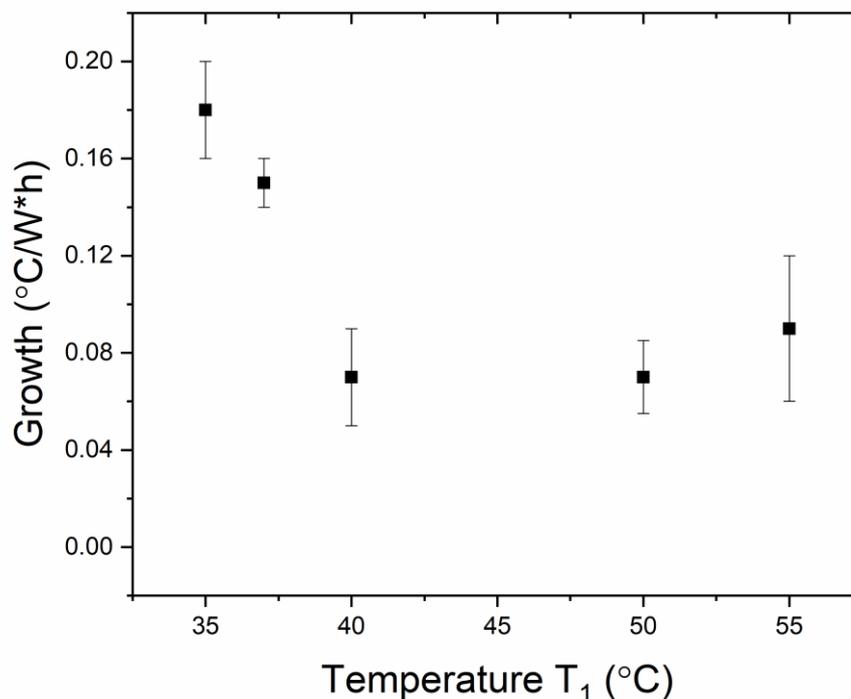


Figure 4. The relative change in thermal resistance per hour when *S. aureus* was grown in nutrient broth at temperatures of 35 °C, 37 °C, 40 °C, and 50 °C, 55 °C. The growth at each temperature was monitored for 2h and the gradient was determined over an average of >600 data points.

Results from the one-way ANOVA in Table 3 indicated that the variable ‘temperature’ (T_1) has a significant impact on cell growth. This result was also confirmed by the small p-values

identified ($p=0.0015$). The LSD test was conducted with a $t(\frac{\alpha}{2}, N-a)$ of 0.03. This test revealed that statistically the temperature range 35-37 °C has the highest impact on cell growth, as it can be seen also from Figure 4 and has been described in literature [31]. Fit statistics indicate the response has a good regression as Predicted R^2 is in agreement with the Adjusted R^2 , while significance is confirmed by the values of adequate precision (>4).

Table 3. ANOVA table for Temperature T_1 impact on cell growth, with SS representing sum of squares, df degrees of freedom and MS mean squares.

<i>Source of variation</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>p-value</i>	<i>Fcrit</i>	<i>F>Fcrit</i>
Between Groups	0.0144	4	0.0036	25.5	0.002	5.2	significant
Within groups	0.0007	5	0.0001				
Total	0.0151	9					

$R^2 = 0.9533$; Adj. $R^2 = 0.9159$; Pred. $R^2 = 0.8132$; Adeq. Precision = 13.9.

It was expected that growth rates of *S. aureus* would decrease after exceeding the optimum growth temperature of 37°C. However, it has been reported that temperatures of 50°C and higher can lead to disintegration of the bacterial cell walls. The consequence of this is the dense packing of dead cells on the surface that can increase the thermal resistance, as previously has been reported for the thermal elimination of yeast cells [22].

3.3 Identification of bacteria present in wastewater samples

Biogas is a promising bioenergy technology offering a two-fold advantage for combining treatment of various organic wastes along with the generation of a versatile and storable energy carrier. To understand the biogas-producing capabilities of the digestate samples, it was necessary to characterise the microbial community. An overview of the bacteria encountered in the sample 2 and sample 3 was determined (Figure 5). Eight colonies were sequenced in sample 2, whereas in sample 3 twenty-two colonies were sequenced.

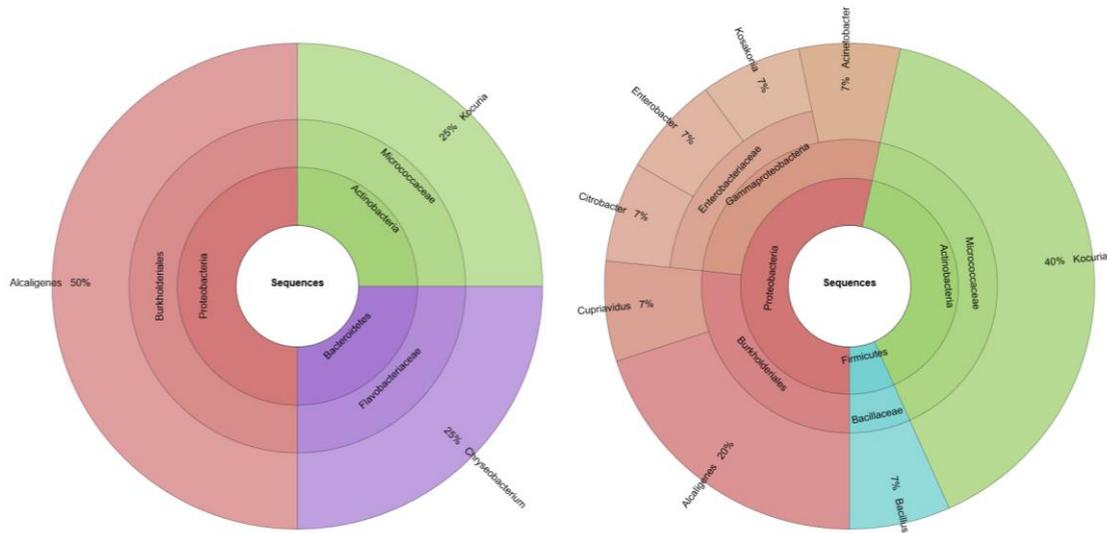


Figure 5. Bacterial compositions of municipal waste digestate (sample 2) and brewery digestate (sample 3) as determined by DNA sequencing

An interesting study [33] conducted to characterise the biogas-producing microbial community by short-read next generation DNA sequencing, revealed the composite microbial consortium developing in a biogas fermenter was characterised by members of the Clostridia (36%) and Bacilli (11%) classes, together with members of the Bacteroidia (3%), Mollicutes (3%), Gammaproteobacteria (3%) and Actinobacteria (3%) classes. Most species identified by Wirth *et al.* were in agreement with those characterised in this study (Figure 5). However, it has to be noted that Wirth *et al.* performed sequencing on the mixtures rather than on the individual colonies as was done in this study. Proteobacteria and Bacteroidetes were found to be the dominant phylum in several samples, which is in line with the results from sample 2 and 3. Both sample 2 and 3 contain a large percentage of Proteobacteria (50 vs 53% and Kocuria (25 vs 40%). The main differences are in the presence of Chryso bacterium (25%) in sample 2 and Bacillus in sample 3 (7%).

Kocuria spp. are isolated from marine sediments [34] and have been successfully used for wastewater treatment as they are excellent for biogas and lowering working temperatures to save energy whilst increasing energy conversion efficiencies. Actinobacteria are used for soil conditioning as the decomposing matter is transformed into nutrients that can be up taken by plant for growth. They are abundant in natural waters, and are a small minority of typical digester consortia [33], as can be seen in these samples.

Chrysobacterium in sample 2 are in line with the study of Wirth. The presence of Bacillus in sample 3 is explained as follows. *Bacillus thuringiensis* are the most well-known biological agents for selective control of pest insects and can be grown on residues post biogas production, as they contain necessary nutritional elements to sustain the growth of microbes. It has been found [35] that the optimum substrate for growth are actually brewer grains, with at least 50% share of this biomaterial in the growing media, and therefore their presence is expected in brewery samples.

These results confirm the complexity and heterogeneity of the used digestate samples, with significant differences encountered between the municipal waste and brewery sample. Within the wastewater community, there is a high demand for the development of sensors that can monitor microbial load *in-situ* since the build-up of microorganisms has been linked to bioaugmentation failure. Furthermore, microbial load is an important parameter for the determination of drinking water quality. Current culturing techniques are time-consuming and cannot be implemented on-site, whereas thermal analysis could provide rapid screening. Therefore, further experiments were conducted with digestate samples and those spiked with *S. aureus*, to demonstrate proof-of-application of the sensor platform in a complex sample.

3.4 Thermal analysis of diluted digestate samples and samples spiked with S. aureus

A fresh digestate sample (sample 1) was added to the flow cell. This sample with an unknown composition and bacterial concentration was used as a first test to determine whether bacterial growth can be monitored in a complex sample.

The results demonstrated that there was a noise of 2.5 % on the signal (Figure S5). This was higher compared to what is previously reported in literature (ref 20, noise on signal ~ 0.5-1%), which could be due to the matrix effect of digestate samples. After an initial period of 30 min, the signal increased for a period of ~5 h with a rate of 0.035 °C/W per hour after which it remained stable. This could be due to complete coverage of the electrode with bacteria, since bacteria further away from the surface or in solution would not have a significant influence on the thermal resistance. The developed platform is not selective towards the bacteria present in the sample but allows for monitoring of bacterial load in a simple and low-cost manner. SEM images (Figure S6) confirmed the presence of a mixture of bacteria on the surface and indicated that the increase in thermal resistance was due to the presence of microorganisms and not caused by build-up of organic material on the electrodes.

Aliquots of the fresh digestate samples (sample 2) with an added *S. aureus* suspension were analysed. After incubating a gold-coated electrode with this sample for 15 min, the R_{th} value stabilized at 3.0 ± 0.1 °C/W (Figure 6).

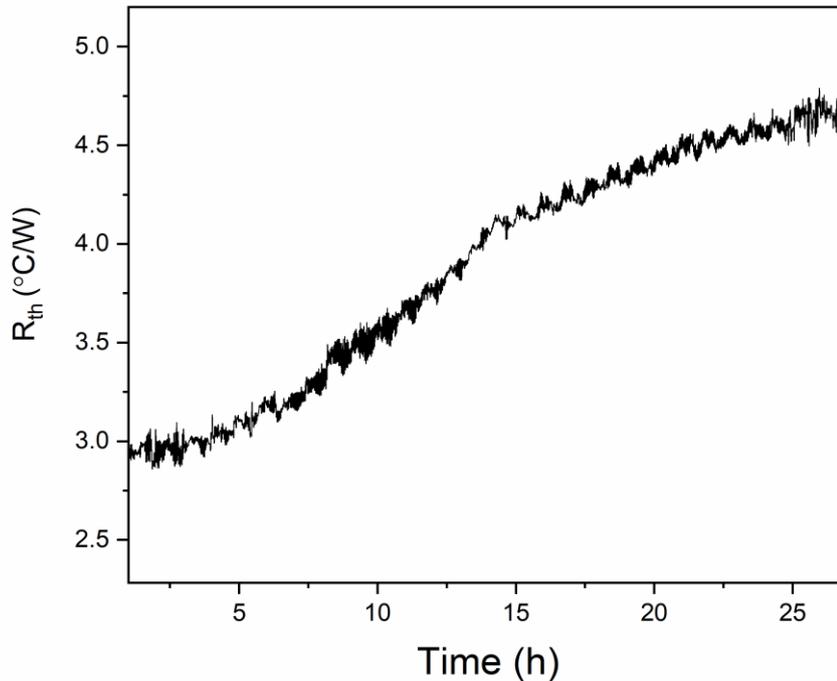


Figure 6. Thermal resistance over time of a 1000x diluted wastewater sample that was mixed in a 1:1 ratio with a suspension of *S. aureus* (1000 CFU/mL) in water. A gentle median filter (50 points, corresponding to 1 measurement point per minute) was applied to the data.

When *S. aureus* was measured as a water suspension, growth started after an incubation time of 30 min. In this measurement however, the thermal resistance did not increase before approximately 4 h. The longer lag time could be due to the lower concentration of *S. aureus* used or due to the inoculation of other microorganisms that can interact with each other. There are limited reports on the impact of other microorganisms on *S. aureus* growth, but most predictive models state that growth is generally led by the most resistant strain, particularly under stressful conditions [36].

After 27 h, the thermal resistance increased to 4.5 °C/W, which corresponds to an increase of nearly 50%. The data was fitted ($R^2=0.95$) with a double linear fit with an initial increase of 0.08 °C/W from 4 to 14 h (average of time period of 10 h), after which the original growth slowed down to 0.03 °C/W. The latter was comparable to what was reported for the growth of

S. aureus in buffered solutions whereas the initial growth was higher compared to buffered solutions. The higher growth rate at the initial stages of the experiment could indicate that the sensor platform is capable of monitoring the overall microbial load instead of solely *S. aureus*. That would be preferential for measurements of digestate samples, which are complex samples containing many microorganism and the overall load is an indication of the efficacy of the organic waste treatment.

Because the *S. aureus* concentration incubated at the start at the measurement was lower compared to other experiments, it could be that saturation takes longer to occur. Furthermore, due to presence of bacteria with different morphologies, it would be possible to form a denser packing in the surface that could overall lead to a higher thermal resistance.

This experiment provides proof-of-application for monitoring bacterial load in complex samples, which could have applications in the field of food safety, organic waste treatment and infection control.

4. Conclusions

In this work, the Heat-Transfer Method (HTM) was employed to monitor bacterial load on electrodes using *S. aureus* as a model organism. Measurements of *S. aureus* suspension in water demonstrated that higher concentrations of the microorganism corresponded to a higher thermal resistance at the solid-liquid interface. The gold-coated electrodes had a lower limit of detection (0.5×10^2 CFU/mL) compared to the glass electrodes and were therefore used in further experiments.

Experiments with *S. aureus* in NB were performed and bacterial growth was linked to the observed increase in thermal resistance. A temperature dependent HTM experiment demonstrated that the thermal analysis technique was suitable for discriminating between different replication rates.

Both digestate samples were composed of eight colonies or more, with the majority of the bacteria belonging to Proteobacteria (~50% for both samples) and Kocuria (respectively, 25 and 40%). The municipal waste also contained commonly encountered *Chrysobacterium* (25%), whereas *Bacillus thuringiensis* (11%) in sample 3 was likely to originate from pesticides used on brewery grains. These results are in line with previous literature reports and highlighted the complexity of the used digestate samples.

Thermal measurements confirmed that growth was encountered in pure digestate samples. When spiking those samples with *S. aureus*, a higher increase in the thermal resistance was found that indicated the sensor platform was capable of determining the overall microbial load

in complex matrices. Considering the simplicity and low-cost of the developed sensor platform, in addition to the ability to implement thermal analysis *on-site*, this could be a useful diagnostic tool for determining microbial loads in water and food samples.

5. Acknowledgements

M.P. would like to acknowledge the EPSRC for funding under grant number EP/R029296/1 and NC3Rs for a CRACK IT grant. Pankaj Singla is thankful to the DST New Delhi (Department of Science and Technology), for INSPIRE Fellowship and Newton Bhabha PhD Placement Programme funded by British Council and DST.

Supporting Information description: contains additional information including raw data, blank measurement, SEM images of *S. aureus* and wastewater, thermal resistance of (non-spiked) wastewater sample.

6. References

- [1] Lever, M.A.; Teske, A.P. Diversity of methane-cycling archaea in hydrothermal sediment investigated by general and group-specific PCR primers. *Appl. Environ. Microbiol.*, **2015**, *81*, 1426-1441
- [2] Lim, S.; Kim, S.; Yeon, K.M.; Sang, B.I.; Chun, J.; Lee, C.H. Correlation between microbial community structure and biofouling in a laboratory scale membrane bioreactor with synthetic wastewater. *Desalination* **2012**, *287*, 209-215.
- [3] Nzila, A.; Razzak, S.; Zhu, J. Bioaugmentation: An emerging strategy of industrial wastewater treatment for reuse and discharge. *Int J Environ Res Public Health*, **2016**, *13*, 846.
- [4] Frigon, D.; Biswal, B.K.; Mazza, A.; Masson, L.; Gehr, R. Biological and physicochemical wastewater treatment processes reduce the prevalence of virulent *Escherichia coli*. *Appl. Environ. Microbiol.* **2013**, *79*(3), 835-844.
- [5] Lyon D.Y., Vogel T. Bioaugmentation for groundwater remediation: An overview. In: Ward C.H., editor. *Bioaugmentation for Groundwater Remediation*. Springer Science and Business Media; New York, NY, USA: 2013. pp. 1–37.
- [6] Yun, M.A.; Yeon, K.M.; Park, J.S.; Lee, C.H.; Chun, J.; Lim, D.J. Characterization of biofilm structure and its effect on membrane permeability in MBR for dye wastewater treatment. *Water res.* **2006**, *40*, 45-52.
- [7] Baquero, F.; Martinez, J.L.; Canton, R. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* 2008, **19**, 260-265.

- [8] Novo, A.; Andre, S.; Viana, P.; Nunes, O.C.; Manaia, C.M. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. *Water res.* **2013**, *47*, 1875-1887.
- [9] Kim, S.; Aga, D.S. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J Toxicol Environ Health B Crit Rev.* **2007**, *10*, 559-573
- [10] Zhang, T.; Zhang, X.X.; Ye, L.; Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PloS one*, **2007**, *6*, e26041.
- [11] Amann, R.I.; Binder, B.J.; Olson, R.J.; Chisholm, S.W.; Devereux, R.; Stahl, D.A. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl. Environ. Microbiol.*, **1990**, *56*, 1919-1925.
- [13] Lemarchand K., Berthiaume F., Maynard C., Harel J., Payment P., Bayardelle P., Masson, L.; Brousseau, R. Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. *J Microbiol Methods* **2005**, *63*, 115–126.
- [14] Varma, M.; Field, R.; Stinson, M.; Rukovets, B.; Wymer, L.; Haugland, R. Quantitative real-time PCR analysis of total and propidium monoazide-resistant fecal indicator bacteria in wastewater. *Water Res.* **2009**, *43*, 4790-4801.
- [15] Fittipaldi, M., Nocker, A. and Codony, F., Progress in understanding preferential detection of live cells using viability dyes in combination with DNA amplification. *Journal of Microbiological Methods*, **2012**, *91*, 276-289.
- [16] Ofiteru, I.D.; Lunn, M.; Curtis, T.P.; Wells, G.F.; Criddle, C.S.; Francis, C.A.; Sloan, W.T., Combined niche and neutral effects in a microbial wastewater treatment community. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 15345-15350.
- [17] Ou, F.; McGoverin, C.; Swift, S.; Vanholsbeeck, F., Near real-time enumeration of live and dead bacteria using a fibre-based spectroscopic device, *Scientific Reports*, **2019**, *9*, Article number: 4807
- [18] Hazan, R.; Que, Y.; Maura D.; Rahme L.G., A method for high throughput determination of viable bacteria cell counts in 96-well plates, *BMC Microbiology*, **2012**, *12*, 259-266
- [19] van Grinsven, B. ; Bon, N. V.; Strauven, H.; Grieten, L.; Murib, M.; Jiménez-Monroy, K.L.; Janssens, S.D.; Haenen, K.; Schöning, K.; Vermeeren, V.; Ameloot, M.; Michiels, L; Thoelen, R.; Ceuninck, W. D; Wagner, W. D., Heat-transfer resistance at solid-liquid interfaces: a tool for the detection of single-nucleotide polymorphisms in DNA, *ACS Nano*, **2012**, *6*, 2712–2721.

- [20] Peeters, M.; van Grinsven, B.; Foster, C.W.; Cleij, T.J.; Banks, C.E. Introducing thermal wave transport analysis (TWTA): a thermal technique for dopamine detection by screen-printed electrodes functionalized with Molecularly Imprinted Polymer (MIP) particles, *Molecules*, **2016**, *21*, 552-560.
- [21] van Grinsven, B.; Eersels, K.; Peeters, M.; Losada-Perez, P.; Vandenryt, T.; Cleij, T.J., Wagner, P., The heat-transfer method: a versatile low-cost, label-free, fast, and userfriendly read out platform for biosensor applications. *ACS Appl. Mater. Interfaces*, **2014**, *6*, 11309–11318.
- [22] Betlem, K.; Hoksbergen, S.; Mansouria, N.; Downa, M.; Losada-Pérezb, P.; Eerselsc, K.; Grinsvenc, B. V.; Cleijc, T.J.; Kelly, P.; Sawtell, D.; Zubko, M.; Banks, C.; Peeters, M., Real-time analysis of microbial growth by means of the Heat-Transfer Method (HTM) using *Saccharomyces cerevisiae* as model organism, *Physics in Medicine*, **2018**, *6*, 1–8.
- [23] Butler, M.T.; Wang, Q.; Harshey, R.M.; Cell density and mobility protect swarming bacteria against antibiotics. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 3776-3781.
- [24] Gill, S.R., Fouts, D.E., Archer, G.L., Mongodin, E.F., DeBoy, R.T., Ravel, J., Paulsen, I.T., Kolonay, J.F., Brinkac, L., Beanan, M. and Dodson, R.J., Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermidis* strain. *Journal of bacteriology*, **2005**, *187*, 2426-2438.
- [25] Enright, M.C., Robinson, D.A., Randle, G., Feil, E.J., Grundmann, H. and Spratt, B.G., The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA). *Proceedings of the National Academy of Sciences*, **2002**, *99*, 7687-7692.
- [26] Goncheva, M.I.; Flannagan, R.S.; Sterling, B.E.; Laakso, H.A.; Friedrich, N.C.; Kaiser, J.C.; Watson, D.W.; Wilson, C.H.; Sheldon, J.R.; McGavin, M.J.; Kiser, P.K.; Heinrichs, D.E.; *Nature Communications*, **2019**, *10*, 1-14.
- [27] Claus, D.; A standardized gram staining procedure, *World Journal of Microbiology & Biotechnology*, **1992**, *8*, 451-452.
- [28] Geerets, B., Peeters, M., Grinsven, B., Bers, K., De Ceuninck, W. and Wagner, P., Optimizing the thermal read-out technique for MIP-based biomimetic sensors: Towards nanomolar detection limits. *Sensors*, **2013**, *13*, 9148-9159.
- [29] Montgomery, D.C., 2017. *Design and analysis of experiments*. John Wiley & sons.
- [30] Valero, A.; Pérez-Rodríguez, F.; Carrasco, E.; Fuentes-Alventosa, J.M.; García-Gimeno, R.M.; Zurera, G.; *International Journal of Food Microbiology*, **2009**, *133*, 186-194.

- [31] Fatta, D.; Achilleos, A.; Nikolaou, A.; Meric, S. Analytical methods for tracing pharmaceutical residues in water and wastewater. *Trends Anal. Chem.* **2007**, *26*, 515-533.
- [32] Sutherland, J.P.; Bayliss A.J., Roberts T.A., Predictive modelling of growth of *Staphylococcus aureus*: The effects of temperature, pH and sodium chloride. *Int J Food Microbiol*, 1994, *21*, 217–236.
- [33] Wirth, R.; Kovacs, E.; Maroti, G.; Bagi, Z.; Rakhely, G.; Kovacs, K.L.; Characterization of biogas producing microbial community by short-read next generation DNA sequencing, *Biotechnol. Biofuels*, **2012**, *5*, 41.
- [34] Kim, S.B., Nedashkovskaya, O.I., Mikhailov, V.V., Han, S.K., Kim, K.O., Rhee, M.S. and Bae, K.S., *Kocuria marina* sp. nov., a novel actinobacterium isolated from marine sediment. *International journal of systematic and evolutionary microbiology*, **2004**, *54*, 1617-1620.
- [35] Zhang, W.; Gong, A.; Lina, Q.; Rulei, Y.; Solid state fermentation of biogas residues for production of *Bacillus thuringiensis* based bio-pesticide, *Transactions of the Chinese Society of Agricultural Engineering*, **2013**, *29*, 212-217.
- [36] Vermeulen A., Gysemans K.P.M., Bernaerts K., Geeraerd A.H., Van Impe J.F., Debevere J., Devlieghere F. Influence of pH, water activity and acetic acid concentration on *Listeria monocytogenes* at 7 °C: data collection for the development of a growth/no growth model, *International Journal of Food Microbiology*, **2007**, *114*, 332-341.