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## Biofilm research using calorimetry – a marriage made in heaven?

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## Abstract

Bacteria growing surface-associated, so-called biofilms, play an important role in technical processes like waste water treatment, bioremediation, or bioprocessing. On the contrary, problems arise when biofilm growth results in e.g. clogged pipes, microbially influenced corrosion or pathogenic contamination, undesired processes that may cause huge financial losses. For observation purposes and to develop efficient control strategies, real time monitoring tools for biofilms are required. Among the large variety of tools used in biofilm research, calorimetry is rarely applied, even though many characteristics qualify it for biofilm investigation and monitoring. Calorimetric measurements are non-invasive and non-destructive and applicable to nearly any kind of samples (including heterogeneous or turbid solutions) without the need of special sample preparation. Online and real time data acquisition reduces the labour and facilitates high throughput measurements.

The following article is meant to introduce and promote calorimetry as a future tool in biofilm research. It attempts an assessment of common, existent monitoring tools and specifically addresses the potential of calorimetry in this field.

### 1) Urgent demand in biofilm monitoring tools

The vast majority of bacteria in natural environments does not occur in well aerated nutrient rich suspensions, as many microbiological laboratory studies appear to suggest, but thrives on surfaces or phase-interfaces. The interaction between bacteria and surfaces had been described in the literature for many years [1-3] but it was in the 1970s that the term “biofilm” for bacteria, living attached to surfaces or interfaces and embedded in a polymeric matrix, was introduced. Not least thanks to this concise and handy term, a blooming research field with ever increasing numbers of publications has developed since then.

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3 Biofilm bacteria exhibit physiological characteristics differing from those of their free-  
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5 floating counterparts. The altered physiological behaviour leads to a better stability against  
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7 extreme and fluctuating environmental conditions such as pH, temperature, salinity, water  
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9 activity, bioavailability of both nutrients and harmful chemical agents like disinfectants or  
10  
11 antibiotics. In some industrial processes, biofilm cultures are preferred as they provide higher  
12  
13 process stability [4, 5]. On the downside, they are very difficult to eradicate wherever their  
14  
15 occurrence is undesired. The enhanced resistance can be attributed to the protective function  
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17 of the extracellular polymeric substances and to other physiological adaptations like slow  
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19 growth, the production of exoenzymes, or the activity of multidrug-efflux-pumps. Besides,  
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21 the occurrence of persister cells is also discussed. Persister cells are non-growing, spore-like  
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23 cells that are nearly impervious to antimicrobial effects. Biofilm persistence may thus rely on  
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25 the random conversion of common cells to persister cells. For detailed review of the different  
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27 protective mechanisms the reader is referred to [6, 7].  
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34 The unusual behavior of biofilm bacteria represents a challenge for the development of  
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36 control strategies. Specific investigation methods are needed for biofilms, which have to  
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38 account for the particular growth mode.  
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41 Since long, it is known that biofilm-bacteria, due to undesired growth on surfaces (e.g. heat  
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43 exchangers, ship hulls, pipes for drinking- and process-water, medical implants), are involved  
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45 in many detrimental processes like corrosion, energy losses or medical complications, thereby  
46  
47 leading to huge economical losses. This gave rise to an intensive search for biofilm  
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49 monitoring and control methods. The interest in such methods was further enhanced by the  
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51 application of surface-associated microorganisms in technical systems (e.g., in  
52  
53 biotechnological or bioremediation processes), municipal wastewater treatment,  
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55 biodegradation of organic waste, bioprocessing and food fermentation procedures. Further  
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57 beneficial and essential aspects of biofilms motivated the desire to better understand these  
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59 bacteria. Additional drivers of the latter research were their involvement in the natural  
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3 purification of water [8], their functions as physiological flora on higher animals, including  
4 humans where they provide protection against pathogens [9, 10] and their roles in the  
5 digestive system of animals where they are responsible for vitamin synthesis or the  
6 degradation of specific substances such as lignin [11, 12]  
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10 Monitoring tools are required under all circumstances where biofilms either cause problems  
11 or are beneficial. They are needed for the improvement of biofilm-based bioprocesses as well  
12 as for the evaluation of the success of countermeasures against biofilms. Furthermore,  
13 techniques are needed to achieve a better scientific understanding of the complex biofilm  
14 communities.  
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## 27 **2) Existing biofilm monitoring tools – different kinds and levels of information**

28 Different situations require different kinds and levels of biofilm control. For example in  
29 industrial systems, biofilm growth cannot be completely prevented and therefore has to be  
30 kept in a range where the system is not negatively influenced. In contrast, complete sterility is  
31 required for most medical applications [13].  
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38 The available monitoring tools can be classified according to the attained level of information  
39 [13, 14]. The following paragraphs will give a short overview on available methods, more  
40 detailed information is given in [13, 14]. It has to be noticed that there are advantages for  
41 every method under certain conditions, although under most practical circumstances broadly  
42 applicable methods are desired.  
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50 The simplest type of monitoring devices detects mass deposition on surfaces, regardless if it is  
51 biotic or abiotic in nature, alive or dead. Common examples are optical methods [15, 16]  
52 which are used to detect changes of biofilm thickness on a surface according to dispersed or  
53 reflected light density. These methods can be influenced by the concentration of particulate  
54 matter, the colour of the biofilm or the chemical composition of the water. Another possibility  
55 is the direct colorimetric measurement of biofilm accumulation on surfaces [17]. This method  
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3 infers cell density from the surface discoloration arising during biofilm growth. It works non-  
4 destructively and provides immediate results, but a calibration to the surface is necessary and  
5 the measurement is influenced by the types of bacteria and chemicals used. Interferences with  
6 coloured abiotic materials can also not be excluded. This makes the investigation of unknown  
7 samples difficult although it might be useful for experiments under well known conditions.  
8 Many more methods of this type are described in [13]. Even though the information provided  
9 by these simple monitoring devices is rather low, so that confirmation by other methods is  
10 required, they might be very useful for early indication of biofouling in technical settings.

11 Somewhat more sophisticated techniques can distinguish between biotic and abiotic  
12 depositions. Commonly used methods of this kind are the (Infrared) IR- or (Fourier  
13 Transform Infrared - Attenuated Total Reflectance) FTIR-ATR spectroscopy of amid bands  
14 and microscopic techniques. IR- or FTIR-ATR of amid bands does not require sample  
15 preparation but can only be applied in systems free or poor of other biological molecules.  
16 Moreover, only smooth surfaces are suited for this kind of analysis. Other methods of similar  
17 information content, i.e. direct light-microscopy of biofilms without the application of  
18 specific dyes, can sometimes hardly distinguish between microorganisms and attached abiotic  
19 material. Biofilm staining with, for instance, crystal violet or safranin followed by washing,  
20 color extraction and absorption measurements of the extract, give a rough estimation of  
21 biofilm mass and differentiate between biotic and abiotic material, but they do not give  
22 information about viability or activity [18].

23 The deepest insight into biofilms is offered by techniques that provide detailed information  
24 about the chemical composition or directly address biofilm microorganisms like e.g. FTIR-  
25 ATR of whole spectra in special flow cells or direct biofilm visualization by confocal-laser-  
26 scanning-microscopy (CLSM) or epifluorescence-microscopy. Modern microscopic  
27 techniques rely on the fluorescence of added dyes or probes. These dyes may interact with the  
28 bacterial metabolism. Furthermore, the exact staining behavior is uncertain when they are  
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3 applied to unknown environmental samples. Moreover, in spite of their high costs, many dyes  
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5 are toxic and require environmentally friendly disposal. The invasive nature of secondary  
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7 staining-procedures excludes the application of modern microscopic techniques for real time  
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9 monitoring in most cases, although the gained snapshot information may be of high diagnostic  
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11 value.  
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15 Microelectrodes are also commonly used in biofilm research. However, for monitoring  
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17 purposes they are not particularly suited. The insertion of electrodes is destructive, even  
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19 though tips with a diameter of a few  $\mu\text{m}$  are nowadays available. The electrodes are analyte-  
20  
21 selective and available for instance for different terminal electron acceptors (e.g. oxygen,  
22  
23 nitrate, and nitrous oxide), electron donors (e.g. sulphide, glucose), nutrients (e.g.  
24  
25 ammonium), toxicants (e.g. chlorine) and environmental conditions (e.g. pH). In any case,  
26  
27 careful calibration is necessary. The provided information only refers to the area where the  
28  
29 electrode is inserted and the origin of the detected substances, biotic or abiotic, is not  
30  
31 distinguished. Specially in environmental biofilm samples of unknown composition, signal  
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33 interpretation is difficult. Furthermore, working with microelectrodes implicates dealing with  
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35 strong responses to interferences and signal drifts.  
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41 Nuclear magnetic resonance (NMR) methods have been used for non-invasive imaging of  
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43 biofilms in porous media and to obtain information about intermediate and metabolite profiles  
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45 [19-21]. For industrial and field applications is NMR too expensive and not flexible enough to  
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47 be integrated into technical and natural systems of interest.  
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51 Calorimetry is directly addressing the activity of biofilm microorganisms by measuring their  
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53 metabolic heat. Despite the advantageous characteristics of calorimetry, which qualifies it as a  
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55 promising monitoring tool for biofilm research, there are only few applications published.  
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57 These examples and their information gain in comparison to conventional methods will be  
58  
59 discussed in detail in chapter 3.  
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3 The most frequently applied technique in biofilm investigations is the determination of colony  
4 forming units (CFU). Here biofilms are removed by scraping, vortexing or sonication,  
5 resuspension, and recultivation on agar-plates followed by counting of growing colonies [22].  
6  
7 The procedure is slow and error-prone due to the possibility of incomplete resuspension  
8 before plating or carryover of substances from the liquid medium [23]. Furthermore, biofilm  
9 cells exhibit a specific physiological state which may result in a situation that most of them do  
10 not grow after plating on agar (W. Costerton, personal communication). In comparison with  
11 cultivation independent methods, CFU typically underestimate cell counts by at least one  
12 order of magnitude [24]. Therefore, all results based on determination of CFU derived from  
13 resuspended biofilms are highly doubtful. Generally, cultivation steps require around 24 h  
14 until results become available. This disqualifies cultivation-based methods wherever quick  
15 responses, e.g. in medical applications, are needed.  
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18 The ideal biofilm monitoring method should work in aqueous systems and function without  
19 sample removal. Secondary procedures like recultivation or staining should be dispensable.  
20 Furthermore, real time and automated online data acquisition should be possible. It should  
21 also be possible to investigate various sample types on various surfaces under various  
22 conditions (environmental/technical samples). Finally, integration into technical systems  
23 should be feasible.  
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### 50 **3) Biofilm monitoring via heat: calorimetry**

#### 51 **3.1) Basic concepts of calorimetry**

52 Calorimetry is defined as any type of measurement of heat, irrespective if it is evolved  
53 (exothermic) or required (endothermic), by any physical, chemical or even biological process.  
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55 Due to the fact that all chemical reactions, including those of the metabolic network, are  
56 accompanied by release or consumption of heat, calorimetry provides real-time information  
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3 about the metabolically active cells. It embraces both, the kinetics as well as stoichiometric  
4 information of the growth reactions. The obtained data have to be interpreted with caution,  
5 because the heat signal also comprises chemical side reactions or physical processes occurring  
6 in the sample, like e.g. phase transitions. However, if all chemical and physical processes are  
7 known and taken into account, the data interpretation is easy [25]. Calorimetric measurement  
8 is non-invasive and non-destructive. The most important feature is that the measured heat  
9 reflects the ongoing processes in real time. The online data acquisition and the easy and fast  
10 operating steps qualify calorimetry as a perfect tool for biofilm analysis and monitoring.  
11 Furthermore the obtained signals have a quantitative meaning and do not require any  
12 calibration, unlike many other methods. Additionally, thermodynamic calculations based on  
13 Hess' law can prove expected or reveal unexpected reactions or side effects. Another  
14 advantage is that calorimetric experiments can be done in a broad range of measuring  
15 conditions regarding for instance pH-value, temperature, electron-acceptor or sample  
16 character. Calorimetry has already been used successfully for the monitoring of suspended  
17 microbial cells for many decades [26, 27].

18 Surprisingly, calorimetry was until now seldom applied to biofilm research. The reasons  
19 might lie in the difficulty of analysing biofilms using conventional and commercially  
20 available calorimeters. However, newly developed calorimeters promise successful and easy  
21 application to biofilm analysis and monitoring. However, pioneering work was done with  
22 conventional calorimeters [28-31].

23 For biofilm measurements, mostly flow systems were chosen [28, 30, 31], even though also  
24 static experiments in ampoules have been conducted [32, 33]. Modifications of fermentation  
25 calorimeters could have been another option for calorimetry in biofilm research. However,  
26 except for experiments with sludge [34, 35] no reports about biofilm research in fermentation  
27 calorimeters can be found in the literature. Recently developed chip-calorimeters, equipped  
28 with exchangeable flow-chambers, allow a higher sample throughput than conventional

1  
2  
3 microcalorimeters. The basic measurement principle of micro- or chip-calorimeters is that the  
4 heat produced in a sample flows through thermopiles (consisting of coupled Peltier-elements)  
5  
6 towards a heat sink with a well defined temperature (thermostat). The Peltier elements act as  
7  
8 thermoelectric generators and convert, according to the Seebeck-effect, the temperature  
9  
10 difference between the sample and a reference point into a voltage signal which is  
11  
12 proportional to the heat. Figure 1 shows a scheme and a photograph of the measurement  
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14 position of a conventional microcalorimeter (A) and a chip-calorimeter (B).  
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### 22 Here Figure 1

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26 In case (A) the sample is located either in a measurement vessel (mainly static conditions) or  
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28 in a flow channel in the wall of the measurement cylinder. In case (B) it is located in a flow-  
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30 cell. The sensitivity of the methods differs, e.g. commercially available microcalorimeter have  
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32 a sensitivity of about 0.05 mW/L, the chip-calorimeter recently developed by Lerchner et al.  
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34 2008 [36] has a sensitivity of about 3 mW/L.  
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38 The main deficiency of established microcalorimeters regarding biofilm research is that either  
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40 the measurements have to be done in a static manner or in a flow channel. Static  
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42 measurements do not reflect the real situation of the biofilm because the heat produced by  
43  
44 planktonic cells adds to the heat produced by the biofilm cells. Flow-through measurements  
45  
46 are time consuming because the biofilm has to develop inside the calorimeter, which can  
47  
48 require days to weeks to reach a stable state. During this time a calorimetric vessel is blocked  
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50 for the measurement of a single biofilm. Any solution overcoming these deficiencies would  
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52 facilitate the application of conventional microcalorimeters in biofilm research.  
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56 As shown in recent publications [36, 37] chip-calorimeters overcome some deficiencies of  
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58 conventional microcalorimeters and are thus developing into a very valuable tool for biofilm  
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60 investigations. They employ flow cells, which are general tools in biofilm research.

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3 Exchangeable flow-cells allow the separation of biofilm cultivation and calorimetric  
4 measurement, thus permitting much higher throughput. A weakness is that the biofilm in the  
5 flow cell can not continuously be flushed with nutrient solution. A stop flow regime is  
6 required to obtain a reference state. Low heat production due to oxygen consumption is  
7 considered as reference state. Details are described in Lerchner et al. 2008 [36].  
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### 17           **3.2) History of biocalorimetry**

#### 18           **General applications of calorimetry in microbiology**

19  
20 Calorimetry has been used as an analytical tool in microbiology for a long time. One of the  
21 earliest calorimetric experiments involving microorganisms took place in 1856 when  
22 Augustine Pierre Dubrunfaut determined the heat of alcoholic fermentation of *Saccharomyces*  
23 *cerevisiae* in an oak vat [38]. The heat production due to other fermentation processes (e.g the  
24 souring of milk) was recorded in 1911 [39]. In 1912, Meyerhoff combined measurements of  
25 heat, consumed O<sub>2</sub> and evolved CO<sub>2</sub> and used the ratio of the produced heat to the consumed  
26 oxygen to distinguish different phases of microbial growth [40]. Today this ratio is referred to  
27 as the oxycaloric quotient [41]. Bayne-Jones et al. in 1929 [42] correlated the heat produced  
28 by bacterial growth and cell counts and found that changes in heat production are related to  
29 changes in the microbial growth rate. Decades later, calorimetry was used for more  
30 sophisticated quantitative investigations of microbial growth. From the relations of  
31 calorimetrically measured enthalpy changes of microbial growth, the calculated Gibb's  
32 energies and the observed growth yields and rates, thermodynamic methods to predict  
33 maximum possible yields, maintenance coefficients, threshold concentrations and maximum  
34 growth rates were derived [43]. Recently, very successful applications of calorimetry in the  
35 medical field (e.g. analysis of bacterial infections of blood, body fluids or implants [44-46])  
36 have been reported. Further application examples are reviewed in [27, 47].  
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3 The amount of heat produced by bacterial cells varies, depending on the prevailing type of  
4 metabolism. Reported values for heat production per cell in suspensions are in the range of  
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6 0.9 pW/cell (methanogenesis) [48] and approx. 7.8 pW/cell (*E. coli* aerobically growing in  
7  
8 complex medium) [49]. For surface-associated cells only very few data are available.  
9  
10 Lerchner et al. reported 0.13 pW per cell [36], whereas from the results of von Rège et al. [29]  
11  
12 a heat output of 0.42 pW per cell can be calculated. Supposedly, the heat output per biofilm  
13  
14 cell varies as much as it does for suspended cells, maybe even more because there are  
15  
16 different maturation stages of biofilms. Therefore, the difference between the reported values  
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18 is not surprising.  
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### 27 **Biofilm related applications of calorimetry**

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29 The first calorimetric approach involving surface attached microbial cultures was to our  
30  
31 knowledge done by Weppen et al. in 1991 [31]. They cultivated microorganisms on the  
32  
33 surface of glass beads and used these microbial cultures in a microcalorimeter for the online-  
34  
35 estimation of the biological oxygen demand of aqueous samples. Short time later Wentzien et  
36  
37 al. in 1994 [30] investigated thiosulfate and tetrathionate degradation by *Thiobacillus* species  
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39 in a flow through-microcalorimeter. They were able to detect metabolic changes due to  
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41 adherence on the surface of the measurement cylinder as well as the contribution of biofilm  
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43 cells to thiosulfate and tetrathionate degradation relative to suspended cells.  
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### 50 **Here Fig. 2**

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55 Both curves in Fig. 2 show the heat output of *T. intermedius* growing on thiosulfate. The  
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57 difference is due to the different treatments of the flow lines in the measurement cylinders. In  
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59 case of the graph marked with triangles (I) bacterial suspension was continuously pumped  
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through the flow line. Thus, the heat signal is the sum of the metabolic heat of planktonic and

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3 surface associated growing bacteria. In case of the graph with rectangles (II) the flow line was  
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5 periodically rinsed with disinfection solution to remove surface associated bacteria. Because  
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7 of the disinfection steps, the measurement was interrupted and the graph is constructed from  
8  
9 separate data points. Graph I shows two maxima and the results of the parallel metabolite  
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11 analysis suggest that it is rather not reflecting two steps of a degradation process. The heat  
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13 could be released due to physical interactions between the bacterial surface and the surface of  
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15 the flow-line, or due to a stimulation of the metabolism, induced by adherence. Since the  
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17 processes are very complicated and overlapping, the reasons for the increase in heat  
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19 production can only be speculated about. However, the difference in the heat output between  
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21 graph I and II clearly indicates that the bacteria adhere to the surface. In the same way the  
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23 impact of medium components or surface material on adhesion and biofilm development can  
24  
25 be evaluated. Bacterial adhesion to surfaces as well as coaggregation were also detected  
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27 microcalorimetrically by Hauser-Gerspach et al. [33] and Postollec et al. [50] who  
28  
29 investigated adhesion and aggregation behaviour of dental streptococci. Morgan et al. [51]  
30  
31 suggested a microcalorimeter with a modified acrylic ampoule as a model system to evaluate  
32  
33 the efficiency of adhesion to different surfaces and of anti-biofilm treatments.

34  
35 In a study of von Rège et al. biocide efficiency was evaluated via a microcalorimetric  
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37 approach [29]. They performed ampoule experiments with biofilms, precultivated on steel  
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39 coupons (static conditions), and flow-through experiments (dynamic conditions). Different  
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41 biocides were tested and decrease in activity was compared to cell counts. These authors  
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43 showed that microcalorimetry has considerable advantages compared to common evaluation  
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45 methods of bactericidal effects. They suggested that microcalorimetry could be a useful  
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47 control tool for biofilm growth in technical systems or for the development and testing of new  
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49 biocides.

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51 Most calorimetric biofilm investigations were performed with mixed cultures. However, this  
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53 complicates the correlation of observed heat signals and metabolic events. This could be a  
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3 reason why before 2007 it was, to our knowledge, never attempted to use the additional  
4 information of an energy-balance for biofilm investigations. In 2007 Peitzsch et al. [28]  
5 performed the first attempt to calculate an energy balance of a single species biofilm of *E. coli*  
6 in a defined medium with a single carbon source (i.e. glycerol). Measurements were carried  
7 out in a TAM-microcalorimeter. An energy consumption rate was calculated from the carbon-  
8 substrate consumption and correlated with the heat production rate. For a younger biofilm  
9 (<150 h) similar patterns for energy consumption rate and heat production rate were reported.  
10 For more mature biofilms the energy consumption rate decreased whereas the heat production  
11 rate remained constant. Thus, the efficiency of energy usage increased. Furthermore, they  
12 reported for the first time about the calorimetric detection of detachment events. The graph in  
13 Fig. 3 (based on the data of Peitzsch et al. [28]) shows the heat production rate of the biofilm  
14 combined with the carbon distribution. In the early phase (until 100 h) the heat signal shows  
15 only a few small peaks, whereas after 100 h many sharp increases in heat production rate can  
16 be observed. These peaks are accompanied by an increase in the ratio between the measured  
17 TOC (Total Organic Carbon) in the outflow and the calculated and expected TOC (carbon-  
18 source glycerol and the known side product acetate). The authors suggested the detachment of  
19 biofilm flocs as an explanation. This can explain both, the increased amount of unknown TOC  
20 as well as the increased heat because of increased metabolic activity in the biofilm flocs due  
21 to better oxygen availability.  
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50 **Here Fig. 3**

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55 Additionally, Peitzsch et al. [28] monitored the toxic influence of 2,4-dinitrophenol (DNP).  
56 The metabolic heat signals, in response to different 2,4-DNP concentrations and to short-term  
57 and continuous intoxication were clearly distinguishable.  
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3 In 2006, Maskow et al. applied for the first time a new calorimetric technique (i.e.  
4 miniaturized- or chip-calorimetry) for biofilm investigations. Advantages of this handy  
5 instrument were higher flexibility and lower costs compared to conventional calorimeters.  
6 They monitored the growth of an *E. coli* biofilm using the chip-calorimeter and a  
7 conventional calorimeter and showed the applicability of chip-calorimetry to qualitative  
8 biofilm monitoring [47]. In 2008, the growth of a *Pseudomonas putida* biofilm was  
9 quantitatively monitored using a recently developed chip-calorimeter [36]. The detected heat  
10 signal was correlated to CLSM cell counts and oxygen consumption, indicating the  
11 quantitative potential of the method. To verify the calorimetric signals, the biofilm responses  
12 to carbon shortage and varied nutrition strategies were investigated.

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With the same technique, bactericidal and bacteriostatic treatments of biofilms were explored  
[37]. Figure 4 shows the heat response of the *Pseudomonas putida* biofilm towards the  
bacteriostatic antibiotic tetracycline, the bactericidal antibiotic kanamycin and the biocide  
formaldehyde. After the addition of the antibiotics to the substrate solution the heat signal  
decreases, in case of tetracycline in a mainly linear way, in case of kanamycin in a slightly  
biphasic way. The different heat responses reflect the different killing mechanisms of the  
antibiotics. The bacteriostatic tetracycline kills only growing cells, and especially the slow  
growing biofilm bacteria are only poorly affected by the antibiotic. Kanamycin actively kills  
bacteria but it is reported to be influenced by extracellular polymeric substances of biofilms.  
Here the decrease of the signal intensity starts later but it is faster than in case of tetracycline.  
To demonstrate the fast response of the calorimetric method, a biofilm was treated with the  
biocide formaldehyde. The immediate inactivation of the biofilm bacteria was reflected in the  
calorimetric signal. Details are given in [37].

**Here Fig. 4**

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3 The study clearly indicates the suitability of calorimetry for the monitoring of disinfection  
4 processes or antibiotic treatments. Further physiological responses were revealed by other  
5 features of the response curve, like for example the increased signal intensity right after the  
6 addition of the antibiotics. This probably reflected the increased energy demand due to  
7 activation of cellular resistance mechanisms.  
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10 All examples presented so far used biofilms which were cultivated under lab conditions, but  
11 calorimetry is also applicable to the investigation of environmental biofilms. One possibility  
12 is the exposure of coupons or other surfaces in natural habitats, which can be collected for  
13 measurements after microorganisms have settled there. Additionally, because calorimetry  
14 does not require a special sample type (liquid-solid, solid etc), samples can be taken with their  
15 natural substratum, transferred in the measurement chamber and measured. Soil particles  
16 represent the largest surface for bacterial settlement and biofilm growth in nature. Thus, it is  
17 not surprising that soil scientists take already advantage of the broad range of sample types  
18 which can be investigated in a calorimeter. Microbial activity in soil is regarded as a measure  
19 for fertility since microbes are the key players in biogeochemical cycles [52]. The activity of  
20 microorganisms is mainly influenced by soil organic carbon which contributes to 20 % to the  
21 increase of atmospheric CO<sub>2</sub> [53] thus linking the loss of soil fertility to global warming.  
22 Therefore, it is increasingly important to understand the soil carbon cycle and the impact of  
23 anthropogenic influence [32, 54]. Calorimetry offers a fast and simple possibility for the  
24 assessment of the fate of soil organic carbon. The limiting factor is the number of active  
25 bacteria, that differs depending on the ongoing metabolic process and the used instrument. In  
26 most studies, soil samples are amended with glucose to promote bacterial growth. According  
27 to Kinura et al. the heat production in a glucose amended soil sample is about 6.7 pW per cell,  
28 [55]. Assuming a thermal detection limit of 100 nW (achievable with modern nano-  
29 calorimeters) and a calorimetric vessel load of 1 g (Elizabeth Sigstad, personal  
30 communication), a detection limit of around 15000 active cells per g is estimated. This  
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3 qualifies calorimetry for the quantification of soils even from extreme habitats, which are only  
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5 sparsely populated with biofilm bacteria.  
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8 Figure 5 is based on the data of Barros et al. [32] where the heat flow from different soil  
9  
10 samples is compared. The soil samples were taken from different sites in the Amazonian  
11  
12 region, varying in the mode of land use. The soil samples differ strongly in their composition,  
13  
14 specially regarding the organic matter content, leading to different microbial communities. In  
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16 response to the addition of glucose all soil samples showed different patterns of heat  
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18 production rates. In combination with the biomass yield, the efficiency of carbon and energy  
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20 conversion was determined. It varied strongly (37 - 84%) depending on the mode of land use.  
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27 **Here Fig. 5**

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31 As assumed, calorimetry offers a fast and easy method to evaluate microbial activity in soils.  
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33 Factors influencing the microbial activity, as for instance soil humidity, soil chemical  
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35 composition and the presence of fertilizers, pesticides or contaminants, can be easily  
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37 investigated in a nearly undisturbed sample [52, 56]. For more details see Barros et al. [57].  
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39 Usually, ammonia besides of glucose is added to induce the metabolism and thus heat flow.  
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41 Considering that not all microorganisms are able to use glucose as substrate, e.g. many  
42  
43 anaerobic bacteria, this approach might provide only limited information. Combined  
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45 measurements of heat production and CO<sub>2</sub>-evolution can provide deeper insights (e.g. indicate  
46  
47 the efficiency of the carbon substrate utilization for growth) [58]. The oxycaloric quotient for  
48  
49 all aerobic heterotrophic growth processes is about  $-455 \pm 15 \text{ kJ mol-O}_2^{-1}$  [59]. Processes  
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51 such as mixed respiratory-fermentative metabolic reactions or enhanced futile substrate  
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53 cycling and uncoupling of oxidative phosphorylation result in a higher value of the oxycaloric  
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55 quotient than the theoretical value for heterotrophic growth.  
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3 Experiments with suspended cells have shown that it is possible to derive the kinetics of  
4 microbial degradation of contaminants from calorimetric data [60, 61] and that this could be  
5 applied in soil investigations to estimate the bio-remediation potential. Advantageous in this  
6 approach is the fact that the kinetics derived from calorimetric experiments reflects the  
7 metabolism, whereas kinetics derived from chemical analysis (e.g. HPLC or GC) reflects only  
8 the contaminant-removal from the aqueous soil phase. Chemical analysis includes also the  
9 disappearance due to sorption to soil matter. In most cases sorption processes do not affect  
10 significantly the heat signal, since the heat of sorption is many times smaller than the  
11 dissipated heat of biodegradation, therefore calorimetry is not biased by sorption processes. In  
12 comparison to chemical analysis calorimetry provides additional information about the fate of  
13 the contaminant (i.e. the bioconversion to incomplete oxidized substances).  
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### 3.3) Potential application fields of calorimetric biofilm monitoring

#### Calorimetry as monitoring tool for disinfection processes or antibiotic susceptibility

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34 Biofilm-directed antibiotic susceptibility tests gained much attention, when it became clear  
35 that the different physiological characteristics of biofilm bacteria result in dramatic increases  
36 (1000 fold and more) in resistance towards antibiotics. For long time, susceptibility tests had  
37 been performed with suspended cells. Suddenly, new methods were required, which  
38 considered the mode of growth. When investigating biofilms, disruption, resuspension and  
39 recultivation should be avoided for reasons mentioned above. Johnston et al. compared the  
40 amount of surviving cells determined by the resuspension method and by the non invasive  
41 impedance method [62]. The amount of surviving cells was much higher than indicated by the  
42 recultivation experiments. This shows the importance of the integrity of the biofilms during  
43 the evaluation of disinfection treatment efficiency and quantification of surviving cells.  
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60 Microcalorimetry has been applied successfully to determine MICs (minimal inhibitory  
concentrations) [63, 64] . In this study the effect of various antibiotics towards *E. coli* and *S.*

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3 *aureus* strains was investigated calorimetrically and the results were compared to the standard  
4 method based on turbidity measurements. It clearly indicated that MIC determination via the  
5 heat signal works and even subinhibitory effects were revealed. First investigations  
6 concerning antibiotic influence on biofilm bacteria were done using chip-calorimetry (Fig. 4).  
7 These results clearly qualify this technique for the monitoring of antibiotic or disinfection  
8 treatments. Further research especially aiming at high throughput measurements is intended to  
9 be conducted in the near future.

### 20 21 22 **Calorimetry for the evaluation of biofilm-growth on surfaces**

23 For the attachment of bacteria, the initial step of biofilm growth, the character of the surface is  
24 very important.[65] Currently, much research is performed to develop materials which  
25 prevent bacterial attachment and biofilm growth [66]. Again, fast and easy methods are  
26 required for evaluating the efficiency of the new antimicrobial surface coatings or  
27 modifications. Regardless of its shortcomings, CFU determination is very often the method of  
28 choice. An attempt has been made for instance by Andersen and Co-workers who developed a  
29 flow-chamber test in which biofilm formation is determined microscopically using the well  
30 established crystal-violet staining [67]. In this approach even a flow regime was kept, which  
31 means a substantial progress compared to the often used microtiter-plate assays. However,  
32 microscopic approaches using special dyes or fluorescence-tagged probes (Fluorescence In-  
33 Situ Hybridization - FISH) [68] can characterize biomass on surface only with limitations  
34 stated before. A replacement of these techniques would be desirable. The non-invasive and  
35 non-destructive character of calorimetry, as well as the smaller effort needed to perform the  
36 measurements, qualifies calorimetry as a tool for the evaluation of biofilm growth on  
37 surfaces. A recent publication applied calorimetry to investigate biofilm growth on different  
38 materials for bone transplantations [69] and further applications of this kind are to be  
39 expected.

### 3.4) The future of calorimetry in biofilm research

For a broader application of calorimetry in biofilm research some technical and scientific challenges have to be overcome. There are several reasons, why this technique is not yet established in biofilm research, e.g. the high costs and the low throughput of the commercially available instruments, the limited flexibility and most likely the lack of expertise required handling and interpreting thermodynamic data.

Steps to improve the situation have been achieved with the development of chip-calorimeters. These instruments are cheaper than the former commercially available systems. They allow the separation of biofilm cultivation and measurement and increase the possible throughput.

A development towards real high-throughput measurements has been made by Torres et al in 2004 [70]. They presented a low cost nano-calorimeter which can detect enthalpies of binding, enzymatic turnover, and other chemical reactions in arrays of 96 positions. Sample volume and measurement time are reduced by three orders of magnitude compared to a conventional calorimeter. They suggested further applications in the field of proteomics or drug discovery. The first commercially available high-throughput-calorimetric system was a device by the Belgian company Vivactis. This calorimeter variation performs measurements in microplate format (96-well). It works completely automated with robotic sample-handling and computerized data acquisition and data handling. The main problems of such multichannel chip-calorimeters are cross-talk effects between the channels and the large influence of water evaporation due to the open design of these arrays. For further information on enthalpy arrays and high throughput developments in calorimetry the reader may consult [71]. One approach of multichannel chip-calorimeters using whole eukaryotic cells was published by Verhaegen et al. in 2000 [72]. To transfer these developments to high throughput biofilm research, these methods have to be adapted carefully.

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3 A scientific challenge is the use of the thermodynamic heat data in combination with other  
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5 methods to calculate energy balances for biofilm bacteria. Furthermore, most investigations  
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7 have been done with lab cultures where the involved bacteria are known. The exposure of  
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9 flow cells or designed substratum to the environment followed by calorimetric measurements  
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11 would prove the applicability for environmental and ecological studies.  
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15 Recent developments in the field of photocalorimetry could even allow the investigations of  
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17 phototrophic biofilms [73].  
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20 In summary, with the progress in modern calorimetry design, characterized by  
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22 miniaturization, ultra sensitive measurement and high throughput ability, a promising biofilm  
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24 monitoring tool entered the scientific scene. This measurement principle provides real time  
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26 information about metabolically active cells. It is able to measure any type of sample and is  
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28 flexible enough to be integrated into technical or natural systems of interest.  
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For Peer Review

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3 **Figure legends**  
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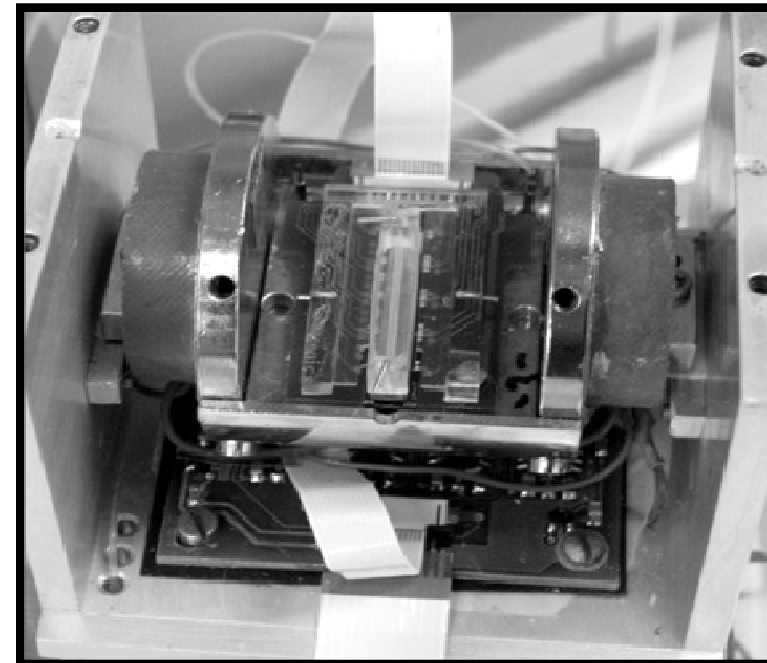
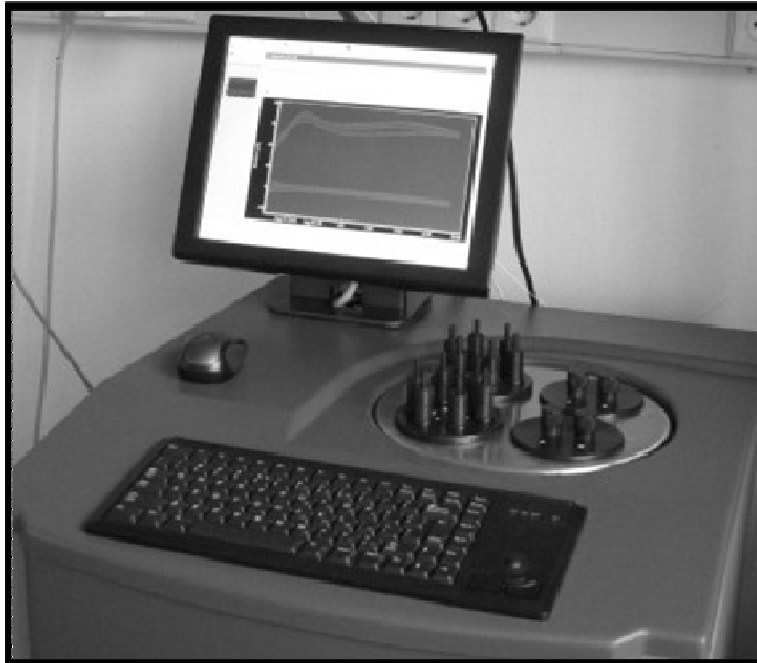
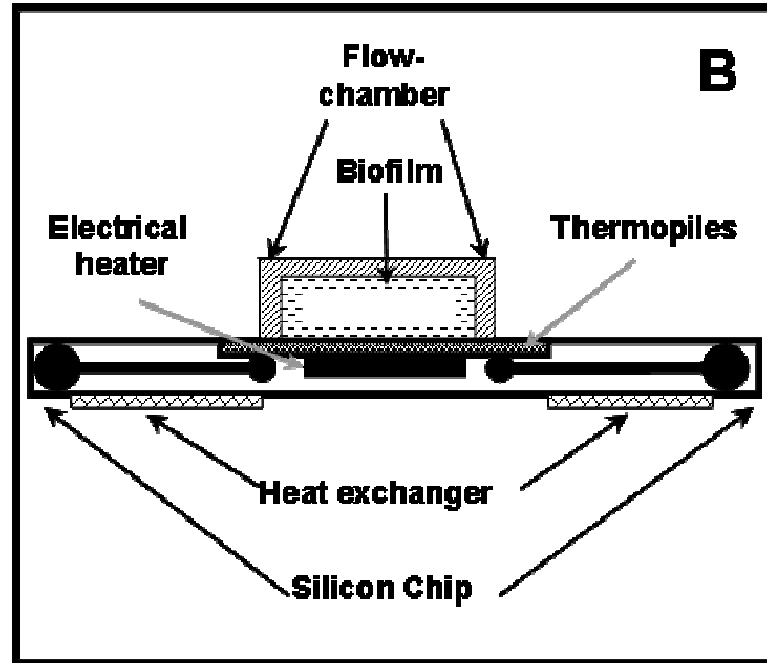
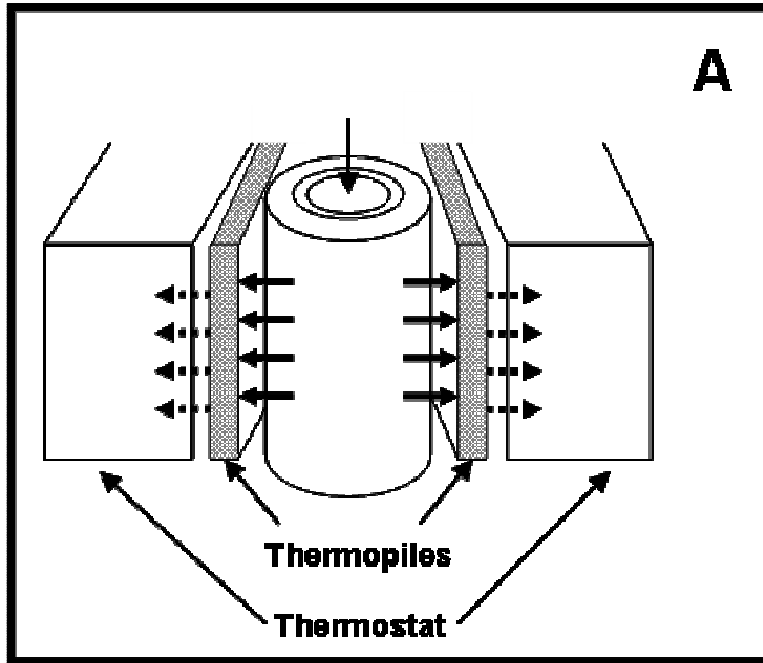
5 Fig.1: (A) TAM series; TA Instruments, USA) and (B) a chip-calorimeter  
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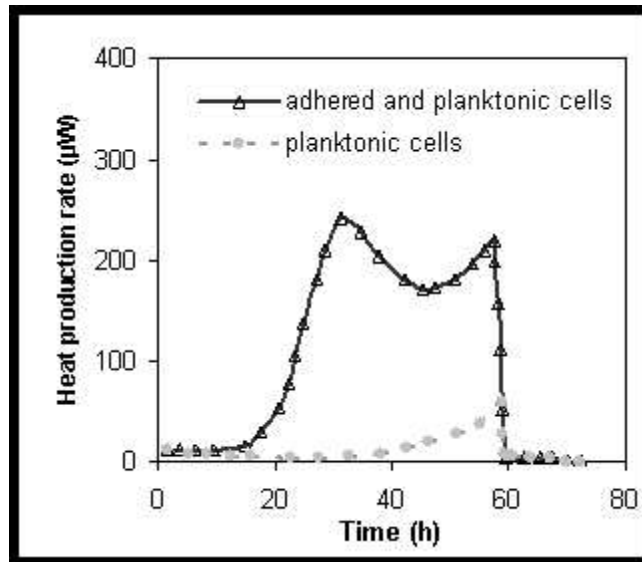
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10 Fig.2: Heat output of *T. intermedius* growing on thiosulfate (reconstructed data from [30])  
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15 Fig. 3: Heat signal of an *E. coli* biofilm growing on glycerol. Rectangles indicate the ratio of  
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17 TOC to the known carbon input (glycerol and acetate) in the outflow [28].  
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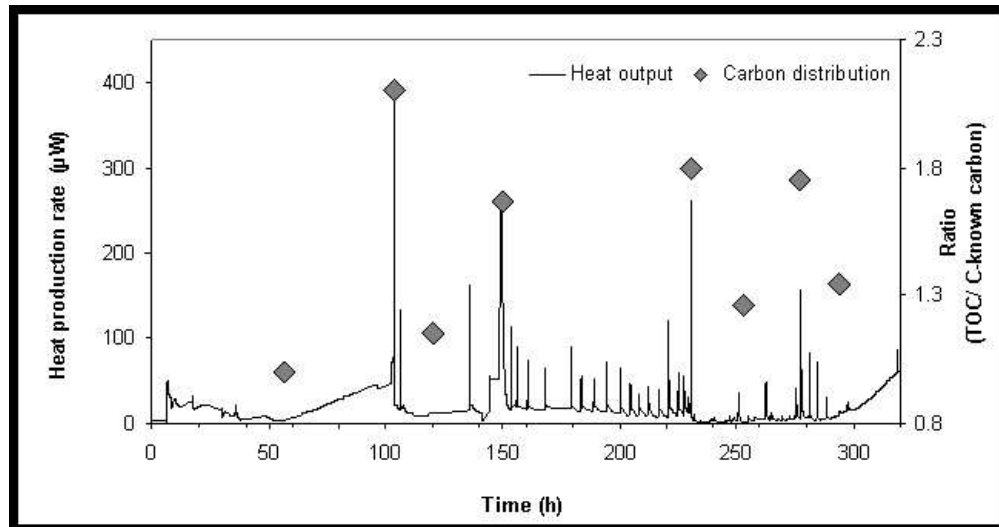
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22 Fig.4: Heat responses of *Pseudomonas putida* biofilms towards the bacteriostatic antibiotic  
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24 tetracycline and the bactericidal kanamycin and the biocide (2% formaldehyde) [37].  
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29 Fig. 5: Heat production rates from different soil samples, graphs reconstructed based on data  
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31 from Barros et al. [32]  
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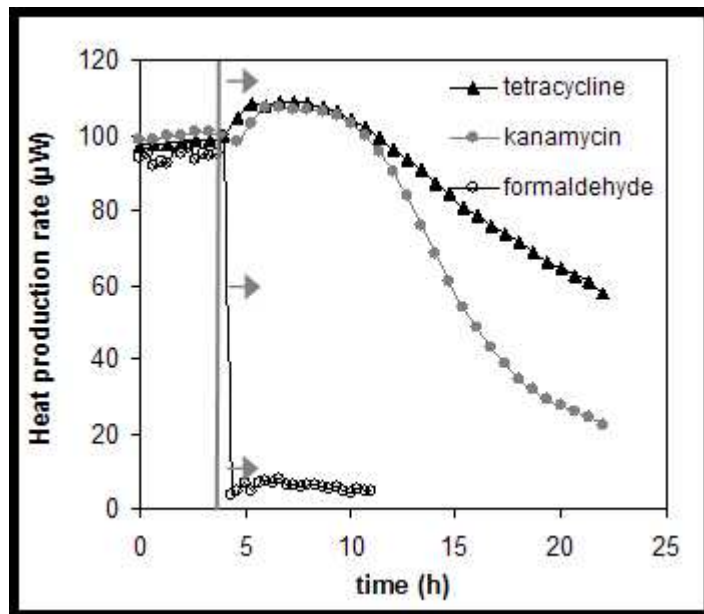




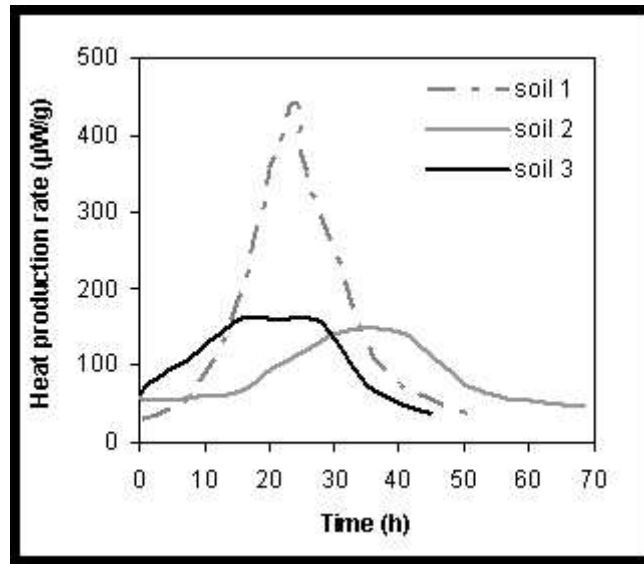
Heat output of *T. intermedius* growing on thiosulfate (reconstructed data from [30])  
85x74mm (96 x 96 DPI)



Heat signal of an *E. coli* biofilm growing on glycerol. Rectangles indicate the ratio of TOC to the known carbon input (glycerol and acetate) in the outflow [28].  
160x84mm (103 x 103 DPI)



Heat responses of *Pseudomonas putida* biofilms towards the bacteriostatic antibiotic tetracycline and the bactericidal kanamycin and the biocide (2% formaldehyde) [37].  
93x80mm (96 x 96 DPI)



Heat production rates from different soil samples, graphs reconstructed based on data from Barros et al. [32]  
85x74mm (96 x 96 DPI)