FEMS MICROBIOLOGY LETTERS

MINIREVIEW

# Use of isothermal microcalorimetry to monitor microbial activities

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Received 24 June 2009; accepted 7 October 2009.
Final version published online 6 November 2009.

DOI:10.1111/j.1574-6968.2009.01819.x

Editor: Simon Silver

**Keywords**
microcalorimetry; environmental microbiology; medical microbiology; microbial activity.

## Abstract

Isothermal calorimetry measures the heat flow of biological processes, which is proportional to the rate at which a given chemical or physical process takes place. Modern isothermal microcalorimeters make measurements of less than a microwatt of heat flow possible. As a result, as few as 10 000–100 000 active bacterial cells in culture are sufficient to produce a real-time signal dynamically related to the number of cells present and their activity. Specimens containing bacteria need little preparation, and isothermal microcalorimetry (IMC) is a nondestructive method. After IMC measurements, the undisturbed samples can be evaluated by any other means desired. In this review, we present a basic description of microcalorimetry and examples of microbiological applications of IMC for medical and environmental microbiology. In both fields, IMC has been used to quantify microbial activity over periods of hours or even days. Finally, the recent development of highly parallel instruments (up to 48 channels) and the constantly decreasing costs of equipment have made IMC increasingly attractive for microbiology. Miniaturization of isothermal calorimeters provides an even wider range of possibilities.

## History of isothermal microcalorimetry (IMC)

The first isothermal calorimeter was devised and used in 1782–1783 by Lavoisier and Laplace to determine the heat produced during chemical changes. This was the 'ice-calorimeter,' in which a sufficient amount of ice was used to keep the temperature constant (Lavoisier & Laplace, 1780). These early scientists realized that the mass of liquid water produced by the melting ice was directly proportional to the heat produced by the reaction taking place atop the ice. Many improvements have of course been made since the early 19th century. In addition, several other types of calorimeters have evolved besides the ones that operate isothermally – for example adiabatic, constant-volume, constant-pressure, heat loss and temperature (differential) scanning calorimeters (van Herwaarden, 2000). Some of them can also be used in the isothermal mode. In the isothermal approach, isothermal titration calorimetry has emerged as the broadly used standard for thermodynamic characterization of relatively fast reactions between molecules – for example ligand binding – especially for molecules of interest in biology (Cooper, 2003). However, because isothermal titration calorimetry is mostly a tool for molecular studies, it is not covered here.

This review focuses on IMC in microbiology for a wide variety of purposes including microorganism detection and discrimination, evaluation of microbial processes and determining the performance of antimicrobial agents. The term IMC is used here to refer to measurements in the microwatt range under essentially isothermal conditions (Wadsö, 2001). The related instruments are often called isothermal microcalorimeters. Most isothermal microcalorimeters are heat conduction calorimeters in which heat produced in the reaction vessel is allowed to flow to a heat sink, usually made of aluminum. Therefore, so-called isothermal microcalorimeters are not truly isothermal, but allow small variations of the sample temperature (up to 0.1 °C). Variation in the sample temperature mostly does not affect the heat sink temperature significantly because the heat sink has a much higher heat capacity than the reaction vessel and its contents (usually $\times 100$). In addition, the heat sink is often placed in a thermostat, ensuring its temperature stability. The heat transfer between the vessel and the heat sink takes place through a thermopile, allowing measurements of the heat produced or consumed (Wadsö & Goldberg, 2001). In other isothermal microcalorimeters, thermoelectric compensation is preferred to maintain isothermal conditions. Heat produced is compensated using Pelletier elements, and

similarly, heat consumed is compensated either by an electric heater or by reversing the polarity of the Peltier elements (van Herwaarden, 2000). In both heat conduction and heat compensation isothermal microcalorimeters, an electric signal can be easily recorded and calibrated to be proportional to the heat flow (van Herwaarden, 2000). To increase sensitivity and accuracy, most isothermal microcalorimeters in use are 'twin instruments,' where heat flow from the reaction vessel is compared with the heat flow from an inert reference ideally having similar heat capacity and heat conductivity as the reaction vessel plus its contents.

The sensitivity of modern isothermal microcalorimeters has, for many years, allowed the investigation of a broad spectrum of relatively slow processes generating microwatts of heat flow in specimens of gram-range (or smaller) amounts of material over a period of hours or days. Examples include food deterioration (Gomez Galindo *et al.*, 2005; Wadsö & Gomez Galindo, 2009) and drug shelf-life (Wadsö, 1997). However, IMC investigations of microbial processes are also becoming increasingly popular. Therefore, the aim of this minireview is to describe the advantages and drawbacks of IMC for such use as well as to provide a brief review of published applications in two fields of microbiology.

## Advantages of isothermal calorimetry in microbiology

### Sensitivity

Table 1 gives the specifications of the sensitivity of several commercial instruments. With a sensitivity on the order of 0.2 μW, IMC can detect the heat produced by a small number of microorganisms. Assuming that a typical single bacterial cell produces ~2 pW when active (Higuera-Guisset *et al.*, 2005, O. Braissant, pers. commun.), only 100 000 bacteria are required to produce a detectable signal in most commercial isothermal microcalorimeters. The typical volume of liquid in an isothermal microcalorimeter measurement vessel (often a disposable glass ampoule) is 1–4 mL. This means the detectable concentration of active microorganisms is between about $2.5 \times 10^4$ and $1.0 \times 10^5$ bacteria $mL^{-1}$. In comparison, the turbidity of such samples would be far below the McFarland standard number, 0.25, which calibrates turbidity for a bacterial concentration of $\sim 0.75 \times 10^8$ CFU $mL^{-1}$ (according to the manufacturer's specifications). In addition, the lower ($10^4$–$10^5$) cell concentrations easily detected by microcalorimetry would not be detectable even using a spectrophotometer (i.e. measuring the turbidity at 600 nm).

**Table 1.** Performances of some common commercially available IMC instruments

| Calorimeter brand | Model | Accuracy (%) | Precision (nW) | Nb* channels |
|---|---|---|---|---|
| TA instruments | TAM 48 | 5 | ± 200 | 48 |
| TA instruments | TAM III | 1 | ± 100 | 1–4 |
| Setaram | C80 | 1 | ± 100 | 1 |
| THT | μMC | Not known | ± 200 | 6†–12‡ |
| Templabs | Not known | Not known | ± 150 | 1 |

*Number of channels.
†Four channels + two references.
‡Eight channels + four references.
THT, thermal hazard technology.

### Accuracy

IMC instrument thermostats can be set at any temperature within an instrument's performance range (e.g. 15–300 °C) with high accuracy, typically within 0.02 °C. Fluctuations around the set point are between $10^{-3}$ and $10^{-5}$ °C. During reactions, the temperature of the ampoule is maintained within 0.1 °C of the set temperature. The dynamic range of reaction-related heat flow that can be measured is very high. Depending on the instrument, it is at least ± 50 mW and can be as much as ± 2000 mW. This is orders of magnitude greater than the range of 0.2–500 μW typically produced by detectable growth of microbial specimens in 1–3 mL media in 4-mL ampoules. The baseline drift of IMC instruments is typically ~0.2 μW per 24 h. Therefore, for intermediate heat flow ranges (e.g. 20–100 μW) over a few days, the IMC accuracy can be expected to be ~1%. This potentially provides a high accuracy for dynamic measurements of bacterial numbers that cannot be achieved with microscopic enumeration, plate counts or protein assays.

### Continuous real-time data

IMC provides a continuous real-time electronic signal proportional to the amount of heat being produced by an ampoule containing microorganisms. Although the signal must be interpreted carefully, it in effect allows to continuously observe the fluctuations in microorganism metabolic activity and replication rates as they occur (Fig. 1).

### Simplicity and passivity

In the simplest form of microorganism IMC, samples containing microorganisms are placed in a disposable glass ampoule, the ampoule is sealed and placed in one of the measuring channels and heat flow measurements are made as long as there is a heat flow signal of interest (e.g. from hours to days). The signal can be evaluated as it occurs and/or recorded for later evaluation. With microorganism cultures in liquid media, flow-through and flow-stop systems can and have been used, but they trade control for experimental complexity (Jespersen, 1982). For example,

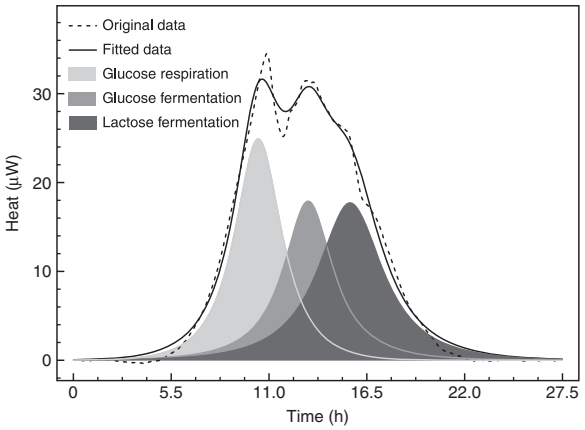


**Fig. 1.** Heat-flow record of the growth of *Escherichia coli* (DSM 10142) in M9 medium containing glucose and lactose (original data are the average of three replicate cultures). Deconvolution yielded individual overlapping peaks that were assigned to glucose respiration, glucose fermentation and lactose fermentation, respectively, according to the observation of Monod (1949). Fitted data represent the addition of the three individual peaks. The heat-flow record was obtained using a TAM 48 (TA instruments) (O. Braissant, unpublished data).

sterilization of flow systems is fastidious and time consuming, and raises safety concerns with pathogenic bacteria. Also, adhesion of microorganisms to the internal surfaces of the flow system potentially compromises the interpretation of results unless one wants to study biofilm formation (von Rège & Sand, 1998). Finally, because heat flow measurements are passive and external, the undisturbed contents of a sealed ampoule are available for other evaluations after IMC measurements are completed.

## Drawbacks of isothermal calorimetry in microbiology

Although IMC presents several interesting advantages, it also has many potential drawbacks.

### Equilibration

To obtain such high sensitivity and accuracy, isothermal microcalorimeters require that the sample and a reference sample (if any) are precisely at the desired temperature during measurements. In most cases, this requires an initial equilibration time of ~1 h, during which data cannot be collected. Flow systems can reduce this time, but introduce the complexities described above.

### Changes in the microorganism environment

As mentioned above, in most IMC studies, samples are placed in closed ampoules. Thus, chemical factors such as oxygen depletion and accumulation of metabolic waste products have to be taken into account in interpreting the results.

Nevertheless, anaerobic processes such as sulfate reduction (Chardin *et al.*, 2002), denitrification (Maskow & Babel, 2003) and fermentation (Antoce *et al.*, 2001) were successfully studied in sealed static ampoules. On the other hand, due to the low solubility of oxygen into aqueous solutions (Stumm & Morgan, 1996), the study of aerobic microorganisms in sealed ampoules is more difficult. For such aerobic microorganisms in sealed ampoules partly filled with unstirred liquid medium in equilibrium with air in the headspace, aerobic respiration will rapidly render the medium anoxic. However, oxygen depletion can be avoided by ensuring that the microorganisms have a direct air interface. This can be accomplished using solid media (e.g. agar or uncompacted soil). For soil studies, Ljungholm *et al.* (1979) have demonstrated that the problem can be readily solved. They closed their ampoules with a 1-mm-thick porous silicone rubber seal because the material readily transmits simple gases. This procedure was shown to allow sufficient gas exchange ($O_2$ and $CO_2$), without significant loss of water, between the calorimetric ampoule and the atmosphere. Similarly, addition of glucose as a powder and not as a solution to soil samples combined with the use of a flow-through cell is also a simple means to achieve calorimetric measurements in soil samples (Sparling, 1983) without reaching oxygen depletion. Finally, it is also possible to calculate the amount of oxygen present in the headspace of the calorimetric ampoule and calculate the amount of substrate that can be consumed using this oxygen. Using such simple calculations, Vor *et al.* (2002) were able to estimate when the transition from oxic to anoxic conditions in soil samples occurred and study changes in the metabolic heat production associated with this transition. Similarly, the use of agar medium or other solid growth substrates allows microorganisms to grow on top of the medium and therefore remain in contact with oxygen present in the headspace (Wadsö *et al.*, 2004). Furthermore, a closed environment can also be analytically advantageous – for mass balance calculations for example.

### Nonspecificity of IMC data

Finally, it must be noted that the heat flow signal is a nonspecific, net signal related to the sum of all chemical and physical processes taking place in an IMC ampoule. As a consequence, unknown phenomena may produce some of the heat measured, and there may be simultaneous exothermic and endothermic processes taking place (Lewis & Daniels, 2003). However, well-described phenomena can be studied under controlled conditions with a high accuracy [see the 'diauxie' (Monod, 1949) example in Fig. 1, Table 2]. Careful planning of IMC experiments is of great

**Table 2.** Measured and calculated heat produced during the growth of *Escherichia coli* (DSM 10142) in M9 medium containing glucose and lactose as carbon sources

| Metabolism | Calculated (mJ)* | Measured (mJ) |
|---|---|---|
| Glucose respiration | 380 | 422 |
| Glucose fermentation | 350 | 349 |
| Lactose fermentation | 420 | 433 |

*Calculations assume the following: (1) $C_6H_{12}O_6$ (glucose) $+6O_2 \rightarrow 6CO_2+6H_2O$ ($\Delta G = 2860\,kJ\,mol^{-1}$), (2) $2C_6H_{12}O_6 \rightarrow 2\text{-}C_3H_6O_3$ (lactic acid) ($\Delta G = 1800\,kJ\,mol^{-1}$) and (3) hydrolysis of lactose provides two hexoses, which are then fermented as described in equation (2). Original oxygen concentration for the calculation was estimated using the Weiss (1970) equation. Measured values were obtained after deconvolution of the heat-flow pattern shown in Fig. 1. Note that the discrepancy for glucose respiration is probably due to oxygen diffusion.

importance. Logical experimental designs must be devised and used that ensure that the observed heat flows are directly related to the processes of interest.

## Applications of IMC in microbiology

IMC has been used in many different fields of microbiology. Medical and environmental applications provide an indication of the possibilities.

### Medical

One noteworthy medical application is rapid isothermal microcalorimetric detection of bacterial infection or contamination, which is of critical importance in quickly implementing the correct treatment. Recent studies have shown that with IMC, it is possible to detect bacterial contamination of donated blood platelets within a few hours (Trampuz *et al.*, 2007). Similarly, it is also possible to determine inhibitory effects and/or the minimal inhibitory concentration for different antimicrobial compounds and microorganisms within hours using IMC (Xi *et al.*, 2002; Yang *et al.*, 2008; von Ah *et al.*, 2009). Also, the IMC heat flow patterns at inhibitory and subinhibitory concentrations provide indications of the nature of inhibition (i.e. bactericidal vs. bacteriostatic) (von Ah *et al.*, 2009). In addition, the bacterial growth-related heat flow patterns observed by IMC can allow rapid discrimination of medically important microorganisms. For example, IMC can be used to differentiate methicillin-susceptible *Staphylococcus aureus* from methicillin-resistant *S. aureus* within 5 h (von Ah *et al.*, 2008; Baldoni *et al.*, 2009). Finally, in connection with dentistry, it has been shown that IMC can measure the growth and the heat of adsorption of mouth bacteria on surfaces (Hauser-Gerspach *et al.*, 2008).

In addition to detection and evaluation of bacterial infection and antimicrobial agents, IMC has proven to be an effective tool in studying viral infections and activities of antiviral compounds (Tan & Lu, 1999; Heng *et al.*, 2005). Heng *et al.* (2005) emphasize that the change in the metabolism of BHK-21 cells infected by the foot and mouth disease virus was easily indicated by the strong heat production of these infected cells compared with uninfected controls.

### Environmental

For environmental microbiology, IMC is of great value in assessing bacterial activities directly without the need to separately culture organisms or add radiolabelled, fluorescent or chromogenic substrates. Therefore, IMC is an excellent complement to molecular studies. For example, early observations of lake and marine sediments have shown that there was a linear relation between the dehydrogenase activity assayed using triphenyltetrazolium chloride (TTC) or iodonitrotetrazolium chloride (INT) and sediment heat production (Pamatmat & Bhagwat, 1973; Pamatmat *et al.*, 1981). In addition, Pamatmat *et al.* (1981) also found a strong correlation between the concentration of ATP in the sediment and the heat production. Similarly, a more recent study on lake sediments has concluded that heat production followed the same trend as radiolabelled leucine and thymidine incorporation. This study concludes that IMC is especially useful with sediments that contain mixed communities of anaerobes, fermenters and aerobes (Haglund *et al.*, 2003). However, it must be noted that the method is somewhat unspecific in distinguishing between metabolic heat and chemical heat, and therefore several controls are required to determine the quantity of chemical heat. The relationships between heat production, ATP and dehydrogenases assay (TTC or INT) have also been observed for larger organisms such as the nematode *Caenorhabditis elegans* (Braeckman *et al.*, 2002). With respect to the size of the aquatic microorganisms considered, IMC has also been useful in investigating allometric relations between mass, surface area and metabolic rate (measured as heat production) of aquatic protists ranging from 1 to $10^6\,\mu m^3$ in size (Johnson *et al.*, 2009). In addition, based on their microcalorimetry results, the authors hypothesized that for these organisms, the cost of motility was low. Potentially, a similar approach might be used for several other features of microorganisms, emphasizing once again the versatility of microcalorimetry.

In soils, IMC has been used to investigate many different processes. Rong *et al.* (2007) identified three major types of IMC studies involving soils. These are: (1) the detection and quantification of microbial activities, (2) the monitoring of organic pollutant toxicity and degradation and (3) the risk assessment associated with heavy metal (and metalloid) contamination. With respect to the detection and

quantification of microbial activities, it was shown that viable cell counts of bacteria and fungi were significantly correlated to IMC-measured heat production (Critter *et al.*, 2002). It was also observed that soil oxygen consumption (i.e. respiration) was highly correlated with heat production when samples were amended with glucose. Such correlations were used to estimate soil microbial biomass (Sparling, 1983; Raubuch & Beese, 1999). In addition to soil biomass estimation, Barros *et al.* (1999) were able to determine an 'apparent' microbial growth rate constant of the microbial populations in different soil samples. The same group also showed that an increasing microbial density resulted in a lower heat production rate per cell. They interpreted the observed negative correlation as indicating a change in microbial strategy toward a more efficient metabolism (Barros *et al.*, 2003). Unfortunately, to our knowledge, no studies performed in soils compared the activity of dehydrogenases (using tetrazolium salts) to activities measured using microcalorimetry. Finally, use of IMC has been demonstrated to be a sensitive tool for studying composting processes (Laor *et al.*, 2004). Nevertheless, in both soil and compost, it was shown that particular attention needed to be paid to methodological aspects such as sample sieving, homogenization and sterilization to avoid systematic errors (Medina *et al.*, 2009; Wadsö 2009).

The previously described studies with sediments emphasize the great versatility of IMC with respect to the nature of the samples that can be evaluated. They also indicate the potential for using different types of media in IMC; for example, utilization of solid culture media has only begun to be explored. Solid media have been shown to be especially useful to facilitate growth of fungi in IMC ampoules and thus enable faster, more accurate studies (Wadsö *et al.*, 2004). For fastidious microorganisms, microorganisms that are difficult to grow in liquid media and filamentous organisms that are difficult to quantify by absorbance, IMC provides a simple and sensitive method to quantify growth.

## Future directions

IMC is a promising tool for medical and environmental microbiology and other areas such as food microbiology. The availability of multicalorimeter instruments allows one to explore many different experimental conditions (except temperature) at once and/or evaluate many replicate specimens at the same time. One current instrument (Table 1) allows simultaneous independent evaluations in 48 measuring channels. It seems likely that IMC will soon become a standard method in clinically related microbiology. The clinical need is actually for multicalorimeter instruments, which are simpler (e.g. having a narrower range of set temperatures) than current multicalorimeter research instruments.

However, for more research-oriented applications, it is, as mentioned earlier, difficult to identify unknown specific phenomena based on IMC only (Lewis & Daniels, 2003). Therefore, to support and interpret nonspecific microcalorimetric results, other analytical measurements are often desirable (Wadsö, 2002). Such analytical capabilities can include added in-line sensors in the case of a flow-cell IMC instrument. However, as stated earlier, such systems are difficult to set up and sterilize. On the other hand, several attempts have been made to add sensors to the measurement ampoule. For example, Johansson & Wadsö (1999) constructed an isothermal microcalorimeter vessel that contained a miniaturized spectrophotometer, plus pH and oxygen electrodes. Johansson & Wadsö (1999) emphasize that many different types of analytical sensors or microsensors are available and could be added. Similarly, Criddle *et al.* (1991) have demonstrated that a device consisting of two microcalorimetric ampoules connected by tubing could be used to measure metabolic heat and $CO_2$ production simultaneously. In this system, one ampoule served as the sample container, and the other contained NaOH and acted as a $CO_2$ trap, with $CO_2$ trapping resulting in measurable heat flow production as well. An additional pressure sensor was added to this system to deduce oxygen concentration from the pressure decrease. Both the approaches presented above, coupling IMC and analytical sensors, seem to be highly promising. However, both of these early setups were 'home-made,' and commercial instruments including such features have not yet emerged. This has probably strongly discouraged other experimenters from supplementing isothermal microcalorimeters in this manner in more recent years. Conversely, it perhaps also indicates how much can already be accomplished with sealed IMC ampoules, followed by postanalysis application of other analytical methods.

Another promising area of IMC instrumentation has emerged with the development of 'calorimeter chips' (van Herwaarden, 2005). These commercially available chips are only a few millimeters in size and are usually encased in an aluminum block that acts as a heat sink. These chips have already been used to monitor bacterial growth from the heat produced (Higuera-Guisset *et al.*, 2005; Maskow *et al.*, 2006). Modified calorimeter chips have also been used as biosensors. Using chip-immobilized glucose oxidase, urease and penicillinase, the heat generated by the oxidation of glucose and the hydrolysis of urea and penicillin were easily detected (Bataillard, 1993; Bataillard *et al.*, 1993). Other chip calorimeters have been used to determine biochemical reactions (mostly enzyme : substrate reactions) by direct mixing in the microcalorimeter chamber (Zhang & Tadigadapa, 2004; Lerchner *et al.*, 2006). Using a similar type of calorimeter chip, Yoon *et al.* (2008) demonstrated that it was possible to detect heat produced during the reaction of

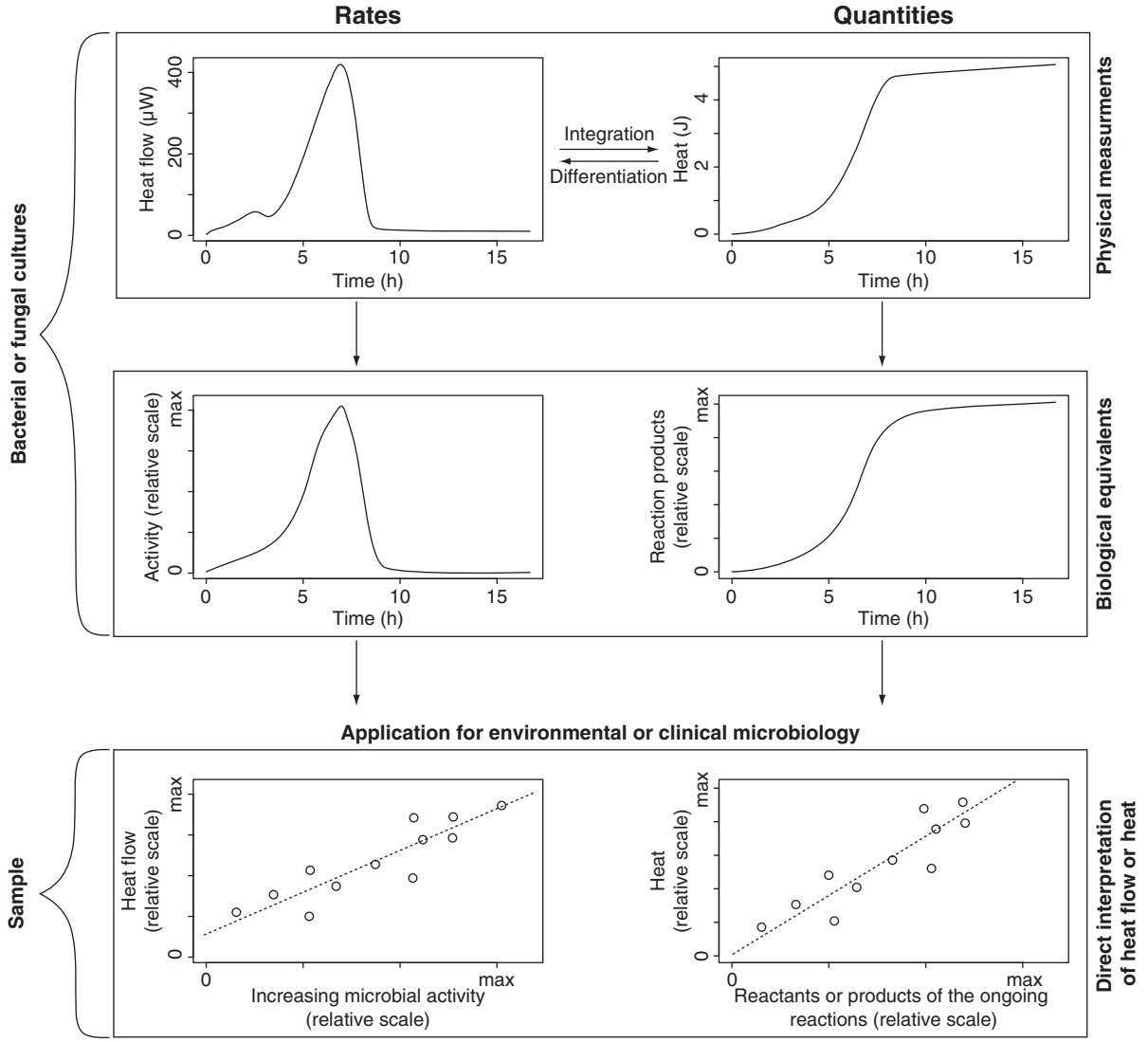


**Fig. 2.** Sketch showing the relationships between actual calorimetric measurements [i.e. heat flow ( ≈ thermal power) and heat], their biological equivalents (microbial activity and products resulting from microbial activity). Note that the heat flow is directly proportional to the microbial activity (see text for further details) measured using TTC assay, INT assay or oxygen consumption rate measurements. Therefore, heat flow and activity represent metabolic rates. On the other hand, heat is directly proportional to the biomass produced, measured by plate counts, protein concentration or OD. Moreover, in certain cases, heat is also directly linked to the electron acceptors consumed, and their reduced counterpart produced. For example, total $CO_2$ produced or oxygen consumed can be related to heat. Similarly, $H_2S$ or $NO_2$ produced by sulfate-reducing bacteria and denitrifying bacteria can also be linked to the heat. In this case, heat is an indication of the quantity of substrate consumed or metabolic product released. Therefore, in samples (soil, sediment or medical sample), there is a correlation between heat flow and microbial activity or between the heat evolved and available carbon (for example).

*Neisseria meningitidis* and its specific antibody HmenB3. It seems likely that chip calorimeter devices could be developed and used in environmental or clinical settings to rapidly check for contamination.

## Concluding remarks

IMC has already been proven to be a highly efficient and versatile tool in several fields of microbiology. It allows monitoring of microbial activity in samples *in situ* without prior preparation and offers a very low detection limit. As heat flow is an excellent proxy for microbial activity, the heat evolved provides valuable information on the global reactions that occur (Fig. 2). Heat flow and activity reflect metabolic rates and, on the other hand, heat is an indication of the quantity of substrate consumed or metabolic product released. Nevertheless, use of IMC is not yet common among microbiologists. This is probably due in part to the

current cost of multichannel isothermal microcalorimeters, which manufacturers indicate is mainly due to the low production volume. Thus, it is likely that the cost of instruments will decrease when increased numbers are being sold and also with further development of calorimeter chip-based instruments. Similarly, the use of other highly promising calorimetric techniques such as enthalpy arrays described by Torres *et al*. (2004) might be of great interest because they may allow the parallel processing of a large number of samples. Such arrays have been successfully used to determine enzymatic reactions for example (Recht *et al*., 2008).

In summary, we believe our review makes it clear that IMC is an increasingly valuable tool for microbiologists. IMC is unique in its ability to easily provide rapid detection and real-time, quantitative monitoring of a wide variety of microbiologic phenomena. There is ample opportunity for IMC to be transformed into a clinical tool having capabilities otherwise unavailable. Finally, with the increasing availability of chip-based sensors and calorimeters, IMC instrumentation seems likely to become both more versatile and more cost efficient.

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