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Direct measurements of heat output by marine microbial and phage communities

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ABSTRACT

An isoperible twin calorimeter was used to monitor effects of viruses, primarily phage, on heat output from a marine aquarium microbial community. The mean heat produced in a microbial reproduction event was approximately 50 nanojoules. Heat production in the presence of phage was larger than in the absence of phage, despite the smaller net increase in microbial cell numbers. A simple model predicts that approximately 33% of the total heat production was due to phage predation. These results show that phage actually cause more heat to be released from the ecosystem, despite a lower net microbial biomass, which is consistent with top-down control.

INTRODUCTION

All biochemical processes produce heat, which can be measured using a calorimeter. This offers a direct approach for monitoring microbial activities via their energy usage. Calorimeters have been used to measure the heat output of living systems for more than two hundred years, but only in recent years has instrumentation become sufficiently sensitive, compact, and robust to perform such measurements on natural samples from microbial communities in ocean environments. Commercial microcalorimeters still require the microbes to be concentrated 1000 times or more to measure the heat output of microbial communities (Pamatmat 1981, Vandenhove 1991, Gustafsson 1987, Larsson 1999, Mukhanov 2003, Mukhanov 2004).

Phage are important microbial predators that influence global biogeochemical cycles. In combination with protist grazing, phage predation is so intense that microbial numbers are maintained at values less than the carrying capacity of the system. This is called top-down control and has important implications for cycling rates of nutrient and energy. Phage are also important mediators of microbial diversity. Most phage are strain-specific predators. As a particular microbial strain becomes dominant in a system, its phage predators will expand exponentially and kill it off. This will leave a niche for another microbial strain to grow into, which will be subsequently killed off by another phage type. Therefore the dominant microbial species within a system will be constantly turned over. This "kill-the-winner" hypothesis may explain much of the observed microbial diversity and community structure.

Much of the available knowledge about the roles of phage in natural environments comes from studies of marine microbial communities. Phage production (i.e., how many phage are "born" per unit time) has been estimated in a number of ways. Initial attempts were based on the observation that mature virus particles are only visible in the cells for a brief period (~10%) just at the end of their life cycle. These phage-filled microbes can be counted under the electron microscope. The frequency of visibly infected microbes is used to estimate the number of microbes actually infected by phage (Proctor and Fuhrman 1990). Phage production has also been estimated by measuring decay rates of free phage particles. In these studies, by poisoning the bacterial cells or removing the cells by filtration, new phage production is inhibited (Heldal and Bratbak 1991). Phage are counted over time, and it is assumed that the replacement rate equals the production rate (i.e., a steady state is maintained). Phage production has been measured by first feeding host microbes radiolabeled nucleotides (Steward et al. 1992; Steward et al. 1992). When the microbes are infected by lytic phage, the labeled nucleotide is drawn from the host's internal pool and incorporated into phage DNA. After the cells are lysed, the labeled phage are released into the environment and production is estimated from the amount of radiation. Another way that phage production has been measured is by diluting microbial communities into phage-free water (Wilhelm et al. 2002; Winget et al. 2005). This reduction of ambient viruses lowers contact rates (i.e., decreases mass action) so that new infections do not happen over the course of the experiment. The increase in the new phage particles reflects the rate of viral production. Finally, phage production has also been measured using

fluorescently labeled virus (FLV) tracers. In this method, the phage are concentrated from an environmental sample, and fluorescently labeled using a nucleic acid stain such as SYBR Green I (Noble and Fuhrman 1998). The labeled viruses are added back to natural water samples at tracer levels (<10% of ambient virus concentration) and after various incubation times both the labeled viruses and total viruses are determined. The decrease in the proportion of labeled viruses over time allows for the calculation of viral production and removal rates.

Using these approaches, it has been shown that pelagic phage production typically ranges from 2 x 10^3 to 3 x 10^6 viruses ml^{-1} h^{-1} (Weinbauer and Rassoulzadegan 2004) with a general decrease in production rates from coastal areas to open waters. Based on these production rates, the total marine phage community turns over every 1-6 days (Wommack and Colwell 2000) and phage kill 4-50% of daily microbial production (Fuhrman 1999; Wilhelm and Suttle 1999; Wommack and Colwell 2000). However, phage production rates, and the subsequent microbial death rate, vary widely in time and space.

An isoperibol twin calorimeter is reported which is capable of measuring marine microbial (heterotrophic Bacteria and Archaea) heat production at natural concentrations (~10^5 cell ml^{-1}) with and without phage predation. This method yields information about growth rates, as well as relative impact on the underlying food sources (i.e., dissolved organic matter (DOM)) and associated energy heat production in natural ecosystems.

MATERIALS AND METHODS

Calorimeter design: A twin calorimeter was constructed with glass vessels of approximately 25 ml capacity. Reaction and reference vessels were placed in separate insulated enclosures within a large water bath. The temperature in the water bath was controlled by a thermostat (Sargent-Welch Co., Model ST; Buffalo, New York). Thermistors were used as the temperature-sensing elements and the reaction and reference vessel thermistors formed two arms of a Wheatstone bridge (Djamali 2005).

A personal computer was used for data collection and control via a 16-bit data acquisition board (Keithley, Inc., Model DAS-1802HR; Cleveland, Ohio). The calorimeter was provided with a calibration heater through which the chemical generation of heat by microbes was simulated by introducing a carefully measured quantity of electrical energy (Djamali 2005). A simplified diagram of the calorimeter is shown in Figure 1.

Aquarium water: Seawater was collected from Scripps Institution of Oceanography's pier (San Diego, CA) and placed in a 200 liter aquarium containing an assortment of macrofauna (corals, fish, and live rock), approximately 5 cm of crushed coral, a protein skimmer, and carbon filter. The aquarium was outfitted with lighting, so both photosynthesis and respiration continued.

Filters of appropriate pore sizes were used to remove particles of different sizes, as summarized in Table 1. An aliquot of the aquarium water was sterilized by filtration through a 0.02 μm pore size, 50 mm diameter Sartorius membrane. This water was stored in a dark place. Levels of dissolved organic matter varied according to the feeding schedules of the macrofauna, so in order to compare biological activity in two samples it

was necessary to collect pairs of samples simultaneously. Microbes from aquarium water samples, before and after each experiment, were directly counted using SYBR Gold . At least 20 fields were counted in each case. Cell counts are reported as the total number of microbial cells in each vessel. Reaction and reference vessel preparation protocols are summarized in Figure 2; the quantities given are those used for the run shown in Figure 3. The experiments were carried out at 23 °C.

RESULTS AND DISCUSSION

Microbial heat output without phage: Figure 3 shows an example of heat output from the differential twin calorimeter containing 20 ml of marine aquarium water in the reaction vessel and an equal volume of saline water from the same source sterilized by filtration in the reference vessel. The microbial cell count changed from 4.6×10^5 to 9.0×10^6 during the experiment. The temperature difference between the two vessels is recorded as a function of time. Microbial heat is generated in the reaction vessel, raising its temperature relative to the reference vessel; the difference levels off as oxygen and nutrients are depleted, or possibly due to quorum sensing among the microbes. The temperature change during the experiment was approximately 0.005 ^{o}C. The power output per microbial cell in these experiments, at the stage where population approaches steady state, was in the range 30 to 200 fW, consistent with the value previously reported (Mukhanov 2003). In a later investigation (Mukhanov 2004) it was concluded that the average power output per microbial cell had an inverse relation with concentration of the microbes present. In the present study, the concentration of microbial cells was 2

to 3 orders of magnitude less per ml than the previous study (Mukhanov 2003)In the absence of phages and presence of excess nutrients, the initial power output per microbial cell from this study was in the range 30 to 90 $pWcell^{-1}$.

In order to check the consistency of heats, further experiments were performed on aquarium water samples at different dilutions and using different sterilization methods. In one case the reference and diluents media were sterilized by filtration (0.02 μm), in the other case they were also autoclaved. The results are shown in Figure 4. While the total heat evolution is very nearly the same (0.63 vs. 0.62 J), the autoclaved sample gave much less power initially, possibly because of structural changes to the nutrients causing slower metabolism without changing the total available energy. The total heat evolution varied from day to day. This implies that the total heat evolution is probably limited by dissolved nutrient concentration, which varies over time according to the aquarium maintenance schedule, rather than by dissolved oxygen content. It also indicates that meaningful comparison of infected and non-infected populations must be limited to samples collected at the same time.

Heat output with a microbial and phage community: At sufficiently high dilutions, phage are not able to infect their target microbes before they degrade. In near-shore marine environments, a dilution of the viral community by 95% inhibits all lytic phage production (Wilcox& Fuhrman 1998, Sano 2004). Therefore the experiments summarized in Figures 3 and 4 constitute a calibration where the heat evolution is essentially all due to microbial growth. A smaller dilution step allows

significant rates of phage infection. An experiment with a 50% dilution step was performed, again using a sterile reference sample; the output is shown in Figure 5.

To compare the heat evolution from communities in the presence and absence of phage a diluent with a live phage medium in the reaction side and a sterilized (autoclaved) phage medium in the reference side were used. The experimental differential heat outputs from microbes, from aquarium water at different initial microbial concentration and in presence of concentrated live vs. autoclaved phage are summarized in Table 2. The results show that independent of initial concentrations, the final cell concentrations were always less and the total heat output was always more when live phage were present (see Table 2 and Figure 6). The results from Figure 6 show also that initial differential power output is inversely proportional to initial concentration.

A model of heat output by microbes: Two biological sources of heat were considered: (1) microbial growth and replication, and (2) phage replication and subsequent lysis of the host. Let q^+ represent the heat associated with one microbial replication and q^- represent the heat associated with one lysis event. Since phage are evolved to utilize every bit of energy from their host cell it is assumed here that a lysis event has similar heat output to the replicative cycle of the host,

$$q^+ \cong q^- \equiv q \qquad (1)$$

Let N^+ represent the number of microbial replication events during the experiment and N^- the number of lysis events. Then the total heat produced is

$$Q^{\text{tot}} = q\,(N^+ + N^-) \qquad (2)$$

with an associated net change in microbial population of

$$\ddot{A}N = N^{+} - N^{-} \quad (3)$$

In the experiments illustrated by Figures 3 and 4 the reaction vessel was inoculated with aquarium water at a dilution level sufficient to render any phage harmless. The dilution was 5% in Figure 3 and 10% in Figure 4. Consequently, in these experiments, we attribute the production of heat exclusively to microbial growth. That is, $N^{-} = 0$ in equations (2) and (3), and thus these experiments can give calculated values of

$$q = Q^{tot}/\Delta N \quad (4)$$

Also, because the reference vessel has no biological activity, the measured calorimetric differential heat output Q^{meas}, can be identified with Q^{tot}, the biological heat in the reaction vessel. The values of q calculated from the experiments in Figures 3 and 4 are q = 0.050, 0.042, and 0.050 μJ cell^{-1} respectively (See Table 3). For the subsequent analysis of Figures 5 and 6 its value is taken as q = 0.050 μJ cell^{-1}.

In the experiment of Figure 5, the dilution of the inoculum was 50%, resulting in considerable phage activity in the reaction vessel. Since the reference cell had no microbes present, we may again identify $Q^{meas} = Q^{tot}$. Equation (2) can then be used to determine $(N^{+} + N^{-})$ while the measured change in cell count, ΔN, determines $(N^{+} - N^{-})$ making it easy to determine the values of $N^{+}$and N^{-}.

The experiments in Figure 6 compared microbial activity with and without phage. Microbes were delivered to both reaction and reference vessels in the form of a 5% inoculum, by itself dilute enough to suppress phage activity. Phage were

introduced separately into the reaction vessel in a concentrated form to guarantee presence of phage in excess. In this case, since the reference vessel contains growing microbes, the determination of $N^+$and N^- is slightly more involved.

We proceed from our value of q and the measured values of Q^{meas}, ΔN_{meas}, and ΔN_{ref}. The heat produced in the reference cell is calculated as

$$Q_{ref}^{tot} = q\Delta N_{ref} \tag{5}$$

and the heat produced in the reaction vessel as

$$Q_{rxn}^{tot} = Q^{meas} + Q_{ref}^{tot} = q\,(N_{rxn}^{+} + N_{rxn}^{-}) \tag{6}$$

Combining this with

$$\Delta N = N_{rxn}^{+} - N_{rxn}^{-} \tag{7}$$

enables us to determine N_{rxn}^{+} and N_{rxn}^{-}.

We use these inferred numbers of replications and lyses to introduce a measure α of phage activity as the fraction of Q_{rxn}^{tot} that can be attributed to lysis

$$\alpha = \frac{N^-}{N^+ + N^-} \tag{8}$$

The values of phage activity α so obtained for the three curves, 6a, 6b and 6c, of Figure 6 are respectively, 0.33, 0.38, and 0.22. The value of α calculated for the curve in Figure 5 is 0.44. To one significant figure, we conclude that about 1/3 of the heat from a naturally growing microbial population is due to phage activity.

Conclusions

The present paper reports the first calorimetric measurements of marine microbes at natural concentration, albeit from an aquarium. The apparatus described makes possible calorimetric measurements on intact natural aquatic microbial communities.

We find that in the presence of phage, the total heat output is increased, while the final microbial cell count is decreased. A simple model indicates that this is due to phage lysis of the microbes, which accounts for about 1/3 of the heat produced.

Table 1. Filters used to select for important biological fractions.

Filter pore size, µm	**Biologically active components in filtrate**
0.02	Dissolved organic matter (DOM)
0.20	Phage* + DOM
0.45	Microbes** + Phage + DOM

* The phage fraction also contains Eukaryotic-specific viruses.

** Microbes refers to both Archaea and Bacteria.

Table 2. Differential heat output from microbes in presence and absence of phage.

expm	Test Cell 10^6 init. Count	Test Cell 10^6 final Count	Test Cell Phage	Reference Cell 10^6 init. Count	Reference Cell 10^6 final Count	Reference Cell Phage	Differential Heat Output (Joules)
6a	0.1	16.7	live	0.1	22.8	Autoclave	1.37
6b	0.4	5.6	live	0.4	8.8	Autoclave	0.63
6c	0.9	17.5	live	0.9	21.4	Autoclave	0.44

Table 3. Calculation of Microbial Energy Use and Phage Activity

	No PHAGE ACTIVITY			PHAGE ACTIVITY			
Figure	3	4a (red)	4b (black)	5	6a (red)	6b (blue)	6c (black)
Q_{meas}	0.43	0.62	0.63	0.5	1.37	0.63	0.44
ΔN_{rxn}	8.54	14.67	12.61	1.3	16.6	5.2	16.60
ΔN_{ref}	0	0	0	0	22.7	8.4	20.5
q	0.050	0.042	0.050	0.05	0.05	0.05	0.05
Q_{rxn}	0.43	0.62	0.63	0.5	2.50	1.06	1.46
Q_{ref}	0	0	0	0	1.135	0.42	1.02
N^+	8.54	14.67	12.61	5.65	33.35	13.2	22.95
N^-	0	0	0	4.35	16.75	8.00	6.35
α	0	0	0	0.44	0.33	0.38	0.22

Figure 1. (A) Simplified Calorimeter Diagram. (B) Reaction and Reference Vessel.

Figure 2. Example of Calorimetric Sample Preparation.

Figure 3. Differential heat output vs. time for twin calorimeter: Reaction vessel, 1 ml (0.45 μm filtered) plus 19 ml (0.02 μm filtered) aquarium water: Reference vessel, 20 ml (0.02 μm filtered) aquarium water. Initial cell count $4.6x10^5$ and final cell count $9.0x10^6$.

Figure 4. Comparison of the heat evolution vs. time from aquarium water samples with diluent sterilized by (0.02μm) filtration and autoclaving.

Figure 5. Differential output for twin calorimeter: Reaction vessel, 10 ml (0.45 μm filtered) plus 10 ml (0.02 μm filtered) aquarium water: Reference vessel, 20 ml (0.02 μm filtered) aquarium water. Initial cell count $2.1x10^6$ and final cell count $3.4x10^6$.

Figure 6. Differential heat output vs. time for microbes in aquarium water in presence of concentrated live and autoclaved phage (see Table 2).

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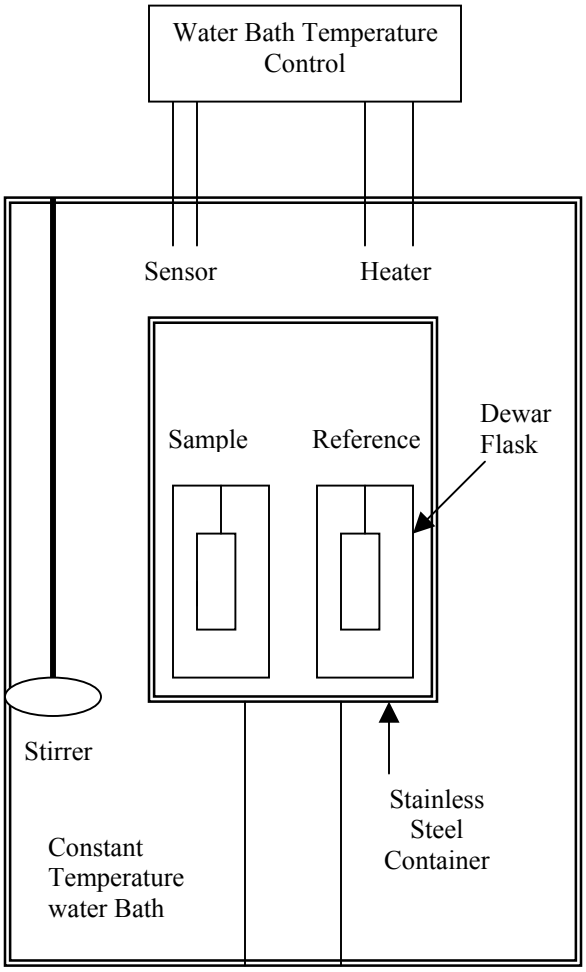
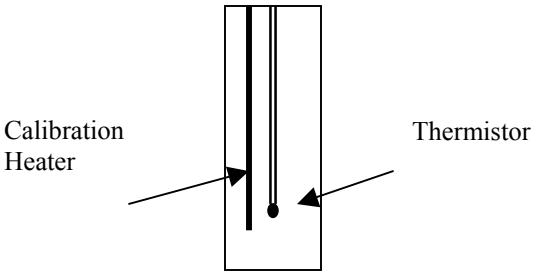
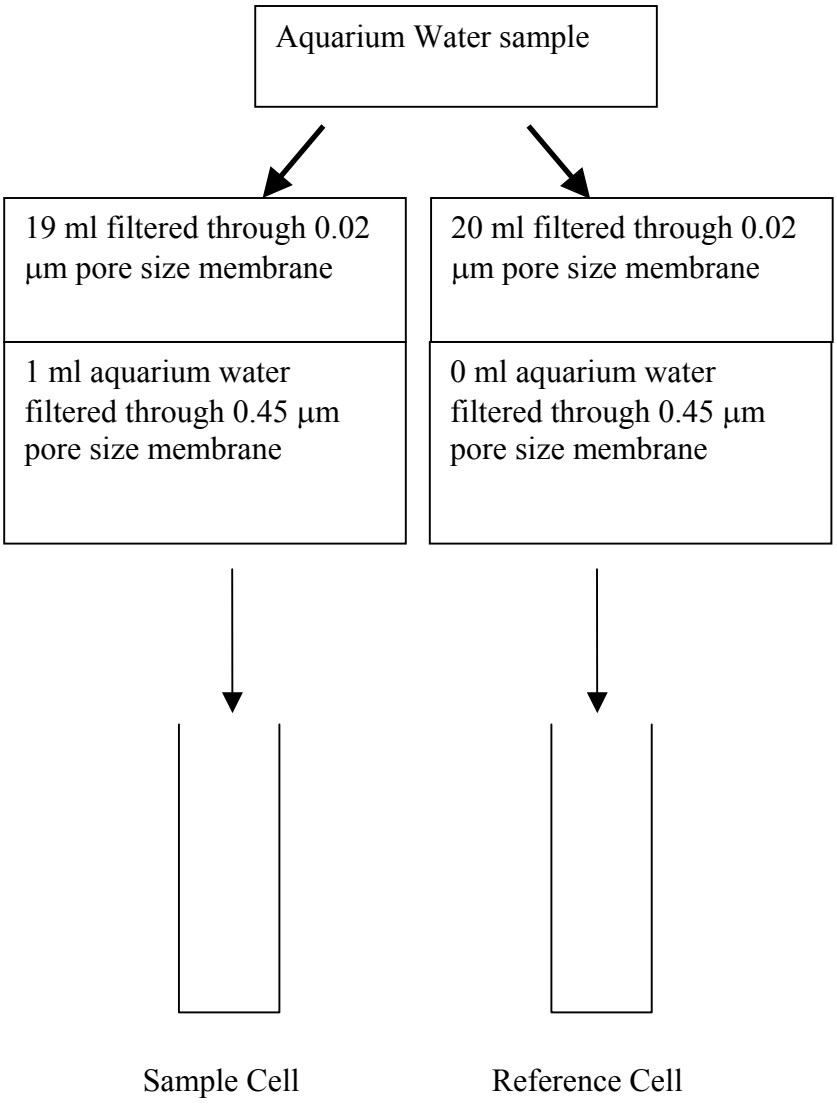
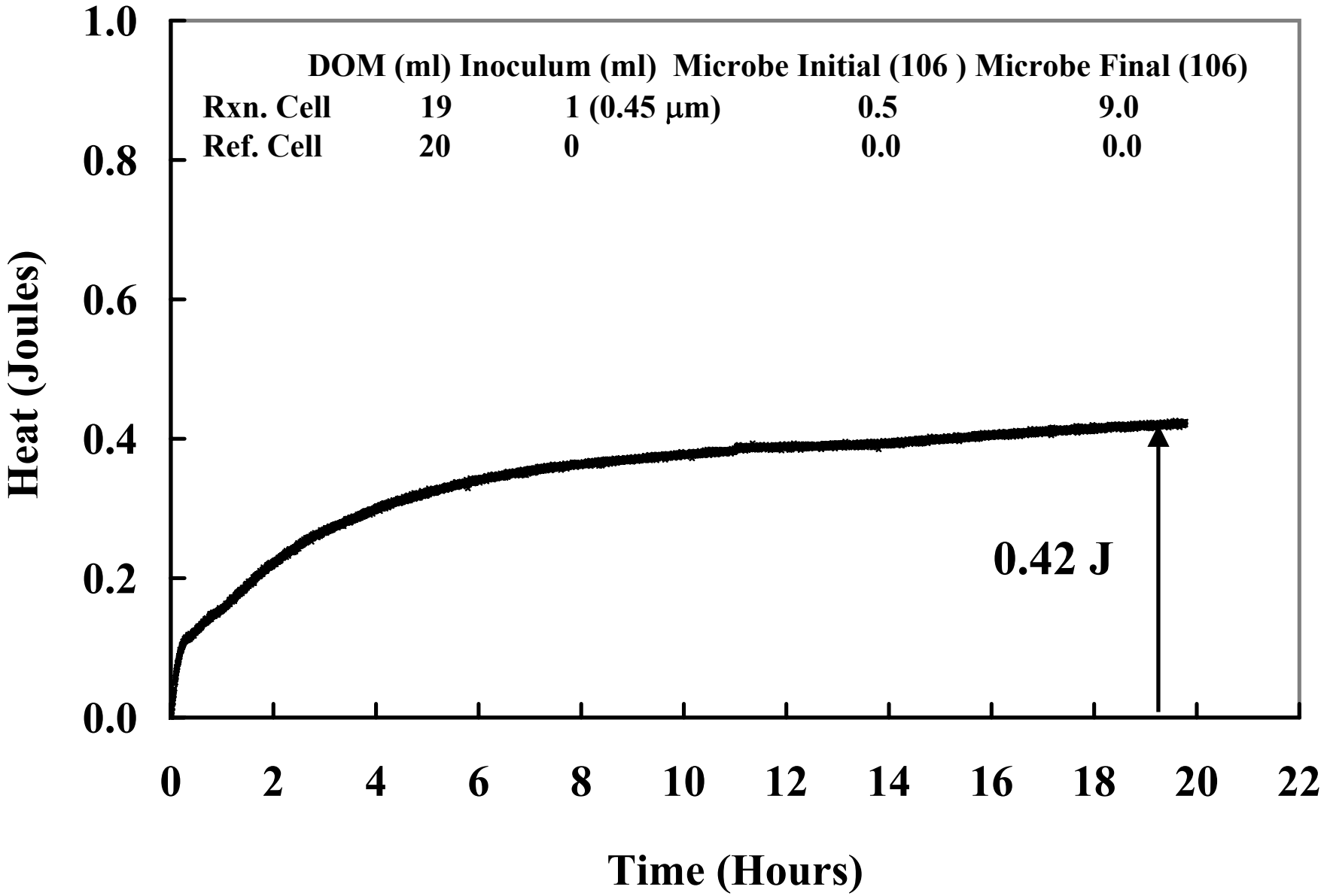


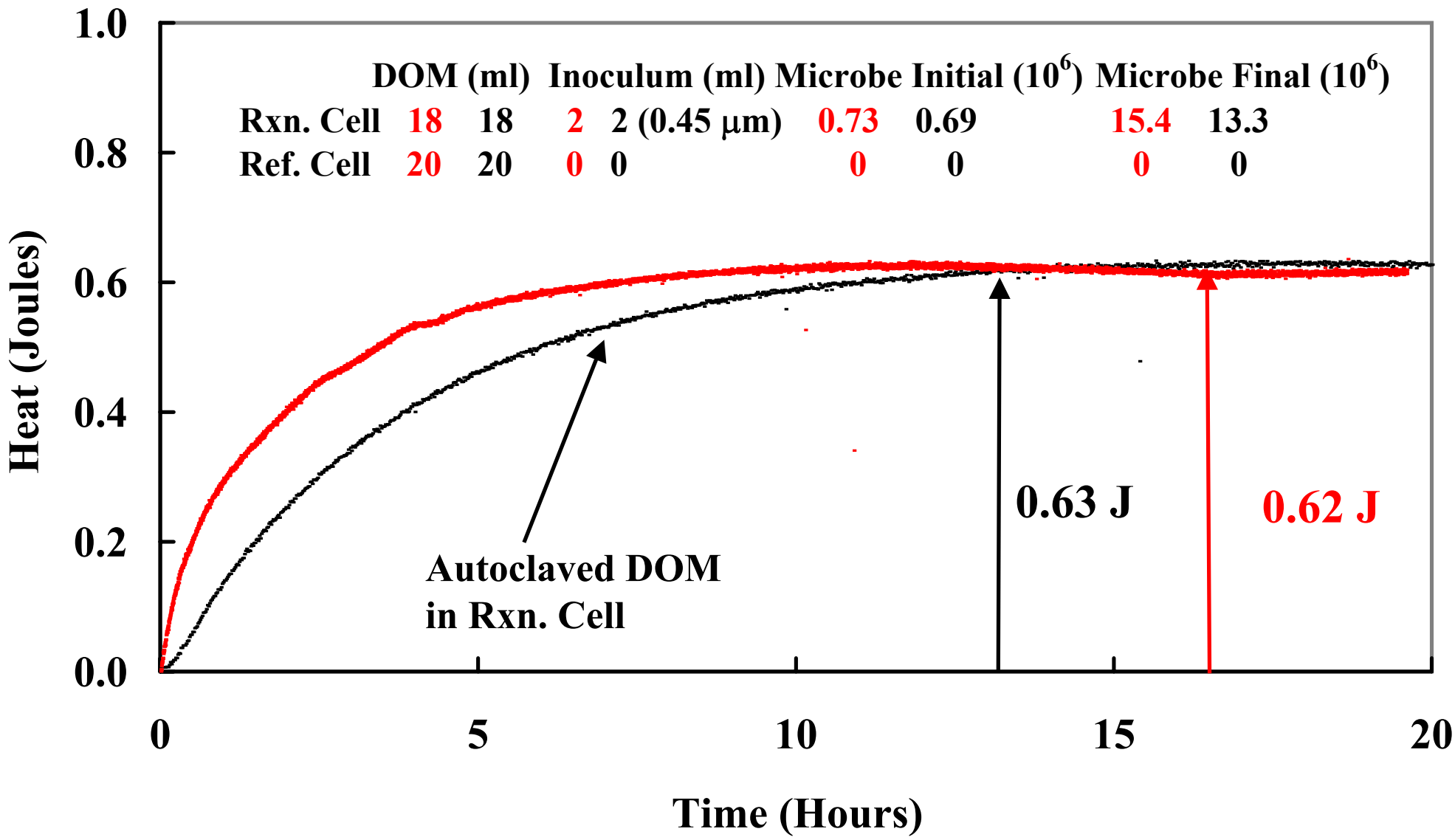
A. Calorimeter.

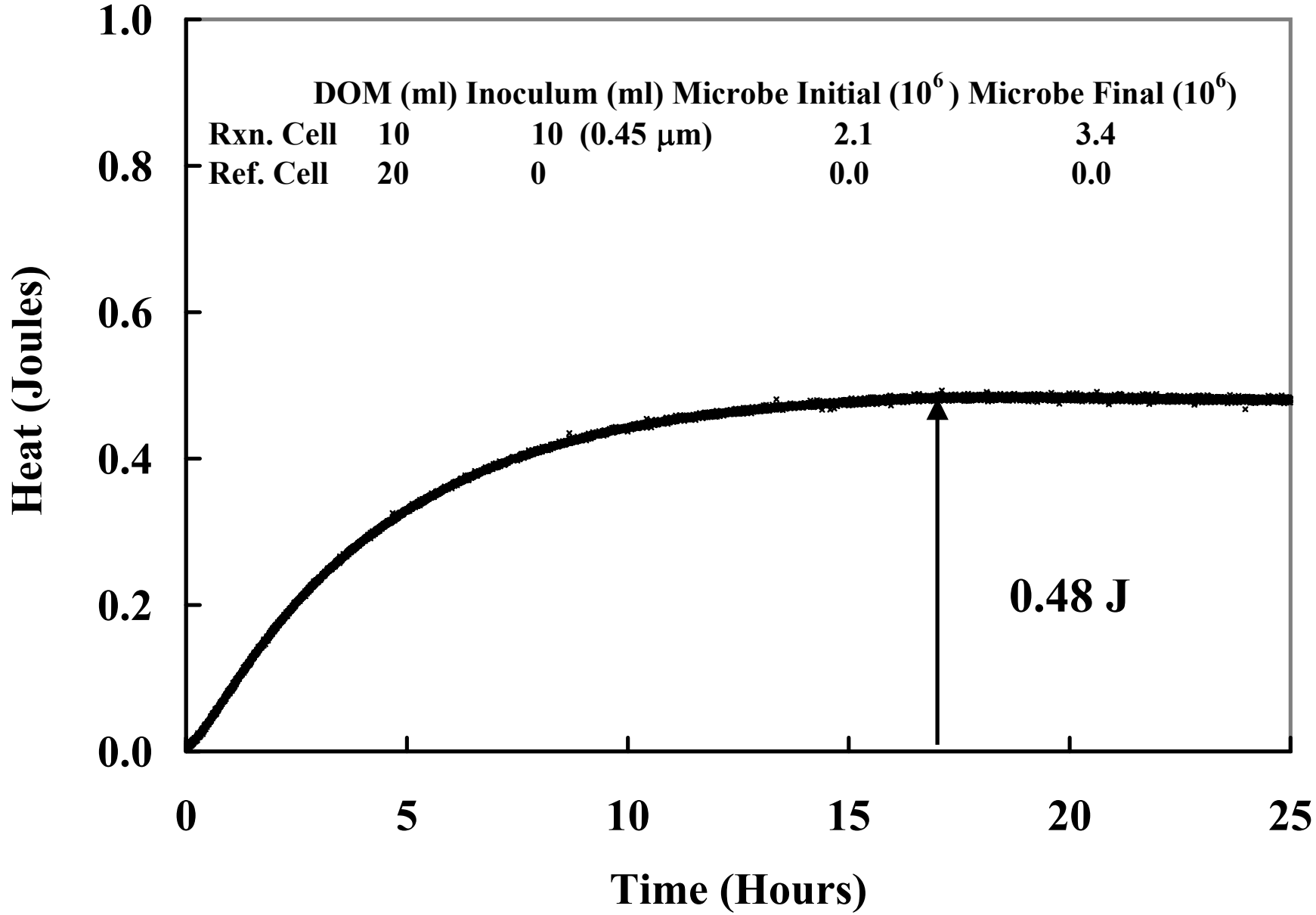


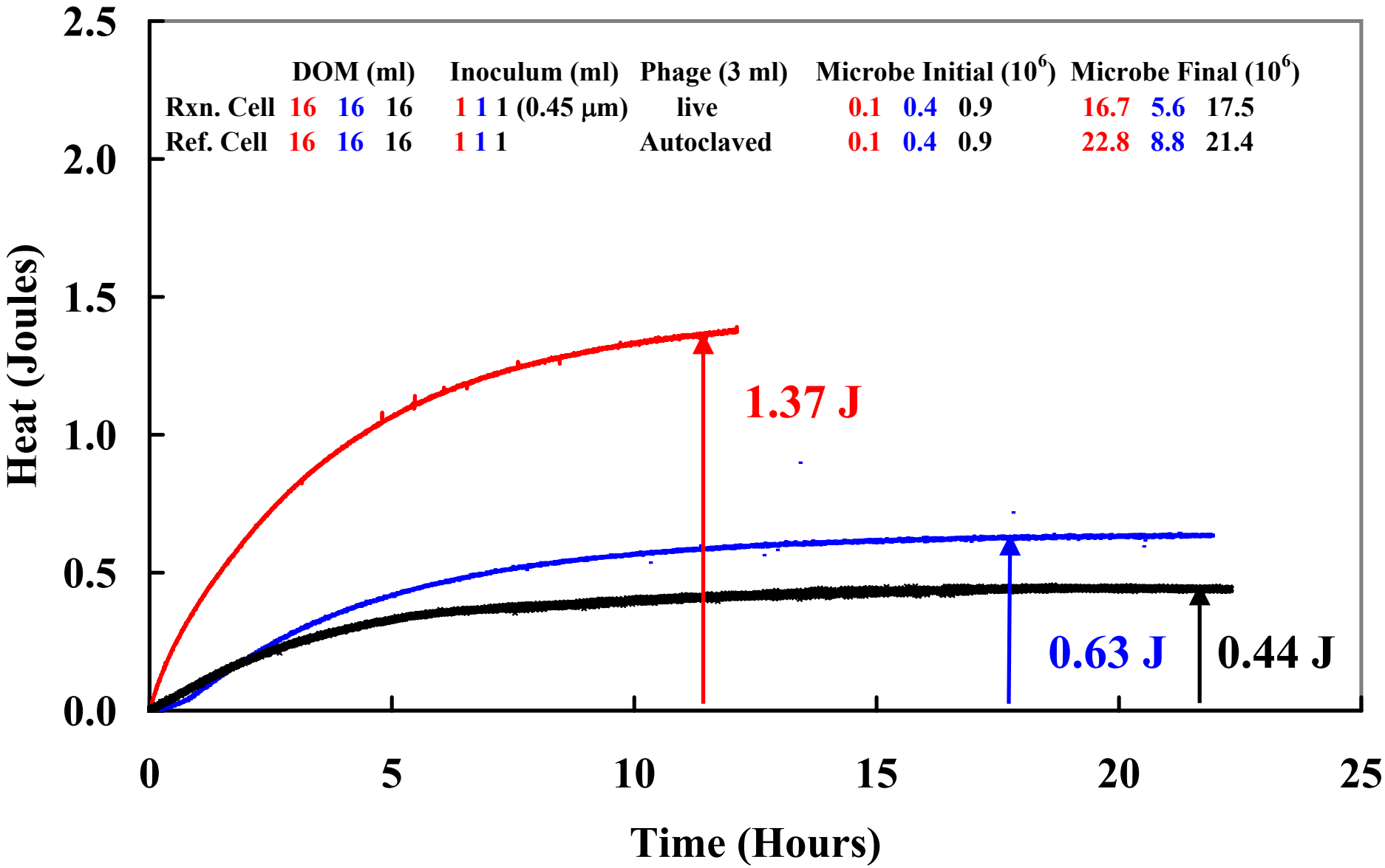
B. Sample Cell.


Aquarium Water sample
19 ml filtered through 0.02 μm pore size membrane
20 ml filtered through 0.02 μm pore size membrane
1 ml aquarium water filtered through 0.45 μm pore size membrane
0 ml aquarium water filtered through 0.45 μm pore size membrane
Sample Cell
Reference Cell


DOM (ml) Inoculum (ml) Microbe Initial (106) Microbe Final (106)
Rxn. Cell 19 1 (0.45 μm) 0.5 9.0
Ref. Cell 20 0 0.0 0.0
0.42 J
Heat (Joules)
Time (Hours)
0.0
0.2
0.4
0.6
0.8
1.0
0
2
4
6
8
10
12
14
16
18
20
22


DOM (ml) Inoculum (ml) Microbe Initial (10^6) Microbe Final (10^6)
Rxn. Cell 18 18 2 2 (0.45 μm) 0.73 0.69 15.4 13.3
Ref. Cell 20 20 0 0 0 0 0 0
1.0
0.8
0.6
0.4
0.2
0.0
Heat (Joules)
0
5
10
15
20
Time (Hours)
Autoclaved DOM
in Rxn. Cell
0.63 J
0.62 J


DOM (ml) Inoculum (ml) Microbe Initial (10^6) Microbe Final (10^6)
Rxn. Cell 10 10 (0.45 μm) 2.1 3.4
Ref. Cell 20 0 0.0 0.0
0.48 J
Heat (Joules)
Time (Hours)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25


DOM (ml)
Inoculum (ml)
Phage (3 ml)
Microbe Initial (10^6)
Microbe Final (10^6)
Rxn. Cell 16 16 16 1 1 1 (0.45 µm) live 0.1 0.4 0.9 16.7 5.6 17.5
Ref. Cell 16 16 16 1 1 1 Autoclaved 0.1 0.4 0.9 22.8 8.8 21.4
1.37 J
0.63 J
0.44 J
Heat (Joules)
Time (Hours)
0.0
0.5
1.0
1.5
2.0
2.5
0
5
10
15
20
25