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MICROCALORIMETRY OF THE SMALLEST PLANKTON FRACTION: IN SEARCH FOR THE SOURCES OF HEAT DISSIPATION

Heat production by the plankton pico (0.2 to 2 μm) and femto (<0.2 μm) fractions was measured in the dark using a microcalorimetric method. It was surprisingly found that the femtofraction (FF) produced more heat per seawater volume (48 ± 24 (95% CI) μW l^{-1}) than the picofraction (25 ± 14 μW l^{-1}). Consequently, we tested two hypotheses which could explain the phenomenon through a differentiation between the potential sources of heat in FF: (i) metabolism of ultramicrobacteria, the only living component in the fraction besides the non-metabolizing virioplankton which are unable to produce heat; (ii) extracellular chemical processes in aquatic environments. Results of microcalorimetry and respirometry combined with the use of antimicrobial agents (ciprofloxacin, glutaraldehide, sodium azide), and microscopical examination (counts and sizing of bacteria in the measuring ampoules) showed that both sources contributed to the overall heat flow by FF but the non-living component was responsible for the bulk of it. Hydrolysis of high molecular weight organic substances dissolved in seawater, associated with bacterial extracellular enzyme activity, is considered the most probable source of the heat. If so, microcalorimetry has great prospective in aquatic microbial ecology and biochemistry as a powerful unspecific analytical method.

Key words: microcalorimetry, heat production, ultramicrobacteria, virioplankton, bacterioplankton, extracellular enzyme activity

Traditionally, hydrobiologists use *respirometry* for quantifying the metabolic activity of aquatic organisms, assuming that: (i) catabolism is totally aerobic; and (ii) in order to obtain thermodynamic information, the dark O_2 uptake is equivalent to the heat production by a factor called the oxycaloric equivalent [10] which is constant for the combustion of a given substrate after allowing for side reactions. Thus, assuming the enthalpy change of anabolism is zero [1, 17], dark O_2 uptake by an aquatic ecosystem and its catabolism are considered synonymous with the ecosystem energy flow. However, there are two serious disadvantages to the respirometric approach, making it an unreliable tool for quantifying energy flows through aquatic ecosystems. Firstly, it is dangerous to define respiration as the only catabolic process in the water column (under relatively adverse *in situ* conditions) and, hence, to equate indirect calorimetry with direct one. Secondly, there may be oxidative chemical processes in aquatic environments, which would be manifest as oxygen uptake (e.g. [22, 23]). In this case, (i) respiration would not be the only oxygen-consuming process, and (ii) the largest measurement error probably would be associated with the smallest, microbial fractions in which it is more probable that the respiration is masked by chemical

reactions. It is a serious experimental problem for quantifying energy flows in aquatic ecosystems as a whole because their microbial (the smallest) component involves the major primary producers (phytoplankton, cyanobacteria, picoalgae) and mineralizers (heterotrophic picoplankton), and, as a rule, contributes most of their energy/carbon budget.

Both of the above difficulties can be overcome to some degree by applying a direct calorimetric approach or, ideally, by combining calorimetry with respirometry. However, as in the case of respirometry, physico-chemical side-reactions in aquatic environments can affect the results of calorimetry. In this context, the fact that all processes, including biogenic ones, are accompanied by changes in heat energy means not only is calorimetry a powerful tool with great potential in aquatic ecology but also its limitations. In this paper, we consider a situation when the "noisy" heat effect of the non-living (extracellular) component of a biogeochemical system is comparable to that of its living (intracellular) component and, hence, the problem of their differentiation becomes principal for interpreting the results.

In contrast to respirometry, calorimetry provides the essential kinetic information on *integral* (aerobic + anaerobic) metabolism, serving as a promising tool for constructing ecosystem network models of material and/or energy flows. In combination with respirometry, it differentiates between the aerobic and anaerobic processes through the calorimetric/respirometric (CR) ratio calculated from the two measurements and compared with the oxycaloric equivalent [10]. A key property of calorimetry, its non-specificity, has made its use compelling in studies of complex biological systems like the natural microbial communities, especially since the advent of microcalorimeters. Investigating the community rather than the individual species within it has been practiced in soil research for over 70 years [11, 21].

One of the reasons for the slow expansion of calorimetry to plankton ecology is its insufficient sensitivity even at the microwatt level: the biomass concentration is not high enough to detect its heat flow. Ideally a nanocalorimeter is required but, nevertheless, this difficulty was surmounted in studies of natural bacterioplankton by a 2-step microfiltration for preparing concentrated samples of picoplankton for batch microcalorimetry [18, 19]. The conceptual basis for interpreting the calorimetric data was similar to that used in respirometry (see e.g., [2]), namely the heat flow (or oxygen uptake) by the picoplankton (precisely, by the *picoseston* because it involved both living and non-living matter) was assumed to be associated exceptionally with microbial metabolism.

For the present study, we applied the same approach for measuring and analysing the heat flow by the smallest (<0.2 μm) planktonic fraction defined by Sieburth et al. [27], as the *femtoplankton*. However, difficulties have arisen in interpreting the results: in general, the femtofraction produced more heat per seawater volume than the picofraction. The living component of the femtofraction involves, firstly, the so-called filterable bacterial cells, or 'ultramicrobacteria' (UMB), whose contributions to the total bacterioplankton biomass and activity are regarded as insignificant [8], and, secondly, non-metabolizing virioplankton. According to the above concept, we would have to explain the phenomenon as due to high UMB metabolic activity but this would contradict the current understanding of their nature. An alternative hypothesis was that extracellular chemical processes in aquatic environments are also a significant heat source, and the heat-producing "reagents" dissolved in seawater (i.e. in the <0.2 μm fraction) can be concentrated onto an ultrafiltration membrane. Consequently, our goals were to measure and compare the heat fluxes and long-term flow patterns produced by the plankton pico- and femtofractions, and to differentiate between the living and non-living heat sources in the femtofraction.

Theory. The heat conduction calorimeter in the present study measures the instantaneous *rate* of heat flow ($\Phi = dQ/dt$, Watts, W) [13],

meaning that it gives the kinetics of the process as well as thermodynamic information relating to state functions such as the enthalpy change, ΔH. The heat flow is properly regarded as the rate of thermal (th) advancement, $d_{th}\xi/dt$, in the energy transformations [9]. It is an important concept in energy transformation because it is expressed explicitly in terms of the stoichiometric coefficients, v_i, of the i-th species in the reaction, i.e. thermal advancement is directly related to the reaction stoichiometry irrespective of the complexity of the reaction, i.e. one as complex as the biotic growth reaction – the metabolic reaction [15].

The rate of the growth reaction, the so-called metabolic rate or "activity", is simply a convenient phrase to denote the rate of advancement of the aggregated biochemical reactions in the growth reaction [14] of living matter, $d\xi_B/dt$. The thermal advancement of energy transformation, $d_{th}\xi$, is related to $d\xi_B$ by the expression [14], $d_{th}\xi = v_i \Delta H_{B,i} d\xi_B$, where $\Delta H_{B,i}$ is the molar enthalpy of the reaction in terms of species i [15]. The change in thermal advancement, $d_{th}\xi$, is exactly equivalent to the change in heat, dQ. The conclusion of this theoretical treatment is that the calorimetrically measured heat flow is a function of the metabolic rate of the living matter under investigation.

It should be realised, though, that part of the microbial catabolic process can be external to the living cells. In particular, high molecular weight biopolymers (proteins, carbohydrates, fats, organic P- or S-compounds) dissolved in seawater and/or adsorbed to suspended particles endocytosed by bacteria, unlike for animal cells, and must be hydrolyzed by extracellular enzymes before they can be assimilated by the bacterioplankton as low molecular weight substrates [6]. Thus, the heat flows by the smallest plankton fractions can involve both intra- and extracellular components of catabolism. Additionally, any other side (bio)chemical reactions in the aquatic environment can potentially affect the calorimetric results if their total enthalpy change is great enough to be comparable with the metabolism-associated heat production.

Materials and Methods. The surface water samples were collected at a station in Sevastopol Bay (the Black Sea, Ukraine) at different seasons over 6 years (1999 to 2004) and in the coastal waters of Cardigan Bay (West Wales, UK) in August 2003. The heat flow and oxygen uptake by the plankton femtofraction were measured after a two-step filtration technique [18, 19]. Initially, 100 ml seawater samples were screened with a 0.2 μm pore size membrane (Sartorius, Cat. # SM 11307-047) to remove the picoseston. The product of this filtration, <0.2 μm filtrate, contains colloidal and dissolved (<1 kDa) organic/inorganic substances [25, 29], and the femtoplankton. At the second filtration step, 70 to 75 ml sub-samples of the <0.2 μm filtrate were concentrated onto a 0.01 μm nitrocellulose membrane (Sartorius, Cat. # SM 11318-047) (see Fig. 1).

In the experiments with "conventional" bacterioplankton (in the picofraction), the same experimental design was chosen, namely the fraction was fractionated and concentrated by 12 μm (Sartorius, Cat. # SM 12500-047) and 0.2 μm pore size nitrocellulose membranes, respectively [18]. A methodological study (manuscript in preparation) has shown that a part of the bacterioplankton did not reach the concentration membrane if a 3 μm pore size filter was used for the fractionation. This was due to the removal of epibacteria attached to suspended detrital and mineral particles. At the same time, the nanoplankton involving fragile ciliates and flagellates were destroyed by water flow shear during direct filtration irrespective of which membrane, 3 or 12 μm pore size, was used for the fractionation. Thus, the 12 μm pore size membrane was more suitable for the fractionation procedure because it removed the fewer of the epibacteria inhabiting planktonic aggregates.

For the microcalorimetric experiments, the wet membrane (or its fragment of known area) with the concentrated fraction was cut into strips

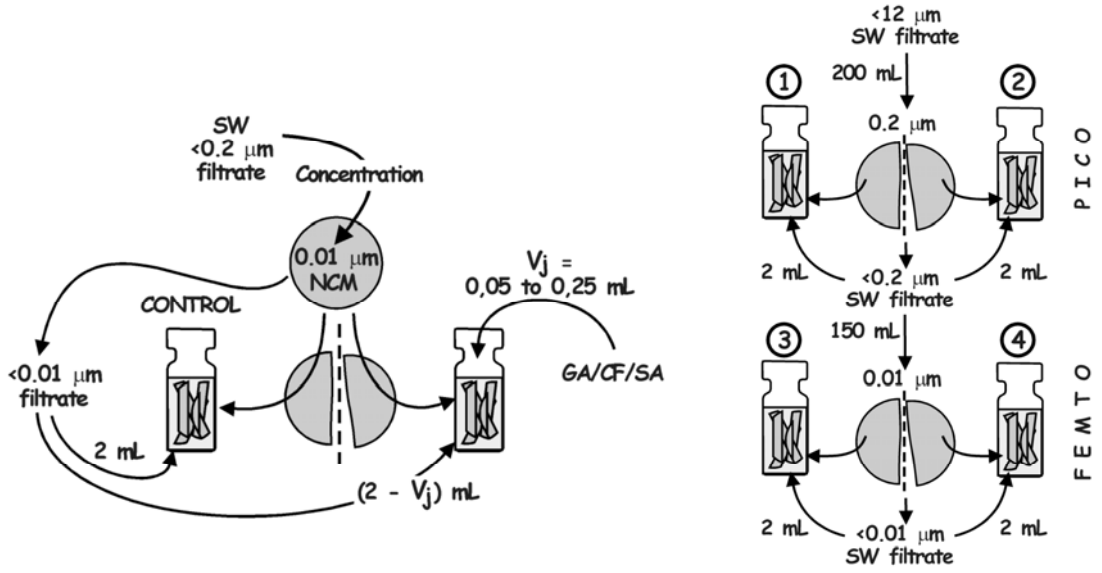

Fig. 1. Left: The 2-step filtration method for concentrating the femtofraction of seawater (SW) sample onto a nitrocellulose membrane (NCM). The membrane fragments of equivalent surface area were used to conduct replicate experiments with the unaltered samples and those subjected to glutaraldehyde (GA), ciprofloxacin (CF), and sodium azide (SA) treatments. V_j is the injection volume. Right: The experimental design used for monitoring the bacterial dynamics in measuring ampoules with the plankton PICO and FEMTO fractions. The numbers of the ampoules (in the circles), nominal pore sizes of the Sartorius nitrocellulose membranes, and the seawater (SW) volumes filtered and poured into the ampoules are shown. The experiment was conducted in 4 replicates.

Рис. 1. Слева: Метод 2-этапной фильтрации для концентрирования фемтофракции пробы морской воды (SW) на нитроцеллюлозную мембрану (NCM). Для постановки повторных экспериментов с контрольными пробами и при воздействии глютаральдегида (GA), ципрофлоксацина (CF) и азида натрия (SA) использовали фрагменты мембраны с равной площадью повеохности. V_j – добавляемый объем. Справа: Схема эксперимента для исследования бактериального роста в измерительных ампулах с пико (PICO) и фемто (FEMTO) фракциями планктона. Показаны номера ампул, номинальный размер пор нитроцеллюлозных мембран, объемы фракций, сконцентрированных на мембранах и фильтратов, используемых для наполнения ампул. Эксперимент ставился в 4 повторностях.

to maximise exposure of the surface to the seawater and placed into a sterile glass measuring ampoule containing 2 ml of the <0.2 μm filtrate obtained at the second filtration step which was hermetically sealed and the heat flow was measured immediately after loading into the batch microcalorimeter. To compare the heat flow patterns produced by both the fractions of the same seawater sample, long-term (up to 72 h) calorimetric experiments were conducted.

The microcalorimetric measurements were carried out at 20ºC with an LKB BioActivity Monitor (BAM), Model 2277 (the successor is the Thermal Activity Monitor (TAM), Thermometric AB, Järfälla, Sweden) equipped with three independent sets of twin differential channels, one of each twin for the test material and the other as the control. Accordingly, only three replicate experiments could be conducted with the same fraction. In the batch mode, the baseline noise was less than ±0.1 μW while the concentrated samples produced up to 35 μW per ampoule.

In the respirometric experiments, a rectangular fragment of the wet membane carrying the fraction was placed in glass vessels of the two chambers filled with 2.3 ml of the particle-free seawater (<0.2 μm filtrate of the same seawater sample). To avoid screening the polarographic

oxygen sensor and disturbing electromagnetic stirrer, the fragment was folded inwards round the vertical walls of the vessel, thus, covering only part of the perimeter (i.e. forming a single layer) and exposing the concentrated fraction inwards. This approach proved to be more efficient than cutting the membrane into small pieces because in the latter case, the sharp edges of them could damage the oxygen sensor membrane. Oxygen consumption rates were measured at 20ºC by a 2-channel Oroboros Oxygraph (Oroboros Instruments, Innsbruck Austria). Besides the experiments with the concentrated samples, oxygen uptake by the non-altered <0.2 μm filtrate (i.e. by the non-concentrated femtofraction) was also measured.

The combination of both the microcalorimetric (1 channel, TAM 2277) and respirometric approaches was used only in the set of the experiments with the seawater samples from Cardigan Bay. It was found in test respirometric experiments with the pico- and femtofractions that oxygen dissolved in the 2.3 ml samples was fully consumed within about 4 to 7 hours while this took longer in some of the calorimetric experiments (see above). To equalise the experimental conditions in both the instruments as close as possible, equivalent amounts of paraffin oil were added to both the experimental and control calorimetric ampoules to form about 3 mm depth oil layer between the liquid sample and the air above it, which prevented oxygen diffusion.

To differentiate between the intracellular (cell metabolism) and extracellular sources of the measured heat flow, a number of antimicrobial agents were added to the ampoules at different stages of the experiments, namely sodium azide (final concentration 0.3% w/v), ciprofloxacin (0.06% to 0.3%), and glutaraldehyde (2%). The method of the sample preparation was the same but the membrane carrying the concentrated fraction was cut into equivalent pieces (with equivalent amounts of the same fraction sample) (Fig. 1, Left scheme). These were placed in the control and agent-treated ampoules for measuring the total (extracellular + intracellular) heat flow and the flow associated with the non-living (extracellular) component of the fraction, respectively.

In the PF, bacteria were counted by epifluorescence microscopy according to Sherr et al. [26]. 2- to 10-ml preserved (1% glutaraldehyde final concentration) seawater sub-sample was filtered onto a polycarbonate black (Sudan-stained) 0.2 μm pore size membrane filter to give 2 ml volume and stained for 7 min with 50 μl of DAPI stock solution (200 μg ml^{-1}; 0.2 μm pre-filtered) at final concentration of 5 μg DAPI ml^{-1} (14 μM). Then the sample was collected onto the filter at low vacuum (<80 mm Hg). A Ziess JENALUMAR-a/d microscope equipped with an HBO-202 mercury burner and a UV light excitation filter set (U 204 + B 226 excitation filters, TS 410 beam splitter and G 243 barrier filter) was used for all the observations. At least 200 cells and 20 fields were counted from each preparation. The volume of the bacteria was calculated using the formula $V = (\pi/4)\ W^2(L - W/3)$, where L is cell length, and W is cell width [26]. The carbon and energy equivalents of the bacterial biomass were calculated using the factors of 0.2 pg C μm^{-3} [4] and 43.5 J mg^{-1} C [7], respectively.

The heat flow by the living component of the fractions depended on the cell abundance concentrated on the membrane and their physiological status. Hence, accuracy of this assay (as a measure of intracellular metabolism) depended on how representative the sample was of the cell population from which it was taken. According to Kirchman's [16] sampling hierarchy and associated statistics, a 100 ml sample is at sampling level 2 (100 ml to >1 l) at which point the variance is equivalent to examining the spatial and temporal variation in natural microbial abundance. Thus, the picoplankton sample on a membrane was representative of the *in situ* assemblage in terms of its abundance. In theory, no replicates were necessary in this case. In the femtofraction, bacterial abundance can be even higher than in the

picofraction (UMB are up to 90% of the total bacterioplankton abundance [24]). Because the concentrated volumes (70 to 75 ml) of the femtofraction were close to those of the picofraction, we assumed that the femtofraction samples also were statically representative.

To estimate a potential contribution of bacterial growth to the long-term heat-producing processes inside the ampoule and the heat flow patterns observed, bacterial dynamics (in terms of abundance and biomass changes in the measuring ampoules) were studied in a complex experiment with both the fractions of the same seawater sample. Bacteria concentrated onto the Sartorius membranes and ultramicrobacteria in the filtrates and not caught by the 0.2 μm membrane were not counted.

The reason for this was evidence that the heat flow by the cell 'population' concentrated on the membrane quickly reduced (our data, manuscript in preparation). At the start point of the experiment, the initial bacterial number/biomass on the membranes were calculated from the data on cell abundance/biomass in the <12 μm filtrate and its volume concentrated. Four replicate experiments were conducted for each of the fractions (Fig. 1, Right scheme). The ampoules were incubated under dark conditions at 20 ºC.

Basic statistics (means, 95% confidence intervals, paired *t*-tests) and cross-correlation coefficients [3] were calculated using STATISTICA 5.5 (StatSoft, Inc).

Results. It is seen in Fig. 3 that the measured rate of heat production converted into the seawater volume-specific quantity (heat flux) varied from about 5 μW l^{-1} to 80 and 160 μW l^{-1} for the pico- (PF) and femtofractions (FF), respectively, with the corresponding means of 23.7 ± 14.1 (95% CI) μW l^{-1} (PF) and 48.0 ± 23.7 μW l^{-1} (FF), and a poor correlation (0.43; $p>0.05$) between the fractions. It was surprising that the FF heat flux was significantly (paired *t*-test, $p<0.05$) the higher value, contributing about 66.0 ± 9% of the total (PF + FF) heat production.

The FF heat production patterns were similar to the PF ones obtained for the same seawater sample (Fig. 3, the samples B to E) that could indirectly indicate the similar nature of the heat-producing processes in both the fractions. However, the heat flow peaks in the FF lagged the PF in time (see Fig. 3), which indirectly indicated delayed bacterial growth in the former at lower start biomass. The maximum (0.48 to 0.94) cross-correlation between the fractions (the FF series lagged during the time series analysis) was obtained for the lags from –0.1 (sample B) to –12.6 hours (sample A).

Microscopical examination showed that intense bacterial growth occurred in the ampoules containing the FF, up to ~5×10^6 cells and ~4×10^6 μm^3 in terms of cell abundance and biovolume, respectively (FEMTO, Replica 2 in Table 1), with the average cell volume of up to 1.1 μm^3 at the end of the experiment (FEMTO, Replica 1). Carbon and energy equivalents of the maximum biomass yield in the FF were 0.79 μg C and 34 mJ, respectively. In the experiments with the PF, 24-h yield of bacterial biovolume (or biomass) in the liquid phase of the measuring ampoule was equal to about the doubled biovolume of the PF bacteria concentrated onto the 0.2 μm membrane (see the PICO replicates in Table 1) and about 5- (Replica 2, Table 1) to 40-fold (replica 1) higher than in the FF. The average cell volume increased up to 0.64 μm^3 (Replica 2, Table 1). The carbon and energy equivalents of the PF yield were 2.8 to 7.9 μg C and 120 to 343 mJ, respectively.

If it is assumed that the bacterial gross growth efficiency is 50%, *a fortiori* less than 34 mJ (the maximum value for FEMTO, Table 1) was dissipated as heat by the growing bacterial population in all the experiments with the FF. It is a small proportion of the total heat quantities (Q_{24}) dissipated by the FF for the first 24 h of the experiment (Table 2), varying between 147 and 1090 mJ. An explanation of this phenomenon is that most of the dissipated heat was associated with some extracellular processes.

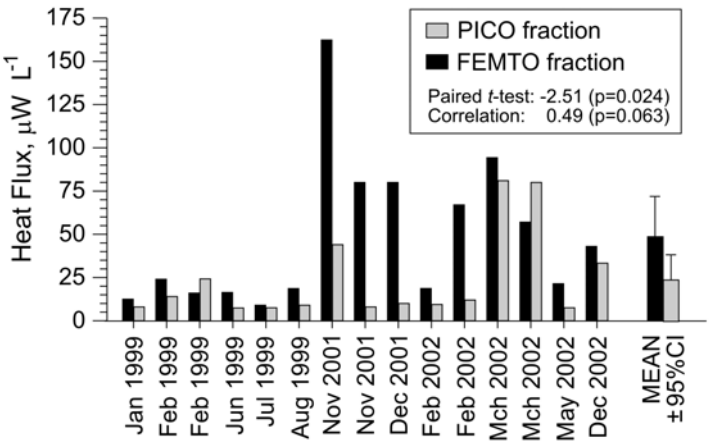

Fig. 2. A comparison of the seawater volume-specific heat fluxes by the pico- and femtofractions from Sevastopol Bay (the Black Sea) over different seasons in 1999, 2001 and 2002.
Рис. 2. Сравнение интенсивности теплопродукции (в единице объема воды) пико- и фемтофракций проб морской воды, отобранных в Севастопольской бухте (Чёрное море) в разные сезоны 1999, 2001, 2002 гг.

Table 1. Bacterial dynamics in the measuring ampoules during a standard microcalorimetric experiment with the pico- and femtofractions. Bacteria were counted and sized in the <12 μm seawater filtrate before the experiment (for calculating the bacterial abundance on the 0.2 μm Sartorius membranes) and in seawater inside the ampoules after the experiments, using the standard method (0.2 μm pore size black polycarbonate membrane, DAPI staining) for quantifying natural bacterioplankton. Means ± 95% confidence intervals are shown.
Табл. 1. Рост бактерий в измерительной ампуле в ходе микрокалориметрических экспериментов с пико- и фемтофракциями. Бактерии были просчитаны и промерены в фильтрате <12 мкм до эксперимента (для расчета численности бактерий на мембране 0.2 мкм после концентрирования) и в воде вокруг мембраны после эксперимента (0.2 мкм черные ядерные мембраны, окраска DAPI). Показаны средние значения и 95% довер. интервал.

Experimental Design (see Fig. 1, Right)		Membrane (t = 0 h)		Seawater (t = 24 h)			
		Cell Number (10^6 cells)	Biovolume (10^5 μm^3)	Cell Number (10^6 cells)	Biovolume (10^5 μm^3)	Biomass[2] (μg C)	Biomass[2] (mJ)
Replica 1 PICO	Ampoule 1	132.3 ± 40.7	129.1 ± 67.0	63.6 ± 6.3	394.1 ± 40.8	7.88	343
	Ampoule 2			43.0 ± 1.6	275.3 ± 10.3	5.51	120
Replica 1 FEMTO	Ampoule 3	ND[1]	ND	0.5 ± 0.1	5.7 ± 1.0	0.11	5
	Ampoule 4			1.1 ± 0.1	11.6 ± 1.5	0.23	10
Replica 2 PICO	Ampoule 1	65.3 ± 8.5	68.0 ± 25.2	32.0 ± 3.5	137.8 ± 18.5	2.76	120
	Ampoule 2			31.2 ± 3.7	152.8 ± 23.0	3.06	133
Replica 2 FEMTO	Ampoule 3	ND	ND	5.1 ± 0.4	39.5 ± 5.9	0.79	34
	Ampoule 4			3.8 ± 0.3	28.9 ± 4.0	0.58	25

[1]ND – non-detectable by the standard method.
[2]Carbon and energy equivalents of the mean bacterial biovolumes, calculated using the factors of 0.2 pg C μm^{-3} [4] and 43.5 J mg^{-1} C [7], respectively

Table 2. Total heat quantities (Q_{24}) dissipated by the fractions for the first 24 h of the experiments presented in Fig. 3.
Табл. 2. Суммарное количество тепловой энергии, продуцируемое фракциями в течение 24 ч эксперимента, представленного на рис. 3

Plot/Sample (see Fig. 3)	Fraction	Concentrated Volume (ml)	Q_{24} (mJ)
A (11.2001)	PICO	250	717
	PICO	250	566
	FEMTO	77	378
B (12.2001)	PICO	250	931
	PICO	250	920
	FEMTO	75	1090
C (02.2002)	PICO	250	885
	PICO	250	1125
	FEMTO	75	789
D (03.2002)	PICO	100	899
	FEMTO	70	531
E[1] (05.2002)	PICO	200	885
	FEMTO	70	758
F (12.2002)	PICO	150	411
	FEMTO	100	147

The same approximations for the PF give 120 to 343 mJ in terms of the produced biomass (Table 1, PICO replicates) versus 411 to 1125 mJ really dissipated as heat energy (Table 2, PICO). The difference is less distinct but a substantial part of the apparent heat production can be explained by metabolic activity of the picoplankton concentrated on the membrane despite the finding that its contribution decreases with time (our data, manuscript in preparation).

In the two experiments combining the respirometric and calorimetric approaches (Fig. 4, Left), the rates of the total oxygen consumption by the concentrated FF varied between 0.015 and 0.9 $\times 10^{-11}$ mol O_2 s^{-1} per ampoule which are equivalent to the heat flux of about 0.1 to 4.2 μW per ampoule (see additional ordinate axes in Fig. 4). Assuming that the FF oxygen uptake rate was associated solely with UMB metabolism, only a portion (<50%) of the total FF heat flow can be explained by bacterial metabolism. This conclusion was supported by the result of sodium azide (SA) injection into the FF in the second combined experiment (Fig. 4, Right): no abrupt decrease in the fraction heat production was observed despite the fact it might be expected owing to SA action and thus supposedly the strongly depressed bacterial metabolism. Addition of two other microbial agents, ciprofloxacin (CF) (Fig. 5, A, B, F) and glutaraldehyde (GA) (Fig. 5, C, D, E), to the FF at different experiment stages resulted in a partially decreased rate of the fraction heat flow. If the residue, i.e. that detected after the agent injection, heat flow by the FF was actually due solely to extracellular processes, the negative heat flows (i.e. heat absorption) measured in the experiments A and F (Fig. 5) could be interpreted as endothermic reactions in the environment (i.e. outside the bacterial cells).

Discussion. The finding that FF gave such an unexpectedly high heat production prompted two hypotheses which could explain the phenomenon: the large FF heat flux is (A) due to the high metabolic activity of UMB; and/or (B) associated with extracellular chemical processes, e.g., enzymatic hydrolysis of high molecular weight organic substances in the water column. Let us consider both the postulates in detail.

In the previous calorimetric studies of picoplankton [18, 19], it was assumed a priori that the heat produced by PF was associated solely with microbial metabolism, by analogy with respirometric studies of the smallest plankton fractions (e.g., [2]). So, it was logical to consider microorganisms, in particular UMB, as the only source of heat in FF. UMB are the smallest heterotrophic bacteria from aquatic environments with a size comparable to those of the largest viruses that allows them to pass 0.2 μm pore size membranes (for this ability they are called “filterable forms”). Sometimes, UMB can contribute most (up to 90%) of the total bacterioplankton abundance [24] but their total biomass is suggested to be insignificant – in fact, they are ignored by the conventional microbiological methods applying 0.2 μm pore size membranes for counting and sizing cells.

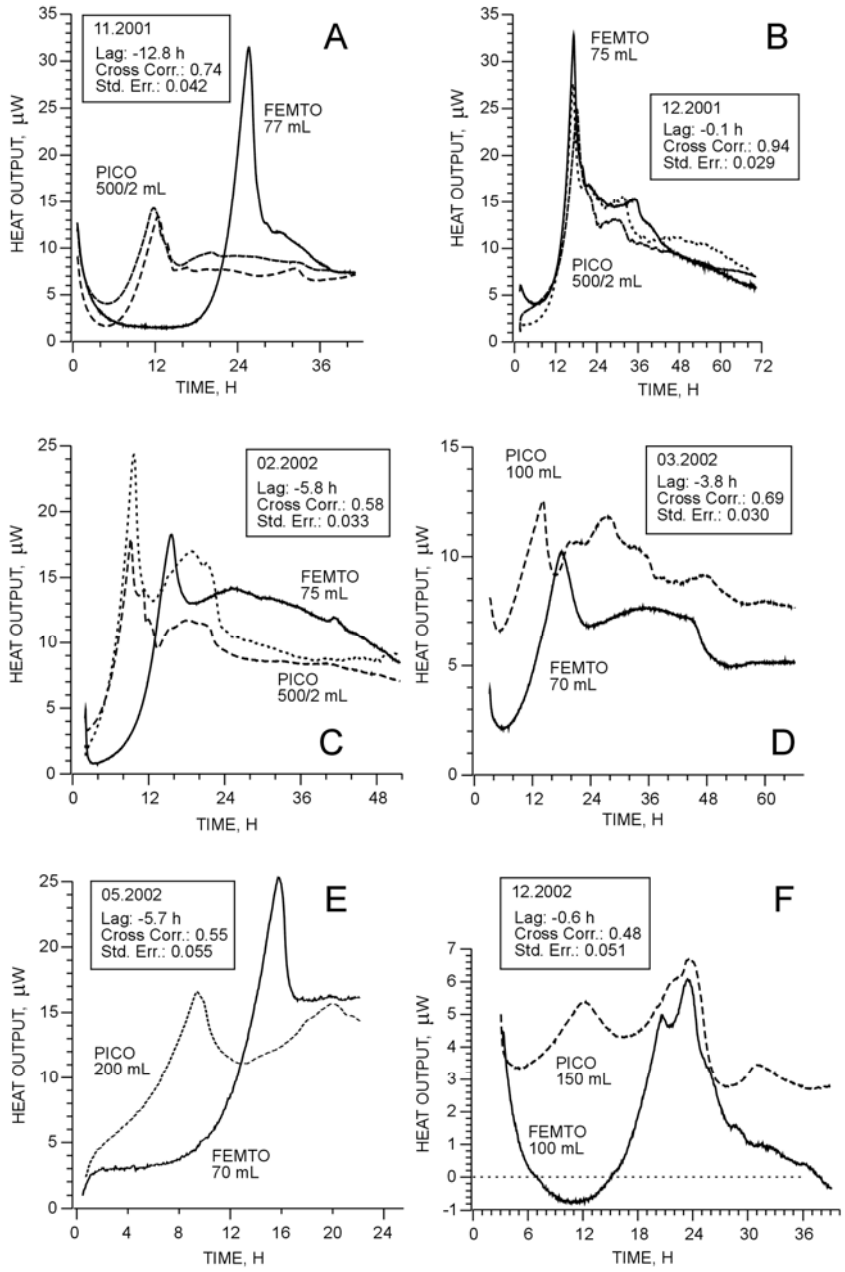

Fig. 3. The similarity between the heat flow patterns obtained for the plankton pico- and femtofractions from Sevastopol Bay (the Black Sea) in different seasons of 2001 and 2002. Each graph represents replicate experiments with the same seawater sample. Dates of the experiments, concentrated volumes (ml) and results of the cross correlation (CC) analysis, namely the maximum CC between the femto- and picofraction heat traces, its lag and standard error are shown. The volume of 500/2 ml means that after concentrating 500 ml of the fraction onto the membrane, the latter was cut into 2 equivalent fragments for conducting the replicate experiments.

Рис. 3. Сходство между теплопродукционными «профилями» для пико- и фемтофракций планктона из Севастопольской бухты (Чёрное море) в разные сезоны 2001 - 2002 гг. Каждый график представляет повторные эксперименты с одной и той же пробой. Показаны даты экспериментов, концентрируемые объемы (мл), результаты кросс-корреляционного (CC) анализа, а именно максимальная величина корреляции между кривыми пико- и фемтофракций, её лаг и стандартная ошибка. Объём 500/2 мл означает, что после концентрирования 500 мл фракции на мембрану, мембрану разрезали на 2 равных фрагмента для постановки повторных экспериментов.

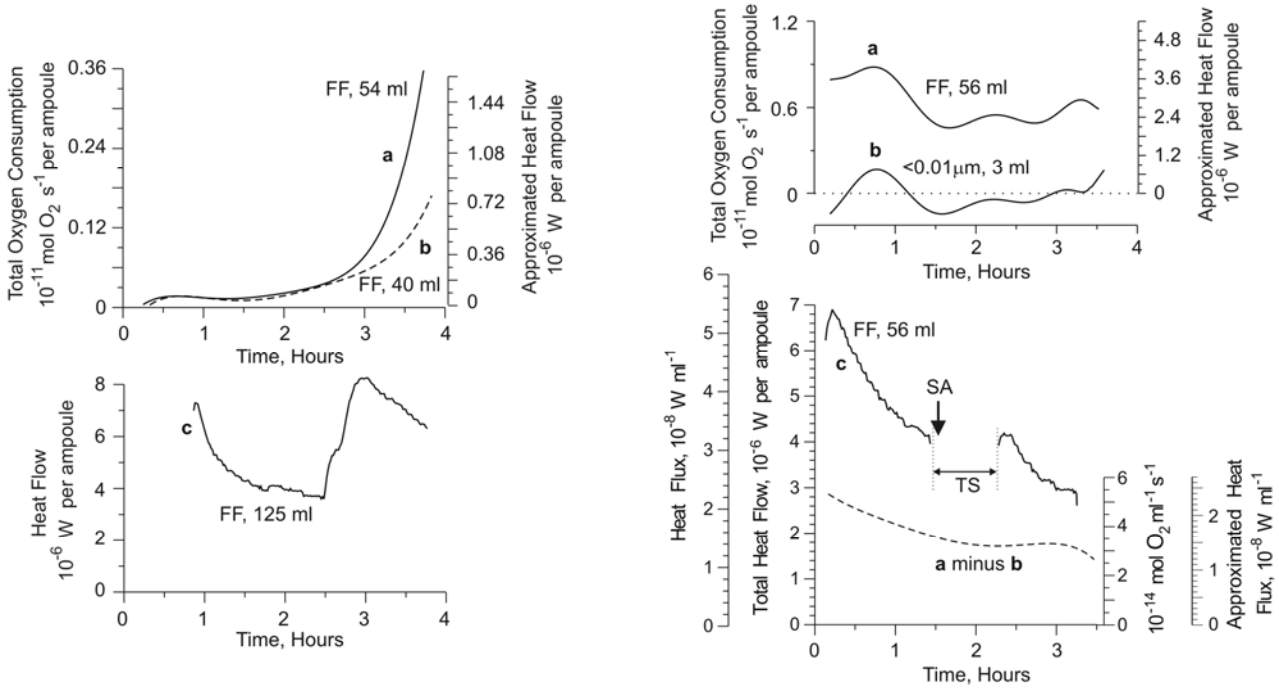

Fig. 4. A combination of the respirometric (curves **a**, **b**) and microcalorimetric (curves **c**) experiments with the concentrated femtofraction from the coastal seawater collected in Cardigan Bay on 21 (left plots) and 25 August 2003 (right plot). The concentrated volumes of femtofraction (FF) are shown. The curve **b** on the right plot demonstrates a replicate with no FF concentrated; <0.01 μm seawater filtrate only was poured into respirometric vessel. "**a** minus **b**" is the difference between the fluxes **a** and **b**, indicating that the heat output was associated exceptionally with FF on the membrane. The moment of the sodium azide (SA) injection into the calorimetric ampoule and the time period of the ampoule thermal stabilization (TS) are indicated by the arrows. The equivalent heat and oxygen fluxes were approximated using the oxycaloric equivalent of –450 kJ mol^{-1} O_2 [10].

Рис. 4. Комбинирование респирометрии (кривые **a**, **b**) и микрокалориметрии (кривые **c**) фемтофракции проб морской воды, отобранных 21 (левый график) и 25 августа 2003 г. (правый график) в прибрежных водах залива Кардиган. Показаны объемы фемтофракции (FF), сконцентрированные на мембрану. Кривая **b** на правом графике иллюстрирует эксперимент без мембраны с фемтофракцией; камера респирометра была наполнена только фильтратом <0.01 мкм. "**a** minus **b**" – разность в скорости потребления кислорода пробами **a** и **b**, демонстрирующая, что процесс происходил на мембране, но не в окружающем её фильтрате. Стрелками показаны момент добавления азида натрия (SA) и период времени, необходимый для термостабилизации пробы (TS). Взаимоэквивалентные скорости теплопродукции и дыхания рассчитаны с помощью коэффициента –450 кДж моль$^{-1}$ O_2 [10].

Protocols for modern techniques using SYBR Gold/Green to count viruses in aquatic samples [20] do not mention UMB at all despite the fact that they allow counts of virus-like particles (VLP) including UMB, not only viruses. UMB are considered to form a pool of dormant bacteria [8, 28] with extremely low metabolic activity. This point of view is prevalent today but it is confirmed only by indirect methods, in particular, using CTC (5-cyano-2,3-dytolyl tetrazolium chloride) as a marker of respiring cells [8].

However, the smaller the cell the more difficult it is to recognize the weak fluorescence of the formazan crystals formed in it, and hence the smallest cells have a greater chance of being described as metabolically inactive. Even if the rate of the volume-specific UMB metabolism is higher or at least not lower than in the 'normal' bacteria as was shown by Button & Robertson, [5], very low UMB biomass concentration makes it difficult to measure their heat production in FF.

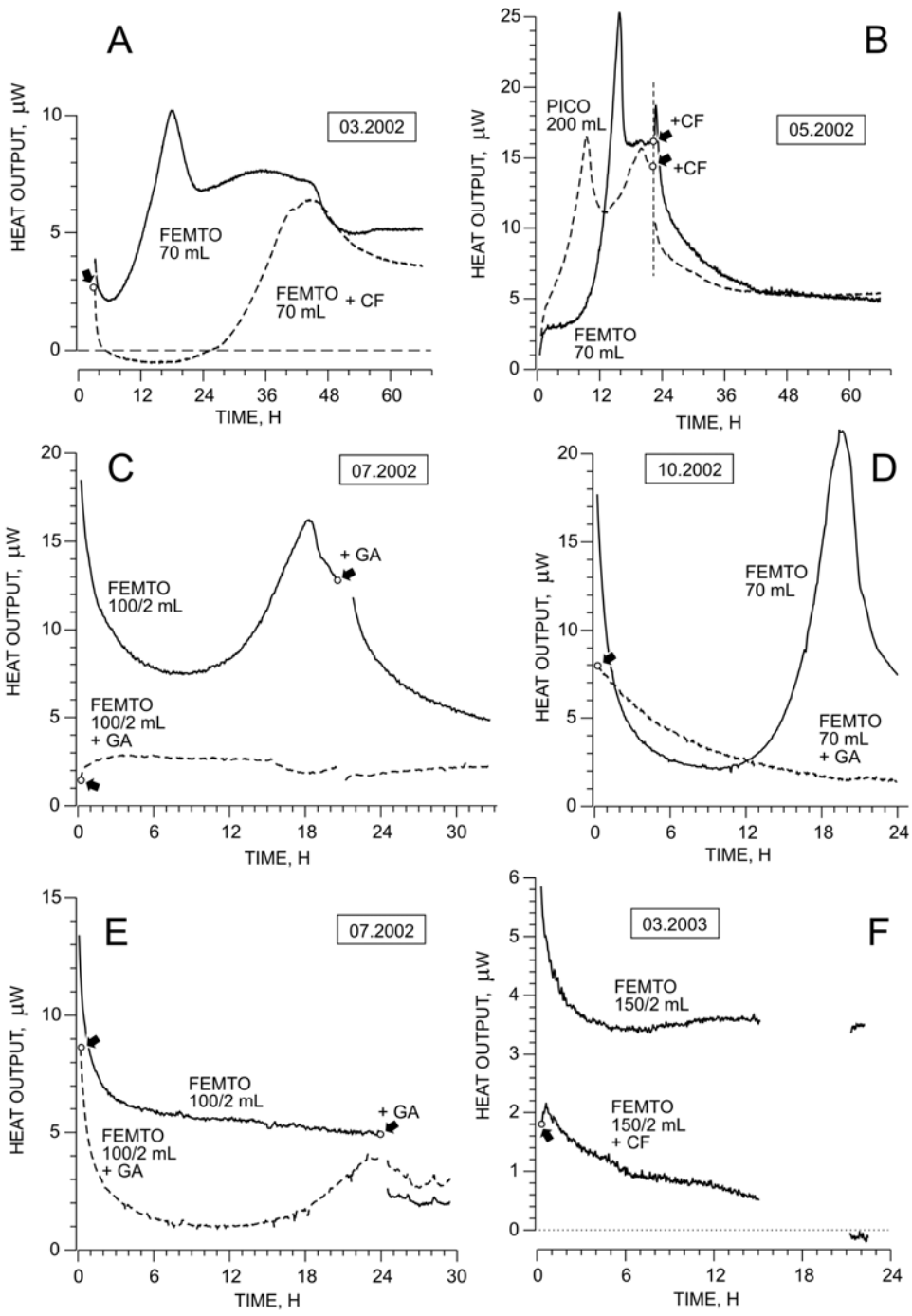

Fig. 5. A set of the microcalorimetric experiments with the concentrated seawater femto- (FEMTO) and picofractions (PICO) collected in Sevastopol Bay (the Black Sea) and subjected to glutaraldehyde (GA), ciprofloxacin (CF) treatments. Arrows indicate the moments of injection of the agents. Each graph represents replicate experiments with the same seawater sample. Other marks are the same as in Fig. 3.

Рис. 5. Серия микрокалориметрических экспериментов с воздействием глютаральдегида (GA) и ципрофлоксацина (CF) на пико- (PICO) и фемтофракции (FEMTO) проб морской воды, отобранных в Севастопольской бухте (Чёрное море). Стрелками показаны моменты добавления агентов. Каждый график представляет повторности для одной и той же пробы. Другие обозначения как на рис. 3.

All the mentioned arguments do not support hypothesis A but nevertheless some of our results provided evidence that potentially UMB could be responsible for at least a minor part of the total FF heat production.

In the firstly place, the similarity observed between the long-term heat flow profiles produced by FF and PF (Fig. 6) indicated that the heat-producing processes had similarities in both fractions. The principal source of heat in PF was bacterial growth. In FF, growth also occurred and it was at least one of the heat sources during the long-term experiments (Tables 1 and 2). Similarity in the profiles could mean the bacterial population were comparable in terms of their physiology while the lagged peaks on the FF curves were explained by the low bacterial biomass (perhaps, only dozens of cells) at the start point of the experiments (Fig. 3). It should be noted that separating UMB from the total bacterioplankton assemblage is artificial just like the conventional approach to use 0.2 μm pore size membranes to concentrate/screen bacteria and particulate matter. In fact, UMB are in the lowest verge of the continued size spectrum of planktonic bacteria.

Secondly, FF consumed oxygen at rates comparable to the heat production aliquots of the same sample (Fig. 4, Right). The results presented in Fig. 4 were controversial though: addition of sodium azide did not reduce the heat flow whereas the observed oxygen uptake meant the UMB respired. This would be possible only if the oxygen consumption was due to some processes different to bacterial respiration. In any case, more replicates are necessary to make this matter clear.

The third point is that antimicrobial agents reduced the FF heat flow (Fig. 5) which could be interpreted as cell death or inhibition of their metabolism. However, natural bacterial assemblages are known to be extremely resistant to antibiotics so, no one can be absolutely certain that the agents acted in expected way. Another problem was how the agents interacted with biogenic and abiogenic substances in the measuring ampoule and whether or not the reactions could affect the heat flow. Such uncertainty has prompted us to search for an alternative way to differentiate between the heat flow components associated with intracellular metabolic processes and extracellular reactions.

The approach we used in this study was in a comparison of the heat energy actually produced (i.e. measured) in a long-term calorimetric experiment with that calculated as the bacterial energy expenditure to yield a particular biomass by the end of the experiment after estimating cell counts and sizing). Huge discrepancies in the values obtained for FF (compare the columns "mJ" and "Q_{24}" in Tables 1 and 2, respectively) indicated that bacterial metabolism contributed a minor part of the total heat measured, i.e. hypothesis B looks more correct. The same conclusion was also supported by the experiments with the agents. Some chemical reactions must occur outside the cells, the compound concentration and enthalpy change of which are large enough to produce much heat.

No attempts were made in this study to identify the reactions responsible for the high heat production by FF. We can only suggest at this stage that hydrolysis of biopolymers dissolved in seawater might potentially be an explanation. The pools of POM and labile DOM in the sea are dominated by high molecular weight substances which cannot be taken up by, or directly incorporated into bacterial cells. To make them available for uptake, bacteria have to "precondition" them by extracellular enzymes [12]. Decomposition of biogenic particles, mediated by bacterioplankton may contribute considerably to the production of organic colloids, 0.2 μm to 1 kD [25, 29], involved into the DOM pool, and FF as well because these terms can be considered synonymous in such context.

Conceptually, there may exist a positive feedback between planktonic bacteria and their hydrolytic capacity: (i) the larger bacterial abundance and activity (PF heat flux), the greater their total extracellular enzyme activity (FF heat flux), and (ii) the higher the rate of hydrolysis of biopolymers inaccessible for bacteria, the faster bacterial growth.

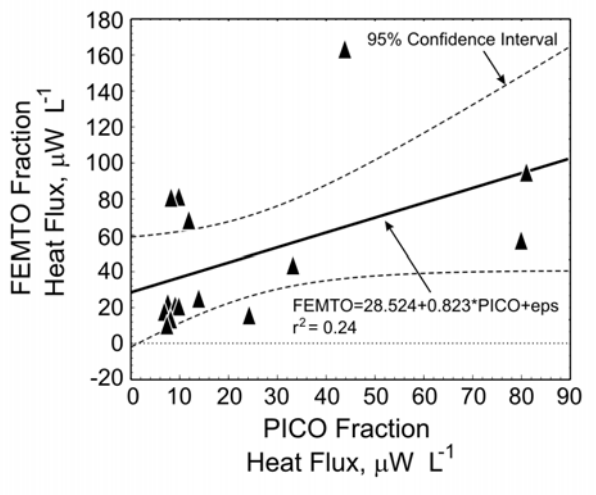

Fig. 6. The femtofraction volume-specific heat flux as a function of those by the picofraction. The data from Fig. 2 (n=15) were used for the regression analysis.

Рис. 6. Зависимость интенсивности теплопродукции фемтофракции от того же показателя пикофракции. В регрессионном анализе использованы данные, представленные на Рис. 2 (n=15).

However, our results have revealed a poor (r^2=0.24, n=15) dependence between the PF and FF heat fluxes (Fig. 4), perhaps, owing to more complicated mechanisms and links involved in the system.

The fact that the enthalpy change of some reactions in the dead component of FF is so high that it even exceeds those of the total microbial metabolism in PF, opens good prospects for applying the microcalorimetric approach to oceanography. The nearest goal still has to be an identification of the heat-producing reactions. If they are the hydrolysis, microcalorimetry can be used as a promising method for measuring bacterial extracellular enzymatic activity in the sea. In contrast to the fluorescence method that requires a set of model fluorogenic substrates [12], an advantage of the microcalorimetric approach would be in its non-specificity, i.e. in the possibility to obtain integral estimates. In any case, there is a need for further methodological studies.

Conclusions. 1. The heat flux produced by FF per seawater volume can be as high as 160 μW l^{-1}, exceeding the maximum flux by the living microrganisms in PF. 2. Both, intracellular (metabolism of alive UMB) and extracellular (associated with dead matter) processes contribute to the total FF heat flow but the latter component is responsible for the bulk of the heat. 3. The heat-producing, extracellular reactions in FF must have relatively large enthalpy changes, so that microcalorimetry can be applied successfully to measure their kinetics. Further studies are necessary to identify the reactions. 4. It is suggested that the source of the heat in FF is bacteria-mediated hydrolysis of high molecular weight organic substances. If so, microcalorimetry can be used as a promising method for measuring bacterial extracellular enzyme activity in the sea, providing integral estimates of the process kinetics.

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Мікрокалориметрія дрібної фракції планктону: пошук джерел теплопродукції. В. С. Муханов, Р. Б. Кемп. За допомогою мікрокалориметричного методу в темних умовах вимірювали теплопродукцію пико- (0.2-2 мкм) і фемто- (<0.2 мкм) фракцій планктону. Фемтофракція (ФФ), як виявилося, продукувала більше теплової енергії в одиниці об'єму проби (48 ± 24 (95% дор. інт.) мкВт $л^{-1}$), ніж пикофракція (25 ± 14 мкВт $л^{-1}$), вміст мікробної біомаси в яку непорівнянно великий. Перевіряли дві гіпотези, що могли б пояснити ці результати, визначивши потенційні джерела тепла у ФФ, а саме: (1) метаболізм ультрамікробактерій, єдиного живого компонента ФФ за винятком віропланктону, нездатного продукувати тепло; (2) позаклітинні хімічні процеси у водяному середовищі. Результати мікрокалориметрії і респірометрії, застосування антимікробних агентів (ципрофлоксацин, глютаральдегид, азид натрію) і мікроскопії (рахунок і вимір клітин у ході експериментів) показали, що обидві гіпотези вірні, але внесок позаклітинних процесів у сумарну теплопродукцію ФФ є визначальним. Гідроліз високомолекулярних органічних сполук, зв'язаний з позаклітинною ферментативною активністю бактеріопланктону, може розглядатися як найбільш ймовірне джерело теплопродукції, що спостерігається. Якщо це припущення вірне, то відкриваються нові перспективи використання мікрокалориметрії у водяній мікробній екології і біохімії в якості могутнього аналітичного методу.

Ключові слова: мікрокалориметрія, теплопродукція, ультрамікробактерії, віропланктон, бактеріопланктон, позаклітинна ферментативна активність

Микрокалориметрия мельчайшей фракции планктона: поиск источников теплопродукции. В. С. Муханов, Р.Б . Кемп. С помощью микрокалориметрического метода в темновых условиях измеряли теплопродукцию пико- (0.2-2 мкм) и фемто- (<0.2 мкм) фракций планктона. Фемтофракция (ФФ), как оказалось, продуцировала больше тепловой энергии в единице объема пробы (48 ± 24 (95% дов. инт.) мкВт $л^{-1}$), чем пикофракция (25 ± 14 мкВт $л^{-1}$), содержание микробной биомассы в которой несопоставимо велико. Проверяли две гипотезы, которые могли бы объяснить эти результаты, определив потенциальные источники тепла в ФФ, а именно: (1) метаболизм ультрамикробактерий, единственного живого компонента ФФ за исключением виропланктона, неспособного продуцировать тепло; (2) внеклеточные химические процессы в водной среде. Результаты микрокалориметрии и респирометрии, применения антимикробных агентов (ципрофлоксацин, глютаральдегид, азид натрия) и микроскопии (счет и измерение клеток в ходе экспериментов) показали, что обе гипотезы верны, но вклад внеклеточных процессов в суммарную теплопродукцию ФФ является определяющим. Гидролиз высокомолекулярных органических соединений, связанный с внеклеточной ферментативной активностью бактериопланктона, может рассматриваться как наиболее вероятный источник наблюдаемой теплопродукции. Если это предположение верно, то открываются новые перспективы использования микрокалориметрии в водной микробной экологии и биохимии в качестве мощного аналитического метода.

Ключевые слова: микрокалориметрия, теплопродукция, ультрамикробактерии, вириопланктон, бактериопланктон, внеклеточная ферментативная активность