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DESIGN AND EXPERIENCE OF USING LIGHT-EMITTING DIODES (LEDs) AS THE INBUILT LIGHT SOURCE FOR A CUSTOMISED DIFFERENTIAL PHOTOMICROCALORIMETER

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In the photomicrocalorimetric module designed by Johansson and Wadsö for a commercial Thermometric TAM heat conduction batch microcalorimeter, the incident light from an external xenon lamp was divided by a beam splitter and directed to the two vessels of the differential system by light guides ideally to give zero heat flow. In practice this proved difficult and so to improve the balance between the vessels in terms of the incident light heat output as well as potentially to give more versatility regarding the choice of wavelengths, the xenon lamp-based system was replaced in the first stage by a pair of cold white LEDs embedded directly in the test and reference vessels. The LEDs had independent electrical circuits to achieve the balance by manual adjustment. As a second stage, the test vessel was equipped with PTFE tubing for changing the liquid phase in it while it was in the middle thermal equilibrium position. This improved the reproducibility of the results.

Keywords: *calorespirometry, differential photocalorimetry, LED, light-emitting diode, photomicrocalorimetry*

Introduction

Direct calorimetry has been used to obtain analytical and thermodynamic data in physical chemistry and biology for many years but its application to photochemical reactions, such as the degradation of pharmaceuticals and photosynthesis, is rare owing to the formidable problem of introducing light with the consequent heat flow into highly sensitive vessels. Mukhanov and Kemp [1] calculated that at the comparatively low photosynthetic photon flux density (PPFD) of up to 100 μmol m^{-2} s^{-1} the light energy for transformation to heat at the vessel walls and in the liquid is milliwatts, outside the range of the microwatt calorimeters used to study biological samples. Possibly for this reason, no commercial isothermal photocalorimeters are available, only customized instruments. In 1939, Magee *et al*. [2] were the first to fabricate a photocalorimeter and obtained good data for the quantum efficiency of photosynthesis in the microalgal Chlorella. There were other scientists studying photochemical reactions, e.g. [3, 4], but they all used a quartz window to shine the light and thus the instruments had relatively low sensitivity. Schaarschmidt and Lamprecht in 1973 [5] were the first to improve sensitivity by using quartz light guides instead of the window to measure optical density changes in their study of yeast cell metabolism using a Tian–Calvet differential photomicrocalorimeter.

In the meantime, Shuvalova *et al*. [6] in 1967 seems to have been the first to employ a Tian–Calvet differential instrument as a photomicrocalorimeter to obtain rates of dark respiration and photosynthesis by cucumber leaves. In this case, the incident light beam from an external 1 kW xenon lamp was guided to the vessels by mirrors. Petrov (reviewed in [7]) used a similar instrument built in Puschino, USSR but changed the light system to a halogen lamp and a series of lens to make similar measurements on Chlorella. Later, he was the first to study photosynthesis and respiration by combining estimates of oxygen uptake/evolution using a Clark electrode with heat flow data from a Tian–Calvet instrument in which the reference vessel contained an inert oil that was Joule heated to compensate for heat from light in the test vessel [7, 8].

In terms of the second generation, differential microcalorimeters described by Suurkuusk and Wadsö in 1982 [9], the first application to photochemical reactions was by Teixeira and Wadsö [10] using two differential thermometric thermal activity monitors (TAM) (successor manufactured by TA Instruments, New Castle, DE 1972, USA) with customised photomicrocalorimeter modules built in Lund University for photochemical studies. The split

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light source for them was a 100 W tungsten lamp in the red part of the spectrum guided to the vessels by fibre-optic bundles and quartz light guides. For the application of photomicrocalorimetry to photosynthesis by spinach leaves, Johansson and Wadsö [11] used one TAM to measure carbon dioxide consumption/evolution by the heat of reaction with sodium hydroxide and the second as the photomicrocalorimeter, i.e. the combined instrument in the dark constituted a calorespirometer. They changed the light source to a xenon arc lamp which gives a broad spectrum of light, similar to daylight.

The photomicrocalorimeter module acquired from Lund was for one TAM instrument and had been modified for each of the two 20-cm^3 stainless steel titration vessels to possess a stirrer shaft with a spindle to a Thermometric KelF turbine [12]. In our studies of photosynthesis in *Dunaliella maritima* [1], a sample of the cells suspended in a Bold basal medium was placed in the stirred test vessel while, based on the differential principle, the reference vessel contained only sterile medium and no photoactive material so that all the incident light was transformed as heat. The optical system was described in detail in [1] but briefly light was generated by a 150 W xenon lamp in a housing and focused with a condenser which also acted to attenuate the PPFD before it passed through a continuously cooled water filter and a glass filter to remove IR and UV radiation, respectively. Light was transferred from the housing exit slit through a bifurcated silica fibre bundle connected to 5-mm quartz rods which introduced the incident light to the test and reference vessels.

Although interesting results were obtained with this setup [1], problems began to show with continued use and these will be discussed in this paper. In addition, it is becoming important to measure photosynthesis at different wavelengths to assess efficiencies across the photosynthetically active spectrum. The use of bandpass filters for the xenon lamp is not a satisfactory solution to this requirement. These two factors will be examined in this paper because they have led us to design and evaluate LED-based changes to the photomicrocalorimeter. This experience also led to a radical solution for introducing samples after light calibration.

The problem of balancing the incident light to the vessels

As mentioned earlier, even at comparatively low PPDF radiance the energy potentially available for transformation to heat is in milliwatts for a microwatt calorimeter. Thus, even a minor imbalance in the radiance to the test and reference vessels produced a large shift in the heat flow from the zero baseline during photomicrocalorimetric measurements. It was thought to be caused by imperfect splitting of the light beam on its way from the source to the vessels. It was minimized by adjusting the position of the lamp in its housing but only rarely was it possible to achieve the perfect balance (no net heat flow). Even if it were achieved initially, there was no stability with time. This was due to fluctuating changes in the shape of the plasma cloud of ionised xenon gas inside the working lamp. They strongly affected the balance between the light input to the test and reference vessels and, as a result, decreased the reproducibility of the measurements.

LED as an alternative

The above problems forced a search for an alternative light source. The idea to use light-emitting diodes (LEDs) for this purpose was grounded on the facts that: (*i*) they are more efficient in terms of producing more light per watt than other alternatives; (*ii*) they can be tuned to a narrow spectrum to emit light of an intended colour without the use of filters which attenuate the light; (*iii*) their output as light intensity as a function of current is very close to linear, i.e. they are current-driven; (*iv*) they do not change their colour tint as the current passing through them is lowered; (*v*) they have a relatively long useful life; and, finally, (*vi*) they are small in size. These advantages make them potentially attractive for photomicrocalorimetry.

LED-based photomicrocalorimeter

Construction

The xenon lamp-based system of the photomicrocalorimeter was replaced by a pair of Toyodo Gosei 5-mm cold white LEDs (part no. EIL51-AWOC-01, Toyodo Gosei Europe NV, Zaventem, Brussels) with emission peaks at 470 (blue) and 578 nm (red) (Fig. 1), mounted

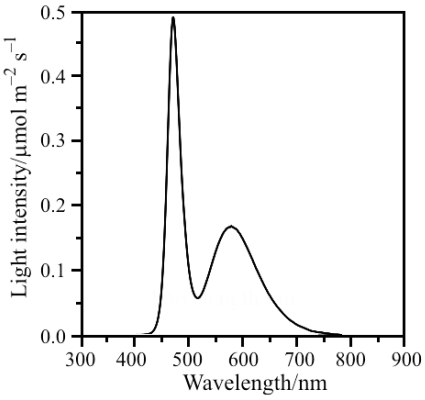

Fig. 1 Light spectrum of the cold white LEDs used in this study

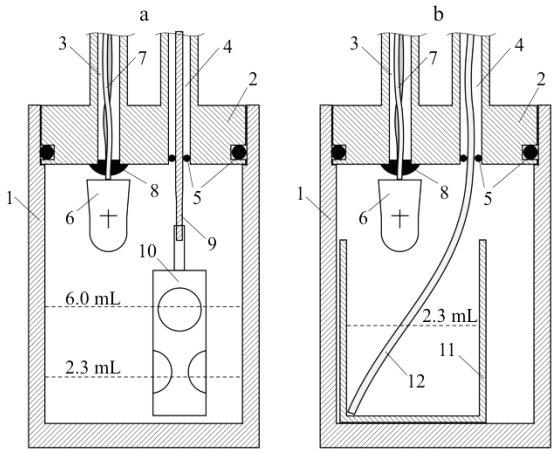

Fig. 2 Outline drawings of a – the standard vessel equipped with an inbuilt LED and b – the modified one with the sub-vessel and the PTFE tubing. The levels of liquid are shown for different volumes. 1 – 20-cm^3 stainless steel vessel, 2 – lid, 3 – shaft previously used for the quartz light guide, 4 – shaft for the stirrer, 5 – rubber o-rings, 6 – LED, 7 – LED electric wiring, 8 – hermetical sealing for the shaft, 9 – stirrer spindle, 10 – turbine stirrer, 11 – sub-vessel, 12 – PTFE tubing

directly in the test and reference vessels. The electrical wiring was passed through the shaft formerly used for the light guides (Fig. 2a). The LED was suspended about 2 mm below the vessel lid and the entrance to the shaft was sealed hermetically. The second stirrer shaft was left unaltered so that a turbine or a propeller could be used to mix the liquid in the vessels.

The LEDs were current-driven by a SkyTronic model 650-685 triple circuit power pack (SkyTronic Ltd., Manchester, M54 3TS) with a Tenma model 72-7735 ammeter (Tenma (UK) Ltd., Cumbernauld, Strathclyde, G68 9LB) and a LED output control box containing a ten-turn potentiometer in each independent electrical circuit to achieve manually the balance (zero heat flow) between the vessels in terms of the heat outputs resultant on the incident light. At constant voltage, the current in the circuits was adjusted between about 4 and 18 mA with an accuracy of 0.01 mA to provide the direct light input to the liquid phase in the test vessel from 4.7 to 16.7 nmol photon s^{-1} at the sample volume of 2.3 cm^3. These values corresponded to the light fluxes from 50 to 150 μmol photon m^{-2} s^{-1}.

Light calibration

Before every experiment, light calibrations had to be performed with the photoinactive incubation medium for the cells in both the test and reference vessels. On the differential principle, each of the light settings in the test vessel was matched by a compensation light flow in the reference vessel to achieve a heat flow baseline as close to zero as possible (Fig. 3). This proved to be a delicate operation requiring patience because of the limitations of the manual (analogue) potentiometer. Then the vessels were extracted from the TAM and opened to change the medium in the test vessel to the cell suspension under investigation and thus start a standard photocalorimetric experiment with the PPDF levels the same as in the light calibration. The calibration results were used later to correct the data obtained in the experiment (Fig. 3). The rates of heating of the LEDs in the test and reference vessels appeared to be different. Thus the heat flow was unbalanced for a short time after switching on and off the light (not shown in Fig. 3). The baseline stabilized in 15–20 min.

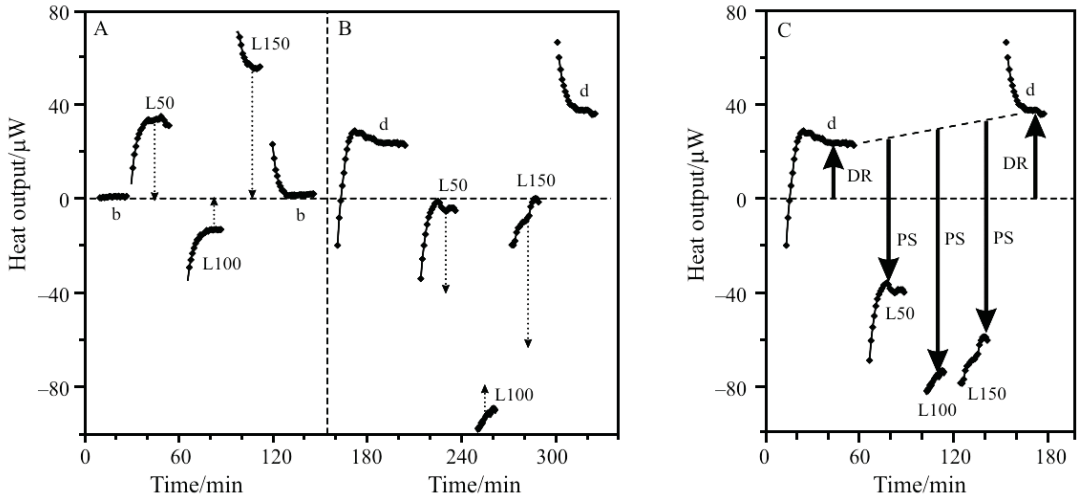

Fig. 3 An example of a photomicrocalorimetric experiment with A – the light calibrations followed by B – the measurement of the net heat flow by the *Dunaliella maritima* motile cells and C – the light correction to the obtained data. L50, L100, L150 – light is on at 50, 100 and 150 μmol photon m^{-2} s^{-1}, respectively, b – dark baseline, d – dark heat flow by the cells, DR – dark respiration, PS – photosynthesis. The dashed arrows show the correction values A – obtained in the calibration and B – applied to the data

Technical problems of combining photomicrocalorimetric and respirometric measurements

Given that it is difficult to combine measurements of heat flow with those of the rate of oxygen uptake and evolution by a Clark electrode in the same vessel of a photomicrocalorimeter, it is most important for accurate estimates of photosynthesis that the total light input per unit time to both the calorimetric and respirometric vessels is the same. The shape of the liquid phase (i.e. the body of the cell suspension) and the distance between it and the light source are also crucial because they determine the incident light intensity and the uniformity of the light conditions in the liquid phase.

In this paper, we experiment with a way to change the configuration of the TAM vessels shown in Fig. 2a for making them geometrically similar to the chamber of an Oroboros Oxygraph (Oroboros Instruments, A-6020 Innsbruck, Austria) [13, 14] which was applied in a previous investigation [1] to measure oxygen uptake in the dark and photosynthetic oxygen evolution in the light in *D. maritima*. The cylindrical glass vessel of the Oxygraph (16 mm id) was filled with a 2.6-cm^3 aliquot of the cell suspension and closed by a piston with a concave conical bottom and a thin vertical channel to remove the trapped air from the vessel and replace it with the suspension (Fig. 4). LED light was guided to the chamber through the horizontal cylindrical window on the side of the chamber.

The photomicrocalorimeter vessels are much larger in size (24 mm id) so the idea was to implant an additional, smaller metal sub-vessel (16.8 mm id, 21.8 mm deep, wall thickness 0.8 mm) into each of them, thus achieving closer similarity of the experimental conditions in both the instruments. The sub-vessel's position in the vessel was asymmetrical (immediately under the LED) but it did not touch the wall of it (Figs 2b and 5). It was fixed to the bottom of the vessel with high thermal conductivity grease. The distance between the LED and the surface of the liquid phase was chosen such that the light intensity and the total light input to the culture was the same as in the Oxygraph. The total light inputs to both the systems were calculated from their spatial configuration and the LED radiation patterns (Figs 4 and 5). The PPDF (μmol photon m^{-2} s^{-1}) and the flow (nmol photon s^{-1}) were measured in a set of spheric sectors specific for each instrument. Then it was possible to estimate the light directly illuminating the liquid phase (termed 'direct light input') and that which potentially can be reflected to the liquid phase by the walls of the vessel ('peripheral light input'). At the incident light fluxes of 50, 100 and 150 μmol photon m^{-2} s^{-1}, the corresponding direct light inputs to the liquid phase were

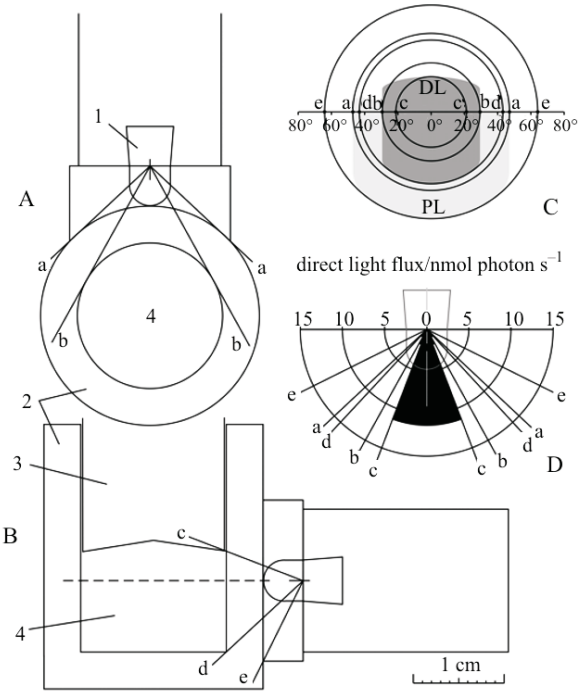

Fig. 4 Representation of the Oxygraph chamber and the light distribution patterns in it: A – top view, B – side view, C – distribution of the direct light (DL, dark grey) illuminating the cell suspension and the peripheral light (PL, light grey) potentially reflected to it in the sphere sectors for which the light flux was measured (D). 1 – LED, 2 – glass vessel, 3 – piston, 4 – cell suspension, a, b, c, d, e – rays delimiting the sphere sectors

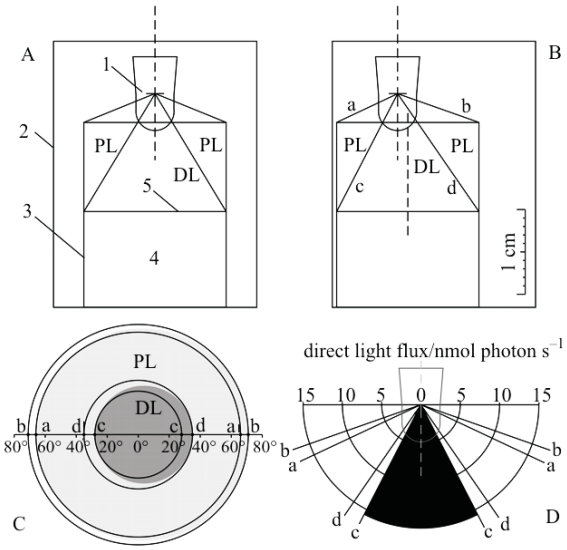

Fig. 5 Representation of the photomicrocalorimetric vessel and the light distribution patterns in it: A and B – side views, C – distribution of the direct light (DL, dark grey) illuminating the cell suspension and the peripheral light (PL, light grey) potentially reflected to it in the sphere sectors for which the light flux was measured (D). 1 – LED, 2 – standard vessel, 3 – sub-vessel, 4 – cell suspension, 5 – suspension-air interface, a, b, c, d – rays delimiting the sphere sectors

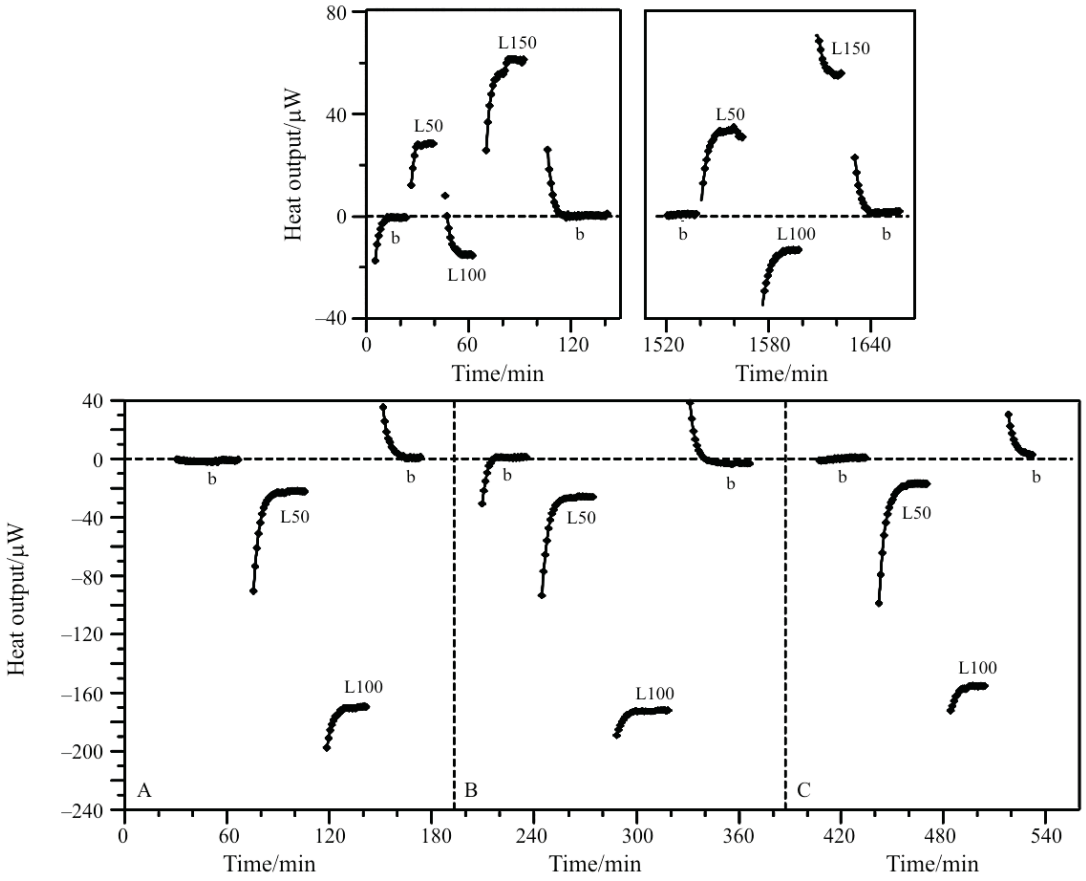

Fig. 6 Reproducibility of the results of the light calibrations distributed in time (upper plots; medium in both the vessels), and also performed with different liquids (lower plots; A, C – distilled water, B – black ink). L50, L100, L150 – light is on at 50, 100 and 150 μmol photon m^{-2} s^{-1}, respectively, b – dark baseline

4.7, 11.9 and 16.7 nmol photon s^{-1} in the photomicrocalorimeter, and 5.2, 10.0 and 14.8 nmol photon s^{-1} in the respirometer, respectively. The peripheral light input was noticeably higher in the photomicrocalorimeter (3.1 to 10.1 nmol photon s^{-1}) than in the respirometer (1.6 to 4.5 nmol photon s^{-1}) owing to the large surface area of the inner walls of the sub-vessel. Thus, the values obtained for the two instruments proved to be close and in the future it will be technically possible to improve the situation by making the sub-vessel smaller to reduce the direct (smaller diameter) and peripheral (smaller height) light inputs. In trials, this double-vessel configuration of the photomicrocalorimeter did not decrease any of the operational properties of the instrument, such as the baseline stability and the time necessary for thermal equilibrium of the sample. In any case, combining two instruments to measure heat absorption and oxygen uptake/evolution is a constrained approach. The technical solution to the problem lies in making measurements of oxygen flows in the photomicrocalorimetric vessel, possibly by using oxygen optrodes [15, 16].

Remote change of the liquid phase in the microcalorimetric vessel

As was stated above, the measurements for every photomicrocalorimetric experiment were divided into two stages: (*i*) the light calibration with the incubation medium in both vessels and (*ii*) the data collection with the biological sample in the test vessel. Between them, the vessels have to be removed from the instrument, opened to change their contents and thermally equilibrated before the next measurement. The procedure requires careful execution and accuracy as it is a potential source of error in the measurement and a factor reducing the reproducibility of the results. This is even more critical in the case of the asymmetric position of both the LED and the sub-vessel inside the calorimetric vessel. A slight turn of the vessel lid with a corresponding horizontal shift in the LED position relative the sub-vessel was found to redistribute the LED radiance in the system and reduce reproducibility of the results. To solve this problem, the test vessel was additionally equipped with PTFE tubing for changing the liquid phase in the sub-vessel. The tube was laid through the

stirrer shaft, replacing the stirrer spindle (Fig. 2b). After the light calibration, both the vessels were lifted to the middle heat sink position, and the test sub-vessel content was evacuated through the tube with a vacuum pump and filled with cell suspension using a syringe. Then thermal equilibrium was achieved before undertaking a standard heat flow measurement. Obviously, this construction eliminated the possibility of stirring the cell suspension, i.e. only motile microorganisms such as *D. maritima* can be studied in this way.

Replicate light calibrations performed with the new system (the sub-vessels+the PTFE tubing in the test sub-vessel) and the new procedure (remote change of the liquid phase) showed a high degree of reproducibility in the results (Fig. 6). The sub-vessel could be rinsed with the culture medium after a standard measurement to finish the experiment with a second light calibration. The use of a blank 'Indian' ink suspension diluted in distilled water for total light absorption in a water–ink–water series of light calibrations produced closely similar results to those obtained for distilled water alone (Fig. 6, lower plots).

Conclusions

- The fast-growing LED technology is highly promising to replace the use of external light sources in the field of photomicrocalorimetry as it provides compact, powerful light sources with accurately adjusted intensity and spectral distribution of light.
- LEDs mounted directly in the photomicrocalorimeter vessels allowed for a high reproducibility of the light calibrations. However, differences in the rates of warming and cooling the test and reference LEDs on switching them on and off initially destabilized the instrument for some minutes. Ways are being sought within LED technology to eliminate this effect.
- An accuracy of 1 μA in adjusting the current in the LEDs circuits (that is about 10 times higher accuracy than in the present setup) would be preferable for achieving the perfect balance between the test and reference vessels under light conditions. There is promise in a computer-based digital feedback control of the current.
- It is typical of differential calorimetry that calibration, in this case for light, has to be performed before every photocalorimetric measurement with a change of the vessel contents between the two operations. The latter caused a mechanical disturbance to the system, exacerbated by the LED milliwatt heat flows, which reduced the reproducibility of the results. The remote change of the liquid phase in the calorimetric vessel (typically from the incubation medium to a cell suspension in that medium) through a PTFE tube at the stage of thermally equilibrating the vessel in the middle heat sink position was found to be the solution to this problem.

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