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A COMPARISON OF THE CLOSED-BOTTLE AND FLOWING-WATER METHODS FOR MEASUREMENT OF RESPIRATION IN AQUATIC INVERTEBRATES

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ABSTRACT

Measurements of the oxygen consumption of three species of aquatic invertebrates were carried out simultaneously by the closed-bottle and flowing-water methods. The results obtained from the closed-bottle technique varied with the length of exposition period. On the other hand, results obtained from the flowing-water respirometer were independent of time in carrying out measurements.

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1. INTRODUCTION

During the last few years there has been a great increase in the number of bio-energetic studies for which the measurement of oxygen consumption of animals is an essential requirement. However, the problem of selecting a suitable and adequate method for the measurement of respiratory rate in a particular species is a difficult one and there is very little comparative information available upon which to base such a decision. ПАТТЭЕ (1965) investigated simultaneously the influence of a rapid increase of temperature on the respiratory rate of nine species of freshwater invertebrates by means of a closed-bottle Winkler technique and a manometric technique in which the water was continuously stirred. The oxygen consumption of the control animals as well as the reaction of the experimental animals to heating in many cases varied accordingly to the method used.

The aim of present work is to compare two methods of measuring respiratory rate of the same species. The two methods of measuring oxygen consumption were the closed-bottle Winkler technique, which is a simple and widely used method, and a flowing-water polarographic method, which is more complicated but has been coming into more general laboratory use in the last few years. To ensure adequate comparison, both methods were operated simultaneously upon each of following three species, *Isoperla buresi* Rauser (Plecoptera), *Cloeon dipterum* (L.)

(Ephemeroptera) and *Bithynia tentaculata*¹ (L.) (Gastropoda). These species were specially selected for their differences in size and to come from different taxonomic and ecological groups (*I. buresi* lives in fast flowing mountain streams whereas the other two species came from shallow lowland standing waters).

2. METHODS

The larvae of *I. buresi* were collected on 29.VI.1967 from the stony bottom of the Olczyski stream in the Tatra mountains; the water temperature was 6.3—6.6°C. As these larvae are aggressive, they were kept separate in glass tubing, 2 cm by 7 cm, together with three small pebbles and with the tube ends covered with netting with holes of 1.5 mm by 1.5 mm. These tubes were placed in an "artificial stream" made of a P.V.C. (polyvinyl chloride) trough, about 90 cm long, 25 cm wide and 15 cm high. This trough had a central partition along its long axis, one end of which was free and the other fixed end of which was pierced by a pipe. In this "stream", the water was continually circulated around the central partition by means of a pump. The "artificial stream" was immersed in a large water thermostat so that its temperature was kept between 7 and 8°C; its water was changed every two days when also the larvae were fed on Tubifex.

The larvae of *C. dipterum* were collected on 7.IX.1967 from amongst the plants growing in the small pools of the Kampinos Forest near Warsaw; the water temperature varied between 19.6 and 22.9°C. All the respiratory measurements by the closed-bottle technique and one of the series using the flowing-water respirometer (19—21.X.1967) were carried out on these larvae. A further series of measurements (15—17.XI.1967) were made on larvae collected on 13.XI. 1967 from the plants growing in a pool on the old glacial river bed of the Vistula, when the water temperature was 5.5°C. In both cases, the larvae together with some plants were placed in glass basins, 18 cm in diameter and with a water depth of 7 cm; the water was ventilated and the basins kept in a thermostat maintained at a temperature of $20 \pm 0.1^\circ\text{C}$.

The specimens of *B. tentaculata* were collected on 24.X.1967 from among the plants in a pool of the old Vistula river bed; the water temperature was 13°C. The animals were kept in the laboratory under the same conditions of *C. dipterum*.

The level of oxygen concentration in the closed-bottle method was determined chemically by Winkler's method. Usually clear glass bottles, 50 ml volume with ground glass stoppers, were used except in one series with *I. buresi* when 100 ml dark bottles were used. The animals were placed directly into the bottles, usually singly, but in one series with *I. buresi*, two individuals per bottle were used, each separated from the other in a well-perforated perspex tube, 2 cm by 1 cm, one end closed by a perforated perspex plate and the other by coarse netting.

The water used for these respiratory measurements was Warsaw tap water, saturated with air but stood long enough to be rid of the chlorine; it was kept at the temperature of the experiment. To this water was added some antibiotic, 32 mg/l streptomycin and 25 mg/l chloromycetin, in order to inhibit

¹ Measurements of the oxygen consumption of *B. tentaculata* were made together with Dr. A. F. ALIMOV from the Zoological Institute, Academy of Sciences, Leningrad, USSR.

as much as possible oxygen consumption by microorganisms. In practice, only a very slight reduction in oxygen concentration occurred in the final control bottles compared with the initial control bottles, usually less than 0.8% of air-saturated water.

Before the experiments proper were carried out, some tests were made to determine the best way to flush out and fill the bottles with the experimental water. RICHMAN (1967) connected six bottles by a series of siphons, allowed the experimental water to flow through all the bottles and determined the oxygen concentration in the first and last bottle of the series. This method was checked by connecting only two bottles together; rubber corks were perforated by two tubes, one, the inflow tube, reaching to the bottom of the bottle and the other, the outflow tube, ended just under the cork. The test conditions were made more severe by reducing the oxygen concentration of the test water. Table I present the oxygen concentration determined in the two bottles after they had been flushed once, twice, three, four or five times.

Table I. Changes in the dissolved oxygen content during the flushing of two bottles connected in series

Series	Bottle I		Bottle II		Number of repetitions
	Water analysed (bottle vol.)	Oxygen conc. mg/l	Water analysed (bottle vol.)	Oxygen conc. mg/l	
a	2nd ¹	0.351	1st ¹	0.698	2
b	3rd	0.273	2nd	0.425	2
c	4th	0.277	3rd	0.316	2
c1 ²	4th	0.187	3rd	0.257	6
d1	5th	0.203	4th	0.251	6

¹ Water analysed (bottle vol.). 1st means that no water was rejected, 2nd — first bottle volume of water was rejected and the second one was analysed, etc.

² Water with another dissolved oxygen concentration was used in the series c1 and d1.

As can be seen in Table I, even in series d1 the amount of oxygen in bottle II is different from that of bottle I and is less than in series c1. The conclusion is that this method of flushing and filling a series of connected bottles gives very uncertain results.

The next test was designed to determine the oxygen concentrations after several flushings and filling of single bottles, unconnected in series. Here, the oxygen concentration was measured in the first, second, third, fourth and fifth bottle volume and was 0.359, 0.242, 0.222, 0.242 and 0.242 mg/l respectively. Therefore, in all the following experiments, bottles were flushed out and filled singly, the first two flushings were rejected and the third re-filling was used for respiratory measurements.

A blank experiment, that is, with water of reduced oxygen concentration treated as described above but without animals, was used to determine the precision of the technique itself. Eleven measurements gave a mean oxygen concentration of 0.196 ± 0.0066 mg/l; this represents a standard error of less than 3.4% of the mean, despite the initial lower dissolved oxygen

concentration increasing the possibility of atmospheric contamination during the handling procedure. In fact, the lowest oxygen concentration determined during the whole experiment was 9⁰/₀ air saturation and, even under such conditions, the above standard error would be 0.8⁰/₀ of the mean value. RICHMAN (1967) in a similar test, obtained a standard error which was 10⁰/₀ of the mean. REBSORF (1966) compared different modifications of the Winkler method. The precision of the measurement was highest when the Pomeroy-Kirshman variation was used with a pure water sample, giving a standard deviation of 0.02 mg/l; the standard deviation of the measurement reported in the present work is also 0.02 mg/l.

The experimental procedure was as follows: first, the initial control bottle was flushed, filled and fixed immediately. Next, one by one, the experimental bottles, containing animals, and the respective final control bottles were filled and placed in the thermostat for periods of 0 to 50 hours. The water temperature was controlled to $\pm 0.015^{\circ}\text{C}$. Finally, the second initial control bottle was filled and fixed. All bottle volumes were measured, all pipettes and burette scales were checked and re-calibrated and 0.01 N sodium thio-sulphate was used for the titration.

In the flowing-water method, the amount of oxygen present was determined polarographically. A detailed description of the apparatus and procedure adopted is given in KLEKOWSKI and KAMLER (1968).

The interior of the perspex animal chamber was a cylinder placed horizontally with cone-shaped inlet and outlet openings but the lower part of the chamber was occupied by a piece of perspex upon whose upper flat surface were placed the animals. Larvae of *C. dipterum* were placed directly into the chamber, 40 larvae for the experiment of 19—21.X.1967 and 30 larvae for that on 15—17. XI.1967. The length of this chamber was 5.5 cm, the width 1.3 cm and the height 0.65 cm. The speed of water flowing through the chamber was about 0.008 cm/sec. Four specimens of *B. tentaculata* were placed in a chamber measuring 7.5 cm by 1.4 cm by 0.7 cm and the speed of water flow was about 0.006 cm/sec. Two larvae of *I. buresi* were placed separately into two chambers connected one behind the other and divided by a net; each chamber measured 1.6 by 0.7 by 0.35 cm and the speed of water flow was about 0.014 cm/sec. The outflowing water from the respirometer contained about 20⁰/₀ less oxygen than the inflowing water in the cases of *C. dipterum* and *B. tentaculata* and 10⁰/₀ less in the case of *I. buresi*. The error in measurement of oxygen content was never more than $\pm 1.5^{\circ}$ /₀. The causes of these errors is discussed by KLEKOWSKI and KAMLER (1968).

The dry weights of the animals were determined after previous drying at 100°C to a constant weight. Oxygen consumption by the larval insects is expressed as $\mu\text{l oxygen/g dry body weight} \cdot \text{hour}$ whereas it is given as $\mu\text{l oxygen/g dry weight of body plus shell} \cdot \text{hour}$ in Bithynia. It proved difficult to separate the body of Bithynia from its shell, either in the dried state or when fresh. Therefore, additional measurements of the dimensions and weights were made on two control series of ten snails in order to calculate the respiratory results in terms of g body weight without shell. The size and living weight of both series of snails were determined; control series 1 of snails were killed in boiling water and digested in a 1⁰/₀ solution of trypsin at 40°C until only shell remained whereas the control series 2 were dried

and the dry weight of the body plus shell measured. From the weight of the dried shell, the weight of the body could be determined.

The weights and sizes of the snails belonging to control series 1 and 2 together with those of the experimental snails whose respiration was measured is given in Table II. A comparison of the height and width of the shell, live body weight with shell and dry body weight with shell shows that all three series of snails belonging to the same population so that it is possible to apply the relationships in body measurements of the control snails to the experimental ones. In fact, the standard errors obtained in the control series are greater than those of the experimental snails. This is very useful as, in the control series 1, the shell formed a similar percentage of the total live weight in both large and small snails and this can be applied with some certainty to the experimental snails².

Table II. The weights and sizes of a sample of *Bithynia tentaculata* from the old Vistula bed (means and standard errors)

Series	Control series 1 (digested snails)	Control series 2 (dried snails)	Experimental series (respiration was measured)
Number of specimens	10	10	28
Height of shell, mm	8.88 ± 0.378 N ¹	8.61 ± 0.384 N	—
Width of shell, mm	4.97 ± 0.183 N	4.92 ± 0.224 N	—
Live weight of body with shell, mg	119.42 ± 14.75 N	117.92 ± 14.04 N	126.45 ± 8.90 N
Dry weight of body with shell, mg	—	50.11 ± 6.75 N	52.18 ± 3.64 N
Weight of shell, mg	38.85 ± 5.792	—	—
Shell weight as % of total live weight	large snails 5 specimens	small snails 5 specimens	—
	34.32 ± 2.072 N	28.86 ± 1.807 N	

¹ N signifies that the difference between columns is not significant at the 1% level.

It is therefore possible to calculate from the given data the mean dry weight of the body: 50.11 – 38.85 mg = 11.26 mg. If the mean dry weight of the body with shell (50.11 mg) is divided by the mean dry weight of the body alone (11.26 mg), a coefficient of 4.45 is obtained. Thus, the oxygen consumption per g dry weight with shell can be multiplied by 4.45 in order to obtain the results in terms of per g dry body weight without shell.

² NOLAN and BRAND (1954) found that the percentage shell weight increases, decreases or remains constant with increasing weight in different species of aquatic animals.

In order to obtain additional information on the influence of different experimental conditions on the larvae of *I. buresi*, the presence and absence of "searching movements" was observed and the frequency of respiratory movements per minute was recorded by means of an electrical tapping key whose signals were registered on a kymograph on which was marked a time scale. FOX and SIDNEY (1953) recorded the frequency of respiratory movements of larval Trichoptera by this means.

3. RESULTS

The rate of oxygen consumption, $\mu\text{l oxygen/g} \cdot \text{hour}$, measured in the flowing-water respirometer, did not vary with time (Figs 1A, 2A and 3A). On the other hand, the rates of oxygen consumption obtained in the closed-bottle method varied considerably depending on the length of the experimental period (Figs 1B, 2B and 3B); the shorter the period, the higher the consumption rate, the longer the period, the lower the consumption rate. The empirically obtained results plotted on a logarithmic scale reveal a relationship described by the general formula,

$$Q = a \cdot t^b$$

where Q is the oxygen consumption, $\mu\text{l oxygen/g} \cdot \text{hour}$, t is the time in hours and a and b are constants, which were determined for each species. The measurements of oxygen consumption obtained with the closed-bottle method were always more dispersed than those for the flowing-water respirometer, particularly for short periods of exposition. These general results applied to all three species.

Isoperla buresi

One measurement in the flowing-water respirometer was carried out, which lasted a short time only for technical reasons; the results are shown in Fig. 1A. The mean and standard error was $282.8 \pm 10.77 \mu\text{l oxygen/g} \cdot \text{hour}$. No respiratory or searching movements were observed during this experiment.

Table III. The behavioural pattern of *Isoperla buresi* in two series of experiments using closed-bottle method

Series	3—4. VII free larvae	10—12. VII larvae in tubes	Statistical difference between columns
Searching movements per min. (N = 80) no. present no. absent	19 10	10 41	significant ($p = 0.001$) (χ^2 test)
Respiratory movements per min. (N = 80) mean and standard error	4.8 ± 2.31	36.6 ± 2.94	significant ($p = 0.01$) (analysis of variance)

Two series of measurements were carried out using the closed-bottle method. Figures 1B1 and 1C1 show the results from experiments carried out on 3—4.VII.1967 using free larvae, one per 100 ml dark bottle. Figures 1B2 and 1C2 give the results of other experiments carried out on 10—12.VII.1967,

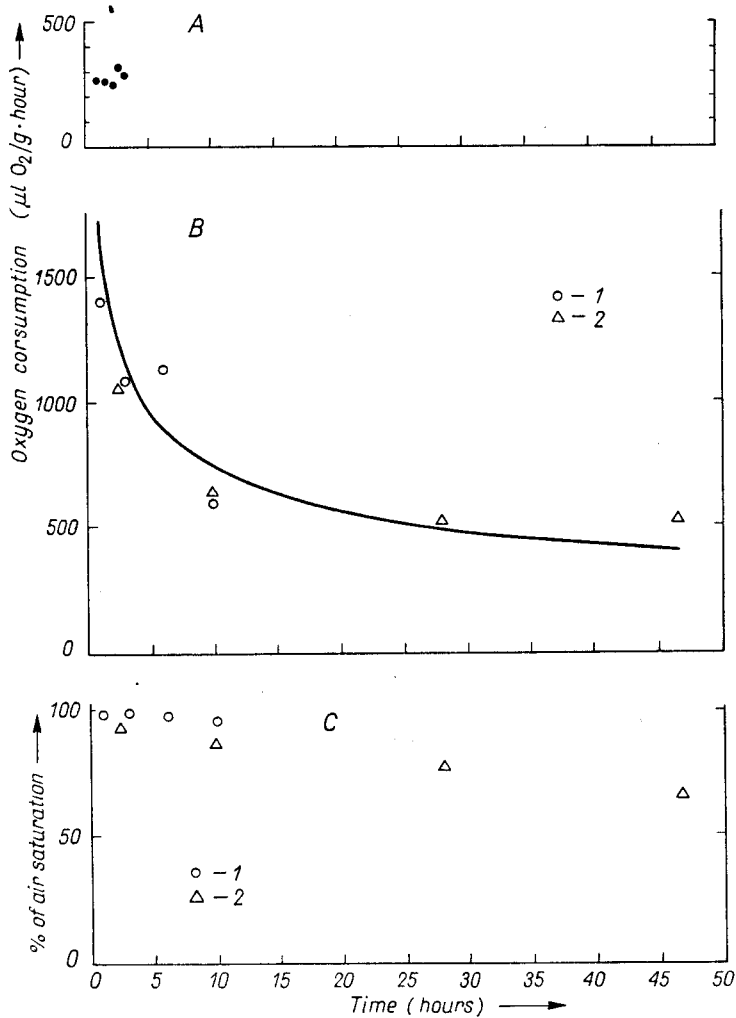


Fig. 1. Oxygen consumption of *Isoperla buresi*, 13 individuals, mean dry weight of one individual 5.34 ± 0.3742 mg; temperature $8 \pm 0.015^\circ\text{C}$. A — flowing-water method; B — closed-bottle method. 1 — 3—4.VII.1967; 2 — 10—12.VII.1967. C — the oxygen content in the bottles after the end of the exposition period. 1 and 2 as in B

using single larvae enclosed in perforated tubes, two larvae per 50 ml clear bottle. Obviously, the oxygen concentration in these bottles were lower at the end of the exposition period, with two larvae in smaller bottles (Fig. 1C2). Table III gives the frequency of respiratory movements per minute and the presence and absence of searching movements, which were recorded in both

sets of experiments. The free larvae renewed the oxygen in the boundary layer near their bodies mainly by searching movements whereas the larvae enclosed in tubes by means of respiratory movements. Despite this difference, an analysis of variance (BAILEY, 1959) of the oxygen consumption of *I. buresi* in these two series of measurements did not differ significantly at the 1% level (see also Fig. 1B). The dependence of rate of oxygen consumption on exposition time for this species is described by the formula,

$$Q = 1750 \cdot t^{-0.377}.$$

Cloeon dipterum

Two series of measurements using the flowing-water respirometer were carried out (Fig. 2A). The mean and standard error of oxygen consumption was 2655 ± 87.9 μl oxygen/g \cdot hour for experiments carried out on 19—21. X.1967 (using the same larvae as in the closed-bottle measurements) and 1859 ± 60.1 μl oxygen/g \cdot hour for experiments carried out on 15—17. XI.1967. The higher respiratory rates of the first series are probably due to the different physiological states of larvae collected at different times, the different periods of acclimatization to the experimental temperature and because different densities of animals were placed in the animal chambers. In neither series was there an increase or decrease in the oxygen consumption with length of experimental period. It seems, however, that the respiration changes with time of a day, at noon it is low and it increases in the evening. This result confirms ELLIOT'S (1968) results on daily activity patterns of larval Ephemeroptera and also of PATTEE (1965) on changes in the metabolism of these animals at different periods of a day.

Four series of measurements were carried out using the closed-bottle method. The series carried out on 14—16. IX.1967 used 10 larvae in a 50 ml bottle (Figs 2B1 and 2C1) whereas 4 larvae were used in the same bottles in all the other series (Figs 2B and 2C: 2, 3, 4). Despite the different experimental conditions, analyses of variance revealed no significant difference at the 1% level in the oxygen consumption from these four series of experiments (see also Fig. 2B). The dependence of oxygen consumption on time of exposition for this species is described by the formula,

$$Q = 7500 \cdot t^{-0.336}.$$

Bithynia tentaculata

The mean and standard error of oxygen consumption of this species measured by the flowing-water respirometer was 74.7 ± 2.62 μl oxygen/g dry body weight with shell \cdot hour (Fig. 3A). This rate did not change during the period of measurement.

One individual per 50 ml bottle was used in all three series of measurements with the closed-bottle method. Two series of experiments were carried out, in which the exposition time was not greater than six hours, on 25. X.1967 (Figs 3B1 and 3C1) and on 30. X.1967 (Figs 3B2 and 3C2) as well as one series with longer periods of exposition, 17—48 hours, on 6—8. XI.1967 (Figs 3B3 and 3C3). However, these differences in the experimental design did not greatly influence the shape of the curve describing the relationship of respiration with time using this method of measurement. This relationship is described by the formula,

$$Q = 192 \cdot t^{-0.450}$$

for all three series, and by the formula,

$$Q = 185 \cdot t^{-0.459}$$

μl oxygen/g dry weight body with shell \cdot hour for the two series of short duration.

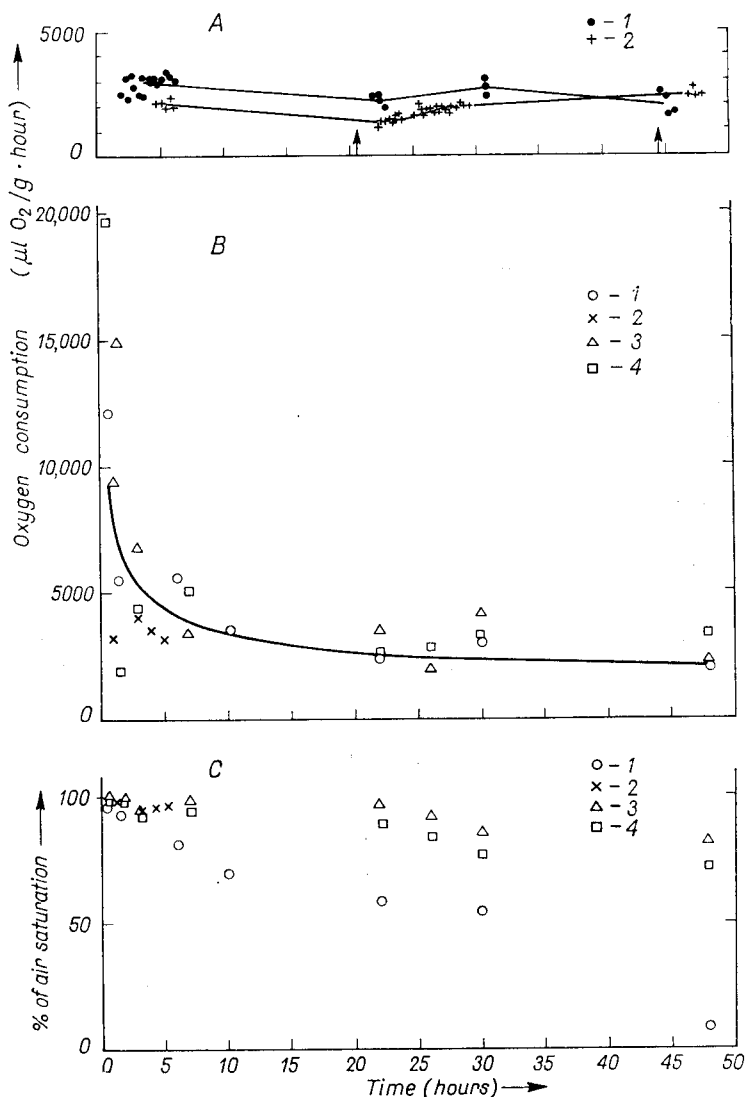


Fig. 2. Oxygen consumption of *Cloeon dipterum*, 215 individuals, mean dry weight of one individual 0.214 ± 0.00445 mg; temperature $20 \pm 0.015^\circ\text{C}$. A — flowing-water method. 1 — 19—21.X.1967; 2 — 15—17.XI.1967; the arrow shows 12 noon. B — closed-bottle method. 1 — 14—16.IX.1967; 2 — 18.IX.1967; 3 — 25—27.IX.1967; 4 — 29—30.IX.1967. C — the oxygen content in the bottles at the end of the exposition period; 1, 2, 3 and 4 as in B

4. DISCUSSION

Ease of measurement and simplicity of equipment in the closed-bottle method compared with the flowing-water respirometer usually influences the decision to use the former method. However, there are several factors causing the results obtained from closed-bottle method to be of little value. Some of these are discussed below.

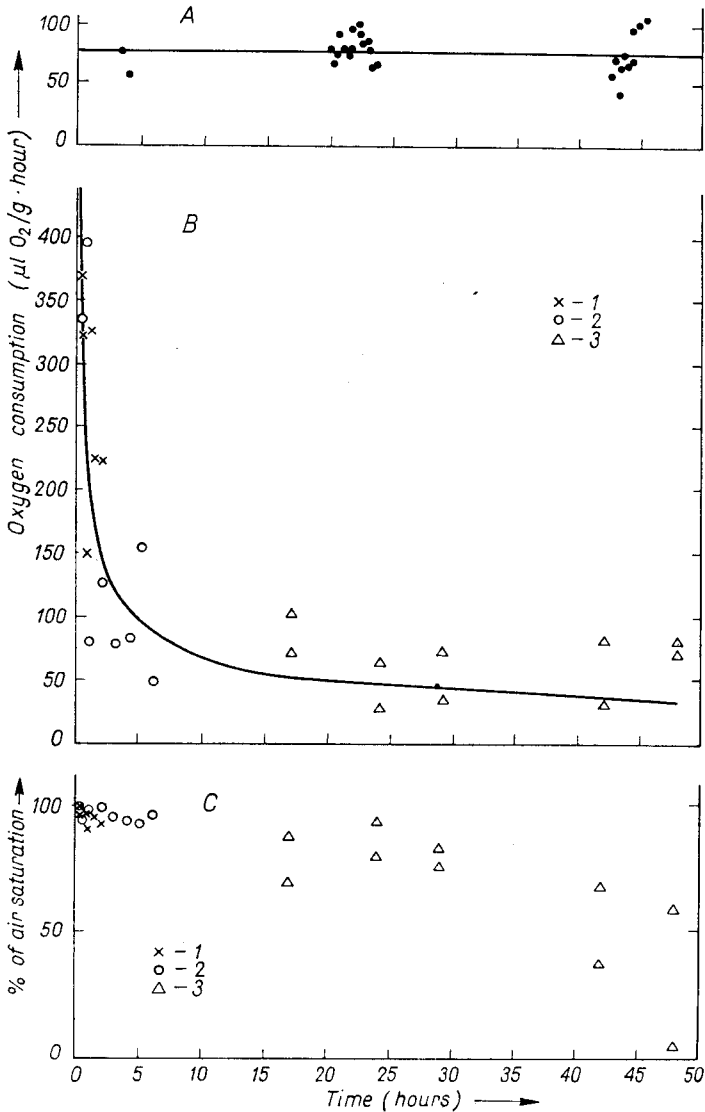


Fig. 3. The oxygen consumption of *Bithynia tentaculata*, 28 individuals, mean dry weight of one individual (body with shell) 52.2 ± 3.64 mg; temperature $20 \pm 0.015^\circ\text{C}$. A — flowing-water method; B — closed-bottle method. 1 — 25.X.1967; 2 — 30.X.1967; 3 — 6—8.XI.1967. C — the oxygen content in the bottles at the end of the exposition period; 1, 2 and 3 as in B

1. In the flowing-water respirometer, conditions are constant and controlled during the experimental period. This is not so in the closed-bottle where conditions change with time of exposition, as the amount of oxygen decreases and that of metabolites increases.

2. The flowing-water respirometer ensures movement of water along the body surface of the animal. Inside the closed bottle which is immersed in a thermostat, there is a complete absence of water movement. It could be said to be an unnatural situation, as in most natural aquatic habitats, even in standing waters, some water movement exists caused by wind, local temperature differences etc. Complete stagnation of water on the body surface of an animal causes the formation of an oxygen gradient. Animals renew these boundary layers by an increase in activity (different kinds of respiratory or searching movements). DAM (1937), PHILIPSON (1954), AMBUHL (1959), KNIGHT AND GAUFIN (1963) all found an increase in frequency of respiratory movements with a fall in the speed of water flow. The results obtained in this paper show a similar relationship, namely, that a lack of respiratory or searching movements in the flowing-water respirometer and their intensification in the closed bottle. In Fig. 4 are given additional observations on changes in the frequency of respiratory movements of two species of Plecoptera in different speeds of water flow. These experiments also show that stagnated water causes a considerable increase in the frequency of respiratory movements.

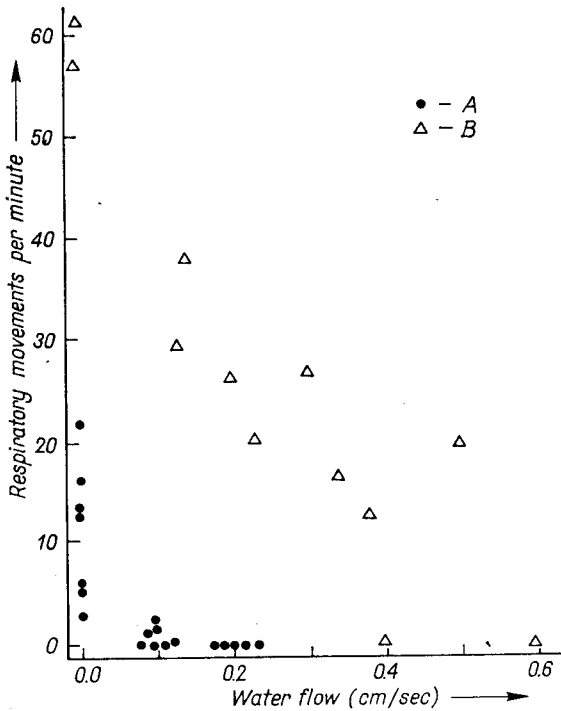


Fig. 4. Frequency of respiratory movements of larval Plecoptera in different speeds of water flow. Room temperature (about 20°C). A — *Arcynopteryx compacta*, 7 larvae, living weight 61—74 mg. B — *Perlodes intricata*, 2 larvae, living weights 103 and 104 mg

3. After placing the animal in the experimental vessel, there follows in general an elevation of the metabolism. Investigating the oxygen consumption in a Barcroft respirometer with water equilibrated with air of larval *C. dipterum* not previously acclimatized to other conditions, HARNISCH (1938) found that the first measurement were higher than those of animals which had stayed in the respirometer for some time. Similarly, PATTEE (1965) (Fig. 14), using a manometric respirometer, found a heightened metabolism in *Ancylus fluviatilis* and *Asellus aquaticus* during the first five hours. ZEISS (1963), using closed bottles, observed that during an exposition period of 2.5 hours, the oxygen consumption of *Calanus finmarchicus* was twice higher than that obtained for a 24 hour period. This heightened metabolism could be the animals, response to handling and being placed in a vessel and is certainly higher than the "normally active state" which TEAL (1967) suggests is the only metabolic measurement that is ecologically useful.

In the flowing-water respirometer, there is the possibility of rejecting the measurements of the initial heightened metabolism. In Fig. 4 of MANN'S (1965) work, it can be seen that the oxygen consumption of gudgeon in the two hours after placing the fish in flowing-water polarographic respirometer was about three times greater than that after five hours. Mann kept the fish in the respirometer for at least five hours before measuring their oxygen consumption. KAMLJUK (1964) measured the oxygen consumption of *Lebistes reticulatus* using also a flowing-water polarographic respirometer. The oxygen consumption measured immediately after placing the animals in the respirometer was about two times greater than that after 1.5—2 hours. In the present study, the initial heightened metabolism is not conspicuous (Fig. 1A, 2A and 3A) because, as a result of such a slow water flow, the time needed to equilibrate the oxygen concentration throughout the apparatus is longer than the acclimatization period of the animal.

On the other hand, in the closed bottles, it is not possible to reject this initial period of adaptation as the oxygen content of the water is determined at the end of the experimental period. Thus, the initial heightened oxygen utilisation results in always giving too high values of oxygen consumption over a period of time. The relationship between the rate of oxygen consumption and the apparent rate as measured by a closed bottle respirometer is described by

$$R_{(t)} = \frac{Q_{(t)} \cdot t - Q_{(t-1)} \cdot (t-1)}{\text{unit of time}}$$

where R represents the real oxygen consumption per unit time, Q the apparent oxygen consumption per unit time, as measured by the closed-bottle technique, and t is a unit of time (two units, three units etc.). The shorter the unit of time utilised, the more accurate are the measurements. Such a formula can be used to calculate $R_{(t)}$ for a species. It would be necessary to make a series of measurements with closed bottles using different experimental periods, to calculate $Q = a \cdot t^b$ (see above) and to determine the curve analogical to that in Figs 1B, 2B and 3B; from these, it is then possible to calculate $R_{(t)}$. This involves a great deal of work and it might be simpler to construct a flowing-water respirometer.

The above relationship is illustrated in the following theoretical example. Let us suppose that four larval *C. dipterum* are placed in each of four bottles;

four control bottles without animals are exposed simultaneously as final controls. In each bottle is placed an oxygen electrode recording the oxygen content every hour. The first pair of bottles are exposed for two hours, the second pair for five hours, the third for ten hours and the fourth pair for twenty hours. After the end of the exposition period, the oxygen content in each bottle is determined by Winkler's method. For simplicity, let us suppose a) there is no individual difference in oxygen consumption between individual animals, b) the dry weight of all individuals is the same, i.e. one individual weighs 0.0002 g, for individuals weigh 0.008 g, c) that only the animals use oxygen and the oxygen content of all final control bottles is the same, that is, equal to 100% air saturation, which is 6.36 ml oxygen/l at 20°C, d) the volume of all bottles is the same, 50 ml, e) the measurement error is zero, f) the adopted unit of time is one hour and is synonymous with an infinitely small unit of time. Figure 5A present the results from these "theoretical" measurements of oxygen concentration in bottles with animals. In all the bottles events proceeded identically; the points on the curve illustrating the electrode readings after one hour really represent four super-imposed points from all four bottles, but because each of the four Winkler determinations used up one bottle, the electrode readings after the sixteenth hour come from only one bottle, the fourth one exposed for the longest time.

From this information, it is possible to calculate two kinds of oxygen consumption, $R_{(t)}$ and $Q_{(t)}$. In Fig. 5B, the curve 5B1 shows the real oxygen utilisation, $R_{(t)}$, in $\mu\text{l oxygen/g}\cdot\text{hour}$, calculated from the electrode readings with the help of the following formula,

$$R_{(t)} = \frac{(C_{(t-1)} - C_{(t)}) \cdot 50 \text{ ml}}{0.0008 \text{ g} \cdot 1 \text{ hour}}$$

where C represents oxygen concentration in ml/l. Thus for example, for the second hour, $R_{(2)} = \frac{(6.239 - 6.169) \cdot 50}{0.0008 \cdot 1} = 4375$. Whereas curve 5B2 presents apparent oxygen consumption, $Q_{(t)}$, in $\mu\text{l oxygen/g}\cdot\text{hour}$ during time t, calculated from the Winkler determinations of the oxygen content at the end of the exposition period with the help of the formula,

$$Q_{(t)} = \frac{(6.360 - C_{(t)}) \cdot 50 \text{ ml}}{0.0008 \text{ g} \cdot t}$$

Thus, for the second hour, $Q_{(2)} = \frac{(6.360 - 6.169) \cdot 50}{0.0008 \cdot 2} = 5968$. Curve 5B1, presenting the real oxygen consumption, shows its initial high level followed by an uniform rate after longer exposition, about 2200 $\mu\text{l oxygen/g}\cdot\text{hour}$, which is close to flowing-water method results, 1859 and 2655. Curve 5B2 shows a consistently higher level than 5B1. This curve 5B2 is exactly the same curve as that given in Fig. 2B for *C. dipterum* and is analogical to those curves in Figs 1B and 3B for the other species.

It is necessary not to forget that the circulating type of flowing-water respirometer such as AMBÜHL (1959) and ERIKSEN and FELDMETH (1967) used prevents only those errors mentioned above in point 2 and not those described in points 1 and 3 unless there is continuous registration of the decrease in oxygen concentration and so the initial heightened metabolism can be rejected.

4. Both methods discussed in this work have their own specific errors. Error involved in determination of oxygen content was discussed earlier in the section on methods. The scatter of results in determination of oxygen consumption by the flowing-water respirometer were not great and were constant. The standard error as percentage of the mean was 3.2% for *C. dipterum* from November and 3.3% from October, 3.5% for *B. tentaculata* and 3.8% for *I. buresi*. FISCHER (unpublished data) measured respiration of a fish, *Ctenopha-*

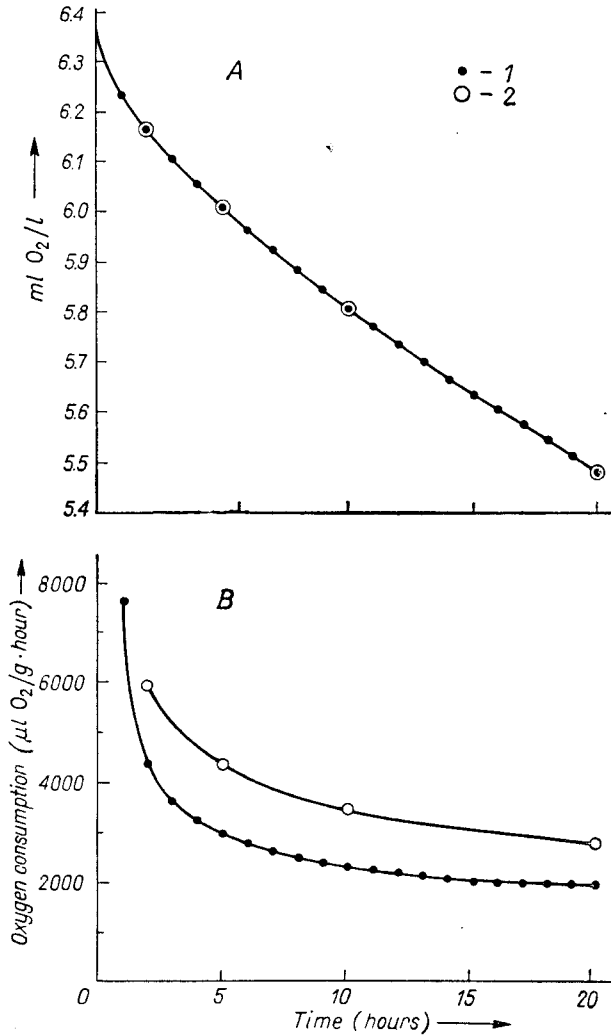


Fig. 5. A theoretical example of oxygen consumption measured by the closed-bottle method. A — changes in the oxygen concentration in the bottles with animals; 1 — “continuous” results obtained from the recorded readings of the oxygen electrode (every hour); 2 — results obtained by the Winkler method (after 2, 5, 10 and 20 hours). B — oxygen consumption by animals; 1 — the real oxygen consumption $R(t)$ calculated from the “continuously” recorded readings of the oxygen electrode; 2 — the apparent oxygen consumption $Q(t)$ calculated from Winkler analyses

ryngodon idella with an another type of flowing-water respirometer; the standard error of her results was 3.5% of the mean. Whereas the scatter of results in the closed-bottle method was always important, particularly when the exposition time was short and so the differences in oxygen concentration small (Figs 1B, 2B and 3B). It may be that here we are dealing not only with errors associated with the method but also due to various specific individual reactions associated with handling and placing into vessels.

Until now our knowledge of recently described species *I. buresi* (RAUŠER, 1962) has been limited to descriptions of its morphology and distribution. There is therefore no data with which to compare the rates of oxygen consumption obtained in the present work.

The magnitude of oxygen utilisation by larval *C. dipterum* in water equilibrated with air is given in several papers by Fox and his co-workers. This information can be compared with the results obtained here only with the greatest care, because the experimental conditions were different. Fox and SIMMONDS (1933) investigated animals with a mean dry weight of 0.28 mg per individual in January. The animals were narcotised and the measurements were carried out with a Barcroft manometric respirometer. At 10°C the mean oxygen consumption was 600 μl oxygen/g dry weight·hour. In Fox, SIMMONDS and WASHBOURN (1935) similar level is given, 606 at 10°C, under similar experimental conditions. The value for Q_{10} for *C. dipterum* recorded by PATTEE (1965) was near to 3. It is possible to calculate that the oxygen consumption of narcotised larvae at 20°C is 1800 or 1818 μl /g·hour on the basis of the information given above. This level is similar to that obtained in the present work when the heightened metabolism associated with the animals' adaptation to the vessel is rejected, that is, to the results obtained when flowing-water method is used (2655 in October, 1859 in November). Results of measurements using the closed-bottle method are contained in the works of Fox, WINGFIELD and SIMMONDS (1936, 1937). Un-narcotised larvae exposed for one to two hours at 10°C during the month of November gave a mean oxygen consumption of 1310 μl /g·hour. Therefore, one can expect that the level of 1310 μl /g·hour at 10°C converted to 20°C would give the level of 3930 μl /g·hour. Such level of oxygen consumption is considerably higher than the results discussed above and it is possible to consider that it is heightened by inclusion of the metabolism of the adaptation period, similarly, as in the results for the closed-bottle method of the present work.

WINBERG (1956) investigated the dependence of oxygen consumption of larval *C. dipterum* and *C. praetextum* on weight. He employed the closed-bottle technique, with an exposure period of two hours and converted the results to 20°C. The oxygen consumption (Q) as μl oxygen/individual·hour was defined by the formula $Q = 0.872 \cdot w^{0.88}$ where w is living weight of one individual, mg. In the larval *C. dipterum* used in the present work, the percentage water content of the body was approximately 85%, obtained from rather a small number of measurements. The mean dry weight of an individual was 0.214 mg, giving a mean living weight of about 1.43 mg. Substituting this value in the above formula gives a rate of 1.195 μl oxygen/individual·hour or a rate of 5584 μl oxygen/g dry weight·hour. This level is similar to that obtained in the closed-bottle results obtained in this work after two hours exposure (5943 μl oxygen/g·hour in Fig. 2B). It appears that both of these results are too high.

WINBERG and BELIAZKAYA (1958, 1959) carried out measurements of the oxygen consumption of ten freshwater Gastropoda species including *B. tentaculata* whose living weight of body without shell was 0.001—0.090 g. The oxygen consumption as ml oxygen/individual·hour at 20°C (Q) for all species was described by one formula, $Q = a \cdot w^{0.75}$, where a refers to the oxygen consumption of one gram weight, w — is living weight of body without shell (in g). These authors carried out their studies on animals kept in the laboratory for about 24 hours and on freshly collected animals. The oxygen consumption of the latter were always higher. In the present work, animals were always kept in the laboratory for 24 hours or more before measurements were made. Thus these results can be compared with those of WINBERG and BELIAZKAYA, using their minimal values for a , $a^{\min} = 0.05$ ml oxygen/g·hour. From Table II, the mean living weight without shell of one experimental animal is $126 - 39 = 87$ mg or 0.087 g; substituting in the above formula, the oxygen consumption obtained is 8 μ l oxygen/individual·hour. The mean dry weight of body with shell was 0.0522 g (Table II); thus the oxygen consumption, calculated by means of WINBERG and BELIAZKAYA's formula, for an animal of similar weight as those used in the present works is $8/0.0522 = 153.2$ μ l oxygen/g dry weight of body with shell·hour. The cited authors carried out their measurements by means of the closed-bottle method and the exposition period varied from one to four hours. In the present work the oxygen consumption at 20°C of *B. tentaculata* using the closed-bottle method for a one hour and a four hour exposure period was 192 and 103 μ l oxygen/g dry weight of body with shell·hour respectively (Fig. 3B). These are very similar results. Undoubtedly, both one and the other are higher values than occur under natural conditions.

BERG (1961) also measured the oxygen utilisation of freshwater snails using closed-bottle technique he does not give the period of exposure but judging from the remarks given in BERG's 1953 work, it seems likely that the exposition period was a short one. Figure 2 in the 1961 paper presents respiratory results for *B. tentaculata* whose living weight without shell was 80 mg which is very close to the weights of animals used in this paper. The temperature (18°C) and dissolved oxygen content (about 18.5% of gas mixture) was also rather similar to those used here. The oxygen consumption by BERG's *Bithynia* was 8 μ l oxygen/individual·hour which is also very similar to that measured in the present work by means of closed-bottle technique.

5. CONCLUSIONS

In the present work, the closed-bottle technique was used in a way that is widely applied, that is, the closure of the bottles and the start of the exposition follows immediately after the placing of the animals in the bottles; the calculation of oxygen consumption is based on the decrease of oxygen caused by the animal during the whole period of exposition. The results obtained suggest that the levels of oxygen consumption from such an application of the closed-bottle method are much higher than the real values and cannot be used as an element in energy balance studies. Besides this, results differ according to the manner of carrying out the measurements, that is, according to the time of exposition utilised. This phenomenon occurs particularly clearly when the exposition time is short.

It seems that this method can be used in comparative investigations, for example, in studies on the influence of different temperatures on an organism when other methods are not available. Then it is necessary to take a suitable period of exposition time. It should be sufficiently long but not so long that the fall in oxygen concentration and the accumulation of metabolites in the bottle can influence the animal being investigated. The exposition time has to be equal in a whole set of measurements. Results so obtained certainly permit inquiry into the dynamics of phenomena, however it is necessary to treat them only as relative values.

Results from the flowing-water method can be applied to both types of investigation. However, there is difficulty in applying this method to certain subjects, for example, to Rotifera. The flowing-water technique can be used also for investigations of the metabolism of benthic communities in the field (PAMATMAT, 1965).

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6. SUMMARY

1. In order to compare two methods of measuring the respiratory rate of fresh-water organisms, namely the closed-bottle-Winkler technique and the flowing-water polarographic technique, a series of long term measurements of the oxygen consumption of three species of aquatic invertebrates was carried out, both methods being applied simultaneously.

2. The test organisms were *Isoperla buresi* (Plecoptera), *Cloeon dipterum* (Ephemeroptera) and *Bithynia tentaculata* (Gastropoda). These species differ taxonomically, ecologically and in their size.

3. The measurements of oxygen consumption obtained by the flowing-water respirometer showed no changes in relation to how long the experiment lasted and revealed very little variation. The elevated metabolism due to the animal's adaptive period did not affect the measurements made after the end of adaptation. On the other hand the results from the closed-bottle method were very variable in relation to exposition period; this relationship can be defined by the formula $Qa \cdot t^b$. All the results obtained by the closed-bottle method include the errors resulting from incorporation of the period of elevated metabolism due to the period of adaptation. The results obtained are higher than the real oxygen consumption of the organism after the passing of the same time from the start of the exposition. This error varies with the length of the period of exposition. The scatter in the results is great.

4. The above relationship between oxygen consumption as measured by closed-bottle technique and length of exposition time occurred in all series of experiments irrespective of species studied or modification in the procedure of the experiment.

5. The following additional measurements were made:

a. Measurements aimed at increasing the accuracy of determination of oxygen concentration.

b. Measurements of the frequency of respiratory movements and the occurrence of searching movements in larval Plecoptera in order demonstrate the changing conditions occurring in the experimental series and the importance of the water movement.

c. Measurements of the size and weight of *B. tentaculata* to permit the oxygen consumption to be expressed in terms of g dry weight of body without shell.

7. STRESZCZENIE

1. Dla porównania metody butelek zamkniętych i metody przepływowej wykonano serie długotrwałych pomiarów zużycia tlenu przez 3 gatunki wodnych bezkręgowców. Obie metody były stosowane równolegle.

2. Jako organizmy testowe wybrano *Isoperla burei* (Plecoptera), *Cloeon dip-trum* (Ephemeroptera) i *Bithynia tentaculata* (Gastropoda). Gatunki te różnią się pozycją systematyczną, wymaganiami ekologicznymi i wielkością.

3. Wyniki pomiarów zużycia tlenu metodą przepływową nie zmieniają się w zależności od tego, jak długo zwierzę przebywało w respirometrze i są mało zmienne. Podniesiony metabolizm okresu adaptacji zwierzęcia do respirometru nie obciąża wyników pomiarów przeprowadzonych po ukończeniu adaptacji. Natomiast wyniki uzyskane metodą zamkniętych butelek zmieniają się w zależności od czasu ekspozycji butelek. Zależność zmierzonej wielkości zużycia tlenu od czasu ekspozycji określa wzór $Q = a \cdot t^b$. Wszystkie wyniki uzyskane metodą butelek zamkniętych są obciążone błędem wynikającym z podwyższonego metabolizmu okresu adaptacji. Uzyskane wyniki są wyższe, niż prawdziwe zużycie tlenu przez zwierzę po upływie tego samego czasu od początku doświadczenia. Ten błąd jest różny w zależności od tego, jak długo trwa ekspozycja. Rozrzut wyników jest duży.

4. Powyższe zależności wystąpiły we wszystkich seriach eksperymentów, niezależnie od badanego gatunku i od modyfikacji w sposobie przeprowadzania pomiaru.

5. Przeprowadzono pomiary dodatkowe:

a. Pomiary, mające na celu zwiększenie dokładności oznaczeń stężenia tlenu.

b. Pomiary częstotliwości ruchów oddechowych i występowania ruchów szukających larw Plecoptera dla zilustrowania odmiennych warunków występujących w seriach eksperymentów i znaczenia ruchu wody.

c. Pomiary wielkości i wagi *B. tentaculata* dla umożliwienia przeliczeń uzyskanych wyników zużycia tlenu.

8. REFERENCES

- AMBUHL, H. 1956. Die Bedeutung der Strömung als ökologischer Faktor. *Schweiz. Z. Hydrol.*, 21, 133—264.
- BERG, K. 1953. The problem of respiratory acclimatization. *Hydrobiologia*, 5, 331—350.
- BERG, K. 1961. On the oxygen consumption of some freshwater snails. *Verh. int. Ver. Limnol.*, 14, 1019—1022.
- DAM, L. van 1937. Über die Atembewegungen von Phryganea-Larven, *Arenicola marina* und *Nereis virens*, sowie über die Sauerstoffausnutzung bei *Anodonta cygnea*, *Arenicola marina* und *Nereis virens*. *Zool. Anz.*, 118, 122—128.
- ELLIOT, J. M. 1968. The daily activity patterns of mayfly nymphs (Ephemeroptera). *J. Zool. Lond.*, 155, 201—221.
- ERIKSEN, C. H., FELDMETH, C. R. 1967. A water-current respirometer. *Hydrobiologia*, 29, 496—504.
- FOX, H. M., SIDNEY, J. 1953. The influence of dissolved oxygen on the respiratory movements of caddis larvae. *J. exp. Biol.*, 30, 235—237.
- FOX, H. M., SIMMONDS, B. G. 1933. Metabolic rates of aquatic arthropods from different habitats. *J. exp. Biol.*, 10, 67—74.
- FOX, H. M., SIMMONDS, B. G., WASHBOURN, R. 1935. Metabolic rates of ephemerid nymphs from swiftly flowing and from still water. *J. exp. Biol.*, 12, 179—184.
- FOX, H. M., WINGFIELD, C. A., SIMMONDS, B. G. 1936. Oxygen consumption of mayfly nymphs in relation to available oxygen. *Nature, Lond.*, 138, 1015.
- FOX, H. M., WINGFIELD, C. A., SIMMONDS, B. G. 1937. The oxygen consumption of ephemerid nymphs from flowing and from still waters in relation to the concentration of oxygen in the water. *J. exp. Biol.*, 14, 210—218.
- HARNISH, O. 1938. Zur Analyse der Sauerstoffverbrauchs der Larven von *Cloeon dipterum* and *Ephemera vulgata*. *Z. vergl. Physiol.*, 26, 548—569.

- [КАМЛЮК, Л. В.] КАМЛЮК, Л. В. 1964. Применение полярографического метода при измерении скорости потребления кислорода водными животными [Application of polarographic method for the measurements of aquatic animals respiration]. *Naučn. Dokl. vysš. Školy, Biol. Nauki*, 3, 49—53 (Russian).
- КЛЕКОВСКИ, R. Z., КАМЛЕР, E. 1968. Flowing-water polarographic respirometer for aquatic animals. *Pol. Arch. Hydrobiol.*, 15, 121—144
- KNIGHT, A. W., GAUFIN, A. R. 1963. The effect of water flow, temperature, and oxygen concentration on the Plecoptera nymph, *Acroneuria pacifica* Banks. *Proc. Utah Acad. Sci.*, 40, 175—184.
- MANN, K. H. 1965. Energy transformations by a population of fish in the river Thames. *J. Anim. Ecol.*, 34, 253—275.
- NOLAN, M. O., BRAND, T. von 1954. The weight relations between shell and soft tissues during the growth of some fresh-water snails. *J. Wash. Acad. Sci.*, 44, 251—255.
- ПАМАТМАТ, М. М. 1965. A continuous-flow apparatus for measuring metabolism of benthic communities. *Limnol. Oceanogr.*, 10, 486—489.
- PATTEE, E. 1965. Sténothermie et eurythermie les invertébrés d'eau douce et la variation journalière de température. *Annls Limnol.*, 1, 281—434.
- PHILIPSON, G. N. 1954. The effect of water flow and oxygen concentration on six species of caddis-fly (Trichoptera) larvae. *Proc. zool. Soc., Lond.*, 124, 547—567.
- RAUSER, J. 1962. *Plecoptera bulgarica*. I. Sb. faun. Praci ent. Odd. nar. Mus. Praha, 8 (70), 67—81.
- REBSDORF, A. 1966. Evaluation of some modifications of the Winkler method for the determination of oxygen in natural waters. *Verh. int. Ver. Limnol.*, 16, 459—464.
- RICHMAN, S. 1967. Laboratory measurements of zooplankton respiration by the Winkler method. *IBP Meeting on Methods on assessment of secondary production in freshwaters*. Prague, 6pp. (Mimeographed copy).
- TEAL, J. M. 1967. Respiration and energy flow. *IBP Meeting on Methods on assessment of secondary production in freshwaters*. Prague. 8pp. (Mimeographed copy).
- [WINBERG, G. G.] ВИНБЕРГ, Г. Г. 1956. О зависимости интенсивности обмена членистоногих от величины тела [On dependence of metabolic rate on body dimension in arthropods]. *Uč. zap. Belorussk. gos. Univ.*, 26, 243—254 (Russian).
- [WINBERG, G. G., BELIAZKAYA, J. S.] ВИНБЕРГ, Г. Г., БЕЛЯЦКАЯ, Ю. С. 1957/1958. Зависимость интенсивности обмена от общих размеров тела у пресноводных брюхоногих моллюсков [Dependence of metabolic rate on total body dimensions in mollusca, Gastropoda]. *Vjul. Inst. Biol. Akad. Nauk Belorussk. SSR*, 3, 273—276 (Russian).
- [WINBERG, G. G., BELIAZKAYA, J. S.] ВИНБЕРГ, Г. Г., БЕЛЯЦКАЯ, Ю. С. 1959. Соотношение интенсивности обмена и веса тела у пресноводных брюхоногих моллюсков [Relation between metabolic rate and body weight in freshwater gastropods]. *Zool. Ž.*, 38, 1146—1151 (Engl. summ.).
- ZEISS, F. R. 1963. Effects of population densities on zooplankton respiration rates. *Limnol. Oceanogr.*, 8, 110—115.