Design and setup of intermittent-flow respirometry system for aquatic organisms

M. B. S. Svendsen*†, P. G. Bushnell‡ and J. F. Steffensen*

*Marine Biological Section, Department of Biology, University of Copenhagen, Strandpromaden 5, DK-3000 Helsingør, Denmark and ‡Department of Biological Sciences, Indiana University South Bend, South Bend, IN 46634, U.S.A.

Intermittent-flow respirometry is an experimental protocol for measuring oxygen consumption in aquatic organisms that utilizes the best features of closed (stop-flow) and flow-through respirometry while eliminating (or at least reducing) some of their inherent problems. By interspersing short periods of closed-chamber oxygen consumption measurements with regular flush periods, accurate oxygen uptake rate measurements can be made without the accumulation of waste products, particularly carbon dioxide, which may confound results. Automating the procedure with easily available hardware and software further reduces error by allowing many measurements to be made over long periods thereby minimizing animal stress due to acclimation issues. This paper describes some of the fundamental principles that need to be considered when designing and carrying out automated intermittent-flow respirometry (e.g. chamber size, flush rate, flush time, chamber mixing, measurement periods and temperature control). Finally, recent advances in oxygen probe technology and open source automation software will be discussed in the context of assembling relatively low cost and reliable measurement systems.

© 2015 The Fisheries Society of the British Isles

Key words: automated respirometry; metabolism; oxygen consumption.

INTRODUCTION

One hundred years after Ege & Krogh (1914) investigated the effect of temperature on gas exchange in goldfish Carassius auratus (L. 1758), the basic technique of measuring metabolism in aquatic organisms still remains an important tool for estimating energy expenditure in a variety of circumstances. For instance, standard metabolic rate (SMR), the minimum aerobic metabolic rate of a poikilothermic animal (at rest, post-absorptive and non-reproducing) at a specific temperature (Beamish, 1964; Beamish & Mookherjee, 1964; Chabot et al., 2016) is often a widely reported value in the literature of many fields (Ultsch et al., 1981; Ultsch & Duke, 1990). It is therefore important that it is measured in a reliable and repeatable fashion (Clark et al., 2013). While this paper will describe how to design an intermittent-flow respirometry system, issues relating to fish physiology or the use of metabolic rate measurements in theoretical modelling are beyond the scope of the topic. Instead, this review focuses on some of the underlying assumptions, technical pitfalls and limitations that affect the design and function of an aquatic intermittent-flow respirometer.
As has been outlined by a variety of authors, each type of respirometry technique has advantages and disadvantages (Keys, 1930; Niimi, 1978; Steffensen, 1989). The three major techniques are summarized below.

Closed-system respirometry involves placing an animal in a closed (sealed) metabolic chamber and measuring the decline in oxygen concentration in the chamber over time. While closed-system respirometers are relatively simple to construct and operate, it is inevitable for the animal to experience progressive oxygen depletion (hypoxia), and a simultaneous increase in carbon dioxide (progressive hypercapnia) and nitrogenous waste (ammonia and nitrite) levels in the chamber, especially if the animal is used to generate hypoxic conditions (Keys, 1930; Steffensen, 1989; Routley et al., 2002; Nilsson & Östlund-Nilsson, 2004). Under these circumstances (progressive hypoxia and hypercapnia), the experimental animal does not experience steady-state conditions. For instance, if the experimenter wants to test the effect of hypoxia by letting the partial pressure of oxygen \( (pO_2) \) decrease by 15–16 kPa in the chamber, carbon dioxide, which has a 25–30 times higher solubility than oxygen, will increase by 0.5–0.6 kPa, a level that is more than even the worst case global change scenario (Stocker et al., 2013). Further, if the closed-system measurement period has resulted in prolonged hypoxia and the fish has had to rely on anaerobic metabolism, this will raise aerobic metabolism during the next flush and measurement cycle as a result of excess post-hypoxia oxygen consumption (EPOC) arising from the anaerobic metabolism (Scarabello et al., 1992; Svendsen et al., 2011; Plambech et al., 2013).

Finally, any of the factors listed can stimulate spontaneous activity, or physiological responses, resulting in oxygen consumption \( (\dot{M}_{\text{O}_2}) \) measurements that do not reflect the animal’s oxygen consumption at SMR (Keys, 1930; Forstner, 1983; Gnaiger, 1983; Steffensen, 1989).

Flow-through respirometry measures oxygen consumption by quantifying the difference between inlet and outlet oxygen concentration and adjusting the flow of water through the respirometer to maintain a certain oxygen content difference. Oxygen consumption is then calculated as the product of water flow through the respirometer per unit time and the difference in oxygen concentration of the water entering and exiting the respirometer.

Although the constant inflow of clean water reduces or eliminates the hypoxia, hypercapnia and nitrogenous waste issues associated with closed-system respirometry, it introduces mixing and equilibration (washout) problems. While washout errors can theoretically be corrected (Niimi, 1978), large errors can occur if there is a small drift or error in oxygen readings or changes in the water flow through the respirometer (Steffensen, 1989; Eriksen, 2002). The former issue can be even more pronounced if both input and output water streams are measured with different oxygen sensors as each will potentially have its own drift and error. Utilizing a valve system that allows the same probe to sample both water streams will reduce this sensor-based error.

Intermittent-flow respirometry combines elements of both methods and generally reduces problems associated with either system. In brief, intermittent-flow respirometry combines short measurement periods in a recirculating, but closed, respirometer, punctuated by clean water flush periods which are long enough to ensure that the water in the respirometer has been thoroughly exchanged to eliminate potential hypoxia, hypercapnia and nitrogenous waste buildup in the chamber (Forstner, 1983; Steffensen, 1989).
One period (loop)

Flush → Wait → Measure

Chamber closed

Fig. 1. During intermittent-flow respirometry, one complete measurement cycle (loop) consists of three timing periods: the flush period where the chamber is open (i.e. water is flowing through it) followed by two closed periods, wait and measure, when the flush pump is off. A short wait period is needed before the measurement period to allow all the water in the chamber to mix and the oxygen content to begin declining in a linear fashion. Data from measurement period are used for determining the oxygen consumption of the organism.

While this method is the best approach currently available for measuring oxygen consumption, it does require more equipment and a slightly more complex experimental setup than closed respirometry. The remainder of this paper will therefore focus on some of the factors that should be considered when using intermittent-flow respirometry.

CALCULATING OXYGEN CONSUMPTION ($\dot{M}O_2$) USING INTERMITTENT-FLOW RESPIROMETRY

Intermittent-flow respirometry is a series of short-term, closed respirometry experiments, interrupted by flushing intervals to replace the respirometer water. One complete measurement cycle (loop) will therefore consist of an open-system flush period and a closed-system, metabolism determination cycle, which itself is made up of a wait period and a measurement period (Fig. 1; see list of abbreviations in Appendix):

$$t_{\text{period}} = t_f + t_w + t_m \quad (1)$$

Changes in oxygen content in the chamber during a complete measurement cycle are illustrated in Fig. 2. When there is no organism present in the respirometer, the air saturation of the water should approach 100% [Fig. 2(a)]. Once an animal is introduced into the chamber, the oxygen level in the chamber will drop to a new, lower equilibrium level, even though the flush pump is still on [Fig. 2(b)]. The magnitude of the decline will be dependent on the size of the chamber, the flush rate and the organism’s metabolic rate. When carrying out an experiment in normoxia, it is recommended that the chamber water air saturation is above 90% at the end of the flushing period, and that it does not decline to <80% saturation at the end of the measuring period lest the animal become hypoxic and stressed. Once the flush pump is turned off, oxygen content in the respirometer will begin to fall in a linear fashion [Fig. 2(c)] until the flush pump is reactivated, at which point the oxygen level in the chamber should rise in an exponential manner [Fig. 2(d)] eventually reaching the pre-measurement period levels [Fig. 2(b)]. When the flush pump is off during the measurement cycle [Fig. 2(c)], the metabolic chamber is functionally closed, so the data points collected during the linear
Fig. 2. Ideally, an intermittent-flow respirometry experiment should consist of a series of linear declines in oxygen content during the measurement period interrupted by exponential increases during the flush period. Before an organism is placed in the chamber, oxygen levels should be near air saturation (a) and will decline slightly and reach a new equilibrium after the animal is introduced (b). Once the flush pump is turned off, a linear decline in oxygen levels should occur (c), the slope of which is used to calculate oxygen consumption. Turning the flush pump back on should cause oxygen levels in the chamber to increase in an exponential fashion (d).

A linear decline can be used to calculate the oxygen consumption rate using the same formula applied to closed-system respirometry (Steffensen, 1989) specifically:

$$\dot{y} = V_{RE} W_o^{-1} \frac{dCO_2}{dt}$$

where \(\dot{y}\) is \(\dot{MO}_2\), \(V_{RE}\) is the effective respirometer volume (l) of water in the respirometer (calculated as total respirometer volume minus the organism volume), \(W_o\) is the mass of the organism and \(\frac{dCO_2}{dt}\) is the slope of the linear decrease in oxygen content during the time the chamber is closed [Fig. 2(c)]. Once the flush pump is turned off, there is a small period of time during which the flush water and chamber water mix completely and a new equilibrium is established. The decline in chamber oxygen during this period is often initially non-linear, due to mixing in the respirometer and due to the response time of the oxygen sensor, so a wait period (\(t_w\); equation 1) should be introduced into the measurement cycle before data for the oxygen consumption measurement is collected.

When the organism is placed into the respirometer, a volume of water equivalent to the organism’s volume will be displaced so the respirometer volume used in calculating oxygen consumption must be reduced accordingly. Because measuring the actual volume of the organism is often difficult and stressful for the organism (and experimenter), a reasonable approximation of \(V_{RE}\) is calculated using the formula:

$$V_{RE} = V_{RT} - W_o \rho_o^{-1}$$

where $V_{RT}$ is the total volume (l) of the empty respirometer including the recirculation loop and $\rho_o$ is the density of the organism. If it is assumed that the animal is neutrally buoyant, then $\rho_o$ is equal to the density of the water which can range between 1.000 kg l$^{-1}$ in fresh water and 1.025 kg l$^{-1}$ in full strength seawater as density is dependent on both salinity and temperature (Fofonoff, 1985). For the sake of expediency, especially with fishes, it is a common practice to simply subtract the mass of the fish in kg from the volume of the respirometer in l to arrive at a reasonable measure of $V_{RE}$ as most fish species can be assumed to be neutrally buoyant.

Finally, depending on the type of oxygen monitoring equipment in use, the output of the instrument may measure the change in $pO_2$ rather than oxygen content or concentration. In this case, the value will have to be converted to concentration units, thereby altering equation (2) to be:

$$y = \beta O_2 V_{RE} W_o^{-1} \frac{\delta pO_2}{\delta t}$$

(4)

where $\beta O_2$ is the oxygen solubility at the given temperature, salinity and atmospheric pressure, and $\delta pO_2$ is the change in partial pressure of oxygen in the chamber. Tables for determining $\beta O_2$ are available from a variety of sources both in print (Green & Carritt, 1967; Benson & Krause, 1984; Garcia & Gordon, 1992) and online (water.usgs.gov/software/DOTABLES/) and at the Aquaresp homepage (www.aquaresp.com/oxygen).

**CALCULATION ASSUMPTIONS**

Inherent in the application of equations (2–4) are the following assumptions: (1) complete water mixing occurs in the respirometer (equations 2 and 4); (2) oxygen solubility is constant (equations 2 and 4) (temperature, atmospheric pressure and salinity are constant); (3) mass of organism is constant (equations 2 and 4); (4) volume of the respirometer is constant (equations 2–4) (density and mass of organism are constant).

Water mixing in the respirometer is a common problem and is considered separately. While it is rare that any of the values from atmospheric pressure through to mass of the organism will change during a single experimental cycle, it is not unusual for small changes to occur in these values over the course of a 2–3 day experiment. Clearly then, the figures used in calculating oxygen consumption would need to be adjusted to reflect this, in order to measure metabolic rate accurately. For practical reasons and if not obviously wrong, some factors must be assumed to be constant, *i.e.* density of the organism, salinity of the water and volume of the respirometer. If for some reason it is suspected that the organism is losing a significant amount of mass during the experiment, then it should be taken into account, and as effective respirometer water volume ($V_{RE}$) depends on fish mass as well, the volume of the respirometer should also be corrected (E. F. Christensen, M. B. S. Svendsen & J. F. Steffensen, unpubl. data).

In instruments that give a choice in how the oxygen measurement is recorded (*e.g.* content, % saturation or other $pO_2$), it must be kept in mind the relationship between oxygen content in the water and partial pressure of oxygen in the air, which is summarized by Henry’s law and can be described as (Hastala & Berger, 1996): $CO_2 = \beta O_2 pO_2$. 

Changes in atmospheric pressure over the course of the experiment will alter $pO_2$ in the air above the water, while temperature and salinity changes will alter $\beta O_2$ (e.g., increasing temperature and salinity will decrease oxygen solubility ($\beta O_2$)). These are all issues that affect the oxygen solubility used in equation (4); temperature and salinity will probably have an effect on the metabolism of the organism as well. Logging the data in kPa, mmHg or % saturation units will reflect the changes in atmospheric pressure and allow the experimenter to apply their own solubility coefficient in equation (4) and correct for changes in temperature or salinity.

**LINEAR REGRESSION**

Assuming that oxygen consumption is constant during the measurement period, a linear regression relating the change in oxygen content over time can be used to calculate the rate of oxygen decline (slope) during the measurement period: $CO_2(t) = \frac{\delta CO_2}{\delta t} t + b$. As this formula describes a line in the form of $Y = mX + b$, $Y (CO_2)$ would be the oxygen content in the respirometer at time $(t)$, $m (\frac{\delta CO_2}{\delta t})$ is the slope, $X (t)$ is time and $b$ is where the fitted regression line intersects the y-axis at time 0 (i.e. the starting level of the $CO_2$ at the end of the wait period). Since the time $(t)$ and $CO_2$ depend on the units of time (e.g. s, min or h), oxygen content (e.g. mg O₂ or ml O₂) and partial pressure (e.g. mmHg or kPa) used, the slope and thus the oxygen consumption will have that same unit. Methods for fitting a linear regression to the decline in oxygen are typically included in freely available software packages such as R (www.r-project.org) and Python (www.python.org) or commercial software such as Microsoft Excel (www.microsoftstore.com). In addition to the linear regression output most software packages provide a term describing the goodness of fit, usually $r^2$ for a regression coefficient between 0 and 1. While a discussion of the statistical meaning of $r^2$ is beyond this paper, it is an important measure for evaluating the resulting slope as it reflects how well the data fits the linear regression. In the case of respirometry, the $r^2$ statistic is important in determining whether the chamber $CO_2$ (or $pO_2$) fell in a linear fashion over the measurement period. An $r^2$ of 1 is the ideal as it indicates that all the data points fit on a straight line and there is no deviation from the predicted drop of oxygen. If the $r^2$ is below 0.9 (or 0.95), it means that >10% (or 5%) of the variance in measured $CO_2$ (or $pO_2$) cannot be explained by the linear model. In general, an $r^2 < 1$ can be attributed to four general causes. (1) A leak that allows new oxygen into the chamber. In this case, the $r^2$ will be reduced as the change in oxygen content in the chamber will not be linear, but more likely exponential in nature (dotted line; Fig. 3) as it is essentially a small oxygen washin (Steffensen, 1989). Interestingly, with this type of error, increasing the measurement period will further decrease the $r^2$ as the oxygen decline trace will be exponentially levelling off to a new equilibrium [Fig. 2(b)]. This has to be solved by finding the leak and removing it. (2) Biologically produced alterations in metabolism during the measurement period. This can occur when the organism’s activity level changes during the metabolism measurement period and is usually due to movement or restlessness in a static respirometer during SMR experiments or inconsistent swimming speed during active metabolism measurements. Reducing the measurement period will sometimes help in this regard as one gets a more homogeneous activity level using a shorter time period. Other steps that can be taken include setting up the experimental system in a quiet room or area, shielding the chamber from visual and vibratory disturbance and allowing the fish sufficient
acclimation time in the respirometer. (3) Measurement noise due to an unstable oxygen probe or electrical interference in the measurement and recording system. As this will result in low signal:noise ratio, tracking down the source of the noise and eliminating it is important. The $r^2$ can also be improved by either decreasing the respirometer to fish-volume ratio or by increasing the measurement period. Applying a moving average to the measured oxygen values will also mitigate this error to some degree. Care must be taken, however, of using a very long running average (e.g. 20 s) as it will prolong the beginning of the recorded linear decline in oxygen at the conclusion of the flush. In this case, the wait time will have to be lengthened to ensure the linear portion of the oxygen decline is truly being recorded. (4) Incomplete mixing of the water in the chamber during the measurement period. Pockets of water that are heterogeneous in their oxygen content (low near the organism and high in other regions) will generate a highly variable decline in oxygen content over the course of the measurement which can dramatically reduce $r^2$. Keeping the water well mixed in the chamber during the measurement period when the flush pump is off will help reduce this error (Rodgers et al., 2016).

Effects of noise and chosen way of analysing data can be seen in Fig. 4. This figure depicts the simulated differences in how data are chosen for the determination of $\dot{M}O_2$. Clearly, the automatic calculation of slopes minimizes human error and ensures that all slopes are calculated the same way, with the same data from the measurement period. This is a clear advantage of using an automated algorithm for choosing and calculating $\dot{M}O_2$. Secondly, it demonstrates the necessity of using a linear regression with many points (Fig. 4). Although automation of experiments is encouraged as a means of minimizing human error, the experimenter must approach automation with a certain amount of caution, thus checking that the slopes are not systematically flawed (e.g. by an oxygen probe’s automatic temperature compensation or similar) and that the correct values are used in the software when calculating oxygen consumption. Automation will lead to a poor ability to understand the experimental details and data, if not approached correctly.
Fig. 4. This figure shows simulated respirometry data. (a) One total loop with flush (F), wait (W) and measurement (M) periods (F: 240 s, W: 60 s and M: 300 s), cf. equation (1), at three different levels of normally distributed random noise [(s.d. = 0), (s.d. = 0.01), (s.d. = 0.02)]. (b) Per cent \( \dot{M} \) deviation from true oxygen consumption (\( \dot{M}O_2 \)) determination at the three different levels of noise, using five different methods for determining the oxygen consumption. The points (+) are outliers outside the 95% c.i. and 75% points (□) represent the 95% c.i. and ■ represents median values. The experiment was simulated to run for 48 h with an \( \dot{M}O_2 \) of 60 mg O2 kg\(^{-1}\) h\(^{-1}\) with fish mass of 0.15 kg and respirometer volume of 2 l with an oxygen solubility of 10 mg l\(^{-1}\). LR Fixed uses the same 300 s measurement period for a linear regression [s.d. \( \dot{M}O_2 \) (%): 0.0, 0.28, 0.52; average \( r^2 \): 1.0, 0.997, 0.992]. LR Var uses 300 s for a linear regression, but not fixed, i.e. simulating post-analysis where the linear part is chosen manually, this comes with the risk of getting points in the wait period, thus some data points will be lower [s.d. \( \dot{M}O_2 \) (%): 0.26, 0.65, 0.74; average \( r^2 \) (box 1, box 2, box 3): 0.997, 0.995, 0.988]. LR short uses 150 s for the linear regression also simulated to be manually chosen [s.d. \( \dot{M}O_2 \) (%): 2.29, 2.45, 2.63; average \( r^2 \): 0.978, 0.970, 0.947]. SE (start-end) represents only choosing two points to get the change in oxygen partial pressure (\( pO_2 \)). The points are chosen in the linear part of the curve, with 300 s apart [s.d. \( \dot{M}O_2 \) (%): 0.0, 1.96, 3.74; average \( r^2 \) : not applicable]. SE short simulates the same, but only 150 s between the points. [s.d. \( \dot{M}O_2 \) (%): 0.0, 3.53, 7.39; average \( r^2 \) : not applicable].

**BACKGROUND RESPIRATION**

Background respiration occurs when oxygen is consumed by sources other than the organism in the chamber. In most cases, bacteria in the water or on the inner surfaces of the respirometer and recirculation loop tubes account for the extraneous oxygen usage. While bacteria suspended in the water column can be minimized by recirculating the water through a UV sterilizer, and using a clean water source instead of recirculated water, it is impossible to prevent the buildup of bacteria on the inner surfaces of the
Fig. 5. Measurement time can have an effect on both the accuracy illustrated with correlation coefficient ($r^2$; ■, ●) and magnitude of bacterial respiration, $MO_{2\text{B}}$ (□, ○), measurement. When background respiration is low (■), measurements periods in excess of an hour may be necessary to achieve an $r^2$ (□) above 0.95 (∙ ∙ ∙ ∙ ∙ ∙ ∙ ∙). With higher background metabolic rates (○), reasonable $r^2$ (●) may be achieved in less time (c. 10 min). Data shown are actual measurements, the 3600 s measurements for the low bacterial load was omitted as the setup had to be taken down to free the space for other researchers in the laboratory. The bacterial cultures are unknown; however, the water is tap water from the Elsinore Community, Denmark. Measurements were completed at 25°C after teaching in a fish biology course, before the measurements of background respiration, either Rutilus rutilus or Carassius auratus had been in the respirometer (both species of fish were held in the same holding tank). The high bacterial load was achieved by letting the bacteria grow on the respirometer material for 3 days. The low level was completed shortly after experimentation.

chamber, pumps and tubing over time. It is, therefore, important to quantify the bacterial contribution to the measured oxygen consumption and subtract it from the $MO_2$ measurement (Via, 1983). While this is a problem common to all experimental systems, there does not appear to be a universally accepted technique for how to measure and mathematically correct for it. It is often stated in papers, for instance, that $MO_2$ been corrected for background respiration, yet details of the procedure are often scant (Plambech et al., 2013) or entirely missing (Scarabello et al., 1992; Schurmann & Steffensen, 1994; Steffensen et al., 1994; Casselman et al., 2012). Even in cases where techniques are mentioned, they appear to vary widely. For instance, Boldsen et al. (2013) measured background respiration during 1 h determination period, while Norin & Malte (2011, 2012) measured it only over the same short period (90–120 s) used for the animal SMR determinations leading them to conclude that background was negligible during the short time interval.

When determining background respiration $r^2$ is important to consider, as it reflects a quality control for the measurements. As illustrated in Fig. 5, extending the measurement time increases both $r^2$ and the oxygen consumption in an asymptotic manner. This implies that determining the background respiration using a very short measurement interval will lead to an underestimation of the bacterial oxygen consumption. As can be seen in Fig. 5, the longer the measurement interval, the further the $CO_2$ drops, and better the signal:noise ($\Delta CO_2$ : noise) ratio becomes, thereby increasing the accuracy of
the measurement (higher $r^2$). Clearly then, when measuring small background oxygen consumptions, a long measurement period is needed to produce a significant decline in oxygen content and generate a precise measurement (as evaluated by $r^2$). Generally, there is not much change in the magnitude of the background respiration measurement when $r^2 > 0.95$, but the closer to 1.0 it can get, the better will be the magnitude of the background respiration. It is not uncommon to expect that measurement periods exceeding hours may be necessary to accurately determine background oxygen consumption when it is very small. In general, it is recommended to put the time and effort into actually quantifying the magnitude of the background metabolism over a series of long measurement periods rather than dismissing it as negligible based on short readings equivalent to that used for $MO_2$ measurements on the animal.

CORRECTING FOR BACKGROUND RESPIRATION

Correcting for background respiration, in general, should be done by converting the two sets of measured oxygen consumptions to non-mass specific measures (e.g. mg O$_2$ h$^{-1}$) which can then be subtracted without any unit bias.

$$RO_{2_{\text{corr}}} = RO_{2_{\text{Animal}}} - RO_{2_{\text{Background}}}$$ (5)

where $RO_{2_{\text{corr}}}$ is the corrected oxygen consumption rate of the animal, $RO_{2_{\text{Animal}}}$ is the whole animal oxygen consumption rate (mg O$_2$ s$^{-1}$) and $RO_{2_{\text{Background}}}$ is the measured background oxygen consumption (mg O$_2$ s$^{-1}$). Because quantifying background respiration is generally achieved by measuring the decline in oxygen content in an empty chamber, equation (2) must be slightly modified to:

$$RO_{2_{\text{Background}}} = V_{RT} \frac{\delta CO_2}{\delta t}$$ (6)

Note that the oxygen consumption calculation resulting from equations (5 and 6) is not mass specific (mg O$_2$ s$^{-1}$) and the volume used in the calculation is the total respirometer volume ($V_{RT}$) as the volume of the animal is missing from equation (4). Because the background respiration as calculated in equation (6) is not mass specific (e.g. mg O$_2$ s$^{-1}$), it is difficult to subtract it from $MO_2$ as calculated by equation (2 or 4) as they are mass-specific $MO_2$ (e.g. mg O$_2$ kg$^{-1}$ h$^{-1}$). In order to obviate this problem, one of the following equations should be used for subtracting background respiration:

$$\dot{MO}_{2_{\text{corr}}} = \dot{MO}_2 - \dot{MO}_{2_{B}} V_{RT} V_{RE}^{-1}$$ (7)

where $\dot{MO}_{2_{B}}$ is a mass-specific background respiration (mg O$_2$ kg$^{-1}$ h$^{-1}$) calculated as if the animal was in the respirometer (equations 2 and 4).

A correction for background respiration can also be made by using equation (8), which uses the measured slopes with an animal ($S_a$) and without ($S_b$) along with the effective respirometer volume $V_{RE}$ and the total respirometer volume $V_{RT}$.

$$\dot{MO}_{2_{\text{corr}}} = \beta O_2 (S_a V_{RE} - S_b V_{RT}) W_o^{-1}$$ (8)
Background metabolism in a respirometry system can range from insignificant (<1% of total measured metabolism) to >20% and will depend on many factors including, but not limited to, volume of the chamber, duration of the experiment, effectiveness of water filtration and sterilization, water temperature and initial bacterial load. If it is assumed that $\dot{M}O_2B$ is constant throughout the experiment, the simplest correction will be to subtract a single measured $\dot{M}O_2B$ from the measured $\dot{M}O_2$ using equation (7). It is likely, however, that background metabolism will increase over the course of the experiment. In this case, it may be useful to quantify $\dot{M}O_2B$ before the organism is introduced to the chamber (hopefully it will be negligible) and again at the conclusion of the experiment. If one assumes, perhaps incorrectly, that background metabolism will increase in a linear fashion as the experiment progresses, the beginning and end point background measurements can be used to calculate the rate at which background metabolism increased over time and subtract an appropriately weighted correction to the $\dot{M}O_2$ measurement. An optimal solution would be to measure background respiration throughout the experimental period, along with the animal measurements. This might be accomplished by setting up an empty chamber in parallel to the measuring chamber (having the measurement period issues in mind) (Rodgers et al., 2016). By using an empty chamber in the same water bath will probably not give representative results as the animal inside the chamber will excrete nutrients, faeces and bacteria of its own, thus making the two systems non-identical. Further investigation of this is, however, needed. So, depending on water quality and the available sterilizing equipment, it is suggested to perform some initial tests before commencing experiments to assess the magnitude of background metabolism (Rodgers et al., 2016). The experimenter can then evaluate whether sterilization of equipment (to keep background low, but growing) is needed between each trial, or if a reasonably low background respiration can be expected without sterilization (and keeping the background low-medium but constant).

Complicating issues further, it has been shown that the bacteria can act as oxygen regulators to a certain point and then become oxygen conformers as ambient oxygen levels decline below a critical level (Tang, 1933). As the break point of the bacteria depends on the particular species (Shoup, 1929; Devol, 1978), it may have a confounding effect if experiments are performed seeking to determine the critical oxygen level of an organism, $P_{\text{crit}}$ (Tang, 1933). That being said, there is at present no way of correcting for this, it could be a subject for future investigation.

**RESPIROMETER DESIGN**

**RESPIROMETER SIZE**

Perhaps one of the most important considerations in designing a respirometer is its size, both in terms of shape and volume. The optimal size with respect to the volume of the respirometer spans both behavioural and technical issues. From a behavioural point of view, the respirometer must be big enough to accommodate the animal comfortably, especially if measuring SMR. Making the chamber too large, however, facilitates spontaneous movements that will have a negative effect on SMR measurements. The shape of the respirometer should also be considered when measuring metabolism in animals with odd shapes such as flatfishes.
When designing an intermittent-flow respirometry system, Forstner (1983) proposed the following equation for determining the appropriate volume of the respirometry chamber:

$$V_{RT} = \omega t_m RO_2 CO_{2}^{-1}$$  \hspace{1cm} (9)

where $\omega$ is the anticipated drop in the oxygen content in the chamber (as a percentage) during the measurement period, $t_m$, and $RO_2$ is the oxygen consumption (mg O$_2$ s$^{-1}$). This assumes, of course, that the experimenter already has an approximate idea of what the metabolic rate of the organism will be. If the experimenter anticipates using the respirometer at different temperatures, this formula can be expanded by adding a $Q_{10}$ component (Reyes et al., 2008) to incorporate the temperature-driven metabolic effect on the necessary chamber volume.

$$V_{RT} = \omega t_m RO_2 CO_{2}^{-1} Q_{10}^{(T_i - T_0) / 10}$$  \hspace{1cm} (10)

Given an estimated range of oxygen consumptions, planning the respirometer volume often comes down to trying to achieve the best compromise between having a large enough drop in oxygen level during the measurement period to achieve a satisfactory $r^2$ (high $\omega$), but doing it over a relatively short amount of time (small $t_m$). While there are no hard and fast rules that can be applied in this regard, it has been the authors’ experience that a respirometer:organism volume ($r_{RO}$) between 20 and 50 appears to be comfortable for most organisms but is small enough to result in a 10% drop in oxygen content or $pO_2$ (Forstner, 1983) within a reasonable amount of time (3–6 min), depending on temperature. The 10% drop in oxygen should not be regarded as a standard, as determinations with a high $r^2$ (>0.95–0.98) with a smaller decline in oxygen levels would suffice. On the other hand, if there are drops of 10% or more, all having low $r^2$ (<0.95), it should be a cause for concern.

The volume of the respirometry system is not only determined by the chamber size, but also includes the volume of the recirculating loop, if one is present. Therefore, external tubing connecting pumps to the respirometer should be as short as possible, not only to minimize volume, but additionally to limit the available surface area for bacterial films which will contribute to background respiration.

When designing a swimming respirometry system, a larger $r_{RO}$ has to be anticipated as the various ancillary portions of the respirometer (turning section, propeller housing, flow straightener and return channel) add significant amounts of volume beyond the swim section. Wherever possible, therefore, the size of the non-swim sections of the respirometer should be kept as small as possible, in order to keep overall volume of the respirometer low. While the higher volume will be necessary when the oxygen consumption increases at higher swimming speed, $\dot{M}O_2$ measurements in resting or slowly swimming fishes will require longer measurement times and will be less precise than results measured in a static system. A swim tunnel with an $r_{RO}$ in the range of 50–150 is a good compromise.

**RECIRCULATION AND MIXING**

Mixing or stirring the water in the unflushed chamber is an important design consideration when building a respirometer. In a swimming respirometer, the water is
Fig. 6. When a recirculating loop is needed, the outflow of the flush pump and recirculating loop can be plumbed in (a) a non-countering manner where the two are in the same direction, or (b) countering where the flows oppose each other. Note that the overflow is above the water surface in order to create an airlock and to prevent transport of water through the tubing when the flush pump is off. Images of dye diffusion are seen in the green channel from a camera, shortly (c. 0.8 s) after a bolus injection of fluorescent dye (injection was in the flush-pump tubing). The colour intensity was monitored in three fixed locations in a respirometer plumbed as a (c) non-countering setup or (d) countering setup. The flush pump is mounted on the left side of each image. Shunting is apparent in the countering setup as the dye enters from both ends of the respirometer. The images are contrast and colour enhanced to show the fluorescent better in print. ❯, edge of the respirometer; □, approximate outline of the baffles.

sufficiently mixed by the propeller, even at low speeds. In a static respirometer, however, mixing is a more critical issue and can be achieved in a variety of ways, which to some degree, depend on the volume of the chamber. In large-volume systems, mixing can be accomplished by mounting a small pump in the respirometer chamber itself or, alternatively, mounting the pump in an external recirculation loop added in parallel to the respirometry chamber as illustrated in Fig. 6. As pointed out above, however, the volume of water in the recirculating loop, while physically separated from the animal, is still part of the total respirometer water volume and must be included when calculating $\dot{M}O_2$. A recirculating loop attached to a small respirometer may be impractical as the volume of the loop may be larger than the volume of the respirometer itself. In this case, mixing may have to be accomplished with a mixing device inside the...
respirometer, such as a magnetic stir bar which is isolated from the animal by a mesh screen or an internal pump (Clark et al., 2013).

The recirculation pump can be kept on or turned off during the flush part of the cycle. If it is turned off, the recirculation loop becomes a functional dead space. If there is significant background respiration, water in this space can become hypoxic and rapidly decrease dissolved oxygen (wash-in) when the recirculation pump starts again. As long as there is not a significant change in the flow characteristics in the chamber that might force the animal to alter its behaviour when the flush period begins (because two pumps are on), it is suggested to leave the recirculation pump on continually.

WASH-IN, WASHOUT AND FLUSHING

Using intermittent-flow respirometry, flushing the chamber is used to replenish its oxygen content and prevent accumulation of waste products such as carbon dioxide and ammonia. The rate at which oxygen levels increase (wash-in) or waste products decrease (washout) in the metabolic chamber during the flush period is also an important consideration when designing the respirometer (volume and flush pump size) and carrying out experiments (flushing frequency and duration). The time course of air-saturated water wash-in during the flushing cycle is described by:

\[ Y_{\text{in}} = 1 - e^{-Dt} \]  

where \( Y_{\text{in}} \) is the fraction of the normoxic level reached, \( t \) is time and \( D \) is the dilution ratio calculated using the flush-pump flow rate \( (F_{F}) \) and respirometer volume \( (V_{RT}) \), \( D = \frac{F_{F}V_{RT}}{V_{RT} - 1} \). Similarly, the washout of chamber water containing higher levels of CO₂ and nitrogenous waste (Steffensen, 1989) can be characterized by:

\[ Y_{\text{out}} = e^{-Dt} \]  

where \( Y_{\text{out}} \) is the fraction left in the chamber. As can be seen, the only difference between equations (11 and 12) is that wash-in (equation 11) rises exponentially to 1, whereas washout (equation 12) declines exponentially to 0.

FLUSH PUMP FLOW DIRECTION

In most static respirometers, the recirculation of water in the chamber is achieved with an external loop driven by a pump. As the water inlets and outlets for the recirculating loop and the inlet for flushing are typically plumbed into the ends of the chambers, a decision has to be made whether to plumb the two water outlets in the same end of the chamber [Fig. 6(a)] to produce parallel streams (non-countering) or in opposite ends [Fig. 6(c)] where they oppose each other (countering).

In order to assess the relative merits of both schemes, a series of experiments were performed where a fluorescent dye was injected into the respirometer and the washout of the dye followed over time [Figs 6(b), (d) and 7]. As the volume of the respirometer and flushing times were the same in both instances, the total washout amounts, \( Y_{\text{out}} \), are identical. Therefore, comparing the effective pumping volumes can
Fig. 7. Fluorescent dye was used to visualize mixing and washout in a respirometer when the recirculating loop was plumbed in the same direction (non-countering) as the flush pump flow (>>) or plumbed in opposite (countering) direction to the flush pump (<<) (cf. Fig. 6). Regardless of the flush rate, a non-countering system results in much more effective flushing. L, M and R, left, middle and right part of the respirometer, respectively. The y-axis is normalized fluorescent intensity, normalized to maximum intensity in each of the regions of interest (L, M and R).

be done by solving for $F_F \left( D = F_F V_{RT}^{-1} \right)$ in equation (11) and finding the time ($t$) to 50% washout. The difference in flushing rate between the two different setups is then equal to $\Delta \% = (t_1 - t_2) t_1^{-1} 100 \%$, where $\Delta \%$ is the difference in per cent and $t_1$ and $t_2$ are the 50% washout times from the non-countering and the countering setups, respectively (Fig. 7).

The experimental trials revealed a reduction in effective flushing by 62% when assembling the recirculating loop in a non-countering manner (1.561 min$^{-1}$) when compared with countering directions (0.601 min$^{-1}$). The drop in effective pumping rate occurred because flush water was being picked up by the inflow of the recirculation loop, shunted around the chamber through the recirculating loop and directed out of the chamber at the exit port. This can be seen visually in Fig. 6(d) where the dye can be clearly seen at both ends of the chamber but not in the middle. In Fig. 7, the last curves to equilibrate are the left and middle of the respirometer of the countering setup (<<L and <<M), indicating a dead space with regards to mixing and flushing that has been created. The dead space in the middle is significant, of course, as this is where the organism would normally be found.

As the calculated error rate between the two setup types is dependent on the physical construction of the respirometer (e.g. presence or absence of internal baffles, placement of the inflow, outflow, recirculation loop ports and pump size), the error rate can be better or worse than the example. To be safe, however, it is recommended to assemble the respirometer in the non-countering manner to avoid any reduction in effective flushing.
DUTY CYCLE OF PUMPS

As stated earlier, a complete measurement cycle consists of three periods: flush time, wait time and measurement time (Steffensen et al., 1984). As each period serves a different purpose, some discussion of their relevance is merited.

Flush time

The length of the flush interval depends on how much waste-product washout and oxygen replenishment the experimenter seeks to achieve. Although a 100% washout of waste products is ideal, it is often impractical as it will take too long. An appropriate flush time can be calculated by \( t_f = \frac{\ln(\alpha^{-1}) V_{RE} F_F^{-1}}{\alpha} \) [rewritten from Steffensen (1989)], where \( \alpha \) is the planned residual water volume in the respirometer chamber expressed as a decimal percentage (i.e. for an exchange of 96% of the water, \( \alpha = 0.04 \)). As can be seen in Fig. 8, the respirometer needs to be flushed with \( c \) five times its volume in order to achieve a washout above 99%.

Wait time

Once the flush pump is turned off and the chamber is effectively sealed, a wait period should be inserted in the cycle to allow thorough mixing of the all the water in the chamber before data are collected for the actual oxygen consumption measurement (Steffensen et al., 1984). The wait period should be long enough to ensure that a linear decline in oxygen content (or \( pO_2 \)) has begun before collecting data points to be used in the \( \dot{M}O_2 \) calculation. While the exact wait time will be dependent on the experimental setup, an initial starting value of 10% of the measurement time has been suggested.
Fig. 9. The approximate time needed to achieve a 5% drop in oxygen content in the respirometer for metabolic rate determinations depends on the size of the animal relative to respirometer (respirometer:organism ratio) and its metabolic rate. This family of curves was constructed based on a metabolic rate approximation adapted from Gillooly et al. (2001) in a 300 g fish at five different temperatures. The smaller the respirometer to organism ratio and the higher its metabolic rate (due to, for example, temperature) the less time the flush pump needs to be off to achieve a reasonable decline in oxygen content in the respirometer. On the x-axis (logarithmic), data start at a respirometer to fish ratio of 20.

(Forstner, 1983). As the exact time for the wait period is independent of the measurement time, the experimenter should determine this on an individual basis for each specific setup (i.e. see how long it takes for the oxygen trace to become linear).

**Measurement time**

Because the rate at which oxygen declines in the sealed chamber will depend on the organism’s activity state and volume of the respirometer, it is also difficult to define an optimal measurement time ($t_m$). On the one hand, the longer $t_m$ the better the $r^2$, but the more waste products will build up. Shorter measurement times reduce waste accumulation but can also increase variability in the measurement and reduce $r^2$. Shorter measurement times, however, also decrease the total measurement cycle thereby increasing the number of $MO_2$ determinations that can be made in a given time period. Lastly, shorter measurement periods also decrease the probability that the animal will exhibit different activity levels during the measurement. By using an estimate of expected $MO_2$, equation (9) can be rewritten to calculate an appropriate measurement time

$$t_m = \omega CO_2 V_{RE} RO_2^{-1}$$

where $\omega$ is the minimum % drop of oxygen content (or $pO_2$).

Information from equations (2, 4, 9, 10 and 13) has been summed up in Figs 9 and 10. The two figures can be used to assess the effect of respirometer:organism ratios, at
different temperatures, on the drop in $pO_2$. They are useful to get an idea about initial measurement times needed for given temperatures or respirometer:fish (organism) ratios ($r_{RO}$), temperature or $r_{RO}$ can be read at the $x$-axis, respectively, on the two figures, and time to 5% drop or drop in $pO_2$ after 5 min can be read from the two $y$-axis on the figures. If a larger drop in $pO_2$ is wanted (e.g. 10%), then simply multiply the ratio (e.g. $10 \times 5^{-1} = 2$) to obtain the time to reach that level. Lastly, post-experimental data analysis will become more trivial, if the duration of total cycle equals a product of 5 min, e.g. 10 or 15 min, as it will be more intuitive to read graphed data.

**EXPERIMENTAL CONTROL**

**EXPERIMENTAL ASSUMPTIONS**

The topics described in the calculation assumptions above apply to the water inside the respirometer when the chamber is closed. Other assumptions are more general, and emerge from the reasons for doing intermittent-flow respirometry instead of closed or flow-through respirometry. (1) The flush or replacement water is free of metabolites (e.g. carbon dioxide and nitrogenous waste). It is important that when fresh, clean water is not available for flushing, the water reservoir used as a source of flush water is vigorously aerated, filtered and recirculated through a UV sterilizer to eliminate bacterial growth that will contribute to background respiration. This is particularly important if there is little water replacement in the whole system and the experiment will be run for longer than 12–18 h (depending on oxygen solubility, water temperature and animal...
metabolism). (2) The flush or replacement water is in equilibrium with atmospheric gases, particularly oxygen and carbon dioxide; vigorous aeration will eliminate this problem. (3) The respirometry chamber and recirculating loop are gas tight. Some types of polymers used in tubing and respirometer construction can be quite permeable to oxygen and are therefore potential sources of oxygen washin during $\dot{M}_{O_2}$ experiments, particularly at low ambient oxygen levels. The oxygen buffering effect, i.e. the ability of these polymers to take up, store and excrete oxygen, can have a significant effect on oxygen levels in the chamber during flush and measurement periods. Generally nylon, acetyl or PVC are preferred building materials for oxygen measurement systems; the commonly available acrylic plastic is average, and polycarbonate and Teflon should be avoided (Stevens, 1992). The ideal gas-tight material for a respirometer would be glass, which may be practical for very small respirometers for larval fishes or zooplankton, for instance. Glass is also transparent and quite thermally conductive. The respirometer’s oxygen permeability can be tested by degassing the water in the respirometer and measuring the oxygen level for 3–4 h to ensure that no (or very little) oxygen is leaking in from the outside (Stevens, 1992).

**MANUAL, TIMING OR AUTOMATED RESPIROMETER OPERATION**

The fundamental idea behind intermittent-flow respirometry is that the water in the respirometer is exchanged at regular intervals by turning a flush pump on and off. Controlling the power output to the pump, and thus the flushing interval, can be done manually, with a timer, or by a PC or microcontroller. Manual flush-pump control provides the most flexibility over flushing and measurement times, and ensures that the experimenter is continuously watching and controlling the experiment so if any unforeseen events occur, immediate action can be taken. This is obviously time consuming and the constant presence of the experimenter may stress the organism and prevent it from settling down during SMR experiments. In swim-tunnel experiments, however, manually altering flushing times may be required to accommodate changes in oxygen consumption as swimming speeds are increased.

While commercially available programmable timers are an inexpensive way to quasi-automate the experimental procedure, the ability to control the exact flush period may be limited by the characteristics of the timer as many of the less expensive timers are only programmable to within 10–15 min periods. This can be problematic in cases where the organism has a very high oxygen consumption requiring measurement periods smaller than the minimum programmable time increment of the timer. Adjusting the size of the respirometer can mitigate this problem.

Using a microcontroller or a PC to automate the pump control and data collection will enable the user to precisely control the flush, wait and measurement periods down to seconds. With a PC-controlled setup, real-time calculations of oxygen consumption for each measurement period is possible, giving immediate information on whether the animal has reached a resting or steady state. In addition to experimental control, a PC-based control system allows external environmental variables (e.g. temperature, oxygen, carbon dioxide and salinity) to be monitored and even controlled to pre-set values. PC-controlled systems typically consist of a PC, an analogue to digital (A–D) interface to collect data from the oxygen measuring device and a digital output to control a relay for the flush pump. As the cost of A–D systems has declined dramatically,
experimenters with minor electronics experience can assemble a system fairly inexpen-
sively. In addition to hardware, software for collecting data and controlling pumps is
also necessary. Free open-source software is available for automated intermittent-flow
respirometry, AquaResp (www.Aquaresp.com) a freeware programme. If the
experimenter has programming experience, interface-based programmes such as
DasyLab (www.dasylab.com), LabTech Notebook (www.omega.com), LabView
(www.ni.com/labview) and MatLab (www.mathworks.com/products/matlab) can be
used to automate the process as well.

When using a PC-controlled setup over long periods of time, it is important to disable
automated, time-sensitive, processes such as hibernation and sleep mode, screen lock,
automatic updates and any other software that interferes with data logging and may
result in an unanticipated shut down and restart of the PC. If the PC is also connected
to a network, the experimenter should also be aware of any nightly updates that are
pushed out by IT service departments. In many cases, it is more expedient to keep the
data collection machine off the network entirely.

CONTROLLING EXPERIMENTAL VARIABLES

There are a variety of variables that can affect oxygen consumption. Some of them
are physical in nature (e.g. temperature, salinity and oxygen) while others are more
biological (e.g. swimming speed, toxins, algae and conspecifics). The ability to mea-
sure and control these types of variables are important in order to manipulate, quantify
(e.g. effect of hypoxia or temperature on metabolism) or control them to eliminate or
limit their effect on metabolism (e.g. diel changes in light or temperature).

Controlling the more common variables such as temperature, salinity, oxygen and
light can generally be done in two ways: using a stand-alone digital controller or a PC.
For example, commercially available digital temperature controllers (PR Electronics
PR5714, PR Electronics; www.prelectronics.com) can activate heaters in a reservoir to
maintain temperature or can be set up to activate a pump from a thermostatically con-
trolled reservoir to drive water through a heat exchanger in the bath which can reduce
temperature variations to ±0.1°C. They can also be used to control oxygen levels by
turning on or off solenoid valves that allow oxygen, air or nitrogen to flow into a water
reservoir when a set point is passed. Controlling the variables via a PC allows for the
same control as described above with the added benefit of allowing the experimenter to
simulate daily variations in environmental factors such as temperature, oxygen, salinity
and light. In general, however, it is recommended to control temperature by stand-alone
regulators because temperature control may be halted when the respirometry software
programme is stopped.

COMMON PROBLEMS IN RESPIROMETRY

Leaks

When a respirometer is submerged in water detecting a water leak can be difficult,
if not impossible, with the naked eye. Detection may be possible by injecting a bolus
of coloured dye in the respirometer and looking for the presence of dye in the sur-
rounding water bath. Sometimes, however, examination of the oxygen trace during a
respirometry experiment can offer clues to the presence of leaks. The non-linear decline
in oxygen consumption in Fig. 3, for instance, could have been generated by a small leak of water, with higher oxygen, from the surrounding water bath.

Gas bubbles

Gas bubbles in the respirometer are to be avoided at all costs as they function as an oxygen buffer in the system and dampen measured oxygen content changes during the closed period. This results from the fact that gas bubbles can either take up oxygen from, or leak oxygen to the surrounding water quite rapidly, depending on the relative $pO_2$ in the gas and water, respectively (Fick, 1855). Allowing bubbles to collect in the vicinity of the oxygen sensor can be even more detrimental as they can dramatically alter the microenvironment around it. Gas bubbles are often introduced when the organism is first placed in the chamber, allowing air to get trapped in the respirometer or associated tubing. Care should therefore be taken to reduce the introduction of gas bubbles into the respirometer and removing them prior to experimentation. A second source of bubble formation in the respirometer is a reduction of water oxygen solubility over time. This most commonly occurs if the atmospheric pressure drops (relevant for long measurement periods), or if the water temperature increases reducing its gas solubility. The very small bubbles produced by these events are very difficult to remove from the respirometer without disturbing the animal and can have a dramatic effect on the measured oxygen consumption. Designing the respirometer properly can reduce the problem of gas bubble accumulation. Firstly, mount the oxygen sensor in a position that will minimize the chance that gas bubbles will collect in its vicinity. Secondly, when possible mount the flush water exit at the highest point in the chamber so that air bubbles that occur in the chamber will collect there and be flushed out. Care should also be taken to make sure that there are no other local high cavities that may collect bubbles.

OXYGEN METERS

It is imperative that the experimenter understands the oxygen measuring principles and behaviour of their oxygen meters in detail when designing the respirometry system. Generally speaking, there are three things to be aware of: noise, oxygen consumption of sensors and automatic correction by sensor software. Noise caused by flow regime, certain types of oxygen sensors (e.g. polarographic and galvanic electrodes) consume oxygen during the measurement process and therefore require a flow of water past them to eliminate buildup of an oxygen-poor microenvironment around the electrode tip. Some fibre-optic sensors require the flow to eliminate a diffusive boundary layer on the sensor. In the absence of a minimal flow, however, the response time of sensors can be severely affected and the output noisy. Typically, if the respirometer itself is mixed properly, the mixing will generate a sufficient water flow to obviate this issue. Sensor drift, as all oxygen sensors have some degree of drift, this type of measurement error requires probe recalibration as the drift cannot be mitigated by using linear regression when calculating the oxygen consumption. Oxygen consumption of the sensor, as mentioned above, polarographic and galvanic electrodes consume oxygen themselves. While typically trivial in comparison to the organism’s oxygen consumption it can become important with small organisms in a small water volume. Automatic corrections, present day modern sensors such as optodes often come with specialized software that includes automatic temperature correction. This can be a troublesome
feature as there is a tendency to get noisier data with temperature compensation on than off, because the measurement error of the temperature probe is added to that of the oxygen sensor. From time to time, for example, the compensation algorithm will create jumps in the oxygen trace because of noise in the temperature sensor. The signal noise introduced by the compensation algorithm can be particularly troubling if it occurs during the measurement period. This phenomenon has been observed in both Presens (www.presens.de) and Pyroscience (www.pyro-science.com) oxygen meters, so it is recommended that the temperature compensation be disabled if feasible and the experimenter, instead, put their efforts into keeping the temperature of the respirometry system constant.

CONCLUSIONS

Intermittent-flow respirometry is a very useful tool to deploy when quantifying the oxygen consumption of aquatic organisms under a variety of conditions. In order to get the most accurate results, the experimenter should be mindful of a number of points when designing the system and carrying out experiments. The respirometer:organism volume ratio needs to be as small as possible to ensure accurate measurements, but should leave a sufficient volume to comfortably accommodate the animal. A ratio of 20–50 for static respirometers and 50–150 for swimming respirometers is suggested. (1) Sufficient flushing and mixing times are critical for accurate measurements on unstressed animals. Flush times that exchange 95–99% of the water in the respirometer between measurement periods should be used. Designing and assembling the recirculation loop and input and output ports to avoid shunting of flush water and providing good mixing will ensure that basic assumptions are met and possibly increase the accuracy of the oxygen consumption measurements. (2) Keep experimental variables constant, especially temperature, as it affects virtually all other variables. If feasible, log variables for quality assurance and post-experiment correction purposes. (3) Reduce or eliminate gas bubbles at all costs. Try to avoid introducing them with the animal, remove them before the experiment starts and position the chamber outflow at a high point in the respirometer so bubbles that collect over time will be flushed out. Be very cognizant of bubbles collecting in the vicinity of the oxygen probe as this can dramatically skew the results. (4) When determining background respiration, make sure that the measurement period is long enough to accurately measure it, as reflected in a high $r^2$.

This paper was an invited review based upon a decision made within the EU COST Action FA1004, Conservation Physiology of Marine Fishes. We would like to thank D. Chabot and the two anonymous referees for thorough feedback and additions to this review.

References


**Electronic Reference**

List of variables, the first column is the variable used, second column holds the descriptions of what the variable signifies and the last column has the SI units of the variable. One special case is when the unit is given as $\text{O}_2$ (except mg $\text{O}_2$), this signifies the oxygen unit used in the given experiment.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_{\text{period}}$</td>
<td>Total duration</td>
<td>s</td>
</tr>
<tr>
<td>$t_f$</td>
<td>Flush period duration</td>
<td>s</td>
</tr>
<tr>
<td>$t_w$</td>
<td>Wait period duration</td>
<td>s</td>
</tr>
<tr>
<td>$t_m$</td>
<td>Measurement period duration</td>
<td>s</td>
</tr>
<tr>
<td>$V_{\text{RE}}$</td>
<td>Effective respirometer volume</td>
<td>l</td>
</tr>
<tr>
<td>$\text{CO}_2$</td>
<td>Total respirometer oxygen content</td>
<td>mg $\text{O}_2$</td>
</tr>
<tr>
<td>$t$</td>
<td>Time variable</td>
<td>s</td>
</tr>
<tr>
<td>$W_o$</td>
<td>Mass of organism</td>
<td>kg</td>
</tr>
<tr>
<td>$V_{\text{RT}}$</td>
<td>Total respirometer volume</td>
<td>l</td>
</tr>
<tr>
<td>$\rho_o$</td>
<td>Organism density</td>
<td>kg l$^{-1}$</td>
</tr>
<tr>
<td>$\beta_{\text{O}_2}$</td>
<td>Oxygen solubility of oxygen in water</td>
<td>mg $\text{O}_2$ l$^{-1}$</td>
</tr>
<tr>
<td>$b$</td>
<td>Intersect in straight line equation</td>
<td></td>
</tr>
<tr>
<td>$R_{\text{O}_2}$</td>
<td>Rate of oxygen consumption</td>
<td>mg $\text{O}_2$ s$^{-1}$</td>
</tr>
<tr>
<td>$\dot{M}_{\text{O}_2}$</td>
<td>Mass-specific oxygen consumption</td>
<td>mg $\text{O}_2$ kg$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>$p_{\text{O}_2}$</td>
<td>Partial pressure of oxygen</td>
<td>e.g. kPa or mmHg</td>
</tr>
<tr>
<td>$S_a$</td>
<td>Slope of oxygen trace, animal</td>
<td>O$_2$ s$^{-1}$</td>
</tr>
<tr>
<td>$S_b$</td>
<td>Slope of oxygen trace, background</td>
<td>O$_2$ s$^{-1}$</td>
</tr>
<tr>
<td>$\omega$</td>
<td>Fraction used in selecting respirometer design</td>
<td></td>
</tr>
<tr>
<td>$Q_{10}$</td>
<td>Temperature coefficient, used for correcting temperature effects</td>
<td></td>
</tr>
<tr>
<td>$T_1$</td>
<td>Temperature endpoint used for correcting temperature effects</td>
<td>°C</td>
</tr>
<tr>
<td>$T_0$</td>
<td>Temperature start point used for correcting temperature effects</td>
<td>°C</td>
</tr>
<tr>
<td>$D$</td>
<td>Dilution ratio</td>
<td>s$^{-1}$</td>
</tr>
<tr>
<td>$F_F$</td>
<td>Flushing rate</td>
<td>l s$^{-1}$</td>
</tr>
<tr>
<td>$t_1$</td>
<td>Time start</td>
<td>s</td>
</tr>
<tr>
<td>$t_2$</td>
<td>Time end</td>
<td>s</td>
</tr>
<tr>
<td>$\alpha$</td>
<td>Residual water fraction</td>
<td></td>
</tr>
</tbody>
</table>