Repeated measurement of $M_{O2}$ in small aquatic organisms: a manual intermittent flow respirometer using off-the-shelf components.

Daniel W. Baker, Michael E. Hudson, Emily J. Frost, and Mary A. Sewell*

School of Biological Sciences, University of Auckland, Auckland 1142, New Zealand.

Running Head: Respirometry in small aquatic organisms

* Corresponding Author: m.sewell@auckland.ac.nz, orcid.org/0000-0002-1595-7951
Phone +649 373-7599 ext. 83758
Fax +649 373-7417
Abstract:
Measurement of rates of oxygen consumption ($M_{O2}$) in small aquatic embryos or larvae (< 1mm) in response to altered environmental conditions has traditionally been challenging. Here, using modifications of a commercially available fluorescent optode flow-through cell (FTC: PreSens™ FTC-PS13) and routine laboratory supplies (syringes, stopcocks, tubing), we have constructed a manual intermittent flow respirometer (MIFR) that allows measurement of $M_{O2}$ in small numbers of individuals when sequentially exposed to different environmental conditions (e.g. changes in seawater pH) through a gravity-driven media replacement perfusion system. We first show that the FTC can be used in ‘static’ mode while incubating small numbers of embryos/larvae contained within the planar oxygen sensor (POS) chamber with Nitex filters. We then demonstrate the use of the MIFR by exposing larval echinoderms (Fellaster zelandiae, Evechinus chloroticus, Centrostephanus rodgersii) to seawater equilibrated with elevated CO$_2$, and measured $M_{O2}$ during acute and chronic exposure to hypercapnia. This MIFR method will allow investigators to address questions regarding the respiratory physiology of small aquatic animals, such as the thresholds for metabolic depression in embryonic and larval forms.

Key words: respiration, echinoderm, larvae.

New and Noteworthy:
A manual intermittent flow respirometer (MIFR) allowing media exchange in a flow-through cell containing small aquatic organisms, permits repeated measurement of $M_{O2}$ of individuals in not only a single media (e.g. technical replication), but also in different media (here, high CO$_2$ equilibrated seawater) enabling measurement of acute physiological responses to changed conditions. This versatile technique has wide-ranging implications for the study of the $M_{O2}$ response of aquatic organisms in the face of climate change.
Introduction:

Organismal metabolic rate ($M_{O_2}$) is an important component of the physiologist’s toolbox and is being increasingly used as a measure of whole-animal performance and fitness, particularly in a climate change context (5, 13, 29, 30). The rate of aerobic respiration is also fundamental to theoretical aspects of animal biology, such as the metabolic theory of ecology (2), and the recently proposed oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis linking animal performance and temperature tolerance (30). The need for cost effective, repeatable, and high throughput techniques to measure $M_{O_2}$ is essential to future progress and has prompted several reviews (e.g. 5, 39) and a recent special issue on metabolic rate measurements in fish (Journal of Fish Biology, vol 88, issue 1, 2016).

Measuring $M_{O_2}$ can be particularly challenging in small aquatic animals (< 1 mm) such as the larval stages of marine invertebrates. A diversity of methods have been used including µBODs and end-point detection with Clark-type micro-cathode oxygen electrodes (18), coulometric respirometry (14), couloximetry (26), and fluorescent optode sensor spots (e.g. 15, 39, 42, 45). In all these approaches, larval $M_{O_2}$ is calculated from the measured decline in oxygen saturation in a sealed volume of seawater either continuously or at the end of an incubation period. None of these approaches lend themselves to the techniques of intermittent-flow respirometry, widely used with aquatic vertebrates and larger invertebrates, where water can be exchanged while the animal(s) are enclosed within the respiration chamber (reviews: 5, 40). Maintaining high oxygen saturation (>80%) through water changes minimizes the chance that $M_{O_2}$ measurements are being influenced by hypoxia-induced metabolic adjustments, reduces build-up of CO$_2$ and other metabolic products (e.g. ammonia), and allows for repeated measures of oxygen consumption in the same individuals (see 5 for example traces).

Here we present a design for a manual intermittent flow respirometer (MIFR) that functions in a similar fashion to the computer-controlled ‘static’ respirometers used with fish (review: 5, 40). Our MIFR acts as a closed system during the $M_{O_2}$ measuring phase, but then uses a manually controlled gravity-fed system that allows seawater exchange without damaging the enclosed larvae. The MIFR is based on an off-the-shelf fluorescent optode flow-through cell (PreSens™ FTC-PS3) with a fluorescent sensor film that allows contact-less measurement, has excellent long-term stability, does not consume oxygen during measurement (16, 31), has...
previously been used in physiological applications (e.g. 7, 12, 25), and allowed high resolution $M_{O2}$ measurements from small aquatic animals including larval fishes (5).

With the addition of simple laboratory supplies (e.g. syringes, stopcocks, tubing) our aim was to build an MIFR for repeated $M_{O2}$ measurements on the same group of larvae, and to provide a method to determine the “tipping point” to metabolic depression by sequentially exposing the larvae to water of different environmental conditions (e.g. pH, salinity). Here we use the early developmental stages of three New Zealand echinoderms [sand dollar *Fellaster zelandiae* (Gray 1855); regular urchins: *Evechinus chloroticus* (Valenciennes 1846), *Centrostephanus rodgersii* (A. Agassiz 1864)] to demonstrate the effectiveness of our MIFR by measuring $M_{O2}$ in response to control and hypercapnic seawater.

**Materials and Methods:**

**Experimental animals**

Adult sea urchins of *E. chloroticus*, *F. zelandiae* and *C. rodgersii* were collected subtidally at Matheson’s Bay (36° 18.10’ S, 174° 48.65’ E), Cheltenham Beach (36° 49.18’ S, 174° 48.39’ E) and the Mokohinau Islands (36° 54.57’ S, 175° 6.37’ E) in the Hauraki Gulf, New Zealand, respectively. Urchins were induced to spawn with coelomic injection of an appropriate volume of 0.55 M KCl (approximately 1 mL per 100g wet weight), the eggs collected over beakers of 1 µm filtered seawater (FSW), and sperm collected using a glass pipette. Eggs were fertilized in a 250 or 500 mL beaker with a dilute sperm suspension at the seawater temperature at the time of collection (*E. chloroticus*, 20°C; *F. zelandiae* 17°C; *C. rodgersii*, 14°C) and the beaker kept on a shaker table for 30-90 mins. Male:female crosses with fertilization $\geq$80% were washed in FSW, and zygotes gently transferred to static 20 L plastic buckets with a central air-lift column containing an air-stone (design modified from ref. 33). For control conditions the air-stone delivered 380 µatm CO$_2$; high CO$_2$ conditions (1000, 1800 µatm CO$_2$) were produced by bubbling with pre-mixed gases generated using mass-flow controllers (SmartTrak, Sierra, Monterey, CA, USA). Cultures were maintained in a temperature-controlled room or water bath matching fertilization temperatures until reaching the required developmental stage.

**Respiration measurements**

$M_{O2}$ measurements were made with an OXY-4 mini multi-channel fiber optic oxygen transmitter (PreSens™, Regensburg, Germany) and a MIFR constructed from a modified
flow-through cell with integrated fluorescent oxygen sensor (FTC-PSt3, PreSens™, Regensburg, Germany). The FTC-PSt3 is a miniaturized fiber-optic chemical sensor integrated in a T-shape flow-through cell, consisting of a small glass planar oxygen sensor (POS) chamber (dimensions: inner diameter = 4 mm, length = 14 mm, liquid volume in FTC ~ 100 ± 10 μl; ref 31) coated on the inner surface with an oxygen-sensitive fluorescing layer and connected to the transmitter via a polymer optical fibre (POF, Fig. 1A). The FTC-PSt3 can be connected via Luer Lock adapters to external tubing and the oxygen tension is measured in fluids (e.g. water, blood) passing through the cell. The distinct advantage of using a fluorescent optode FTCs in this application is that no oxygen is consumed during measurement and there is no hypoxic boundary layer formed at the sensor surface (15, 30).

Off-the-shelf FTCs were modified to allow continuous recording from larvae in a static volume of media by the addition of fine-mesh Nitex filters (NF), gas-impermeable PTFE tubing, and fold-back clips attached to either end of the POS chamber (Fig. 1B, C). NF (~8-10 mm in diameter) were cut from sterilized Falcon™ Cell Strainers (60-100 μm, dependent on developmental stage; Fisher Scientific, USA), and placed flush against the rim of the opening of the POS chamber using fine forceps. A double layer of NF at each end of the POS chamber was secured with the sealing O-ring and the female and male Luer Lock adapters provided in the FTC (Fig. 1B). Clamping the inflow and outflow PTFE tubing of the FTC prevented oxygen diffusion from up- or down-stream media (Fig. 1C). Fold back clips were positioned as close to the LL ends of the FTC as possible, creating a visible “squash” point in the PTFE tubing that allowed consistent positioning of the clip; the total static volume was determined using a 200 μl Hamilton syringe and the weight change between empty and full FTCs (range: 100-150 μl). NF were soaked in 70% ethanol after each experiment and discarded when exhibiting signs of deformation. Fold back clips were replaced when corrosion reduced clamping strength.

The MIFR presented here is perfused by a gravity-fed system that allows for exchange of media within a FTC. This system consists of a “header tank” (60 ml syringe without plunger), which was either attached to a vertical backboard with a cable-tie or secured via a metal adjustable clamp, connected in series to: a 2-way stop-cock (SC); a 3-way SC with a 5 ml syringe attached to the horizontal output; and finally via PTFE tubing to the inflow end of the FTC (Fig. 1C). After the 60 ml syringe was filled with air-saturated FSW at the temperature of incubation, the 2-way and 3-way SCs were opened to the 5 ml syringe, then 4 ml of system water drawn into the syringe; any air bubbles were expelled by detaching,
depressing, and then reattaching the 5 ml syringe. The 3-way SC was then opened between the 5 ml syringe and the FTC to permit filling the chamber via depressing the syringe and eliminating all air bubbles. Once filled, the inflow tubing was clamped and the FTC removed for the recommended 2-point calibration (31). FTCs were first flushed with 70% ethanol to eliminate microbial growth, then calibrated with sodium sulfite (20 g l\(^{-1}\) in de-ionized water) as a 0% dissolved oxygen standard, followed by calibration with 100% air-saturated FSW at the incubation temperature. The FTC was then drained, reconnected to the inflow tubing, and the Luer Lock male adapter removed from the outflow end (Fig. 1B) ready for insertion of the test organisms.

To load the FTC, sea urchin embryos/larvae were transferred from cultures to a glass petri dish (10 cm) containing FSW. Between 20 and 100 individuals (stage-dependent) were collected under a dissecting microscope in aliquots of FSW (15-40 µl) using a 20 or 200 µl pipette (stage-dependent) with the last 5 mm of the pipette tip removed with a sterile, single use razor-blade. Test animals were then transferred into the outflow end of the FTC which remained moist from the FSW used in calibration; the NF at the inflow end prevented animals from exiting the chamber (Fig. 1B). After successful loading of the entire aliquot of fluid, the outflow NF was placed with fine forceps on the glass edge of the POS chamber, the sealing O-ring added, and the Luer Lock male adapter tightened (Fig. 1B, C). The FTC was then oriented vertically with the outflow end “up”, and any air bubbles removed by gently depressing the 5 ml syringe; as water entered the chamber from the bottom, air was pushed out through the top (cf. a syringe being cleared of air prior to injection). The fold back clips were then applied to the outflow tubing to create a closed system (Fig. 1B, C), and the pipette tip was closely examined to determine the number of larvae in the FTC. If loading was deemed unsuccessful, either by visual inspection or O\(_2\) saturation irregularities in the recorded output, the larvae were discarded and the loading process repeated. For successful loadings, after equilibration of oxygen tensions within the chamber (<5 min), oxygen saturation was measured every 15 seconds for a period of 10-120 mins (dependent on rate of O\(_2\) depletion) using a PC running the OXY-4 software (PreSens). During recording, each FTC was covered with tinfoil to reduce ambient light (as recommended by ref 31) and all FTCs were immersed in a horizontal position in either a single water-filled container located in a temperature controlled environmental chamber (for *E. chloroticus* and *F. zelandiae*) or a single wet table provided with flow-through seawater (*C. rodgersii*) to maximize thermal stability.
To exchange media in the FTC containing the embryos/larvae, the new media (control or hypercapnic FSW) maintained at the temperature of incubation was loaded into the 60 ml syringe, and the 5 ml syringe used to fill the MIFR upstream of the 3-way SC with bubble-free new media. Both SCs were then opened slightly, allowing the new media to perfuse the FTC by gravity and so avoiding substantial pressure changes associated with syringe use that could damage the enclosed larvae. After complete media exchange in the FTC (flushing with a volume 30-50x greater than the chamber volume = 3 to 5 ml, and confirmed with a return to readings of 100% saturation), the SCs were closed, and the fold back clips re-applied for the next $M_{O_2}$ measurements (Fig. 1C).

After each trial was complete, the 3-way SC was opened to the 5 ml syringe, and the inflow fold-back clip and outflow adapter with the NF were removed from the FTC to allow the animals to be flushed from the respiratory chamber using the 5-ml syringe (Fig. 1C). The FTC was then re-perfused with the same media to verify the absence of oxygen producing or consuming micro-organisms in the respiratory chamber. At no time was detectable oxygen consumption observed in a chamber without larvae. The entire perfusion setup was rinsed with 70% ethanol between discrete trials to eliminate micro-organism growth.

**Calculations and data analysis**

$M_{O_2}$ was calculated from the negative slope of the best-fit linear regression of oxygen depletion over time adjusted for the static volume of the FTC and tubing to the clamped area (Fig. 1C), and oxygen solubility constants appropriate for the salinity and temperature of incubation. Oxygen depletion was converted to moles hr$^{-1}$ and divided by the number of test animals. Only those trials with $r^2$ values above a pre-determined threshold (>0.80 for developmental experiments, and >0.97 for both chronic and acute hypercapnia experiments) were used in subsequent statistical analyses (criteria met by >95% of trials). Statistical analyses were performed using Sigmaplot Ver. 13.0 ($\alpha = 0.05$).

**Results and Discussion:**

The development and validation of our MIFR for use with embryos/larvae used the sea urchin species with available gametes – *E. chloroticus*, November-March (43); *C. rodgersii*, July-September (27); *F. zelandiae*, small numbers of fertilizable eggs available almost year-round (23).
1. Can a FTC be sealed for use as a static chamber to measure $M_{O_2}$?

We first needed to show that the FTC could perform in ‘static mode’: specifically, could the POS chamber be sealed with the use of Nitex filters, the gas-impermeable PTFE tubing and the fold back clips? To test this, we prepared oxygen depleted autoclaved seawater (DASW) by adding 0.2M sodium sulphite, as recommended for the 0% calibration (31). In Run 1, we loaded DASW into one POS chamber, and added the NF and OR (Fig. 1B). Oxygen enriched autoclaved seawater (ASW) was then loaded on the external side of the NF and OR surrounding the POS (i.e. in the region of the FTC labelled LL♀ and LL♂ in Fig. 1B). The oxygen saturation of the FTC containing DASW was then recorded for 20 mins in parallel to an independent ASW sample in a second FTC (Fig. 2A). After the recording period, the ASW external to the NF was removed using a Hamilton gas-tight syringe, and the oxygen saturation determined by placing this water sample into a third FTC (Fig. 2A – independent sample). Oxygen saturation was stable at both 0% and 100% saturation in both the FTC and in the water external to the POS chamber in the DASW cell (Regression slope NS for 100%, cannot be calculated for 0% as all values 0), suggesting that there was no substantial flux of $O_2$ across the NF, or through the gas-impermeable PTFE tubing between the FTC and the fold back clips.

In a separate series of validation runs, we depleted $O_2$ from seawater by bubbling with instrument grade N$_2$. In Run 2, we included ~100% and two low levels of % saturation (ca. 20%, Fig. 2B). The high saturation level in this run showed a very slow decline in saturation (Regression slope -0.0125 % saturation/min) indicating very slight $O_2$ leak out of the POS chamber, while the low saturation FTCs showed a slow increase in saturation over time (Regression slope +0.0527, +0.0521 % saturation/min). In Run 3, we included two traces at the minimum range over which $M_{O_2}$ was to be measured (~80%), and another at low saturation (Fig. 2B). At 80% saturation, one FTC had no significant regression slope, the other had a slight positive slope (0.0155 % saturation/min); the low saturation 27% FTC, as in the previous run, had a higher increase in % saturation over time (slope 0.0264) than at higher levels of saturation.

Why might we see slight changes in % $O_2$ saturation over time in our FTCs? Two factors are generally considered to be causing such changes: (1) background respiration, primarily from bacteria and other micro-organisms, which can over-estimate oxygen usage, and (2) oxygen washin from the surrounding water bath, particularly at low oxygen levels (40, 41).
address the former and prevent bacterial growth we routinely used 70% ethanol rinses of the FTC, the Nitex filters and tubing between trials, rinsed the FTC immediately before calibration, and performed runs without embryos/larvae to check for bacterial contamination of the chambers. All seawater used was 1 µm FSW to remove any micro-organisms. The second factor, changes in oxygen saturation due to minor leakage of oxygen into/out of chambers, is a routine issue in respirometry as a result of oxygen-permeable materials, poorly sealed chambers, or chambers constructed from inappropiate materials, such as some plastics (4, 37). In the trials shown in Fig. 2 we only saw levels of washin >0.05% saturation/min when O₂ saturation was extremely low (ca 20%) and with a large difference in partial pressure between the fluid in the FTC and the exterior. We have presented all these runs, rather than showing “representative” traces to be fully transparent on the need to check for sealing of the LL of the FTC. We suggest that, if runs in a particular FTC without embryos/larvae showed evidence of oxygen leakage, then the entire FTC or O-rings should be replaced. Application of a biologically inert oil or grease to the O-rings of the FTC might also improve sealing, but with the disadvantage that this material might be washed downstream and contaminate parts of the FTC. In our FTC we minimized the potential impacts of minimal oxygen leakage by measuring M₀₂ only at high levels of saturation, between 100 and 90%, for short incubation periods with sufficient embryos/larvae to produce high rates of oxygen decline and, following suggestions from Svendson and colleagues (39, 40), we also excluded any runs with non-linear declines in oxygen saturation and with r²s less than our thresholds (0.8 for developmental stages, >0.97 for our hypercapnia experiments). Runs failing these benchmarks became infrequent as our technique was refined.

We next needed to show that the FTC in ‘static mode’ could be used to measure a significant decline in oxygen saturation when embryos/larvae were present, and that the smaller chamber volume of the FTC (~100 µl) was not impacting measurements by impeding swimming (chamber volume for echinoid larvae is generally ≥400 µl; refs 18, 21, 38, 44). To this end we measured M₀₂ during the early development of embryos/larvae of *F. zelandiae* and *E. chloroticus* (Fig. 3A). For each time point we used the 4 channels of the OXY-4 to measure M₀₂ simultaneously in 4 groups of pooled embryos/larvae (range: 19-101 individuals dependent on developmental stage). M₀₂ measurements were made on developmental series for three independent male/female crosses (N=3 biological replicates).
E. chloroticus and F. zelandiae showed a steady increase in $M_{O_2}$ during embryonic development (Fig. 3A), a pattern usually attributed to increases in cell numbers with time (18). $M_{O_2}$ in F. zelandiae ($17^\circ C$) increased from ~3 pmol individ$^{-1}$ hr$^{-1}$ in 4- or 8-cell embryos (3 hours post-fertilization, hpf) to just under 25 pmol individ$^{-1}$ hr$^{-1}$ in 4-arm echinoplutei (96 hpf). $M_{O_2}$ in E. chloroticus ($20^\circ C$) showed a similar pattern, increasing from ~15 pmol individ$^{-1}$ hr$^{-1}$ in the blastula stage (12 hpf) to almost 60 pmol individ$^{-1}$ hr$^{-1}$ in 4-arm echinoplutei (72 hpf). These $M_{O_2}$ values are in the same range, and with the same relative increase between developmental stages as those obtained in E. chloroticus using alternate measurement methods: (i) the Marsh and Manahan (18) $\mu$BOD method (vial size 420-520 $\mu$l) and end-point detection using a Strathkelvin Clark-type oxygen electrode (Blastula 11.9 pmol individ$^{-1}$ hr$^{-1}$, 72 hpf echinoplutei 33.4 pmol individ$^{-1}$ hr$^{-1}$ at $20^\circ C$; M.A. Sewell, unpublished data), or (ii) a Unisense (Denmark) microrespiration system with 400 $\mu$l chamber (72 hpf echinoplutei, 64 pmol individ$^{-1}$ hr$^{-1}$ at $15^\circ C$; E. Frost, unpublished data).

For E. chloroticus, with the most complete data-set (3 biological replicates, 6 time-points, Fig. 3A) the coefficient of variation (CV: SD/mean) on $M_{O_2}$ for 4 FTC within a male/female cross was 12.5-21.4% (grand mean 16.0%, N=18), and there was no significant difference in the CV through time (One-way ANOVA on ranks: $H = 4.493$, df = 5, NS), suggesting that this method is suitable for measuring $M_{O_2}$ in both embryos and larvae.

We next investigated $M_{O_2}$ changes in response to an experimental treatment: altered CO$_2$ levels in the media contained within the FTC. $M_{O_2}$ was measured in sibling larvae of C. rodgersii chronically exposed (i.e., reared from fertilization) under three CO$_2$ regimes (380, 1000 or 1800 $\mu$atm CO$_2$) at $14^\circ C$. Larval C. rodgersii (96 hpf) reared at 1800 $\mu$atm CO$_2$ exhibited a significantly reduced $M_{O_2}$ ($F_{2,21}= 6.38$, p<0.01) compared to age-matched siblings reared under control conditions (Fig. 3B), demonstrating the sensitivity of this method to detect changes in $M_{O_2}$ of larval animals.

The FTC, with the addition of the Nitex filters to retain embryos/larvae, performed well as a ‘static’ chamber. We found evidence for negligible diffusion of oxygen from the water outside the POS chamber or through the gas-impermeable PTFE tubing. The chamber design proved suitable for rotating embryos, echinoderm larvae of diverse shapes (from 4-arm high angled echinoplutei in E. chloroticus and F. zelandiae to the 2-arm low-angled echinopluteus of C. rodgersii; see images in ref 17, 10, 34 respectively), Artemia nauplii (data not shown), and single zebrafish embryos (data not shown).
2. Can we manually change the media in the FTC? i.e. MIFR performance

The next step was to show that gravity-fed media via the MIFR would allow \( M_{O2} \) to be measured repeatedly from larvae in either the same or different media. Initial testing of the MIFR used \( F. \) zelandiae larvae and a representative trace of 4 media exchanges (5 \( M_{O2} \) measurements), and a final testing of the FTC with the larvae removed is shown in Figure 4A. In control conditions ANCOVA revealed a significant difference in slopes between repeat measurements (Equal slopes test: \( p < 0.05 \)), but the calculated values of \( M_{O2} \) shown in Fig. 4B are consistent (Grand Mean ± SD: 23.63 ± 1.92 pmol O\(_2\) individ\(^{-1}\).h\(^{-1}\)). In hypercapnia (Fig. 4C) there was no significant difference in slopes (Equal slopes test: \( p = 0.391 \)) and, in this single test, a higher value for \( M_{O2} \) (Grand Mean ± SD: 28.15 ± 0.49 pmol O\(_2\) individ\(^{-1}\).h\(^{-1}\)).

Further focused validation of the MIFR was based on 3 repeated readings of 5d \( E. \) chloroticus larvae from a single FTC after media exchange. Data from 4 trials are shown in Figure 5. Although ANCOVA reveals significantly different slopes between repeat measurements within each trial (Equal slopes test: \( p < 0.05 \)), there was no correlation between the slope and sequential order (i.e. Repeat 1-3), and the CV on the calculated \( M_{O2} \) is of a similar magnitude to that seen without media exchange (mean for Trials 1-4 = 12.6%, range 5.5-20.1%).

To test for the acute response of echinoderm larvae to altered media we cultured \( F. \) zelandiae larvae from 4 separate male/female crosses in control conditions (380 \( \mu \)atm). 5-day old echinothuri from each cross were loaded into four FTC chambers and \( M_{O2} \) measured during exposure to either a) 380 \( \mu \)atm as in culture, or b) seawater equilibrated with 1800 \( \mu \)atm CO\(_2\) in random order (Total N=16 before/after measurements). \( M_{O2} \) of larval \( F. \) zelandiae was significantly higher (~30%) after exposure to water equilibrated with 1800 \( \mu \)atm CO\(_2\) (paired t-test: \( t = -6.055, \text{df} = 15, p < 0.001; \) Fig. 3C). As individual FTC provide independent measurements of \( M_{O2} \) we can determine the frequency distribution of the change in \( M_{O2} \) when exposed to high CO\(_2\) [Change = (\( M_{O2} \) 1800 \( \mu \)atm CO\(_2\)) minus (\( M_{O2} \) 380 \( \mu \)atm CO\(_2\))]; Fig. 3D] and, for example, examine differences in \( M_{O2} \) response between male/female crosses (One-way ANOVA: \( F_{3,12} = 0.052, \text{NS} \); individual crosses not shown in Fig. 3D for clarity). Of interest is that, although there was a modal increase in metabolic rate of 5-10 pmol O\(_2\) individ\(^{-1}\).h\(^{-1}\) when groups of individuals in a FTC were exposed to high CO\(_2\), there was considerable variability in response, with 1/16 groups showing a decrease in metabolic rate, and 1/16 groups increasing \( M_{O2} \) by 15-20 pmol O\(_2\) individ\(^{-1}\).h\(^{-1}\) (Fig. 3D).
Visual observation of larvae recovered from the FTCs at the end of a series of repeated media exchanges confirmed the suitability of the system as it provided no evidence of physical damage to the larvae and confirmed post-measurement swimming activity following MIFR use.

Implications of the ‘static’ FTC and MIFR for respiration studies in small aquatic organisms.

Here we have presented two methodological advances: (1) a simple modification to a commercially-available FTC which allows containment of the larvae and continuous recording of oxygen consumption, and (2) an MIFR constructed from common laboratory supplies that allows for exchange of water within the FTC, without damaging the larvae.

Marsh and Manahan (18) described the need in larval biology for methods to measure oxygen consumption rates that are: (a) accurate, (b) robust (e.g. for field use), and (c) methodologically simple to use. The method they developed, incubating known number of embryos/larvae in small sealed glass vials (μBOD vials) and end-point detection of oxygen consumption using polarographic oxygen sensors, has been widely used in larval biology (e.g. 9, 22, 24, 25, 44). In recent years, with the greater availability of fluorescent optode technology, measurement of the oxygen concentration at the end of the μBOD incubation has shifted to the use of optode probes (e.g. 6, 19, 38).

Here we have provided a methodological improvement, by incubating the embryos/larvae within a ‘static’ FTC containing an integrated fluorescent optode and recording oxygen continuously (i.e. combining an equivalent of the sealed μBOD chamber, with more than end-point detection). The advantage of a ‘static’ FTC is that we were able to simultaneously take 4 replicate measurements of respiration rate in individuals obtained from the same biological source (cultures, or male/female crosses), as each FTC includes a separate group of echinoderm embryos/larvae. This provides a statistical improvement on the original Marsh and Manahan (18) technique, which generates only a single respiration rate (with a S.E.) based on the slope of the line of oxygen consumed (Y-axis) in multiple μBOD vials with varying numbers of larvae (X-axis).

Use of multiple ‘static’ FTCs is an ideal way to collect time-matched data from two treatments; for example, a common larval scenario is comparing physiological parameters in fed and unfed treatments (e.g. 9, 22, 24, 25). Using our four-channel system, we could
compare oxygen consumption rates in two fed and two unfed treatments, and by blocking consecutive runs, use a traditional one-way ANOVA to compare $M_{O2}$ between feeding treatments. Similar and greater replication of individual chambers is provided by other technologies that were not available to us, including the PreSens™ OXY-10 system that can record 10 FTCs, or the Loligo Systems 24-chamber microplate that, as here, uses fluorescent optode sensor spots in the base of a glass chamber. We have presented our design for a modified FTC to provide “fresh thinking” on how existing oxygen sensing equipment in a laboratory can be repurposed to measure $M_{O2}$ in a new way, and as an essential component of the MIFR that we consider below.

One potential disadvantage of using a FTC is that we cannot see the larvae inside the chamber: first, because the plastic of the FTC is opaque (Fig. 1A); and, second, following the recommendations of PreSens (31), we covered the FTC with tinfoil to reduce the influence of ambient light. We have assumed, as in other sealed respirometers used for larvae and smaller zooplankton, that adequate stirring of the water in the FTC is provided by the movement of the animals (e.g. 1, 4, 32). Echinoderm embryos and larvae are active swimmers and, although varying with species, developmental stage and seawater temperature, swimming speeds are generally in the order of 0.15-0.40 mm/sec (3, 20). In glass μBOD vials, oriented horizontally as in the FTCs used here, all Evechinus developmental stages are observed swimming to and fro within the sealed space (MA Sewell, pers. obs.). A back-of-the-envelope calculation suggests that, at an average speed of 0.25 mm/sec, each larva could swim the length of the 14 mm POS chamber in ~60 seconds. During a 20 minute respiration run (Fig. 4, 5), with multiple embryos/larvae each swimming the length of the chamber, we expect that animal activity would preclude the development of oxygen gradients that might occur in larger respirometers, despite the slower diffusion rate of oxygen in water (approximately 10000 fold less than in air) (39, 40). Additionally, with actively swimming embryos/larvae, and an optode sensor that is not consuming oxygen (15, 30), the requirement for additional mixing devices to eliminate hypoxic boundary-layer effects as may be created with polarographic oxygen sensors is absent (4, 10). The linear declines that we observed in oxygen saturation with high $r^2$ values (see Fig. 4, 5) indicate that the ‘static’ FTC chamber provides a reliable measure of $M_{O2}$ in echinoderm larvae and, by modification of an established, commercially available product, fits Marsh and Manahan’s (18) criteria for measuring larval metabolic rates (accurate, robust, simple to use).
Our second, and more significant, methodological contribution is in the design of our MIFR, which provides a system for repeatable measurements of $M_{O_2}$ in embryonic and larval invertebrates with only a single handling when loading the chamber. This allows for two significant advances: (1) We can obtain technical replication of respiration rates in the same group of embryos/larvae. After the initial recording of oxygen consumption, the seawater can be exchanged and the POS chamber returned to 100% $O_2$ saturation for a second period of measurement. This, as shown here (Fig. 4) can be repeated multiple times, without the need for additional handling of delicate organisms and would be particularly useful when measuring $M_{O_2}$ in species with $O_2$-dependent respiration rates or when the research question required long incubations (e.g. if one wanted to measure $M_{O_2}$ across the blastula/gastrula transition). (2) We can change the chemical attributes of the water (e.g. pH, salinity), so that differences in $M_{O_2}$ can be measured between different water types (e.g. Fig. 4), allowing performance curves and transition points, such as the threshold to metabolic depression and the pejus to the pessimum (as defined in refs 8, 29, 30, 35, 36) to be determined on the same individuals/groups of individuals.

The advantage of our gravity-fed MIFR is two-fold. Firstly, in other larval respiration methods involving a sealed chamber (e.g. µBOD, couloxiometer) repeat measurements of $M_{O_2}$ in the same individual(s) would require either transfer of the larvae using some form of pipette, or retaining the larvae in the bottom, perhaps through the use of gentle centrifugation, and replacing the water on top; both approaches have considerable potential to damage the embryos/larvae during water exchange. Secondly, measurements of respiration rates in small organisms can be extremely variable, especially when measured at an individual level (19, 26, 38, 42). Understanding the physiological response of a species/population to changing environmental conditions might be significantly advanced by conducting before/after experiments, such as shown in Fig. 4A, where the mean $M_{O_2}$ (based on repeated measurements) can be compared to the mean $M_{O_2}$ when exposed to a stressor.

Measuring metabolic rate in small invertebrate embryos and larvae has always been challenging and the method we present here is not intended to replace existing techniques. Our aim is to provide an alternative “physiological tool” for measuring $M_{O_2}$ in laboratories that contain the appropriate instrumentation and provide a “new” way to use what one already has. As a caveat, this method might prove most useful for small organisms that are actively motile within the POS of the FTC during incubation; we recommend that future users run preliminary tests to see if our modified FTC is suitable for their target species. In addition,
though not directly tested here, thermal performance curves could be produced by sequential
introduction of warmer 100% saturated water via the MIFR, adjusting the temperature of
incubation and setting the appropriate temperature within the OXY-4 software (31). We hope
that this simple yet highly versatile, technique will be applied to study of larval physiological
ecology, and particularly for study of the $M_{O_2}$ response of aquatic organisms in the face of
environmental change.

Acknowledgements:

We thank B. Doak and the crew of the RV Hawere for assistance in collecting
Centrostephanus; A. Gower, A. Leonard and K. Jenkins for laboratory assistance; I.
MacDonald for Fig. 1A, V. Ward for Figs. 1B and 1C; Robert Meier (PreSens) for technical
advice, and the anonymous reviewers whose comments significantly improved the
manuscript. Daniel Baker is currently in the Department of Fisheries and Aquaculture at
Vancouver Island University, Nanaimo, Canada.

Grants:

The University of Auckland Faculty Research Development Fund (equipment); the Royal
Society of New Zealand Marsden Fund (to MAS); School of Biological Sciences PBRF fund
(field costs).

Disclosures:

No conflicts of interest, financial or otherwise, are declared by the authors.

References:

1. Belman BW, Childress JJ. Oxygen consumption of the larvae of the lobster Panulirus
   interruptus (Randall) and the crab Cancer productus Randall. Comp. Biochem Physiol

2. Brown JH, Gillooly JF, Allen AP, Savage VM, West GB. Toward a metabolic theory


28. Polymeropoulos ET, Plouffe D, LeBlanc S, Elliott NG, Currie S, Frappell PB. Growth hormone transgenesis and polyploidy increase metabolic rate, alter the...


31. **PreSens.** *Instruction Manual OXY-4, Software Version OXY4v2_11FB.*


41. **Svendsen MBS, Bushnell, PG, Christensen EAF, Steffensen, JF.** Sources of variation in oxygen consumption of aquatic animals demonstrated by simulated constant oxygen consumption and respirometers of different sizes. *J Fish Biology* 88: 51-64, 2016.


Figure legends:

Figure 1: Details of manual intermittent flow respirometer (MIFR).

(A). Photographic image of FTC-PSt3 (PreSens, Germany) used in the MIFR. Note the black O-rings pictured in (B). Scale bar = 15 mm.

(B). Diagrammatic representation of the flow through cell (FTC) showing the polymer optic fibre (POF) that connects to the OXY-4, the integrated planar oxygen sensor (POS), the Luer Lock adapters (LL♂ and LL♀), the O-ring (OR) that seals the POS, and the Nitex filter (NF) internal to the OR. Double NFs were inserted into both ends of the POS to prevent loss of larvae during gravity-fed water changes. When the LL♂ is inserted into the LL♀ the ORs seal the integrated POS (colored pink) so that there is no direct contact of the liquid sample with the plastic of the T-Part of the FTC.

(C). Gravity-fed MIFR showing 60 ml syringe as a “header” tank, connected to 2-way and 3-way stopcocks (SC), and the 5 ml syringe used to ensure air bubbles are not introduced into the FTC. The “header” tank (a 60 ml syringe with the plunger removed) was filled with media at the incubation temperature immediately before use, and was affixed to a backing board with a cable-tie or held in place with an adjustable clamp. Fold-back clips at the inflow and outflow ends are used to create a closed system for $M_{O_2}$ measurements (static volume includes tubing down-stream from inflow fold-back clip, the FTC, and tubing upstream from outflow fold-back clip; volume 100-150 µl).

Figure 2: Validation of the FTC in ‘static’ mode.

(A) Oxygen saturation measured in two independent FTCs containing oxygen depleted autoclaved seawater (DASW) and oxygen enriched autoclaved seawater (ASW). At the end of the 20 minute incubation a sample of the ASW water from outside the NF and OR of the DASW-containing FTC was injected into a third FTC to confirm that there had been no change in $O_2$ saturation (triangle at end of Run 1).

(B) Oxygen saturation measured in a FTC containing seawater depleted of oxygen by bubbling with instrument grade N$_2$. Data are shown from two separate runs (Runs 2, 3). Asterisk adjacent to Run number indicates that there is a significant regression between % $O_2$ saturation and time (see text for details).
Figure 3: **Use of the MIFR in measuring \( M_{O_2} \) in echinoderm embryos/larvae.**

(A) \( M_{O_2} \) during development (hours post fertilization) in *Evechinus chloroticus* (20°C) and *Fellaster zelandiae* (17°C). Mean ± S.E. based on 3-4 FTC at each time point for 3 male/female crosses in *E. chloroticus* (N=11-12 \( M_{O_2} \) measurements per time), and 1-4 FTC at each time point for 4 male/female crosses in *F. zelandiae* (N= 7-16 \( M_{O_2} \) measurements per time). Filled symbols indicate embryological stages; open symbols indicate larvae.

(B) \( M_{O_2} \) in 5-day *Centrostephanus rodgersii* (14°C) larvae after rearing in elevated CO\(_2\). Mean ± S.E. for each CO\(_2\) level based on two male/female crosses and 4-6 FTCs (380, 1000 µatm CO\(_2\), 2 crosses, N=10; 1800 µatm CO\(_2\), 1 cross, N=4). * indicates a significant difference.

(C) \( M_{O_2} \) in 5-day *Fellaster zelandiae* larvae (17°C) sequentially exposed to 380 and 1000 µatm CO\(_2\) seawater. Mean ± S.E. based on four male/female crosses and 4 FTC (N=16). * indicates a significant difference.

(D) Frequency histogram of magnitude of change in \( M_{O_2} \) (1800 minus 380 µatm CO\(_2\)) in the 5-day *Fellaster zelandiae* larvae (17°C) depicted in Panel C (N=16) binned by 5 pmol individual\(^{-1}\) h\(^{-1}\) categories. Although the modal increase in \( M_{O_2} \) was + 5-10 pmol individual\(^{-1}\) h\(^{-1}\) in 1800 µatm CO\(_2\), there was a decrease in \( M_{O_2} \) in one of the trials.

Figure 4: **Validation of media exchange using the MIFR.**

A. Representative trace of change in oxygen saturation (raw data) by 5-day old larval *Fellaster zelandiae* at 20°C in FTC under sequential replacements of media: control, 380 µatm CO\(_2\) (N=3), hypercapnia, 1800 µatm CO\(_2\) (N=2), no larvae. Total media exchanges=5; N=20 larvae in FTC.

B. Decline in \( O_2 \) saturation by 5 day larvae of *Fellaster zelandiae* in control conditions (380 µatm CO\(_2\)) in 3 repeated media exchanges at 20°C. Rates of oxygen consumption were calculated from the slopes of the best fit linear regression (slope and \( r^2 \) shown in legend; \( M_{O_2} \) value to right of graph).
C. Decline in O$_2$ saturation by 5 day larvae of *Fellaster zelandiae* in hypercapnic conditions (1800 µatm CO$_2$) in 2 repeated media exchanges at 20°C. Rates of oxygen consumption were calculated from the slopes of the best fit linear regression (slope and $r^2$ shown in legend; $M_{O2}$ value to right of graph).

Figure 5: **Decline in O$_2$ saturation by 5 day larvae of *Evechinus chloroticus* in four FTC at 20°C** (shown in A-D). For each of the four FTC we show 3 traces representing sequential recordings from a single group of larvae after changing media using the MIFR. Rates of oxygen consumption were calculated from the slopes of the best fit linear regression (slope and $r^2$ shown in legend; $M_{O2}$ value to right of graph). Note that differences in values of the slope between the four FTC are a result of differences in the numbers of larvae incubated.
A

% O₂ Saturation

Run 1 ASW

Run 1 DASW

Time (mins)

B

% O₂ Saturation

Run 2*

Run 3*

Run 3

Run 2*

Run 2*