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Flow-through chamber method for clearance rate measurements in bivalves: design and validation of individual chambers and mesocosm

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Abstract

There is an ongoing discussion in the scientific literature about methodological aspects of clearance rate (CR) measurement with regard to bivalves, especially when the CR is measured by flow-through chamber method. In the present paper, an experimental chamber, a mesocosm system, and a validation protocol have been developed for determining the CR using the flow-through method. The procedure consisted of a preliminary analysis of the fluid dynamics in the interior of the chamber and a statistical analysis of the CR measurement in the mussel *Mytilus galloprovincialis* L at different water inflows. This allowed the performance of the chamber for each flow to be identified. The performance of the chamber for all the flows studied was also modeled simultaneously by means of Ivlev curve. The protocol, applied to an individual cylindrical experimental chamber (ICEC) (radius 71 mm, height 76 mm, volume 1200 mL), established that the ICEC complies with all the requirements for CR measurement using the flow-through chamber method, provided that the percentage of particles cleared is approximately 20% (minimum 13%, maximum 25%). In agreement with the allometric relationship between length and volume of *Mytilus galloprovincialis*, 3 types of ICEC were designed for CR measurement on individuals of 20 to 85 mm length. After validation of the ICEC, the performance of a mesocosm system used regularly by our group (box raft experimental chamber or BREC) was evaluated. Three comparative measurements were carried out for the ICEC and BREC, two in situ and one in the laboratory. No statistically significant differences were observed between the experimental systems for the CR determinations, which validates the BREC for CR measurement using the flow-through chamber method.

Introduction

There is ongoing discussion in the scientific literature regarding clearance rate (CR) determination in bivalves. This has led to the development of numerous methods for measuring the CR (e.g., direct method, flow-through chamber, clearance method, biodeposit method, suction method, InEx method) as well as studies focused on method intercomparison and validation (Widdows 1985; Urrutia et al. 1996; Pouvreau et al. 1999; Navarro and Velasco 2003; Bayne 2004; Petersen et al. 2004; Yahel et al. 2005). Moreover, there is an additional debate regarding the conceptual understanding of the bivalve filtration process, which has been considered to be a process subject to physiological regulation (Hawkins et al. 1996; Bayne 1998, 2001) or as an essentially autonomous process (Jørgensen et al. 1986, 1988; Jørgensen 1990). Recent differences of opinion on this matter have questioned experimental results and ecological projections derived from physiological parameters in bivalve mollusks, in particular for those studies in which the flow-through chamber method has been used to determine CR. When used under optimal conditions, flow-through presents great advantages for in situ measurements, because it permits the use of natural seawater and exhaustive control of experimental conditions.

The measure of the CR in bivalves by means of the flow-through chamber method dates from the early 1970s (Haven and Morales-Alamo 1970; Bayne et al 1971; Widdows and Bayne 1971). Nevertheless, the detailed study of the experimental requirements, and therefore the theoretical base for the suitable use of the flow-through chamber method, was carried out later (Bayne et al 1976; Hildreth and Crisp 1976; Riisgård 1977). Recently, several studies have revised the methodologies of measurement of the CR, emphasizing the reliability or accuracy of CR measurements (Riisgård 2001; Cranford 2001; Widdows 2001) and the intercalibration experiments (Petersen et al. 2004; Bayne 2004; Riisgård 2004).

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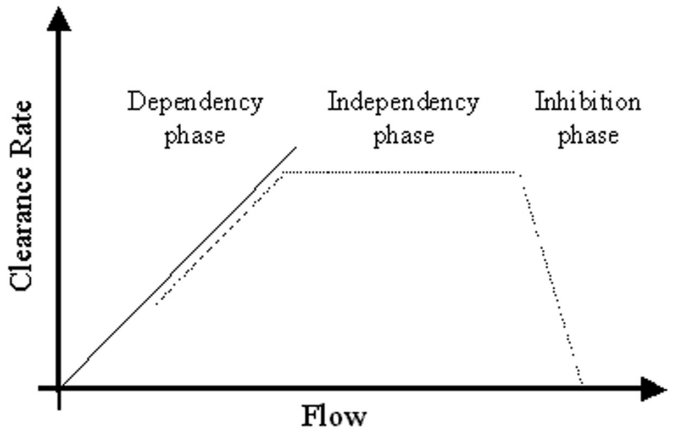

Fig. 1. Performance of an optimal geometry chamber (dashed line). The continuous line represents the points in which the clearance rate is equal to the input flow. At high input flow the CR falls rapidly because of physical stress.

In agreement with the latter studies, the requirements for the suitable use of the flow-through chamber method are that (1) the food crossing the chamber is completely available to the mussel, otherwise the available food will be less than the theoretical value; (2) the geometry of the chamber minimizes water recirculation to prevent dilution of the incoming food concentration; and (3) the food is completely retained by the gills to reduce underestimation of the measurement. On this way, the relationship CR vs. inflow of an optimal geometry chamber would show a characteristic pattern (Figure 1). Therefore, the suitability of the experimental chamber could be tested by comparing the experimental relationship with the expected by the optimal geometry chamber.

In recent years, our group has repeatedly used the flow-through chamber method with the individual cylindrical experimental chamber (ICEC) (Iglesias et al. 1996; Navarro et al. 1996; Labarta et al. 1997) and box raft experimental chamber (BREC) (Babarro et al. 2000; Pérez Camacho et al. 2000), a mesocosm designed to measure the CR in situ. From the point of view of the debate about methodological aspects, in the present survey we propose a way for designing experimental chambers and a protocol for evaluating them for CR measurement with the flow-through chamber method. In addition, chamber design as a function of the mussel size-volume relationship is discussed, as well as the discussion between the equations to calculate the CR.

Materials and procedures

Experimental system: ICEC—A cylindrical chamber of 1200 mL volume (radius 71 mm, height 76 mm) was designed with a water inflow in the lower part and water outflow in the upper opposite side (Figure 2A). Validation studies and CR estimations were made on mussels of 60 mm length.

The mussel was placed in the chamber so that the input flow was directed on the inhalant aperture and the exhalant aperture was directed toward the water outflow, thus preventing refiltration. In each of the experiments, a chamber without mussel served as a blank for the calculation of the CR.

The food supply to the chamber depended on the required flow:

- Flows < 200 mL min^{-1}: Ismatec MCP standard peristaltic pump equipped with a 12-channel pump head (model CA).
- Flows > 200 mL min^{-1}: Waterfall distribution system (Figure 2A) directly connected to a submersible pump (Eheim type 1261, maximum flow 3400 L h^{-1}). This system allows simultaneous use of 16 chambers.

Experimental mesocosm: BREC—The mesocosm (rectangular box: 45 by 40 by 14 cm) of 19 L capacity has PVC tubing bordering the upper part of the chamber, which distributes the input flow uniformly (3 L min^{-1}). The only water outflow is situated in the upper part of the box. A frame with 16 individual spaces is placed in the box interior, each holding an individual mussel. In each experiment session, 3 boxes are needed, 2 with individuals and 1 with no mussels as a blank to calculate the CR (Figure 2B). The 3 boxes take the diet from a common tank

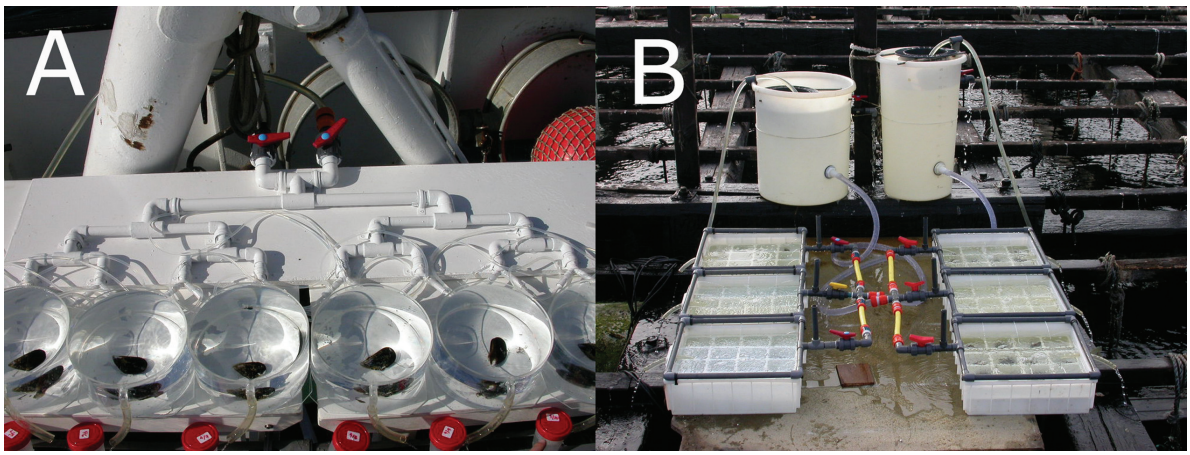

Fig. 2. (A) Individual cylindrical experimental chamber and waterfall distribution system with 16 outlets. (B) Box raft experimental chamber. Six boxes grouped in a number of 3 joined to the food tank.

situated at a higher level, therefore distributing food by gravity.

Basis to validation protocol of the ICEC—In this work, an experimental chamber is considered valid for determining CR using flow-through chamber method if the CR performance for different input flows corresponds with the optimal geometry chamber. The CR performance for different input flows in an optimal geometry chamber consists of 2 phases:

- Dependency phase of the CR with the input flow at low water flow (CR = flow). For testing the chamber suitability, the experimental results are fitted to a linear regression model II, and the slope of the fit is compared (Zar 1984) with the theoretical slope ($\beta = 1$) of the optimal geometry chamber. The chamber is considered to comply with the requirements of an optimal geometry chamber when there are no significant differences between the slopes.
- Independency phase, during which CR deviates from CR = flow above a critical flow and tends to form a plateau. At this point, the CR is representative of the maximum that a bivalve can undertake. For testing suitability, the experimental results are fitted to a linear regression model II. If the regression is not statistically significant, the chamber has optimal performance. The flow employed must be sufficiently high to satisfy the filtration requirements of the bivalve, and sufficiently low to avoid an excessive dilution of the outflow, which could hide differences between inflow and outflow because of analytical error of the Coulter. Therefore, we propose to use flows that allow at least a percentage of particles cleared of 10%, since a higher flow (lower percentage) could result in a loss of accuracy.

When suitability of the chamber performance is not strictly fulfilled, the identification of the critical flow is complicated due to the transitional phase between the 2 phases, rather than a rapid change. In this case, a protocol is proposed for the identification of the phases based on the grouping of the cases in discrete groups as a function of the input flow. The CR measurements of the groups are compared, identifying groups with similar performance in the CR vs. input flow relationship as homogeneous subgroups. The homogeneous subgroups are assigned to the distinct phases as follows:

- Dependency phase: assigned to homogeneous subgroups whose linear regression model II fit is indistinguishable (Zar 1984) from the theoretical slope ($\beta = 1$).
- Independency phase: assigned to homogeneous subgroups that employ larger flows and whose fit to a linear regression model II is not statistically significant. The flows should be sufficiently high to satisfy the maximum requirements of the bivalve (10% percentage of particles cleared). Additionally, the range of flows within the homogeneous subgroup should be sufficiently wide to avoid statistical artifacts. It is proposed that minimum inflow of the homogeneous subgroup is 66% of the maximum flow as the highest value; in other words, 33% of the flows should be within the plateau.
- Transitional phase: assigned to the remaining homogeneous subgroups.

Invalidation of the chamber design for use in the flow-through method arises when no homogeneous subgroup can be assigned to the dependency or independency phase.

Basis to validation protocol of the BREC—The validation of the CR results for the BREC was performed by comparison with those obtained for the ICEC.

Clearance rate—The CR was estimated from the reduction in particulate concentration, measured as particulate volume ($mm^3\ L^{-1}$), between the water surrounding the mussel and the outflow of the experimental chamber. The particulate concentration was measured in triplicate with an electronic Multisizer Coulter II counter with a 100-μm orifice. The lower limit for particulate detection was set at 3.1 μm, which includes practically all the diet offered. *Isochrysis galbana* has a diameter of 4.7 ± 1.15 μm, and 99% of the particulate volume of the pulverized sediment was between 3.1 and 50 μm. The equation of Hildreth and Crisp (1976) was used for the calculation of the CR:

$$CR = f \times [(C_i - C_o) / C_s] \quad \text{(Eq. 1)}$$

where CR is the clearance rate (mL min^{-1}), f is the flow through the experimental chamber (mL min^{-1}), C_i and C_o are the particulate concentrations in the inflow and outflow of the chamber, respectively, and C_s represents the concentration surrounding the mussel. Assuming the chamber to be theoretical, C_s is equal to C_i, since all the inflow is available to the individual and recirculation is absent (Eq. 2: $CR = f \times [(C_i - C_o) / C_i]$). Hildreth and Crisp (1976) suggest that if C_s cannot be measured in the experimental chamber, it may be more correct to assume that C_s equals C_o (Eq. 3: $CR = f \times [(C_i - C_o) / C_o]$). When construction of an experimental chamber without recirculation is not possible, Widdows (1985) proposes that the CR can be calculated with Eq. 3. This equation is valid at low inflows since (1) the mussel can remove particulates at a greater velocity than their addition to the chamber (Wildish and Kristmanson 1984), and (2) at low inflow, recirculation gains more importance, implying that C_s tends to approximate C_o rather than C_i. Therefore, Eq. 2 was used for verification of chamber suitability in our case, which a priori assumes theoretical performance of the chamber.

Standardization of the CR—In all cases, CR was standardized to a mussel size of 60 mm length with the following formula:

$$CR_{std} = CR_{exp} \times (L_{std}/L_{exp})^b$$

where CR_{std} is the standardized clearance rate, CR_{exp} is the experimental clearance rate, L_{std} is the standardization size, L_{exp} is the size of the experimental individual, and b is the exponent relating the clearance rate with size. In this study, a value of 1.72 was employed as a size standardization exponent of the CR (unpublished data). This allometric exponent differs from those in the literature and those obtained with different methods for CR measurement (2.14, Kiørboe and Møhlenberg 1981; 1.57, Pérez Camacho and González 1984; 2.19, Jones et al. 1992).

Experimental conditions—The mussels used for the experiments, *Mytilus galloprovincialis* (Lamarck 1819), were collected

Table 1. Characteristics of the experimental diet used in the laboratory.

Experiment	TPM, mg L^{-1}	POM, mg ORG L^{-1}	%ORG	Part. Vol. (mm^3 L^{-1})
1	1.2 ± 0.01	0.6 ± 0.03	52.0 ± 2.20	1.0 ± 0.12
2	1.3 ± 0.03	0.7 ± 0.01	51.4 ± 1.44	1.0 ± 0.18
3	1.3 ± 0.05	0.7 ± 0.02	51.6 ± 0.96	0.9 ± 0.03
Mean	1.3 ± 0.05	0.6 ± 0.04	51.6 ± 1.42	1.0 ± 0.15

TPM indicates total particulate matter; POM, particulate organic material; %ORG, percentage organic material; Part. Vol., particulate volume

from a cultivation raft in the Ría de Arousa (Galicia, NW Spain). Individuals of 60 mm length were selected (61 ± 2.1 mm length and 1.3 ± 0.37 g dry meat weight) and epibionts removed. The mussels were maintained for 7 days in 19-L tanks with 20 to 25 individuals per tank. The tank was of open-flow design using filtered (10 μm) seawater (Cartridge CUNO Super Micro-Wynd 10 μm), between 15 and 16 °C and 35.5‰ salinity. The filtered seawater was enriched with a mixture of microalgae (Tahitian *Isochrysis aff. Galbana*, T-ISO) and sediment from the seafloor below the rafts (40:60 microalgae:sediment, by weight) supplied with a peristaltic pump at constant flow, so that particulate material load was maintained at 1.2 mg L^{-1} with a percentage organic content of 50%, which simulates the average diet observed in the Ría de Arousa. The same diet was used during the experiments (Table 1).

The seawater used for the preparation of the diet was filtered with a cartridge filter system (CUNO Super Micro-Wynd 10, 5, and 1 μm; CUNO Betapure 0.5 μm) with an effective mesh size of 0.5 μm, and treated with ultraviolet light. The diet was maintained in an aerated tank to generate a homogeneous mixture and prevent sedimentation.

The total (TPM) and organic (POM) content of the diet was determined gravimetrically on Whatman GF/C 25-mm ashed filters (450 °C for 4 h). After filtering in triplicate 1 L of diet, the salts were eliminated by washing with 100 mL isotonic solution of 0.5 M ammonium formate. Subsequently, the filters were dried at 110 °C for 24 h and weighed to determine the TPM. The determination of POM was carried out after ashing the filters for 4 h at 450 °C. A Sartorius micro M3P analytical balance was used.

Statistical analysis—Several *t* tests for homogeneous variance were performed. When homogeneity of variance was rejected (Levene test), a *t* test was carried out for nonhomogeneous variance (Snedecor and Cochran 1989). Statistical analyses were carried out with the computer package SPSS 11.5.

Assessment

Individual Cylindrical Experimental Chamber—**Study of the fluid dynamics.** The fluid dynamics in the interior of the chamber was simulated using a mathematical computer program (Fluid Flow Analyzer 4 [Fluids4], Raczynski Consulting, Raczynski 2003). Fluids4 resolves the Navier-Stokes equation for an incompressible liquid flowing through a 3D channel with obstacles. The model simulated the experimental chamber in which an ellipsoidal obstacle was placed to simulate the mussel in the center of the chamber. The obstacle was placed perpendicular to the flow to simulate an orientation with the inhalant and exhalant apertures toward the inflow and outflow, respectively. The simulated fluid density and viscosity were set to seawater values (1000 kg m^{-3} and 0.001 kg m^{-1} s^{-1}, respectively). Constant fluid flows (50, 100, 200, 400, and 800 mL min^{-1}) were used as boundary conditions for each of the 5 simulations.

Figure 3 shows fluid vorticity for the different flows simulated with Fluids4 in the vertical section of the experimental

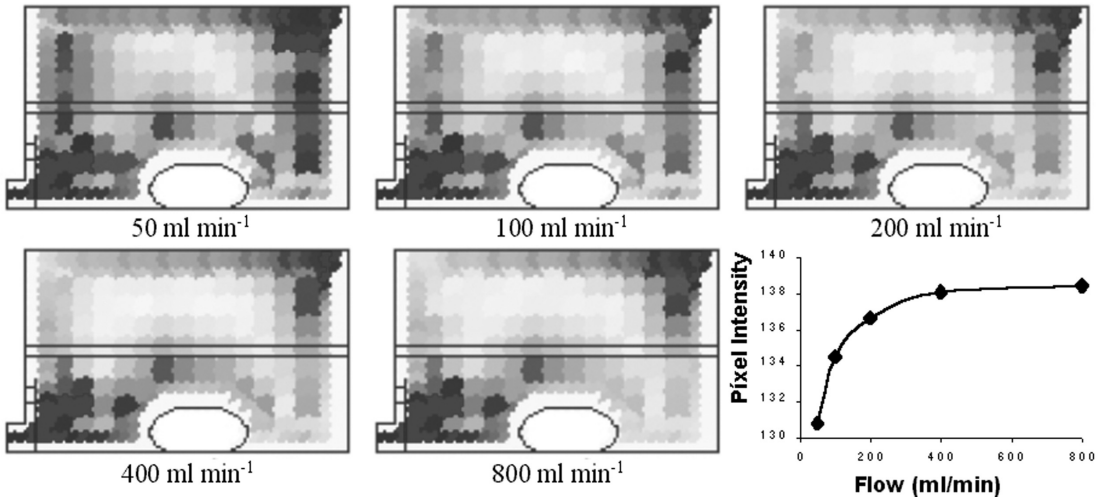

Fig. 3. Vorticity in the vertical section of the experimental chamber that includes the inflow and outflow for different flows (50, 100, 200, 400, and 800 mL min^{-1}) simulated with Fluids4. The vorticity scale and pixel intensity are described in the text. The horizontal lines in the middle of the chamber represent the principal duct, which is necessary to design the model; nevertheless, it is only a representation, not a physical duct.

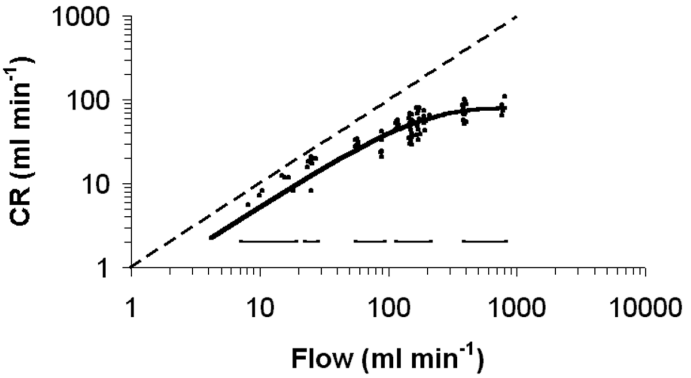

Fig. 4. Clearance rate (CR) vs. water input flow in the ICEC. The dashed line represents the points in which the CR equals input flow. The horizontal lines represent the limits of the homogeneous subgroups formed from the *t* test (Table 2), and the continuous line represents the fit to an Ivlev function ($CR = 78.87 \times [1 - e^{-0.00685 \times Flow}]$; $n = 88$, $r^2 = 0.784$, $P < 0.001$; normality of the residuals: $Z = 0.762$, $P = 0.608$).

chamber that includes both inflow and outflow. The vorticity scale is represented by a gray scale that ranges from 0 (white) to 1.8×10^{-3} s^{-1} (black). The graphical outputs were analyzed using Adobe Photoshop 6.0.1, and the average pixel intensity was measured. The pixel intensity is measured in an arbitrary scale that ranges from 0 (black) to 255 (white). Therefore, an inverse relationship between pixel intensity and vorticity could be established. The relationship pixel intensity vs. flow shows that turbulence progressively disappears as the input flow increases, obtaining a very similar solution for the 2 large flows of 400 mL min^{-1} and 800 mL min^{-1}. Therefore, in a preliminary way, these results indicate that the chamber could be suitable for the CR measurement at higher flows than 400 mL min^{-1}. This flow is the threshold for the turbulence decrease, and therefore, the minimum flow that satisfies the requirements of the optimal geometry chamber.

CR measurement at different water inflows. In total, 19 experiments were performed, each with a different input flow between 8.25 mL min^{-1} and 812 mL min^{-1}. A total of 88 mussels were analyzed. Each experiment lasted 3 h, and samples were taken after 2 and 3 h for the CR measurement. The first hour was considered to be a stabilization period.

Figure 4 shows the CR obtained for the various flows, whereby the dashed line represents CR equal to input flow. From this figure the critical flow separating the dependency and independency phase cannot be determined. Therefore, the identification of the flow range associated with each phase was carried out with the protocol described previously. The flows are combined in discrete groups (Table 2), and their CRs are compared to identify homogeneous subgroups and simplify posterior analyses. The data do not fulfill the ANOVA assumptions, therefore the CR between groups is compared with *t* test (Bonferroni correction has not been applied because it protects excessively against the possibility of rejecting erroneously some of the null hypotheses at the expense of diminishing the power of the test; see Perneger 1998) having established 5 homogeneous subgroups (Table 2 and Figure 4). The cases belonging to homogeneous subgroups 1 and 2 are significantly fitted to $CR = 0.82 \pm 0.08 \times flow - 3.29 \pm 1.61$ ($r^2 = 0.70$, $P < 0.001$), whose slope does not differ statistically from the theoretical slope ($\beta = 1$) of an optimal geometry chamber ($P < 0.05$). Therefore, these homogeneous subgroups are associated with the dependency phase of an optimal geometry chamber. The cases in homogeneous subgroup 5 (flow > 381.3 mL min^{-1}) do not fit significantly to a straight line ($CR = 0.02 \pm 0.02 \times flow + 63.92 \pm 10.1$, $r^2 = 0.07$, $P = 0.260$), and this homogeneous subgroup is associated with the CR vs. input flow independency phase. Homogeneous subgroups 3 and 4 are associated with the transitional phase, which indicates that the experimental chamber does not fit completely to an optimal geometry chamber.

The description of the optimal geometry chamber performance as composed by 2 linear dependent and independent phases separated by a singular critical level is based on theoretical arguments. To compile the information of all the flows tested, and give a better description of the real performance of the chamber, the experimental results of the CR vs. input flow relationship are fitted to an Ivlev exponential function (Figure 4). The fit indicates that the CR reaches an asymptotic value of

Table 2. Number of mussels (*n*), and mean, minimum, maximum flow and homogeneous subgroup.

Groups	*n*	Mean, mL min^{-1}	Minimum, mL min^{-1}	Maximum, mL min^{-1}	Homogeneous subgroup
1	10	14.0 ± 4.19	8.3	18.3	1
2	9	25.3 ± 1.13	23.7	27.7	2
3	7	57.6 ± 1.12	56.3	59.0	3
4	5	89.6 ± 0.60	88.7	90.0	3
5	7	118.4 ± 2.77	115.3	122.7	4
6	13	151.1 ± 3.14	146.7	157.3	4
7	10	172.7 ± 3.27	168.0	178.0	4
8	7	195.7 ± 8.10	190.0	212.7	4
9	15	394.0 ± 9.15	381.3	408.0	5
10	5	787.2 ± 15.07	772.0	812.0	5

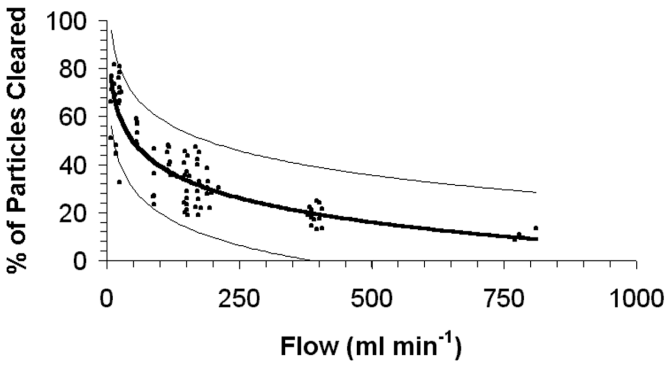

Fig. 5. Percentage of particles cleared as a function of the input flow in the ICEC. The equation of the logarithmic fit (thick line) is described in the text. The fine lines represent the 95% confidence interval.

78.87 mL min^{-1}, which is not significantly different from the CR in the homogeneous subgroup 5 (Table 2, group 9: $t = 1.604$, df = 14, $P > 0.05$; group 10: $t = 0.298$, gl = 4, $P > 0.05$). This corroborates that at flows > 381.3 mL min^{-1} there is no dependency of CR on input flow. In other words, the maximum filtration capacity of the 60-mm mussel is reached, and therefore the CR measurement is representative of the real CR.

Following the criteria proposed, the chamber complies with the requirements for CR measurement using the flow-through chamber method. The results show the existence of deviations with respect to theoretical characteristics, however:

(1) In the dependency phase, CR is expected to be equal to input flow because at low inflow, recirculation (perhaps generated by the individual mussel) may increase the phenomenon of food concentration reduction due to refiltration (Newell et al. 2001). Nevertheless, in our study the CR is less than the input flow, which could be from the bypass produced when part of the input flow directly falls on the animal.

(2) A transitional phase is observed rather than the expected rapid behavioral change. This phenomenon is a consequence of the bypass, which causes the input flow to be insufficient to satisfy the maximum filtration capacity. The effect decreases as the adequate flow rate is approached.

The existence of a bypass brakes the characteristics of an optimal geometry chamber, although it contributes to the disappearance of recirculation. In fact, a high bypass could reduce the recirculation to negligible levels (Larsen 2001). The bypass could become problematic if the particulate concentrations at the chamber inflow and outflow were similar, which would decrease the accuracy of the measurement (Larsen 2001). Such a scenario could arise if the analytical error of the Coulter counter were larger than the mussel-induced reduction in particulates. However, in the experimental conditions that we describe as optimum (flow between 381.3 and 408.0 mL min^{-1}, group 9, Table 2), the inflow and outflow concentrations were statistically different in all cases (t test: in 5 cases $P < 0.05$, in 20 cases $P < 0.001$). Consequently, the analytical error is insufficient to modify the CR. Therefore, the high inflow and the consequent bypass contribute to adequately reduce the recirculation, acting like a physical barrier, which prevents the mussel from recirculation currents.

As has been demonstrated with the described protocol, the experimental chamber complies with the requirements for CR measurements. Nevertheless, to check the correct use of the experimental system during the experiments, the percentage of particles cleared could be used as internal control of the chamber's performance. The percentage of particles cleared and the 95% confidence intervals shown in Figure 5 are fitted to a logarithmic function, % particles cleared = –14.66 × Ln (Flow) + 107.05 ($n = 88$, $r^2 = 0.753$, $P < 0.001$; normality of the residuals: $Z = 0.946$, $P = 0.332$). Based on the fit in Figure 5 and the range of flows of the homogeneous subgroups in Table 2, a percentage of particles cleared of 19.9% (about 20%) is obtained for a flow of 381.3 mL min^{-1} (the minimum flow for the independency phase of CR vs. input flow). This percentage of particles cleared would be the maximum permitted to maintain independency of CR on input flow. We suggest the interval 13% to 25% as confidence intervals, minimum and maximum observed in the experiment for a flow of 381.3 mL min^{-1}. The 95% confidence interval reported by the adjustment was rejected because is influenced by the wide dispersion observed at lower flows.

The validation protocol of an experimental chamber for CR measurement using the flow-through chamber method is based on a preliminary analysis of the fluid dynamics in the interior of the chamber and on statistical analysis. The results establish that the proposed experimental chamber (radius 71 mm, height 76 mm, and volume 1200 mL) is valid for CR measurements using the flow-through chamber method, provided that the percentage of particles cleared is

Table 3. Characteristics of the individuals and the diet employed in each of the three comparative experiments (1 and 2 in natural medium and 3 in the laboratory) for the 2 experimental systems.

Exp	Weight, g	Size, mm	TPM, mg L^{-1}	POM, mg ORG L^{-1}	%ORG	Part. Vol., mm^3 L^{-1}
1	0.40 ± 0.110	45.8 ± 1.01	1.2 ± 0.43	0.6 ± 0.19	48.5 ± 5.60	1.5 ± 0.33
2	1.10 ± 0.356	65.6 ± 0.96	1.2 ± 0.43	0.6 ± 0.19	48.5 ± 5.60	1.5 ± 0.33
3	0.81 ± 0.297	60.7 ± 1.49	1.1 ± 0.27	0.6 ± 0.20	48.8 ± 7.14	0.9 ± 0.07

Weight indicates dry meat weight; TPM, total particulate matter; POM, particulate organic matter; %ORG, percentage organic material; Part. Vol., particulate volume.

Table 4. Comparison of the clearance rate of the ICEC and the BREC. The mean value and typical deviation of the clearance rate, the number of replicates (*n*), and the *t* test results are shown: *t* value (*t*), degrees of freedom (d.f.) and probability (*P*).

Experiment	System	CR, mL min^{-1}	*n*	*t* test *t*	d.f.	*P*
1	ICEC	75.0 ± 13.00	8	0.716	14	0.486
	BREC	80.0 ± 19.67	8			
2	ICEC	70.0 ± 20.17	6	0.917	12	0.377
	BREC	61.7 ± 11.17	8			
3	ICEC	51.7 ± 7.50	7	1.778	8.998	0.109
	BREC	56.7 ± 4.00	4			

around 20% (13% to 25%). This value is lower than that proposed by Hawkins et al. (1999), Smaal and Widdows (1994), and Cranford and Gordon (1992). From theoretical arguments, Larsen (2001) also suggests the maximum reduction of the outflow concentration with respect to the inflow should be 30%.

Box raft experimental chamber—The validation of the mesocosm was carried out after validation of the individual chamber. Three similar experiments were carried out under different conditions, simultaneously using the ICEC and the BREC systems. The experimental conditions of the 3 tests are summarized in Table 3. The first 2 experiments were performed in the sea, with no acclimation period or experimental diet. The third experiment was carried out in the laboratory, employing the diet described above.

Table 4 summarizes the results of the 3 comparative experiments between the ICEC and BREC systems. No statistically significant differences in the CR measurements (Table 4) were obtained between the ICEC and the mesocosm system (BREC, 45 × 40 × 14 cm). Therefore, the BREC design thus fulfils the same requirements to estimate the CR using the flow-through chamber method.

ICEC scaling. The validity of the CR measurement is implicitly based on a percentage of particles cleared between 13% and 25%. Because CR depends on the size of the organism

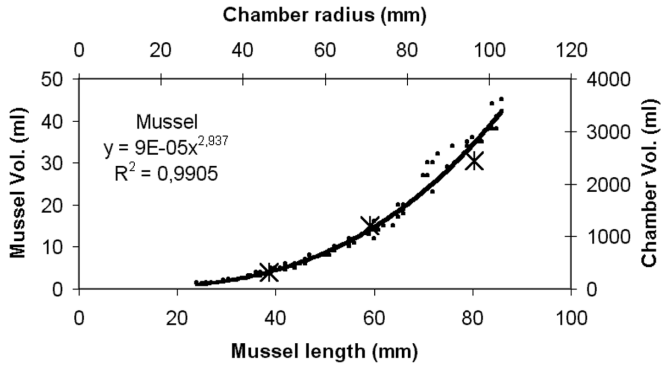

Fig. 6. Allometric relationship of mussel volume (mL) vs. mussel size (mm) for ICECs (asterisks) designed for different mussel sizes.

Table 5. Mussel size range (mm) and the radius (mm) and volume (mL) of the experimental chambers.

Mussel size range, mm	Chamber radius, mm	Approximate chamber volume, mL
25 to 45	46.5	300
50 to 65	71.0	1200
70 to 85	96.5	2400

or, more specifically, on the gill surface area, different inflows are required as a function of mussel size. However, the use of different inflows could cause deviations from optimal geometry chamber performance. Besides, the use of small chambers with large mussels may cause "wall effects," which implies an overestimation of CR as a consequence of the increase of the mixture within the chamber caused by the exhalant siphonal current (Ackerman 1999). Therefore, a further 2 chambers of different dimensions (radius and volume) are proposed which permit optimal experimental conditions to be applied to a wide range of sizes (25 to 85 mm). The dimensions of these chambers are determined from the allometric size-volume relationship of the mussel (Figure 6), and from this relationship the adequate dimensions of the experimental chambers for different mussel sizes are obtained (Table 5).

The application of the experimental conditions described in this work for the ICEC allow the CR of *Mytilus galloprovincialis* to be experimentally determined under optimum conditions over a size range of 25 to 85 mm, provided that the flow used is adjusted so that the percentage of particles cleared is around 20% (13% to 25%).

Discussion

The main reason for conflicting data on filtration rates of bivalves is partly due to use of different methods. Therefore, the design and development of a validation protocol to check the flow-through chamber would clarify doubts on the methodological aspects to obtain robust and reliable data. Doubts about methodology actually impede a fluid discussion regarding the conceptual understanding of the bivalve filtration process. Besides, the validation protocol would allow to analyze previous works, validating or rejecting the methodology in each case.

On the other hand, the mesocosm validation would allow establishment of a system designed especially to measure the CR in situ. The validation of the mesocosm—carried out under controlled experimental conditions and based on a chamber that complied with the requirements for CR measurements using flow-through chamber method—gives rigor to the validation process to obtain reliable data in the field. In this way, Yahel et al (2005) developed the InEx method, a new method for measuring the CR in situ; however, the methodology does not seem suitable for organisms without siphons such as *Mytilus* genus.

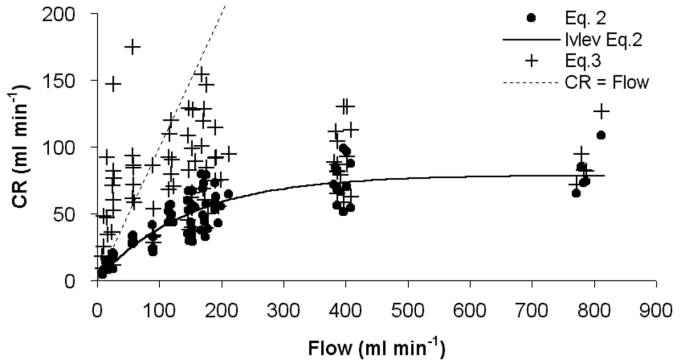

Fig. 7. Clearance rate (CR) vs. input flow in the ICEC. The dashed line represents the points in which the clearance rate equals input flow. The circles represent the CR calculated with Eq. 2. The continuous line shows the Ivlev fit of the results using Eq. 2 (see Figure 4). The crosses represent the CR calculated with Eq. 3.

Comments and recommendations

Flow-through chamber method or steady-state method?—The flow-through chamber method is based on the principles of optimal flow with no recirculation of filtered water, whereas the steady-state method is based on momentary mixing of exhalant water in the whole water volume of the flow-through chamber. Because both methods could be used in the same kind of chamber, it is necessary to validate the chamber performance for discerning between the correct equation to use, flow-through chamber (Eq. 2: $CR = f \times [(C_i - C_o) / C_i]$) or steady state (Eq. 3: $CR = f \times [(C_i - C_o) / C_o]$). An a priori election of the equation without an experimental check could cause error in the CR estimates.

Figure 7 represents the CR obtained in our experiments using both equations, as well as the Ivlev fit for the CR obtained with Eq. 2. It is clear that Eq. 3 increases data dispersion, especially at low flows. Table 6 shows the coefficient of variation (CV) of the CR of the groups described in Table 2 for Eq. 2 and Eq. 3. The CV show a considerable decrease of the precision with Eq. 3, particularly at low flows which should, however, facilitate homogeneous mixing by increasing the water residence time in the chamber. Due to the large dispersion in our data using Eq. 3 as demonstrated in Figure 7, constant CR for all the flows tested cannot be verified. Based on these results, where there was no observable momentary mixing of the water in the chamber, we conclude that the CR calculation in the experimental chamber described should employ Eq. 2 ($CR = f \times [(C_i - C_o) / C_i]$), the correct equation for the flow-through chamber method.

Table 6. Coefficient of variation of the CR using Eq. 2 and Eq. 3 for the groups described in Table 2.

Groups	CV Eq. 3	CV Eq. 2
1	78	31
2	55	22
3	44	9
4	51	30
5	22	11
6	44	30
7	43	32
8	22	17
9	24	20
10	23	21

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