A method for the continuous monitoring of active and passive ion transport in aquatic animals

Signal analyses
Ion permeability
Passive ion fluxes
Active ion fluxes

Analyse de signaux
Perméabilité aux ions
Flux passifs d'ions
Flux actifs d'ions

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ABSTRACT

A mathematical procedure for concentration-signal analyses is developed, which makes it possible to monitor continuously various ion exchange characteristics of aquatic organisms.

Changes of medium ion concentrations can be described as being the result of passive and active transport processes, in which the passive transport depends on the permeability and the concentration difference between the extracellular fluids and the medium. The unknowns, active flux and permeability, can be determined from time series of medium ion concentrations.

The requirements for such calculations are continuous, precise measurements of ion concentrations in a fixed volume of medium containing the animal. Either conductivity (total ion concentration) or single ion measurements (with ion-selective electrodes) may be made.

Autocorrelation of subsequent measurements during a certain period of time yields information on the ion permeability and the active ion flux. Other exchange characteristics (*e.g.* passive ion flux; net ion flux; concentration gradient across the body wall; biological residence time for an ion; half time for adaptation to a change in external ion concentration; area-specific ion permeability; energy requirements for ion transport) can subsequently be derived.

The method may prove to be useful in the characterization of the functioning of aquatic organisms in their normal environments (if desired in comparison with that in polluted environments).

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RÉSUMÉ

Une méthode de mesure continue des flux passifs et actifs d'ions chez les animaux aquatiques

Cet article présente les développements mathématiques d'une méthode d'analyse qui permet de suivre, de manière (semi-) continue, l'évolution des caractéristiques d'échange de diverses espèces ioniques chez les organismes aquatiques.

Les variations des concentrations ioniques du milieu environnant y sont décrites comme le résultat de mouvements passifs et de l'activité de mécanismes de transport actif, les mouvements passifs dépendant des propriétés de perméabilité et du gradient de concentration entre les fluides extracellulaires et le milieu. Les inconnues que sont le flux actif et la perméabilité peuvent être déterminées par la mesure des concentrations ioniques environnantes en fonction du temps.

Ces calculs nécessitent des mesures précises et intermittentes des concentrations ioniques d'un volume déterminé du milieu qui contient l'animal. Les mesures consistent en des déterminations de conductivité (pour la concentration ionique totale) ou de concentrations d'espèces ioniques individuelles (au moyen d'électrodes sélectives).

Les informations sur la perméabilité et sur les flux actifs d'ions proviennent d'autocorrélations établies entre des séries de mesures réalisées sur certaines périodes de temps. En outre, d'autres caractéristiques d'échanges peuvent également être dérivées, comme, par exemple, le flux passif d'ions, le flux net d'ions, le gradient de concentration au

niveau de la paroi du corps, le temps de résidence biologique d'une espèce ionique donnée, la période (demi-durée) d'adaptation à une variation de la concentration ionique environnante, la perméabilité spécifique à une espèce ionique, le coût énergétique du transport ionique.

La méthode s'est avérée bien adaptée à la définition des caractéristiques fonctionnelles d'un organisme aquatique dans son milieu normal (en comparaison par exemple avec celles rencontrées dans un environnement pollué).

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INTRODUCTION

Aquatic organisms continuously extract chemical substances from their environment which are used for growth, maintainance and as a source of energy for vital activities. By the action of internal biochemical processes, numerous chemical conversions take place and after some time the absorbed substances will leave the organism again. Solute fluxes are partly driven by passive (diffusion) processes and partly by active (*viz.* energy consuming) processes. The biochemical conversions at the cellular level require very stable ionic conditions. The ion fluxes are therefore controlled in such a way that in all organisms the intracellular ion composition remains extremely constant. In most organisms, also, the extracellular ionic composition is strongly stabilized. Lower invertebrates, especially those inhabiting marine and estuarine environments, show the weakest regulation of their extracellular body fluids under conditions of osmotic challenge.

In principle, the exchange of solutes takes place anywhere across the body surface area. In metazoa, however, the exchange has become more or less confined to specialized areas of the body surface, *e.g.* the digestive tract, gills and excretory organs. When an organism is exposed to a change in external ion concentration, if the extracellular ion concentrations are not stabilized completely, water, dissolved ions and also organic solutes will move across the body wall. In a closed volume the solute exchange will be reflected in (small) changes in the medium concentrations. Accurate analyses of the medium concentrations will permit determination of the net flux of a solute (provided that no substantial movements of water between animal and medium occur).

Also, when an organism is completely adapted to a certain environment there is often no perfect balance in the solute fluxes, especially when a certain gradient across the body wall is maintained and passive fluxes are counterbalanced by active, metabolically determined, fluxes. Then one may frequently observe a certain net flux which after some time is balanced by a net flux in the opposite direction. This phenomenon can be explained by the different routes a solute takes for entering and leaving the body. If, for instance, a continuous salt input via the gills is compensated by the output of salts via the excretory organs the temporary accumulation of urine in the bladder will delay the output. Although over a longer period the output may be in equilibrium with the input, the momentary output will not continuously balance the input. In hyperregulating estuarine species, such as shore crabs, adapted to a hypotonic medium, one may therefore observe, even when the animals are completely adapted to their medium, a continuous net salt influx, from time to time interrupted by sudden events of net salt efflux (the production of urine isotonic to the blood).

The present paper describes a method for the analyses of the ion concentrations kinetics in a closed volume of medium containing an animal. The presence of momentary net fluxes make it possible to characterize the active and passive fluxes separately.

MATERIAL AND METHODS

Animals, measuring chamber

Shore crabs, *Carcinus maenas* (L.) were collected in the western part of the Dutch Wadden Sea, near the island of Texel. In the laboratory the animals, males as well as non-oviferous females, with weights varying between 10 and 40 g, were placed in natural sea water (salinity about 28).

For the measurements individual specimens were taken from the storage container, wiped dry and weighed (to the nearest milligram and transferred to a thermostated measuring chamber containing a known volume (200 ml) of seawater with a salinity equal to that to which the animal was adapted. After a few minutes of acclimatization the salinity in the measuring chamber was decreased about 6 by replacing a certain medium volume with demineralized water.

Data collection

After dilution of the medium and a short (2 mn) incubation time for complete mixing, removal of adhering salts and temperature equilibration, the medium salinity was recorded automatically during 2.5 mn periods with a sampling frequency of 1 measurement per 5 s. Medium salinity was measured by means of a flow-through conductivity electrode (Philips, type PW 9513) connected to a Wayne Kerr (type B 642) conductivity bridge reaching a sensitivity of 0.01%. The conductivity cell was continuously flushed with the medium using an Eheim aquarium pump at a rate of 4 l/mn.

Temperature fluctuations in the measuring chamber never exceeded 0.1°C. With the temperature coefficient for sea water (1.80%/K), the actual temperature fluctuations only slightly affected the fourth digit of the conductivity reading. Most modern laboratory conduc-

tivity meters can give a reading of 3 to 4 significant digits and offer a possibility for automatic temperature compensation. Their sensitivity can be further increased by using a reference electrode in the measuring chamber, which also perfectly compensates temperature fluctuations.

Data analyses

In the general procedure for the concentration-signal analyses as given below, the term "salt" may also read "ion" if, instead of measuring the total ion concentration by means of a conductivity electrode, the activity of a specific ion is measured by means of a ion selective electrode. The results described in the next section are obtained using a conductivity electrode.

Derivation of permeability and active fluxes

When an animal, kept in a closed medium volume, loses salts from its internal body fluids, then the concurrent increase in the medium salinity multiplied with the medium volume, *viz.* the increase in the salt content (Q_e) of the medium, will equal the net salt loss of the animal. In (probably all) multicellular animals, the intracellular ion composition will remain approximately constant as changes in the extracellular osmolarity are intracellularly compensated by organic compounds. This intracellular osmotic adaptation probably occurs rapidly, at rates conducive to the production of intracellular osmotic pressure changes matching those occurring extracellularly to prevent cellular shrinkage or swelling by osmotic transfer of water. Therefore, the extracellular body fluids respond, by good approximation, as a single compartment (*e.g.* Comar and Brunner, 1967; Simon, 1986). In most regulating species, but especially in Crustacea (having a rigid exoskeleton), body volume changes due to water movements between animal and medium are very limited. A change in the salt content of the medium (dQ_e/dt) will then be the result of passive (J_p) and active (J_a) fluxes:

$$dQ_e/dt = J_p + J_a. \tag{1}$$

The passive salt flux follows from the product of the permeability (p) and the concentration difference ($C_i - C_e$) across the body wall. Adopting a sign convention assigning, quite arbitrarily, positive values to fluxes from the animal to the medium, we can write:

$$dQ_e/dt = p(C_i - C_e) + J_a. \tag{2}$$

During the experiment, the total salt content in the extracellular space (V_i) and in the medium (V_e) will remain constant (Q_t):

$$V_i C_i + V_e C_e = Q_t. \tag{3}$$

The internal concentration (C_i) in equation (2) can thus be replaced by:

$$C_i = (Q_t - V_e C_e)/V_i. \tag{4}$$

Substituted in equation (2) this yields:

$$dQ_e/dt = V_e\, dC_e/dt = p(Q_t - V_e C_e - V_i C_e)/V_i + J_a \tag{5}$$

or:

$$dC_e/dt = -(V_i + V_e)\, p\, C_e/V_i V_e + (J_a V_i + Q_t p)/V_i V_e. \tag{6}$$

Solving this differential equation yields an (inverted) exponential equation (*e.g.* Shaw, 1961; Simon, 1986) of the general form:

$$C_e(t) = (A - B)\exp(kt) + B. \tag{7}$$

In this equation, A represents the initial salt concentration in the medium. B, which equals $(V_i J_a/p + Q_t)/(V_i + V_e)$, represents the final salt concentration of the medium (to be expected when active and passive fluxes reach equilibrium). The time constant, k, follows from $-(V_i + V_e)p/V_i V_e$. For *Carcinus* the (steady state) volume of the extracellular fluids, V_i, amounts to approximately 31.1% of the body volume (Zanders, 1980). Changes in the extracellular volume will occur during acclimation to hypo- and hyperosmotic conditions (Harris and Andrews, 1982). In these measurements the animals are only exposed to salinities very close or equal to their adaptation salinity. Variations in V_i will therefore be neglected here.

With measurements for $C_e(t)$ equally spaced in time, an elegant way to derive the time constant (k) and the equilibrium concentration (B) is to calculate the regression between subsequent measurements $C_e(t+dt)$ and $C_e(t)$.

$$C_e(t) = (A - B)\exp(kt) + B$$

$$\text{or } \exp(kt) = (C_e(t) - B)/(A - B) \tag{8}$$

$$C_e(t+dt) = (A - B)\exp[k(t+dt)] + B$$

$$= (A - B)\exp(kt)\exp(kdt) + B. \tag{9}$$

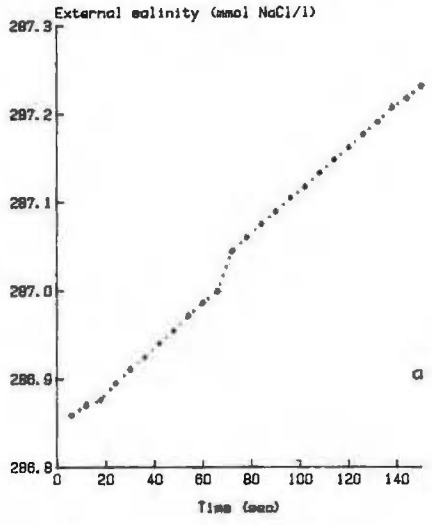
Substitution of $\exp(kt)$ from equation (8) into equation (9) yields:

$$C_e(t+dt) = \exp(k\,dt)\, C_e + B[1 - \exp(k\,dt)]. \tag{10}$$

Correlations of $C_e(t+dt)$ and $C(t)$ values, obtained within short (2.5 mn) sampling periods (*e.g.*, Fig. 1), show linear relationships (Fig. 1 *b*). According to equation 10 the slope represents the term $\exp(k\,dt)$, which is related to the permeability, and the Y-intercept represents the term $B\,[1\text{-}\exp(k\,dt)]$ which is related to the active flux.

Strictly speaking the slope, $\exp(k\,dt)$, and the Y-intercept, $B[1\text{-}\exp(k\,dt)]$ are no constants. In the calculation of the regression line one obtains time averages over the sampling period. Using sampling periods of 2.5 mm the linear correlation coefficients are rarely lower than 0.99. Figure 1 also demonstrates that a sudden change (due to urine production at time $\approx$ 70 s) has almost no effect on the slope and the Y-intercept of the autoregression line.

Slope and Y-intercept can most rapidly be derived using a matrix least square method (*e.g.* Deming and Morgan, 1987). The natural logarithm of the slope equals the product of the time constant k and the time



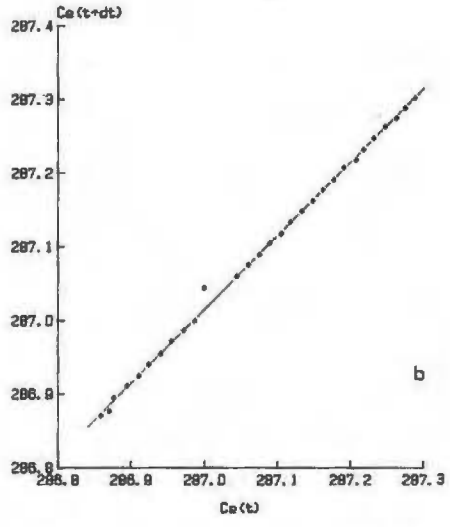


Figure 1

Example of the change in medium concentration in the measuring chamber as a function of time (a) and an autocorrelation plot (equation 10: cf. text) of subsequent measurement values (b). Slope and Y-intercept of the autocorrelation plot yield information on the whole animal permeability and the active ion transport. For further explanation cf. text.

Exemple d'évolution, en fonction du temps, de la concentration en ions dans la chambre de mesure (*a*) et de représentation graphique d'une autocorrélation [équation (10) : *cf.* texte] des valeurs mesurées (*b*). La pente du tracé [autocorrélation (*b*)] et son intersection avec l'axe des ordonnées (*y*) fournissent des informations sur la perméabilité globale de l'animal et sur le transport actif d'ions. Pour de plus amples informations : cf. le texte.

periods (dt) between subsequent measurements. Hence:

$$k = -(V_i + V_e)\,p/V_i V_e = \ln(\text{slope})/dt \qquad (11)$$

from which it follows:

$$p = -V_i V_e \ln(\text{slope})/(V_i + V_e)\,dt. \qquad (12)$$

From the Y-intercept, B (1-slope), follows the final concentration (B) which equals $(V_i J_a/p + Q_t)/(V_i + V_e)$. The total (exchangeable) salt content of the system [Q_t, equation (3)] can be assessed by assuming that the salt concentration of the extracellular space, C_i, is about the same as that present in the adaptation medium, C_a, [hence equation (3): $Q_t = V_i C_a + V_e A$], as in natural sea-water the body fluids of shore crabs are approximately isotonic to the medium. At hypotonic medium concentrations the exact value for Q_t of a hyperregulating species will be slighly higher. The inaccuracy introduced by this approximation is not very large as: 1) the differences between the internal and external concentrations are always fairly small; and 2) the contribution of internal salts to the total salt content of the system is only small as the volume of the extracellular space is much smaller than the medium volume. The actual value for Q_t can, of course, also be derived by using experimentally determined values for the salt concentration in the extracellular space.

Hence, from the Y-intercept and the value for p, derived from the slope, an approximation of the active flux can be derived:

$$J_a = ((V_i + V_e)\,B - Q_t)\,p/V_i. \qquad (13)$$

Derivation of the net and passive fluxes

The net fluxes (J_n) during the course of salinity adaptation are given by the first derivative of the medium concentration [equation (7)] multiplied with the medium volume:

$$dQ_e/dt = J_n = k V_e (A - B) \exp(kt). \qquad (14)$$

During equilibration the net fluxes will gradually decrease, eventually reaching zero. The initial net flux, immediately after a change in external concentration, follows from:

$$J_n(0) = k V_e (A - B). \qquad (15)$$

The passive flux, finally, follows from the difference between the net flux [equation (14)] and the active flux [equation (13)]. If the active flux is based on an estimated value for the total salt content of the system, then the value obtained for the passive flux should also be considered to be an approximation.

Hence, from the analyses of kinetic data of the concentrations in a closed medium, values can be derived for the whole animal permeability for pasive salt transport, and for the net and active fluxes. Subsequently, passive flux values follow from the differences between net and active fluxes. Permeability and animal size determine the halftime value for salinity equilibration. Permeability and body surface area may yield a (relative) value for the area specific permeability.

RESULTS

Figure 2 gives an example of a continuous registration of the salt exchange characteristics in the shore crab *Carcinus maenas*. The values for the permeability, fluxes and concentration difference across the body wall were calculated repetitively (each 2.5 mn) from medium concentration measurements according to the

equations given in the previous section. Medium concentrations were measured as conductivities, sampled every 5 s. By lowering of the salinity at the beginning of the experiment the example demonstrates both the adaptation to a change in salinity (during the first few hours) as well as the steady−state response (near the end of the observation period). Other recordings, not illustrated here, show similar phenomena as described below for this particular example.

Whole animal permeability in *Carcinus* as a function of time

From Figure 2*a* it becomes evident that the whole animal permeability of a species does not have a rigidly fixed value, but may, as a function of time, show a very broad range of variation. In this respect, the registration shows a striking resemblance with the continuous registrations of other physiological phenomena, for instance of heart rate or scaphognathite rate (Uglow, 1973; Cumberlidge and Uglow, 1977*a*; Hagerman and Uglow, 1981). Long periods (several minutes) of low permeability are alternated with periods of higher permeability. It is very likely that this resemblance is not merely accidental. This supports the idea that the passive transport of salts is strongly determined by the perfusion (and ventilation) of the gills. During long periods of cardiac arrests, commonly found in *C. maenas* (Cumberlidge and Uglow, 1977*a*, *b*; Spaargaren, 1982), the exchange of salts drops to very low values. Although permeability, defined as salt flux per unit of concentration gradient, is in principle a passive parameter determined by membrane characteristics, whole animal permeability turns out to be a quantity which is actively regulated under nervous control.

At the beginning of the experiment, during the first hour after the salinity change, permeability remains very low until it rises abruptly to very high values. From this time onwards, permeability alternates between periods of high and low values. The initial drop in permeability (*cf.* Spaargaren, 1989) probably has an adaptive function in stabilizing (*e.g.* Winkler *et al.*, 1988) the extracellular concentrations.

Net salt efflux in *Carcinus* as a function of time

Despite the low permeability during the first hour after a change in salinity, the initial net salt efflux (Fig. 2*b*) is very high. The reason for this is, of course, the steep concentration gradient shortly after the salinity change (Fig. 2*d*). Concurrent with a decrease in the concentration gradient, the net efflux drops to low values up to the moment when the permeability rises suddenly. Then also the net flux increases for a short period, in this case not because of a steep concentration gradient across the body wall, but due to the higher permeability. From that time onwards, the net efflux decreases again and after about 4 hours of adaptation the net efflux becomes negative: between 4 hours after the salinity change to the end of the observation period (about 8 hours) a net salt influx is found. During this time the active influx surpasses the passive efflux. Concurrently, the concentration gradient across the body wall (Fig. 2*d*) increases to a new steady state value.

Net flux and permeability are of course connected to each other. Generally, however, the net flux values are very poorly related to the permeability (Fig. 3*c*), due to the significant interference of active transport.

Even when the net fluxes are very low, which also means that the medium concentrations show very little variation in time, the various characteristics can still be

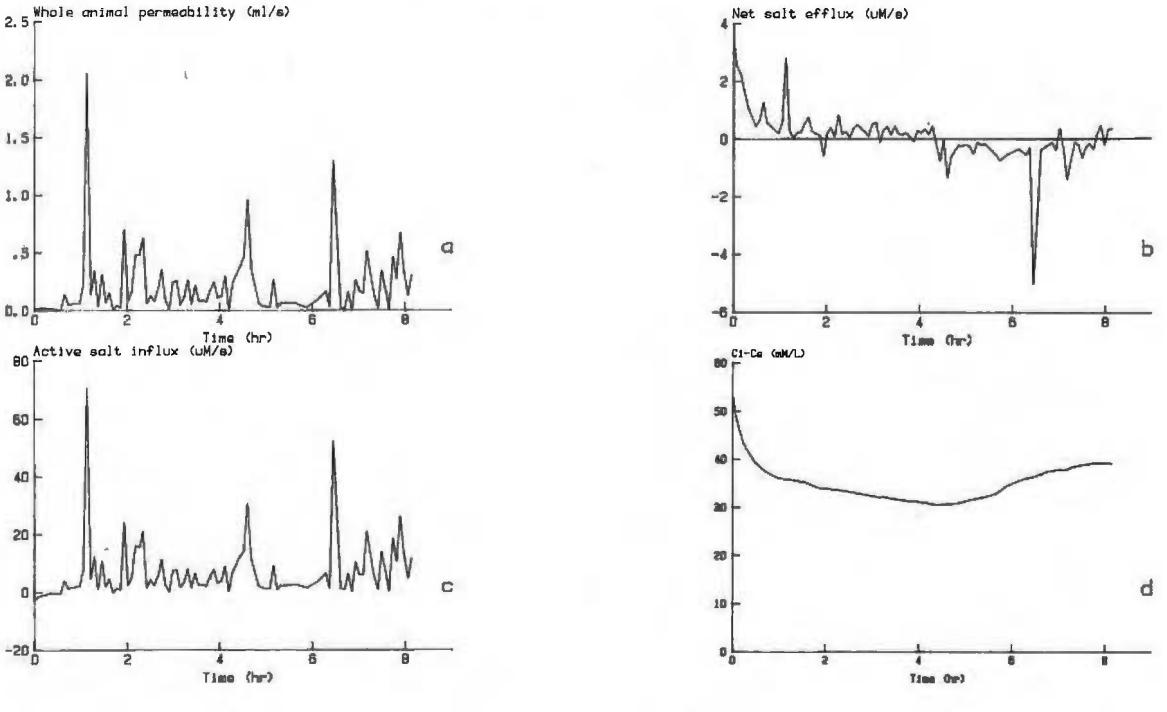


Figure 2

Whole animal permeability (*a*); *net salt efflux* (*b*); *active salt influx* (*c*); *and concentration difference between the body fluids and the medium* (*d*), *in* Carcinus maenas *adapted to* 28.5 *salinity and at time zero exposed to a medium salinity of* 22.2. *Temperature* 19.5°C; *animal weight* 39.3 *g*.

Perméabilité globale de l'animal (*a*), flux net sortant de sel (*b*), flux entrant actif de sel (*c*) et gradient de concentration entre les fluides corporels et le milieu (*d*) chez le crabe *Carcinus maenas* acclimaté à une salinité de 28,5 et, au temps zéro, exposé à un milieu de salinité 22,2. Température : 19,5°C; poids de l'animal : 39,3 g.

determined. The values for the concentration gradient across the body wall (Fig. 2 *d*) do not become unstable. Hence, the calculations, as required in the signal analyses procedure as presented here, do not suffer seriously from truncation errors at very low net efflux levels.

Active transport in *Carcinus* as a function of time

Shortly after exposure to the lower salinity, the active efflux values (Fig. 2 *c*) are very low, associated with low permeability values. During the whole observation period a very close relation (Fig. 3 *a*, *b*) can be found between the active and passive fluxes and whole animal permeability, already evident from Figure 2 *a* and Figure 3 *c*. The connection between active transport and permeability is probably indirect. Both are related to the gill perfusion/ventilation rates. For active transport this connection may be based on the required oxygen supply.

During the first hours, the net efflux remains positive, indicating that the passive efflux is still higher than the active influx and that the concentration gradient (Fig. 2 *d*) is still decreasing. After about 4 hours this is no longer the case.

The active flux, during the whole observation period ranging on the average between 5 and 10 uM/s (for an animal of 39.3 g), represents the transport of salts against the existing concentration gradient. Hence, the product of both values will be proportional to the energy demand for ion regulation.

Concentration gradient across the body wall in *Carcinus* as a function of time

The difference between the internal and external salt concentration (Fig. 2 *d*) rapidly decreases within the first hour after exposure to a lower salinity. After about 4 hours, when the net flux becomes negative, the gradient increases slightly. The time course clearly demonstrates the undershoot of the extracellular salt concentrations.

DISCUSSION

The procedure as presented in the section data analyses permits rapid determination of flux and permeability values. In the example described, the measuring periods were 2.5 mn (in which 30 measurements were taken). Other recordings showed that the measuring periods can be reduced to 1 mn (30 measurements, sampling frequency 2 s; shorter periods not tried). It is clear that with small differences in measured C_e values (either due to very low net fluxes or by short measurement intervals), the regressions between subsequent medium concentration values will show lower correlation coefficients related to the scatter in the measured values. It is useful therefore to add in the calculations a condition that sets a limit to acceptable values. In the example illustrated in Figure 2, those (rare) measurement series which showed a correlation coefficient of less than 0.99 (and/or a regression $> = 1$) were automatically skipped. This precaution also cancels the irregularities induced by incidental production of urine during a sampling period. The rapid determination of the salt transport variables makes it possible to monitor the values almost continuously and to detectect the variations in time as they occur in the undisturbed animal.

As in the case of many other physiological (rate) functions (*e.g.* McHahon and Wilkens, 1983), the flux values appeared to be very unstable with time. To characterize a flux under specific circumstances, it is always necessary to monitor the flux over a certain period of time. The flux value can then be presented as an average level together with the variation around this level. For comparative purposes, data on the average levels and their variability are more interesting.

Figure 4 gives an example illustrating the effect of elevated CO_2 ($TCO_2 = CO_2$, carbonic acid, bicarbonate and carbonate) concentration in the medium on the exchange characteristics of *Carcinus maenas*. Permeability and net efflux (Fig. 4 *a*, *b*) increase at higher

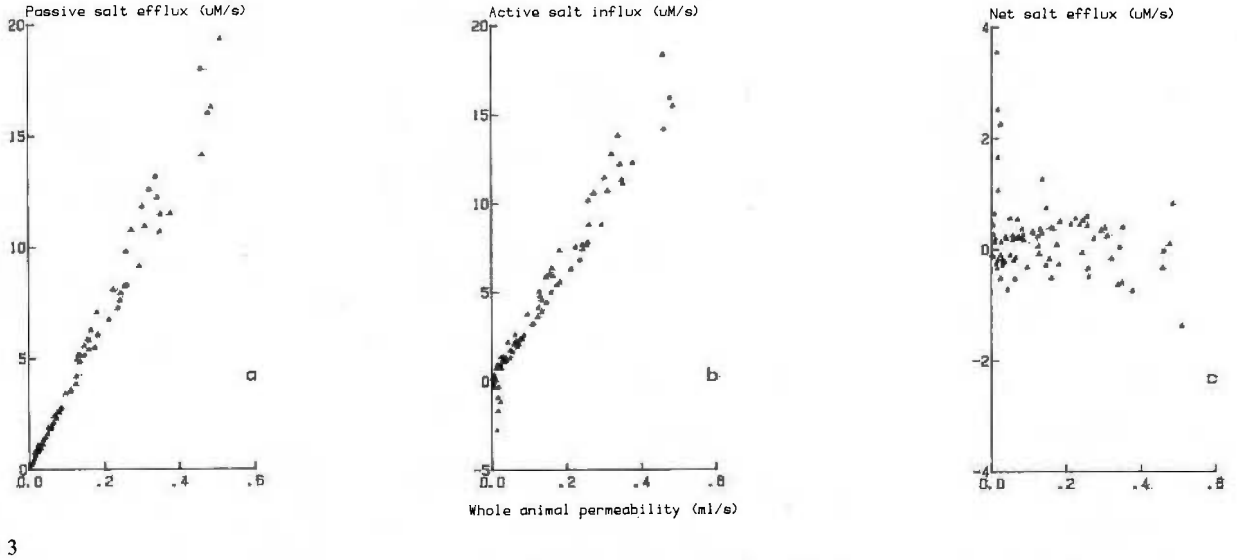


Figure 3

Passive salt efflux (a); active salt influx (b); and net salt efflux (c), of Carcinus maenas, *during the recording illustrated in Figure* 1, *in relation to whole animal permeability.*

Flux sortant passif (*a*), flux entrant actif (*b*) et flux net sortant de sel mesurés au cours de l'expérience illustrée à la figure 1 en fonction de la perméabilité globale du crabe *Carcinus maenas*.

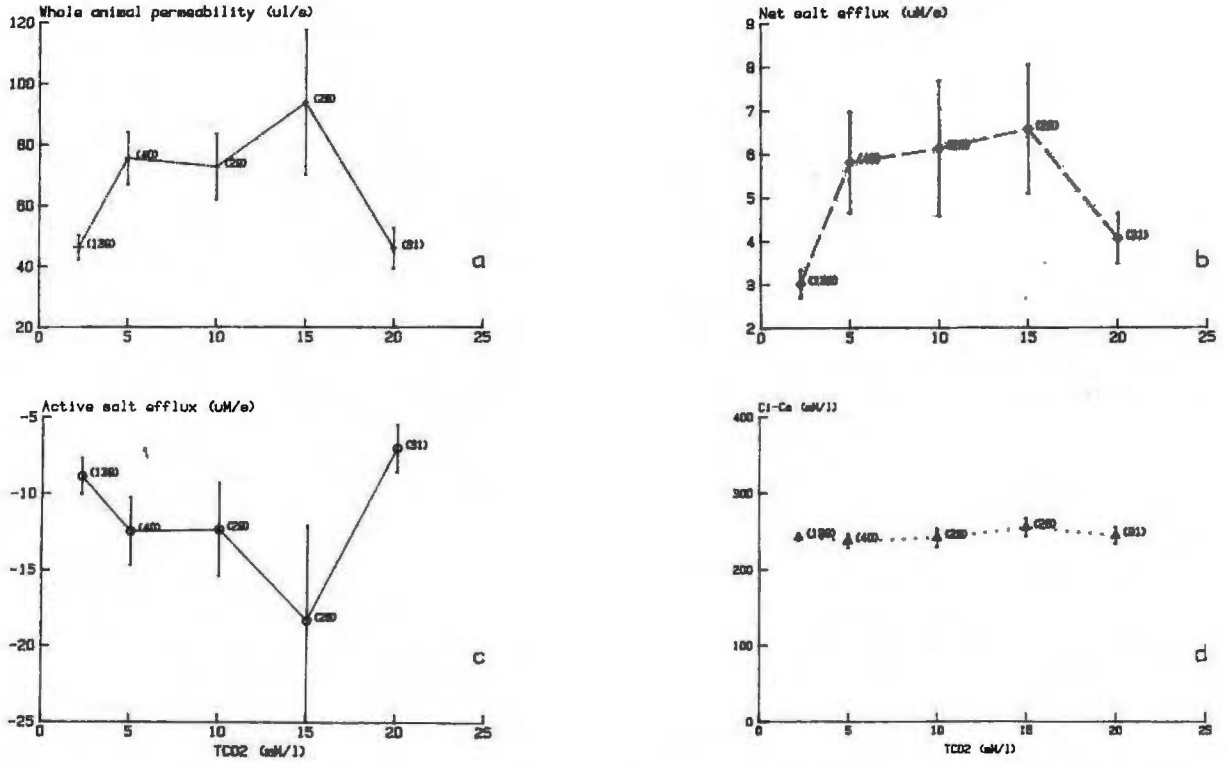


Figure 4

Whole animal permeability (a); net salt efflux (b); active salt efflux (c); and concentration difference between the body fluids and the medium (d), in Carcinus maenas *as a function of the external CO_2 concentration (TCO_2 = carbon dioxide, carbonic acid, bicarbonate and carbonate). Temperature 17.5°C; salinity 12.6; 18 animals, fresh weight 18-39 g.*

The numbers in parenthesis next to the symbols refer to the number of observations; the vertical lines through the symbols represent the standard errors of the means.

Perméabilité globale du crabe *Carcinus maenas* (*a*), flux net sortant (*b*), flux sortant actif de sel (*c*) et gradient de concentration entre les fluides corporels et le milieu (*c*) en fonction de la concentration en CO_2 du milieu extérieur (TCO_2 : CO_2, H_2CO_3 et CO_3^{--}). Température 17,5°C; salinité 12,6; expérience réalisée sur 18 crabes d'un poids frais variant entre 18 et 39 g. Les nombres entre parenthèses à proximité des symboles indiquent le nombre d'observations; les lignes verticales qui traversent les symboles figurent les erreurs standard sur les moyennes.

external TCO_2 concentrations until (at $TCO_2 > 15$ mM/l) the medium TCO_2 level exceeds the internal TCO_2 level. The changes in the active fluxes (Fig. 4*c*) are not sufficient to compensate the passive losses. In the short – term (30 mn) experiments, a slight decrease in the concentration gradient across the body wall (Fig. 4*d*) becomes already visible.

It is to be hoped that the method will provide a useful tool in ecophysiological studies for the characterization of solute exchange in different species living in various habitats or in relation to their body size, moulting stage, activity, *etc.* A practical limitation however, may, be set by the body size of the animals under study. Other applications may be found in pollution studies. The method can be used to study the effect of a specific pollutant on one of the most basic metabolic processes, *viz.* the exchange of solutes between an animal and its environment. The control of internal stability is of vital importance for all metabolic activities.

In principle, the method of data analyses can also be adapted for the measurement of the flux rates and permeabilities of particular ions. A (small) number of ion selective electrodes is at present available to monitor directly the specific ion activities in fresh and sea water. On the other hand, electrodes that require sample pretreatment, to mask interferences of other ions, can not be used. Up to now, the use of ion-selective electrodes has not been tested, but will be the subject of further work.

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