DOES NEUTRAL RED SUIT THE REQUIREMENTS OF BEING A RELIABLE INDICATOR IN CLEARANCE RATE MEASUREMENTS OF SUSPENSION-FEEDING BIVALVES? AN EMPIRICAL REFUTATION

Enrique Navarro*, Kristina Arranz and Iñaki Urrutxurtu.

GIU 17/061, GI 544, Departamento de Genética, Antropología Física y Fisiología Animal, Facultad de Ciencia y Tecnología, Universidad del País Vasco / Euskal Herriko Unibertsitatea, UPV/EHU, Apartado 644, 48080 Bilbao, Spain

*Corresponding author, e-mail: enrique.navarro@ehu.eus

Keywords: clearance rate, neutral red, filter feeders, *Mytilus galloprovincialis.*

Abstract:

One basic requirement in the measurements of feeding rates in suspension-feeding bivalves when using clearance methods is that the suspended particles that are used in the determination of these rates must reach size compatibility with full retention by the gill. In spite of this requirement, clearance of neutral red (NR)—a soluble vital dye—has long been used for this purpose and recourse to this method has even increased over the last years for determining the filtration rate as a specific physiological response to stress in ecotoxicological and biomonitoring studies. The aim of this study was to produce empirical evidence that would dismiss the suitability of the NR method for providing reliable measurements for feeding rates in suspension feeders. Experiments that were designed to this end in the blue mussel *Mytilus galloprovincialis* led to three main conclusions: 1) Temporal dynamics of NR retention by the mussels point to a diffusion mechanism whereby the marker would be up-taken by gill tissue rather than filtered by the muco-ciliary action of this organ. 2) The NR method largely underestimates the clearance rate (by 90% on average) when compared with conventional methods that use microscopic particles in the size-range of gill retention. 3) The close similitude between rates of retention of NR by intact mussels and mussels that had been sacrificed by sectioning apart the valves adductor muscle proves outright the lack of a quantitative relationship between this retention process and the pumping activity.

1.- Introduction:

Suspension feeders constitute a characteristic functional group that plays a key role in aquatic ecosystem processes. Populations of marine bivalves, in particular, are important components of benthic communities in estuarine and coastal areas where, given their high abundance and filtering capacity, they stand as the most important biogeochemical agents driving benthic-pelagic coupling (Dame, 2012). These features have also resulted in several characteristic species of bivalves becoming model sentinel organisms in biomonitoring (e.g., mussel watch) programmes (Goldberg, 1975; Widdows et al., 1995). Thus, appropriate determinations of the filtering activity in species of bivalves are key factors in research programmes that aim to model these processes in the ecosystem for different purposes. Quite understandably, reported values of this physiological parameter in bivalves are the subject of periodical revision and lively debate concerning the different procedures that are involved in its determination (Bayne, 1998; 2001; 2004; Cranford, 2002; Filgueira et al., 2006; Jørgensen, 1996; Petersen et al., 2004; Riisgård, 2001a, b; 2004; Widdows, 2001).

Filtration in bivalves is a muco-ciliary process (Ward et al., 1993; but see Jørgensen1990) whereby microscopic particles that are suspended in the water column are conveyed to the gill by the pumping activity of this organ, where they are retained and concentrated for feeding purposes. Methods of recording the clearance rate -the volume of water cleared from particles per unit of time- were designed to approach the more problematic direct measurement of the pumping rate, or flow rate of water provided by the biological pump (Riisgård, 2001a), with both measurements becoming coincident only when suspended particles are fully retained by the gill. Thus, in concerning the use of the clearance rate as a proxy for the feeding activity, one methodological requirement is that the tracer particles attaina critical size that is compatible with retention by the gill. This has been estimated to fluctuate between 3 and 5 μm in different species of bivalves (Vahl, 1972; 1973; Møhlenberg and Riisgård, 1978; Riisgård 1988) depending on the development of the dorso-frontal ciliation in gill filaments (Jørgensen, 1990). It is generally assumed that for particles above these critical diameters (in the range of nanophytoplankton), the pumping rate is properly

determined through the clearance rate, but this later measurement would underestimate the pumping rates for lesser sizes as a consequence of the progressive decline of gill retention efficiency

Given these general requirements regarding the filtering activity measurements, it becomes quite remarkable that the method using neutral red (NR) solutions had been successively reported over more than 60 years in studies that measure the clearance rate of this trophic category of animals in both marine and freshwater environments. In a recent review, Martínez-Haro et al. (2016) documented 23 studies using NR in CR determinations since Cole and Hepper (1954) proposed this method as an alternative to classical procedures on account of its simplicity.

Among the different procedures proposed (reviewed by Riisgård, 2001a),the one that computed clearance rate from recording the exponential decline in particle concentration over the time in a closed system (as described by Coughlan 1969)has been most commonly used and is possibly less problematic (Riisgård, 2001a). The method based in NR also uses Coughlan principles, as CR determinations are computed recording the changing NR concentration over a given interval of time, but the problem is that NR being a soluble marker its retention by the gill could hardly be associated with the muco-cilliary action on particles of the appropriated size to be filtered. Rather, as already suggested by Ward and Aiello (1973), the recorded change in the NR concentration in the presence of animals would result from the uptake of NR molecules that are dissolved in water by gill cells (and possibly cells of other tissues, such as the mantle, exposed to water circulation). This behaviour would in fact be in accord with functions that are attributed to a vital staining dye. Rates of NR uptake in this case would be driven by the concentration gradient through the plasma membrane of these cells and, even if this gradient might keep some marginal relationship with the pumping rates (Cole and Hepper, 1954),it becomes clear that NR uptake by tissues in contact with water currents can, in no way, be considered to represent the filtering activity.

According to the above statements, there appears to be not much of a rationale for considering the clearance of NR solutions to be an appropriate procedure for measuring the filtration rate of suspension feeders, with the only remaining reason being the availability of the technique in laboratories that are not specifically equipped for the proper recording of this physiological

parameter. Nevertheless, resort to this method has considerably increased over the last years for determining the clearance rate as a specific physiological response to stress in ecotoxicological or biomonitoring studies (Faria et al. 2009; Palais et al. 2012; Parolini et al. 2014; Magni et al. 2016). The recent proposal of a "clearance index" (sic) based on NR as a reference indicator of physiological stress in bivalves (Martínez-Haro et al. 2016) is particularly concerning.

Since no theoretical arguments of the type that is invoked in this section have resulted in dismissing the use of NR solutions, as should have been the case, the aim of this study was to provide an empirical refutation of the suitability of the NR method for measuring the filtration rates. To this end, two experiments were conducted in mussels *Mytilus galloprovincialis*, a species taken to be a representative of the suspension feeding bivalves: In the first experiment, the CR values recorded with standard Coughlan procedures using natural suspensions (phytoplankton), and those using NR solutions, were compared in the same individuals. In the second experiment, the NR clearance (retention) was compared between intact mussels and mussels that had been sacrificed by cutting off the adductor muscle to open the valves make the gill and mantle directly accessible to the surrounding water. Both experiments were aimed to provide empirical evidence to test the hypothesis that NR clearance does not constitute a suitable measurement of the filtration rate in suspension feeders.

2.- Materials and methods:

The mussels, *Mytilus galloprovincialis,* were collected for this study during low tide along a rocky shore of the Biscay coast (43º25´LN; 02º92´LE). The collection dates were February 10thand May 20th2018, for experiments 1 and 2, respectively. Individuals measuring some 50 mm shell length were carefully detached from the rock by cutting the byssus and were transported to the laboratory where they were cleaned of epizoa and were maintained in a re-circulating sea-water system (salinity 34 ‰) that was regulated to the temperature that had been recorded during the recollection (12 and 14 ºC in February and May, respectively). In

the period prior to the measurements (12-16 hours),the mussels were fed phytoplankton (*Isochrysis galbana*) dosed at a concentration of $12x10^6$ cells per litre.

2.1.- Experiment 1

Individual CR determinations based on Coughlan (1969) principles using neutral red and microscopic particles that were susceptible to full branchial retention (i.e., phytoplankton cells of the species *I. galbana* measuring4.5 μmin diameter)were compared using the same group of nine uniformly sized mussels (*M. galloprovincialis*): The mean shell length was 49.5 (±5.2) mm. All measurements were conducted at 14.5 ± 0.5 °C inside temperature-regulated water baths. CR determinations using phytoplankton were performed in closed systems in which the mussels were individually placed in beakers filled with 3 L seawater and were provided with gentle aeration to avoid the sedimentation of particles. For each set of 9 experimental flasks, an additional beaker without an animal was set as the control. At the beginning of the experiment, the required volume of a pure culture of the microalga *I. galbana* (T-Iso clone) was added to each flask to achieve an initial concentration of 12,000 particlesml^{-1}. From this point, two different approaches to the CR determination using the same animals were undertaken, which resulted in two sets of values that were both based on microalgal particles(Figure 1): a) The declining particle concentration over the time was recorded at 7-10 min intervals for approximately 70 min, and the curves of the exponential decay were fitted to these values to estimate CR. b) To prevent the possible effects of reducing the particle concentration on CR measurement, the initial particle concentrations were restored after its reduction was recorded at intervals of 15 min. The CR was then estimated as the mean of 5 of these repeated measurements. Particle concentrations were measured with a Beckman Coulter Z1 particle counter.

CR determinations using neutral red were conducted as described by Cole and Hepper(1954) and optimized by Martínez-Haro et al. (2016). Once the CR measurements using microalgae were concluded, the same mussels that were used in these determinations were transferred to 1L beakers with aerated seawater containing neutral red (2 mg L^{-1}). This NR concentration was chosen on account that, according to Martínez-Haro et al. (2016), it maximizes the rates of filtration. Then, 50 ml samples of every chamber (including a chamber without an animal for the control) were taken for colorimetric quantification at the start and after 1, 2 and 17h.

The samples were adjusted to pH 5.0 using a solution of HCl 0.1 M prior to the spectrophotometric analysis (Martínez-Haro et al., 2016). The NR concentrations of these samples were estimated from their absorbance values read at 454 nm in a Shimadzu UV-160A spectrophotometer, using a standard curve that was prepared in a range of 0-8 mgL^{-1} NR concentrations.

The clearance rates were estimated, according to Coughlan (1969), through the following formula:

$$CR\ (Lh^{-1}) = \frac{ln\ C_0 - ln\ C_t}{t(h)} x\ Vol\ (L)$$

where *CR* is the clearance rate, C_0 and C_t are concentrations of particles or NR at t=0 and at a given time (t=t), respectively; t is the time elapsed since t=0 and *Vol* is the volume of water.

2.2.-Experiment 2

CR determinations using NR were performed in May under the same conditions that were reported for Experiment 1 (i.e., 14.5 ± 0.5 °C) and in mussels of the same size (50.04±4.64 mm shell length). On this occasion, rates of NR retention were compared between two groups (n=5) of mussels: one group of intact mussels and one group of mussels sacrificed by cutting off the adductor muscle to allow wide separation of the valves. Of each chamber (including the control), 50 ml samples were taken at the start and after 10, 70 and 130 min for spectrophotometric quantification of the NR concentration, as previously described.

2.3.- Data treatment.

The individual rates that were obtained with the different methods were compared by using regression analysis. The mean values of the different groups were compared by one-way ANOVA. To remove the effects of the small differences in the size of the specimens, all of the rates that were used in the mean computations were standardized to a common size (1g soft- body dry weight individual) by using the following formula:

$$Yst = Ye\ (1/We)^{\wedge b}$$

where *Yst* and *Ye* are the standardized and actual (recorded) values of CR, respectively; *We* is the dry soft-body weight, in g, of the individual whose rate is *Ye*; and *b*is the power value scaling clearance rate of the body size in mussels (*b* = 0.67; Møhlenberg and Riisgård, 1979). This size was chosen to facilitate comparisons with data of *Mytilus* that were reported in the literature.

3.- Results

3.1.-Dynamics of marker retention (phytoplankton vs. neutral red)

The temporal evolution of the concentrations of both markers, *I. galbana* cells (in the continuous measurements) and NR molecules, has been plotted in Figure 2A, in a relative scale (as fraction of initial or t_0 concentrations) and over similar time intervals to make both trends comparable. Fulfilling the theoretical expectations for clearance processes of suspended particles (Coughlan, 1969), the microalgae concentration data points fit the exponential decay trends that are accountable by constant pumping rates. Although the change in NR concentration over time can also be described by an exponential decaying function (Figure 2A), involving the constant removal of the marker, some essential information would be missed by assuming this fitting model: namely, that the rates of NR reduction appear to lessen with time. This was further confirmed (Figure 2B) by comparing rates of NR removal given by the exponent values of functions fitted to data for the first 2 h interval (b = -0.27) and for the longest period (17 h) of exposure to the dye (b = -0.20). Consequently, the clearance rate values that were estimated by this method showed dependence on the time intervals over which measurements are being performed (see Table 2), with the maximum CR values corresponding to shorter exposure times and vv.

Statistical comparisons, using a mixed-model ANOVA, of the CR that was estimated during the first and second hours using NR confirmed significant time-dependent differences (P = 0.037), while no such differences could be found between consecutive time intervals in CR determinations with microalgae (P = 0.574). Differences between repeated measurements for each 15 min interval using phytoplankton were found to be highly significant (P = 0.001) but, unlike the NR data, no definite time tendency was evident in this case.

200 *3.2.- Comparison of CR based on both markers (phytoplankton vs. neutral red)*

201 CR measurements with microalgae using two different approaches were compared by using
202 a regression analysis (Figure 3). There is a significant correlation between both CR
203 determinations (R^2 = 0.62; P = 0.01),although the values obtained through repeated
204 measurements (CR_{RM}) were consistently higher: the slope of the regression line through the
205 origin indicates a 53% increment in values recorded with this method compared with
206 continuous measurements of the decline in particle number (CR_C). In this comparison, the
207 CR recorded with NR (CR_{NR}; averaged values) did not exhibit a correlation with the CR_C
208 estimations (a significant correlation was achieved only for values based on the first hour)
209 and were remarkably low and constant (Figure 3).

210 The mean 1g-standardized values together with the ratios comparing different CR
211 measurements are presented in Table 1. The two CR values recorded with phytoplankton
212 were significantly different (P = 0.014), and each differed highly significantly from the CR
213 values that were recorded with NR, either1st hour measurements, mean or averaged values
214 (P < 0.001).

215 Computed ratios in Table 1 would indicate that, for comparison with CR_{RM}, the continuous
216 recording method using phytoplankton would underestimate CR by 35%, while in the case
217 of the NR method, this underestimation would reach to 89 to 93% depending on the time
218 interval that was considered.

219 Results of Experiment 2 are summarized in Figure 4. The curves of NR retention did not
220 significantly differ between intact mussels and mussels dissected to expose the soft body
221 tissues (P = 0.87). As shown before (see Figure 2), the rates of retention are time-dependent
222 (P = 0.001).

223 In both groups of mussels, the gill appeared intensely dyed by NR, and the coloration was
224 resistant to washing, thus confirming the dye incorporation by the tissue.

225

226

4.- Discussion

One primary aim of this work was to set a standard method of the clearance rate determination with natural particles (phytoplankton) as a reference to evaluate the NR method using the same individuals. Reported discrepancies between both approaches to CR determination with microalgae should, thus, be addressed to establish such standards. As previously stated, one problem with recording the particle decay in a closed system during the prolonged measurements that are required to achieve an integrated response is that the pumping activity might become affected by the changing ambient particle concentration (Riisgård, 1991). In this respect, a discontinuous method (involving the periodic restoration of initial concentration) might be considered more reliable for CR determination in a closed system because of its potential to provide measurements under a more stable particle concentration. For instance, Pascoe et al. (2009) reported that *Mytilus edulis* fed variable concentrations of *Isochrysis galbana*in a flow-through system reduced the CR by up to 1/3 when the concentration drops from 10,000 to 5,000 cells ml^{-1}, which is nearly the range of reduction that was achieved in our Coughlan measurements during continuous recording using the same microalgal species. However, the absence of any definite tendency of mean CR values over time (Figure 5 in Supplementary Materials) does not support the hypothesis that differences in CR estimated with both methods are attributable to the effects of time-dependent reduction in ambient particle concentration.

The magnitude of these discrepancies, on the other hand, fall within the range that is encountered for differences between the filtration rates that were measured under comparable conditions by the different commonly used methods (bio-deposition, flow through and clearance or Coughlan methods; see Petersen et al., 2004 for an interesting inter-calibration experience with mussels *Mytilus edulis*). Clearly, the values that were recorded with the NR method are in a different order of magnitude and deserve a different consideration. As already stated (see Introduction),the pretence that this method might constitute an alternative to standard procedures of CR determination based on measuring the concentrations of suspended particles is untenable, since the clearance of a soluble marker cannot provide an effective measurement of the pumping rates. In what follows, this discussion is aimed at

outlining three main issues of empirical evidence against the suitability of this method in performing filtration rate measurements:

1) Temporal dynamics of the marker (NR) within the filtering system reveals a saturation kinetics that is inconsistent with its retention by the gill being a direct measurement of the pumping rate.

This limitation can be traced back to the foundational study of the NR method (Cole and Hepper, 1954), where the percentage of neutral red that was removed from the suspension by mussels (*Mytilus edulis*) is shown to increase with time at a decreasing rate (see Figures 3 and 4 in their study), while a constant rate would be expected, should the pumping be involved in clearance (Coughlan, 1969). Similarly, data from the present study indicates that the rates of retention of NR declined with time (Figures2 and 4), as was expected if the marker would be up-taken by diffusion into the mussel tissues rather than filtered.

In line with the foregoing discussion, a neat account of the problems posed by NR and the limitations of the method for measuring the clearance rates is already given in one early ecotoxicological study that is based on the use of this indicator (Abel, 1976):

> The equation [Coughlan] further assumes that the dye extraction by the animal is 100% efficient. In fact, the efficiency of dye extraction was found to decrease markedly with increasing dye concentration……Furthermore, the dye extraction efficiency may continue to change during the test as the dye concentration decreases. Therefore, the filtration rate values determined by this technique are probably underestimates and should not be accepted as absolute values.

Thus, as clearly stated in this critical evaluation of the method, the rates of NR retention would be affected by the change in dye concentration and, hence, be inherently dependent on the initial concentration, the time interval and, as an implication, the water volume in the measurement chambers, as a determinant of the rate of change. Determinations of only the initial and final points in routine measurements of the "filtration rates" with NR (Faria et al., 2009; Martínez-Haro et al., 2016; Palais et al., 2012) have possibly caused these important shortcomings of the method to be overlooked.

2) The NR method largely underestimates the CR; values that were obtained with this procedure come to represent approximately10% of those that were recorded with the conventional Coughlan method using suspended particles.

In the present comparison based on the same individuals, the "CRs" that were recorded with the NR during the first hour of exposure (the highest values) represented only 11% of the highest value that was recorded with phytoplankton (i.e., in repeated measurements) and dropped to 7% in the longer-term recordings. The magnitude of this underestimation is consistent with the suggested mechanism of NR retention by the gill, and possibly other tissues of mussels. Dye uptake by gill cells would also account for the persistent (wash-resistant) coloration of this organ in mussels that are exposed to NR in all conditions.

The availability of an extensive bibliographical recording on filtration rates in bivalve molluscs offers a broad scope for the comparative assessment of data that were obtained with the NR method *vs.* more conventional methods of particle clearance, even if such comparisons might become limited by differences in the measuring conditions and size of the individuals. In Table 2, we gathered a number of studies on different species of bivalves for which the measuring conditions are defined and the rates could be standardized to a common size. Despite the limitations of this comparison, a cursory analysis of the data confirms the conclusions of the present study as to the degree of underestimation (90% on average) of the clearance rates that were measured with NR compared to that of any other of conventional method(clearance, biodeposition, flow-through or suction).

3) The similitude in rates of retention ("clearance") of neutral red by filtering live mussels and mussels sacrificed by sectioning apart the valves clearly proves that there is absolutely no quantitative relationship between this process of retention and the pumping activity.

The only functional link between both parameters, NR retention and pumping (= filtering activity) is that NR is incorporated into the pallial cavity in the water current and is, in this way, put in contact with the gill (and mantle), just as this contact is achieved in dissected mussels by the direct exposure of these tissues to the dye solution. From this point, the NR retention becomes, as was already stated, a passive uptake process that is driven by diffusion, such as was long ago evidenced in isolated gill preparations of mussels (Ward and Aiello, 1973). Indeed, it is hard to conceive how the filtering activity-the characteristic attribute of filter-feeders- might be identified with and measured by a process (NR retention)able to survive intact after the disruption of the organism as a functional unit.

A final discussion concerning the use of NR-based rates of filtration as a biomarker might be opportune, as most recent applications of the method have occurred in the field of ecotoxicological studies, particularly in freshwater systems (Faria et al., 2009; Palais et al., 2012; Parolini et al., 2014; Magni et al., 2016). In this regard, the present study was not intended to put in question whether NR retention by bivalves could be properly used as an end point in environmental stress evaluation and even serve as the basis for a particularly suitable and sensitive bioassay; stress response to toxicants or other forms of environmental deterioration is largely ethological in bivalves, involving valve closure and a consequent reduction of the capacity for NR retention. Rather, the aims of this critical evaluation of the NR method were to state that such retention in no way represents a measurement of the filtration rate. Consequently, the use of parameters (e.g., clearance rates) based on the NR method to quantify the feeding activity should be considered misguided, thus precluding any possible application of these rates in computing the energy exchanges of the population and its alteration in an ecotoxicological context.

Acknowledgments

This research was funded by the GIU 17/061 project from the UPV/EHU. Manuscript confection was benefited from a sabbatical leave to E. Navarro.

References:

Abel, P.D., 1976. Effect of some pollutants on the filtration rate of *Mytilus*. Mar. Pollut. Bull. 7, 228–231.

Bayne, B.L., 2004. Comparisons of measurements of clearance rates in bivalve molluscs. Mar. Ecol. Prog. Ser. 276, 305–306.

Bayne, B.L., 2001. Reply to comment by HU Riisgård. Ophelia 54, 211–211.

Bayne, B.L., 1998. The physiology of suspension feeding by bivalve molluscs: an introduction to the Plymouth “TROPHEE” workshop. J. Exp. Mar. Biol. Ecol. 219, 1–19.

344 Bayne, B.L., Hawkins, A.J.S., Navarro, E., 1987. Feeding and digestion by the mussel
345 *Mytilus edulis* L.(Bivalvia: Mollusca) in mixtures of silt and algal cells at low
346 concentrations. J. Exp. Mar. Biol. Ecol. 111, 1–22.

347 Berg, D.J., Fisher, S.W., Landrum, P.F., 1996. Clearance and processing of algal particles by
348 zebra mussels (*Dreissena polymorpha*). J. Great Lakes Res. 22, 779–788.

349 Cole, H.A., Hepper, B.T., 1954. The use of neutral red solution for the comparative study of
350 filtration rates of lamellibranchs. ICES J. Mar. Sci. 20, 197–203.

351 Coughlan, J., 1969. The estimation of filtering rate from the clearance of suspensions. Mar.
352 Biol. 2, 356–358.

353 Cranford, P.J., Hill, P.S., 2001. Evaluating the 'reliability' of filtration rate measurements in
354 bivalves. Mar. Ecol. Prog. Ser. 215, 303–305.

355 Dame, R.F., 2012. Ecology of marine bivalves: an ecosystem approach. 2nd Ed., CRC Press,
356 Boca Raton.

357 Fanslow, D.L., Nalepa, T.F., Lang, G.A., 1995. Filtration rates of the zebra mussel
358 (*Dreissena polymorpha*) on natural seston from Saginaw Bay, Lake Huron. J. Great
359 Lakes Res. 21:489-500.

360 Faria, M., Carrasco, L., Diez, S., Riva, M.C., Bayona, J.M., Barata, C., 2009. Multi-
361 biomarker responses in the freshwater mussel *Dreissena polymorpha* exposed to
362 polychlorobiphenyls and metals. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.
363 149, 281–288.

364 Filgueira, R., Labarta, U., Fernandez-Reiriz, M.J., 2006. Flow-through chamber method for
365 clearance rate measurements in bivalves: design and validation of individual chambers
366 and mesocosm. Limnol. Oceanogr. Methods 4, 284–292.

367 Goldberg, E.D., 1975. The mussel watch-A first step in global marine monitoring. Mar
368 Pollut. Bull. 5, 111.

Ibarrola, I., Larretxea, X., Navarro, E., Iglesias, J.I.P., Urrutia, M.B., 2008. Effects of body-size and season on digestive organ size and the energy balance of cockles fed with a constant diet of phytoplankton. J. Comp. Physiol. B 178, 501–514.

Iglesias, J.I.P., Pérez-Camacho, A., Navarro, E., Labarta, U., Beiras, R., Hawkins, A.J.S., Widdows, J., 1996. Microgeographic variability in feeding, absorption, and condition of mussels (*Mytilus galloprovincialis* Lmk.): a transplant experiment. J. Shellfish Res., 15, 673-680.

Iglesias, J.I.P., Urrutia, M.B., Navarro, E., Ibarrola, I., 1998. Measuring feeding and absorption in suspension-feeding bivalves: an appraisal of the biodeposition method. J. Exp. Mar. Biol. Ecol. 219, 71–86.

Jansen, H.M., Strand, Ø., van Broekhoven, W., Strohmeier, T., Werdegem, M.C., Smaal, A.C., 2019. Feedbacks from filter feeders: Review on the role of mussels in cycling and storage of nutrients in oligo- meso- and eutrophic cultivation areas. In, Smaal, A.C., Ferreira, J.G., Grant, J., Petersen, J.K. Strand, Ø (Eds.), Goods and Services of Marine Bivalves. Springer Nature Switzerland AG, pp. 143-177.

Jørgensen, C.B., 1990. Bivalve filter feeding: hydrodynamics, bioenergetics, physiology and ecology. Olsen & Olsen, Fredensborg, Denmark.

Jørgensen, C.B., 1996. Bivalve filter feeding revisited. Mar. Ecol. Prog. Ser. 142, 287–302.

Kryger, J., Riisgård, H.U., 1988. Filtration rate capacities in 6 species of European freshwater bivalves. Oecologia 77, 34–38.

Madon, S.P., Schneider, D.W., Stoeckel, J.A., Sparks, R.E., 1998. Effects of inorganic sediment and food concentrations on energetic processes of the zebra mussel, *Dreissena polymorpha*: implications for growth in turbid rivers. Can. J. Fish. Aquat. Sci. 55, 401–413.

Magni, S., Parolini, M., Binelli, A., 2016. Sublethal effects induced by morphine to the freshwater biological model *Dreissena polymorpha*. Environ. Toxicol. 31, 58–67.

Martinez-Haro, M., Pais-Costa, A.J., Verdelhos, T., Marques, J.C., Acevedo, P., 2016. Optimising a clearance index based on neutral red as an indicator of physiological stress for bivalves. Ecol. Indic. 71, 514–521.

Matthiessen, G.C., 1960. Observations on the ecology of the soft clam, *Mya arenaria*, in a salt pond. Limnol. Oceanogr. 5, 291–300.

Møhlenberg, F., Riisgård, H.U., 1978. Efficiency of particle retention in 13 species of suspension feeding bivalves. Ophelia 17, 239–246.

Møhlenberg, F., Riisgård, H.U., 1979. Filtration rate, using a new indirect technique, in thirteen species of suspension-feeding bivalves. Mar. Biol. 54, 143–147.

Navarro, E., Iglesias, J.I.P., Ortega, M.M., Larretxea, X., 1994. The basis for a functional response to variable food quantity and quality in cockles *Cerastoderma edule* (Bivalvia, Cardiidae). Physiol. Zool. 67, 468–496.

Palais, F., Dedourge-Geffard, O., Beaudon, A., Pain-Devin, S., Trapp, J., Geffard, O., Noury, P., Gourlay-Francé, C., Uher, E., Mouneyrac, C., Biagianti-Risbourg, S., Geffard, A., 2012. One-year monitoring of core biomarker and digestive enzyme responses in transplanted zebra mussels (*Dreissena polymorpha*). Ecotoxicology 21, 888–905.

Parolini, M., Magni, S., Binelli, A., 2014. Environmental concentrations of 3, 4-methylenedioxymethamphetamine (MDMA)-induced cellular stress and modulated antioxidant enzyme activity in the zebra mussel. Environ. Sci. Pollut. Res. 21, 11099–11106.

Pascoe, P.L., Parry, H.E., Hawkins, A.J.S., 2009. Observations on the measurement and interpretation of clearance rate variations in suspension-feeding bivalve shellfish. Aquat. Biol. 6, 181–190.

Petersen, J.K., Bougrier, S., Smaal, A.C., Garen, P., Robert, S., Larsen, J.E.N., Brummelhuis, E., 2004. Intercalibration of mussel *Mytilusedulis* clearance rate measurements. Mar. Ecol. Prog. Ser. 267, 187–194.

422 Riisgård, H.U., 1988. Efficiency of particle retention and filtration rate in 6 species of
423 Northeast American bivalves. Mar. Ecol. Prog. Ser. 45, 217–223.

424 Riisgård, H.U., 1991. Filtration rate and growth in the blue mussel, *Mytilus edulis* Linnaeus,
425 1758: dependence on algal concentration. J. Shellfish Res. 10, 29-35.

426 Riisgård, H.U., 2001a. On measurement of filtration rates in bivalves—the stony road to
427 reliable data: review and interpretation. Mar. Ecol. Prog. Ser. 211, 275–291.

428 Riisgård, H.U., 2001b. Comment: Physiological regulation versus autonomous filtration in
429 filter-feeding bivalves: Starting points for progress. Ophelia 54, 193–209.

430 Riisgård, H.U., 2004. Intercalibration of methods for measurement of bivalve filtration rates-
431 a turning point. Mar. Ecol. Prog. Ser. 276, 307–308.

432 Riisgård, H.U., Seerup, D.F., 2003. Filtration rates in the soft clam *Mya arenaria*: effects of
433 temperature and body size. Sarsia 88, 416–428.

434 Roditi, H.A., Caraco, N.F., Cole, J.J., Strayer, D.L., 1996. Filtration of Hudson River water
435 by the zebra mussel (*Dreissena polymorpha*). Estuaries 19, 824–832.

436 Sprung, M., Rose, U., 1988. Influence of food size and food quantity on the feeding of the
437 mussel *Dreissena polymorpha*. Oecologia 77, 526–532.

438 Vahl, O., 1972. Particle retention and relation between water transport and oxygen uptake in
439 *Chlamys opercularis* (L.) (Bivalvia). Ophelia 10, 67–74.

440 Vahl, O., 1973. Porosity of the gill, oxygen consumption and pumping rate in *Cardium edule*
441 (L.) (Bivalvia). Ophelia 10, 109–118.

442 Ward, J.E., MacDonald, B.A., Thompson, R.J., Beninger, P.G., 1993. Mechanisms of
443 suspension feeding in bivalves: resolution of current controversies by means of
444 endoscopy. Limnol. Oceanogr. 38, 265–272.

445 Ward, M.E., Aiello, E., 1973. Water pumping, particle filtration and neutral red absorption
446 in the bivalve mollusc *Mytilus edulis*. Physiol. Zool. 46, 157–167.

Widdows, J., 1978. Physiological indices of stress in *Mytilus edulis*. J. Mar. Biol. Assoc. U. K. 58, 125–142.

Widdows, J., 2001. Bivalve clearance rates: inaccurate measurements or inaccurate reviews and misrepresentation? Mar. Ecol. Prog. Ser. 221, 303–305.

Widdows, J., Donkin, P., Brinsley, M.D., Evans, S.V., Salkeld, P.N., Franklin, A., Law, R.J., Waldock, M.J., 1995. Scope for growth and contaminant levels in North Sea mussels *Mytilus edulis*. Mar. Ecol. Prog. Ser. 127, 131–148.

Table 1: Mean values (±2SE) of CR (standardized to 1g. dry tissue wt: DTW) recorded with different methods and approaches: (1) and (2): using phytoplankton, estimated as average of repeated measurements at 15 min intervals (1) and by continuous recording of particle decline (2); (3),(4) and (5): different measurements using NR, considering the 1st hour (3), the mean value for different intervals (4) and the rate averaged over a period of 17 h (5). Different letters denote significant differences between the means (Tukey post hoc test). Ratios between different CR estimations are given in columns 6-9.

$CR_{standardized\ to\ 1g\ DTW}$ ($L\ h^{-1}$)					Ratios			
CR_{RM} (1)	CR_C (2)	CR_{NR} 1st h (3)	CR_{NR} (mean) (4)	CR_{NR} (average) (5)	(2)/(1)	(3)/(1)	(4)/(1)	(5)/(1)
6.293^a ±1.405	3.960^b ±0.890	0.773^c ± 0.515	0.466^c ± 0.156	0.379^c ± 0.043	0.653 ± 0.112	0.114 ± 0.041	0.075 ± 0.013	0.068 ± 0.019

Table 2.- Clearance rates using neutral red and conventional methods are reported in different species of bivalve molluscs. For comparative purposes, CR values reported for each species have been standardized to a common size.
Mytilus edulis and Mytilus galloprovincialis: standard size = 1g SBDW (soft body dry weight); scaling power b = 0.67 (Møhlenberg and Riisgård, 1979)*
Mya arenaria: no standardization was necessary (groups to compare have identical size)
Cerastoderma edule: standard size 25 mm SH (shell height) = 0.3 g SBDW; scaling power b= 0.57 (Ibarrola et al., 2008).
Dreissena polymorpha: standard size 20 mm SL (shell length) = 0.018 g SBDW; scaling power b = 0.88 (Kryger and Riisgård, 1988).

Species	Method	Temperature	Body size	Aims of the study or context	CR (ml h^{-1})	Reference
Mytilus edulis	Neutral red	2-10 ºC	75 mm SL	Temperature and salinity effects	100-300	Cole and Hepper, 1954
		12 ºC	30-50 mm SL	Toxicological effects	1070-1344 (control groups)	Abel, 1976
	Flow-through	10 ºC	20-80 mm SL	Multifactor effects	2550 (adjusted intercept)	Widdows, 1978
	Suction	10-13ºC	0.01-1.3 g (SBDW)	Size-scaling relationships	7450 (adjusted intercept)	Møhlenberg and Riisgård, 1979
	Flow-through	9 –15 ºC	45-50 mm SL	Food quality and seasonal effects	3700-4120	Bayne et al., 1987
	Biodeposition, Flow through, Clearance (Coughlan)	15 ºC	45-47 mm SL	Intercalibration methods and sites	2200-11000	Petersen et al. 2004
Mytilus galloprovincialis	Neutral red	14 ºC	50 mm SL	Methodological	378-773	This study
	Biodeposition	15ºC	80 mm SL	Micro-geographic comparison	2000-4500	Iglesias et al., 1996
	Clearance (Coughlan)	14 ºC	50 mm SL	Methodological	3900 -6300	This study
Mya arenaria	Neutral red	22ºC	27 mm SL	Salinity effects	100-125	Matthiesen, 1960
	Clearance and suction	22 ºC	27 mm SL	Temperature and body size effects	3160 (from regression equations)	Riisgård and Seerup, 2003
Cerastodermaedule	Neutral red	20 ºC	25 mm SH	Methodological	80-170	Martínez-Haro et al., 2016
	Clearance (Coughlan)	17 ºC	22 mm SH	Food ration and quality effects	1220-2450	Navarro et al., 1994
	Biodeposition and flow-through	20 ºC	22 mm SH	Methodological	750-2400	Iglesias et al., 1998
Dreissenapolimorpha	Neutral red	20ºC	20 mm SL	Toxicological effects	7-8	Faría et al., 2009
		10-20 ºC	21 mm SL	Toxicological / seasonal effects	1.4 (winter) -5.3 (summer)	Palais et al., 2012
		?	15 mm SL	Toxicological effects	1.5-3.8 (baseline levels)	Parolini et al., 2014
	Clearance (SPM reduction)	20 ºC	20 mg (SBDW)	Field energetics	45-72	Madon et al., 1998
	Flow-through (SPM and Chlorophyll)	21 ºC	13-14 mm SL	Field filtration	233-350	Roditi et al., 1996
	Flow-through (Chlorophyll)	6-25 ºC	5-9 mm SL	Seasonal/inter-annual variation	127-510	Fanslow et al., 1995
	Clearance (Coughlan)	19-20 ºC	2-66 mg (SBDW)	Size-scaling relationships	120 (from regression equations)	Kryger and Riisgård, 1988
	Clearance (Coughlan)	12 ºC	25-30 mm SL	Feeding behaviour	105-118	Sprung and Rose, 1988
	Clearance (Coughlan)	22 ºC	10-15/ 22-25 mm SL	Feeding behaviour	120-140	Berg et al., 1996

*See Jensen et al. (2019) for a more extensive list of CR values (standardized to 1g.SBDW) in species of *Mytilus.*

FIGURE CAPTIONS

Figure 1.- Two recordings of the changing particle concentration over the time (on a semi-logarithmic scale) in clearance chambers illustrating the two procedures followed in the CR determination with phytoplankton (see text). Open circles: Values of the particle concentration in the continuous recording. Full circles: Values of the particle concentration in the discontinuous recording (repeated measurements).

Figure 2.- A) Temporal evolution of marker concentration (phytoplankton or NR). Values (mean ±2 SE) are plotted in a relative scale to make trends comparable. Solid symbols: NR; hollow symbols: phytoplankton. Dotted lines and equations represent exponential functions fitted to both sets of data. B) Changes in the NR concentration in different time intervals over 17 hrs. Exponential curves and equations are presented for the first two hours interval (solid line) and the 17 h interval (dotted line).

Figure 3.- Regression analysis for different CR measurements with phytoplankton and NR using the same individual mussels. CR_{RM} : clearance rate based on repeated measurements; CR_C : clearance rate based on continuous measurement (both using phytoplankton); CR_{NR}: clearance rates using neutral red (values averaged over 17 h exposure). Equations of the lines were: $CR_{RM} = 0.88 + 1.17\ CR_C$; $R^2 = 0.62$; $p = 0.01$ (regression through the origin: $CR_{RM} = 1.53\ CR_C$;; $R^2 = 0.55$; $P = 0.02$); $CR_{NR} = 0.20 – 0.00\ CR_C$; $R^2 = 0.00$.

Figure 4.- Time curve of the NR concentration in the presence of intact mussels (hollow symbols and grey line) and dissected mussels (full symbols and black line). Error bars represent 2SE (n = 5).

Figure 1.

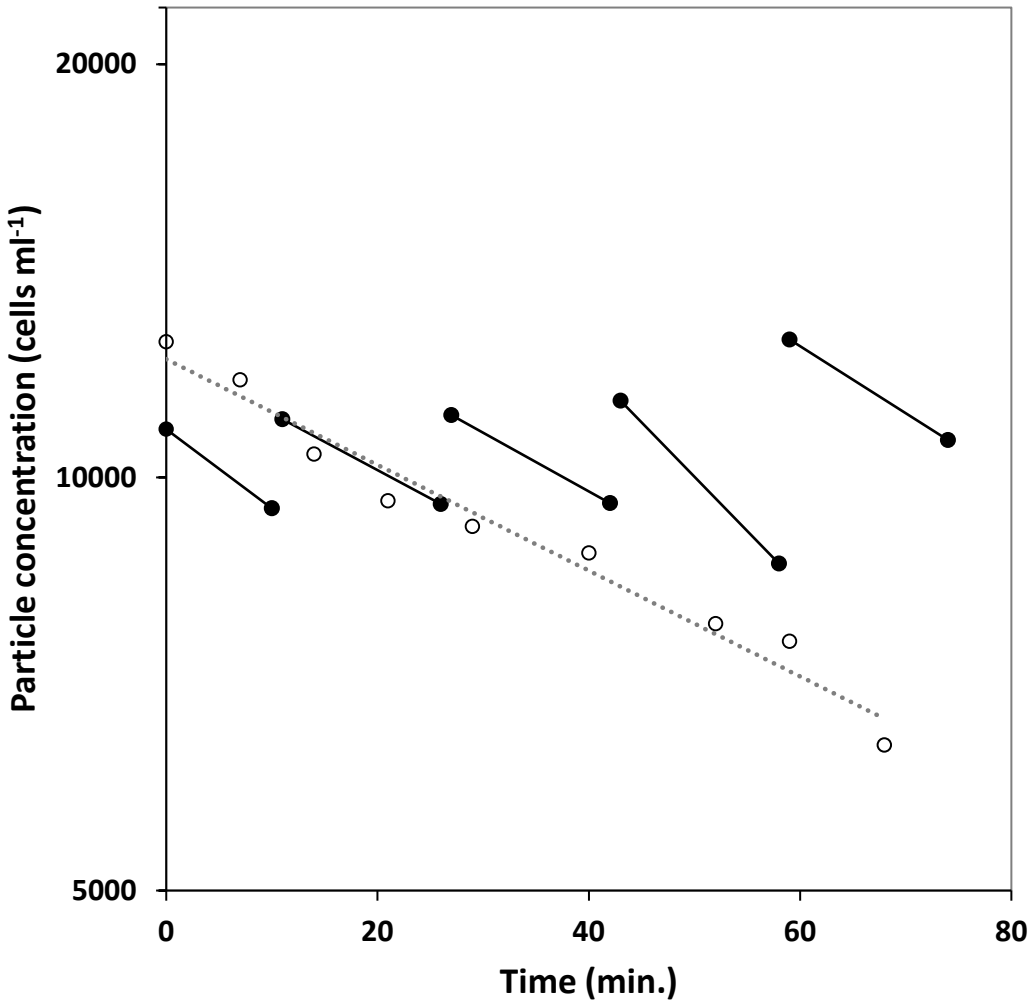
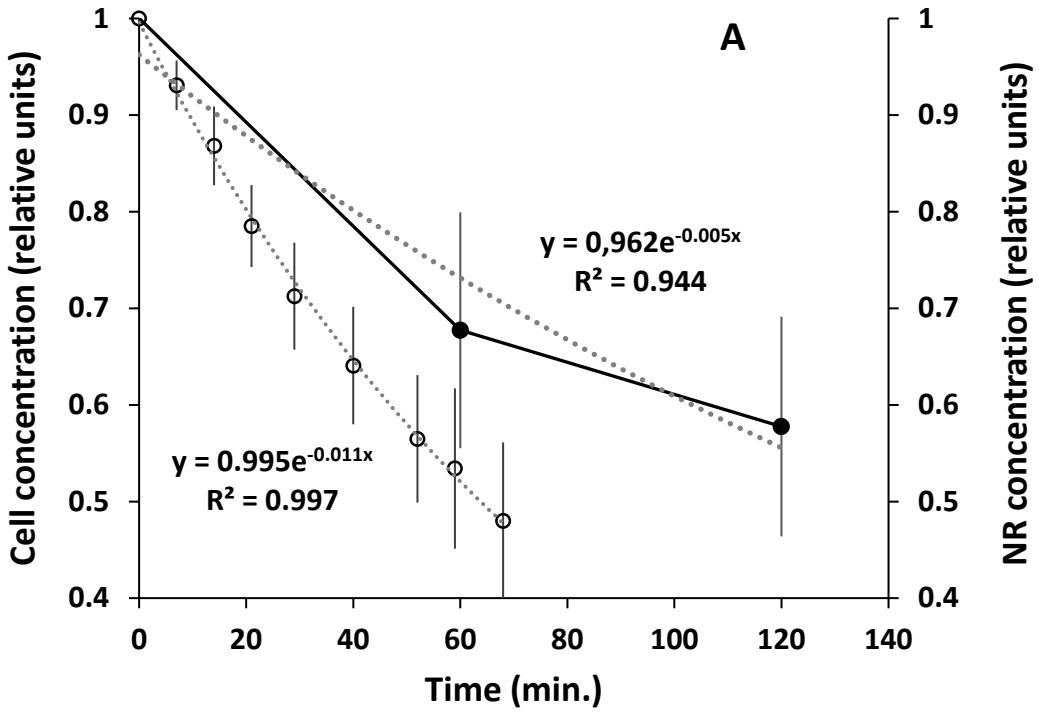

20000
10000
5000
Particle concentration (cells ml-1)
0
20
40
60
80
Time (min.)

Figure 2.


A
Cell concentration (relative units)
NR concentration (relative units)
Time (min.)
y = 0,962e-0.005x
R² = 0.944
y = 0.995e-0.011x
R² = 0.997
1
0.9
0.8
0.7
0.6
0.5
0.4
0
20
40
60
80
100
120
140

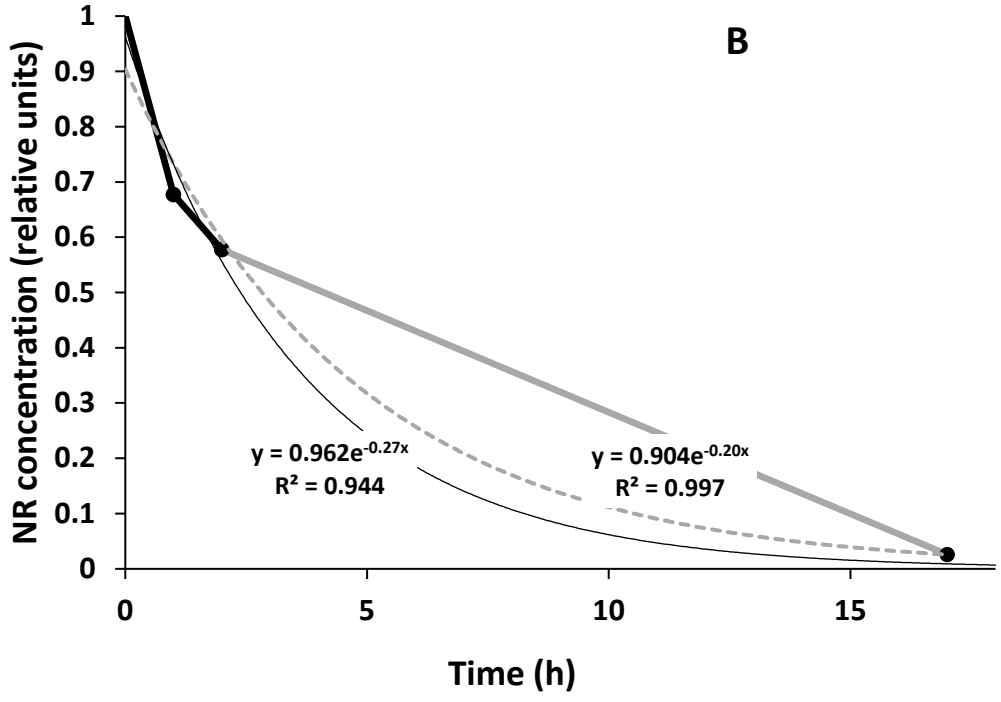

B
NR concentration (relative units)
Time (h)
y = 0.962e-0.27x
R² = 0.944
y = 0.904e-0.20x
R² = 0.997
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15

Figure 3.

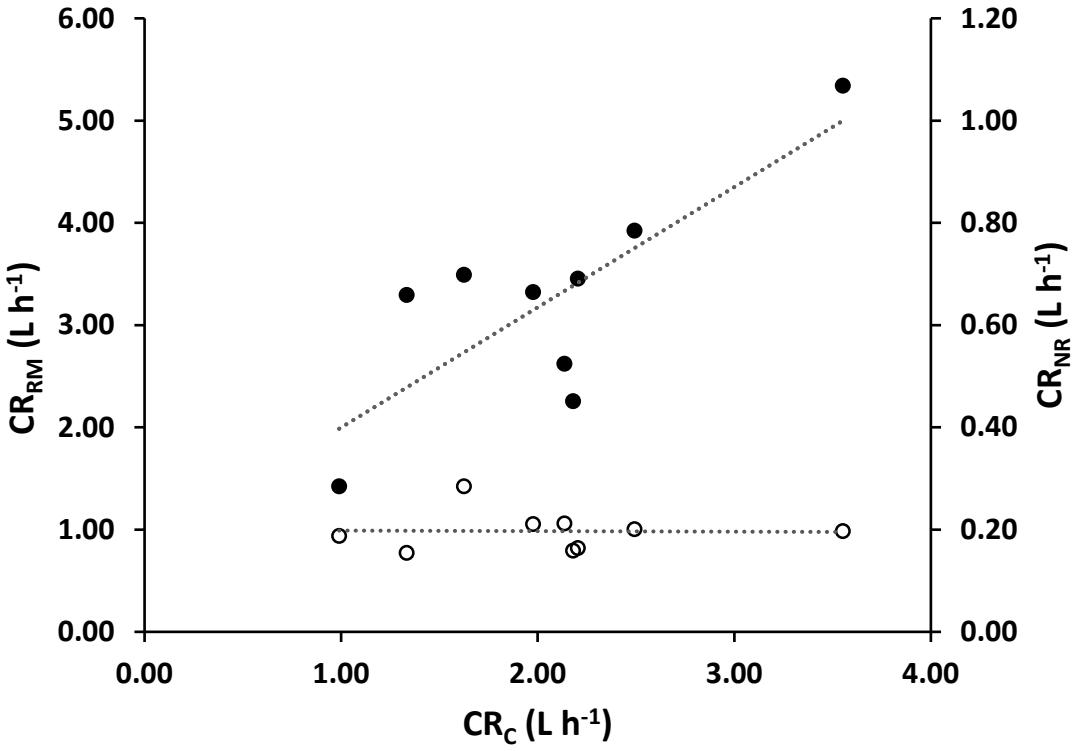
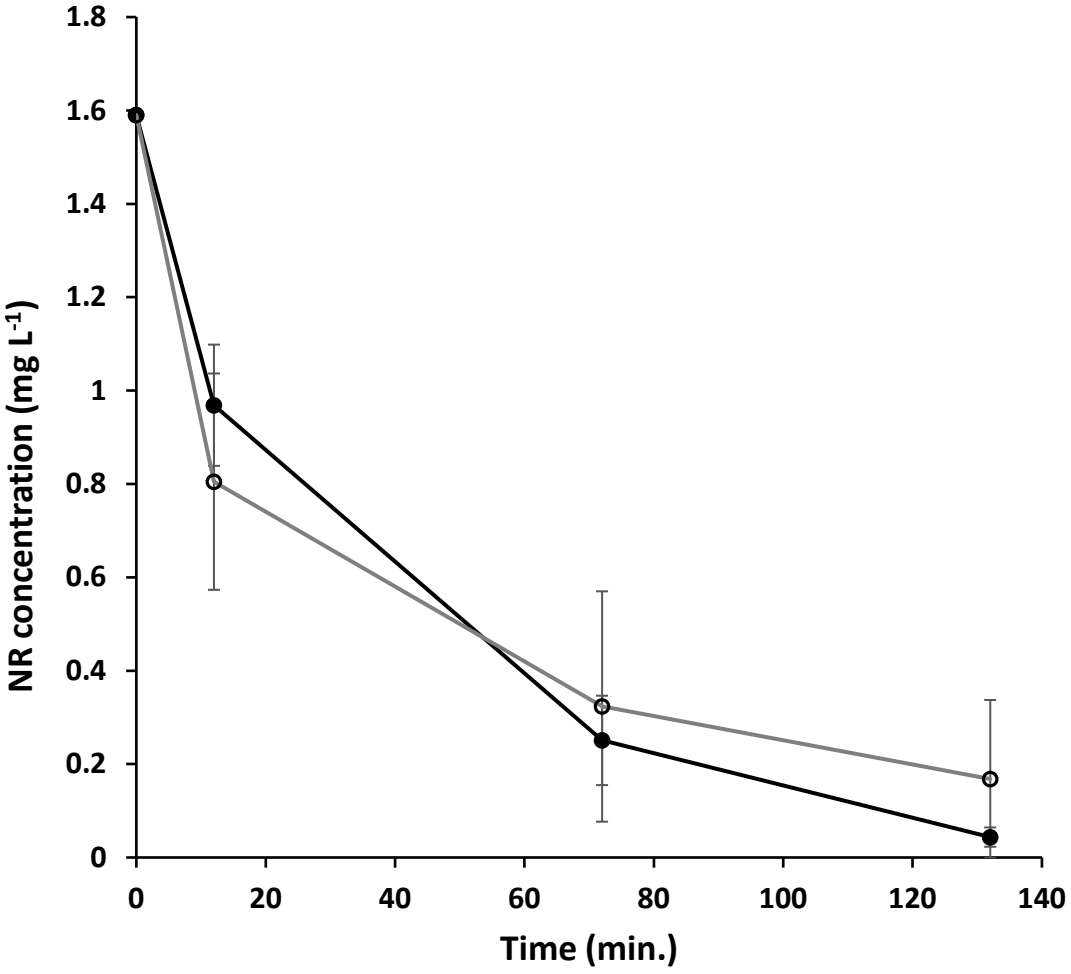

6.00
5.00
4.00
3.00
2.00
1.00
0.00
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0.00
1.00
2.00
3.00
4.00
CR_RM (L h-1)
CR_NR (L h-1)
CR_C (L h-1)

Figure 4.


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
NR concentration (mg L-1)
0
20
40
60
80
100
120
140
Time (min.)