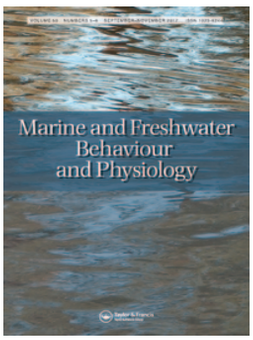

Marine and Freshwater Behaviour and Physiology

ISSN: 1023-6244 (Print) 1029-0362 (Online) Journal homepage: http://www.tandfonline.com/loi/gmfw20

Modelling hydrolysis of leaf litter by digestive enzymes of the snail Melanopsis praemorsa: combination of response surface methodology and in vitro assays

Juan Rubio-Ríos, Encarnación Fenoy, J. Jesús Casas & F. Javier Moyano

To cite this article: Juan Rubio-Ríos, Encarnación Fenoy, J. Jesús Casas & F. Javier Moyano (2017) Modelling hydrolysis of leaf litter by digestive enzymes of the snail Melanopsis praemorsa: combination of response surface methodology and in vitro assays, Marine and Freshwater Behaviour and Physiology, 50:5-6, 313-328, DOI: 10.1080/10236244.2017.1404429

To link to this article: https://doi.org/10.1080/10236244.2017.1404429

MARINE AND FRESHWATER BEHAVIOUR AND PHYSIOLOGY, 2017
VOL. 50, NOS. 5–6, 313–328
https://doi.org/10.1080/10236244.2017.1404429

Modelling hydrolysis of leaf litter by digestive enzymes of the snail *Melanopsis praemorsa:* combination of response surface methodology and *in vitro* assays

Juan Rubio-Ríos, Encarnación Fenoy, J. Jesús Casas and F. Javier Moyano

Departamento de Biología y Geología, Facultad de Ciencias. Campus de Excelencia Internacional del Mar (CEI-MAR), Universidad de Almería, Almeria, Spain

ABSTRACT

The objective of the present study was to test the application of an *in vitro* assay simulating the digestive hydrolysis of leaf litter by the freshwater snail *M. praemorsa,* as well as to determine the possible influence of different factors in the efficiency of such process to release biologically available C and N under the forms of reducing sugars and amino acids from two different substrates. A novel approach to construct a model explaining the effect of three main factors (temperature, total reaction time and enzyme:substrate ratio) in the digestive hydrolysis of cellulose and protein present in leaf litter of different nutritive value is used. The methodology combines a factorial design based in the response surface methodology (RSM) and *in vitro* digestibility assays adapted to the physiology of both plant substrates used (alder and poplar leaves). The model revealed a different influence of the factors in the hydrolysis of two plant substrates, poplar and alder leaves and the main effect was produced by the time available for hydrolysis. A compensation response based in a longer gut retention time for the lower quality substrate was observed in the feeding assays. The use of *in vitro* assays and RSM provides a useful insight on the effect of factors and mechanisms underlying the observed differences in nutritional value of leaf litter for an aquatic invertebrate, being such differences linked to the whole bioavailability of carbon and nitrogen in headwater streams.

ARTICLE HISTORY
Received 26 May 2017
Accepted 6 November 2017

KEYWORDS
In vitro digestion; leaf litter; model; *Melanopsis praemorsa*

Introduction

Environmental enzymology, based in describing the role of enzymes as drivers of the bioavailability of some main elements as carbon and nitrogen in ecosystems, is a topic of increased interest. Measurement of enzyme activities is oriented to assess both their participation in nutrient fluxes and their suitability as indicators, provided their high sensitivity to biotic and abiotic factors (Tate 2002). These issues are of a particular interest in aquatic environments, mainly in streams, since in such ecosystems the primary production is very limited and leaf litter decomposition mediated by enzymes is the main source providing

CONTACT F. Javier Moyano fjmoyano@ual.es

carbon and other nutrients for secondary production (Wallace et al. 2015). These enzymes are produced mainly by microbial decomposers (Bärlocher 1992; Gulis and Suberkropp 2003) but also by invertebrate detritivores (Graça 2001). Nevertheless, while a great number of papers have assessed the effect of factors affecting the activity of ectoenzymes produced by fungal decomposers in aquatic environments (Chróst 1991; Gessner et al. 2007) much less information is available on which ones affect the net efficiency of endogenous enzymes present within the digestive systems of invertebrate shredders. Thus, the evaluation of factors affecting the efficiency of gut processing of leaf litter by invertebrate shredders is of primary interest when assessing the potential bioavailability of C and N for stream food webs. Although physical and chemical traits of leaf litter are key factors governing consumption rates by shredders (Graça et al. 2015), there are other factors with an important effect on the net efficiency of the digestion. They are mainly related to the process of enzyme hydrolysis and further absorption of products by the organism; as an example, water temperature, gut retention time or total production of digestive enzymes. Temperature exerts an important direct effect on the digestion process through the modification of enzyme kinetics, and also influencing whole metabolism and therefore food transit rates within the gut, this determining the available time for enzyme hydrolysis (Logan et al. 2002). Variation in food consumption linked to its availability also determines changes in the enzyme/substrate ratio within the gut and hence the kinetics and net efficiency of the enzymes.

The evaluation of the above-mentioned effects and other factors on the efficiency of enzyme hydrolysis within the gut of a given species is a difficult task if performed using *in vivo* assays, but it potentially can be determined from *in vitro* simulation of the digestion process, an approach commonly used in nutritional studies with animals and humans. Within this perspective, the digestive system can be considered as a more or less complex bioreactor with a specific configuration and functionality. In fact, Penry and Jumars (1986, 1987) and several other authors (Martínez del Rio et al. 1994; Logan et al. 2003) have developed a complete theory to establish operating similitudes between animal digestive systems and reactors as well as on species-specific strategies followed to maximize gain of energy and nutrients. From an applied perspective, a great number of physiologists and nutritionists have used a variety of reactors and combinations of enzymes to simulate *in vitro* the guts of a wide range of species. These models, ranging from quite simple batch reactors (Saunders et al. 1973; Parsons 1991) to the very complex multi-compartment devices used in human nutrition studies (Minekus et al. 1995), have been routinely used to perform digestibility assays, as well as to assess different aspects related to the hydrolysis of food substrates (Boisen 2000; Moyano et al. 2014). Nevertheless, up to date *in vitro* assays have been scarcely used to model the digestion process of invertebrates. Langdon (1989) simulated the digestion of different artificial food sources by the bivalve *Crassostrea gigas*, while Hamdan et al. (2013) optimized a digestion model for the evaluation of ingredients in diets for *Octopus vulgaris*. However, the use of this approach may have a wider application in ecological studies, as demonstrated by Areekijseree et al. (2006) and Supannapong et al. (2008), who evaluated the hydrolysis of different types of phytoplankton by the digestive enzymes of the bivalve *Hyriopsis bialatus*. When considered together these studies show that *in vitro* simulation of digestive enzyme hydrolysis may be used to evaluate the relative effect of factors affecting the efficiency of gut processing of different types of plant material by detritivores, this helping to assess differences in nutritional value and its effect in their growth and survival, in addition to the potential bioavailability of C and N at the ecosystem level.

A suitable framework to develop this kind of assay should consider not only the particular type of bioreactor used (Moyano et al. 2014), but also the possibility of testing the effects of several main factors affecting the evaluated response. Thus, the use of a factorial design in a similar approach to that used by chemical engineers to optimize hydrolysis conditions in reactors may be helpful. In factorial experiments, different levels of multiple factors are investigated simultaneously and one factor can be examined at different levels of the rest. One of the most powerful tools is the design of such experiments is the response surface methodology (RSM), a group of mathematical and statistical techniques that generates a mathematical model after defining the relationships between a selected response and several independent variables. RSM is routinely applied in the optimization of industrial processes based in enzyme hydrolysis (Zhang et al. 2013; Dey and Dora 2014), but more recently, it has also been used in the assessment of optimal conditions to be used when modelling human digestion *in vitro* (Hollebeeck et al. 2013) and also to assess the effect of several factors on protein hydrolysis by digestive enzymes of fish (Gilannejad et al. 2017). One of the more widely used designs to develop response surface models is the Box–Behnken Design (BBD) (Box and Behnken 1960). In BBD, the level of one of the selected factors is fixed at the centre level, while combinations of all levels of the rest of factors are applied (Kocabaş 2001; Myers and Montgomery 2002). Giving a number of factors, the BBD approach often requires fewer experimental points as compared to other response surface designs, being therefore more suitable when there are limitations imposed by the cost of the assays or by the amount of available biological material.

The aims of the present study were: (a) to develop an *in vitro* assay suitable to simulate gut hydrolysis of cellulose and protein of leaf litter by an aquatic invertebrate shredder, (b) to assess the effects of three different factors (temperature, time of gut residence and relative digestive enzyme:substrate (E:S) concentrations) on such hydrolysis and (c) to assess the sensitivity of the model, testing the response when using leaf litter showing potential differences in nutritional quality. The selected species was the freshwater snail *Melanopsis praemorsa* (L.), because it is widespread throughout the Mediterranean region and plays an important role in leaf litter decomposition in many lowland headwaters (Chergui and Pattee 1991; Heller and Abotbol 1997; Casas et al. 2006, 2011).

Material and methods

The present study comprised three steps: (1) assessment of some physiological features of the target species required to design the *in vitro* experiment, (2) running *in vitro* digestibility assays on two selected types of leaf litter following the afore-mentioned BBD and (3) validation of the results by an *in vivo* nutritional assay.

Biological material

Specimens of *Melanopsis praemorsa* (L). were collected from different streams at two different regions in southern Spain – Sierra de Grazalema (36° 48′–36° 49′ N, 5° 22′–5° 24′ W) and the semi-arid lowland of Almeria (36° 50′–37° 05′ N, 2° 00′–2° 38′ W) – and acclimated to laboratory conditions in aquaria at 14 °C under a 10:14 (light:dark) photoperiod during seven days, being fed on leaf litter obtained in their stream of origin. Two leaf litter species of contrasting quality, black alder (*Alnus glutinosa* L. Gaertn) and white poplar (*Populus alba* L.),

were used in the experiments to assess potential differences in their *in vitro* hydrolysis by the digestive enzymes of the snail. This species possesses the range of enzymes already described for other gastropods, including a high activity of cellulase produced by the snails themselves (Flari and Charrier 1992) as well as Alder leaf litter was selected for being widely recognized to have high nutritional value for invertebrate shredders, thus serving as a reference material, while poplar was selected for being a common species in the riparian vegetation of streams where inhabit the snail used in the experiments. Senescent leaves were collected from the riparian vegetation of one stream in Sierra Nevada (southeastern Spain), picked from the ground just after abscission, air-dried to constant mass and stored at room temperature until required. These leaves were later incubated in a stream for 21 days (mean temperature 12 ± 0.6 °C) in bags of 1 mm mesh size to allow microbial colonization and hence to obtain a litter material similar to that present in streams as food for the snails. After incubation, bags were withdrawn from the stream, placed in individual sealed plastic bags with stream water and transported in a cool box to the laboratory, where leaf litter was carefully rinsed with filtered (0.45 μm) stream water to remove fine particles and invertebrates. These leaf litter samples were used both as substrate for the *in vitro* assays and as food source for the *in vivo* assays. Selected physical and chemical parameters of leaf litter were evaluated. Toughness was measured on hydrated leaves (n = 20 per species), avoiding major veins, using a texturometer (TA.XT2 Plus, Stable Micro Systems, London, UK) equipped with a cylindrical steel sounding line with a puncture surface of 0.38 mm^2. Portions (n = 3) of dried (60 °C, 72 h) and ground (particle size < 1 mm) leaf litter were used to analyse chemical composition. Contents in hemicellulose, cellulose and lignin were measured using an ANKOM 200/220 fibre analyser (ANKOM Technologies, Macedon, NY, USA) (Fenoy et al. 2016). Total carbon and nitrogen concentrations were determined using a Leco TruSpec CN elemental analyser (Leco Corporation, St Joseph, MI). Phosphorus concentration was determined using an ICP-OES (IRIS Intrepid II XDL; Thermo Fisher Scientific Inc.). Silicon concentration was determined using inductively coupled plasma atomic emission spectroscopy (Thermo ICAP 6500 duo, Thermo Fisher Scientific, Cambridge, UK), after microwave sample digestion in nitric acid (65%) and hydrogen peroxide (30%).

The methodology of the BBD requires an assessment of the range values of the different factors involved in the model. For this purpose, some preliminary assays were conducted to determine such reference values for gut evacuation time and enzyme production by *M. praemorsa*. Gut evacuation time after feeding on each leaf litter species was measured on 10 snails (mean ± 1SD individual dry mass = 43.0 ± 24.2 mg) per diet maintained in aquaria at 14 °C. The snails were fed for 2 days on an artificial diet prepared using a mixture of agar and cellulose powder that produced white faecal pellets. After this time, leaf discs from either alder or poplar substituted the food. The snails were visually monitored every 15 min to assess the time lapse between the first sign of leaf consumption (i.e. evidence of nibbling on the leaf disc) and the appearance of dark faecal matter; this was considered the gut residence time and was assumed to be the time lapse on which the enzyme hydrolysis was taking place.

To estimate the physiological enzyme:substrate ratio present in the gut of the snail, average cellulase and protease activities were measured in enzyme extracts prepared by manual homogenization of the digestive glands of 10 snails in distilled water (1/3 weight:volume). After measurement of pH in crude extracts (pH Basics 20, Crison instruments, Barcelona,

Spain), the homogenates were centrifuged (12,500 × g, 4° C, 15 min) and the obtained supernatants were stored at −20 °C until required. The activities of cellulase and protease were determined by the Somogy-Nelson method and the ortho-phthalaldehyde method to assess the production of reducing sugars (RS) and total amino acids (AA), respectively. On the other hand, average food consumption required to estimate the amount of substrate present in the gut was estimated by specific feeding trials detailed in the next section.

The range of water temperatures used on *in vivo* and *in vitro* experiments was obtained from one-year records obtained in the streams (spring 2013–spring 2014) by means of HOBO Pendant (Onset Computer Corporation, Bourne, MA, USA) loggers.

In vitro assays

Samples of each leaf litter species to be used as substrate in the bioreactors were dried (60 °C, 72 h), and ground to pass through a 0.5 mm sieve to ensure uniformity of particle size. Previous assays indicated that a dialysis of the resulting powder (48 h at 4 °C using a Spectra/Por ® Biotech Cellulose Membrane of MWCO 1000 Da) was required in order to remove reducing sugars already present in the samples that may interfere with further measurement of those produced by the enzymatic hydrolysis when using the Somogy-Nelson method. Amicon® Ultra 4 cartridges with a MWCO of 10 kDa, from Millipore (Bedford, MA, USA) were used as bioreactors. These units maintain the reaction mixture formed by the enzyme extract and the substrate particles in the internal compartment, allowing regular removal of the products released to the external chamber by centrifugation. Once the enzyme extract and the substrate were placed into the cartridge, the pH was set at 5.5 (average value measured in the gland extracts) by adding 3.5 ml of citrate buffer (pH 5.5 0.1 M). Bioreactors were then placed in a rotary shaker located inside an incubation chamber maintained at the desired temperatures in the range of 10–25 °C and samples were taken every hour. To get the samples, each bioreactor was centrifuged at 4000 g for 7 min to remove the reducing sugars and total amino acids released after hydrolysis during such time. The removed volume of filtered solution was always replaced with an equal volume of fresh citrate buffer prior to continue with the incubation. Blanks were prepared in a similar way, but enzyme extracts were inactivated by the addition of trichloroacetic acid before incubation. The amount of reducing sugars and total amino acids in dialysates resulting from the hydrolysis of cellulose and protein present in leaves were analysed by the same methods described for enzyme activities.

In vivo assays

Feeding trials were carried out using discs of leaf litter from each species that were freeze-dried, weighed individually and stored at −20 °C until required. Food consumption and growth of snails on each leaf litter species was assessed using 30 individual snails that were maintained, at 14 °C (average winter temperature in the streams of origin) and under a 10:14 light:dark cycle, in individually aerated cylindrical containers (5 cm ø, 7 cm height) with filtered stream water, that was replenished every five days. In each container, a septum of 1 mm mesh size, located at 0.5 cm from the base, prevented the access of the snail to its faeces. Two discs of the corresponding leaf litter were provided to each snail and replaced every five days, during a 30-day period. A pair of leaf discs was submerged in a mesh bag

near the bottom of the container unavailable to the snail, and served to account for leaf mass loss due to microbial activity. Leaf litter consumption was estimated from loss of disc mass offered at the beginning and the end (discounting microbial-driven mass loss) of the experimental period, using the following expression (e.g. Danger et al. 2012): Feeding rate ($mg_{leaf\ litter}\ g_{individual}\ day^{-1}$) = ($DM_t$ – DM_i)/($DM_{individual}$ × t); where DM_t is final discs dry mass, DM_i is initial dry mass of leaf discs, t is the exposure time (five days) to consumption and $DM_{individual}$ is the initial or final dry mass of the snail (see below) for estimating feeding rates at the beginning or the end of the experiment, respectively.

Growth of the snails was measured to calculate several indices of the nutritional efficiency of each leaf litter species to be compared with results from the *in vitro* assays. Growth rate was estimated as the difference of snail size between the beginning and the end of the feeding experiment. Each individual was photographed at the beginning and the end of the experiment, and their shell height measured to the nearest mm using a *SigmaScan Pro v 5.0* image analyser. A linear regression model for shell height-dry mass conversion DM=(0.0088 shell height-0.0619) was previously constructed, subtracting incinerated shell mass (450 °C, 5 h) from total DM (60 °C, 72 h). Thus, daily growth rate was calculated using the following expression: Growth rate (mg d^{-1}) = (SDM_t) – (SDM_i)/t; where SDM_i and SDM_t are the initial and final snail dry mass, respectively and t is the time (30 days). Daily instantaneous growth rate was estimated as: DIGR (d^{-1}) = (Ln(SDM_t)-Ln(SDM_i))/t. Gross production efficiency (GPE) and food conversion efficiency (FCE) were estimated from the increase in snail biomass and the leaf dry mass (LDM) ingested, using the following expressions: GPE = ΔBiomass (g SDM d^{-1})/Total leaf ingested (g LDM d^{-1}); FCE = 100 × (ΔBiomass (g SDM)/Total leaf ingested (g LDM)); respectively. Food conversion rate (FCR) was estimated as the quantity of ingested leaf litter that was transformed in biomass, using the following expression: FCR = Total leaf ingested (g LDM)/ΔBiomass (g SDM). These results were compared to the estimated carbon and nitrogen bioavailability obtained with the *in vitro* assays.

Experimental design and statistical analysis

Gut evacuation time, physical and chemical characteristics of the two types of leaf litter, as well as all growth-related indices measured in snails were compared using two-sample *t*-test at $p < 0.05$. The effect of gut residence time, temperature and enzyme:substrate ratio (E:S) on the enzymatic hydrolysis of cellulose and protein present in the two leaf litter species was assessed by a three-level BBD with two replicates using Minitab 17 trial version software (Minitab Inc., State College, PA). The result was a one-block face-centred ($a = 1$) BBD for the three numerical and one categorical (leaf litter species) factors. The combinations of variable values (coded and uncoded) are detailed in Table 3. Orthogonal least-squares calculation on factorial design data was used to obtain empirical equations describing the release of either reducing sugars or amino acids as dependent variable related to the effects.

The general form of the polynomial equations is:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_I + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i<j=1}^{3} b_{ij} X_i X_j$$

where Y is the response variable (Y_1 and Y_2, reducing sugars and amino acids, respectively), b_0, b_i, b_{ii} and b_{ij} are the intercept, linear, quadratic and interaction coefficients, respectively; and X_i and X_j are the independent variables (the numerical factors considered in the model). One-way ANOVA was used to evaluate the statistical significance of the regression coefficients. Coefficient of determination R^2, adjusted R^2 and predictive R^2 were determined for the generated model. The lack of fit of the model was measured using a p-value < 0.05. Once the fitted regression model was determined, response surface plots were obtained maintaining each factor constant at a central point, while the other two factors varied within the experimental range.

Results

Physical–chemical characterization of the leaf litter

Alder leaf litter showed significantly higher C concentration than poplar, which can be related to its higher (1.8 times) lignin concentration, no significant differences between species were detected for hemicellulose and cellulose (Table 1). Significant differences between leaf litter species were also detected for the concentrations of N, P, Si and toughness (Table 1). While N concentration in alder was almost triple that of poplar, P concentration was just 1.5 times higher in poplar. The main difference detected between species lies in the 15 times higher Si concentration in poplar than in alder, which likely determined higher toughness of the first species. In conclusion, considering the variables with greater divergence between species (Si, N and toughness) we can attribute a potential lower nutritive value to poplar leaf litter compared to that of alder.

In vitro hydrolysis

The range of values estimated for temperature and for enzyme/substrate ratio was between 10 and 25 °C, and 2.06 and 5.15 units/g substrate, respectively. Gut retention times showed a great variability among individuals and also were significantly affected by the type of plant litter (Table 2). Using these values as a reference, a range from one to four hours was considered representative. A total of 30 assays were carried out to test the production of RS and AA after hydrolysis of the two types of leaf litter by the digestive enzymes of

Table 1. Physical and chemical characteristics (mean ± 1SE) of alder (*Alnus glutinosa*) and poplar (*Populus alba*) leaf litter.

	Plant species			
Trait	Alder	Poplar	*t*-value	*p*-value
C (%)	47.76 ± 0.23	42.98 ± 0.48	34.09	<0.0001
N (%)	2.45 ± 0.10	0.86 ± 0.03	19.90	<0.0001
P (%)	0.04 ± 0.01	0.06 ± 0.01	−3.63	0.01
Ratio C/N	19.55 ± 0.80	50.11 ± 1.83	−34.27	<0.0001
Ratio C/P	1097.44 ± 156.80	713.33 ± 89.05	4.76	0.001
Ratio N/P	56.02 ± 6.81	14.25 ± 1.88	13.21	<0.0001
Hemicellulose (%)	25.81 ± 4.66	20.21 ± 6.69	1.54	0.16
Cellulose (%)	18.94 ± 0.91	18.65 ± 2.26	0.26	0.80
Lignin (%)	13.01 ± 2.80	7.28 ± 1.06	4.28	0.003
Si (%)	0.064 ± 0.002	0.050 ± 0.010	2.31	<0.0001
Toughness (g)	50.31 ± 8.62	67.81 ± 14.95	−7.239	<0.0001

Table 2. Gut retention time (min) calculated for *M. praemorsa* (n = 30; mean ± 1SD dry mass: 43.0 ± 24.2 mg) fed on *A. glutinosa* and *P. alba* leaf litter.

Species	Range	Mean ± SD	*p* value
A. glutinosa	103–240	173 ± 68	0.007
P. alba	173–300	193 ± 68	

Table 3. Experimental values obtained for the response variables in the three-level Box–Behnken factorial design.

	Independent variables[a]								Dependent variables[b]	
	Coded level				Uncoded level					
Run	X_1	X_2	X_3	X_4	A	B	C	D	Y_1	Y_2
1	0	0	0	1	2.5	17.5	2.94	Poplar	18.16	370.49
2	0	−1	−1	1	2.5	10	2.06	Poplar	11.60	291.04
3	0	0	0	2	2.5	17.5	2.94	Alder	36.46	541.49
4	0	1	1	1	2.5	25	5.15	Poplar	14.96	338.88
5	1	1	0	1	4	25	2.94	Poplar	19.34	591.72
6	1	−1	0	1	4	10	2.94	Poplar	27.13	623.37
7	0	0	0	1	2.5	17.5	2.94	Poplar	16.00	420.55
8	0	−1	−1	2	2.5	10	2.06	Alder	21.25	371.94
9	0	0	0	2	2.5	17.5	2.94	Alder	19.98	450.58
10	−1	−1	0	2	1	10	2.94	Alder	7.83	99.69
11	1	−1	0	2	4	10	2.94	Alder	23.28	560.12
12	−1	1	0	2	1	25	2.94	Alder	8.60	132.23
13	0	1	−1	2	2.5	25	2.06	Alder	16.45	402.80
14	0	−1	1	1	2.5	10	5.15	Poplar	19.29	448.77
15	1	0	1	1	4	17.5	5.15	Poplar	31.76	713.83
16	−1	0	−1	1	1	17.5	2.06	Poplar	9.23	267.80
17	−1	1	0	1	1	25	2.94	Poplar	7.49	235.26
18	−1	−1	0	1	1	10	2.94	Poplar	8.11	153.39
19	0	1	−1	1	2.5	25	2.06	Poplar	13.95	428.52
20	−1	0	−1	2	1	17.5	2.06	Alder	6.97	212.52
21	0	1	1	2	2.5	25	5.15	Alder	23.09	506.33
22	1	0	−1	1	4	17.5	2.06	Poplar	30.49	722.46
23	−1	0	1	1	1	17.5	5.15	Poplar	13.91	238.26
24	0	0	0	2	2.5	17.5	2.94	Alder	30.28	514.27
25	1	0	−1	2	4	17.5	2.06	Alder	20.49	642.32
26	1	1	0	2	4	25	2.94	Alder	26.83	509.13
27	0	0	0	1	2.5	17.5	2.94	Poplar	15.64	431.90
28	−1	0	1	2	1	17.5	5.15	Alder	9.20	207.08
29	0	−1	1	2	2.5	10	5.15	Alder	23.72	659.94
30	1	0	1	2	4	17.5	5.15	Alder	26.35	588.18

[a]Independent variables X_1 and *A*, incubation time (hours); X_2 and *B*, hydrolysis temperature (°C); X_3 and *C*, E/S ratio (enzymatic activity/gr of substrate), X_4 and *D*, kind of substrate.
[b]Dependent variables Y_1, reducing sugars (μmol); Y_2, total amino acids (μg).

M. praemorsa. Results varied considerably depending on the different combinations of the considered factors (gut evacuation time, temperature and E:S ratio) with total RS ranging between 6.97 and 36.46 μmol and AA ranging between 99.69 and 722.46 μg (Table 3). The regression coefficients of the models of cellulose and protein hydrolysis by the enzymes of *M. praemorsa* in coded form are presented in Table 4 and the initial regression models with coefficients in uncoded form were as follows:

$$\begin{aligned} RS_{Alnus} = &- 52.5 + 25.30\ \text{time} + 14.2\ \text{E:S} + 2.30\ \text{temp} - 3.92\ \text{time}^2 - 1.81\ \text{E:S}^2 \\ &- 0.0613\ \text{temp}^2 - 0.39\ \text{time} \times \text{E:S} + 0.062\ \text{time} \times \text{temp} - 0.090\ \text{E:S} \times \text{temp} \end{aligned} \quad (1)$$

Table 4. Regression coefficients, R^2 and lack-of-fit test for the two-dependent variables; Y_1: reducing sugars (µmol); Y_2 and total amino acids (µg) obtained with *in vitro* digestion assays of two plant substrates by the enzymes of *M. praemorsa*. Significant coefficients in bold letters.

Factors	Coefficients							
	A. glutinosa				*P. alba*			
	Y_1	*p*-value	Y_2	*p*-value	Y_1	*p*-value	Y_2	*p*-value
Constant	**28.91**	0.000	**502.1**	0.000	**16.75**	0.000	**407.6**	0.000
Time	**8.04**	0.009	**206.0**	0.001	**8.75**	0.000	**219.6**	0.000
Temperature	−0.14	0.946	−17.6	0.555	−1.30	0.124	9.7	0.422
E:S ratio	−2.15	0.320	−41.5	0.198	**−1.83**	0.048	−3.7	0.750
Time^2	**−8.82**	0.028	**−124.8**	0.029	2.58	0.055	**51.1**	0.026
Temperature^2	−3.45	0.283	−52.1	0.261	**−3.81**	0.014	**−57.7**	0.017
E:S^2	−4.33	0.191	35.2	0.431	2.02	0.109	26.9	0.162
Time*Temp	0.69	0.811	−20.9	0.620	−1.79	0.132	−28.4	0.131
Time*E:S	−0.91	0.755	12.2	0.770	0.85	0.431	−5.2	0.755
Temp*E:S	−1.04	0.721	46.1	0.296	1.67	0.154	**61.9**	0.011
R^2	0.859		0.934		0.975		0.986	
R^2 adjusted	0.605		0.814		0.931		0.968	
Lack of fit		0.975		0.202		0.368		0.570

$$\mathrm{RS}_{Populus} = 23. + 1.55\ \mathrm{time} - 10.72\ \mathrm{E{:}S} + 2.078\ \mathrm{temp} + 1.149\ \mathrm{time}^2 + 0.845\ \mathrm{E{:}S}^2 - 0.0678\ \mathrm{temp}^2 + 0.368\ \mathrm{time} \times \mathrm{E{:}S} - 0.1593\ \mathrm{time} \times \mathrm{temp} + 0.1441\ \mathrm{E{:}S} \times \mathrm{temp} \quad (2)$$

$$\mathrm{AA}_{Alnus} = 75 + 428\ \mathrm{time} - 216\ \mathrm{E{:}S} + 20.3\ \mathrm{temp} - 55.5\ \mathrm{time}^2 + 14.7\ \mathrm{E{:}S}^2 - 0.926\ \mathrm{temp}^2 + 5.3\ \mathrm{time} \times \mathrm{E{:}S} - 1.86\ \mathrm{time} \times \mathrm{temp} + 3.98\ \mathrm{E{:}S} \times \mathrm{temp} \quad (3)$$

$$\mathrm{AA}_{Populus} = 208 + 85.2\ \mathrm{time} - 171.5\ \mathrm{E{:}S} + 24.3\ \mathrm{temp} + 22.69\ \mathrm{time}^2 + 11.27\ \mathrm{E{:}S}^2 - 1.027\ \mathrm{temp}^2 - 2.24\ \mathrm{time} \times \mathrm{E{:}S} - 2.52\ \mathrm{time} \times \mathrm{temp} + 5.34\ \mathrm{E{:}S} \times \mathrm{temp} \quad (4)$$

The various regression models fit the data well (R^2 ranging from 0.86 to 0.99 and $p > 0.05$ in all cases for the lack-of-fit test, Table 4). Response surface plots resuming the combined effect of each pair of factors on the release of either RS or AA when fixing the third factor at the central value (temp 17.5 °C; time 2.5 h; E:S 2.94U/mg) are presented in Figure 1. The ANOVA test showed that release of RS from alder was only significantly affected only by time both in the linear and quadratic terms, while in poplar, release was significantly influenced by time but also by the E:S ratio (linear term) and by temperature (quadratic term). The release of AA from both litter species was significantly affected by time (both in the linear and quadratic terms), but in poplar also by temperature (quadratic term) and by the interaction between temperature and E:S. More simple regression models were recalculated considering only these significant terms affecting release of both RS and AA:

$$\mathrm{RS}_{Alnus} = -11.91 + 23.74\ \mathrm{time} - 3.67\ \mathrm{time}^2 \quad (5)$$

$$\mathrm{RS}_{Populus} = -10.45 + 5.832\ \mathrm{time} - 1.185\ \mathrm{E{:}S} + 2.404\ \mathrm{temp} - 0.0736\ \mathrm{temp}^2 \quad (6)$$

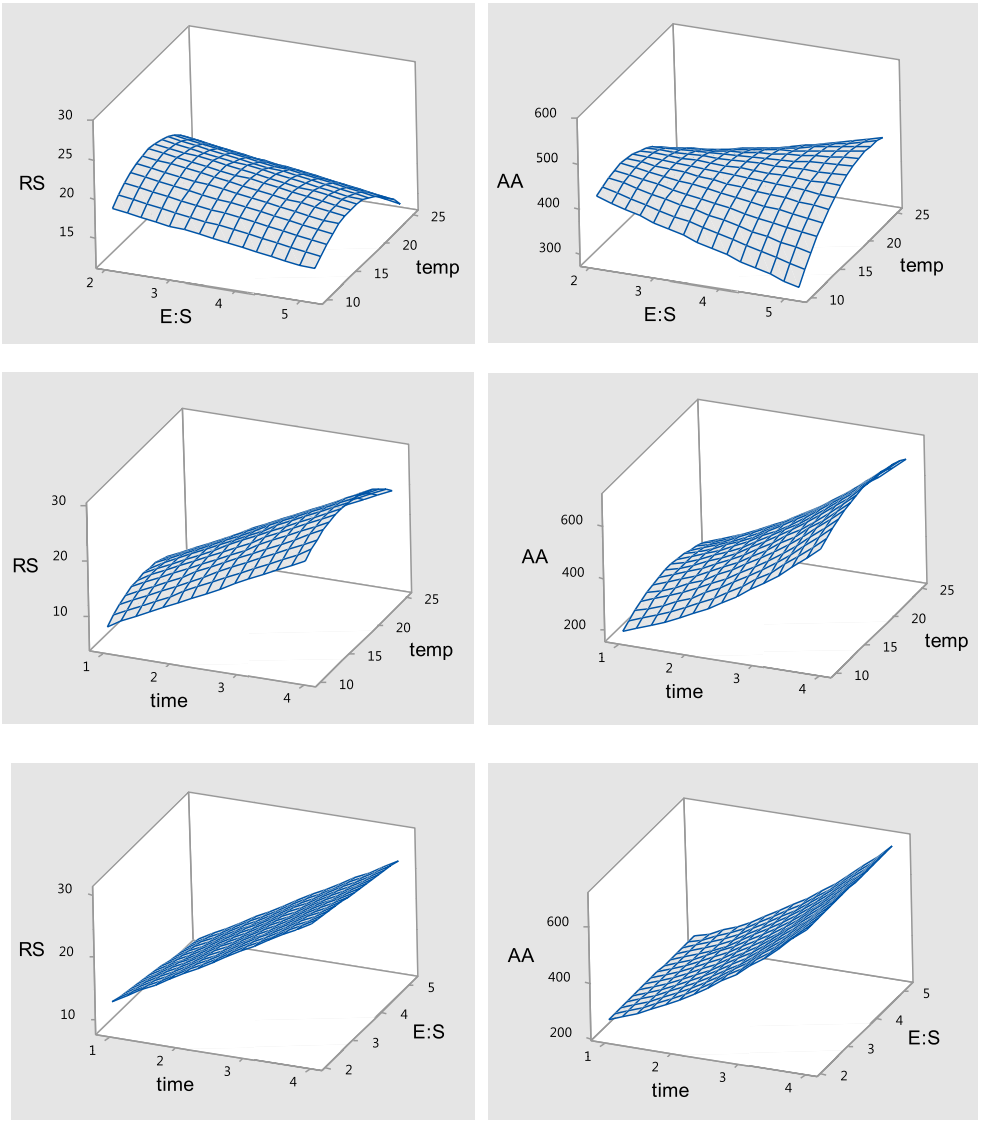

Figure 1. Response surface plots of the release of (A) reducing sugars and (B) amino acids, from samples of Poplar leaf litter hydrolyzed by the digestive enzymes of *M. praemorsa* as a function of the considered factors (temperature, time and enzyme:substrate ratio). Hold values in each case were: temp 17.5 °C; time 2.5 h; E:S 2.94U/mg). RS: reducing sugars; AA; amino acids.

$$\mathrm{AA}_{Alnus} = -194 + 412.0\ \mathrm{time} - 54.9\ \mathrm{time}^2 \tag{7}$$

$$\mathrm{AA}_{Populus} = 191 + 37.5\ \mathrm{time} - 95.8\ \mathrm{E{:}S} + 19.3\ \mathrm{temp} + 21.77\ \mathrm{time}^2 - 1.063\ \mathrm{temp}^2 + 5.34\ \mathrm{E{:}S} \times \mathrm{temp} \tag{8}$$

Although lack-of-fit can occur if important terms from the model, such as interactions or quadratic terms are not included, fitting was maintained even in the simplified version of the models presented in Equations (5–8). The relative influence of each of the above

significant factors on the release of either RS or AA, based on the regression coefficients of the polynomial models in coded values (Table 4), was as follows:

RS in *Alnus*; Time^2 > Time

RS in *Populus;* Time > Temp^2 > E:S

AA in *Alnus;* Time > Time^2

AA in *Populus;* Time > Temperature*E:S > Temperature^2 > Time^2

These simplified models revealed important differences between plant litter species in the factors influencing the release of either RS or AA. In the case of alder, the only significant factor was the time of incubation, which showed a quadratic response and hence a maximum release of both ES and AA after 3.5 h hydrolysis. However, in poplar, the final result of the hydrolysis was significantly affected by more factors. The simplified models were used to predict results obtained when values from 1 to 6 were given to the variable 'time' for both leaf litter species (Figure 2). This gave clear differences in the profile of release of either RS or AA were obtained for the two kinds of leaf litter. In the case of alder, the net effect of time offered the above-mentioned curve with a maximum release at 3.5 h. However, in poplar a more complex response was obtained, being characterized by a sharp decrease in

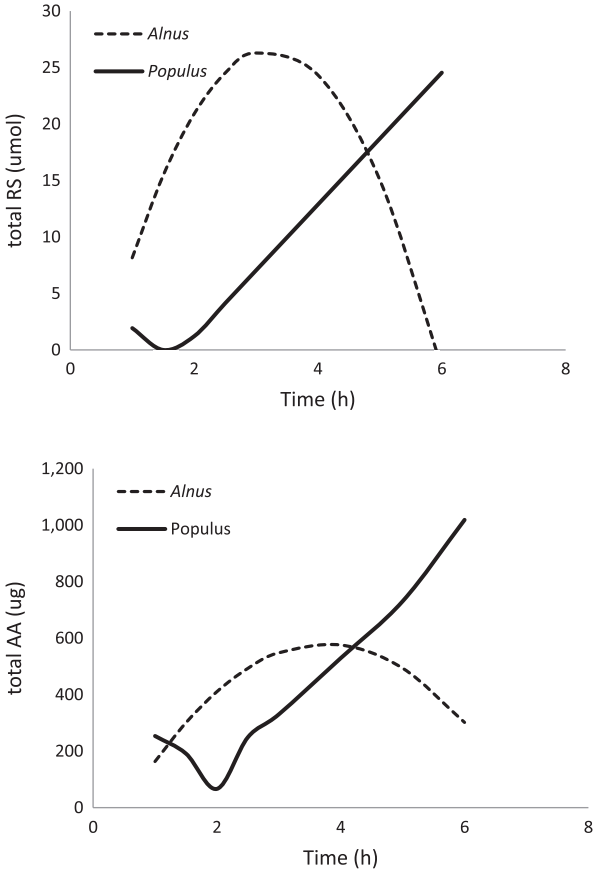

Figure 2. Plots of the estimated release of reducing sugars (RS) and amino acids (AA) by the digestive enzymes of *M. praemorsa* from alder and poplar leaf litter based in the regression models obtained using *in vitro* assays and Box–Behnken design.

the release of both nutrients when increasing incubation time from 1 to 2 h, followed by a sharp and continuous increase from that time onwards. The amount of RS released from poplar only reached that from alder after 5 h, while a similar release of AA required 4 h. Total release of both nutrients within the interval between 1 and 4 h was estimated by integrating the functions that defined the regression models (5–8) using the online calculation tool Symbolab. The values obtained – 64.0 µmol RS and 2900 µg AA for alder leaf litter and 4.85 µmol RS and 1528 µg AA for poplar – confirmed the total lower nutrient release from this latter substrate.

Results obtained with the *in vivo* assay are shown in Table 5. Although growth indexes showed lower values for the snails fed on poplar, no significant differences were observed between leaf litter species.

Discussion

The objective of the present study was to test the potential application of an *in vitro* assay to simulate the digestive hydrolysis of leaf litter by the freshwater snail *M. praemorsa,* as well as to determine the possible influence of different factors in the efficiency of such process to release biologically available C and N in the forms of reducing sugars and amino acids from two different substrates. The first objective was successfully achieved using a configuration based in the use of a bioreactor provided with a semipermeable membrane allowing the use of small amounts of reaction mixture (enzyme extracts + substrates) and the regular separation of the end products of enzyme hydrolysis, this preventing saturation and partial inactivation of the enzymes. As indicated in the Introduction, there are not many papers dealing on the development and application of *in vitro* digestion models to invertebrates; the present one combines simplicity and feasibility as well as the possibility of being adapted to different organisms.

The initial model showed a main effect of the incubation time on the release of both products from the two types of leaf litter, but also the significant effect of other factors in the hydrolysis of poplar within the ranges of the variables considered in the assays. The suitability of the model was confirmed by the lack-of fit test, which showed values larger than 0.05, this suggesting that they fitted properly to the data.

Results obtained with the simplified model offered an interesting picture of the differences between both substrates in the expected hydrolysis of carbohydrates and proteins. Under the range of conditions used in the present assays, hydrolysis of components present in alder leaf litter was only dependent on time and not on temperature or the relative amount of

Table 5. Results of growth and food efficiency obtained when feeding *M. praemorsa* on alder or poplar leaf litter. DIGR = Daily instantaneous growth rate; GPE = Gross production efficiency; FCE = Food conversion efficiency; FCR = Food conversion rate.

	DIGR	GPE	FCE	FCR
	(d-1)	Δ Biomass (g/d)/Total leaf ingested (g/d)	100*(Δ Biomass (g)/ Total leaf ingested (g))	Total leaf ingested (g)/ΔBiomass (g)
Alder	0.0022 ± 0.00030	0.0025 ± 0.00028	7.358 ± 0.828	25.134 ± 4.445
Poplar	0.0018 ± 0.00027	0.0021 ± 0.00038	6.402 ± 1.135	33.841 ± 6.718
t-value	0.865	0.666	0.666	−1.057
p-value	0.391	0.508	0.508	0.295

substrate mixed with the enzymes, with maximum release of RS and AA reached at 3.5 h and decreasing after this time. In contrast, hydrolysis of components in poplar leaf litter followed a more complex pattern with a first stage (up to 1.5–2 h) mainly dependent on temperature and amount of substrate, and characterized by a very low release of nutrients. A second stage (from 2 h onwards) showed a continuous release directly correlated with the incubation time. This points to a higher bioaccesibility of nutrients in alder leaf litter when compared to that in poplar and suggests that a much longer gut retention time should be required in the first case to provide an equivalent amount of either RS or AA to that provided by alder. This lower nutritional value of poplar compared to alder is well supported by its physical–chemical traits; higher toughness and Si concentration and lower N concentration of the first species. These features have been shown to negatively affect growth and fitness of freshwater shredders (Tuchman et al. 2002; Graça and Cressa 2010; Flores et al. 2014) and folivore terrestrial insects (Massey and Hartley 2009). Nevertheless, none of the indicators of nutritional efficiency measured in the *in vivo* assays offered significant differences between leaf litter species and this result may be produced by a compensation mechanism based in the different gut transit rate observed for both substrates. The mean retention time (MRT) of ingesta is usually understood as a species-specific parameter that is determined by the species-specific body mass and characterizes the digestive efficiency of a vertebrate herbivore (Demment and Van Soest 1985; Illius and Gordon 1992; Robbins 1993). However, central in the existing digestion models on rate maximization is the trade-off between gut passage rate and digestive efficiency; the slower food is pushed through the gut, the longer its gut retention time, and the better it is digested if other parameters are held constant (Karasov 1996; Karasov and Martínez del Rio 2007). Results of growth and nutritional efficiency obtained in the present study suggest that such compensation was achieved to a great extent by *M. praemorsa* inasmuch as snails fed on poplar leaf litter presented a higher retention time and did not differ significantly in growth when compared to snails fed on alder.

The model also suggests that exist important differences in gut retention times required to release an equivalent amount of RS from both substrates. As depicted in Figure 2, maximum release is reached in alder after 3 h hydrolysis, but a similar amount requires 6 h for poplar leaves. Such great differences did not exist for AA and similar values of release for the two substrates were obtained after 4 h hydrolysis. These differences could be explained considering that reactive kinetics of the main components present in foods eaten by an organism (carbohydrates, proteins and lipids) may be quite different (Karasov and Douglas 2013). It follows that modulation of MRT in the live animal to maximize hydrolysis of carbohydrates will entail enough supply of AA to cover its nutritional needs. Considering the aforementioned, a good agreement was found between results obtained with the *in vitro* and the *in vivo* assays; while results obtained *in vitro* point that time appears to be the main factor affecting the extent of hydrolysis of carbon and nitrogen substrates, results obtained *in vivo* evidenced a compensatory response also based on the modulation of the retention time of food within the gut.

Nevertheless, it is clear that the course of the digestion in a real gut is much more complex and that, in the live organism, a number of adaptive responses may modify to a great extent some of the results observed in these simplified *in vitro* experiments. For example, the theoretical change in enzyme:substrate ratio used in the *in vitro* assays may not exist in the live snail if enzyme secretion is modulated in such a way to be maintained constant. On

the other hand, the effect of temperature on the hydrolysis may be more complex; besides a direct and positive effect on the activity of cellulase and proteases enzymes within the tested range it may exert a negative effect through an increase in the metabolic rate and therefore in the gut transit rate, this influencing MRT (Logan et al. 2002). In addition, while in the present study pH was stated as a fixed factor, in other cases it may be considered an additional variable. This is possible considering that different species may present differences in their modulation of this pH, as it has been demonstrated in some terrestrial and aquatic vertebrates (Secor 2009; Hlophe et al. 2014).

In summary, the combination of *in vitro* assays and factorial design used in the present paper provides a useful insight on the effect of different factors affecting enzyme hydrolysis of carbon and nitrogen compounds present in leaf litter within the gut of an aquatic invertebrate (specifically if their effect on the response is direct or inverse, if they are independent or dependent on any type of interaction, among other insights). The study used the aquatic snail *M. praemorsa* only as an example, but the use of this approach may be highly valuable to assess differences in the sensitivity of enzyme hydrolysis of plant substrates consumed by different aquatic invertebrates present in a given habitat to variations in some environmental factors (i.e. water temperature, available food type). This information may help to construct more complex models explaining potential changes in total carbon and nitrogen bioavailability within a specific habitat i.e. under a global warming scenario.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Spanish Ministry of Science and Innovation [grant number project RIBARID CGL2012–39635]; MINECO-FEDER.

References

Areekijseree M, Engkagul A, Kovitvadhi S, Kovitvadhi U, Thongpan A, Rungruangsak-Torrissen K. 2006. Development of digestive enzymes and in vitro digestibility of different species of phytoplankton for culture of early juveniles of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus* Simpson, 1900. Invertebr Reprod Dev. 49:255–262.

Bärlocher F. 1992. The ecology of aquatic hyphomycetes. Berlin: Springer-Verlag.

Boisen S. 2000. In vitro digestibility methods: history and specific approaches. In: Moughan PJ, Verstegen MWA, editors. Feed evaluation: principles and practice. Wageningen: Wageningen pers; p. 153–168.

Box GE, Behnken DW. 1960. Some new three level designs for the study of quantitative variables. Technometrics. 2:455–475.

Casas JJ, Gessner MO, Langton PH, Calle D, Descals E, Salinas MJ. 2006. Diversity of patterns and processes in rivers of eastern Andalusia. Limnetica. 25:155–170.

Casas JJ, Gessner MO, López D, Descals E. 2011. Leaf-litter colonisation and breakdown in relation to stream typology: insights from Mediterranean low-order streams. Freshw Biol. 56:2594–2608.

Chergui H, Pattee E. 1991. An experimental study of the breakdown of submerged leaves by hyphomycetes and invertebrates in Morocco. Freshw Biol. 26:97–110.

Chróst RJ. 1991. Environmental control of the synthesis and activity of aquatic microbial ectoenzymes. In: Chróst RJ, editor. Microbial enzymes in aquatic environments. New York (NY): Springer; p. 29–59.
Danger M, Cornut J, Elger A, Chauvet E. 2012. Effects of burial on leaf litter quality, microbial conditioning and palatability to three shredder taxa. Freshw Biol. 57:1017–1030.
Demment MW, Van Soest PJ. 1985. A nutritional explanation for body-size patterns of ruminant and nonruminant herbivores. Am Nat. 125:641–672.
Dey SS, Dora KC. 2014. Optimization of the production of shrimp waste protein hydrolysate using microbial proteases adopting response surface methodology. J Food Sci Technol. 51:16–24.
Fenoy E, Casas JJ, Díaz-López M, Rubio J, Guil-Guerrero JL, Moyano-López FJ. 2016. Temperature and substrate chemistry as major drivers of interregional variability of leaf microbial decomposition and cellulolytic activity in headwater streams. FEMS Microbiol Ecol. 92(11):fiw169.
Flari V, Charrier M. 1992. Contribution to the study of carbohydrases in the digestive tract of the edible snail *Helix lucorum* L. (Gastropoda: Pulmonata: Stylommatophora) in relation to its age and its physiological state. Comp Biochem Physiol A Physiol. 102:363–372.
Flores L, Larrañaga A, Elosegi A. 2014. Compensatory feeding of a stream detritivore alleviates the effects of poor food quality when enough food is supplied. Freshw Sci. 33:134–141.
Gessner MO, Gulis V, Kuehn KA, Chauvet E, Suberkropp K. 2007. Fungal decomposers of plant litter in aquatic ecosystems. In: Kubicek CP, Druzhinina IS, editors. Environmental and microbial relationships. 2nd ed. Berlin: Springer; p. 301–324.
Gilannejad N, Martínez-Rodríguez G, Yúfera M, Moyano FJ. 2017. Estimating the effect of different factors on the digestive bioaccessibility of protein by the Senegalese sole (*Solea senegalensis*); combination of response surface methodology and in vitro assays. Aquaculture. 477:28–34.
Graça MAS. 2001. The role of invertebrates on leaf litter decomposition in streams – a review. Int Rev Hydrobiol. 86:383–393.
Graça MAS, Cressa C. 2010. Leaf quality of some tropical and temperate tree species as food resource for stream shredder. Int Rev Hydrobiol. 95:27–41.
Graça MAS, Ferreira V, Canhoto C, Encalada AC, Guerrero-Bolaño F, Wantzen KM, Boyero L. 2015. A conceptual model of litter breakdown in low order streams. Int Rev Hydrobiol. 100:1–12.
Gulis V, Suberkropp K. 2003. Effect of inorganic nutrients on relative contributions of fungi and bacteria to carbon flow from submerged decomposing leaf litter. Microb Ecol. 45:11–19.
Hamdan M, Tomás-Vidal A, Martínez S, Cerezo-Valverde J, Moyano FJ. 2013. Development of an *in vitro* model to assess protein bioavailability in diets for common octopus (*Octopus vulgaris*). Aquac Res. 45:2048–2056.
Heller J, Abotbol A. 1997. Litter shredding in a desert oasis by the snail *Melanopsis praemorsa*. Hydrobiologia. 344:65–73.
Hlophe SN, Moyo NAG, Ncube I. 2014. Postprandial changes in pH and enzyme activity from the stomach and intestines of *Tilapia rendalli* (Boulenger, 1897), *Oreochromis mossambicus* (Peters, 1852) and *Clarias gariepinus* (Burchell, 1822). J Appl Ichthyol. 30:35–41.
Hollebeeck S, Borlon F, Schneider YJ, Larondelle Y, Rogez H. 2013. Development of a standardised human *in vitro* digestion protocol based on macronutrient digestion using response surface methodology. Food Chem. 138:1936–1944.
Illius AW, Gordon IJ. 1992. Modelling the nutritional ecology of ungulate herbivores: evolution of body size and competitive interactions. Oecologia. 89:428–434.
Karasov WH. 1996. Digestive plasticity in avian energetics and feeding ecology. In: Carey C, editor. Avian energetics and nutritional ecology. New York (NY): Chapman and Hall; p. 61–84.
Karasov WH, Douglas AE. 2013. Comparative digestive physiology. Compr Physiol. 3:741–783.
Karasov WH, Martínez del Rio C. 2007. Physiological ecology: how animals process energy, nutrients, and toxins. Princeton: Princeton University Press.
Kocabaş Z. 2001. An application and interpretation of second order response surface model. Ankara University J Agric Sci. 7:121–128.
Langdon CJ. 1989. Preparation and evaluation of protein microcapsules for a marine suspension-feeder, the Pacific oyster *Crassostrea gigas*. Mar Biol. 102:217–224.

Logan JD, Joern A, Wolesensky W. 2002. Location, time, and temperature dependence of digestion in simple animal tracts. J Theor Biol. 216:5–18.
Logan JD, Joern A, Wolesensky W. 2003. Chemical reactor models of optimal digestion efficiency with constant foraging costs. Ecol Modell. 168:25–38.
Martínez del Rio C, Cork SJ, Karasov WH. 1994. Modeling gut function: an introduction. In: Chivers DJ, Langer P, editors. Digestive system in mammals: food, form and function. Cambridge: Cambridge University Press; p. 213–240.
Massey FP, Hartley SE. 2009. Physical defences wear you down: progressive and irreversible impacts of silica on insect herbivores. J Anim Ecol. 78:281–291.
Minekus M, Marteau P, Havenaar R, Huis in't Veld J. 1995. A multi-compartmental dynamic computer-controlled model simulating the stomach and small intestine. ATLA. 23:197–209.
Moyano FJ, Saénz de Rodrigáñez M, Díaz M, Tacon AGJ. 2014. Application of *in vitro* digestibility methods in aquaculture: constraints and perspectives. Rev Aquacult. 6:1–20.
Myers RH, Montgomery DC. 2002. Response surface methodology: process and product optimization using designed experiments. 2nd ed. New York (NY): Wiley.
Parsons CM. 1991. Use of pepsin digestibility, multienzyme pH change and protein solubility assays to predict *in vivo* protein quality of feedstuffs. In: Fuller MF, editor. In vitro digestion for pigs and poultry. Wallingford: CAB International Publishing; p. 105–115.
Penry DL, Jumars PA. 1986. Chemical reactor analysis and optimal design. BioScience. 36:310–315.
Penry DL, Jumars PA. 1987. Modelling animal guts as chemical reactors. Am Nat. 129:69–96.
Robbins CT. 1993. Wildlife feeding and nutrition. 2nd ed. San Diego (CA): Academic Press.
Saunders RM, Conner MA, Booth AN, Bickoff EM, Kohler GO. 1973. Measurement of digestibility of alfalfa protein concentrates by *in vivo* and vitro methods. J Nutr. 39:530–535.
Secor SM. 2009. Specific dynamic action: a review of the postprandial metabolic response. J Comp Physiol B. 179:1–56.
Supannapong P, Pimsalee T, Teerasak A, Engkagul A, Kovitvadhi U, Kovitvadhi S, Rungruangsak-Torrissen K. 2008. Digestive enzymes and *in vitro* digestibility of different species of phytoplankton for culture of the freshwater pearl mussel, *Hyriopsis (Hyriopsis) bialatus*. Aquac Int. 16:437–453.
Tate RL. 2002. Microbiology and enzymology of carbon and nitrogen cycling. In: Burns R, Dick R, editors. Enzymes in the environment: activity, ecology and applications. New York (NY): Dekker; p. 227–248.
Tuchman NC, Wetzel RG, Rier ST, Wahtera KA, Teeri JA. 2002. Elevated atmospheric CO_2 lowers leaf litter nutritional quality for stream ecosystem food webs. Glob Chang Biol. 8:163–170.
Wallace JB, Eggert SL, Meyer JL, Webster JR. 2015. Stream invertebrate productivity linked to forest subsidies: 37 stream-years of reference and experimental data. Ecology. 96:1213–1228.
Zhang H, Zu H, Wang S, Wang W. 2013. Investigation of hydrolysis conditions and properties on protein hydrolysates from flatfish skin. Front Chem Sci Eng. 7:303–311.