

Growth and nutrient budgets (C-N-P) of the manila clam *Venerupis philippinarum* in a commercial pond system.

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Abstract

To study potential mechanism(s) causing the reduced growth of the clam *Venerupis philippinarum* at the end of the production cycle, observed in the culture ponds at Zeeland Aquaculture in 2011, specific studies on eco-physiology and nutrient dynamics of individual clams were performed in the culture ponds during the production period in 2012. The study was divided in two sub-projects: Sub-project I: which focused on the uptake of the clam in terms of clearance rates and absorption efficiencies, Sub-Project II: focused on the internal nutrient fluxes of carbon, nitrogen and phosphorus, of the clams in the culture ponds in relation to food availability and environmental conditions, described in this report.

Shell length increased from 11.6 to 28.2 mm in the period May-August, over the same period tissue weight in DW and AFDW increased from 26.3 and 0.02 to 220.0 and 0.18 mg respectively. Similar to findings of 2011 reduced growth rates were observed in this study towards the end of the experimental period, however an overall higher growth rate of 1.15 mm week⁻¹ was observed. Nutrient contents of the food (POC: 0.1–0.8, PON: 0.01–0.1, POP: 0.1–0.2) were found to be rather low, and comparable to the Oosterschelde. Alongside with reduced growth rates and low food availability a stoichiometric imbalance between C:N of the food ($C:N_{\text{Food}} \sim 10$) and the tissue ($C:N_{\text{Tissue}} \sim 5$) was observed. Although tissue elemental composition of carbon (359–390 mg C g⁻¹) and phosphorus (7–8 mg P g⁻¹) was kept relatively stable, in comparison with nitrogen (63–97 mg N g⁻¹) which increased significantly throughout the study period in response to reproductive preparation. The stoichiometric imbalance was therefore not necessarily observed as a negative effect on growth, rather by need of higher nitrogen accumulation.

The physiological activities: *i* Ingestion, *ii* Egestion, *iii* Respiration and *iv* Excretion, were regulated to the nutrient requirements of the clams. Ingestion rates (8–32 mg h⁻¹ g⁻¹), and ingestion of carbon (0.4–1.8 mg C h⁻¹ g⁻¹), nitrogen (0.05–0.23 mg N h⁻¹ g⁻¹) and phosphorus (0.01–0.03 mg C h⁻¹ g⁻¹) were found to be overestimated since for filtration rate calculation, resting individuals were excluded from data analyses. Absorption efficiencies (81%) were found to be relatively high for carbon, nitrogen and phosphorus (>90%), however absorption of carbon and nitrogen were found to be considerably lower in June, by a lower digestibility of *Tetraselmis suecica*. This was also seen by higher egestion rates (1.52–6.8 mg h⁻¹ g⁻¹) of carbon (75–217 C µg h⁻¹ g⁻¹), nitrogen (14–42 N µg h⁻¹ g⁻¹) and phosphorus (2–5 P µg h⁻¹ g⁻¹). Respiration (16–37 µmol h⁻¹ g⁻¹) and TAN excretion (0.8–3.4 µmol h⁻¹ g⁻¹) were significantly correlated to temperature, however phosphate excretion (0.2–1.0 µmol h⁻¹ g⁻¹) was found to be independent to temperature. TAN and P excretion were also found to be strongly correlated to the PON and POP concentrations of the food. Towards the end of the production cycle it was found that larger clams subjected difficulties on subtracting nutrients from smaller algae, with the result that nutrient demand could not have been met. This resulted in negative growth rates and increased TAN excretion indicating that internal nitrogen sources in the tissue were catabolized. After this period of negative growth tissue growth was increased again towards August, however shell growth rates even further decreased, indicating that acquired energy was primarily used for tissue growth rather than shell growth. Concluding that the reduced growth at the end of the production cycle was not due to a single mechanism, but clam growth rates was primarily affected by the nutrient content and digestibility of the algae. Finally the Growth Rate Hypothesis could not have been applied for the clams, since it can only be detected under maximal growth rates, and not under resource limitation.

Scope For Growth calculations were found to be higher than the measured growth, caused by overestimated ingestion rates. From the allocation of nutrients over the physiological rates it was found that after carbon and phosphorus were acquired, they were primarily lost by $C_{\text{Respiration}}$, $P_{\text{Excretion}}$ followed by storage in the tissue $> C_{\text{Growth}}$ and least by faeces C_{Egestion} . Nitrogen was mainly accumulated in the body ($\sim 50\%$), concluding the higher nitrogen demand of the clams throughout the study period, followed by $> N_{\text{Excretion}} > N_{\text{Egestion}}$. From these results it was seen that a high amount of acquired nutrients are regenerated directly to the culture ponds again, making them available for algae growth. From decomposition of the faeces by microbial bacteria and by nitrifying bacteria in the sediment more nutrients originating from the clams could be made available for algae. These regenerated nutrients can be used for growth of macroalgae or on the other hand can be used for the growth of their own food source: the microalgae creating a less expensive and sustainable culture cycle.

Introduction

Background

The manila clam *Venerupis philippinarum* (Adams & Reeve, 1850: before 2012 commonly known as *Ruditapes philippinarum*), is a worldwide cultured marine bivalve species. The manila clam is of high commercial value since it became one of the main cultured species in the world (FAO, 2011: 3 million tonnes) with China, South Korea and the USA respectively as their main producers (Gouletquer, 1997; FAO, 2012). In Europe Italy, France and Spain are the main clam producing countries (FAO, 2012). *V. philippinarum* also occurs in Dutch waters, however in too low numbers to sustain a profitable fishery. Since 2011 culture of commercial clams was made possible in the Netherlands through a land-based culture system (small scale) in the commercial / research shellfish farm "Zeeland Aquaculture" (Stichting-Zeeuwse-Tong, 2011).

In 2011 a study was conducted by IMARES to monitor the growth of *V. philippinarum* in the culture ponds of "Zeeland Aquaculture" (Stichting-Zeeuwse-Tong, 2011). Growth of the clams and food availability were measured every second week from May till October 2011. The length of the clams was approximately 9mm at start and a size of 35mm was reached in October (Figure 1, red line). Additionally, a Dynamic Energy Budget model (DEB-model) was developed to simulate individual growth of the clam over time as a function of food concentration and water temperature (Fig. 1, blue lines). From Fig. 1 it can be concluded that towards the end of the production cycle the actual growth of the clams was lagging behind from what was predicted by the DEB-model. The DEB-model has previously shown to be a successful and accurate model for the prediction of growth in different bivalve species (Filgueira, 2011; Filgueira, et al. 2011). It is therefore likely that the actual growth of the clams towards the end of the production cycle is suppressed. The main question originating from the growth study is which mechanism(s) caused this reduced growth towards the end of the production cycle.

Bivalve growth in relation to nutrients, acquired and lost by physiological activities

While studying the growth of bivalves, individual growth rates can be used as a tool to assess their physiological fitness and response to surrounding environmental conditions (Dame, 1996), since growth is not constant but changes in relation to physiological activities influenced by fluctuating environmental factors (Bayne, 1976; Dame, 1996;

Newell, 2004). Because bivalves are poikilotherms, the metabolism of bivalves is strongly influenced by temperature (Bayne, 1976). Next to thermal influences physiological activities are mainly controlled by food availability (quantity & quality) (Bayne, 1976; Dame, 1996; Newell, 2004).

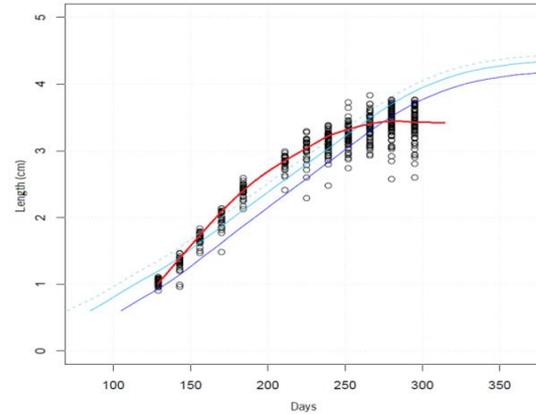


Fig. 1: Growth of the clam *Venerupis philippinarum* in length (cm) over time. The blue lines represent the DEB-model growth predictions. The open circles represent actual measurements with the red line as an average 'curve line'. (unpublished data, Zeeuwse Tong Project 2011)

A useful tool in the study of bivalve growth is the Scope For Growth method (SFG) (Winberg, 1960)). The SFG method is based on physiological measurements on *i* Food uptake, *ii* Growth, *iii* Egestion, *iv* Respiration and *v* Excretion, and calculates the energy/material available for growth as the difference between the energy/material acquired and lost through the physiological activities. A positive SFG indicates energy/material available for growth and a negative SFG can be interpreted as a detrimental effect indicating the use of reserves, resulting in reduced tissue mass (Naylor, et al. 1989; Smaal & Widdows, 1994). The SFG-method has shown to be a successful and accurate method for the prediction of growth in bivalves (Filgueira, 2011; Filgueira, et al. 2011), and can detect even small stressors on growth that were difficult to measure with actual growth measurements (Grant & Cranford, 1991; Smaal & Widdows, 1994). The SFG method can therefore be an important information tool for commercial bivalve farming, since the growth of bivalves is directly linked to the profitability of farming. The SFG is also widely used for research on, for example environmental stressors such as nutritionally poor conditions or toxicity, the growth processes of populations, effects on physiological rates and behaviour on different diets (Hawkins & Bayne, 1985; Widdows & Johnson, 1988; Grant & Cranford, 1991; Smaal & Vonck, 1997; Sobral & Fernandes; Filgueira, et al. 2011).

The SFG method can also be applied to nutrients, by calculations on uptake, storage and loss of the macro nutrients(carbon, nitrogen and phosphorus) in bivalves. The amount of carbon, nitrogen and phosphorus ingested can either be *i* stored in the body tissue resulting in tissue growth, *ii* lost by excretion or respiration, or *iii* lost as faeces (Fig. 2).

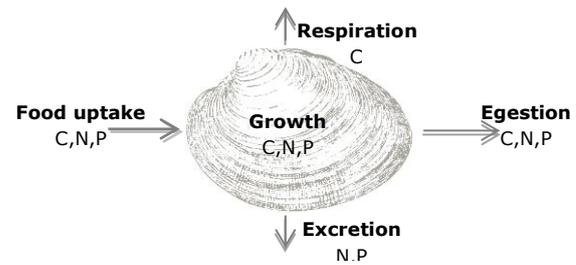


Fig. 2: Nutrients flow, through the clam. Nutrients are acquired through the food filtered from the water, stored in the tissue, lost by respiration (carbon) and excretion (nitrogen and phosphorus) and through the faeces.

If nutrient ratios (stoichiometry) in the food are similar to that of the tissue, it is referred as a "balanced interaction" (Sterner & Elser, 2002). However, if there are changes in either the body composition or the food, the relation changes to a stoichiometric imbalance (Sterner & Elser, 2002; Vrede, et al. 2004) Stoichiometric imbalance is often caused by differences in the food since the stoichiometric variation in algae is very high (Elser, et al. 2002), whereas stoichiometric variability in higher trophic levels is low due to improved homeostatic regulation (Sterner & Elser, 2002).

There are three ways in which consumers can cope with imbalanced resources and thereby try to maintain their stoichiometry: *i* the consumer can compensate by selecting different or supplemental food items *ii* consumers can alter their absorption efficiencies, and *iii* consumers can alter their amount of waste, for example higher P excretion and egestion when a diet is rich in P (Shumway et al., 1985; Sterner & Elser, 2002). Consumers will probably use these different methods simultaneously with a preference for one of them. According to Foster-Smith (1975) an Newell (2004) mussels and oysters use primarily the selective feeding method and thus the generation of pseudofaeces, in response to changes in food concentrations, while clams mainly regulate their ingestion rates by reducing their filtration rates.

A broad studied hypothesis on the growth rate of organisms in relation to their nutrient composition is described by the Growth Rate Hypothesis (GRH). The GRH states that the P-content of organisms is linked to their growth rates through RNA and protein synthesis (Elser et al., 1996; Main et al., 1997; Sterner & Elser, 2002; Vrede et al., 2004) With increasing growth rates high ribosomal RNA needs to be synthesized (Vrede et al., 2004). Ribosomal RNA is rich in P, thus an increased growth rate will result in an increase of the P content in body tissue. An increase of P will subsequently lead to a decrease of the N:P and C:P ratios (Elser, et al. 1996; Sterner & Elser, 2002). Smaller species have in general higher growth rates than larger species (Peters, 1983). This implies that body N:P is negatively correlated with growth rate, and body P-content is negatively correlated with body size (Sterner & Elser, 2002; Vrede et al., 2004). Several studies have been conducted to test the GRH on different taxa, and the GRH is often

used as an explanation of nutrient growth processes in ecological stoichiometry research. For example, (Main et al., 1997) tested the GRH for different species and sizes of zooplankton and found an increased P and decreasing N:P ratios with increasing growth rates (Fig.3). Similar results were obtained for several other classes/species such as, bacteria (Caldwell et al., 1950), insects (Sutcliffe Jr, 1970; Liess & Hillebrand, 2005), microbes (Sutcliffe Jr, 1970), snails and crustacean (Liess & Hillebrand, 2005). Liess and Hillebrand (2005) also found a significant positive relation between C:P, N:P ratios and size for the zebra mussel (*Dreissena polymorpha*, Bivalvia).

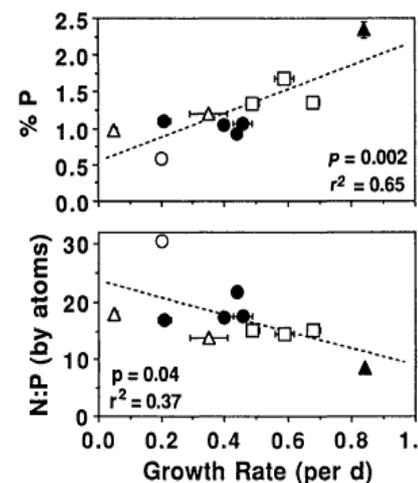


Fig. 3: Growth Rate Hypothesis: with increasing growth rates different species, and size classes of zooplankton showed an increasing P, what resulted in a decreasing N:P (Main et al., 1997)

Objectives

To study potential mechanism(s) causing the reduced growth of the clam *Venerupis philippinarum* at the end of the production cycle in the commercial ponds at Zeeland Aquaculture (see Fig. 1), specific studies on eco-physiology and nutrient dynamics of individual clams were performed in the culture ponds during the

production period in 2012. This study was divided in two sub-projects: *i* Sub-project I, focussed on the uptake and absorption efficiency, including clearance rates. This part was executed by Bastien Debeuf from the Rennes University for his MSC internship; *ii* Sub-project II focussed on the nutrient cycle of clams in the culture ponds, and is further described in the current report. Although the study was divided into two sub-projects, the sub-projects were linked as much as possible to create an integrated overview of the driving mechanisms behind clam growth in the culture ponds throughout a culture cycle.

The objectives of the Sub-project II were to: *i* Quantify the nutrients fluxes (C, N, P) involved in: *a* Food uptake, *b* Growth, *c* Egestion, *d* Respiration and *e* Excretion, in relation to temperature and food conditions. *ii* Compare the elemental composition and stoichiometric ratios for C:N:P in the food, body tissue, faeces and excreta over time to determine whether there is a balanced or imbalanced nutrient acquisition. *iii* Calculate the Scope for Growth and evaluate if there is a significant correspondence between SFG and measured growth of for C,N,P. *iv* Test if the Growth Rate Hypothesis applies to the clams cultured in commercial ponds systems. *v* Test whether food quality might be a reason for the reduced growth towards the end of the culture cycle.

Material and Methods

Study site and experimental design

The study was conducted at Zeeland Aquaculture, a 2ha commercial/research shellfish farm (Yerseke, the Netherlands), which focusses on the development of an integrated land based aquaculture cultivation system for shellfish (*Ostrea edulis*, *Crassostrea gigas*, *Mytilus edulis*, *Venerupis philippinarum*, *Tapes decussatus*) and algae (Stichting-Zeeuwse-Tong, 2011). The farm consist of 18 100 m³ algae ponds in which the algae *Chaetoceros muelleri*, *Skeletonema costatum*, *Tetraselmis suecica* and *Phaeodactylum tricornutum* were cultured. A mixture of these algae were fed to the clams with filtered seawater (50µm) dispersed over the shellfish ponds (water renewal, 2m³/per kg clams/week). Commercial clams were cultured in six 100 m² ponds, containing a 15cm sand layer, and stocked in different densities adjusted to their size (500 – 2000 per m²). Juveniles of the suspension feeding Manila clam; *V. philippinarum* were obtained in March 2012 from a shellfish hatchery, with an initial size of approximately 7.5mm. Per 20 individuals the clams were held in

buckets filled with sand which were placed in the bivalve ponds.

In the period May-August, six samplings were performed on the clams (May: 11, 29, June: 20, July: 9, 30 and August: 20) to determine the nutrient fluxes of carbon, nitrogen and phosphorus for the following physiological processes: *a* Food uptake, *b* Growth, *c* Faeces production, *d* Respiration, and *e* Excretion (Fig. 2). Additionally, the absorption efficiency was calculated. A three week sampling interval was applied, which was determined after a pilot study that showed little daily variability in individual oxygen consumption and nutrient excretion (Fig. 4).

Measurements of Sub-project II (nutrient dynamics), were conducted on the same batch of clams, observing the same sampling days/period as sub-project I (feeding physiology) which made integration of data easier. In this way a detailed overview of the eco-physiology of the clam and nutrient cycling in commercial pond systems could be made.

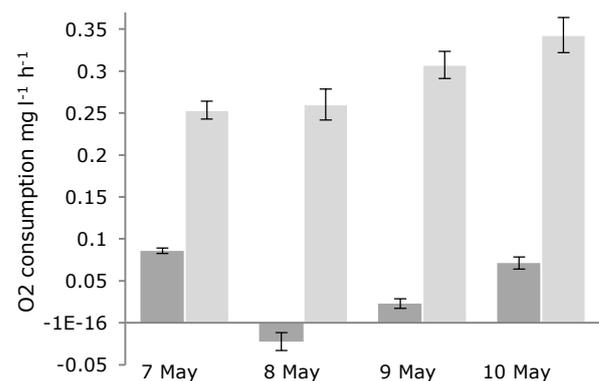


Fig. 4. Respiration variability of the same individuals of *Venerupis philippinarum* over time in mg l⁻¹ h⁻¹. The light grey bars indicate the experimental chambers, each containing one individual, the dark grey bars the controls, without clams. All data are expressed as averages (±standard error).

Environmental conditions

Environmental conditions were determined simultaneously with the physiological measurements. Temperature (°C) was measured by a respiratory meter during respiration measurements. Water samples to determine food quantity and quality in the ponds, were collected from the header tank (20 L), which was used for the physiological measurements (Fig. 5). To assure similar environmental conditions, water was pumped in the header tank at mid water depth at close proximity of where the experimental bivalves were situated. Food availability was determined by suspended particulate matter (SPM), particulate organic matter (POM), particulate inorganic matter

(PIM), particulate organic carbon (POC), nitrogen (PON), phosphorus (POP), chlorophyll *a* and phytoplankton abundance. Water samples (500-1000ml) were filtered onto four pre-dried/ashed and weighed 1.2 µm Whatman GF/C filters. One filter was dried for 48h at 70°C to determine SPM, the filter was further combusted at 450°C (muffle furnace) to determine the organic (POM) and inorganic (PIM) fractions. Another filter was analysed for POC and PON using a Fisons NA-2500 automatic elemental analyzer (column: Haysep-Q, mesh 80-100) according to the method described by Nieuwenhuize *et al.* (1994). POP was analysed from the third filter based on destruction methods described in Standard Methods 4500P-J (Eaton *et al.*, 2005), followed by phosphate measurements adapted for the auto-analyzer (Standard method, Autoanalyzer Skalar San++) (also read Box. 1). The last filter was used for determination of Chlorophyll *a* concentrations, based on the fluorescence method proposed by Arar & Collins (1997). Phytoplankton abundance, in terms of total number of particles between 4–20µm (which were assumed to be primarily algae and algae chains) was determined by Sub-project I. Additionally, three water samples were taken on the bottom of the pond, at mid water depth and at the surface which were analysed by an Algae Online Analyser (AOA, bbe Moldaenke, Germany) to quantify the main groups of algae: green algae, blue/green algae, diatoms and cryptophyceae present in the water (Standard measure: bbe, AOA). These samples were also microscopically analysed to specify the main algae groups present.

Absorption efficiency

The absorption efficiency was calculated according to the Conover ratio method (Conover, 1966):

$$AE = \frac{(F' - E')}{(1 - E')} \cdot F' \quad (1)$$

Where *F* = the ash free dry weight (AFDW) : dry weight (DW) ratio of the food and *E* = the AFDW:DW of the faeces. The amount of faeces from individual clams was used in mg, collected by Sub-project I.

Faeces for nutrient content measurements were collected separately in flow-through chambers (*n*=3) with a volume of 400 ml and a flow of approximately 300 ml min⁻¹. To ensure sufficient faeces material to execute the nutrient analysis, three to six clams were held per chamber. The flow-through chambers were connected to the header tank (Fig. 5). After 24 hours faeces was collected from the flow-through chambers using a syringe, and were analysed for organic carbon, nitrogen and phosphorus contents in mg by the methods described in previous section: *i Food uptake*. To

Box 1: Underestimation POC and PON concentrations

Determination of POC and PON was conducted by an independent laboratory. However, by an incorrect assumption of their methodology, the filters were not provided properly to the laboratory. It was thought that for this method, initial weight of the filters was not needed. 25 filters from the same batch as the sample filters, were weighed and averaged, and used as the initial weight of the filters of the sampling dates May 11, 29 and June 20. However the standard error of the filters weight approximately the weight of the filtrate of 1L. For this reason the POC and PON of the food on May 11, 29 and June 20, could have been overestimated or underestimated.

The particulate organic matter of the food (POM%) varied between 52 – 70% (Fig. 7B). POP concentrations followed the trend of the POM% in the food, however POC and PON were found to be very low in May and June and were almost four times higher in July and August (Fig. 7C). The percentages of carbon, nitrogen and phosphorus together were much lower than the total POM%. Additionally, PON of the food was also measured based on the destruction method (used to analyze POP). However this method is not officially approved yet, PON was found to be much higher for May – June, and only slightly higher for July- August. For this reason it was assumed that the POC and PON concentrations for May and June were underestimated.

calculate the absorption efficiency of carbon nitrogen and phosphorus, the Conover equation was modified by Smaal & Vonck (1997) as:

$$AE = 1 - (e/f) \quad (2)$$

where AE = the absorption efficiency in percentage (%), *e* = the elemental content: ash weight ratio of the faeces, *f* = the elemental content: ash weight ratio of the food.

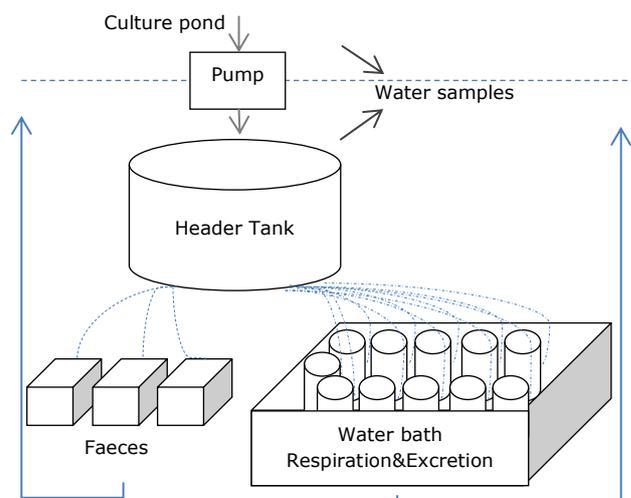


Fig. 5. Set-up faeces collection, excretion rate, respiration rate and water samples for food concentration and algae specification.

Physiological measurements

i Food uptake: total amount of nutrients consumed

The ingestion rates and the ingestion of C, N and P of the clams was calculated by multiplying clearance rates (CR) obtained by Sub-project I, with the SPM or nutrient concentrations of the food (POC, PON and POP) in mg h^{-1} . The ingestion rates were standardized to an individual of 1 gram tissue DW by the weight standardization exponent (see section: *Size standardization*).

ii Growth: Biomass and nutrient composition

Individual growth was measured by shell length in mm and tissue dry weight (DW) and ash free dry weight (AFDW), shell dry weight (SDW) organic shell weight (OSW) in gram, and C,N,P content of the tissue material in mg. Each sampling date 43 clams were collected (as a reference of the 'total population') and individual length, width and height was measured with a digital calliper. Subsequently tissue of 28 clams from the collected group was removed from the shell and dry weight (DW, SDW) and ash free dry weight (AFDW, OSW) was automatically determined by the prep-ASH (Precisa, series 340) using a set protocol (DW: 70°C , $<1\%$,

AFDW: 520°C , $<0,1\%$). The remaining 15 clams were used for determination of nutrient tissue composition. Therefore tissue of five individuals were pooled resulting in three samples per sampling date which were subsequently freeze-dried and homogenized with a mortar and pestle. Sub-samples were analysed for organic C, N and P by the method described in the section: *Environmental conditions* (POC, PON, POP). The growth rates in length, DW, AFDW and nutrients was calculated the difference between over consecutive sampling points in mm of mg per day. Additionally repeated measurements on length, width and height was performed on a group of 12 clams (further described in *Box 2: A*).

iii Egestion rates and nutrient concentration in faeces

The amount of faeces egested and the egestion rates of nutrients was calculated using the calculated ingestion and absorption efficiencies in:

$$E = (1 - AE) \cdot IR \quad (3)$$

Where E = the egestion rate $\text{mg h}^{-1} \text{g}^{-1}$ or $\text{mg C, N or P h}^{-1} \text{g}^{-1}$, AE = the absorption efficiency, IR = the ingestion rate in $\text{mg h}^{-1} \text{g}^{-1}$ or $\text{mg C, N or P h}^{-1} \text{g}^{-1}$.

Box 2: Repeated measurements on individuals (reference group)

A. The initial research plan consisted of the identification of length, respiration- and excretion rates over time by repeated measurements on eight individual clams (reference group). The clams were naturally differentiated by their shell figure and color pattern. Respiration and excretion measurements were conducted according to the protocol described in section *iv & v Respiration and inorganic nutrient excretion*. The reference group was measured for shell length, width and height with a digital caliper after the respiration and excretion measurements. Subsequently weight (DW, AFDW) was determined by length-weight correlation from the 43 individual measured clams described in section *ii Growth*. In June four clams from the reference group were lost (presumably eaten by birds), which led to a different sampling design. For July – August, eight random clams were now collected after respiration and excretion measurements, and measured on length and weight. To couple both methods respiration rates were also measured for eight reference individuals: four clams which had been followed throughout the study period (May-August) and four that were measured after the disappearance (July-August). Next to these eight reference clams, four additional clams were measured for length (July - August).

The average shell length of the reference group (12 clams), increased significantly over time, from 11.2 ± 0.5 mm begin May to 29.8 ± 0.3 mm on the end of August. The measurements on growth of the reference group might have been affected by the repeatedly disturbance during sampling. It was noticed that the clams of the reference group had clear 'growth rings' on the dates they were measured. No significant differences between the average length and growth of the reference group and the 'total population' were observed for any of the sampling points ($p>0.05$).

B. The reference group oxygen consumption rates measured on July – August were significantly higher than for the random group of clams for the same dates (Fig. 6). Temperature was also found to be significantly higher ($p<0.05$) during reference group measurements in comparison with the random group. Measurements on the reference group were always performed following the random group, what resulted in higher temperatures of the water from the culture ponds (0.7, 0.6, 1.3 respectively). It was found that the respiration rates of the clams were significantly positively correlated with temperature ($p<0.0001$, $r^2 = 0.62$, $n = 48$), which was also shown in other studies (further described in section: *Discussion*). Therefore the conclusion can be made that the observed higher respiration rates in the reference group are a response to higher temperature, and that the significant relationships are changed due to the amount of temperature differences on the sampling date.

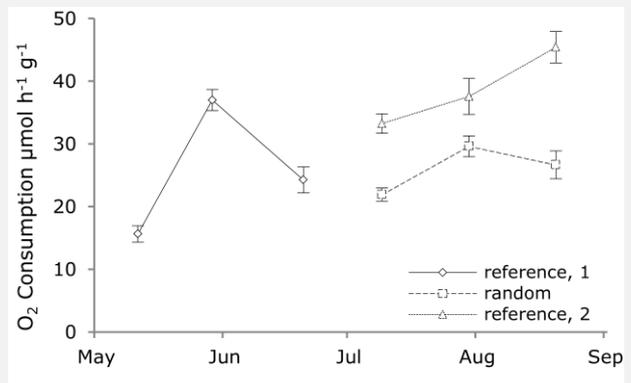


Fig. 6 Oxygen consumption of the clam *Venerupis philippinarum* in $\mu\text{mol per hour per gram of tissue dry weight}$. Comparison of measurements on the reference group and random group. All data are expressed as averages (\pm standard error).

iv & v Respiration and inorganic nutrient excretion

Respiration (C) and excretion (N, P) rates of the clams were determined using individual incubation chambers based on the protocol described by Jansen *et al.* (2011). Ten experimental chambers (300 ml) received water from the header tank with a flow of approximately 300 ml min⁻¹. The experimental chambers were placed in a water bath with running water from the header tank to ensure a similar temperature in the incubation chambers compared to the culture pond (Fig. 5). The incubation chambers were covered with dark foil to prevent potential nutrient uptake by phytoplankton. Eight of the experimental chambers contained one individual clam and the other two chambers (without clams) served as controls. Prior to the incubations the clams were held in this set-up for at least 30 minutes to let the clams acclimatize. Incubations were started by closing the in- and outflow taps at the experimental chambers. Incubations were terminated when oxygen concentration had decreased approximately 10-15% compared to initial concentrations, which was monitored in an additional chamber which was not used for further calculations. At the start and end of each incubation oxygen measurements (Hach Lange, HQ30D, probe, dissolved oxygen meter) and water samples for dissolved inorganic nutrient concentrations were taken in each chamber. Water samples were filtered through a Whatman FP 0.45µm membrane filter and stored in the freezer (-18 °C) until analysis. Dissolved inorganic nutrients (TAN, NO₂, NO₃ and PO₄) concentrations were measured with an Auto analyzer (Standard method, Autoanalyzer Skalar San++).

Oxygen consumption and excretion rate of the dissolved inorganic nutrients was calculated as the difference between the initial and final concentrations multiplied by chamber volume and standardised to one hour/day, according to:

$$E = \frac{(C_{t0} - C_{tx})}{(t_x - t_0)} \cdot V \quad (4)$$

Where E = the O₂ consumption, TAN or PO₄ excretion rate in µmol h⁻¹, C_{t0}= the initial concentration and C_{tx}= the final concentration in µmol l⁻¹, V = the volume of the incubation chamber in l, t₀ = is the time starting incubation and t_x the final time in h. The excretion and respiration rates were corrected for the control measurements. From the respiration rates, carbon metabolism was calculated by converting the oxygen consumption to carbon excretion (in CO₂) using a mean Respiratory Quotient (RQ=CO₂ eliminated/O₂ consumed) of 0.8 (Bayne & Thurberg, 1988). Resulting in a conversion factor of 0.32 (assuming that per mole O₂ 1 C atom is released; A.Smaal personal communication), giving the C excretion in µmol h⁻¹.

The excretion of C, N and P was standardized to an individual of 1 gram tissue DW (see: *Size standardization*). Additionally repeated measurements on O₂ consumption was performed on a group of 8 clams (further described in *Box 2: A, B*).

Size standardization

To exclude variability in physiological rates by the size of the clams, the physiological rates *a* ingestion (and therefore egestion) *d* respiration and *e* excretion, were standardized to an individual of 1 gram tissue dry weight using:

$$Y_s = (W_s / W_e)^b \cdot Y_e \quad (5)$$

Where Y_s represent the standardized rate in mg or µmol per hour per gram of tissue DW, Y_e = the physiological rate, W_s =1 gram of tissue DW, W_e = the tested clam weight in gram, and *b* = the exponent, standardizing the physiological rate to 1 gram of tissue DW. The exponent *b*= 0.68 was used in this study for all physiological rates (Marin *et al.*, 2005; Moschino *et al.*, 2011).

Scope for growth calculations

Prior to SFG calculations, all standardized physiological rates (ingestion, growth, egestion, respiration and excretion) were converted to mg carbon, nitrogen and phosphorus per gram tissue weight per day (mg C g⁻¹ d⁻¹). The SFG was calculated as:

$$\text{SFG (P)} = C - (F + (E \text{ or } R)) \quad (6)$$

Where SFG = the scope for growth for carbon, nitrogen or phosphorus, C = nutrients consumed, F = nutrients egested by faeces, E = inorganic nutrients (TAN, PO₄) excreted and R = carbon excretion by respiration, all expressed in mg individual⁻¹ d⁻¹. P (production) is the actual amount of nutrients in the tissue measured as growth. SFG was calculated for all sampling points and compared with the actual growth (Production).

Stoichiometric ratios

The stoichiometric ratios were calculated by converting the carbon, nitrogen and phosphorus contents in molar units, C:N:P. Stoichiometric ratios in the food (food quality) were compared with the general C:N:P ratio of 106:16:1 for phytoplankton (Redfield, 1936). The stoichiometric ratios of the food were compared to the stoichiometry of the body tissue of the clam and their faeces, to assess how these ratios modify in terms of balance, food concentration, temperature and body size. Subsequently the growth rate hypothesis (see: *Introduction*) was tested.

Statistical analysis

All statistical analyses were performed using SPSS 19.0. Physiological rates, clam length and weight (DW, AFDW), C, N and P composition of tissue and egestion and C:N and N:P ratios, were tested for normality of variable distributions and homogeneity by the Shapiro-Wilk test and Levene test. If a parameter was not normally distributed, data were transformed by LOG or square root. Subsequently a One-Way analysis of Variance (ANOVA) was used to test significance over the different sampling points. In case of significance the one-way ANOVA was followed by the Tukey HSD post-hoc test to test which data points differed significantly. Whenever there was a violation of the assumption of homogeneity of variances (Levene's test) the Welch test was used in comparison with the Games-Howell post-hoc test. The ANOVA assumptions of normality and homogeneity of variance were not met for weight (DW, AFDW) and length of the clams and phosphate excretion. In this case the non-parametric Kruskal-Wallis test for significance together with Mann-Whitney for the comparison of more than two independent groups was used. The relationship with the environmental parameters: POC, PON, POP, Chlorophyll *a*, SPM, temperature and particle abundance was tested by correlation analyses in case of normality by Spearman. To avoid assuming a significant correlation due to random processes the Bonferroni correction was applied (<0.007).

Results

Environmental factors

Water temperature varied from 14.3 °C begin May to 25.7 °C in August (Fig. 7A). Chlorophyll *a* peaked early May with a concentrations of 34.7 $\mu\text{g l}^{-1}$ while latter data points varied between 1 - 12 $\mu\text{g l}^{-1}$ (Fig. 7A). The phytoplankton population fed to the clams consisted in May primarily of the diatoms *Skeletonema constatum* and *Phaeodactylum tricorutum*, in June only *Tetraselmis suecica*, and July and August a mix of *T.suecica*, *S.constatum* and *Chaetoceros muelleri*. The amount of algae cells (between 4-20 μm) fluctuated daily with a minimum of 10,888 cells ml^{-1} measured in June and a maximum of 134,780 cells ml^{-1} the end of May (Fig. 7B). Particulate organic matter (POM) varied from 70% the end of May to 53% in August. Suspended particulate matter (SPM) concentrations fluctuated around 3.5 mg l^{-1} . Particulate organic carbon (POC) increased significantly from 0.099 mg l^{-1} in June to 0.825 mg l^{-1} begin July and decreased slightly towards the end of August. Particular organic nitrogen (PON) followed a similar trend compared to POC, with low concentrations in May

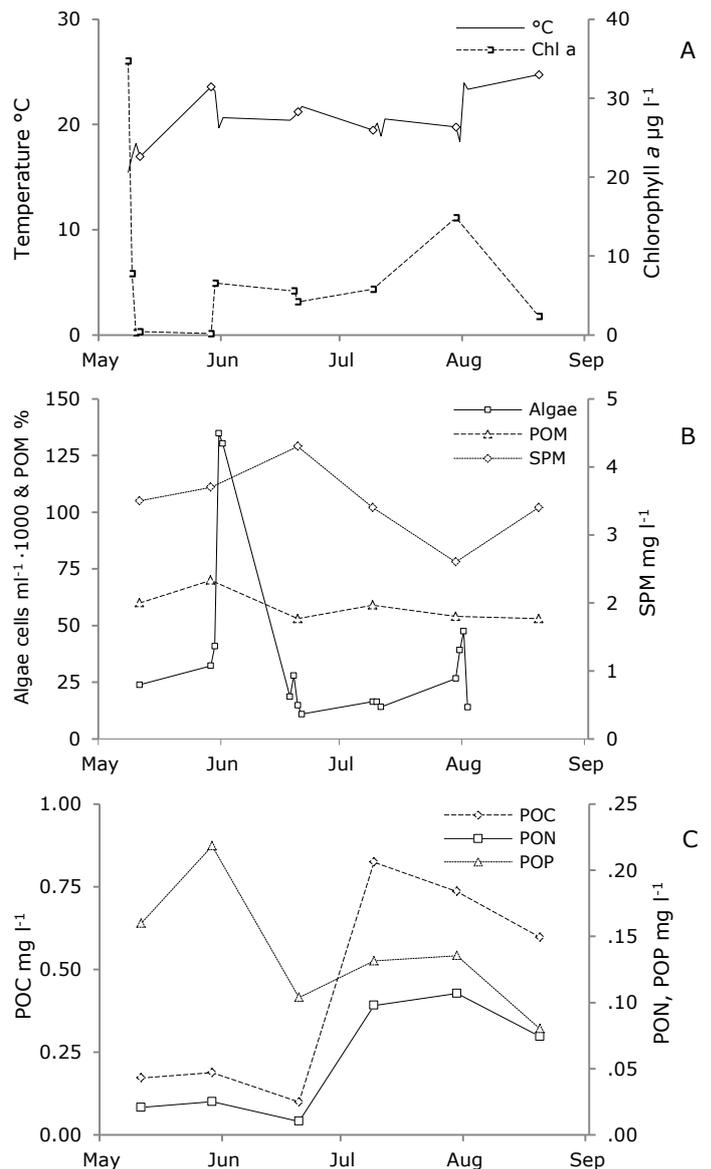


Fig. 7. Environmental factors, culture ponds. A, Temperature °C and Chlorophyll *a* in $\mu\text{g l}^{-1}$: the diamond markers represent the temperature during physiological measurements. B, algae cells per ml (4-20 μm) divided by 1000, POM in % and SPM in mg l^{-1} . C, Particulate organic carbon (POC), nitrogen (PON) and phosphorus (POP) in mg l^{-1} .

and June of approximately 0.01 mg l^{-1} , followed by a steep increase early July resulting in an average of approximately 0.1 mg l^{-1} in July and August (Fig. 6C). Particulate organic phosphorus (POP) showed an opposite pattern compared to POC and PON, with higher concentrations in May of 0.2 mg l^{-1} , which decreased to 0.1 mg l^{-1} from June onwards.

SPM was significantly negatively correlated with Chlorophyll *a* ($p < 0.0001$, $r^2 = -0.61$, $n = 48$). POC and PON were strongly correlated with each other ($r^2 = 0.94$, $n = 48$), and also with chlorophyll *a* (POC: $p < 0.0001$, $r^2 = 0.54$, $n = 48$, PON: $p < 0.0001$, $r^2 = 0.60$, $n = 48$) but not with POP.

Growth and elemental tissue composition

Shell length increased from 11.6 ± 0.3 mm in May to 28.2 ± 0.3 mm in August. Tissue weight increased from 26.3 ± 2.4 mg to 220.0 ± 8.4 mg and 0.0234 ± 0.002 mg to 0.175 ± 0.007 mg between May and August for dry weight (DW) and ash free dry weight (AFDW) respectively (Fig. 8A). Although, average tissue weight (DW and AFDW) did not significantly differ between early and late July ($p > 0.05$), length of the clams, on the other hand, increased significantly between these dates ($p < 0.05$). Width and height of the shell were strongly correlated to shell length (width: $r^2 = 0.98$, height: $r^2 = 0.99$, $n = 239$).

Growth rates of tissue and shell varied highly over the different sampling points. Highest growth rates were found begin July of 0.025 , 0.021 mg d^{-1} and 1.89 mm d^{-1} of DW, AFDW and shell length respectively. Negative growth of tissue weight was observed the end of July with -0.0002 and -0.0026 mg d^{-1} for DW and AFDW respectively. Growth in shell length was not found to be negative over time but had lowest rates the end of August 0.35 mm d^{-1} (Fig. 8B).

The organic carbon content in clam tissue varied between 359 and 390 mg g^{-1} DW without any apparent pattern through time. Organic nitrogen in clam tissue showed a significant increasing trend over time from 62.8 in May to 97.4 mg g^{-1} DW in August (except between the end of May and June, and the two samplings in July). The organic phosphorus content in clam tissue was approximately 8.3 mg g^{-1} DW, and only begin May with a significantly lower concentration of 6.9 mg g^{-1} (Fig. 8C).

Table 1. Spearman correlation matrix, of ingestion, egestion, respiration and excretion rates of *Venerupis philippinarum*, against the environmental variables. Only significant data are presented. na: not applicable (Bonferroni corrected significant correlations: $p < 0.007$)

	POC	PON	POP	Chla	SPM	Temp	Algae
Ingestion	-0.58	-0.67		-0.51	.53		
Ingestion C	na	na	na		-0.57	-0.45	
Ingestion N	na	na	na		-0.62	-0.46	
Ingestion P	na	na	na	-0.59			
Egestion	-0.65	-0.76		-0.65	.65		
Egestion C	na						
Egestion N			na	.43			-0.49
Egestion P							
Respiration O ₂						.62	.54
Excretion TAN		.51			-0.57	.43	.59
Excretion P			.71				.68

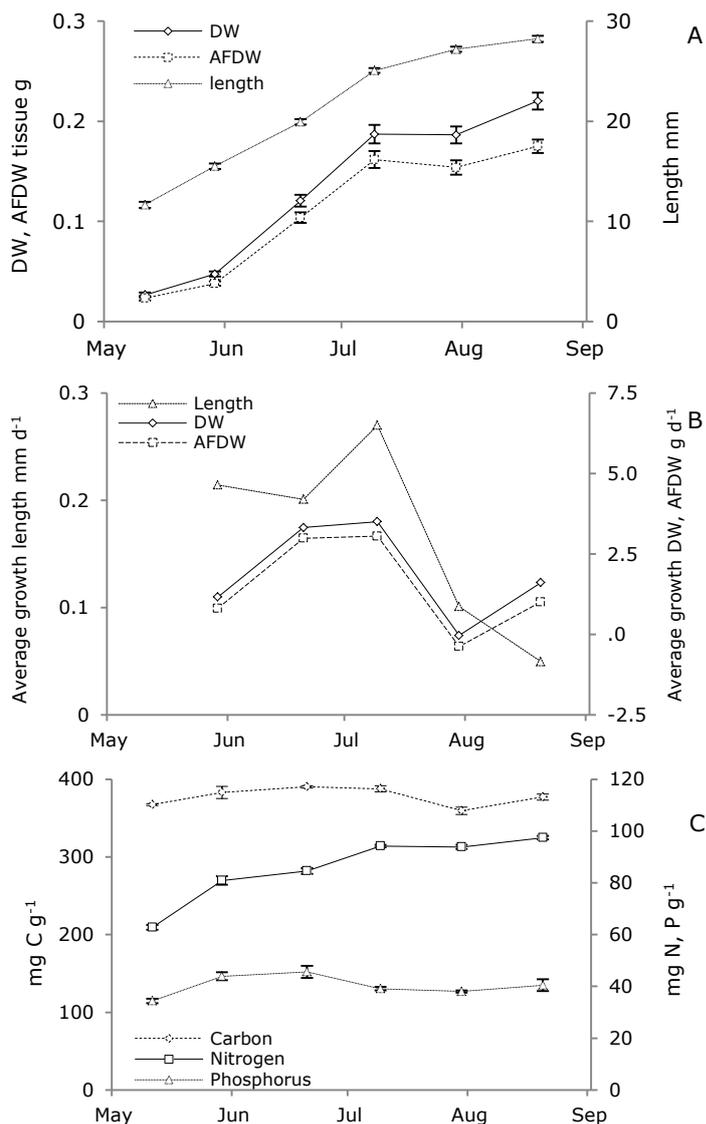


Fig. 8. Growth of *Venerupis philippinarum*. A. Tissue dry weight (DW) and ash free dry weight (AFDW) in mg, and shell length in mm B. Growth of tissue DW in mg and shell length in mm, C. Organic carbon (C) nitrogen (N) and phosphorus (P, multiplied by 5) concentrations in tissue material in mg per gram DW. All data are expressed as averages (\pm standard error).

Ingestion of nutrients

The average ingestion rates over the different sampling points showed two significantly higher rates begin May, of 31.5 ± 6.8 mg DW particles $h^{-1} g^{-1}$ and in June of 18.1 ± 3.5 mg DW particles $h^{-1} g^{-1}$ (not significantly different from each other). Ingestion rates on the remaining sampling points showed an average of approximately 8 mg DW particles $h^{-1} g^{-1}$ (Fig. 9A). A positive correlation exist between ingestion and SPM ($p < 0.001$), and negative correlations between ingestion and chlorophyll *a* ($p < 0.0001$), POC ($p < 0.0001$) and PON ($p < 0.0001$) (Table 1). There was no significant correlation observed between ingestion and temperature ($p > 0.21$) nor with algae particles per ml ($p > 0.31$).

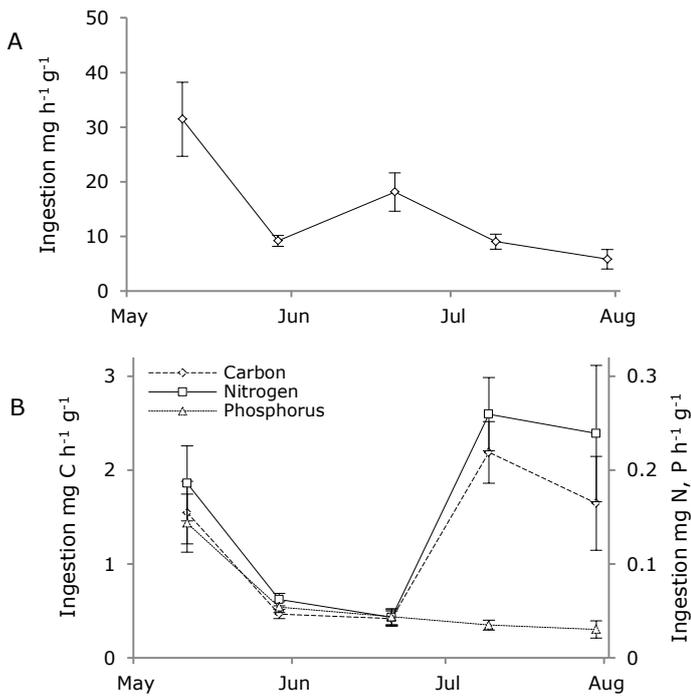


Fig. 9. Ingestion rates of *Venerupis philippinarum* throughout the entire study period. A, Ingestion rates in mg DW particles per hour per gram of tissue DW (based on Debeuf, 2012 unpublished data). B, Ingestion of carbon, nitrogen and phosphorus in mg per hour per gram of tissue DW. All data are expressed as averages (\pm standard error).

Ingestion of carbon and nitrogen were significantly lower the end of May and June, with an average of $0.44 \text{ mg C h}^{-1} \text{ g}^{-1}$ and $0.05 \text{ mg N h}^{-1} \text{ g}^{-1}$ against averages of $1.79 \text{ mg C h}^{-1} \text{ g}^{-1}$ and $0.23 \text{ mg N h}^{-1} \text{ g}^{-1}$ during the other sampling points. Ingestion of carbon and nitrogen were negatively correlated with temperature and SPM (Table 1). Average phosphorus ingestion was $0.04 \pm 0.01 \text{ mg P h}^{-1} \text{ g}^{-1}$ throughout the entire study period with only significant higher rates on the end of May, $0.14 \pm 0.03 \text{ mg P h}^{-1} \text{ g}^{-1}$.

Egestion of nutrients

Average faeces production was $1.52 \pm 0.35 \text{ mg DW h}^{-1} \text{ g}^{-1}$, and showed similar to ingestion rates, higher rates begin May $6.80 \pm 0.32 \text{ mg DW h}^{-1} \text{ g}^{-1}$, and June $3.55 \pm 0.33 \text{ mg DW h}^{-1} \text{ g}^{-1}$ (Fig. 10A). Egestion rates were like ingestion rates (from which they were calculated) also positively correlated with SPM ($p < 0.0001$, $r^2 = 0.65$, $n = 39$) and negatively correlated to chlorophyll *a* ($p < 0.0001$, $r^2 = -0.65$, $n = 39$), POC ($p < 0.0001$, $r^2 = -0.65$, $n = 39$) and PON ($p < 0.0001$, $r^2 = -0.76$, $n = 39$).

The carbon, nitrogen and phosphorus contents in the faeces was significantly higher the end of July in comparison with the other sampling points (except for the sampling in June). Nutrient concentrations were on average $16.3 \pm 3.07 \text{ mg C}$, $1.23 \pm 0.33 \text{ mg N}$, and $0.39 \pm 0.04 \text{ mg P}$, and,

$35.13 \pm 0.50 \text{ mg C}$, $3.97 \pm 0.03 \text{ mg N}$ and $0.89 \pm 0.03 \text{ mg P}$ the end of July (Fig. 10B).

Combining egestion rates with faeces composition resulted in comparable carbon and nitrogen egestion rates with averages of $119.5 \mu\text{g C h}^{-1} \text{ g}^{-1}$, $13.6 \mu\text{g N h}^{-1} \text{ g}^{-1}$. A gain significant higher concentrations were observed in June with 217.3 ± 42.2 , $16.0 \pm 3.10 \mu\text{g h}^{-1} \text{ g}^{-1}$ for carbon and nitrogen respectively. The phosphorus egestion rates varied slightly around $3.3 \pm 0.7 \mu\text{g P h}^{-1} \text{ g}^{-1}$, without any apparent pattern through time (Fig. 10C). The egestion of carbon was negatively correlated with POC ($p < 0.002$, $r^2 = -48$, $n = 39$), while nitrogen egestion was not correlated to PON ($p = 0.014$) (Table 1). Egestion of phosphorus was not correlated to any of the environmental variables.

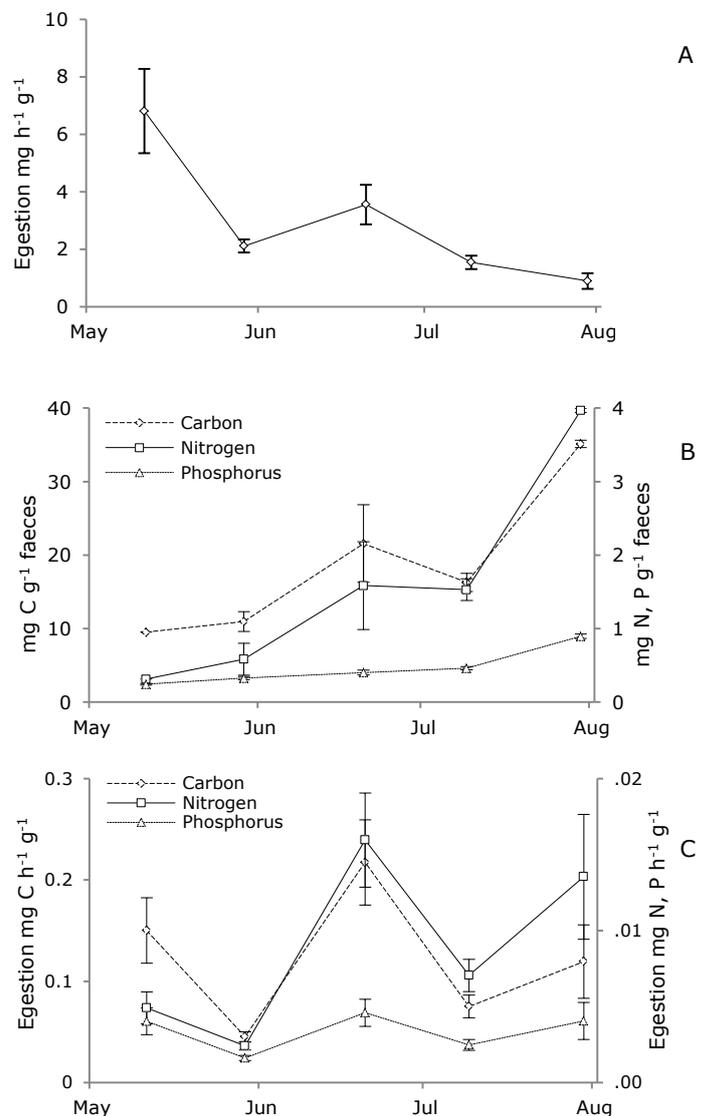


Fig. 10. Egestion rates of *Venerupis philippinarum* throughout the study period. A, Egestion rates in mg faeces DW per hour per gram of tissue DW, (based on Debeuf, 2012 unpublished data). B, mg carbon, nitrogen and phosphorus per gram of faeces DW. C, Egestion rates of carbon, nitrogen and phosphorus in mg per hour per gram of tissue DW. All data are expressed as averages (\pm standard error).

Absorption efficiency

The average absorption efficiency was $81 \pm 1\%$, and did not show significant differences between sampling points. The absorption efficiency of carbon, nitrogen and phosphorus did not show significant differences between the two sampling points in May, but all other data were found to be significantly different for each other. The absorption efficiency of carbon and nitrogen in June were considerably lower with percentages of 48% C and 63% N in comparison with the averages of $92 \pm 1\%$ and $96 \pm 0.7\%$ for the remaining sampling dates on absorption of carbon and nitrogen, respectively (Table 2). The absorption efficiency of phosphorus remained stable over the sampling points with an average of $93 \pm 2\%$.

Table 2. Absorption efficiencies and the absorption efficiencies of carbon, nitrogen and phosphorus of *Venerupis philippinarum* over the different sampling points. All data are expressed as averages (\pm standard error).

Sampling points	Absorption efficiency %	Absorption efficiency Carbon %	Absorption efficiency Nitrogen %	Absorption efficiency Phosphorus %
May 11	78.4 ± 2.3	90.3 ± 0.2	97.4 ± 0.1	97.3 ± 0.1
May 29	77.0 ± 3.4	90.3 ± 1.0	96.1 ± 0.4	97.5 ± 0.3
June 20	80.4 ± 1.3	48.0 ± 0.6	62.9 ± 0.4	90.8 ± 0.1
July 9	82.9 ± 4.0	96.6 ± 0.2	97.3 ± 0.1	93.9 ± 0.3
July 30	84.6 ± 1.1	92.7 ± 0.5	94.3 ± 0.4	90.0 ± 0.7

Respiration and excretion

A significant peak in oxygen consumption was observed in May with rates of $15.6 \pm 1.3 \mu\text{mol h}^{-1} \text{g}^{-1}$ begin May and $15.6 \pm 1.3 \mu\text{mol h}^{-1} \text{g}^{-1}$ to $37.0 \pm 1.7 \mu\text{mol h}^{-1} \text{g}^{-1}$ the end of May. From June to August the oxygen consumption rate remained stable around approximately $25 \mu\text{mol h}^{-1} \text{g}^{-1}$ (Fig. 11A). Oxygen consumption was significantly correlated with temperature ($p < 0.0001$, $r^2 = 0.62$, $n = 48$) and algae particles abundance ($p < 0.0001$, $r^2 = 0.59$, $n = 40$).

Excretion of dissolved inorganic nitrogen comprised mainly the excretion of Total Ammonia Nitrogen (TAN) with an average of $2.1 \pm 0.4 \mu\text{mol h}^{-1} \text{g}^{-1}$, while nitrate and nitrite concentrations were below $< 0.15 \mu\text{mol h}^{-1} \text{g}^{-1}$ throughout the study period. TAN excretion showed a significant increase from $0.80 \pm 0.23 \mu\text{mol h}^{-1} \text{g}^{-1}$ in June to $3.40 \pm 0.48 \mu\text{mol h}^{-1} \text{g}^{-1}$ in August (Fig. 11B). TAN excretion was positively correlated with temperature ($p < 0.002$, $r^2 = 0.43$, $n = 48$) and PON ($p < 0.0001$, $r^2 = 0.51$, $n = 48$) (Table 1).

The peak in oxygen consumption observed the end of May was also detected in phosphate excretion rates, with a maximum rate of $1.06 \pm 0.10 \mu\text{mol h}^{-1} \text{g}^{-1}$. This was significantly higher from all other sampling points which showed average rates of $0.17 \pm 0.04 \mu\text{mol h}^{-1} \text{g}^{-1}$ (Fig. 11C). Phosphate excretion was strongly correlated with POP concentrations in the food ($p < 0.0001$, $r^2 = 0.65$, $n = 40$) and the algae particles abundance ($p = 0.000$, $r^2 = 0.71$, $n = 48$). Phosphate excretion was not correlated to temperature.

C:N and N:P ratios

C:N ratios of the food remained relatively stable with an average of 9.6 (Fig. 12A). The steep increase of N:P in tissue from 2.5 in June towards

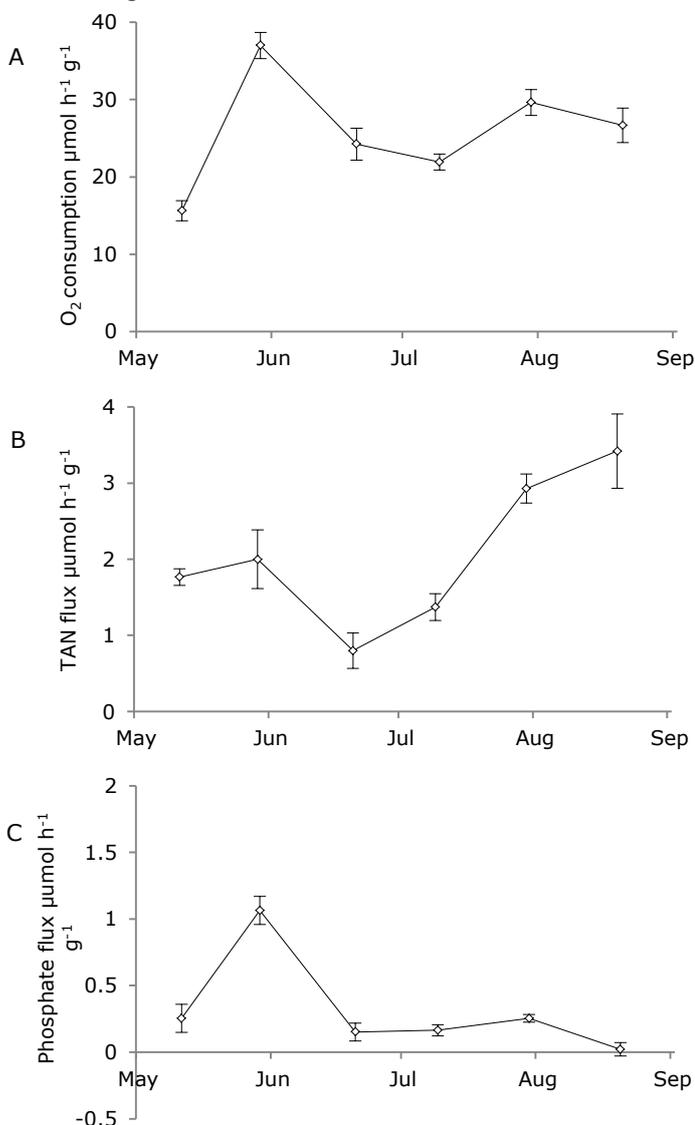


Fig. 11. Respiration and excretion of *Venerupis philippinarum* throughout the study period. A, Oxygen consumption B, Total Ammonia Nitrogen (TAN) excretion C, Phosphate excretion. All data are expressed as averages in μmol per hour per gram of tissue DW (\pm standard error).

17.0 in July was due to the steep increase in PON concentrations and a stable POP (Fig. 7).

Tissue C:N ratios decreased significantly over time, from 6.8 ± 0.1 begin May to 4.5 ± 0.2 towards the end of August (Fig. 12B), due to the increasing nitrogen content in clam tissue (Fig. 8). Consequently an opposite pattern was observed for tissue N:P ratios, increasing from 20.4 ± 0.1 in May-June to 26.0 ± 0.2 in July – August.

The C:N ratio in the faeces showed a similar pattern compared the C:N ratio in tissue and decreased significantly from 35.7 begin May towards 11.0 on July – August (Fig. 12C). N:P ratios in faeces on the end of July were significantly higher with an average ratio of 9.8 in comparison with the N:P ratios begin May of 2.9.

The C:N ratios of the excretion of C and N had an average of 17 ± 4.2 . The peak observed in the C:N ratios on June, was only significantly different to the end of May and August, no further significant trend was observed (Fig. 12D). The N:P ratios had an average of 17 ± 4.8 . The N:P ratios for August were removed from the results, since they were unrealistic variable and different from the other data points.

Scope for growth

Tissue growth in terms of nutrients is shown as accumulation or loss of nutrients over consecutive sampling points in mg of carbon, nitrogen or phosphorus per day per gram of tissue dry weight (Table 3). Tissue growth of carbon, nitrogen and phosphorus were positive in May, June begin July and August but negative on the end of July. The SFG estimates were considerably higher for carbon, nitrogen and phosphorus in comparison with the actual measured tissue growth. Differences were particularly profound in July when high SFG estimates were found whereas negative tissue growth was observed by the empirical measurements. The allocation of carbon, nitrogen and phosphorus ingested was ranked as followed: respiration or excretion > egestion > growth (Table 3).

Growth Rate Hypothesis

A slightly negative trend in N:P ratios and a slightly positive trend in tissue %P was seen in relation to growth rates of the clams (Fig. 13). However, no significant correlations were found (N:P, $p = -0.25$, $r^2 = 0.37$, $n = 15$; %P, $p = 0.30$, $r^2 = 0.28$, $n = 15$).

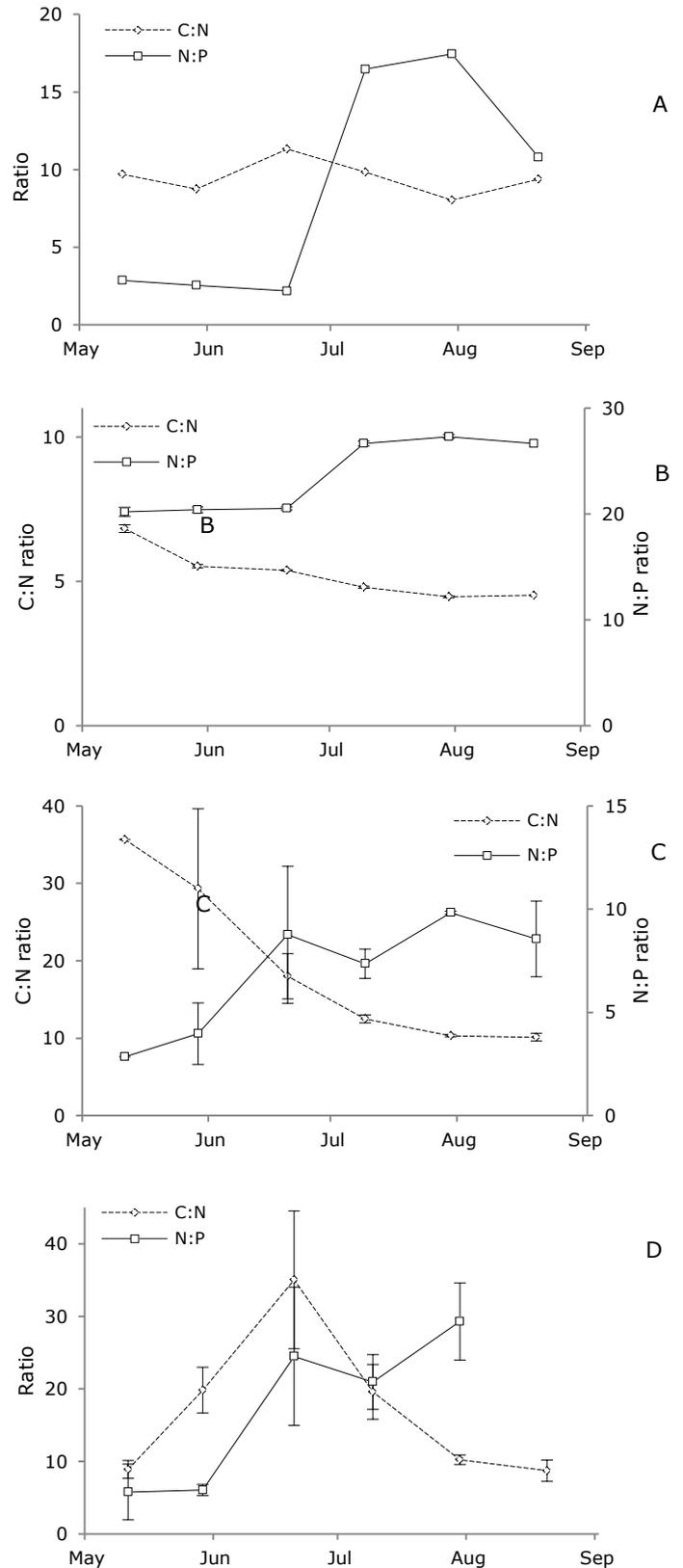


Fig. 12. C:N and N:P ratios of physiological processes in *Venerupis philippinarum*. A, Food B, Tissue, C, Faeces, D, Excretion and Respiration. All data are expressed as averages (\pm standard error).

Discussion

The main objective of this study was to assess how the growth of the clam *Venerupis philippinarum* was regulated by physiological processes as a function of food availability (quality & quantity) and temperature, and thereby gain insights/knowledge in causes for reduced growth of clams cultured in a commercial pond system.

Effects on growth rates

Similar to 2011, reduced growth rates in shell length were observed towards the end of the study period and even negative tissue growth the end of July. Although average growth rates of *V. philippinarum* in the culture ponds was 1.15 mm week⁻¹, which is 1.3 times higher than findings of 2011 (0.91 mm week⁻¹, calculated over the same time-span) and even 2.3 times higher than found by Gallager & Mann (1981) under similar conditions for *V. philippinarum* around 20 cm. Growth of the shell and tissue were found to be uncoupled since tissue growth rates after the negative growth period in July, increased again towards August, but decreased further for rates on shell growth. This indicated that after a period of negative tissue growth, acquired energy is primarily stored in growth of tissue rather than the shell. The end of July also negative elemental tissue growth rates were found for carbon, nitrogen and phosphorus. Despite variable and negative growth rates, clams were able to absorb adequate amounts of nutrients to maintain a relatively stable tissue elemental composition throughout the sampling period. Elemental composition of the clams in this study were comparable with findings of Nizzoli *et al.* (2006) and Mann (1979) for *V. philippinarum*, who reported concentrations of 377-380, 86-89 and 8 mg g⁻¹ DW for carbon, nitrogen and phosphorus

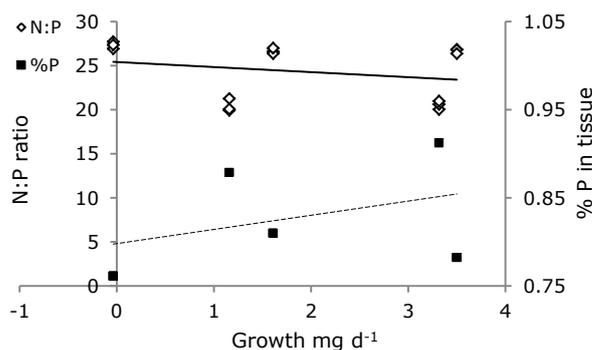


Fig. 13. Relationship between specific tissue growth rate in mg per day and N:P (diamonds) and %P (dark squares) of the tissue of *Venerupis philippinarum*. A linear trendline was added to both of the data series.

respectively. Additionally the organic content of the shell in this study was $2.47 \pm 0.08\%$ which mainly consisted of carbon (Nizzoli *et al.*, 2006).

On the other hand an increasing nitrogen content in the tissue throughout the sampling period (May-August) was observed. Ansell (1974) and Ansell *et al.* (1964) also found increasing nitrogen contents in scallops and the hard clam *Mercenaria mercenaria*, which was related to their reproductive status, since tissue nitrogen content increased to a maximum prior spawning followed by a drastic decrease post spawning. It seems therefore probable that the increasing nitrogen content in the tissue of the clams was a result of preparations for spawning, and since no drastic decrease in nitrogen tissue was observed, and eggs and sperm were found to be not ripened enough, it was unlikely that the clams spawned during the study period.

Besides effects of spawning activity (Bayne, 1976; Kanazawa & Sato, 2008), growth is mainly affected by food availability and temperature (Bayne, 1976; Dame, 1996; Newell, 2004). The environmental conditions in the culture ponds were

Table 3; Average carbon, nitrogen and phosphorus, tissue growth, ingestion, egestion, respiration, calculated scope for growth and total averages of *Venerupis philippinarum*. Data are expressed in mg per day per gram of tissue (DW)

mg d ⁻¹ g ⁻¹	11 May	29 May	20 June	9 July	30 July	20 August	Averages
Carbon							
Ingestion	37.11	11.19	10.04	52.55	39.52		30.08
Egestion	3.60	1.09	5.22	1.80	2.87		2.92
Respiration	3.72	8.83	5.68	6.58	8.02	8.60	6.91
Scope for growth	29.77	1.28	-0.86	44.16	28.63	-	20.60
Nitrogen							
Ingestion	4.46	1.49	1.03	6.23	5.74		3.79
Egestion	0.12	0.06	0.38	0.17	0.33		0.21
Excretion	0.61	0.74	0.36	0.44	0.96	1.15	0.71
Scope for growth	3.73	0.70	0.29	5.62	4.46	-	2.96
Phosphorus							
Ingestion	3.45	1.30	1.05	0.84	0.73		1.47
Egestion	0.97	0.04	0.11	0.06	0.10		0.08
Excretion	0.18	0.77	0.11	0.12	0.18	0.02	0.23
Scope for growth	3.17	0.49	0.83	0.66	0.45		1.12

comparable to conditions in the Oosterschelde (Table 4), which was considered to be relatively low. However Gallagher & Mann (1981-1982) reported a stronger relation in growth for *V. philippinarum* and quality (nutrient content and stoichiometric relation) rather than the quantity of the food. The latter study also reported that there was a higher correlation to dietary nitrogen rather than carbon, since nitrogen is mainly used for tissue growth and carbon for immediate or long-term energy. Therefore Gallagher & Mann (1981-1982) observed highest growth with a C:N_{Tissue} 4~5 under food ratios between C:N_{Food} 8~10, under low and high food quantities. This indicates that the stoichiometric imbalance which is also observed in this study C:N_{Tissue} ~ 5, C:N_{Food} ~10, does not necessarily mean a negative effect on growth, as described in the *Introduction* (Sterner & Elser, 2002). Rather a higher demand for nitrogen, since more nitrogen was needed to be accumulated in the tissue, accompanied with lower nitrogen egested and excreted, C:N_{Egestion, Excretion} ~10.

Physiological activities in relation to environmental conditions and regulation on stoichiometric differences

As described in the introduction, bivalves can regulate their physiological processes to cope with different food concentrations and stoichiometric differences by: *i* adjusting filtration / ingestion rates, *ii* selective feeding (absorption efficiency) *iii* and by all means, by metabolic processes. Therefore the physiological activities will be described over these three approaches: *i* ingestion and egestion, *ii* absorption efficiency and *iii* respiration and excretion.

***i* Filtration / ingestion rates**

Debeuf (Sub-project I, unpublished data, 2012) found for smaller individuals (<15 mm), with increasing temperatures, decreasing clearance and ingestion rates, which concurred with findings of Gouletquer *et al.* (1989) and Bernard (1983), but was the opposite from findings of Han *et al.* (2008). In this study ingestion of carbon and nitrogen were negatively correlated to temperature, however total ingestion rates were not correlated to temperature. Bayne (1976; 2009), concluded that filtration rates of bivalves in relation to food quality and temperature are observed differently and often contradicting over different studies, because of wide variation within food quality, quantity, size distribution and changes in metabolic rates due to temperature.

In natural waters the organic content of the 'seston' is usually decreasing with increasing SPM concentrations by dilution of increased inorganic

Table 4; Average concentrations of the environmental parameters; SPM, Chla, POC, PON all in mg l⁻¹ except for Chla in µg l⁻¹ and Temperature in °C, for the Oosterschelde (overviewed by Jansen *et al.* 2012) and the culture ponds of Zeeland Aquaculture 2012

Parameters	Oosterschelde	Culture pond
SPM mg l ⁻¹	5-6	3.5
Chla µg l ⁻¹	5	5
POC mg l ⁻¹	1	0.099 -0.825
PON mg l ⁻¹	0.1	0.01-0.1
Temp. °C	5 - 18	14.3 - 25.7

particles (e.g. silt) (Prins & Smaal, 1989), however in this study a lower organic content of the food was the result from different algae species with different nutrient contents (quality).

By increasing feeding rates during low nutrient availability, bivalves can meet their nutrient requirements (Bayne, 2009). However in this study no significant difference was found in the total ingestion from the end of May-August. Additionally the significant higher ingestion of carbon and nitrogen on July were probably the results from underestimations of the carbon and nitrogen contents of the food (see *Box 1*).

On the other hand the end of July negative growth rates were observed, accompanied with increased egestion and respiration / excretion rates, indicating that the nutrients required were not subtracted from the food. It was found that in July the algae *Chaetoceros muelleri* and *Skeletonema costatum* were fed to the clams. *C. muelleri* is a small sized algae (2-6 µm) and *S. costatum* can occur in long chains, which could have underestimated the algae quantity which was measured between 4-20µm. Debeuf (Sub-project I, unpublished, 2012) reported that it is conceivable that larger individuals would not be able to filter particles smaller than 2µm, because of different particle retention by the gills over size, agreed by Newell (2004) for < 3µm. For this reason clearance rates might have been overestimated, what could have resulted in overestimated ingestion rates of C, N and P and underestimated egestion rates on the end of July. By these observation it can be probable that the reduced growth observed the end of July was caused by a lower gain of nutrients due to a lower ability of filtering small particles, and thereby the nutrient requirements of growth an even maintenance could not have been met.

***ii* Absorption efficiency**

Absorption efficiency (AE) (81%), in our study was relatively high compared to findings reported by Munari & Mistri (2007), who found AE varying between 40 - 60% for *V. philippinarum* fed on *T. Isochrysis*. Under similar conditions compared to the latter study, Han *et al.* (2008) observed AE ranging between 70 to 80%. Stead *et al.* (2003) stated that bivalves which feed on natural seston

generally have AE varying between 30 – 60%, whereas suspension feeding bivalves feeding on algal diets reach AE >80%, provided under low algae concentrations. Elemental absorption efficiencies for C, N and P (>90%) were in general higher than the 'total' absorption efficiency. This was in agreement with findings of Bayne (2002), who reported AE_N 94-96% for oysters, (Grant & Cranford, 1991) for scallops, AE_N 92% and AE_C 85% and Prins & Smaal (1989) for mussels, AE_N & AE_C >90%. The latter two also reported higher AE for nitrogen in comparison with carbon, which was in agreement with findings of this study and probably related to the higher nitrogen demand of the clams. Temperature did not affect the absorption efficiency which was in agreement with findings of Han *et al.* (2008). On the other hand, a bias may occur in AE estimates as food conditions are measured at one occasion and egestion rates are the integrated result over a longer time span (Li *et al.*, 2001 found maximum gut retention times of 10.5 hours). Additionally decomposition of the faeces could have resulted in lower total nutrient contents of the faeces (Carlsson *et al.*, 2010; Jansen *et al.*, 2012b; Newell, 2004), and hence an over estimation of AE.

Several studies reported a significant relation between AE and food quantity/quality (Prins & Smaal, 1989; Cranford & Grant, 1990; Grant & Cranford, 1991; Rueda & Smaal, 2004; Bayne, 2009). Cranford (1995) even concluded that food quality in terms of POC and PON explained ~80% of the variance in absorption efficiencies in sea scallops.

In June a considerably lower AE for carbon and nitrogen was observed in comparison with the other sampling data. Since no increased ingestion rates were observed in June the lower absorption efficiency could not be explained based on decreased gut residence times (Dame, 1996; Jørgensen, 1996; Bayne, 2002; Shumway & Parsons, 2003; Bayne, 2009). It was found that for a period in June only the green algae *Tetraselmis suecica* was fed to the clams. *T. suecica* is a common algae in feed for bivalves (Walne, 1970; Sarkis, 2007), but is known for his low nutritional value and lower digestibility (Epifanio, 1979), and is often used only in combination with other algae (preferably diatoms) (Romberger & Epifanio, 1981; Sarkis, 2007). On June lowest carbon and nitrogen contents were measured, however a higher $C:N_{Food}$, indicating a lower nitrogen content of the food. The higher demand for nitrogen under low nutrient availability, was primarily regulated by means of selective feeding rather than by adjustments on filtration rates. From Fig. 14 it can be observed that in June $C:N_{Food}$ was higher than the $C:N_{absorbed}$ ($C:N_{absorbed}$ was calculated by multiplying the $AE_{C, N, P}$ by the ingestion rates of C, N and P), indicating that

more nitrogen was absorbed in relation to carbon (Bayne & Svensson, 2006).

The growth of the clams seemed not affected following the period when a mono-algal diet of low in quality *T. suecica* was fed. It is been proposed that bivalves are able to compensate for differences in food availability, by maintaining high AE, to moderate negative effects on growth. Since absorption efficiencies were found to be considerably lower, it was thought that *T. suecica* was only fed for a short time-span, which was agreed by the farm.

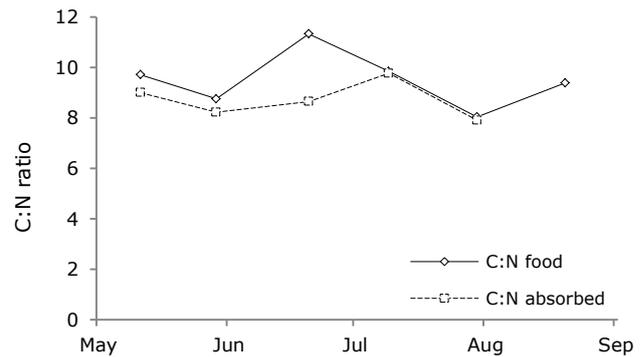


Fig. 14 C:N ratios of the food and absorbed by *Venerupis philippinarum* throughout the sampling period

iii Metabolic processes

Effect of temperature on respiration and excretion rates in bivalves is well documented (Bayne, 1976; Dame, 1996; Kraeuter & Castagna, 2001). Respiration rates in this study were also positively correlated with temperature, which was agreed by Laing *et al.* (1987) and Albentosa *et al.* (1994) for clams and by Han *et al.* (2008) for *V. philippinarum* with comparable results to this study.

The excretion of TAN was also correlated to temperature which was agreed by findings of Zhu *et al.* (1999) for *V. philippinarum*. However TAN excretion obtained by Han *et al.* (2008) were approximately 2.5 times higher than measured in this study, and even 4 times by Magni *et al.* (2000). TAN excretion was also found to be significantly correlated to the PON of the food.

The significant lower TAN excretion observed in June could be explained by the lower nitrogen availability and higher tissue nitrogen demand (see, *Absorption efficiency*). The high TAN excretion observed the end of July was the result from the utilization of body reserves for maintenance of the clams (see *filtration / ingestion rates*).

Phosphate excretion was independent of temperature fluctuations but was strongly correlated to the POP of the food. Although phosphate excretion obtained by Magni *et al.* (2000) were almost a factor 10 higher than observed in this study. Although respiration and excretion rates were standardized to an average

individual of 1 gram of tissue DW by a weight exponent of $b = 0.68$. For bivalves the exponent b varies between 0.62 and 0.75 (Riisgård, 2001). Using a maximal exponent $b = 0.75$, Respiration and TAN excretion rates were found to be 10 – 30% higher. However the trend of the different rates did not change by different exponents.

Scope for growth

Similar to the DEB-model which was used for growth prediction of the clams in the culture ponds last year, the Scope For Growth (SFG) method, also predicted higher growth rates than were measured (Fig. 15). Since SFG estimates were based on the calculations of physiological activities, it was thought that more insight could have been provided in the growth limiting mechanism(s). Overall the SFG calculations gave positive but rather high unrealistic growth rates. Grant & Cranford (1991) and Smaal & Vonck (1997) found also relative high scope for growth rates in comparison with their actual measurements on elemental composition of C, N and P on scallops and mussels. Smaal & Vonck (1997) suggested that their SFG which was based on total organic matter as in this study, would give better estimations on elemental growth if calculated based on the phytoplankton nutrient content, using the conversion: Chlorophyll * 40 = Phyto-C which was based on Bakker *et al.* (1994). When applying this method to our data this still resulted in high SFG estimates. Physiological rates can be either over- or under-estimated as been described in earlier sections. Summarizing below the main discrepancies, which were mainly explained from the method used for determination of the physiological rates (see Table 3).

Non-standardized average filtration rates were approximately $4.8 \text{ L h}^{-1} \text{ g}^{-1}$ (unpublished, not standardized by weight exponent for better comparison with literature). This rate is 1.4 times higher than described by Han *et al.* (2008), and 2.8 times higher than reported by Nizzoli *et al.* (2006) for *V. philippinarum* of 30 and 25 mm respectively. For calculation on filtration, only filtering individuals were included, and 'resting' individuals were excluded from data analysis (unpublished data, Debeuf, 2012). In retrospect, approximately half of the individuals used for our clearance rate experiments were actively filtering and thus included in data analysis, what could have overestimated the ingestion rates. Corrected filtration rates, by including also non-filtering clams, still resulted in high but also low unrealistic SFG rates. Filtration rates measured begin May were even 2 to 3 times higher in comparison with the filtration rates from June-August. Since clams were small in comparison with the filtration rate

measuring chambers used by Sub-project I, algae reduction was difficult to measure. Therefore the assumption was made that this filtration rate was overestimated, and as a result the nutrient ingestion. Additionally ingestion rates on the end of May and June were considerably lower in comparison with the other samplings. These rates were probably underestimated since the POC, PON and POP concentrations of the food were underestimated see Box 1.

Egestion rates for carbon, nitrogen and phosphorus were found to be highest on June and the end of July. In June caused from lower absorption efficiencies from the lower digestibility of the green algae *Tetraselmis suecica* and in July probably from the inability of the absorption of the algae *Chaetoceros muelleri*.

Respiration and excretion rates were clearly higher in relation to elevated temperature on the end of May and August. However also high rates were found the end of July which was explained by the utilization of body reserves.

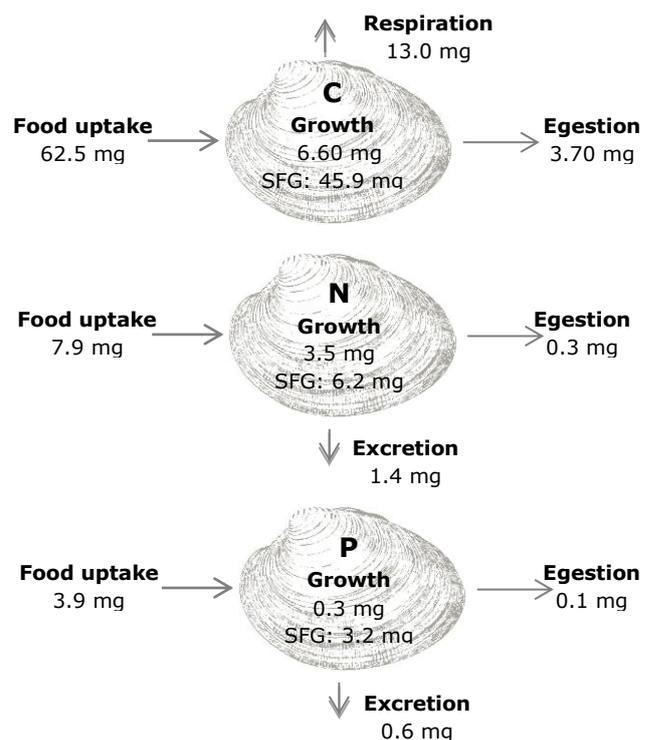


Fig. 15 average, carbon, nitrogen and phosphorus allocation in *Venerupis philippinarum* in mg per day per average individual.

Nutrient budgets

From Table 5 it was shown that carbon and phosphorus were mainly lost by respiration and excretion followed by tissue growth and the least by egestion. Mainly half of the acquired nitrogen was accumulated in growth (43.6%) thereafter excreted and only 4% was lost by egestion. The sum of

egestion, respiration / excretion and measured tissue growth, resulted for carbon and phosphorus on approximately 1/3 of the total calculated amount ingested, which was also found by Jansen (2012) who observed it as the difference between

individual on community based measurements. For nitrogen the amount was almost twice as high which was explained from the higher accumulation of nitrogen. Partitioning of carbon, nitrogen and phosphorus over different physiological processes of other studies reviewed by Jansen (2011) reported an overall division of: biodeposition > excretion > tissue growth. However most studies were based on yearly cycles, with lower growth and food availability in winter.

Growth rate hypothesis

With increasing growth rates no increased P content of the tissue was observed throughout the study. Elser *et al.* (2003) reported that high growth rates require high P uptake, to produce P-rich RNA for protein synthesis, which was agreed by Vrede *et al.* (2004) who also stated that fast growing organisms have higher P contents than slower growing organisms, since they have to synthesize more RNA. Indicating that there would be a difference between the detection of the GRH in zooplankton species, in which the GRH was mainly tested, and in clams which have in comparison far lower growth rates. Vrede *et al.* (2004) further stated that since protein synthesis is a temperature depended process, growth rates increase with increasing temperatures, but under similar RNA contents. In this study growth and the phosphorus content was not related to temperature, making this statement not applicable to this study. Sterner & Elser (2002) stated that the GRH was mainly based on maximal growth rates and not under resource limitation. This was agreed by findings of Elser *et al.* (2003), who found no correlation of RNA and P content with growth rates for *Daphnia* grown at extremely low food levels or under N limitation. In this study nutrient food concentrations were found to be low, and nitrogen was found to be the limiting nutrient for the clams. The GRH was therefore not agreed for the clam *V. philippinarum* in commercial pond systems described in this study.

Conclusion

Similar to findings in 2011 reduced growth rates were observed in our study towards the end of the experimental period. However growth of tissue was inconsistent with shell growth rates, and after a period of negative tissue growth, acquired energy

Table 5. Partitioning of carbon, nitrogen and phosphorus over the different physiological processes: Ingestion, Egestion (E), Respiration / excretion (R.E.), Tissue growth (T) and the sum of the latter three, all expressed in percentages %.

	Carbon %	Nitrogen %	Phosphorus %
Ingestion	100	100	100
Egestion	5.9	4	2.4
Resp. / Excr.	20.7	17.5	15.8
Tissue growth	10.6	43.6	8.3
E + R.E. + T	37.2	65.1	26.6

was primarily used for tissue growth rather than shell growth. Alongside with reduced growth rates, low food availability a stoichiometric imbalance between C:N of the food and the tissue was observed. The imbalance became more profound towards the end of the study period which could be related to reproductive preparation of the clam by higher accumulation of nitrogen in the tissue and was therefore not necessarily negative on the growth of the clams.

During periods of low nitrogen quantity in the food in comparison with the higher nitrogen demands by the clam, increased absorption of nitrogen was primarily regulated by means of selective feeding and suppressed TAN excretion, rather than increased ingestion rates. Towards the end of the production cycle it was found that larger clams subjected difficulties on subtracting nutrients from smaller algae, with the result that nutrient demands could not have been met. The latter was also indicated by increased TAN excretion indicating that internal nitrogen sources in the tissue were catabolized.

The Scope For Growth method predicted higher growth rates than measured, however these were mainly the result of overestimated ingestion rates since resting individuals were excluded from data analysis. Egestion rates were mainly affected by the ability of absorption and digestibility of ingested food and respiration and excretion rates increased considerably with elevated temperatures. Additionally TAN and P excretion were also regulated by the PON and POP levels in the food. The allocation of nutrients after ingestion was similar for carbon and phosphorus: $C_{Respiration}, P_{Excretion} > C_{PGrowth} > C_{PEgestion}$. Nitrogen was primarily (~50%) accumulated in the body N_{Growth} followed by $>N_{Excretion} > N_{Egestion}$. It was found that the reduced growth at the end of the experimental period could not be attributed to one mechanism, but was regulated by the absolute nutrient content and stoichiometric ratios of the algae and algae species composition rather than only food quantity. Finally the Growth Rate Hypothesis could not have been applied for the clams, since it can only be detected under maximal growth rates, and not under resource limitation.

Perspectives

Although growth rates in this study were found to be higher in comparison with the study in 2011, the desired commercial size of ~40 mm was not reached during this experimental / production period (May to August ~11-28 mm). It was also found that growth rates of the shell and tissue were uncoupled, since after the period of negative growth the end of July, tissue growth rates increased again towards August, while shell growth rates decreased, suggesting that clams used their acquired energy primarily for tissue growth rather than shell growth. Subsequently resulting in a higher condition index of the clams. From this study it can be concluded that growth was not suppressed due to a single mechanism, but clam growth rates was primarily affected by the nutrient content and digestibility of the algae. Although it is known that energy costs for growth is mainly met by carbon in comparison with nitrogen (Flaak & Eplfanio, 1978; Bayne, 2009), nitrogen was the main limiting nutrient for tissue growth of the clams in this study.

On the other hand, it is known that larger clams have lower physiological rates in relation to body size. This mechanism is generally known as the allometric scaling (Moschino *et al.*, 2011), additionally reduced food uptake can be enhanced by morphological limitation of the siphon diameter, as stated by Debeuf (Sub-project I, unpublished 2012). Additionally it was reported that bivalves stop filtering under a certain threshold, to avoid higher losses than could have been required (Riisgård *et al.*, 2003).

To improve growth of the clams, further research should focus on: *i* the ability of absorption and digestibility of different algae over size in relation to the nutrient content of the algae rather than the optimal food concentration (eg. in cells per ml), *ii* additionally, since water warms up quickly in the shallow culture ponds, research can be conducted on the susceptibility to stress by fluctuating temperatures and the maximum temperature they can withstand. Since for the clam *Ruditapes decussates* temperatures above 27 °C resulted in declined SFG and were above 32 °C even negative (Sobral & Fernandes, 2004). Which was also agreed by Han *et al.* (2008), which additionally reported an optimal temperature of 20 °C for *V. philippinarum*. Higher current speeds (and thus lower residence times) can then be used to regulate temperature to a certain extend.

Clams cultured in the ponds are not only consumers of nutrients but also producers, since nutrients are lost by excretion of metabolic waste products and egestion. As the costs of discharge of nutrient-rich water to the surface water is high and can also be seen as loss of expensive nutrients, the farm design

can be modified to profit from the released nutrients by bivalves.

Dissolved inorganic nitrogen and phosphate excretion are directly available for algae growth. In this study it was found that 60% of phosphorus and 27% of nitrogen acquired (calculated by Egestion + Excretion + Tissue growth) is directly regenerated back into the system by excretion of inorganic nutrients. The average N:P ratio of inorganic nutrients excreted by the clams was approximately 17 which in close proximity to the Redfield ratio (16) and to the N:P in the food in July-August (17). These nutrients can for example be used for the growth of macroalgae, since a macroalgae-filter is already installed at Zeeland Aquaculture to reduce nutrient discharge. On the other hand nutrients can also be used for the growth of their own food source: the microalgae. These may in turn be used for the growth of the clams again.

Nutrients egested in the (pseudo) faeces are not directly available for algae growth. However, these nutrients may become available through degradation of faeces by microbial activity. Jansen *et al.* (2012b) reported that the mineralization of carbon and phosphorus was preferred over nitrogen. Remaining faeces will accumulate in the sediment and will degraded on longer time spans. In which form nutrients may become available is depending on the oxygen conditions of the pond sediment: e.g. to ammonia under aerobic conditions and to nitrite, nitrate or nitrogen gas by nitrifying and denitrifying bacteria under anaerobic conditions. Nitrogen gas is not available for algae and therefore considered as a loss in the system. During severe anaerobic conditions the sediment will turn blackish which was also observed in the sediment of the culture ponds at Zeeland Aquaculture. If the sediment is not enriched with enough oxygen less nitrogen will be regenerated into the system but might be lost as gas. By increasing the oxygen concentration of the ponds and the sediment, more nutrients will be regenerated and will thus be available for the micro and/or macroalgae growth. The nutrients excreted and egested by the clam should therefore not be seen as a negative flux, but as a useful tool for sustainable growth of algae. Baud *et al.* (1992) suggested for the culture of *V. philippinarum* in marine culture ponds that a high density together with high food concentrations will result in the highest growth, since it concurs with high excretion rates, which in turn stimulated phytoplankton growth again. However the realization of such a recirculation system requires further research on *i* sediment-water interactions of the ponds in relation to bivalve density and water renewal time, and *ii* algae growth as a function of waste water and nutrient additives to reach desired nutrient stoichiometry for algae growth.

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