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Neuroenzymatic activity and physiological energetics in Manila clam, *Ruditapes philippinarum*, during short-term sublethal exposure to harmful alga, *Heterocapsa circularisquama*

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ABSTRACT

The harmful alga, *Heterocapsa circularisquama*, causes recurrent mortalities of bivalve molluscs in Japan, with demonstrated hemolysis and cytotoxicity in rabbit erythrocytes, HeLa cells, and bivalve tissues. Nonetheless, the effects of exposure to sublethal cell densities on the physiological energetics of bivalves have not been investigated, nor the potential involvement of neurotoxicity.

In the present study, two sets of experiments were conducted with adult clams, *Ruditapes philippinarum*. In the first set, the clearance rate (CR), respiration rate (RR), absorption efficiency (AE), ingestion rate (IR), and absorption rate (AR) were examined in clams exposed to *H. circularisquama* to quantify the scope for growth (SFG) as an indicative of the bioenergetic status of clams (5, 50, 2.5×10^2, and 5×10^2 cells ml^{-1}; under 15 °C and 20 °C). In the second set, the activity of the biomarker of neurotoxic exposure, acetylcholinesterase (AChE), was monitored following 3, 6, 24, and 48 h of exposure (5, 50, 5×10^2, and 10^3 cells ml^{-1}, at 20 °C) in gills of *R. philippinarum*, and compared to that in Mediterranean mussels, *Mytilus galloprovincialis*; a species also affected by *H. circualrisquama* and in which AChE activity was more extensively studied.

At 15 °C, CR, IR, and AR were decreased for exposures to 50–5×10^2 cells ml^{-1} resulting in a significant decrease in the absorbed energy (A), and a significant decrease in SFG at 5×10^2 cells ml^{-1}. At 20 °C, AE was null for exposures to 2.5×10^2 and 5×10^2 cells ml^{-1}. RR was decreased at 2.5×10^2 and 5×10^2 cells ml^{-1}, CR, IR, and AR were decreased at 5–5×10^2 cells ml^{-1}, and the AE was null for 2.5×10^2–5×10^2 cells ml^{-1} resulting in a significant decrease in the respired energy (R), but mainly in (A) especially at 2.5×10^2 and 5×10^2 cells ml^{-1} decreasing the SFG over the entire range of cell density with negative values for 2.5×10^2 and 5×10^2 cells ml^{-1}. The activity of AChE in both clams and mussels was significantly reduced following 3–48 h of exposure to 5–5×10^2 cells ml^{-1}.

Reduction in the energy available for clams to grow and reproduce was induced mainly via decreased energy acquisition, and delayed and/or reduced functions of the digestive organs. In addition, this is the first report of decreased neuroenzymatic activity in two bivalve molluscs induced by exposure to *H. circularisquama* potentially via either neurotoxic compounds affecting the activity of gill cilia and/or gill muscles, and/or potentially via either neurotoxic compounds affecting the activity of gill cilia and/or gill muscles, and/or non-cholinergic affects associated with other functions; both resulting in decreased SFG. The relationship between the decreased AChE and decreased SFG should be corroborated in future research.

1. Introduction

The dramatic increase in the incidence, frequency, complexity, and extent of harmful algal blooms (HAB) caused by the overgrowth of microscopic algae in aquatic systems over the past three decades

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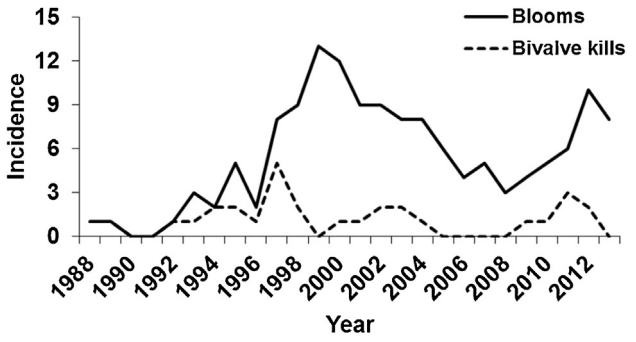

Fig. 1. Bloom incidence of *Heterocapsa circularisquama* and associated incidence of bivalve mortalities in Japan from 1988 to 2013. (Data compiled after the annual reports on harmful algae of the Seto Inland Sea Fisheries Coordination Office, Kyushu Sea Fisheries Coordination Office, and Fisheries Research Division of Mie Prefectural Science and Technology Promotion Center, 2004–2013).

Table 1
Effects of the density of *Heterocapsa circularisquama* and temperature on the physiological and energetic rates of adult clams, *Ruditapes philippinarum*.

	Parameters of evaluation		
Physiological and energetic rates	*D*	*T*	*D* × *T*
$CR^{(\theta)}$	*	NS	–
$RR^{(\dagger)}$	*	NS	NS
$AE^{(\theta)}$	NS	NS	NS
$IR^{(\theta)}$	*		–
$AR^{(\dagger)}$	*		*
$A^{(\dagger)}$	*		*
$R^{(\dagger)}$	*	NS	NS
$SFG^{(\dagger)}$	*	*	NS

CR: Clearance rate, *RR*: Respiration rate, *AE*: Absorption efficiency, *IR*: Ingestion rate, *AE*: Absorbed energy, *R*: respired energy, *SFG*: Scope for Growth. *D*: density of *H. circularisquama*. *T*: Temperature, *T* × *D*: Interaction. (*): significant effect, $P < 0.05$. NS: non-significant effect. (–): not-applicable. (**θ**): non-parametric analysis, Wald-Wolfowitz Runs test, (†): parametric analysis, univariate ANOVA.

has been paralleled with a dramatic increase in the effects of their blooms and associated toxins on the health of aquatic organisms, population growth, ecosystem integrity, as well as human health, and several industries and economies (Burkholder, 1998; Van Dolah, 2000; Landsberg, 2002; Glibert et al., 2005). The diversity of the causative agents and the impacts of HAB have become a challenge to both scientists and managers of coastal resources and ecosystems (Zingone and Oksfeldt Enevoldsen, 2000; Anderson, 2009). In particular, a diversity of dinoflagellate species has been responsible for both toxic and non-toxic HAB events worldwide, a number of which has caused extensive mortalities in wild and cultured populations of fish and shellfish (Landsberg, 2002).

The dinoflagellate, *Heterocapsa circularisquama*, is one of the most notorious shellfish-killing HAB that has occurred for the first time in Japan, in Uranouchi Bay, in southern Shikoku Island in 1988, and caused mass mortality of short-neck (Manila) clam, *Ruditapes philippinarum*, estimated at 1560 tons (Matsuyama et al., 1996). In 1989, it occurred further to the south of Japan, in Fukuoka Bay, northern Kyushu Island, and caused mass mortality of at least five species of bivalve molluscs (Yamamoto and Tanaka, 1990). Since then, *H. circularisquama* has been forming recurrent blooms with associated bivalve mortality in several localities in western Japan, and in 2009 it expanded northward to Eastern Japan, in Niigata Prefecture, where it also caused mortality of bivalves (Kondo et al., 2012). The incidence of blooms of *H. circularisquama* and associated shellfish kills has decreased over the years 2000–2008, although both have been on a course of revival since 2009 (Fig. 1).

Field and laboratory studies showed that exposure to *H. circularisquama* impacted bivalves in many ways, from alerted feeding and respiration, extensive cytotoxicity, to impaired early-life development (Matsuyama et al., 1996, 1997a,b; Matsuyama 2003a; Nagai et al., 1996, 2006; Yamatogi et al., 2005; Basti et al., 2009, 2011a,b, 2013, 2015). Photosensitizing hemolytic toxins (Oda et al., 2001; Sato et al., 2002; Miyazaki et al., 2005) and cytotoxins (Hiraga et al., 2002) were isolated from several strains of *H. circularisquama*. Nonetheless, the instability of the toxins associated with *H. circularisquama* rendered their extraction and characterization challenging (Oda et al., 2001; Hiraga et al., 2002; Sato et al., 2002; Miyazaki et al., 2005; Kim et al., 2008).

Blooms of *H. circularisquama* generally form in warm coastal waters, from early summer to late autumn (June–November), favored by upwelling, seawater mixing events, and the die-off of diatom blooms during the rainy season (Matsuyama, 2003a). In the wild, mortality of bivalve molluscs was reported for cell densities generally exceeding 10^3 cells ml^{-1} (Annual reports of harmful algae of Kyushu Sea Fisheries Coordination Office, 2004–2013). Under controlled laboratory conditions, *H. circularisquama* induced mortalities of bivalves at lower cell densities (Nagai et al., 1996; Matsuyama, 2003b), and at as low as 50 cells ml^{-1} in the case of *R. philippinarum* (Basti and Segawa, 2010). Both wild and cultured populations of bivalve molluscs can, however, be exposed to sublethal cell densities of *H. circularisquama* that might impact their growth and reproduction without causing their death (see Table 1 in Matsuyama, 2003b for cell densities during blooms without shellfish kills). The recent reports of field mortality of bivalves at temperature lower than previously reported (Kondo et al., 2012; Fujimoto et al., 2013), and the mortality of juvenile and adult clams, *R. philippinarum*, under controlled laboratory temperature below the thermal optimal of *H. circularisquama* (15 °C), compels to investigate the combined effects of temperature and sublethal densities of the toxic alga on bivalves.

The scope for growth, or SFG, was introduced in fish bioenergetics (Warren and Davis, 1967), and then applied to the energetics of filter-feeding bivalves and other invertebrates (reviewed in Jørgensen, 1990). It is a sensitive measure of the physiological energetic balance between the energy acquired via feeding and absorption of food, and the energy expended via metabolism and excretion, reflecting an estimation of the energy available for an organism to grow and reproduce from easily-measured physiological rates converted into energy equivalents (Widdows and Staff, 2006). Growth being a sensitive parameter that reflects the suitability of an environment, the description of its bioenergetics, the SFG, has been a useful approach to study the sublethal effects of anthropogenic pollutants on aquatic organisms, in both short- and long-term frames, and was first applied to the blue mussel, *Mytilus edulis*, in marine pollution studies covering the accumulation, immobilization, and metabolism of discharged toxic substances, and later on adopted as a biomarker of water quality in coastal monitoring programs (Widdows et al., 1995a,b; Kesarcodi-Watson et al., 2001a,b; Widdows et al., 2002). The integration of several physiological processes in the estimation of SFG over short-term experiments makes it, therefore, a non-specific, highly-sensitive, and ecologically relevant measure of growth and reproductive potentials of bivalves exposed to a toxic and lethal agent like *H. circularisquama*. Therefore, SFG was adopted in the present study as a measure of the bioenergetic balance in adult clams, *R. philippinarum*, following short-term exposure to sublethal cell densities of the toxic alga.

The potential involvement of neurotoxicity in the extensive damage caused by *H. circularisquama*, specifically in the quantitative alteration of the shell valve movement and in the paralysis coupled with contraction of the mantle and/or siphons preceding death of bivalves (Nagai et al., 2006; Basti et al., 2009; Basti and Segawa, 2010), has never been investigated. Acetylcholinesterase

(AChE; EC 3.1.1.7) is a key enzyme in the nervous system that catalyzes the hydrolysis of the ubiquitous neurotransmitter, acetylcholine, in synaptic clefts and at neuromuscular junctions thereby reducing or terminating nerve impulses and muscular stimulations (Payne et al., 1996). The inhibition of AChE is one of the first validated biomarkers in environmental monitoring of neurotoxic xenobiotics, in both vertebrates and invertebrates (Day and Scott, 1990; Bouquené and Galgani, 1998; Kirby et al., 2000). Extensively used in terrestrial monitoring programs, in aquatic environments AChE is considered a relevant tool for investigating the biological effects of many neurotoxic contaminants and has been extensively studied in bivalves, specifically mussels of the genus *Mytilus* (Lionetto et al., 2011). Additionally, the recent discovery of the non-cholinergic functions of AChE makes the assessment of its activity a suitable measure and/or a potential biomarker of exposure and/or effect to/of other stressors (Lionetto et al., 2011). Thus, the activity of AChE in gills of *R. philippinarum* following exposure to *H. circularisquama* at realistically low cell densities was adopted in the present study, to assess the potential neurotoxicity of this harmful alga. It was also assessed in adult Mediterranean mussel, *Mytilus galloprovincialis*, collected from the same sampling site as Manila clams, to confirm the potential neurotoxic effect in another bivalve species, also affected by *H. circularisquama* and in which the AChE activity has been more extensively studied.

2. Materials and methods

2.1. Collection and acclimation of clams and mussels

Adult short-neck clams, *Ruditapes philippinarum*, and Mediterranean mussels, *Mytilus galloprovincialis*, were sampled from Hakkeijima, Tokyo Bay (35°20′N, 139°38′E), at low tide, during winter (once in November and once in December). The monthly temperature at the sampling site ranged from 9.6 °C in February (coolest temperature) to 26.6 °C in July (hottest temperature), with an average annual temperature of 17.5 ± 6.1 °C. The water temperature at the sampling site was 15.7 °C in November and 14.2 °C in December. Clams and mussels were transported to the laboratory within two hours of collection in aerated tanks. Upon arrival, they (200–300 individuals, 80 individuals/tank) were cleaned and placed in closed-recirculating 70-l tanks filled with filtered (Carbon Fiber NTM −037, Slide Filter 600, NISSO) and continuously aerated offshore seawater, regulated at 15 °C. During acclimation (7–10 days), the water temperature in the acclimation tank was either maintained or gradually raised to the experimental water temperature by no more than 0.5 °C per day. Once the experimental temperature reached, clams and mussels were maintained at that temperature for at least 3 days. Clams sampled in November were used for the 15 °C and 20 °C experiments to assess the scope for growth, and clams and mussels sampled in December were used for the 20 °C experiments to assess the acetylcholinesterase activity. They were fed daily on a single ration of the non-toxic microalga *Isochrysis galbana* distributed manually and regulated at an initial cell density of 5×10^4 cells ml^{-1} (0.42% of clam tissue dry weight). All of the water in the tanks was replaced every 48 h and new clean tanks were used every 4–5 days. Prior to experiments, clams and mussels were allowed to clear their gut contents for 24–48 h in static 20-l tanks filled with filtered (GF/C Whatman®, pore size ca 1.2 μm) and continuously aerated seawater.

2.2. Algal cultures

The toxic dinoflagellate, *Heterocapsa circularisquama* (strain 92HC; average cell length = 23.9 m, average cell width: 17.3 m, biovolume: 3745 μm^3), and non-toxic microalga, *Isochrysis galbana* (average cell seize = 4.3 m, biovolume: 41.63 μm^3), were cultured in modified SWM-3 medium, at 25 °C (Chen et al., 1969; Itoh and Imai, 1987). Cultures of *I. galbana* were carried in 6-l batches with aeration to maximize the yield, and were used to feed clams and mussels during acclimation. Toxic of *H. circularisquama* was cultured in 2-l flasks. The non-axenic cultures were maintained under a 12 h L: 12 h D cycle. The algae were collected at late exponential to early stationary phases, for both the acclimation of bivalves and the experimental exposures.

2.3. Exposure protocol and measurement of the scope for growth

2.3.1. Measurement of clearance rate (CR) and respiration rate (RR)

The experimental set-up adopted for the measurement of the CR and RR of clams was similar to the one used in previous experiments in our laboratory, in 1-L closed chambers (Basti et al., 2011b). For the estimation of the CR, five adult clams (N = 150, shell length = 31.99 ± 1.64 mm, tissue dry weight = 0.25 ± 0.09 g) were placed individually in pre-aerated transparent glass vessel containing 900 ml filtered seawater (Whatman® GF/C filter, pore size 0.45 μm). Four concentrations of *H. circularisquama* were used (5, 50, 2.5×10^2, and 5×10^2 cells ml^{-1}). The total density of food particles in every treatment was adjusted to 10^5 cell ml^{-1}. Adequate volumes of cultures of *I. galbana* were added to the experimental seawater of each treatment to a total concentration of 5×10^4 cells ml^{-1}, a concentration below the threshold value of pseudofeces production (Foster-Smith, 1975; Savina and Pouvreau, 2004). The CR and RR of clams exposed to *I. galbana* at 5×10^4 cells ml^{-1} was defined as the control. Controls without animals regulated at the same algal concentration were used to correct for any spontaneous change in the algal densities due to algae settlement or division, and for any change in the oxygen concentration due to microrespiration. All experiments were run in triplicate under two temperatures, 15 °C and 20 °C. The salinity, pH, and DO were set at the beginning of the experiments to 33, 8.00 ± 0.05, and 6 mg O_2 l^{-1}, respectively. Aeration and stirring were not performed to avoid any disturbance of the filtering process of clams, and not to allow *H. circularisquama* to transform into temporary cysts. Animals were allowed to resume filtration and measurements started when all clams opened their shells or extended their siphons.

The CR was measured using the indirect method of Coughlan (1959). Chlorophyll content of triplicate samples of experimental waters, for each time-measurement (every 30 min for 3 h, 5 ml each), was immediately extracted with 6 ml DMF (*N*,*N*-dimethylformamide) and stored at −5 °C, in complete darkness for less than one month (Suzuki and Ishimaru, 1990). Chlorophyll *a* content was then determined with a fluorometer (Turner Design 10R).

The *CR* was calculated using the following equation (Coughlan, 1959):

$$CR = ln(F_0/F_t)V/t$$

where, *CR* is the clearance rate (l h^{-1} indiv^{-1}), *V* is the total volume of ambient water (l), F_0 and F_t are the fluorescence values between two sampling times, and *t* is the time increment (h). Any small changes in the fluorescence value of the control were subtracted from the experimental rates.

The decline rate of oxygen was measured with calibrated oxygen electrodes connected to oxygen meters (DKK, TOA Corp., Japan). The decline in oxygen concentration was monitored every 30 min for a total exposure duration of 3 h. The respiration rate was then calculated using the following equation:

$$RR = [C_{t0} - C_{ti}] \times V/(t_i - t_0)$$

where, *RR* is the rate of oxygen uptake (mg O_2 h^{-1}), C_t is the concentration of oxygen in the water (mg O_2 l^{-1}) at time *t*, *V* the total volume of water in the sealed chamber (l), and t_0 and t_i are the initial and end times (h) for the measurement period.

In order to relate the CR and RR to the dry tissue weight of the clam, the soft parts of the clams were removed from the shell and dried for 48 h at 80 °C to constant dry weight, measured to the nearest 0.1 mg (Sobral and Widdows, 1997; Pouvreau et al., 1999). The CR and RR were then expressed in terms of weight-specific clearance rate (CR; l h^{-1} g^{-1}) and weight specific respiration rate (RR; mg O_2 h^{-1} g^{-1}). The real pumping rate (l h^{-1}), which is equal to the volume of water processed through the pallial cavity by the animal per unit time, was assumed to be equal to the CR. In fact, *R. philippinarum* has been shown to retain and feed on picocyanobacteria (1 μm long) and thus effectively retain *I. galbana* (4 μm) (Brown et al., 1997; Nakamura, 2001).

In order to compare the physiological rates on the same basis, the CR and the RR were converted to a standard animal of 1 g tissue dry weight using the following equation (Bayne and Newell, 1983):

$$Ys = (Ws/We)^b Y$$

where, *Ys* is the specific physiological rate for an animal of standard weight, *Ws* is the standard weight (1 g), *We* is the observed weight of the animal (g), *Y* is the measured physiological rate, and *b* is the weight exponent for the physiological rate function. The average weight exponents (*b*) for the *CR* and *RR* calculated for several bivalves showed that they are generally around 2/3 and 3/4 for filtration and respiration, respectively (Savina and Pouvreau, 2004). Thus we used these values for the clearance rate and respiration rate.

The organic ingestion rate (IR, mg AFDW g^{-1} h^{-1}) was obtained by multiplying the CR by the concentration of diet (expressed in mg AFDW l^{-1}). The organic weight was considered as the difference between the dry weight and the ashed weight of the diet, and was estimated as described below for the AE.

2.3.2. Measurement of absorption efficiency (AE) and absorption rate (AR)

The AE was measured using the ratio method of Conover (1966), which represents the efficiency with which material is absorbed from the ingested food. It assumes that the animal can digest and absorb the organic components of the food but not the inorganic fraction. The Conover method is based on the ratio between the organic content of the food and feces, and is calculated from the equation:

$$AE = (F - E) \times 100/[(1 - E)F]$$

where, *AE* is the absorption efficiency (%), *F* is the organic content of the ingested food and corresponds to the ash-free dry weight/dry weight ratio of the food, and *E* is the organic content of the feces which corresponds to the ash-free dry weight/dry weight ratio of the feces.

To measure the organic contents, the ash-free dry weight/dry weight ratio of both food and feces were determined according to the following procedures: the algal culture (10 ml) of known cell concentration was filtered through washed, ashed, and pre-weighed 45 mm glass-fiber filters (GF/C, Whatman®). Feces were collected 4 h after the end of the experiments, by pipetting onto washed, ashed, and pre-weighed GF/C filters. Salts were then washed out with 0.5 M ammonium formate (5 ml). Blank GF/C filters, washed, ashed, and pre-weighed were used with each batch of filters to correct for any small weight changes between filters. The filters were oven-dried at 80 °C for 48 h, weighed, and then ashed in a muffle furnace at 450 °C for 4 h and weighed again to the nearest 0.01 mg to calculate the weight of organic material combusted. Filters were cooled in desiccators before weighing (Grodzinski et al., 1975; Sobral and Widdows, 1997; Bayne, 1998).

The absorption rate (AR, mg AFDW g^{-1} h^{-1}) was obtained by multiplying the ingestion rate by the absorption efficiency.

2.3.3. Assessment of the scope for growth (SFG)

The SFG reflects the balance between energy acquisition from feeding and digestion, and energy expenditure via metabolism and excretion. Many laboratory studies showed that the energy lost via excreta was a small portion of the total energy budget, and accounts for less than 5%. Therefore the SFG was calculated by the following simplified equations (Widdows, 1993; Smaal and Widdows, 1994; Widdows and Staff, 2006):

$$C = CR \times \text{particleconcentration} \times \text{energyof} I.galbana$$

$$A = C \times AE$$

$$R = RR \times \text{energyof1 mgO}_2$$

$$SFG = A - R$$

where, *C* is the total energy consumed, *CR* is the specific clearance rate (l h^{-1} g^{-1}), *A* is the total energy absorbed, *AE* is the absorption efficiency (%), *RR* is the respiration rate (mgO_2 g^{-1} h^{-1}) *R* is the energy respired (J h^{-1} g^{-1}). Each standardized physiological rate was converted to energy equivalents (J h^{-1} g^{-1}). The energy of *I. galbana* was assumed to be 17.7 J mg DW^{-1} (see Savina and Pouvreau, 2004 for literature review). Since the fraction of *H. circularisquama* in the total mixture of food was very small, the possible difference in energy value of *H. circulariquama* and *I. galbana* was ignored. The energy conversion factor for oxygen was 13.98 J mg $O_2{}^{-1}$ (Smaal and Widdows, 1994).

2.4. Exposure protocol and measurement of acetylcholinesterase (AChE) activity of clams and mussels

Clams and mussels were separately exposed to *H. circularisquama* at 0, 5, 50, 500 or 10^3 cells ml^{-1} in 6-l tanks, under a 12 h L: 12 h D photocycle, at 20 °C. In previous studies, *H. circularisquama* was shown to induce mortalities over the temperature range of 15–25 °C, with the lowest mortalities occurring at 15C and the highest at 25. Therefore, 20 °C was chosen as an intermediate temperature. In each tank, 20 animals were initially placed, and three clams or three mussels were removed following 3, 6, 24 and 48 h of Cexposure to each cell density of the toxic microalga. The gills were excised and immediately thawed on dry ice in eppendorf microtubes and preserved at − 80 °C until analysis.

The gills were homogenized in 1:1 (w/v) 0.1 M Tris-HCl buffer (pH 7.0) with 0.1% Triton X-100 and centrifuged at 10,000*g* at 4 °C for 20 min (Lionetto et al., 2003). The resulting supernatants were used for the AChE measurements. The activity of AChE was spectrophotometrically determined according to the method of Ellman et al. (1961) adopted to the microplate reader (Bocquené and Galgani, 1998), by measuring the increase in absorbance of the sample at 412 nm in the presence of 1 mM acetylthiocholine as substrate, and 0.1 mM 5,5′-dithiobis-2-dinitrobenzoic acid (DTNB), as follows:

$$AChE = (A \times Vtot \times 10^3)/(1.36 \times 10^4 \times Vsn \times Depth \times Cprot)$$

where, *AChE* is the acetylcholinesterase activity (nmol min^{-1} mg total protein^{-1}); *A* is the change in absorbance per min (nmol min^{-1}); *V*tot is the total volume of the assay (300 μl); 1.36×10^4 is the extinction coefficient for the yellow product (M^{-1} cm^{-1}); *Vsn* is the volume of the sample used (50 μl); *Depth* is

the depth of the microplate (1 cm); and *Cprot* is the concentration of total protein in the sample (mg ml^{-1}).

The enzymatic reaction rate was quantified against a blank without substrate for each activity measurement. In order to subtract the spontaneous hydrolysis of the substrate, a second blank was performed without sample. Each AChE activity measurement was performed in duplicate. The activity of AChE activity was expressed as nmoles of product developed per minute per mg of total proteins (nmol min^{-1} mg tot $protein^{-1}$). Protein concentration was measured using the Bio-Rad (Richmond, CA) protein assay kit1, based on the Bradford dye binding procedure, with bovine serum albumin (BSA) as standard (Bradford, 1976).

2.5. Statistical analysis

Normality and homogeneity of variance were tested a priori. Data expressed in percentage was transformed by the angular transformation (arcsin$\sqrt{}$percentage) prior to analysis to ensure normality. To test the combined effect of the experimental factors on the physiological and energetic rates, the multivariate analysis of variance, MANOVA, was used (Wilks test). Univariate analysis of variance (ANOVA) was used to test the impacts on single physiological and energetic rates, and on the activity of acetylcholinesterase, followed by Fisher's least significant difference test (LSD) or Newman-Keuls Test (N-K). Non-parametric data was analyzed with Kruskal-Wallis ANOVA. A 5% significance level was adopted for all analyses.

3. Results

3.1. Physiological energetics

The physiological and energetic rates of adult clams, *Ruditapes philippinarum*, were sigificantly affected by temperature, exposure to the harmful alga, *Heterocapsa circularisquama*, and their interactions (MANOVA, Wilks test, $P < 0.001$). The CR, RR, IR, AR, A, and SFG were affected by exposure to *H. circularisquama* (Table 1).

The AR was decreased at 15 °C, over the cell density range of $50 - 5 \times 10^2$ cells ml^{-1} by 28–34%, and at 20 °C over the cell density range of $5 - 5 \times 10^2$ cells ml^{-1} by 63–100%. Indeed, although the AE of both control and exposed clams was not affected, clams that were exposed to 2.5×10^2 cells ml^{-1} and 5×10^2 cells ml^{-1} of *H. circularisquama* did not produce any feces and their AE was null. The RR of control clams and those exposed to *H. circularisquama* at 15 °C was not affected by temperature; however, at 20 °C, exposure to 2.5×10^2 cells ml^{-1} and 5×10^2 cells ml^{-1} resulted in a significant decrease of the RR by up to 88% of the control. Temperature affected IR, AR, A, and SFG. Interactive effects of exposure and temperature were significant for AR and A (Table 1). The CR, IR, and AR of control clams increased significantly from 15 °C to 20 °C, whereas temperature did not affect AE of control clams (Fig. 2). Exposure to *H. cricularisquama* at 15 °C induced a 28–32% decrease of the CR for the cell density of $50 - 5 \times 10^2$ cells ml^{-1}, whereas exposure at 20 °C induced a decrease of the CR for the cell densities of $5 - 5 \times 10^2$ cells ml^{-1} by as much 56–62%. Similarly, at 15 °C and 20 °C, IR decreased following exposure to $50 - 5 \times 10^2$ cells ml^{-1} by 28–35%, and to $5 - 5 \times 10^2$ cells ml^{-1} by 57–62%, respectively.

The absorbed energy (A) increased significantly with temperature for control clams, whereas their respired energy (R) and scope for growth (SFG) were not affected by temperature (Fig. 3). Exposure to *H. circularisquama* at 15 °C did not affect R, induced a significant decrease in A at $50 - 5 \times 10^2$ cells ml^{-1}, and a significant decrease of SFG only at 5×10^2 cells ml^{-1}. At 20 °C, R and A decreased significantly following exposure to $5 - 5 \times 10^2$ cells ml^{-1} by 32–88%, and $5 - 5 \times 10^2$ cells ml^{-1} by 67–100%, respectively, resulting in a significant decrease of the SFG at $5 - 5 \times 10^2$ cells ml^{-1} by 34–111% of the control. The A was null following exposures to 2.5×10^2 cells ml^{-1} and 5×10^2 cells ml^{-1} resulting in negative values of SFG.

3.2. Acetylcholinesterase (AChE)

The AChE activity in gills of both clams and mussels was affected by exposure to *H. circularisquama* (Figs. 4 and 5). In gills of clams, the AChE activity decreased following exposure to 5×10^3 cells ml^{-1} by as much as 19–95% within 3 h, 93–97% within 6 h, and 40–98% within 24 h. Following 48 h of exposure, the AChE was decreased by 22–94% for exposure to 50×10^3 cells ml^{-1}. In mussels, 3 h and 6 h of exposure induced a decrease in the AChE at 50×10^3 cells ml^{-1} by 22–18% and at 10^3 cells ml^{-1} by 35%, respectively. After 24 h and 48 h, however, the decrease was significant under all cell densities of *H. circularisquama*, and the AChE activity was reduced by 19–47% and 19–41%, respectively.

4. Discussion

4.1. Physiological energetics

In the present study, control short-neck clams, *Ruditapes philippinarum*, compensated for the temperature-induced increase in energy expenditure (R) related to increased respiration rate (RR) with increased food particle capture and ingestion, i.e. increased clearance rate (CR) and ingestion rate (IR), and maintenance of an optimal absorption efficiency (AE) to conserve a relatively constant scope for growth (SFG) over the temperature range of 15–20 °C. The responses of control clams to the increase in temperature, when other parameters including food quantity and quality are maintained constant, are in line with numerous previous studies. The effects of temperature on the clearance rates and oxygen consumption rates of coastal bivalves have been well-documented and discussed in a host of previous papers. Two types of responses to temperature have been described for variously acclimated bivalves: a gradual increase in both the clearance rate (Walne, 1972; Kusuki, 1978; Hutchinson and Hawkins, 1992; Bougrier et al., 1997) and the respiration rate (Widdows, 1973; Newell et al., 1977; Kim, 1980) with increasing temperature; or an increase of the clearance rate and respiration rate up to an optimum temperature beyond which they start to decrease (Newell et al., 1977; Winter, 1978; Widdows, 1978; Buxton et al., 1981; Goulletquer et al., 1989 for clearance rate; and Newell et al., 1977; Buxton et al., 1981; Laing et al., 1987; Albentosa et al., 1994; Sicard et al., 1999; Savina and Pouvreau, 2004; Zhuang, 2005 for respiration rate). The absorption efficiency in bivalve molluscs is believed to be relatively independent of temperature (Laing et al., 1987; Wilbur and Hilbish, 1989; Albentosa et al., 1994) and above all dependent on the quality and quantity of ingested food (Winter, 1978). The effects of temperature on the SFG differ considerably between species and between populations of the same species. Whereas the SFG is held relatively constant over a wide range of low temperature, it often decreases drastically for high temperature generally exceeding 20 °C (Bayne and Newell, 1983). In the present study, all measured physiological and energetic rates of control clams were in accordance with the reported results for the same species, over similar temperature range (e.g. Bodoy et al., 1986; Goulletquer et al., 1989; Defossez and Daguzan, 1995; Nakamura, 2001). The SFG values at both 15 and 20 °C were superior to 15 J g^{-1} h^{-1}; a value which corresponds to energetic balance of a bivalve with low stress (i.e. the stress includes both natural stress, such as lack of feeding, and anthropogenic stress such as pollutants) (Widdows et al., 1995b).

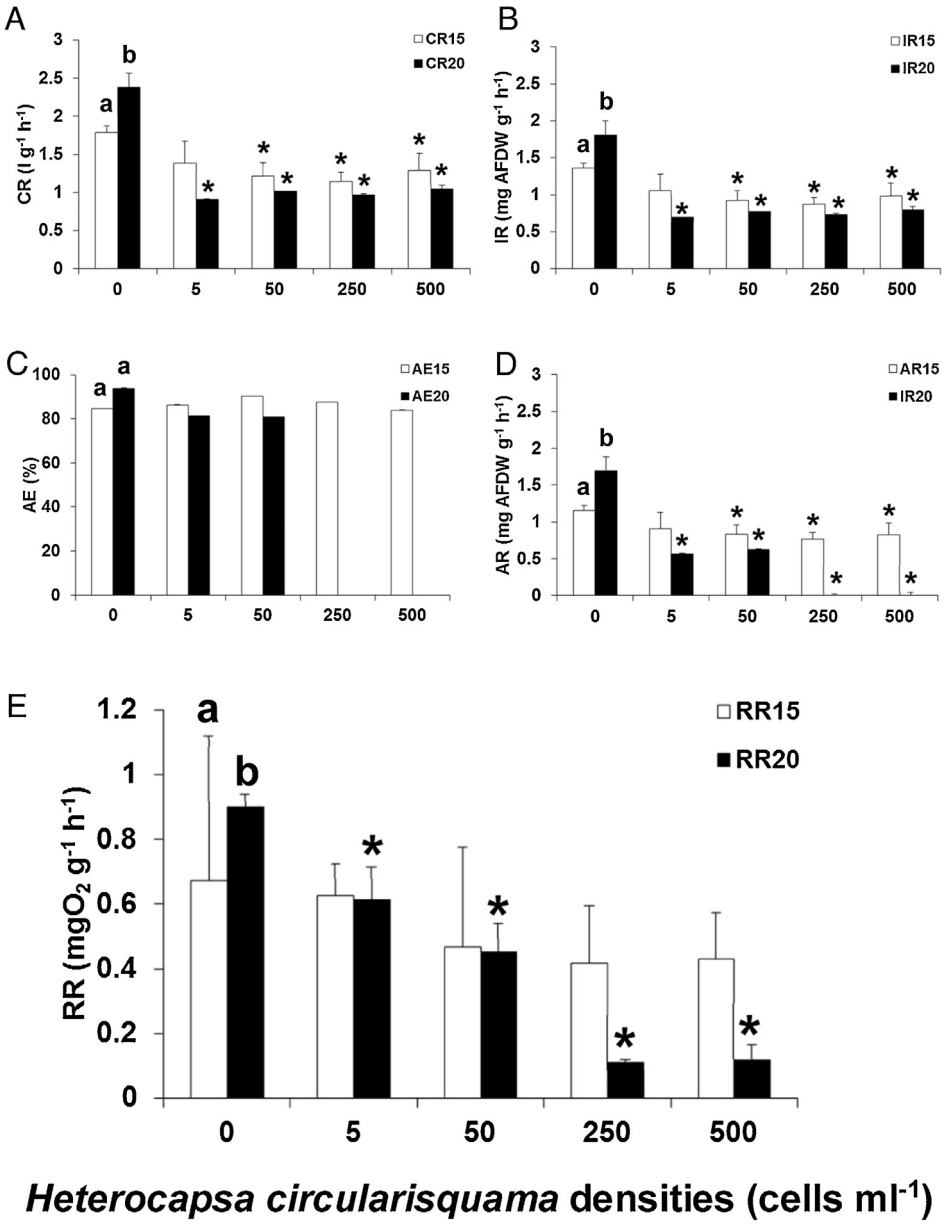

Fig. 2. Physiological responses of clams, *Ruditapes philippinarum*, exposed to different cell densities of *Heterocapsa circularisquama*, at two temperatures (15 or 20 °C). Physiological rates were standardized for an animal of 1 g dry meat weight. (A) Clearance rate, (B) Ingestion rate, (C) Absorption efficiency, (D) Absorption rate, (E) Respiration rate. Vertical bars represent the standard error of the mean (mean ± SE). Different letters indicate significant difference among controls ($P < 0.05$). (*) Indicates significant difference from the respective control ($P < 0.05$).

Exposure to the toxic dinoflagellate *Heterocapsa circularisquama* affected the SFG of clams with three distinct levels of severity: (1) At low temperature (15 °C), the absorption efficiency (AE), respiration rate (RR), and energy expended (R) were not affected; however, exposure to the density range of 50–5 × 10^2 cells m^{-1} induced a decrease in the clearance rate, ingestion rate (IR), and energy absorbed (A) resulting in a decreased SFG at 5 × 10^2 cells m^{-1}; (2) At higher temperature (20 °C), exposure to the density range of 5–50 cells m^{-1} did not affect AE; however, it induced a further decrease in the CR, IR, A and SFG in spite of a decrease in RR and thus R; (3) At 20 °C, exposure to cell densities ≥2.5 × 10^2 cells m^{-1} induced further decrease in CR and IR coupled with a null AE resulting in a null AR, and thereafter a null A and a negative SFG, in spite of a further decrease in RR and R. These results clearly show that the harmful alga *H. circularisquama* affects the energy budget of adult short-neck clams either via an induction of regulations of physiological functions resulting in decreased energy acquisition, and/or following toxin(s)-induced impairments and/or inhibition of food ingestion and absorption, thereafter energy intake and SFG.

It has been demonstrated that the absorption rate, AR, is the most influential physiological component of the energy balance in bivalves (Albentosa et al., 1994; Beiras et al., 1994), which is in turn related to both the ingestion rate and the absorption efficiency of food (Toro et al., 2003). Nonetheless, the process of feeding and absorption of food, that would dictate the efficiency with which an individual suspension-feeding bivalve maximizes the acquisition of energy, involves sequential and interdependent steps of processing of particulate matter by all of the feeding organs, both the pallial and the digestive organs. Several intrinsic and extrinsic factors could influence the feeding process, including the physical and chemical quality of the food particles (reviewed in Ward and Shumway, 2004). The reduction of the CR and the resultant decrease in the

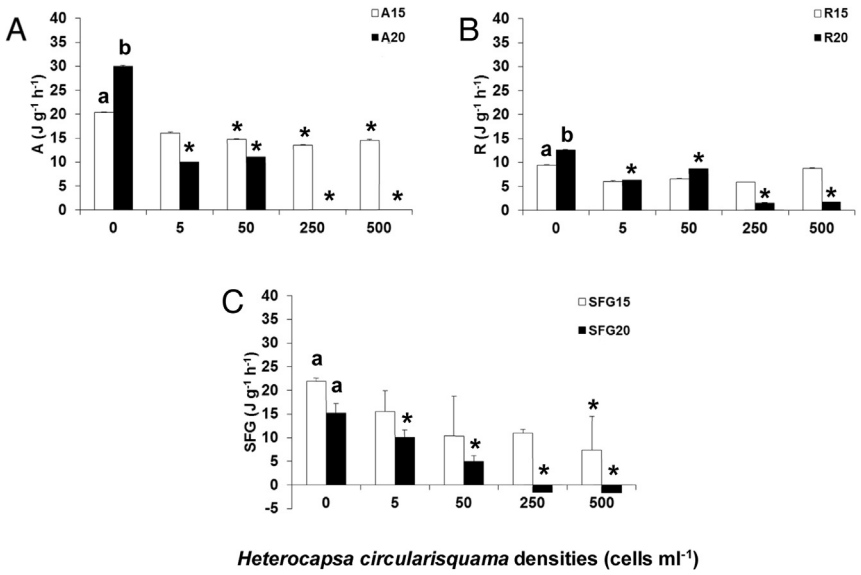

Fig. 3. Bionergetics of clams, *Ruditapes philippinarum*, exposed to different cell densities of *Heterocapsa circularisquama*, at two temperatures (15 or 20 °C). (A) Absorbed energy, (B) Respired energy, (C) Scope for growth. Vertical bars represent the standard error of the mean (mean ± SE). Different letters indicate significant difference among controls ($P < 0.05$). (*) Indicates significant difference from the respective control ($P < 0.05$).

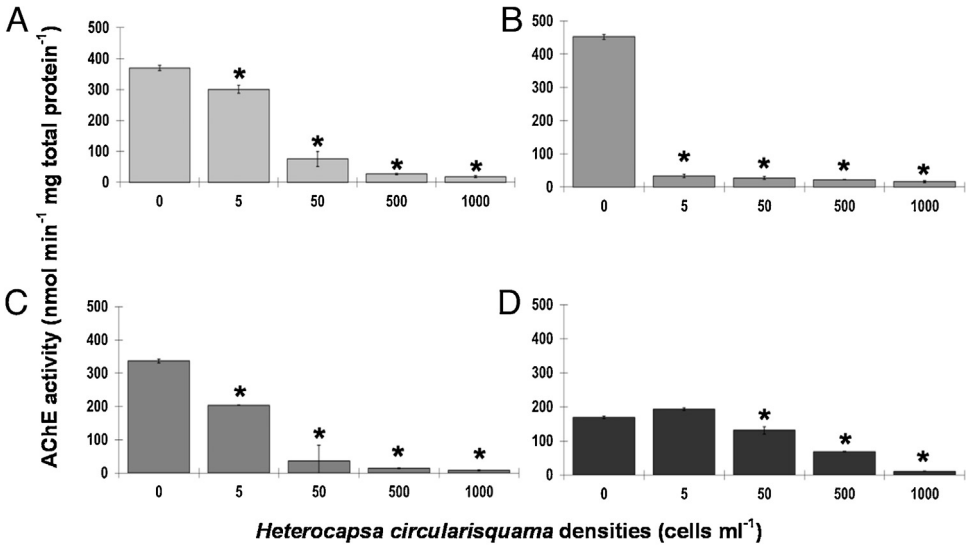

Fig. 4. Acetylcholinesterase (*AChE*) activity in gills of adult mussels, *Ruditapes philippinarum*, following exposure to *Heterocaspa circularisquama* at 20 °C. (A) 3 h post exposure, (B) 6 h post exposure, (C) 12 h post exposure, (D) 48 h post exposure. Vertical bars represent the standard deviation of the mean (mean ± SD). (*) Indicates significant effect (ANOVA, Newman-Keuls, $P < 0.05$).

ingestion rate without changes to the AE in the presence of *H. circularisquama*, as summarized above in (1) and (2), could be explained by regulations in the process of particle uptake by the gills of clams, and the labial palps, to enhance the quality of food material ingested, opting for a preingestive selection against the toxic alga or avoidance response through a closure of the shell valve and/or the production of pseudofeces. Depressed or inhibited feeding, as a result of avoidance response, in various bivalve species have been reported for exposure to some toxic dinoflagellates (Shumway and Cucci, 1987; Lassus and Bethrome, 1988; Wikfors and Smolowitz, 1993; Lassus et al., 1996). In the earliest studies, the CR of blue mussel, *Mytilus galloprovincialis*, and Pacific oyster, *Crassostrea gigas*, were reported to decrease during exposure to *H. circularisquama* at 50 cells ml^{-1} (Matsuyama et al., 1997b; Matsuyama,1999). The results of the present study are also corroborated by results of a previous study in our laboratory, in which both the CR and RR of adult *R. philippinarum* were decreased following 2 h of exposure to 50 cells ml^{-1} of the same strain of *H. circularisquama* at 20 °C (Basti et al., 2011b). Refusal behavior previously reported for *R. philippinarum* in the presence of *H. circularisquama* may have resulted in the lower CR. The decreased CR would have accounted for lower IR and the reduction in SFG. Nonetheless, the quantitative analyses of the valve movement behavior of adult short-neck clams showed that *H. circularisquama* had no effect on the duration the shell valves of clams were actively open, although this does not exactly reflect the activity of the gills and other feeding organs (Basti et al., 2009). Exposure to *H. circularisquama* induces hypersecretion of mucus and increased production of pseudofeces in short-neck clams (Basti and Segawa, 2010); however, in the present experiments, clams did not produce any pseudofeces or mucus. On the other hand, it has been reported that cell contact is the mechanism through which unstable toxin(s) located on the cell surface of *H. circular-*

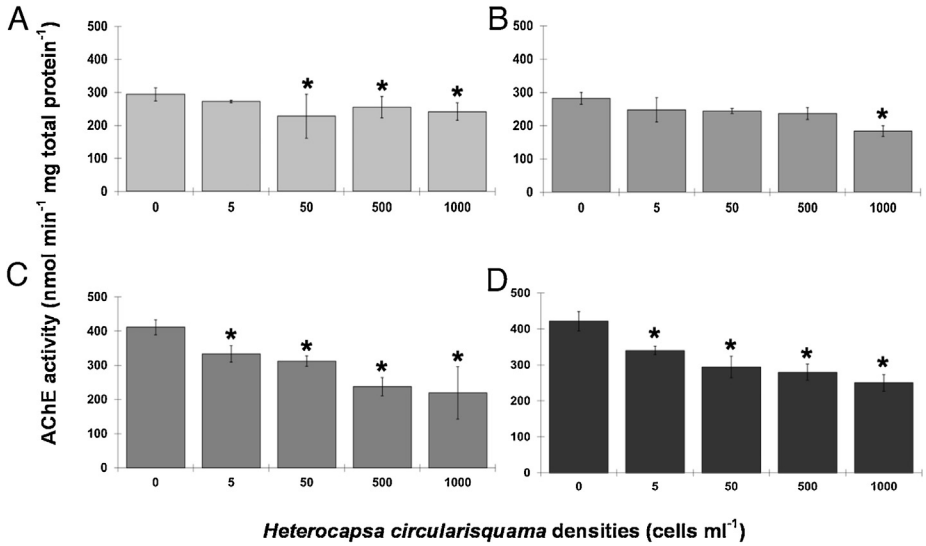

Fig. 5. Acetylcholinesterase (*AChE*) activity in gills of adult mussels, *Mytilus galloprovincialis*, following exposure to *Heterocaspa circularisquama* at 20 °C. (A) 3 h post exposure, (B) 6 h post exposure, (C) 12 h post exposure, (D) 48 h post exposure. Vertical bars represent the standard deviation of the mean (mean ± SD). (*) Indicates significant effect (ANOVA, Newman-Keuls, $P < 0.05$).

isquama are liberated and initiate hemolysis and/or cytotoxicity in phytoplankton and several life stages of bivalve molluscs (Uchida et al., 1995; Matsuyama et al., 1997b; Yamasaki et al., 2011; Basti et al., 2011a, 2013). Extensive cytotoxicity and pathological alterations were observed in both *R. philippinarum* (Basti et al., 2011b) and *M. galloprovincialis* (Basti et al., 2015) exposed to *H. circularisquama*. A 3 h of exposure to the same strain of *H. circularisquama* under similar laboratory conditions induced no pathological alterations in adult *M. galloprovincialis* at 15 °C, whereas, at 20 °C, a 3 h of exposure to densities of 5–10^3 cells ml^{-1} induced a significant increase in both the prevalence and the intensity of pathologies in six organs of mussels, including the gills, labial palps, mantle, hepatopancreas, stomach, and intestines (Basti et al., 2015). In the case of *R. philippinarum*, the 3 h of exposure to 10^3 cells ml^{-1} induced a slight increase in the prevalence and intensity of pathology in the gills represented by hemocytic infiltration of the connective and epithelial tissues, but mainly by ciliary matting (Basti et al., 2011b). Particle encounter, retention, and sorting by the gills and labial palps ensure the selection of food material for ingestion, transported in mucus strings or mucus-water slurries (Ward and MacDonald, 1996; Ward et al., 1993). The process of food uptake by the gills relies mainly, but not only, on the activity of the lateral cilia, and the laterofrontal cilia and their cirri (Riisgård and Larsen, 2000; Riisgård et al., 1996; Ward et al., 2000), and cytotoxicity of *H. circularisquama* should have affected the cilia and cirri of the gills, reducing the CR, the IR, and thereafter A and SFG.

The effects of exposure to toxic dinoflagellates on the RR of bivalves have been less studied and variable responses have been reported including increased and decreased RR (Shumway et al., 1985; Mardsen and Shumway, 1992; Marsden and Shumway, 1993). In a previous study, exposure of short-neck clams to *H. circularisquama* induced a clear decrease in the RR that the authors attributed to the pathological alterations induced by the harmful alga in the gills, including the matting of cilia (Basti et al., 2011a,b). In addition, the reduction in RR could have been related to a reduction in energy consumption co-occurring with the decrease in ingested energy to favor an energy balance reflecting situations of nutritive stress or starvation (Albentosa et al., 2007, 2012); i.e. the stress induced by exposure to *H. circularisquama* resulted in a reduction of the energy expenditure to compensate for the reduction in energy acquisition.

The AE was inhibited in clams exposed to *H. circularisquama* at cell densities $\geq 2.5 \times 10^2$ cells m^{-1} and at 20 °C. This complete cessation of food absorption could have been related to cytotoxicity in the digestive organs of clams. Generally, AE depends on the gut content, the gut passage time and the enzyme activity in the digestive system, all of which are conditioned by the ingested food and the speed at which the food passes through the gut (Iglesias et al., 1992). The inhibition of AE at the highest cell densities of *H. circularisquama* may have been associated with the lower CR and IR, reducing the quantity of food ingested and possibly delaying the gut passage time and thus the production of enzymes in the digestive organs. Enzymatic digestion leading to the absorption of energy from the ingested food, and thus determining the SFG, is produced in two stages in bivalve molluscs: an extra-cellular digestion in the stomach mediated by the crystalline style, and an intra-cellular digestion in the digestive gland (Morton, 1983). The efficiency of absorption of food was found to be regulated mainly by the enzymatic activity of the digestive gland (Albentosa and Moyano, 2009; Albentosa et al., 2012), and cytotoxic effects in both the digestive gland and the stomach were previously reported for *M. galloprovincialis* exposed to *H. circularisquama* over the same cell density range and at the same experimental temperature (Basti et al., 2015).

So far, a very few studies have examined the impacts of HAB exposure on the energetic balance of bivalve molluscs. Reduced SFG was demonstrated for some bivalve mollucs following exposure to species of *Alexandrium* producers of paralytic shellfish toxins. Clams, *R. philippinarum*, and mussels, *Perna viridis*, exposed to the toxic *A. tamarense*, exhibited reduced SFG caused by decreased CR and AE (Li et al., 2002). The SFG of Chilean mussels, *M. chilensis*, was also decreased following a few days of exposure to a toxic strain of *A. catenella* due to decreased CR, IR, and AE (Navarro and Contreras, 2010), albeit a recovery after three days. The reduction in the SFG of clams, in the present study, was likely caused by a reduction in the capacity of the gills to clear food particles, reducing the organic food ingested and thereafter energy acquisition. At high temperature and cell densities of *H. circularisquama*, the SFG became negative reflecting energy expenditure greater than the energy obtained from food due to both a reduction in the energy ingested and either a delayed absorption of energy and/or an inhibition of energy absorption related to damage in the digestive tracts. In this case, the animals have to rely on catabolism to survive and

their somatic and gonadal growth would be greatly reduced, if they fail to recover from short-term exposure to *H. circularisquama*, or if blooms of *H. circularisquama* become persistent.

4.2. Acetylcholinesterase (AChE) activity

Significant decrease in AChE activity in gills of *R. philippinarum* and *M. galloprovincialis* is reported for the first time in response to exposure to *H. circularisquama*. The activity of AChE continued to decrease in gills of clams and mussels 48 h following the initial exposure. Numerous studies demonstrated the effectiveness of AChE as a biomarker of exposure to neurotoxic compounds in aquatic organisms (Cajaraville et al., 2000). The enzyme is present in most animals and is responsible for the rapid hydrolytic degradation of the neurotransmitter acetylcholine (ACh). When an effective compound inactivates AChE, the enzyme is no longer able to hydrolyze ACh, and the concentration of ACh in synaptic clefts and neuroeffector junctions remains high. Continuous stimulation of the muscle or nerve fibers then occurs, resulting in tetanus and eventually paralysis and death. In vertebrates AChE is abundant in nervous tissues, brain, red blood cells and muscles, while in bivalves it is usually best measured in the adductor muscle and gills (e.g. Bocquené and Galgani, 1998).

Bivalve gills insure both feeding and respiration functions and are responsible for most of the water flow through the mantle cavity (Morton, 1983; Jones and Richards, 1993). The rate of water flow can be altered through changes in the beating of the lateral cilia and through muscular changes in the dimensions of the gills, which are both under the control of the nervous system (Gainey et al., 2003; Gainey and Greenberg, 2005; Ward and Shumway, 2004). Extensive neural networks serve the plicated gills of eulamellibranch bivalves like *R. philippinarum,* and the more structurally simple gills of filibranch bivalves like *M. galloprovincialis*. Neural elements occur within the filaments of both filibranch and eulamellibranch gills, and in the latter type they are also associated with gill septa, blood vessels, and interlamellar muscles (e.g. Setna, 1930; Aiello, 1990; Atkins, 1937). The ciliated cells of the gill filaments are innervated by catecholaminergic and serotonergic neurons. The neural regulation of the metachronal beating of the ciliary structures of bivalve gills is not yet fully explained, in spite of the extensive literature, mainly on *M. edulis* (Frank et al., 2015). The direct nervous control of gill lateral cilia, has been shown in several bivalve species, with serotonergic systems acting as cilio-excitatory (e.g. Aiello, 1970, 1990; Jørgensen, 1974, 1976; Catapane, 1983) in most studied bivalves, and dopaminergic systems acting as cilio-inhibitory in some but not all studied bivalves (Catapane, 1983; Paparo, 1985a, 1985b; Gainey and Shumway, 1991; Carroll and Catapane, 2007). Modulation of the activity of the ctenidial lateral cilia in adult *M. edulis* by ACh was shown (Jones and Richards, 1993), with excitatory effects at low concentrations and inhibitory effects at high concentrations (Aiello and Paparo, 1974). It was postulated that ACh affects the activity of lateral cilia via the release of endogenous mediators and via direct action on other elements of the nervous system (Stefano and Aiello, 1975). In hard clam *M. mercenaria*, isolated demibranch were found to contract in response to serotonine (5HT), dopamine (DA), but also ACh (Gainey et al., 2003). The authors proposed that 5HT is the excitatory transmitter of gill muscles, and that DA and ACh exert their excitatory effect by stimulating 5HT motor nerves. They also noted that ACh may be an inhibitory transmitter of the gill muscles. Whether the inhibition of AChE and the resulting increase in ACh affected the activity of the lateral cilia and/or the muscles of the gills, the decreased food uptake and respiration observed in *R. philippinarum* following short-term exposure, preceding the potential cytotoxicity in gills, could have also been associated with alterations in the neural activity of the gills.

AChE inhibition is commonly used as a biomarker of pollution by pesticides, since these often contain neurotoxic organophosphates and carbamates as effective compounds (Day and Scott, 1990; Bocquené and Galgani, 1998; Kirby et al., 2000). Inhibitory effects in organisms collected from areas with no pesticide contamination, however, have been recorded, leading to suspect that AChE may have a wider spectrum of responsive potential than previously thought (Payne et al., 1996; Guilhermino et al., 1998; Forget et al., 1999). Non-enzymatic functions have recently been demonstrated for AChE, and several studies have shown non-cholinergic functions of AChE involved in proliferation, differentiation and responses to various stresses (Grisaru et al., 1999, 2006; reviewed in Lionetto et al., 2011). In addition, significant correlation between AChE inhibition and alterations in the antioxidant enzyme activity, following exposure to neurotoxic pesticides, was found for several aquatic species, including mussels (Lionetto et al., 2003) and fish (Lionetto et al., 2003; Kavitha and Rao, 2008). In more recent studies, laboratory exposure to toxic species of HAB has been found to alter the antioxidative responses and lipid peroxidation of bivalves (Estrada et al., 2007; Amado and Monserrat, 2010; Fabioux et al., 2015). Further studies on the effects of exposure to *H. circularisquama* on the antioxidative response and lipid peroxidation of bivalve molluscs coupled with AChE activity and the activity of other dopaminergic and cholinergic neurotransmitters would help understand whether *H. circularisquama* toxins directly affect the gill cilia or the gill muscles or both, and/or whether this effect is also coupled with other responses including the antioxidative enzymes.

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