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The toxic dinoflagellate *Alexandrium minutum* disrupts daily rhythmic activities at gene transcription, physiological and behavioral levels in the oyster *Crassostrea gigas*

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ABSTRACT

The objective of the present work was to study the effect of the harmful alga *Alexandrium minutum* on the daily rhythm of the oyster *Crassostrea gigas*. Many metabolic and physiological functions are rhythmic in living animals. Their cycles are modeled in accordance with environmental cycles such as the day/night cycle, which are fundamental to increase the fitness of an organism in its environment. A disruption of rhythmic activities is known to possibly impact the health of an animal. This study focused in *C. gigas*, on a gene known to be involved in circadian rhythmicity, cryptochrome gene (*CgCry*), on putative clock-controlled genes involved in metabolic and physiological functions, on the length cycle of the style, a structure involved in digestion, and on the rhythmicity of valve activity involved in behavior. The results indicate that daily activity is synchronized at the gene level by light:dark cycles in *C. gigas*. A daily rhythm of valve activity and a difference in crystalline style length between scotophase and photophase were also demonstrated. Additionally, *A. minutum* exposure was shown to alter cyclic activities: in exposed oysters, gene transcription remained at a constant low level throughout a daily cycle, valve opening duration remained maximal and crystalline style length variation disappeared. The results show that a realistic bloom of *A. minutum* clearly can disrupt numerous and diverse molecular, physiological and behavioral functions *via* a loss of rhythmicity.

1. Introduction

In oceans, planktonic microalgae primarily occupy coastal and lagoon areas, where they can proliferate and reach up to millions of cells per liter. Although proliferation of most species is beneficial, as they serve as food in the first link of the trophic chain, some species produce toxins and are therefore harmful for shellfish, fish and humans (Hallegraeff et al., 2003; ICES, 2007). Proliferations of such algae are called harmful algal blooms (HABs). They have major impacts on marine ecosystems, tourism and shellfish harvesting. Among them, the dinoflagellate species *Alexandrium minutum* is particularly known for producing Paralytic Shellfish Toxins (PSTs) and it has been widely observed, including in Europe, the west coast of the USA, Asia, Australia and New Zealand (Anderson et al., 2012; Bricelj and Shumway, 1998; Hallegraeff et al., 2003).

The oyster *Crassostrea gigas* is a bivalve of high commercial interest worldwide. As a filter-feeder, it is highly susceptible to HAB exposure, with potentially grave public health consequences. It is therefore important to better understand and describe determinants of PST accumulation in oysters. Many recent studies have investigated cellular and physiological effects of PSTs in bivalves. Briefly, bivalves such as oysters or mussels exposed to *Alexandrium* species show inflammatory responses and modification of lipid content in the digestive gland, increased mucus production in gills, impairment of the cellular immune response, myopathies, paralysis in the adductor muscle and decreased spermatozoa motility (Galimany et al., 2008; Hégaret et al., 2007; Haberkorn et al., 2010a,b; Mello et al., 2013). Exposure of the Pacific oyster *C. gigas* to *Alexandrium* species inhibited feeding activity, filtration and biodeposition rates (Lassus et al., 1999; Navarro and Contreras, 2010; Wildish et al., 1998). Interestingly, PST accumulation was reduced during a simultaneous exposure to both *A. minutum* and metals (Cd and Cu) (Haberkorn et al., 2014). Triploid oysters accumulate more PSTs during the pre-spawning period than do diploids (Haberkorn et al., 2010b; Guéguen et al., 2012). Finally, *C. gigas* valve

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behavior is deeply modified by *A. minutum* exposure (Tran et al., 2010; Haberkorn et al., 2011). Despite this extensive literature on *A. minutum* effects on *C. gigas*, it remains important to investigate the impact of HABs in terms of potential disruption of biological rhythms which are a fundamental property of life, play a key role in animal fitness and are governed by cycles of environmental factors. Tetrodotoxin (TTX) which has a mechanism of action very close to that of STX, has been shown to repress clock gene expression (in this article the term gene expression is used to indicate gene transcription, although it is acknowledged that gene expression can in addition be regulated, *e.g.* by translation and mRNA and protein stabilities) in *Drosophila melanogaster* (Van Den Pol and Obrietan, 2002). It can be thus hypothesized that STX could directly influence the clock molecular mechanism and consequently have an effect on rhythmic physiological and metabolic activities. Most metabolic, physiological and behavioral activities are rhythmic and controlled in such a way as to produce optimum fitness of an organism in its environment (Yerushalmi and Green, 2009). The organization of biological activities into different cycles (*e.g.* circadian, circannual . . .) is well known in all studied phyla (Bell-Pedersen et al., 2005). Rhythms have a double origin: an endogenous component, which consists of clock genes that generate the rhythmicity (De Haro and Panda, 2006), and environmental factors, called "zeitgebers" ("time giver" in German), which synchronize the rhythmicity, such as light for the circadian rhythm (Aschoff, 1981). The output of the rhythm generated by clock genes is likely through clock-controlled genes (*ccgs*), which are responsive to the cyclic activities of metabolic and physiological functions. Thus, molecular or environmental disruption of biological rhythms can result in decreased fitness of the organism in its environment (Emerson et al., 2008). Some of previously reported biological responses upon *A. minutum* exposure may indeed result from disruption of biological rhythms.

Over the last years, the chronobiology of *C. gigas* valve behavior has been studied. It was shown *in situ* that submerged *C. gigas* express a strong tidal rhythm, whose intensity is modeled by synodic and anomalistic lunar cycles. In parallel, there is a daily *in situ* rhythm, which is nocturnal in autumn and winter and becomes diurnal in spring and summer (Tran et al., 2011). In the laboratory, it was shown that this daily rhythm was driven by a circadian clock, whereas the apparent tidal rhythm was not supported by a tidal clock, though the mechanisms involved remain to be discovered (Mat et al., 2012, 2013).

The objective of the present study was two-fold. First to show if, at the gene level, the *C. gigas* daily rhythm is synchronized by light:dark cycles. If yes, the second aim was to determine whether *A. minutum* impacted *C. gigas* cyclic activity at gene transcription, physiological and behavioral levels. The hypothesis is that STX could directly repress clock genes and consequently impact rhythmic activities.

We focused on expression of one gene known to be involved in circadian rhythmicity, cryptochrome (*CgCry*) (Cashmore, 2003), and on others involved in metabolic and physiological functions which might be clock-controlled. On the physiological and behavioral levels, the focus was on the length of digestive crystalline style and valve movement activity.

2. Materials and methods

2.1. General conditions and experimental setup

The experiment was performed on 112 diploid Pacific oysters *C. gigas* (1.5 years old, 73 ± 1 mm shell length; mean ± SE) from the Bay of Arcachon, France. Analyses were conducted at the Arcachon Marine Station in December 2010, outside of the period of oyster gametogenesis. Oysters were acclimated for 10 days prior to treatments; they were isolated from external vibrations using an anti-vibrating bench and the experiment was conducted in an isolated blind room to minimize any external influences on animal behavior. Oysters were placed in four tanks (55 × 35 × 15 cm) containing 15 L of seawater. The tanks were fed with running seawater at 100 ml min^{-1}. Upstream, two tanks in series (45 and 0.2 m^3), with different retention times, were used to homogenize seawater pumped from the bay of Arcachon. Each tank was continuously supplied with seawater of constant composition, measuring: T °C = 17.0 ± 0.5 °C; [Chla] = 0.10 ± 0.07 μg l^{-1}; pH = 7.90 ± 0.05, salinity = 31.0 ± 0.2 ‰ (mean ± SE). Twenty eight oysters were placed in each of the 4 experimental tanks, including 8 oysters equipped with valvometric electrodes per tank. The pH value and salinity were measured daily with a R301 pH meter (Consort, Belgium) and a Cond 330 I conductivity probe, respectively (WTW, Germany). Central air-lifts were used to homogenize microalgae and seawater in the tanks and an alternation of light and dark periods of 12 h each was imposed. Irradiance (Photosynthetically Active Radiation, PAR), measured with the PAR (Biospherical Instruments Inc., San Diego, CA, USA), was 20 μE m^{-2} s^{-1} at the air-water interface during photophase (neon light MASTER TL-D Xtra 36 W/865 1SL, Philips) and 1 μE m^{-2} s^{-1} during scotophase. Finally, during the acclimation and experimental periods, no oyster mortality was observed.

2.2. Experimental protocol

After the 10-day acclimation, oysters (n = 28 per tank) in the 4 tanks were supplied with the non-harmful algae *Heterocapsa triquetra* for 7 days at 100 ml min^{-1} at a density of 1500 cells ml^{-1}. Cell concentration was measured by a Beckman Coulter Z2 (Beckman Coulter Inc., USA). Then, during the final 24 h cycle, 2 tanks remained exposed to *H. triquetra* in the same conditions, whereas the 2 other tanks were exposed to the harmful algae *A. minutum* supplied at the same rate and density as *H. triquetra*. This algal density and exposure time was representative of an *A. minutum* bloom event (Bricelj and Shumway, 1998). During this last day, 7 sampling times (12 h, 16 h, 20 h, 0 h, 4 h, 8 h, 12 h; local time, UTC + 1) were defined. At each time, 4 oysters per tank were sampled to measure Paralytic Shellfish Toxins (PSTs) accumulated in their digestive gland (primary target organ of trophic contamination and PST accumulation). The digestive gland (50–200 mg) was weighed, placed in 1 ml HCL 0.1 mol l^{-1} and stored at −80 °C for quantification of PSTs. Gills (approx. 50 mg) were sampled, kept in RNA later (Qiagen) and placed at −80 °C for transcriptional analysis. Finally, the length and the weight of the digestive crystalline style were measured for each oyster sampled.

2.3. Microalgal culture

The PST-producing dinoflagellate *A. minutum* (Halim, strain AM89BM) and the dinoflagellate *H. triquetra* (strain HT99PZ – Ehrenberg, 1840) were grown in 10-l and 80-l phytoreactors using autoclaved seawater filtered to 1 μm and supplemented with *f*/2 medium (Guillard, 1975). Cultures were maintained at 17 ± 1 °C and 100 μmol photon m^{-2} s^{-1}, with a dark:light cycle of 10:14 h. In the exponential growth phase, *A. minutum* produced 1.3 ± 0.1 pg eq. STX per cell, measured by the method of Oshima (1995). The non-toxic dinoflagellate *H. triquetra* was chosen as a control because of its similarity to *A. minutum* in terms of size and shape: *H. triquetra* cell size (19–28 μm) is similar to *A. minutum* cell size (23–29 μm).

2.4. Paralytic Shellfish Toxins (PSTs)

PSTs (saxitoxin and its derivatives) were measured in the digestive gland using a direct competitive ELISA assay (Abraxis,

Table 1
Nucleotide sequences of specific primer pairs used in the present study.

Gene	Function of the encoded protein	Accession number	Sequence 5′-3′
28S	28S RNA making part of the large ribosomal subunit	Z29546	AAACACGGACCAAGGAGTCT[a] AGGCTGCCTTCACTTTCATT[b]
Cat	Catalase, involved in the neutralization of the oxidative stress	EF687775	AACTACTTCGCTGAGGTG[a] GGTCTTGGCTTTGTATGG[b]
Cox1	Cytocrome *c* oxidase subunit 1, involved in the mitochondrial respiration	NC_001276	TTACGCTTCACGACACT[a] AACTACTCGACGTGGT[b]
Cgcry	Cryptochrome, involved in the circadian rhythm	GQ415324	ATCTACGCTTTGGCTG[a] CCTCGTATCTGAGCTGC[b]
Gpx	Glutathione peroxidase, involved in the neutralization of the oxidative stress	EF692639	GACCGTGGAACCAATGGACATC[a] GTTGGATTCGGACACAGATAGGG[b]
Ilk	Interleukin 17, involved in the immune response	EF190193	AGCATCAAAGCCATCAC[a] ACTCTCACTGGCCTGTA[b]
Mxr	Multixenobiotic resistance protein, involved in the detoxification process	EU073425	CACGGCAGTCATGTTC[a] TCCTCGGAGTAAGGGT[b]
Mt2	Metallothionein, involved in zinc and copper homeostasis and in the general stress response	AJ297818	TCCGGATGTGGCTGCAAAGTCAAG[a] GGTCCTTTGTTACACGCACTCATTT[b]

[a] Forward primer.
[b] Reverse primer.

Novakits, France) following manufacturer's instructions. Samples were crushed on ice and then centrifuged at 4 °C and 3500 × *g* for 10 min. Samples were then diluted from 1/200 to 1/1000 depending on the sample. Standard solutions and samples were added in duplicate (50 μl) into wells of an ELISA microtiter plate coated with a secondary sheep anti-rabbit antibody. Then, 50 μl of the saxitoxin-horseradish peroxidase (saxitoxin-HRP) conjugate solution and of the primary antibody solution (rabbit anti-saxitoxin) were added successively. Plates were incubated for 30 min at room temperature and washed four times using 300 μl of washing solution. Then 100 μl of substrate solution containing tetramethylbenzidine was added to each well and plates were incubated for 30 min at room temperature. Finally, a stop solution (100 μl) containing sulfuric acid was added to the wells and absorbance was read at 450 nm. The intensity of the blue color is inversely proportional to the concentration of the PSTs present in the sample.

2.5. Total RNA extraction and reverse transcription of RNAs

Total RNA was extracted from approximately 50 mg of gill tissue using the "Absolutely RNA extraction" kit (Stratagen, Agilent) according to manufacturer's instructions. First-strand cDNA was then synthesized from RNA using the "AffinityScript Multiple Temperature cDNA Synthesis" kit (Stratagen, Agilent) according to manufacturer's instructions. Furthermore, a step of phenol:chloroform:isoamylic alcohol (25:24:1) extraction was added to eliminate the excess of lipids and proteins before loading the homogenate on the RNA-binding column. The cDNA was then stored at −80 °C until the real-time PCR reaction.

2.6. Real-time quantitative PCR

Real time PCR reactions were performed using the Stratagen apparatus (model Mx3000) and cDNA amplification was monitored using the DNA intercalating dye SYBR-Green I (Stratagen, Agilent). Each 20 μl reaction contained 2 μl of cDNA, 2 μl of the gene-specific primer pair (final concentration 3 μM for each) and 16 μl of PCR mix. The expression of the following genes was studied in oyster gills: genes encoding ribosomal unit 28S (*28S*), catalase (*cat*), cytochrome oxidase 1(*cox1*), cryptochrome (*Cgcry*), glutathione peroxidase (*gpx*), interleukin (*ilk*), multidrug resistance protein (*mdr*), metallothionein type 2 (*mt2*) and superoxide dismutase (*sod*). Primer pairs used to determine gene expression are listed in Table 1. The PCR mix consisted of 11.8 μl of PCR grade water, 3.2 μl of 25 mM $MgCl_2$ and 1 μl of Roche solution including the polymerase, dNTPs and the SYBR-Green I fluorescent dye. The thermal program consisted of one warming step of 10 min at 95 °C followed by 50 amplification cycles at 95 °C for 5 s, 60 °C for 5 s and 72 °C for 20 s. Reaction specificity was controlled using dissociation curves, which were obtained by following the SYBR-Green I fluorescence level during a progressive heating of the PCR products from 60 to 95 °C. Relative quantification of each gene expression level was normalized to the *28S* ribosomal RNA gene expression and determined by calculating $2^{\Delta Ct}$ where $\Delta Ct = Ct(28S) - Ct(gene)$ where Ct is the cycle threshold from which the amplification enters in the exponential phase. The *28S* gene was used as the reference gene. The stability of the Ct of the reference gene for control and exposed oysters was also verified ($p = 0.129$).

2.7. C. gigas valve activity measurement

Valve activity of *C. gigas* was studied using HFNI (High Frequency – Non Invasive) valvometers. Lightweight electromagnets (0.1 g) were glued on each valve of each animal. These electrodes were connected to the valvometer by flexible wires, which allowed the oysters to move their valves without constraint. The measurement principle is based on the application of Maxwell's Law, $\varepsilon = -N \cdot (d\phi_B/dt)$, where ε is the electromotive force (in volts), N is the number of turns of wire, ϕ_B is the magnetic flux (in Webbers), and t the time. The apparatus measures an induced voltage that varies according to the distance between the electromagnetic electrodes. The sampling frequency for each individual was 0.2 Hz. Data were processed using LabView 8.0 software (National Instruments, Austin, TX, USA).

Mean hourly opening duration of each individual and of the group was expressed as the percentage of time oysters spent with their valves opened, and ranged from 100% (valves open for the entire hour) to 0% (valves closed throughout the hour). Additionally, number of valve micro-closures, which corresponds to the number of partial and fast valve closure was measured and expressed as number of micro-closures/hour.

2.8. Chronobiological analysis

2.8.1. Data analysis and quality

Chronobiological analyses were done with the software Time Series Analysis Serial Cosinor 6.3 (http://www.euroestech.com/). Several steps were performed to first verify the quality of the data, then determine the periodicity of the behavior of the oysters, if any, and finally model the potential rhythm (Gouthière et al., 2005a; Gouthière et al., 2005b). First, the absence of randomness in the data set were controlled using the autocorrelation diagram and then for

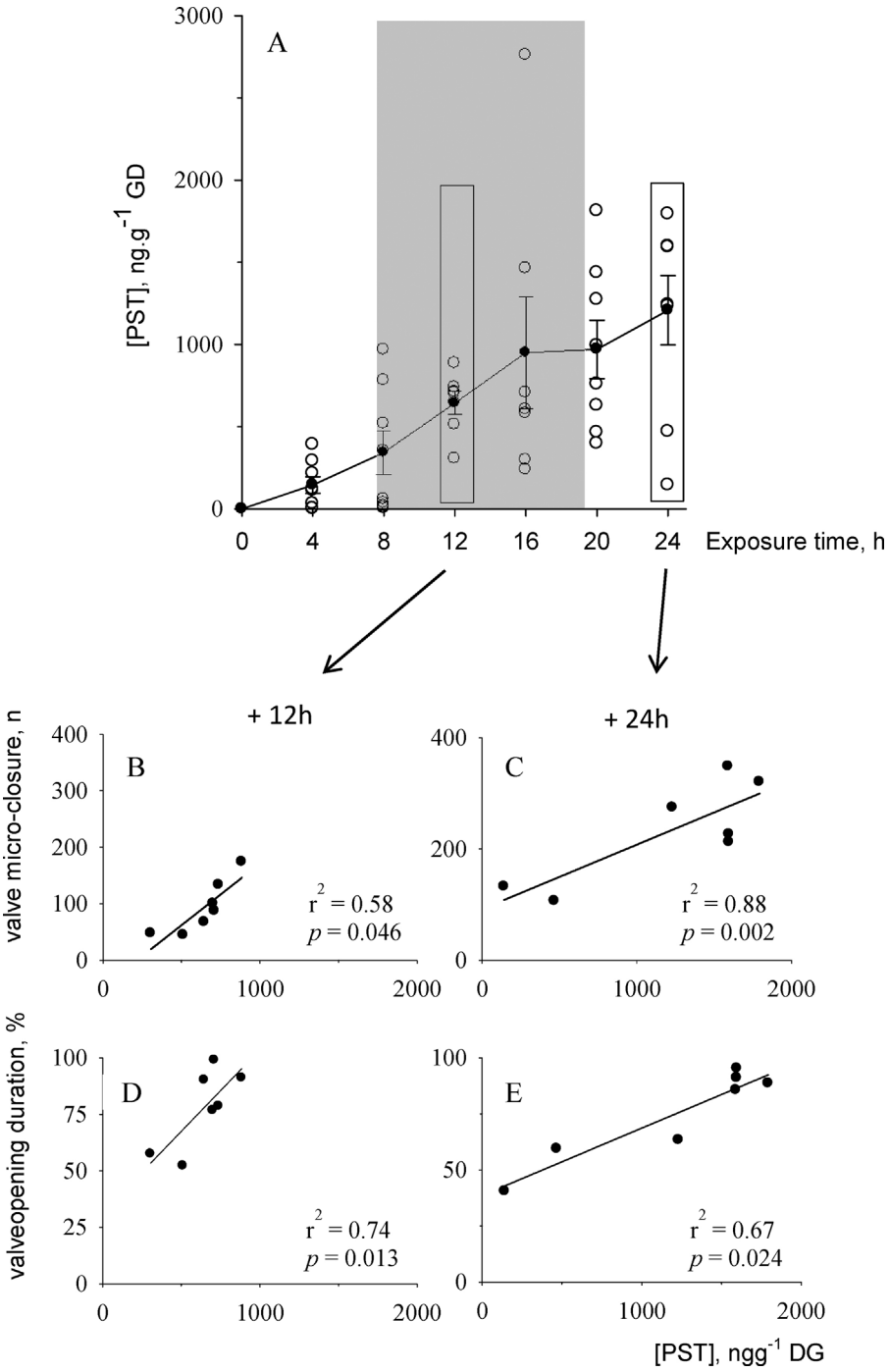

Fig. 1. A–E quantification and behavioral effects of PST accumulation in digestive gland of *C. gigas* exposed to *A. minutum* during a daily cycle. (A) Kinetics of individual PST accumulation and mean values (±SE, $n = 8$) at each 4-h sampling time during a daily cycle. Time 0 started at 12-h local time (UTC + 1). Gray area corresponds to scotophase. Individual relationship between PST accumulation and valve micro-closures after 12 h (B) and 24 h (C) of exposure; or valve opening duration after 12 h (D) and 24 h (E) of exposure.

the absence of a stationary character by using a Partial Autocorrelation Function (PACF) calculation (Box et al., 1994). These checks indicated a real biological or physical phenomenon.

2.8.2. Search for periodicity

The method used to determine the period in our equispaced data was the Lomb and Scargle periodogram (Scargle, 1982), which allows determination of statistical significance ($p = 0.95$) of a period. The confidence interval of the period was determined using the method of Halberg (1969).

2.8.3. Modeling and statistical validation

When a period was validated, rhythmicity was then modeled with the Cosinor model, which uses a cosine function calculated by regression (Nelson et al., 1979; Bingham et al., 1982). The model for a given period is written as: $Y(t) = A\cos(2\pi t/\tau + \varphi) + M + \varepsilon(t)$ where A is the amplitude, φ is the acrophase, τ is the period, M is the mesor and ε is the relative error (Gouthière et al., 2005a). Two tests have to be performed to validate the calculated model and the existence of a rhythm: the elliptic test (Bingham et al., 1982) must be rejected and the probability for the null amplitude hypothesis must be lower

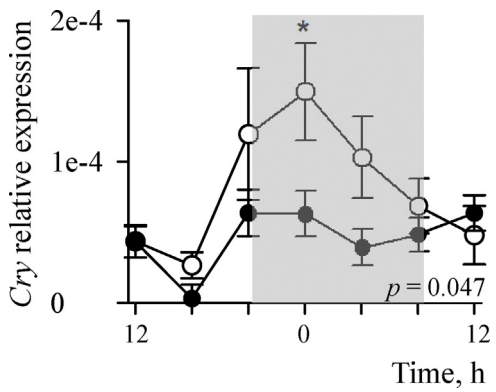

Fig. 2. Effect of *A. minutum* on expression of the circadian gene *Cgcry*. Mean (±SE, $n = 8$) *Cgcry* expression during a 24-h cycle in gills of control oysters fed on the non-harmful algae *H. triquetra* (○), and of tested oysters exposed to the harmful algae *A. minutum* (●). The 24-h cycle is expressed in local hours (UTC + 1), gray area corresponds to scotophase. *Statistically different from the control condition at the same time, p-value < 0.05.

than 0.05. These tests have always been validated when a rhythm is mentioned.

2.9. Statistical analyses

Data are expressed as mean ± SE. Differences between variables were investigated using One-Way ANOVA after checking assumptions (normality of data and equal variance tests). When assumptions were not validated, the non-parametric Kruskal–Wallis One-Way ANOVA on rank was applied. For all statistical results, a probability of $p < 0.05$ was considered significant. Analyses were performed with SigmaStat (Version 3.5, Systat, Chicago, USA).

3. Results

3.1. Quantification of PST accumulation in digestive gland of C. gigas

Fig. 1 presents PST accumulation in the digestive gland (DG) of oysters exposed to a simulated bloom of the harmful algae *A. minutum* (concentration maintained at 1500 cells ml^{-1} at 100 ml min^{-1} in the experimental tank flow-through system) during one daily cycle (light:dark regime 12 h:12 h). Fig. 1A shows the kinetics of PST accumulation in DG, at the individual level ($n = 8$ at each 4 h time sampling), and the mean value ± 1 SE. Results show increased accumulation with time ($p < 0.001$). At the individual level, Fig. 1B–E shows the effect of PST accumulation on valve behavior, demonstrating a significant dose-response effect of PST on valve micro-closures (Fig. 1B–C) and valve opening duration (Fig. 1D–E). These effects are first present at 12 h exposure and remain significant at 24 h.

3.2. Rhythmic activity of the circadian gene Cgcry and its disruption by the harmful algae A. minutum

The expression of cryptochrome (*Cgcry*), a gene involved in the generation and/or synchronization of circadian rhythmic activities was assessed. Fig. 2 shows that in the control group (white circles) fed with non-harmful algae *H. triquetra*, *Cgcry* expression is not constant during a circadian cycle. Instead, the rhythm is an increase of expression just before and at the beginning of scotophase, followed by a decrease at the end of scotophase and during photophase. On the contrary, oysters exposed to the *A. minutum* bloom (black circles) did not express a similar cyclic *Cgcry* activity: the transitory increase during scotophase was not present. The *Cgcry* expression was significantly different ($p = 0.047$) between control and tested groups according to time. As this disruption of circadian gene expression should have major consequences at the level of rhythmic activities of the oyster, the associated valve behavior was first studied.

3.3. Daily rhythm of behavior disrupted by A. minutum

Fig. 3A shows the mean valve hourly opening duration in control and tested groups before and during the *A. minutum* bloom. During the 24-h cycle before *A. minutum* exposure, both groups exhibited the same rhythmic valve activity ($p = 0.37$), with a quick increase in valve opening duration at the beginning of scotophase followed by a progressive decrease down to a basal level during photophase. During the next 24-h cycle, the control exhibited the same rhythmic pattern while the *A. minutum*-exposed group exhibited a disrupted rhythm with valve opening durations that remained maximal during the entire circadian cycle. Mean hourly opening duration was 47.9 ± 4.3% in the control group and 74.3 ± 3.7% (+28.4%) in the exposed group, a significant difference ($p < 0.0001$). These results were validated by chronobiological analyses. Fig. 3B shows a spectral analysis (Lomb and Scargle periodogramm) that allows the determination of significant rhythmic periodicity in valve behavior. The results show valve activity with a significant circadian period (range: 20–28 h) before exposure in both groups and during exposure in controls, whereas during *A. minutum* exposure, there was no significant period (the arrow in the lower right panel). Moreover, all the significant periods for the different conditions were tested in

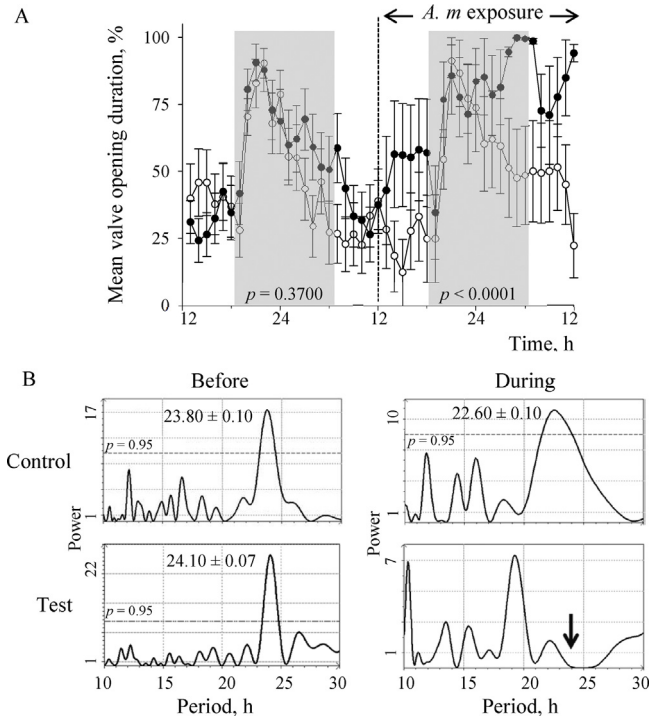

Fig. 3. *A. minutum* effect on the behavioral rhythm of *C. gigas*. (A) Mean (±SE) hourly opening duration (%) of the control group (○) and tested group (●) during two circadian cycles: the 24-h cycle before *A. minutum* exposure ($n = 16$ oysters per group) and the 24-h cycle during *A. minutum* exposure ($n = 8$ oysters per group). The 24-h cycle is expressed in local hours (UTC + 1), gray area corresponds to scotophase. (B) The rhythmic activity is determined by the period of the group (±SD) with a spectral analysis for each condition (Lomb and Scargle periodogram; dotted line determines significant period for a p-value = 0.95). "Before" means 24-h cycle before *A. minutum* exposure whereas "During" means 24-h cycle during *A. minutum* exposure, for control and tested groups.

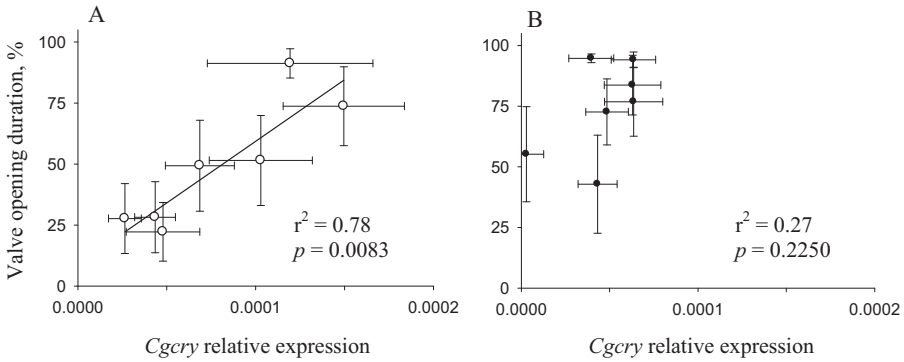

Fig. 4. Relationship between *Cgcry* expression and valve behavior. Mean (±SE) valve opening duration as a function of mean *Cgcry* expression in gills at each sampling time ($n = 7$). (A) Control oysters (○) fed on the non-harmful algae *H. triquetra*. (B) Tested oysters (●) exposed to the harmful algae *A. minutum*. p-value < 0.05.

the chronobiological model (Cosinor model), which validated the existence of a circadian rhythm.

The relationship between *Cgcry* expression and the synchronization and/or genesis of rhythmicity and the daily rhythm of valve activity was also investigated. Fig. 4 shows valve opening duration as a function of *Cgcry* expression during a 24-h cycle. In the control group (Fig. 4A), a significant correlation ($r^2 = 0.78$, $p = 0.0083$) between increased *Cgcry* expression and valve opening duration is observed. This relationship (Fig. 4B) disappears in oysters exposed to the *A. minutum* bloom ($p = 0.2250$).

3.4. Daily rhythm of digestive functions disrupted by A. minutum

Fig. 5A shows the length of the crystalline style, involved in the digestive process, during a 24-h cycle. No significant difference can be observed between the control group (fed with non-harmful algae *H. triquetra*) and the tested group, exposed to the *A. minutum* bloom. However, Fig. 5B demonstrates a significant difference of digestive crystalline style length between scotophase and photophase of the circadian cycle in the control group ($p = 0.026$) but not in the group exposed to *A. minutum* ($p = 0.163$).

3.5. Daily rhythm of gene expression disrupted by A. minutum

To address the question of *A. minutum* effect on putative ccgs, mRNA expression of genes involved in essential metabolic functions, we measured selected genes such as genes encoding proteins involved in oxidative stress defense (catalase *Cat* and glutathione peroxidase *Gpx*), immunity (interleukin *Ilk*), respiration (cytochrome oxidase *Cox*) and detoxification (metallothionein type 2 *Mt2* and multi-xenobiotic resistance *Mxr*) during a daily cycle. The results are shown in Fig. 6.

In the control group (white circles), there is a rhythmic pattern to all gene expressions (except for *Mxr* expression which remains constant). This pattern is characterized by an increase at the beginning of scotophase followed by a progressive decrease to a basal level during photophase. On the contrary, the group exposed to the *A. minutum* bloom (black circles) did not express any rhythmic gene expression. In particular, the transient increase of gene expression during early scotophase was not observed.

4. Discussion

The objective of this study was to determine whether an *A. minutum* HAB impacts the daily rhythm of *C. gigas* gene transcription, digestive activity and behavior. Indeed, many *A. minutum* effects have been shown on oyster physiology and metabolism (Haberkorn et al., 2010a,b; Lassus et al., 1999; Navarro and Contreras, 2010; Wildish et al., 1998), yet possible effects on biological rhythms remain unknown. Biological rhythms are a fundamental property of living organisms, driving the entire life of animals, and maximizing their fitness in the environment (Emerson et al., 2008). This function is upstream of all other metabolic and physiological functions,

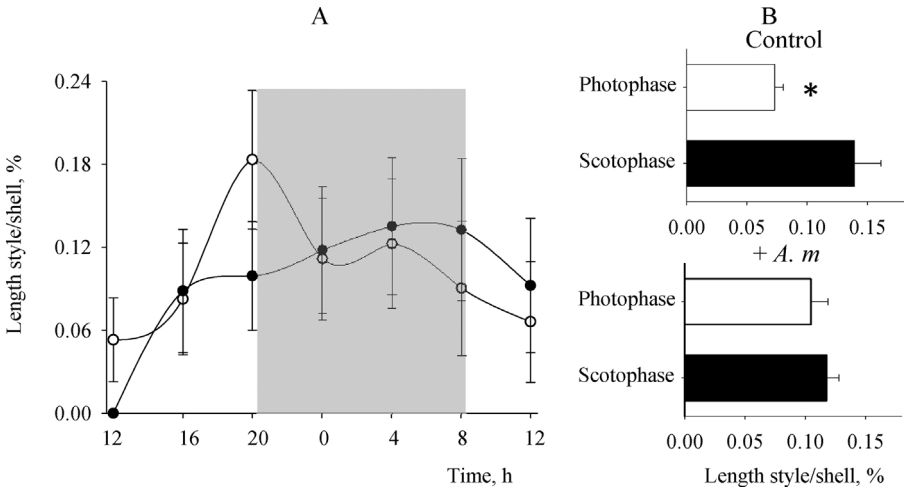

Fig. 5. Effects of *A. minutum* expsosure on the digestive rhythm in *C. gigas*. (A) Mean (±SE, $n = 8$) digestive crystalline style length weighted by shell length of the control group (○) fed on the non-harmful algae *H. triquetra* and the tested group (●) exposed to the harmful algae *A. minutum* during a 24-h cycle. The 24-h cycle is expressed in local hours (UTC + 1), gray area corresponds to scotophase. (B) The mean (±SE, $n = 24$) of digestive style length weighted by shell length of the control group and the group exposed to *A. minutum* during photophase and scotophase of the circadian cycle. *Statistically different from the scotophase condition. p-value < 0.05.

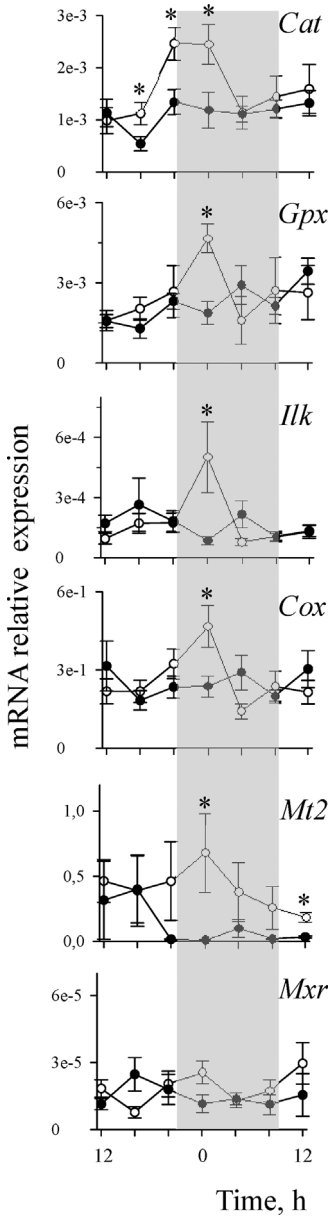

Fig. 6. Effect of *A. minutum* exposure on cyclic gene expressions in *C. gigas*. Mean (±SE, $n = 8$) expression in oyster gills of genes *Cat* (catalase), *Gpx* (glutathione peroxidase), *Ilk* (interleukin), *Cox* (cytochrome oxidase), *Mt2* (metallothionein type 2) and *Mxr* (multi-xenobiotic resistance) during a 24-h cycle, of control oysters (○) fed on the non-harmful alga *H. triquetra*, and of tested oysters (●) exposed to the harmful alga *A. minutum*. The 24-h cycle is expressed in local hours (UTC + 1), gray area corresponds to scotophase. *Statistically different from the condition *A. minutum* exposure at the same time with a p-value < 0.05.

and specifically drives the daily cycle (produced by the circadian clock) (Panda and Hogenesch, 2002). Overall, major disruptions of biological rhythms would impair its fitness and normal metabolic functioning and could lead to disease and in fine death.

This study is the first clear demonstration of an HAB disrupting the daily rhythm of *C. gigas* at very diverse physiological levels: transcriptional, digestive and valve activity levels.

The results first demonstrate, in control oysters, the synchronization of increased *CgCry* transcription, involved in biological clock function, with increased: (i) expression of genes involved in metabolic functions, (ii) crystalline style length (digestive process), and (iii) valve movement (behavior). This result is consistent with previous *in situ* (Tran et al., 2011) and laboratory (Mat et al., 2012) observations, showing that oysters have a nocturnal circadian valve behavior rhythm in winter (as opposed to the diurnal summer rhythm), with a peak of activity at the beginning of the night. This study, clearly demonstrates that the peak in valve movement activity is correlated with increased physiological functions such as gene expression and digestive processes; all occurring at night. In bivalves, only a few studies have been devoted to cyclic activities, especially at the gene level. Only for the mussel *Mytilus californianus*, transcriptome profiling studies have shown that most gene expression is rhythmic, thus suggesting that many metabolic and physiological pathways are subjected to daily fluctuations (Gracey et al., 2008; Connor and Gracey, 2011). The results illustrate that temporal fine tuning of gene transcription, metabolism and physiology act in concert to optimize metabolic efficiency by anticipation, to synchronize and order physiological functions and to limit energy expenditure to when it is needed.

During the *A. minutum* bloom, oysters lost all observed cyclic activities. Gene expression remained relatively constant and on the low end of the range during a daily cycle. Mat et al. (2013) already observed a repression of *C. gigas* gene expression after a 48-h exposure to *A. minutum*. First, a gene involved in the circadian rhythm was studied. At the time this experiment was performed (December 2010), only the cryptochrome gene (*CgCry*) of *C. gigas* was sequenced (Genbank no GQ415324). The cryptochrome family is known to be involved in the circadian rhythm of all living organisms (Cashmore, 2003). According to the species, and following the class of Cry orthologs, Cry is involved either in the light transduction and synchronization of the circadian clock (*e.g.* in insects such as *D. melanogaster*; Tomioka and Matsumoto, 2010), or in a negative feedback loop of the circadian clock, (for example in mammals and fishes; Zhang and Kay, 2010). Until now, evidence for a role of *Cg*Cry in the circadian rhythm of the oyster remained to be elucidated, although phylogenetically *Cg*Cry is closer to the *Drosophila* type of Cry than to the mammalian one. In 2012, the complete genome of *C. gigas* was sequenced (Zhang et al., 2012), which allows the study of the expression of all circadian genes following exposure to a bloom of *A. minutum*. The algae *A. minutum* produces saxitoxin (STX) and derivatives, toxins with a mechanism of action very close to tetrodotoxin, produced by the fish Fugu. Tetrodotoxin has been shown to repress clock gene expression in the fly *D. melanogaster* (Van Den Pol and Obrietan, 2002), thus we hypothesize that STX could impact the oyster by a direct repression at the clock gene level. Consequently, this disruption of CgCry expression should be associated with disruption of the rhythmic activities of the oyster in terms of either synchronization and/or generation of the daily rhythm. This study shows an effect at transcription level. Indeed, the output of the clock is to activate/repress genes called clock-controlled genes (ccgs) responsible for the cyclic activities of metabolic and physiological functions (Dunlap, 1999). In this study, the transcription of genes encoding proteins involved in oxidative stress (catalase, glutathione peroxidase and superoxide dismutase), mitochondrial metabolism (cytochrome oxidase 1), immunity (interleukin) and detoxification process (multidrug resistance protein, metallothionein) were affected; most of these seem good candidates for ccgs. Indeed, except for the gene encoding the multidrug resistance protein, they all have a daily cycle, with an increase of expression at the beginning of the night. These cycles vanished during *A. minutum* exposure. Similar to *CgCry* expression, the expression of these genes remained stable at a low level. The consequence of stable gene expression would be metabolic and physiological functions always occurring at the same "tempo", which would not allow for transient activity adaptation necessary to maximize fitness with environmental cycles.

To assess the effect of *A. minutum* on cyclic physiological functions, this study focused on the digestive process, particularly on extracellular digestion in the stomach due to digestive enzymes in

the style (Alyakrinskaya, 2001). This digestive style dissolves when food arrives into the stomach to be digested, and then reforms between digestive episodes. This dissolution-reformation of the style is known to be cyclic in *C. gigas*, following the tidal and diurnal cycles (Morton, 1977). Environmental cycles are also correlated with the intracellular digestion cycle in the mussel *Mytilus galloprovincialis* (Zaldibar et al., 2004). The results here showed that for control *C. gigas* the length of the style was not constant during a 24-h cycle, increasing before and in the beginning of the night and decreasing during and toward the end of the night and in photophase. When exposed to an *A. minutum* bloom, there was no significant effect on the style diurnal cycle compared to the control, probably due to high individual variability. However, the pattern of formation-dissolution of the digestive crystalline style suggests a transitory increase of the crystalline style length just before the beginning of scotophase in the control group that does not appear in the exposed group. However, when results of style length during scotophase and photophase were pooled, a significant difference in the control condition was seen. This difference in style length between scotophase and photophase clearly disappeared when oysters were exposed to *A. minutum*. The disappearance of this clock-driven physiological cyclic activity may be related to the disruption of ccgs transcription by *A. minutum*.

Finally, the effect of *A. minutum* on cyclic valve activity behavior was also assessed. First, a daily cycle of valve opening duration was clearly shown to be nocturnal, reaching a maximum in the beginning of scotophase. This result is consistent with those obtained by Mat et al. (2012), Mat et al. (2013). The effect of *A. minutum* on valve activity of *C. gigas* was already investigated, showing that during a bloom, *C. gigas* increased its duration of valve opening during the day, with a significant decrease of the amplitude of valve opening (Tran et al., 2010). An increase of valve micro-closures, dependent on the *A. minutum* concentration was also reported by Haberkorn et al. (2011). In the present study, the daily rhythm was shown to vanish. Indeed, in oysters exposed to harmful algae, duration of valve opening remained near the maximum during scotophase and photophase, without the decrease observed in the control group. This result is supported by the spectral analysis, which showed a statistical circadian period in the control group and before HAB exposure, but which disappeared during exposure. This result is also consistent with the fact that the disruption of clock gene transcription induces the disruption of *ccg* transcription, which in turn leads to a disruption of rhythmic behavior in the bivalve.

In conclusion, the present study clearly demonstrated for the first time that the harmful algae *A. minutum* in conditions of a realistic bloom concentration, disrupted oyster rhythmic activities patterned by its environment. The question as to whether this loss of rhythm could have consequences in terms of fitness or if it could transiently minimize deleterious effects when harmful algal bloom occurs remains to be investigated.

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References

Alyakrinskaya, I.O., 2001. The dimensions, characteristics and functions of the crystalline style of molluscs. Biol. Bull. 28, 523–535.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012. The globally distributed genus *Alexandrium*: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae 14, 10–35.

Aschoff, J., 1981. Handbook of Behavioral Neurobiology: Biological Rhythms, vol. 4. Plenum Press, New York.

Bell-Pedersen, D., Cassone, V.M., Earnest, D.J., Golden, S.S., Hardin, P.E., Thomas, T.L., Zoran, M.J., 2005. Circadian rhythms from multiple oscillators: lessons from diverse organisms. Nat. Rev. Genet. 6, 544–556.

Bingham, C., Arbogast, B., Cornélissen, G., Lee, J.-K., Halberg, F., 1982. Inferential statistical methods for estimating and comparing cosinor parameters. Chronobiologia 9, 397–439.

Box, G.E.P., Jenkins, G.M., Reinsel, G.C., 1994. Time Series Analysis: Forecasting and Control, 3rd ed. Prentice Hall, New York.

Bricelj, V.M., Shumway, S.E., 1998. Paralytic shellfish toxins in bivalve molluscs: occurrence, transfer kinetics, and biotransformation. Rev. Fish. Sci. 6, 315–383.

Cashmore, A.R., 2003. Cryptochromes: enabling plants and animals to determine circadian time. Cell 114, 537–543.

Connor, K.M., Gracey, A.Y., 2011. Circadian cycles are the dominant transcriptional rhythm in the intertidal mussel *Mytilus californianus*. Proc. Natl. Acad. Sci. 108, 16110–16115.

De Haro, L., Panda, S., 2006. Systems biology of circadian rhythms: an outlook. Biol. Rhythm Res. 21, 507–518.

Dunlap, J.C., 1999. Molecular bases for circadian clocks. Cell 96, 271–290.

Emerson, K.J., Bradshaw, W.E., Holzapfel, C.M., 2008. Concordance of the circadian clock with the environment is necessary to maximize fitness in natural populations. Evolution 62, 979–983.

Galimany, E., Sunila, I., Hégaret, H., Ramon, M., Wikfors, G.H., 2008. Experimental exposure of the blue mussel (*Mytilus edulis*, L.) to the toxic dinoflagellate *Alexandrium fundyense*: histopathology, immune responses, and recovery. Harmful Algae 7, 702–711.

Gouthière, L., Claustrat, B., Brun, J., Mauvieux, B., 2005a. Complementary methodological steps in the analysis of rhythms: search of periods, modelling. Examples of plasma melatonin and temperature curves. Pathol. Biol. 5, 285–289.

Gouthière, L., Mauvieux, B., Davenne, D., Waterhouse, J., 2005b. Complementary methodology in the analysis of rhythmic data, using examples from a complex situation, the rhythmicity of temperature in night shift workers. Biol. Rhythm Res. 36, 177–193.

Gracey, A.Y., Chaney, M.L., Boomhower, J.P., Tyburczy, W.R., Connor, K., Somero, G.N., 2008. Rhythms of gene expression in a fluctuating intertidal environment. Curr. Biol. 18, 1501–1507.

Guéguen, M., Baron, R., Bardouil, M., Haberkorn, H., Soudant, P., Truquet, P., Lassus, P., 2012. Influence of *Crassostrea gigas* (Thunberg) sexual maturation stage and ploidy on uptake of paralytic phycotoxins. Toxicon 60, 40–43.

Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Chanley, M.H. (Eds.), Culture of Marine Invertebrates Animals. Plenum Press, New York, pp. 29–60.

Halberg, F., 1969. Chronobiology. Annu. Rev. Physiol. 31, 675–725.

Hallegraeff, G.M., Anderson, D.M., Cembella, A.D., 2003. Manual on Harmful Marine Microalgae, Monographs on Oceanographic Methodology. UNESCO, Paris, pp. 793.

Haberkorn, H., Lambert, C., Le Goïc, N., Moal, J., Suquet, M., Guéguen, M., Sunila, I., Soudant, P., 2010a. Effects of *Alexandrium minutum* exposure on nutrition-related processes and reproductive output in oysters *Crassostrea gigas*. Harmful Algae 9, 427–439.

Haberkorn, H., Lambert, C., Le Goïc, N., Guéguen, M., Moal, J., Palacios, E., Lassus, P., Soudant, P., 2010b. Effects of *Alexandrium minutum* exposure upon physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. Aquat. Toxicol. 97, 96–108.

Haberkorn, H., Tran, D., Massabuau, J.C., Ciret, P., Savar, V., Soudant, P., 2011. Relationship between valve activity, microalgae concentration in the water and toxin accumulation in the digestive gland of the Pacific oyster *Crassostrea gigas* exposed to *Alexandrium minutum*. Mar. Pollut. Bull. 62, 1191–1197.

Haberkorn, H., Lambert, C., Le Goïc, N., Quéré, C., Bruneau, A., Riso, R., Auffret, M., Soudant, P., 2014. Cellular and biochemical responses of the oyster *Crassostrea gigas* to controlled exposures to metals and *Alexandrium minutum*. Aquat. Toxicol. 147, 158–167.

Hégaret, H., Wikfors, G.H., Soudant, P., Lambert, C., Shumway, S.E., Bérard, J.B., Lassus, P., 2007. Toxic dinoflagellates (*Alexandrium fundyense* and *A. catenella*) have minimal apparent effects on oyster hemocytes. Mar. Biol. 152, 441–447.

ICES, 2007. Report of the ICES-IOC Working Group on Harmful Algal Bloom Dynamics , International Council for the Exploitation of the Sea, Riga, Latvia, 10–13 April 2007.

Lassus, P., Bardouil, M., Beliaeff, B., Masselin, P., Naviner, M., Truquet, P., 1999. Effect of a continuous supply of the toxic dinoflagellate *Alexandrium minutum* Halim on the feeding behavior of the pacific oyster (*Crassostrea gigas* Thunberg). J. Shellfish Res. 18, 211–216.

Mat, A.M., Massabuau, J.C., Ciret, P., Tran, D., 2012. Evidence for a plastic dual circadian rhythm in the oyster *Crassostrea gigas*. Chronobiol. Int. 29 (7), 857–867.

Mat, A.M., Massabuau, J.C., Ciret, P., Tran, D., 2013. Looking for the clock mechanism responsible for circatidal behavior in the oyster *Crassostrea gigas*. Mar. Biol. 161, 89–99.

Mello, D.F., Da Silva, P.M., Barracco, M.A., Soudant, P., Hégaret, H., 2013. Effects of the dinoflagellate *Alexandrium minutum* and its toxin (saxitoxin) on the functional activity and gene expression of *Crassostrea gigas* hemocytes. Harmful Algae 26, 45–51.

Morton, B.S., 1977. The tidal rhythm of feeding and digestion in the Pacific oyster, *Crassostrea gigas* (Thunberg). J. Exp. Mar. Biol. Ecol. 26, 135–151.
Navarro, J.M., Contreras, A.M., 2010. An integrative response by *Mytilus chilensis* to the toxic dinoflagellate *Alexandrium catenella*. Mar. Biol. 157, 1967–1974.
Nelson, W., Tong, Y.L., Lee, J.K., Halberg, F., 1979. Methods for cosinor-rhythmometry. Chronobiologia 6, 305–323.
Oshima, Y., 1995. Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. J. AOAC Int. 78, 528–532.
Panda, S., Hogenesch, J.B., 2002. It's all in the timing: many clocks, many outputs. J. Biol. Rhythms 19, 374–387.
Scargle, J.D., 1982. Studies in astronomical time series analysis. II – Statistical aspects of spectral analysis of unevenly spaced data, AA (NASA, Ames Research Center, Space Science Div., Moffett Field, CA). Astrophys. J. 1, 835–853.
Tomioka, K., Matsumoto, A., 2010. A comparative view of insect circadian clock systems. Cell. Mol. Life Sci. 67, 1397–1406.
Tran, D., Haberkorn, H., Soudant, P., Ciret, P., Massabuau, J.C., 2010. Behavioral responses of *Crassostrea gigas* exposed to the harmful algae *Alexandrium minutum*. Aquaculture 298, 338–345.
Tran, D., Nadau, A., Durrieu, G., Ciret, P., Parisot, J.P., Massabuau, J.-C., 2011. Field chronobiology of a molluscan bivalve: how the moon and sun cycles interact to drive oyster activity rhythms. Chronobiol. Int. 28, 307–317.
Wildish, D., Lassus, P., Martin, J., Saulnier, A., Bardouil, M., 1998. Effect of the PSP-causing dinoflagellate, *Alexandrium* sp. on the initial feeding response of *Crassostrea gigas*. Aquat. Living Resour. 11, 35–43.
Yerushalmi, S., Green, R.M., 2009. Evidence for the adaptive significance of circadian rhythms. Ecol. Lett. 12, 970–981.
Van Den Pol, A.N., Obrietan, K., 2002. Short circuiting the circadian clock. Nat. Neurosci. 5, 616–618.
Zhang, E.E., Kay, S.A., 2010. Clocks not winding down: unravelling circadian networks. Nat. Rev. Mol. Cell Biol. 11, 764–776.
Zhang, G., et al., 2012. The oyster genome reveals stress adaptation and complexity of shell formation. Nature 490, 49–54.
Zaldibar, B., Cancio, I., Marigómez, I., 2004. Circatidal variation in epithelial cell proliferation in the mussel digestive gland and stomach. Cell Tissue Res. 318, 395–402.