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Abstract

Artificial Light At Night (ALAN) masks the natural light cycles and thus can disturb the synchronization of organisms' biological rhythms with their environment. Although coastlines are highly exposed to this growing threat, studies concerning the impacts of ALAN on coastal organisms remain scarce. In this study, we investigated the ALAN exposure effects at environmentally realistic intensities (0.1, 1, 10, 25 lux) on the oyster *Crassostrea gigas*, a sessile bivalve subject to light pollution on shores. We focused on the effects on oyster's daily rhythm at behavioral and molecular levels. Our results showed that ALAN disrupts the oyster's daily rhythm by increasing valve activity and annihilating day / night differences of expression of circadian clock and clock-associated genes. ALAN effects occur starting from 0.1 lux, in the range of artificial skyglow illuminances. We concluded that realistic ALAN exposure affects oysters' biological rhythm, which could lead to severe physiological and ecological consequences.

Keywords: ALAN, biological rhythm, circadian clock, *Crassostrea gigas*, behavior

Introduction

Natural light cycles play an important role in biological timings (Gaston et al., 2017). Indeed, predictable variations of light intensity over a day, a month, or a year are used as signals by organisms to synchronize their biological rhythms with their environment (Bradshaw and Holzapfel, 2010; Gaston et al., 2017). These biological rhythms are ubiquitous and find their origin in each cell with an endogenous clock, which uses environmental cues, such as natural light cycles, to synchronize organisms' physiological processes and behavior with their environment. This synchronization enables organisms to be fully adapted to their environment and to anticipate its changes (Cermakian and Sassone-Corsi, 2000; Partch et al., 2014). However, Artificial Light At Night (ALAN) affects the natural nocturnal lighting levels, which can disrupt the organisms' perception of natural variations of light, and affect organisms' biological rhythms (daily, lunar, seasonal) and their physiology (Falchi et al., 2011; Gaston and Bennie, 2014). For example, it can disrupt migration, reproduction, foraging, or prey/predator interactions (Davies et al., 2014; Gaston et al., 2017; Longcore and Rich, 2004; Navara and Nelson, 2007). These ALAN's effects on organisms' physiology and behavior occur in a large range of species, including diurnal ones, and at low intensities (below 1 lux) (Sanders et al., 2021). Thus ALAN can have consequences at the individual, population, and ecosystem scale. Nocturnal artificial lighting affects the whole world (Falchi et al., 2016) and spreads fast with a 6% increase in the sky luminosity per year (Hölker et al., 2010). The negative effects of ALAN on organism behavior or physiology become increasingly studied over the years (Davies and Smyth, 2018) but they remain widely investigated for terrestrial organisms. However, ALAN effects on marine organisms, and especially benthic organisms, recently received more attention. Studying the impacts of ALAN on species living in coastal ecosystems is of great importance considering that already 22.2 % of the world's coasts are exposed to ALAN as well as 35% of marine protected areas

(Davies et al., 2014, 2016), percentages that certainly will increase since the human population living in coastal areas is predicted to grow (Kummu et al., 2016). Furthermore, ALAN's negative effects could be worsened by the increasing use of LED (Light-Emitting Diode) in public lighting (Gaston, 2018; Zissis and Bertoldi, 2018). Indeed, LED lights spectrum contains more short-wavelength than spectra of other lighting types such as HPS (High-Pressure Sodium) lighting (Bierman, 2012; Falchi et al., 2011; Longcore et al., 2018; Luginbuhl et al., 2014). Considering that short-wavelength, such as blue wavelength, go further into seawater (Davies et al., 2014; Grubisic et al., 2019) and that marine organisms would be highly sensitive to blue wavelength (Grubisic, 2018), the negative effects of ALAN could be amplified on the coastal fauna. Therefore, ALAN becomes over the years a growing threat to marine life.

The oyster *Crassostrea gigas* is a marine bivalve of commercial importance with a large distribution. Oysters are also sessile organisms living in benthic areas, thus inevitably exposed to ALAN through direct or indirect sources of artificial light. The direct sources of lighting on the coast are for example streetlights on a pier, harbors, etc. The indirect source of ALAN is called skyglow, with a lower light intensity but much more spatially extensive than direct lighting. It takes its source mostly in big cities from which artificial lights are diffused into the atmosphere, resulting in a global lightening of the sky at night (Gaston, 2018). Both of these ALAN sources could disrupt oysters' biological rhythms. These oysters' biological rhythms related to daily (Mat et al., 2012), tidal (Tran et al., 2011), lunar (Payton and Tran, 2019), and seasonal (Payton et al., 2017b) natural cycles have already been described. Concerning their daily rhythm, oysters have a plastic endogenous circadian rhythm generated by a molecular clock (Mat et al., 2012; Mat et al., 2014; Payton et al., 2017a; Perrigault and Tran, 2017; Tran et al., 2020). This plasticity enables oysters to easily adapt to new environments but it also

could make them vulnerable to disruption by ALAN, which could have consequences on their physiology and ecology.

The objective of this study is to evaluate the effects of several ALAN intensities encountered in the environment on the daily rhythm of the oyster *C. gigas*. To achieve that, valve behavior rhythm and circadian clock genes expression were studied. We exposed oysters during 7 days to ALAN intensities ranging from 0.1 to 25 lux with the lowest intensity representing a skyglow intensity (Gaston, 2018) and the highest intensity being in the range of direct ALAN due to a lighted parking lot (Rich and Longcore, 2006). We hypothesized that ALAN would disrupt oysters' daily rhythm at the behavioral and molecular levels. We assumed that ALAN exposure disrupts the oysters' circadian clock machinery, impairing the clock's outputs such as clock-associated genes' expression and rhythmic behavior, and may also lead to a direct behavioral response during nighttime. Finally, we hypothesized that some of these effects would occur in an intensity-dependent manner.

Materials and methods

General conditions

The experiment was conducted from January to April 2021 in the Marine Station of Arcachon on 160 oysters (85.8 ± 0.7 mm shell length; 48.4 ± 0.5 mm shell width; mean ± SE) coming from an oyster's farm source of the Arcachon bay (France). During the acclimation and experimental duration, oysters were placed into tanks (L x W x H: 74.8 x 54.8 x 40.8 mm) continuously supplied with natural seawater from the Arcachon bay, which was filtered (< 1µm), oxygenized, and the temperature was monitored (T = 15.0 ± 0.1 °C). Tanks were placed in an isolated room and equipped with an antivibration bench to minimize external disturbances to animal behavior. The oysters were not fed during the experiment.

Experimental protocol

Oysters were maintained under a L:D 10:14 cycle with daytime from 7:00 to 17:00 h (all times noted in local time, UTC +1) (Fig. S1A). The light intensity during daytime varied gradually to mimic the natural cycle of light using programmable white (413–688 nm, peak at 551 nm; Fig. S2A) LED light bars (MH3SP3 DSunY). The maximum intensity during daytime was 1473.42 ± 106.21 lux (mean ± SE) (Tab. S.1) between 11:30 to 12:30 h. After 7 to 9 days of acclimation to the experimental setup, the proper experiment started for a duration of 7 days. There were 5 conditions: a control condition and 4 conditions where oysters were exposed to several ALAN intensities (Tab. S1): 0.1, 1, 10, and 25 lux. In the 4 ALAN conditions, oysters were exposed to ALAN from 17:30 to 6:30 h (Fig. S1B) using white (411–687 nm, peak at 563 nm; Fig. S2B–S2E) LED strips (MiBoxer Mi-Light WL5). Illuminances underwater were measured at five positions in the tank (Fig. S3) using a handheld spectroradiometer (Blue-Wave UVN-100, StellarNet Inc.). The control group was in the complete dark at night, with a light intensity inferior to the detection limit of the spectroradiometer (0.05 lux), mentioned in the study as 0 lux for convenience. For each of the 5 experimental conditions, 32 oysters were placed in the same tank, and the valve activity of 16 of them was continuously measured using a High Frequency–Non Invasive (HFNI) valvometer technology (Andrade et al., 2016). For each experimental conditions, 32 different oysters were used. On the 7^{th} day of the experiment, the gill tissue of 8 oysters were sampled during daytime at 12:00 h and during nighttime at 19:00 h and stored in Tri Reagent (Invitrogen) at –80 °C for further molecular analysis.

Behavioral measurements

For valve activity measurement using HFNI valvometer technology, lightweight's electromagnets were glued on each valve of oysters and linked to a valvometer device using flexible wires (Andrade et al., 2016). The electromagnetic current generated between the two

electromagnets allows to measure oyster valve activity. For each individual, the signal was recorded every 4.8s. Data were processed using Labview 8.0 (National Instrument, Austin, TX, USA). The study focused on the hourly Valve Opening Duration (VOD) meaning that we determined the percentage of time each individual spent with its valve open for each hour. For example, an individual with its valves open during a whole hour corresponds to a VOD of 100 %, while an individual with its valves closed during one hour corresponds to a VOD of 0 %.

Chronobiological analyses

Chronobiological analysis of the hourly VOD data were performed at groups and individual levels using the software Time Series Analysis Serie Cosinor 8.0 (Expert Soft Technologies). First, data quality was evaluated using the autocorrelation diagram to control the absence of random repartition of data, and the Partial Autocorrelation Function (PACF) calculation to assess the absence of stationary character (Gouthière and Mauvieux, 2003). Then the Lomb and Scargle periodogram was used to search for periodicity in the data (Scargle, 1982). A significant period was accepted for $p > 0.95$. Data rhythmicity was then modeled using the Cosinor model, which uses a cosine function calculated by regression (Bingham et al., 1982). For a given period, the model is written as: $Y(t) = Acos\left(\frac{2\pi t}{\tau} + \phi\right) + M + \varepsilon(t)$ where A is the amplitude (difference between the average level and the highest value of the rhythm), ϕ the acrophase (the highest value of the rhythm), τ the given period (interval between two identical events), M the mesor (average level of the rhythm), and $\varepsilon(t)$ the relative error. Then the calculated model and the existence of rhythmicity were validated by two tests: the ellipse test had to be rejected, and the probability for the null amplitude hypothesis had to be lower than 0.05. The percent rhythm (PR), a chronobiometric parameter, had been calculated and represents the percentage of cyclic behavior explained by the model, meaning the strength of the rhythm.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from gills using Tri reagent (Invitrogen) and an SV Total RNA Isolation System kit (Promega). The total RNA quantity and quality were assessed by spectrophotometry (OD230, OD260, OD280). RNA reverse transcription was realized using Goscript™ Reverse Transcription System kits (Promega).

mRNA expression analysis by Real-Time PCR

Real-Time qPCR was realized using GoTaq® qPCR Master Mix kit (Promega). Primers sets of clock and clock-associated genes (*CgClock, CgBmal, CgCry, CgPer, CgTim1, CgCry1, CgRev-erb, CgRor, CgHiomt-like, CgOctβ2, CgRhodopsin-like 1, CgRhodopsin-like 2, CgRhodopsin-like 3*), and housekeeping genes (*CgEf1, Cg28S, CgGadph*) are listed in the table S2. qPCR reactions were realized as follows: 95 °C for 2 min to activate the GoTaq polymerase, 40 cycles of 95 °C for 15 seconds (denaturation), and 60 °C for 1 minute (annealing and extension) for amplification of target cDNA, followed by 2 minutes at 60 °C for the final elongation. In the end, melting curves were generated by gradually decreasing the temperature from 95 °C to 60 °C to control the primer specificity. The comparative Ct method $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) was used to determine the relative transcript level of clock and clock-associated genes, where ΔCt = Ct(target gene) – Ct(housekeeping gene). Gene's expression was normalized with the geometric mean of the housekeeping genes *CgEf1* and *Cg28S*, based on stability values (Xie et al., 2012).

Statistical analysis

All statistical analysis were performed using SigmaPlot software (version 13.0; Systat Software, USA). T-tests were performed for two groups' comparisons after checking assumptions (normality of data and equal variance) and if they were not validated the non-parametric Mann-Whitney rank sum test was performed. Two groups' comparisons were used for molecular analysis results. For multiple comparisons, one-way ANOVA were used after

checking assumptions (normality of data and equal variance). When assumptions were not validated, the non-parametric Kruskal-Wallis One-Way ANOVA on ranks test was performed. In case of significant differences, a Tukey test was realized for all pairwise multiple comparisons unless the groups did not have equal size, in which case the Dunn's test was realized. Multiple comparisons were performed for behavioral analyses. On the whole molecular data, a two-way ANOVA was also performed after checking assumptions (normality of data and equal variance), and in case of significant differences, a Tuckey test was used for all pairwise multiple comparisons. For all test results, a difference was considered significant when $p < 0.05$.

Results

Figure 1 shows the behavioral mean daily pattern of oysters in the control condition, and exposed to ALAN ranging from 0.1 to 25 lux. In the control condition, we show a diurnal pattern with a maximal VOD at 11–12 h (peak showed by the black arrow). When exposed to ALAN, the overall daily pattern of oysters' behavior is modified, with an increase of the hourly VOD during nighttime and a 7-hours shift of the daily VOD peak, delayed from daytime to nighttime, for all ALAN intensities starting from 0.1 lux. Statistical analysis of the mean daily VOD shows a significant increase from 0.1 lux, of the mean VOD when oysters are exposed to ALAN for all of the tested intensities ($p = 0.002$; Fig. 2A), without an intensity effect. The figure 2B shows no significant differences in VOD during daytime between all conditions. However, ALAN causes a significant increase of the mean VOD during nighttime starting from 0.1 lux. The significant difference of VOD between daytime and nighttime observed in the control condition decreases at 0.1 lux and significantly disappears at 1, 10, and 25 lux. Figure 3 shows chronobiological analysis, characterizing the effects of ALAN exposure on the oysters' daily behavioral rhythm at the group and individual levels (detailed

results in Table S3 and Table S4). At the group level, a significant daily rhythm persists in all conditions with no significant differences of period length between the conditions (Fig. 3A). However, ALAN affected several parameters of this rhythm. Indeed, there is a significant decrease ($p < 0.001$) of the percent rhythm (PR, %) of the daily rhythm when oysters are exposed to ALAN starting from 1 lux (Fig. 3B). Furthermore, the amplitude of the daily rhythm decreases significantly ($p < 0.001$) in all ALAN conditions, starting from 0.1 lux and without significant ALAN intensity-effect (Fig. 3C). At the individual level, results show an ALAN intensity-dependent decrease of the percentage of oysters having a significant daily rhythm (Fig. 3D). Figure 3E shows a decrease ($p < 0.001$) of the individual rhythmic period lengths, getting out the daily range for many individuals exposed to ALAN (Table S3). The mean period of oysters exposed to 1 lux and 25 lux are 17.9 ± 2.1 and 15.2 ± 1.7 h respectively, under the circadian range (20-28 h), explaining the global loss of rhythmicity at the daily scale shown in figure 3D. Furthermore, the PR of oysters having a daily rhythm tends to decrease starting from 0.1 lux, and it decreases significantly ($p = 0.015$) when they are exposed to 10 lux (Fig. 3F). Finally, the amplitude of oysters having a daily rhythm show no significant differences ($p = 0.169$) but a trend to decrease when oysters are exposed to ALAN (Fig 3G). Figures 4 and S4 shows the mRNA level during daytime and nighttime of thirteen circadian clock (*CgClock*, *CgBmal*, *CgCry*, *CgPer*, *CgTim1*, *CgCry1*, *CgRev-erb*, *CgRor*) and clock-associated (*CgHiomt-like*, *CgOctβ2*, *CgRhodopsin-like 1*, *CgRhodopsin-like 2*, *CgRhodopsin-like 3*) genes for all of the tested ALAN intensities. No significant differences of gene expression are observed between the control and the ALAN conditions neither at daytime nor at nighttime. However, results show significant differences of mRNA expression levels between daytime and nighttime in the control condition for five genes (two circadian clock genes: *CgTim1* and *CgRev-erb*; and three clock-associated genes: *CgHiomt-like*, *CgOctβ2* and *CgRhodopsin-like 1*), with an increase of expression during nighttime (Fig.

4). This daytime / nighttime difference of gene expression significantly disappears in all of the five genes for all ALAN intensities tested. Finally, the table 1 shows the results of a two-way ANOVA, testing the effects of the period of the day (daytime and nighttime), ALAN intensities, and their interaction on the overall relative mRNA levels of the thirteen genes studied. These results reveal a significant difference ($p = 0.046$) of the overall mRNA level between daytime and nighttime only for the control condition ($p = 0.014$). In all ALAN conditions, the daytime / nighttime difference of gene expression is abolished.

Discussion

Our study shows that ALAN affects the daily behavioral rhythm and the molecular clock of oysters starting from the lowest ALAN intensity tested, 0.1 lux, in the range of artificial skyglow. Multiples ALAN's effects on oyster's behavioral rhythm are observed: a decrease of the percentage of oysters having a daily rhythm, a decrease of the rhythm's robustness, a decrease of the rhythmic amplitude, an increase of the mesor (mean VOD), and a shift from a diurnal to a nocturnal peak activity. At the molecular level, the overall difference of clock and clock-associated gene expression observed in control condition between daytime and nighttime is abolished when oysters are exposed to ALAN. While a clear ALAN intensity-effect is observed at the individual level regarding the percentage of oysters having a daily behavioral rhythm, most of ALAN's effects at both behavioral and molecular levels occur from 0.1 to 25 lux without significant intensity-effect.

We showed that ALAN exposure leads to an increase of oysters' VOD, explained by an increase of activity during nighttime leading to a decrease (at 0.1 lux) or a loss (at 1, 10, and 25 lux) of daytime / nighttime differences of valve behavior and a shift of peak activity from daytime to nighttime. Activity increase during nighttime as an effect of ALAN has been also shown in other marine organisms, such as the diurnal fish *Girella laevifrons* exposed to 70 lux

at night (Pulgar et al., 2019). On the contrary, the nocturnal crayfish *Pacifastacus leniusculus* showed a decrease of activity during nighttime when exposed to ALAN at 12 lux using a HPS streetlight bulb (Thomas et al., 2016). ALAN effects on organisms' behavior have also been investigated in juvenile gastropods *Concholepas concholepas*, in which ALAN at an intensity of 329.9 lux (LED technology) induces a less efficient search for prey during nighttime (Manriquez et al., 2019). Moreover, some previous results of bivalve behavior disruption by ALAN was shown by Christoforou (2022), which investigated the effects of several ALAN wavelengths at an intensity of 19.86 lux using LED lightings on the behavior of the mussel *Mytilus edulis*. The exposure of mussels to white ALAN induces a decrease during both nighttime and daytime of the open/close frequency valve events. However, in this study, no chronobiological analysis were performed and the relatively invasive experimental device used to record mussels' behavior could have mask ALAN effects on mussels valve activity. In our study, we went further in the investigation of ALAN's impacts on daily behavioral rhythm using chronobiological analysis and lower ALAN intensities, using a non-invasive biosensor (Andrade et al., 2016).

Daily rhythms and more generally biological rhythms are of great importance since they enable organisms to anticipate cyclic changes in their environment and regulate accordingly the temporal organization of their physiological processes such as feeding, respiration, immunity, or growth (Helm et al., 2017; Yerushalmi and Green, 2009). Thus, the clear disruption of oysters' daily rhythm by ALAN observed in this study, starting from 0.1 lux, could have deep consequences on organism's physiology and fitness. The oysters were not fed during the experiments to avoid an effect of food supply that may fluctuate, and thus acts as a zeitgeber of daily rhythm. However, during our experimentation duration in the control condition, we did not observe a modification of behavior, showing that food absence did not affect ALAN's effects on oysters' daily rhythm. Furthermore, the experiments were

conducted in winter, when the food supply is very low in the environment. Moreover, the rhythm at the daily scale could not be the only rhythm that can be disrupted by ALAN. It has been shown that oysters have other biological rhythms. For example, oysters showed a lunar rhythm i.e. their valve behavior is synchronized with lunar cycles by detecting moonlight illumination cycle (Payton and Tran, 2019). This lunar rhythm could be affected by ALAN since the maximal intensity of the moonlight ranges from 0.1 to 0.3 lux (Rich and Longcore, 2006), thus the natural variations of moonlight intensity can be easily masked by ALAN intensities, such as the skyglow. The masking effect of lunar cycles by ALAN using LED has been investigated in the corals *Acropora millepora* and *Acropora digitifera*, in which exposition to ALAN delayed or masked their gametogenesis leading to a spawning desynchronization (Ayalon et al., 2021). Another example of ALAN effect on lunar rhythm is the disruption of the monthly nocturnal foraging pattern of the gastropod *Nucella lapillus*, based on the lunar cycle, by ALAN produced by LED lighting at an intensity of 10 lux (Tidau et al., 2022). Moreover, oysters' seasonal rhythms could be disrupted by ALAN. Oysters use the variation of photoperiod through the year to anticipate seasonal changes and synchronize physiological processes such as behavior, growth, and spawning (Bernard et al., 2016; Payton et al., 2017b). However, ALAN being often present during dusk and dawn, it can mask these two natural phenomena, preventing oysters to detect the variation of photoperiod along the year, which could affect several seasonal physiological processes and decrease the fitness of organisms (Gaston et al., 2017). ALAN's effects on organisms' physiological processes and fitness have been shown for example in the amphipod *Orchestroidea tuberculate*, in which ALAN (halogen light) induces a decrease of growth (Luarte et al., 2016), and in barnacles, in which ALAN (LED lighting) affects the settlement process (Lynn et al., 2021; Manriquez et al., 2021). Moreover, ALAN's effects on organisms' fitness have also been observed in the juvenile crabs *Neohelice granulate* since it increases mortality, mainly by increasing

cannibalistic interactions with adults at an intensity of approximately 40 lux (LED lighting) (Nunez et al., 2021).

In addition to the effects on the oyster's behavioral daily rhythm, this study also showed that ALAN disturbs the circadian clockwork of *C. gigas* by annihilating differences between daytime and nighttime in clock and clock-associated genes expression starting from the intensity 0.1 lux. Genes for which their expression was mainly affected by ALAN are *CgTim1*, *CgRev-erb*, *CgHiomt-like*, *CgOctβ2*, and *CgRhodopsin-like 1*. *CgTim1* and *CgRev-erb* are both involved in the regulation's feedback loops of the oysters' molecular clock and thus are core clock genes (Perrigault and Tran, 2017). On the other hand, *CgHiomt-like*, *CgOctβ2*, and *CgRhodopsin-like 1* are clock-associated genes. The first one is a homologue of the mammal gene *Hiomt* involved in the final step of the synthesis of melatonin, known to be a key molecule of the day / night rhythm and a potent antioxidant with a proposed role in immune function (Jones et al., 2015; Pevet et al., 1980). *CgOctβ2* is the gene of the octopamine receptor β-2R. Octopamine is a biogenic amine well studied in arthropods and gastropods where it functions as a neurotransmitter and hormone, fulfilling the roles played by adrenalin in vertebrates. It is an extremely pleiotropic substance, participating in invertebrate development such as growth, maturation, and reproduction by activating their corresponding G protein-coupled receptors (GPCRs), and may interact with the circadian clock (Roeder, 2020). Finally, the last gene to show a disrupted expression in presence of ALAN is the gene of the rhodopsin (*CgRhodopsin-like 1*), a photoreceptor involved in oysters' light-sensitivity (Wu et al., 2018). Thus, ALAN affects five of the thirteen genes studied here. This relative low transcriptional response observed might be due to the fact that oysters have been sampled at only two sampling times over the daily cycle. Indeed, a stronger transcriptional disruption might possibly have been revealed by sampling at another time of the day. However, our results still show that ALAN can affect genes of the molecular clock of

C. gigas (*CgTim1*, *CgRev-erb*), suggesting that the observed disruption of oyster's daily behavioral rhythm would be a real consequence of the circadian clock disruption and not only a direct reaction of light perception in presence of ALAN. ALAN also affects essential clock-associated genes (*CgHiomt, CgOctβ2*, *CgRhodopsin-like 1*) involved in physiological processes regulation. Thus, we can imagine that, by cascading effect, the expression of other genes involved in several physiological functions can also be disrupted by ALAN. For example, ALAN at 0.1–0.3 lux using a fluorescent bulbs induces the suppression of the increase of melatonin during nighttime in *Salvelinus* (Liu et al., 2019). Moreover, in the European perch *Perca fluviatilis* and the roach *Rutilus rutilus* the impairment of circadian melatonin pattern by ALAN (HPS lamps) is associated with a decrease of the mRNA expression of gonadotropins, which could disrupt their reproduction (Bruning et al., 2018).

Conclusion

This study is the first to investigate ALAN effects at environmentally realistic intensities on oysters, a key species of the benthic coastal ecosystems. Our results reveal deep impacts on the daily behavioral rhythm of *C. gigas*, from ALAN intensities starting from 0.1 lux, comparable to the skyglow intensities. We also found that the expression of some of the clock and clock-associated genes of the oyster's molecular clock was affected by ALAN, suggesting a disruption of the endogenous clock and potential cascading physiological consequences. However, studies focusing on the ALAN's effects on oysters' biological rhythms and the consequences of their disruption on their fitness and physiology are needed. Furthermore, given that ALAN spreads fast in coastal environments in which oysters are a key species, ALAN's effects on this organism could have ecological consequences that should be investigated to get a global picture of how ALAN will affect coastal ecosystems.

Ethics: All experiments complied with the laws in effect in France and they conformed to international ethical standards.

Data accessibility: The data underlying this study are available on Supplementary data.

Author contributions: Study design and methodology, D.T., A.B., L.P.; experimentation, A.B., D.T.; molecular analysis: A.B., L.P.; data treatment, A.B.; interpretation, A.B., D.T., L.P.; manuscript writing, A.B.; review and editing, all authors; funding, D.T. All authors contributed critically to the drafts, and gave final approval for publication.

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Figures & legends

Figure 1. Behavioral daily pattern of oysters (n = 15–16 oysters) in control condition (0 lux) and exposed to different ALAN intensities (0.1 lux, 1 lux, 10 lux, and 25 lux). Mean hourly VOD data are shown as mean ± SE (n = 7 days). White backgrounds indicate daytime and yellow and black backgrounds indicate nighttime. Arrows indicate the VOD peak during the day.

Figure 2. Effect of ALAN intensity on (A) the mean daily valve opening duration (VOD), and (B) the mean VOD during the daytime and the nighttime. Data are expressed in mean ± SE (n = 7 days). Different letters indicate significant differences between ALAN conditions and between daytime and nighttime ($p < 0.05$).

Figure 3. Effect of ALAN intensity on oysters daily rhythm characteristics: (A) the period at group level, (B) the percent rhythm (PR) at group level, (C) amplitude of the rhythm at group level, (D) the percentage of oysters having a daily rhythm, (E), period of individual rhythmic oysters, dotted lines define the daily range, (F) the PR at individual level of oysters having a daily rhythm, (G) amplitude of the rhythm at the individual level of oysters having a daily rhythm. Data are expressed in mean ± SE. For A-C, the SE shows the daily variability of the group's significant rhythm parameters (n = 7 days). For E-G the SE shows the individual variability (n = 15-16 oysters). Different letters indicate significant differences ($p < 0.05$).

Figure 4. Effect of ALAN intensity on the difference in relative mRNA expression level (mean ± SE, n = 8) on oyster gills tissues, between daytime and nighttime of five clock and clock-associated genes. White bars indicate the gene level during daytime and, colored bars during nighttime. Asterisks indicate significant differences between daytime and nighttime ($p < 0.05$).

Table 1. Two-Way Analysis of Variance testing the effect of Daytime/Nighttime and the ALAN intensity (0 lux, 0.1 lux, 1 lux, 10 lux, 25 lux) on the relative mRNA expression level on gill tissues of 13 clock and clock-associated genes. In bolt, significant *p*-values ($p < 0.05$).

Source of variation	***p*-value**	**df**	**F**
Daytime/Nighttime	**0.046**	1	3.996
ALAN intensity	0.886	4	0.288
Daytime/Nighttime x ALAN intensity	0.462	4	0.902
Daytime/Nighttime within 0 lux	**0.014**		
Daytime/Nighttime within 0.1 lux	0.743		
Daytime/Nighttime within 1 lux	0.652		
Daytime/Nighttime within 10 lux	0.903		
Daytime/Nighttime within 25 lux	0.261		

Artificial light at night at environmental intensities disrupts daily rhythm of the oyster *Crassostrea gigas*

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Abstract

Artificial Light At Night (ALAN) masks the natural light cycles and thus can disturb the synchronization of organisms' biological rhythms with their environment. Although coastlines are highly exposed to this growing threat, studies concerning the impacts of ALAN on coastal organisms remain scarce. In this study, we investigated the ALAN exposure effects at environmentally realistic intensities (0.1, 1, 10, 25 lux) on the oyster *Crassostrea gigas*, a sessile bivalve subject to light pollution on shores. We focused on the effects on oyster's daily rhythm at behavioral and molecular levels. Our results showed that ALAN disrupts the oyster's daily rhythm by increasing valve activity and annihilating day / night differences of expression of circadian clock and clock-associated genes. ALAN effects occur starting from 0.1 lux, in the range of artificial skyglow illuminances. We concluded that realistic ALAN exposure affects oysters' biological rhythm, which could lead to severe physiological and ecological consequences.

Keywords: ALAN, biological rhythm, circadian clock, *Crassostrea gigas*, behavior

Introduction

Natural light cycles play an important role in biological timings (Gaston et al., 2017). Indeed, predictable variations of light intensity over a day, a month, or a year are used as signals by organisms to synchronize their biological rhythms with their environment (Bradshaw and Holzapfel, 2010; Gaston et al., 2017). These biological rhythms are ubiquitous and find their origin in each cell with an endogenous clock, which uses environmental cues, such as natural light cycles, to synchronize organisms' physiological processes and behavior with their environment. This synchronization enables organisms to be fully adapted to their environment and to anticipate its changes (Cermakian and Sassone-Corsi, 2000; Partch et al., 2014). However, Artificial Light At Night (ALAN) affects the natural nocturnal lighting levels, which can disrupt the organisms' perception of natural variations of light, and affect organisms' biological rhythms (daily, lunar, seasonal) and their physiology (Falchi et al., 2011; Gaston and Bennie, 2014). For example, it can disrupt migration, reproduction, foraging, or prey/predator interactions (Davies et al., 2014; Gaston et al., 2017; Longcore and Rich, 2004; Navara and Nelson, 2007). These ALAN's effects on organisms' physiology and behavior occur in a large range of species, including diurnal ones, and at low intensities (below 1 lux) (Sanders et al., 2021). Thus ALAN can have consequences at the individual, population, and ecosystem scale. Nocturnal artificial lighting affects the whole world (Falchi et al., 2016) and spreads fast with a 6% increase in the sky luminosity per year (Hölker et al., 2010). The negative effects of ALAN on organism behavior or physiology become increasingly studied over the years (Davies and Smyth, 2018) but they remain widely investigated for terrestrial organisms. However, ALAN effects on marine organisms, and especially benthic organisms, recently received more attention. Studying the impacts of ALAN on species living in coastal ecosystems is of great importance considering that already 22.2 % of the world's coasts are exposed to ALAN as well as 35% of marine protected areas

(Davies et al., 2014, 2016), percentages that certainly will increase since the human population living in coastal areas is predicted to grow (Kummu et al., 2016). Furthermore, ALAN's negative effects could be worsened by the increasing use of LED (Light-Emitting Diode) in public lighting (Gaston, 2018; Zissis and Bertoldi, 2018). Indeed, LED lights spectrum contains more short-wavelength than spectra of other lighting types such as HPS (High-Pressure Sodium) lighting (Bierman, 2012; Falchi et al., 2011; Longcore et al., 2018; Luginbuhl et al., 2014). Considering that short-wavelength, such as blue wavelength, go further into seawater (Davies et al., 2014; Grubisic et al., 2019) and that marine organisms would be highly sensitive to blue wavelength (Grubisic, 2018), the negative effects of ALAN could be amplified on the coastal fauna. Therefore, ALAN becomes over the years a growing threat to marine life.

The oyster *Crassostrea gigas* is a marine bivalve of commercial importance with a large distribution. Oysters are also sessile organisms living in benthic areas, thus inevitably exposed to ALAN through direct or indirect sources of artificial light. The direct sources of lighting on the coast are for example streetlights on a pier, harbors, etc. The indirect source of ALAN is called skyglow, with a lower light intensity but much more spatially extensive than direct lighting. It takes its source mostly in big cities from which artificial lights are diffused into the atmosphere, resulting in a global lightening of the sky at night (Gaston, 2018). Both of these ALAN sources could disrupt oysters' biological rhythms. These oysters' biological rhythms related to daily (Mat et al., 2012), tidal (Tran et al., 2011), lunar (Payton and Tran, 2019), and seasonal (Payton et al., 2017b) natural cycles have already been described. Concerning their daily rhythm, oysters have a plastic endogenous circadian rhythm generated by a molecular clock (Mat et al., 2012; Mat et al., 2014; Payton et al., 2017a; Perrigault and Tran, 2017; Tran et al., 2020). This plasticity enables oysters to easily adapt to new environments but it also

could make them vulnerable to disruption by ALAN, which could have consequences on their physiology and ecology.

The objective of this study is to evaluate the effects of several ALAN intensities encountered in the environment on the daily rhythm of the oyster *C. gigas*. To achieve that, valve behavior rhythm and circadian clock genes expression were studied. We exposed oysters during 7 days to ALAN intensities ranging from 0.1 to 25 lux with the lowest intensity representing a skyglow intensity (Gaston, 2018) and the highest intensity being in the range of direct ALAN due to a lighted parking lot (Rich and Longcore, 2006). We hypothesized that ALAN would disrupt oysters' daily rhythm at the behavioral and molecular levels. We assumed that ALAN exposure disrupts the oysters' circadian clock machinery, impairing the clock's outputs such as clock-associated genes' expression and rhythmic behavior, and may also lead to a direct behavioral response during nighttime. Finally, we hypothesized that some of these effects would occur in an intensity-dependent manner.

Materials and methods

General conditions

The experiment was conducted from January to April 2021 in the Marine Station of Arcachon on 160 oysters (85.8 ± 0.7 mm shell length; 48.4 ± 0.5 mm shell width; mean ± SE) coming from an oyster's farm source of the Arcachon bay (France). During the acclimation and experimental duration, oysters were placed into tanks (L x W x H: 74.8 x 54.8 x 40.8 mm) continuously supplied with natural seawater from the Arcachon bay, which was filtered (< 1µm), oxygenized, and the temperature was monitored (T = 15.0 ± 0.1 °C). Tanks were placed in an isolated room and equipped with an antivibration bench to minimize external disturbances to animal behavior. The oysters were not fed during the experiment.

Experimental protocol

Oysters were maintained under a L:D 10:14 cycle with daytime from 7:00 to 17:00 h (all times noted in local time, UTC +1) (Fig. S1A). The light intensity during daytime varied gradually to mimic the natural cycle of light using programmable white (413–688 nm, peak at 551 nm; Fig. S2A) LED light bars (MH3SP3 DSunY). The maximum intensity during daytime was 1473.42 ± 106.21 lux (mean ± SE) (Tab. S.1) between 11:30 to 12:30 h. After 7 to 9 days of acclimation to the experimental setup, the proper experiment started for a duration of 7 days. There were 5 conditions: a control condition and 4 conditions where oysters were exposed to several ALAN intensities (Tab. S1): 0.1, 1, 10, and 25 lux. In the 4 ALAN conditions, oysters were exposed to ALAN from 17:30 to 6:30 h (Fig. S1B) using white (411–687 nm, peak at 563 nm; Fig. S2B–S2E) LED strips (MiBoxer Mi-Light WL5). Illuminances underwater were measured at five positions in the tank (Fig. S3) using a handheld spectroradiometer (Blue-Wave UVN-100, StellarNet Inc.). The control group was in the complete dark at night, with a light intensity inferior to the detection limit of the spectroradiometer (0.05 lux), mentioned in the study as 0 lux for convenience. For each of the 5 experimental conditions, 32 oysters were placed in the same tank, and the valve activity of 16 of them was continuously measured using a High Frequency–Non Invasive (HFNI) valvometer technology (Andrade et al., 2016). For each experimental conditions, 32 different oysters were used. On the 7^{th} day of the experiment, the gill tissue of 8 oysters were sampled during daytime at 12:00 h and during nighttime at 19:00 h and stored in Tri Reagent (Invitrogen) at –80 °C for further molecular analysis.

Behavioral measurements

For valve activity measurement using HFNI valvometer technology, lightweight's electromagnets were glued on each valve of oysters and linked to a valvometer device using flexible wires (Andrade et al., 2016). The electromagnetic current generated between the two

electromagnets allows to measure oyster valve activity. For each individual, the signal was recorded every 4.8s. Data were processed using Labview 8.0 (National Instrument, Austin, TX, USA). The study focused on the hourly Valve Opening Duration (VOD) meaning that we determined the percentage of time each individual spent with its valve open for each hour. For example, an individual with its valves open during a whole hour corresponds to a VOD of 100 %, while an individual with its valves closed during one hour corresponds to a VOD of 0 %.

Chronobiological analyses

Chronobiological analysis of the hourly VOD data were performed at groups and individual levels using the software Time Series Analysis Serie Cosinor 8.0 (Expert Soft Technologies). First, data quality was evaluated using the autocorrelation diagram to control the absence of random repartition of data, and the Partial Autocorrelation Function (PACF) calculation to assess the absence of stationary character (Gouthière and Mauvieux, 2003). Then the Lomb and Scargle periodogram was used to search for periodicity in the data (Scargle, 1982). A significant period was accepted for $p > 0.95$. Data rhythmicity was then modeled using the Cosinor model, which uses a cosine function calculated by regression (Bingham et al., 1982). For a given period, the model is written as: $Y(t) = Acos\left(\frac{2\pi t}{\tau} + \phi\right) + M + \varepsilon(t)$ where A is the amplitude (difference between the average level and the highest value of the rhythm), ϕ the acrophase (the highest value of the rhythm), τ the given period (interval between two identical events), M the mesor (average level of the rhythm), and $\varepsilon(t)$ the relative error. Then the calculated model and the existence of rhythmicity were validated by two tests: the ellipse test had to be rejected, and the probability for the null amplitude hypothesis had to be lower than 0.05. The percent rhythm (PR), a chronobiometric parameter, had been calculated and represents the percentage of cyclic behavior explained by the model, meaning the strength of the rhythm.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from gills using Tri reagent (Invitrogen) and an SV Total RNA Isolation System kit (Promega). The total RNA quantity and quality were assessed by spectrophotometry (OD230, OD260, OD280). RNA reverse transcription was realized using Goscript™ Reverse Transcription System kits (Promega).

mRNA expression analysis by Real-Time PCR

Real-Time qPCR was realized using GoTaq® qPCR Master Mix kit (Promega). Primers sets of clock and clock-associated genes (*CgClock, CgBmal, CgCry, CgPer, CgTim1, CgCry1, CgRev-erb, CgRor, CgHiomt-like, CgOctβ2, CgRhodopsin-like 1, CgRhodopsin-like 2, CgRhodopsin-like 3*), and housekeeping genes (*CgEf1, Cg28S, CgGadph*) are listed in the table S2. qPCR reactions were realized as follows: 95 °C for 2 min to activate the GoTaq polymerase, 40 cycles of 95 °C for 15 seconds (denaturation), and 60 °C for 1 minute (annealing and extension) for amplification of target cDNA, followed by 2 minutes at 60 °C for the final elongation. In the end, melting curves were generated by gradually decreasing the temperature from 95 °C to 60 °C to control the primer specificity. The comparative Ct method $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) was used to determine the relative transcript level of clock and clock-associated genes, where ΔCt = Ct(target gene) – Ct(housekeeping gene). Gene's expression was normalized with the geometric mean of the housekeeping genes *CgEf1* and *Cg28S*, based on stability values (Xie et al., 2012).

Statistical analysis

All statistical analysis were performed using SigmaPlot software (version 13.0; Systat Software, USA). T-tests were performed for two groups' comparisons after checking assumptions (normality of data and equal variance) and if they were not validated the non-parametric Mann-Whitney rank sum test was performed. Two groups' comparisons were used for molecular analysis results. For multiple comparisons, one-way ANOVA were used after

checking assumptions (normality of data and equal variance). When assumptions were not validated, the non-parametric Kruskal-Wallis One-Way ANOVA on ranks test was performed. In case of significant differences, a Tukey test was realized for all pairwise multiple comparisons unless the groups did not have equal size, in which case the Dunn's test was realized. Multiple comparisons were performed for behavioral analyses. On the whole molecular data, a two-way ANOVA was also performed after checking assumptions (normality of data and equal variance), and in case of significant differences, a Tuckey test was used for all pairwise multiple comparisons. For all test results, a difference was considered significant when $p < 0.05$.

Results

Figure 1 shows the behavioral mean daily pattern of oysters in the control condition, and exposed to ALAN ranging from 0.1 to 25 lux. In the control condition, we show a diurnal pattern with a maximal VOD at 11–12 h (peak showed by the black arrow). When exposed to ALAN, the overall daily pattern of oysters' behavior is modified, with an increase of the hourly VOD during nighttime and a 7-hours shift of the daily VOD peak, delayed from daytime to nighttime, for all ALAN intensities starting from 0.1 lux. Statistical analysis of the mean daily VOD shows a significant increase from 0.1 lux, of the mean VOD when oysters are exposed to ALAN for all of the tested intensities ($p = 0.002$; Fig. 2A), without an intensity effect. The figure 2B shows no significant differences in VOD during daytime between all conditions. However, ALAN causes a significant increase of the mean VOD during nighttime starting from 0.1 lux. The significant difference of VOD between daytime and nighttime observed in the control condition decreases at 0.1 lux and significantly disappears at 1, 10, and 25 lux. Figure 3 shows chronobiological analysis, characterizing the effects of ALAN exposure on the oysters' daily behavioral rhythm at the group and individual levels (detailed

results in Table S3 and Table S4). At the group level, a significant daily rhythm persists in all conditions with no significant differences of period length between the conditions (Fig. 3A). However, ALAN affected several parameters of this rhythm. Indeed, there is a significant decrease ($p < 0.001$) of the percent rhythm (PR, %) of the daily rhythm when oysters are exposed to ALAN starting from 1 lux (Fig. 3B). Furthermore, the amplitude of the daily rhythm decreases significantly ($p < 0.001$) in all ALAN conditions, starting from 0.1 lux and without significant ALAN intensity-effect (Fig. 3C). At the individual level, results show an ALAN intensity-dependent decrease of the percentage of oysters having a significant daily rhythm (Fig. 3D). Figure 3E shows a decrease ($p < 0.001$) of the individual rhythmic period lengths, getting out the daily range for many individuals exposed to ALAN (Table S3). The mean period of oysters exposed to 1 lux and 25 lux are 17.9 ± 2.1 and 15.2 ± 1.7 h respectively, under the circadian range (20-28 h), explaining the global loss of rhythmicity at the daily scale shown in figure 3D. Furthermore, the PR of oysters having a daily rhythm tends to decrease starting from 0.1 lux, and it decreases significantly ($p = 0.015$) when they are exposed to 10 lux (Fig. 3F). Finally, the amplitude of oysters having a daily rhythm show no significant differences ($p = 0.169$) but a trend to decrease when oysters are exposed to ALAN (Fig 3G). Figures 4 and S4 shows the mRNA level during daytime and nighttime of thirteen circadian clock (*CgClock*, *CgBmal*, *CgCry*, *CgPer*, *CgTim1*, *CgCry1*, *CgRev-erb*, *CgRor*) and clock-associated (*CgHiomt-like*, *CgOctβ2*, *CgRhodopsin-like 1*, *CgRhodopsin-like 2*, *CgRhodopsin-like 3*) genes for all of the tested ALAN intensities. No significant differences of gene expression are observed between the control and the ALAN conditions neither at daytime nor at nighttime. However, results show significant differences of mRNA expression levels between daytime and nighttime in the control condition for five genes (two circadian clock genes: *CgTim1* and *CgRev-erb*; and three clock-associated genes: *CgHiomt-like*, *CgOctβ2* and *CgRhodopsin-like 1*), with an increase of expression during nighttime (Fig.

4). This daytime / nighttime difference of gene expression significantly disappears in all of the five genes for all ALAN intensities tested. Finally, the table 1 shows the results of a two-way ANOVA, testing the effects of the period of the day (daytime and nighttime), ALAN intensities, and their interaction on the overall relative mRNA levels of the thirteen genes studied. These results reveal a significant difference ($p = 0.046$) of the overall mRNA level between daytime and nighttime only for the control condition ($p = 0.014$). In all ALAN conditions, the daytime / nighttime difference of gene expression is abolished.

Discussion

Our study shows that ALAN affects the daily behavioral rhythm and the molecular clock of oysters starting from the lowest ALAN intensity tested, 0.1 lux, in the range of artificial skyglow. Multiples ALAN's effects on oyster's behavioral rhythm are observed: a decrease of the percentage of oysters having a daily rhythm, a decrease of the rhythm's robustness, a decrease of the rhythmic amplitude, an increase of the mesor (mean VOD), and a shift from a diurnal to a nocturnal peak activity. At the molecular level, the overall difference of clock and clock-associated gene expression observed in control condition between daytime and nighttime is abolished when oysters are exposed to ALAN. While a clear ALAN intensity-effect is observed at the individual level regarding the percentage of oysters having a daily behavioral rhythm, most of ALAN's effects at both behavioral and molecular levels occur from 0.1 to 25 lux without significant intensity-effect.

We showed that ALAN exposure leads to an increase of oysters' VOD, explained by an increase of activity during nighttime leading to a decrease (at 0.1 lux) or a loss (at 1, 10, and 25 lux) of daytime / nighttime differences of valve behavior and a shift of peak activity from daytime to nighttime. Activity increase during nighttime as an effect of ALAN has been also shown in other marine organisms, such as the diurnal fish *Girella laevifrons* exposed to 70 lux

at night (Pulgar et al., 2019). On the contrary, the nocturnal crayfish *Pacifastacus leniusculus* showed a decrease of activity during nighttime when exposed to ALAN at 12 lux using a HPS streetlight bulb (Thomas et al., 2016). ALAN effects on organisms' behavior have also been investigated in juvenile gastropods *Concholepas concholepas*, in which ALAN at an intensity of 329.9 lux (LED technology) induces a less efficient search for prey during nighttime (Manriquez et al., 2019). Moreover, some previous results of bivalve behavior disruption by ALAN was shown by Christoforou (2022), which investigated the effects of several ALAN wavelengths at an intensity of 19.86 lux using LED lightings on the behavior of the mussel *Mytilus edulis*. The exposure of mussels to white ALAN induces a decrease during both nighttime and daytime of the open/close frequency valve events. However, in this study, no chronobiological analysis were performed and the relatively invasive experimental device used to record mussels' behavior could have mask ALAN effects on mussels valve activity. In our study, we went further in the investigation of ALAN's impacts on daily behavioral rhythm using chronobiological analysis and lower ALAN intensities, using a non-invasive biosensor (Andrade et al., 2016).

Daily rhythms and more generally biological rhythms are of great importance since they enable organisms to anticipate cyclic changes in their environment and regulate accordingly the temporal organization of their physiological processes such as feeding, respiration, immunity, or growth (Helm et al., 2017; Yerushalmi and Green, 2009). Thus, the clear disruption of oysters' daily rhythm by ALAN observed in this study, starting from 0.1 lux, could have deep consequences on organism's physiology and fitness. The oysters were not fed during the experiments to avoid an effect of food supply that may fluctuate, and thus acts as a zeitgeber of daily rhythm. However, during our experimentation duration in the control condition, we did not observe a modification of behavior, showing that food absence did not affect ALAN's effects on oysters' daily rhythm. Furthermore, the experiments were

conducted in winter, when the food supply is very low in the environment. Moreover, the rhythm at the daily scale could not be the only rhythm that can be disrupted by ALAN. It has been shown that oysters have other biological rhythms. For example, oysters showed a lunar rhythm i.e. their valve behavior is synchronized with lunar cycles by detecting moonlight illumination cycle (Payton and Tran, 2019). This lunar rhythm could be affected by ALAN since the maximal intensity of the moonlight ranges from 0.1 to 0.3 lux (Rich and Longcore, 2006), thus the natural variations of moonlight intensity can be easily masked by ALAN intensities, such as the skyglow. The masking effect of lunar cycles by ALAN using LED has been investigated in the corals *Acropora millepora* and *Acropora digitifera*, in which exposition to ALAN delayed or masked their gametogenesis leading to a spawning desynchronization (Ayalon et al., 2021). Another example of ALAN effect on lunar rhythm is the disruption of the monthly nocturnal foraging pattern of the gastropod *Nucella lapillus*, based on the lunar cycle, by ALAN produced by LED lighting at an intensity of 10 lux (Tidau et al., 2022). Moreover, oysters' seasonal rhythms could be disrupted by ALAN. Oysters use the variation of photoperiod through the year to anticipate seasonal changes and synchronize physiological processes such as behavior, growth, and spawning (Bernard et al., 2016; Payton et al., 2017b). However, ALAN being often present during dusk and dawn, it can mask these two natural phenomena, preventing oysters to detect the variation of photoperiod along the year, which could affect several seasonal physiological processes and decrease the fitness of organisms (Gaston et al., 2017). ALAN's effects on organisms' physiological processes and fitness have been shown for example in the amphipod *Orchestroidea tuberculate*, in which ALAN (halogen light) induces a decrease of growth (Luarte et al., 2016), and in barnacles, in which ALAN (LED lighting) affects the settlement process (Lynn et al., 2021; Manriquez et al., 2021). Moreover, ALAN's effects on organisms' fitness have also been observed in the juvenile crabs *Neohelice granulate* since it increases mortality, mainly by increasing

cannibalistic interactions with adults at an intensity of approximately 40 lux (LED lighting) (Nunez et al., 2021).

In addition to the effects on the oyster's behavioral daily rhythm, this study also showed that ALAN disturbs the circadian clockwork of *C. gigas* by annihilating differences between daytime and nighttime in clock and clock-associated genes expression starting from the intensity 0.1 lux. Genes for which their expression was mainly affected by ALAN are *CgTim1*, *CgRev-erb*, *CgHiomt-like*, *CgOctβ2*, and *CgRhodopsin-like 1*. *CgTim1* and *CgRev-erb* are both involved in the regulation's feedback loops of the oysters' molecular clock and thus are core clock genes (Perrigault and Tran, 2017). On the other hand, *CgHiomt-like*, *CgOctβ2*, and *CgRhodopsin-like 1* are clock-associated genes. The first one is a homologue of the mammal gene *Hiomt* involved in the final step of the synthesis of melatonin, known to be a key molecule of the day / night rhythm and a potent antioxidant with a proposed role in immune function (Jones et al., 2015; Pevet et al., 1980). *CgOctβ2* is the gene of the octopamine receptor β-2R. Octopamine is a biogenic amine well studied in arthropods and gastropods where it functions as a neurotransmitter and hormone, fulfilling the roles played by adrenalin in vertebrates. It is an extremely pleiotropic substance, participating in invertebrate development such as growth, maturation, and reproduction by activating their corresponding G protein-coupled receptors (GPCRs), and may interact with the circadian clock (Roeder, 2020). Finally, the last gene to show a disrupted expression in presence of ALAN is the gene of the rhodopsin (*CgRhodopsin-like 1*), a photoreceptor involved in oysters' light-sensitivity (Wu et al., 2018). Thus, ALAN affects five of the thirteen genes studied here. This relative low transcriptional response observed might be due to the fact that oysters have been sampled at only two sampling times over the daily cycle. Indeed, a stronger transcriptional disruption might possibly have been revealed by sampling at another time of the day. However, our results still show that ALAN can affect genes of the molecular clock of

C. gigas (*CgTim1*, *CgRev-erb*), suggesting that the observed disruption of oyster's daily behavioral rhythm would be a real consequence of the circadian clock disruption and not only a direct reaction of light perception in presence of ALAN. ALAN also affects essential clock-associated genes (*CgHiomt, CgOctβ2*, *CgRhodopsin-like 1*) involved in physiological processes regulation. Thus, we can imagine that, by cascading effect, the expression of other genes involved in several physiological functions can also be disrupted by ALAN. For example, ALAN at 0.1–0.3 lux using a fluorescent bulbs induces the suppression of the increase of melatonin during nighttime in *Salvelinus* (Liu et al., 2019). Moreover, in the European perch *Perca fluviatilis* and the roach *Rutilus rutilus* the impairment of circadian melatonin pattern by ALAN (HPS lamps) is associated with a decrease of the mRNA expression of gonadotropins, which could disrupt their reproduction (Bruning et al., 2018).

Conclusion

This study is the first to investigate ALAN effects at environmentally realistic intensities on oysters, a key species of the benthic coastal ecosystems. Our results reveal deep impacts on the daily behavioral rhythm of *C. gigas*, from ALAN intensities starting from 0.1 lux, comparable to the skyglow intensities. We also found that the expression of some of the clock and clock-associated genes of the oyster's molecular clock was affected by ALAN, suggesting a disruption of the endogenous clock and potential cascading physiological consequences. However, studies focusing on the ALAN's effects on oysters' biological rhythms and the consequences of their disruption on their fitness and physiology are needed. Furthermore, given that ALAN spreads fast in coastal environments in which oysters are a key species, ALAN's effects on this organism could have ecological consequences that should be investigated to get a global picture of how ALAN will affect coastal ecosystems.

Ethics: All experiments complied with the laws in effect in France and they conformed to international ethical standards.

Data accessibility: The data underlying this study are available on Supplementary data.

Author contributions: Study design and methodology, D.T., A.B., L.P.; experimentation, A.B., D.T.; molecular analysis: A.B., L.P.; data treatment, A.B.; interpretation, A.B., D.T., L.P.; manuscript writing, A.B.; review and editing, all authors; funding, D.T. All authors contributed critically to the drafts, and gave final approval for publication.

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Figures & legends

Figure 1. Behavioral daily pattern of oysters (n = 15–16 oysters) in control condition (0 lux) and exposed to different ALAN intensities (0.1 lux, 1 lux, 10 lux, and 25 lux). Mean hourly VOD data are shown as mean ± SE (n = 7 days). White backgrounds indicate daytime and yellow and black backgrounds indicate nighttime. Arrows indicate the VOD peak during the day.

Figure 2. Effect of ALAN intensity on (A) the mean daily valve opening duration (VOD), and (B) the mean VOD during the daytime and the nighttime. Data are expressed in mean ± SE (n = 7 days). Different letters indicate significant differences between ALAN conditions and between daytime and nighttime ($p < 0.05$).

Figure 3. Effect of ALAN intensity on oysters daily rhythm characteristics: (A) the period at group level, (B) the percent rhythm (PR) at group level, (C) amplitude of the rhythm at group level, (D) the percentage of oysters having a daily rhythm, (E), period of individual rhythmic oysters, dotted lines define the daily range, (F) the PR at individual level of oysters having a daily rhythm, (G) amplitude of the rhythm at the individual level of oysters having a daily rhythm. Data are expressed in mean ± SE. For A-C, the SE shows the daily variability of the group's significant rhythm parameters (n = 7 days). For E-G the SE shows the individual variability (n = 15-16 oysters). Different letters indicate significant differences ($p < 0.05$).

Figure 4. Effect of ALAN intensity on the difference in relative mRNA expression level (mean ± SE, n = 8) on oyster gills tissues, between daytime and nighttime of five clock and clock-associated genes. White bars indicate the gene level during daytime and, colored bars during nighttime. Asterisks indicate significant differences between daytime and nighttime ($p < 0.05$).

Table 1. Two-Way Analysis of Variance testing the effect of Daytime/Nighttime and the ALAN intensity (0 lux, 0.1 lux, 1 lux, 10 lux, 25 lux) on the relative mRNA expression level on gill tissues of 13 clock and clock-associated genes. In bolt, significant *p*-values ($p < 0.05$).

Source of variation	***p*-value**	**df**	**F**
Daytime/Nighttime	**0.046**	1	3.996
ALAN intensity	0.886	4	0.288
Daytime/Nighttime x ALAN intensity	0.462	4	0.902
Daytime/Nighttime within 0 lux	**0.014**		
Daytime/Nighttime within 0.1 lux	0.743		
Daytime/Nighttime within 1 lux	0.652		
Daytime/Nighttime within 10 lux	0.903		
Daytime/Nighttime within 25 lux	0.261		

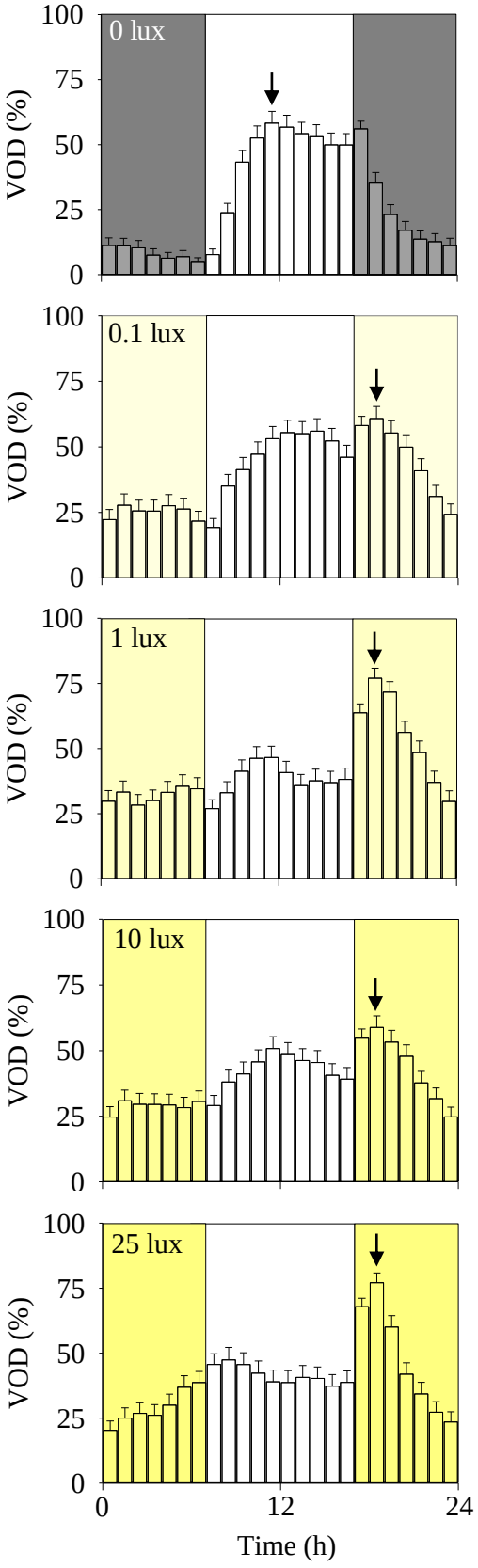

Figure 1. Botté et al.

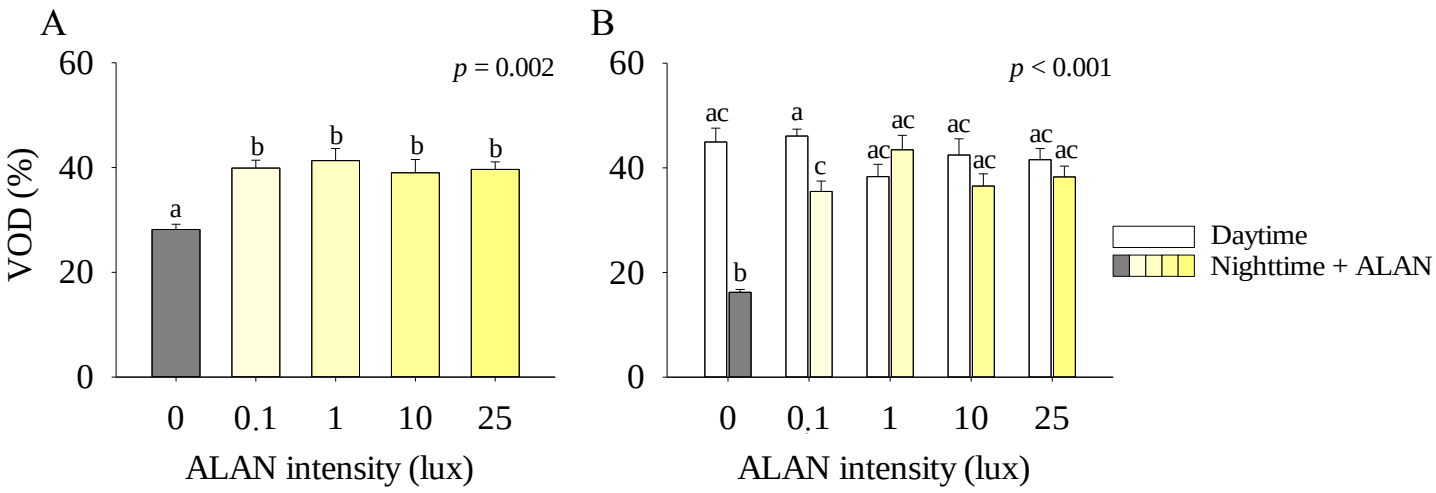

Figure 2. Botté et al.

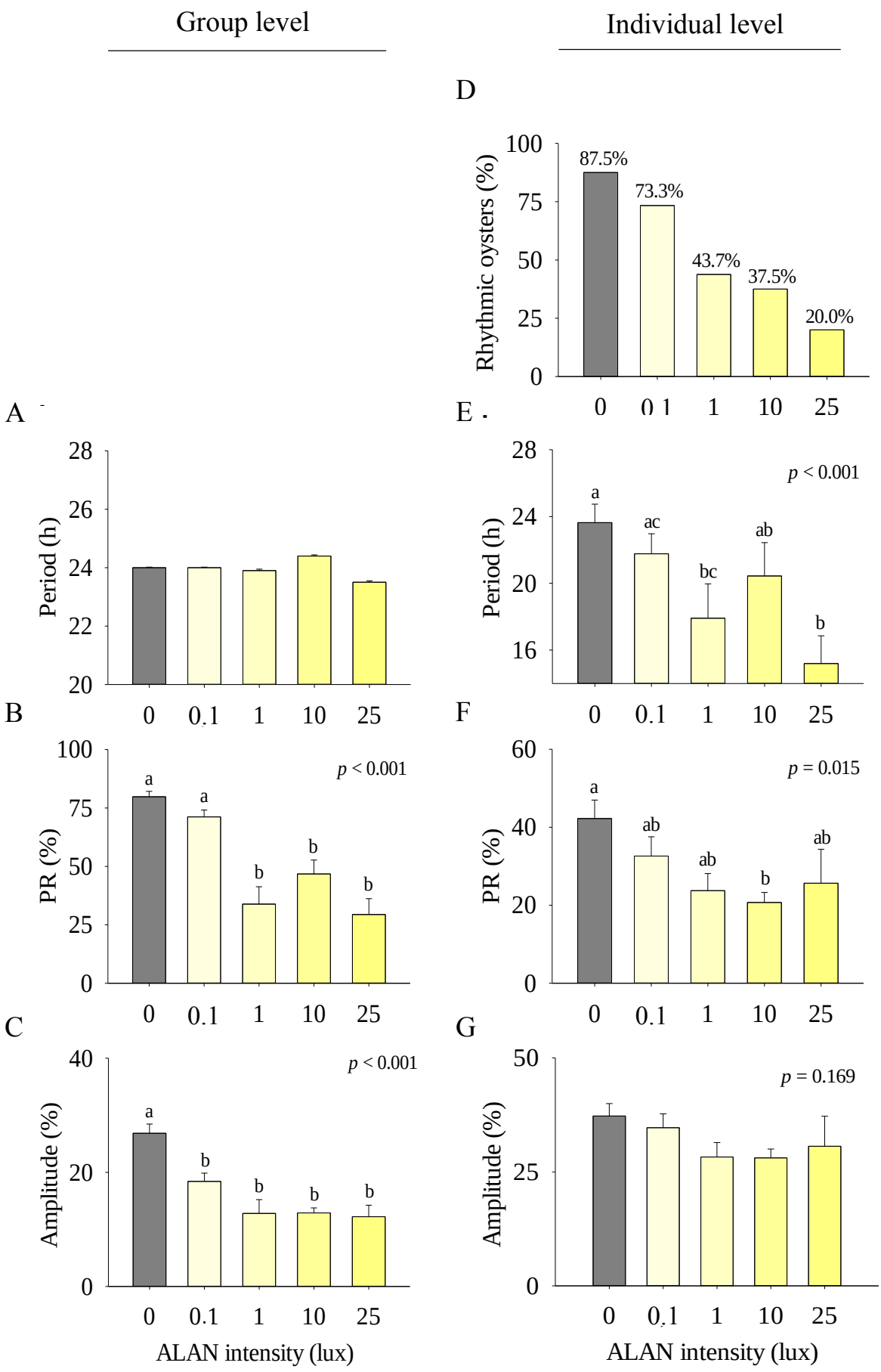

Figure 3. Botté et al.

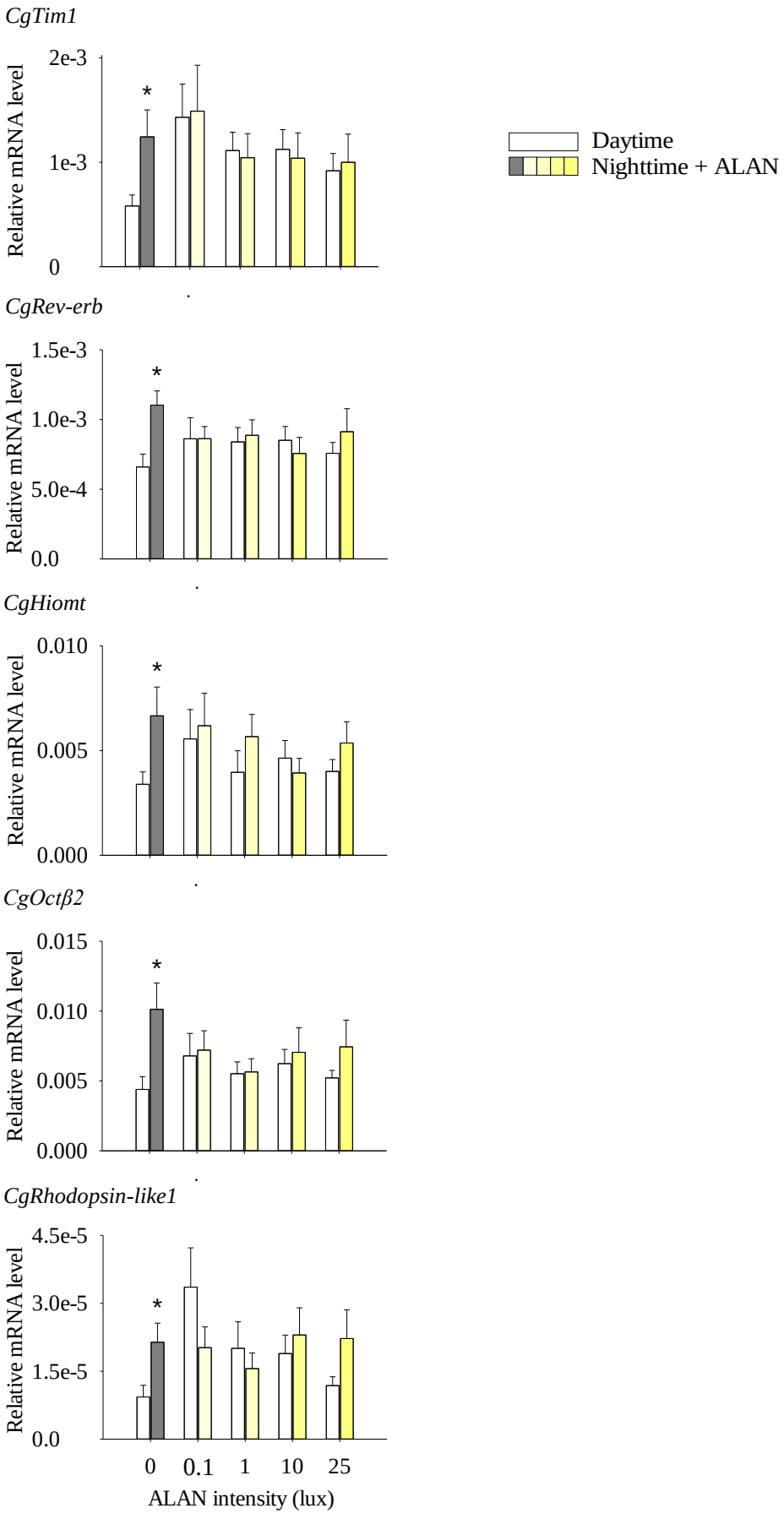

Figure 4. Botté et al.

Supplementary material

Artificial light at night disrupts daily rhythm of the oyster *Crassostrea gigas*

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The following items are provided:

Table S3. Results of chronobiological analysis for the control condition and ALAN conditions at the group level. Rhythmic parameters are expressed as mean ± standard error, where the standard error shows the daily variability (n = 7 days).

Table S4. Results of chronobiological analysis for the control condition and ALAN conditions at the individual level (n = 15-16 oysters / condition). The table indicates for the control condition and ALAN conditions: the period of rhythmic individuals (the periods out of the daily range of 24 – 28 h are in italic); and the percent rhythm (PR, %) and rhythm's amplitude of individuals having a daily rhythm.

Figure S4. Relative mRNA expression level (expressed as mean ± SE) between daytime and nighttime of eight clock and clock-associated genes according to ALAN intensities exposure. White bars indicate the gene level during daytime and, colored bars during nighttime. Differences between dautime and nighttime are significant for a *p*-value = 0.05.

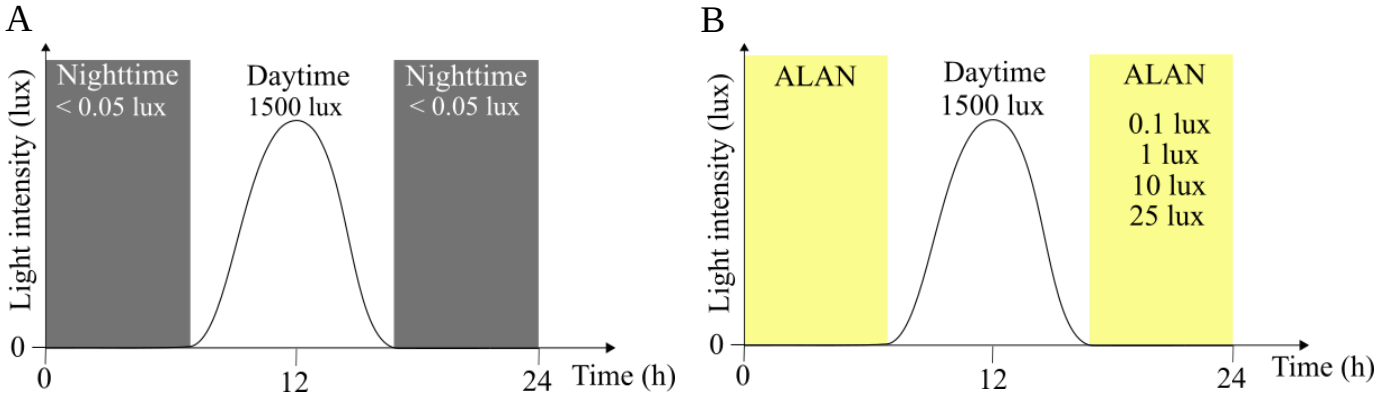

Figure S1. Daily light exposure of the control condition (A) and the ALAN conditions (B).

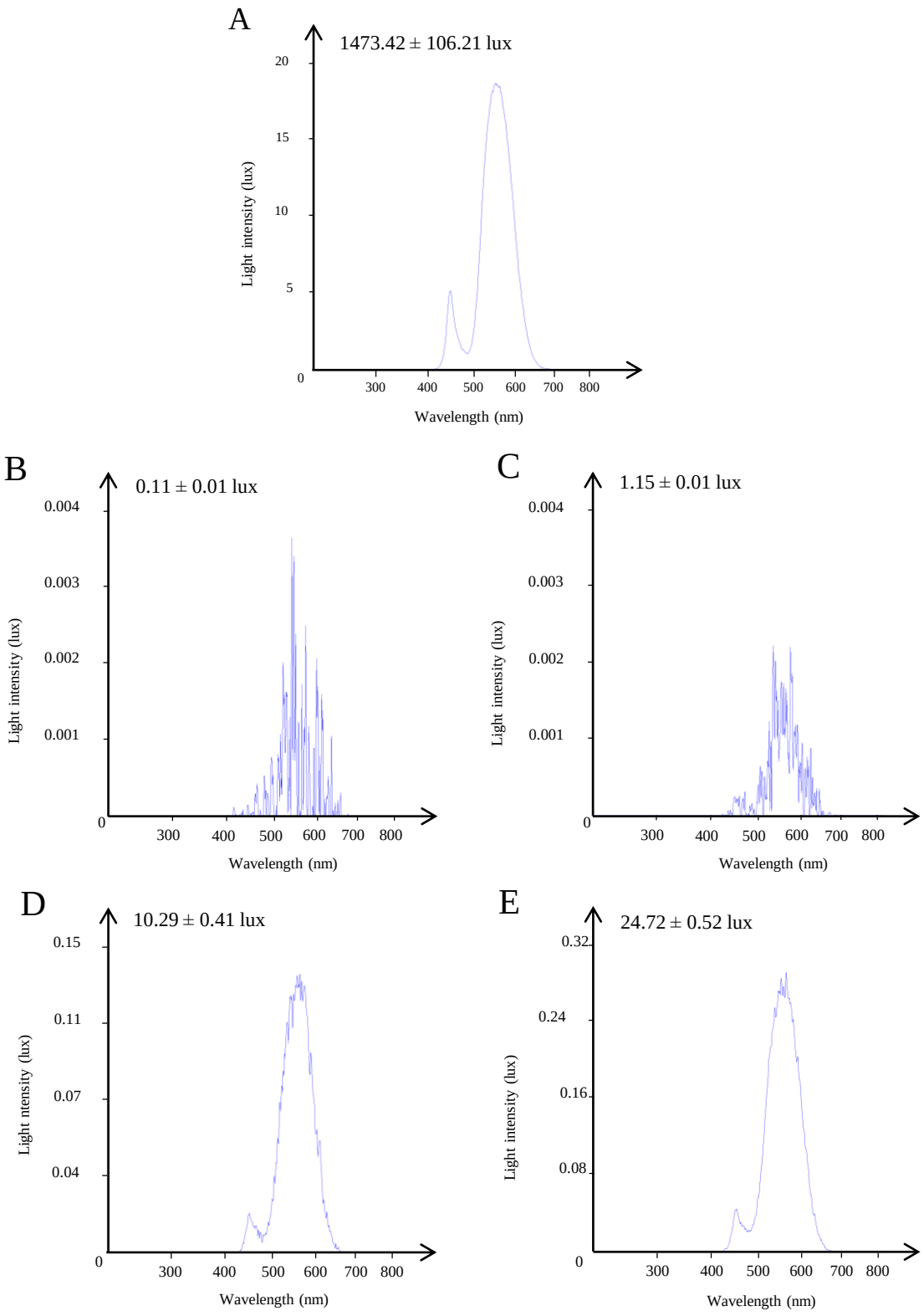

Figure S2. Spectrum of the maximum light intensity during daytime (A), and light spectra of ALAN intensities measured underwater near the oysters: 0.1 lux (B), 1 lux (C), 10 lux (D), and 25 lux (E) conditions.

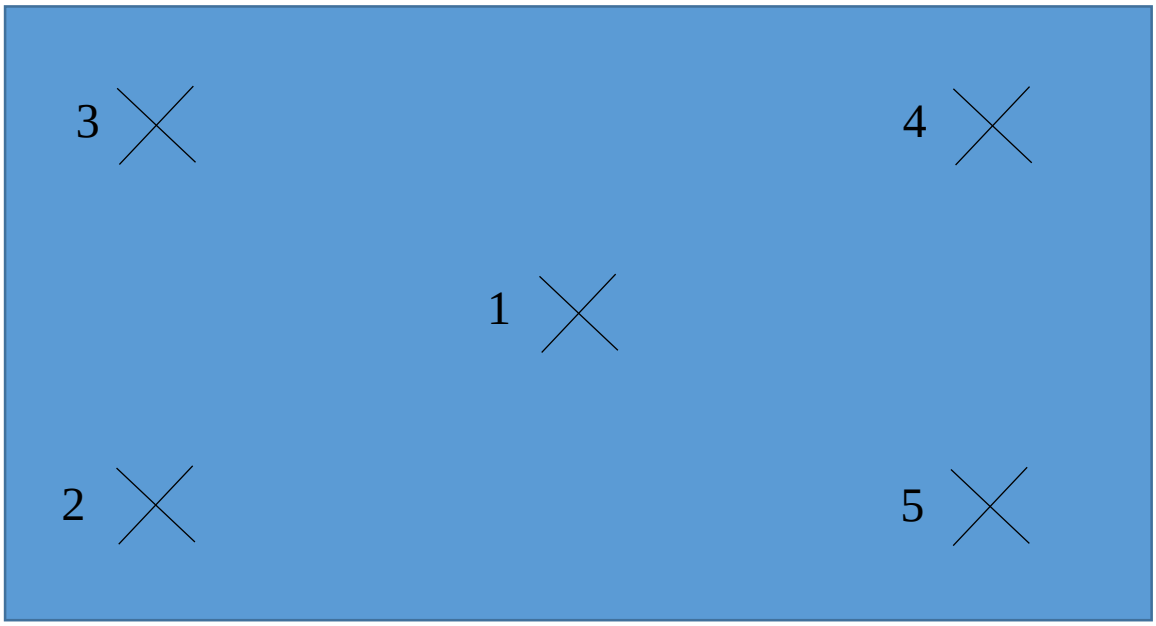

Figure S3. Five locations in the experimental tank used for underwater intensity measurements. The depth of the measure is done at the oyster level.

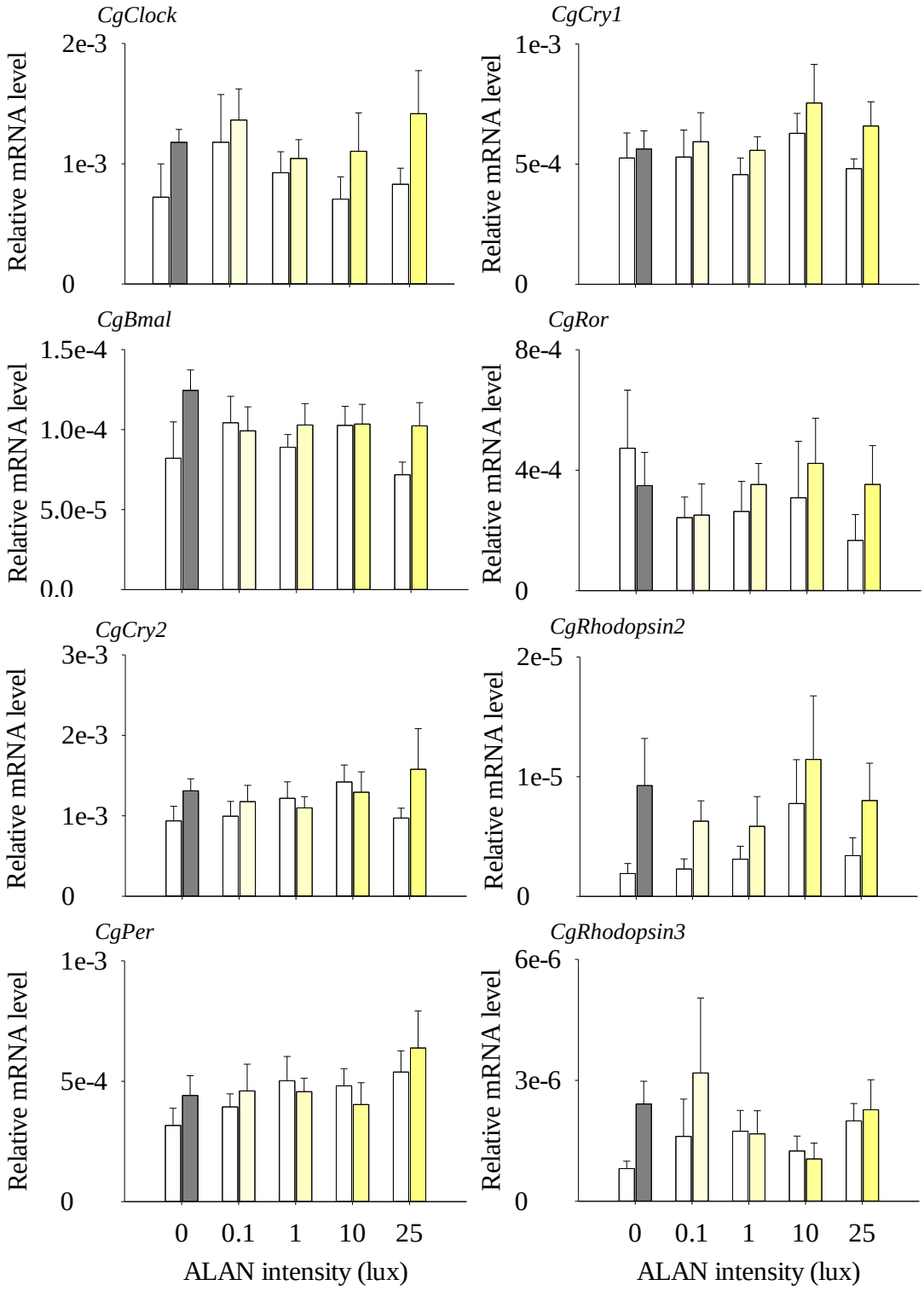

Figure S4. Relative mRNA expression levels (expressed as mean ± SE) between daytime and nighttime of eight clock and clock-associated genes according to ALAN intensities exposure. White bars indicate the gene level during daytime and, colored bars during nighttime. Differences between dautime and nighttime are significant for a *p*-value = 0.05.

Location in the tank	ALAN intensity (lux)				Maximum intensity during daytime (lux)
	0.1 lux	1 lux	10 lux	25 lux	
1	0.12	1.46	11.86	26.64	1813.99
2	0.11	1.23	10.33	23.82	1591.36
3	0.12	1.11	9.79	24.87	1192.62
4	0.11	0.84	9.73	23.94	1407.54
5	0.10	1.11	9.75	24.31	1361.59
Mean ± SE	**0.11 ± 0.01**	**1.15 ± 0.01**	**10.29 ± 0.41**	**24.72 ± 0.52**	**1473.42 ± 106.21**

Table S1. Values of underwater measurements of the maximum light intensity during the daytime and ALAN intensities in the tank for each position in the tank, and the mean ± SE.

GeneBank access	Gene name	Forward primer	Reverse primer
Crassostrea gigas core clock genes			
KX371073	*CgClock (Clock)*	5’-TGGGAATGATGTCCAACAGAG-3’	5’-GGTCCATCAATGACAGGAAGT-3’
KX371075	*CgBmal (Bmal)*	5’-CACAAGTTCAGGTCAGAGTGTAG-3’	5’-TCACCTGAGGTAGACTGGTTAT-3’
KX371074	*CgCryptochrome 2 (Cry2)*	5’-AACCTTACAGCAAGCACGAA-3’	5’-TGACATCTGGCTGTGGTTTC-3’
KX371076	*CgPeriod (Per)*	5’-CCGATGACAGAAATCCCAGTAG-3’	5’-CCATCCTATTCTCCTGCTCTTG-3’
KX371077	*CgTimless 1 (Tim1)*	5’-AAAGATCCCGGACACAGTATG-3’	5’-TGGAACTCGTTCCTGACTTG-3’
KT991835	*CgCryptochrome 1 (Cry1)*	5’-TCATGAAGCAGCTCAGATACG-3’	5’-ACCTCCCAGTTCAACCAAAG-3’
KJ188106	*CgRev-erb (Rev-erb)*	5’-GACTTTGCTGATCGCTTCAAC-3’	5’-CTTTCCAACTGCTCCACATTTC-3’
EKC18621	*CgRor (Ror)*	5’-CTACGTGAGCAGGTGTTTGA-3’	5’-CGTCCGCTATGTCCTTCAAT-3’
Crassostrea gigas clock-controlled genes			
EKC41768	*CgHiomt-like*	5’-CGGGTGGATCAGTGTTAGTAATG-3’	5’-TCTCTTGGCCCTGTGATAGA-3’
XM_011433587	*CgRhodopsin-like 1*	5’-TAGTTCGGCGTCGGAATTTATC-3’	5’-CTGTTCGAATCTCTGCTCTCAC-3’
XM_011448766	*CgRhodopsin-like 2*	5’-CCCTGAGTCATCCCAAATTCA-3’	5’-GATGTTCTCGGCGTAGCTTTA-3’
XM_020065754	*CgRhodopsin-like 3*	5’-TGACTTTGACGGCGATACTG-3’	5’-ATAGATCCGCCACCGAAATG-3’
XM_011427386	*CgOctβ2*	5’-AATCCAGCACACACTCCATAG-3’	5’-TCTGAGTCTCATCTGCGTTTG-3’
Crassostrea gigas housekeeping genes			
AB122066	*CgElongation Factor 1 (Ef1)*	5’-ACCACCCTGGTGAGATCAAG-3’	5’-ACGACGATCGCATTTCTCTT-3’
CAD67717	*CgGadph*	5’-CGTACCAGTTCCAGATGTTTCC-3’	5’-GCCTTGATGGCTGCCTTAATA-3’
Z29546	*Cg28S*	5’-AAACACGGACCAAGGAGTCT-3’	5’-AGGCTGCCTTCACTTTCATT-3’

Table S2. Forward, reverse primers sequences used for Real-Time PCR analysis in *Crassostrea gigas*.

ALAN intensity (lux)	Period (h)	PR (%)	Amplitude (%)
0 lux	24.00 ± 0.02	79.80 ± 0.02	26.86 ± 1.61
0.1 lux	24.00 ± 0.02	71.20 ± 0.03	18.41 ± 1.45
1 lux	23.90 ± 0.04	33.90 ± 0.07	12.79 ± 2.43
10 lux	24.40 ± 0.04	46.80 ± 0.06	12.88 ± 0.87
25 lux	23.50 ± 0.05	29.40 ± 0.07	12.22 ± 2.01

Table S3. Results of chronobiological analysis for the control condition and ALAN conditions at the group level. Rhythmic parameters are expressed as mean ± standard error, where the standard error shows the daily variability (n = 7 days).

ALAN intensity (lux)														
0 lux			0.1 lux			1 lux			10 lux			25 lux		
Period (h)	PR (%)	Amplitude (%)	Period (h)	PR (%)	Amplitude (%)	Period (h)	PR (%)	Amplitude (%)	Period (h)	PR (%)	Amplitude (%)	Period (h)	PR (%)	Amplitude (%)
23.7	62.62	46.7	24.3	38.78	40.2	23.9	20.46	18	23.8	15.40	24.6	20.9	13.67	21.7
23.3	18.57	22.8	23.7	20.28	29.4	23.5	26.14	31.6	22.4	18.10	26.7	23.8	42.58	43.5
24	75.95	56.9	24	38.74	37.2	24.1	15.37	25.9	23.5	17.51	24.7	22.8	20.74	26.7
25.9	24.84	28.5	23.9	60.09	49.8	22.9	16.00	25.5	25.3	18.12	26.3	*12.3*	–	–
23.7	47.88	38.8	23.7	18.23	26.4	23.4	15.35	25.2	24	32.67	37.2	*7.55*	–	–
24	28.21	25.7	24.1	16.64	27.4	23.3	24.82	26.7	24.4	22.51	29	*10.1*	–	–
24	36.84	38.7	24.2	60.73	51.1	24	48.08	45	*8.6*	–	–	*12.2*	–	–
24	61.35	49.9	23.8	26.86	28.5	*11.8*	–	–	*17.4*	–	–	*17.1*	–	–
23.9	62.35	48.7	24	16.71	19.6	*7.96*	–	–	*33.2*	–	–	*12.1*	–	–
25.1	33.97	28.9	24.9	39.95	41.8	*9.69*	–	–	*18.1*	–	–	*10.9*	–	–
24	23.76	30.8	23.1	21.88	30.3	*12*	–	–	*12*	–	–	*17.4*	–	–
24.2	41.95	37.1	*16.5*	–	–	*8.37*	–	–	*12.6*	–	–	n.s.	–	–
24.3	46.16	37.8	*12.5*	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–
24	26.95	30.3	*12.1*	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–
31.4	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–
8.7	–	–				n.s.	–	–	n.s.	–	–			

Table S4. Results of chronobiological analysis for the control condition and ALAN conditions at the individual level (n = 15-16 oysters / condition). The table indicates for the control condition and ALAN conditions: the period of rhythmic individuals (the periods out of the daily range of 24 – 28 h are in italic); and the percent rhythm (PR, %) and rhythm's amplitude of individuals having a daily rhythm.