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

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Crassostrea gigas

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21 **Abstract**

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3 22 Artificial Light At Night (ALAN) masks the natural light cycles and thus can disturb the
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5 23 synchronization of organisms' biological rhythms with their environment. Although
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8 24 coastlines are highly exposed to this growing threat, studies concerning the impacts of ALAN
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10 25 on coastal organisms remain scarce. In this study, we investigated the ALAN exposure effects
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12 26 at environmentally realistic intensities (0.1, 1, 10, 25 lux) on the oyster *Crassostrea gigas*, a
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15 27 sessile bivalve subject to light pollution on shores. We focused on the effects on oyster's daily
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17 28 rhythm at behavioral and molecular levels. Our results showed that ALAN disrupts the
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20 29 oyster's daily rhythm by increasing valve activity and annihilating day / night differences of
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22 30 expression of circadian clock and clock-associated genes. ALAN effects occur starting from
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25 31 0.1 lux, in the range of artificial skyglow illuminances. We concluded that realistic ALAN
26
27 32 exposure affects oysters' biological rhythm, which could lead to severe physiological and
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30 33 ecological consequences.

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33 34 Keywords: ALAN, biological rhythm, circadian clock, *Crassostrea gigas*, behavior
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44 Introduction

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3 45 Natural light cycles play an important role in biological timings (Gaston et al., 2017). Indeed,
4
5 46 predictable variations of light intensity over a day, a month, or a year are used as signals by
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7 47 organisms to synchronize their biological rhythms with their environment (Bradshaw and
8
9 48 Holzapfel, 2010; Gaston et al., 2017). These biological rhythms are ubiquitous and find their
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11 49 origin in each cell with an endogenous clock, which uses environmental cues, such as natural
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13 50 light cycles, to synchronize organisms' physiological processes and behavior with their
14
15 51 environment. This synchronization enables organisms to be fully adapted to their environment
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17 52 and to anticipate its changes (Cermakian and Sassone-Corsi, 2000; Partch et al., 2014).
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19 53 However, Artificial Light At Night (ALAN) affects the natural nocturnal lighting levels,
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21 54 which can disrupt the organisms' perception of natural variations of light, and affect
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23 55 organisms' biological rhythms (daily, lunar, seasonal) and their physiology (Falchi et al.,
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25 56 2011; Gaston and Bennie, 2014). For example, it can disrupt migration, reproduction,
26
27 57 foraging, or prey/predator interactions (Davies et al., 2014; Gaston et al., 2017; Longcore and
28
29 58 Rich, 2004; Navara and Nelson, 2007). These ALAN's effects on organisms' physiology and
30
31 59 behavior occur in a large range of species, including diurnal ones, and at low intensities
32
33 60 (below 1 lux) (Sanders et al., 2021). Thus ALAN can have consequences at the individual,
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35 61 population, and ecosystem scale. Nocturnal artificial lighting affects the whole world (Falchi
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37 62 et al., 2016) and spreads fast with a 6% increase in the sky luminosity per year (Hölker et al.,
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39 63 2010). The negative effects of ALAN on organism behavior or physiology become
40
41 64 increasingly studied over the years (Davies and Smyth, 2018) but they remain widely
42
43 65 investigated for terrestrial organisms. However, ALAN effects on marine organisms, and
44
45 66 especially benthic organisms, recently received more attention. Studying the impacts of
46
47 67 ALAN on species living in coastal ecosystems is of great importance considering that already
48
49 68 22.2 % of the world's coasts are exposed to ALAN as well as 35% of marine protected areas
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69 (Davies et al., 2014, 2016), percentages that certainly will increase since the human
70 population living in coastal areas is predicted to grow (Kummu et al., 2016). Furthermore,
71 ALAN's negative effects could be worsened by the increasing use of LED (Light-Emitting
72 Diode) in public lighting (Gaston, 2018; Zisis and Bertoldi, 2018). Indeed, LED lights
73 spectrum contains more short-wavelength than spectra of other lighting types such as HPS
74 (High-Pressure Sodium) lighting (Bierman, 2012; Falchi et al., 2011; Longcore et al., 2018;
75 Luginbuhl et al., 2014). Considering that short-wavelength, such as blue wavelength, go
76 further into seawater (Davies et al., 2014; Grubisic et al., 2019) and that marine organisms
77 would be highly sensitive to blue wavelength (Grubisic, 2018), the negative effects of ALAN
78 could be amplified on the coastal fauna. Therefore, ALAN becomes over the years a growing
79 threat to marine life.

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

93 could make them vulnerable to disruption by ALAN, which could have consequences on their
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65
94 physiology and ecology.

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

107 **Materials and methods**

108 *General conditions*

109 The experiment was conducted from January to April 2021 in the Marine Station of Arcachon
110 on 160 oysters (85.8 ± 0.7 mm shell length; 48.4 ± 0.5 mm shell width; mean \pm SE) coming
111 from an oyster's farm source of the Arcachon bay (France). During the acclimation and
112 experimental duration, oysters were placed into tanks (L x W x H: 74.8 x 54.8 x 40.8 mm)
113 continuously supplied with natural seawater from the Arcachon bay, which was filtered (<
114 $1\mu\text{m}$), oxygenized, and the temperature was monitored ($T = 15.0 \pm 0.1$ °C). Tanks were
115 placed in an isolated room and equipped with an antivibration bench to minimize external
116 disturbances to animal behavior. The oysters were not fed during the experiment.

117 *Experimental protocol*

1
2 118 Oysters were maintained under a L:D 10:14 cycle with daytime from 7:00 to 17:00 h (all
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5 119 times noted in local time, UTC +1) (Fig. S1A). The light intensity during daytime varied
6
7 120 gradually to mimic the natural cycle of light using programmable white (413–688 nm, peak at
8
9 121 551 nm; Fig. S2A) LED light bars (MH3SP3 DSunY). The maximum intensity during
10
11 122 daytime was 1473.42 ± 106.21 lux (mean \pm SE) (Tab. S.1) between 11:30 to 12:30 h. After 7
12
13 123 to 9 days of acclimation to the experimental setup, the proper experiment started for a
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15 124 duration of 7 days. There were 5 conditions: a control condition and 4 conditions where
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17 125 oysters were exposed to several ALAN intensities (Tab. S1): 0.1, 1, 10, and 25 lux. In the 4
18
19 126 ALAN conditions, oysters were exposed to ALAN from 17:30 to 6:30 h (Fig. S1B) using
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21 127 white (411–687 nm, peak at 563 nm; Fig. S2B–S2E) LED strips (MiBoxer Mi-Light WL5).
22
23 128 Illuminances underwater were measured at five positions in the tank (Fig. S3) using a
24
25 129 handheld spectroradiometer (Blue-Wave UVN-100, StellarNet Inc.). The control group was in
26
27 130 the complete dark at night, with a light intensity inferior to the detection limit of the
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29 131 spectroradiometer (0.05 lux), mentioned in the study as 0 lux for convenience. For each of the
30
31 132 5 experimental conditions, 32 oysters were placed in the same tank, and the valve activity of
32
33 133 16 of them was continuously measured using a High Frequency–Non Invasive (HFNI)
34
35 134 valvometer technology (Andrade et al., 2016). For each experimental conditions, 32 different
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37 135 oysters were used. On the 7th day of the experiment, the gill tissue of 8 oysters were sampled
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39 136 during daytime at 12:00 h and during nighttime at 19:00 h and stored in Tri Reagent
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41 137 (Invitrogen) at -80 °C for further molecular analysis.
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51 *Behavioral measurements*

52 138 For valve activity measurement using HFNI valvometer technology, lightweight's
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54 139 electromagnets were glued on each valve of oysters and linked to a valvometer device using
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56 140 flexible wires (Andrade et al., 2016). The electromagnetic current generated between the two
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142 electromagnets allows to measure oyster valve activity. For each individual, the signal was
1 recorded every 4.8s. Data were processed using Labview 8.0 (National Instrument, Austin,
2 143 TX, USA). The study focused on the hourly Valve Opening Duration (VOD) meaning that we
3
4 144 determined the percentage of time each individual spent with its valve open for each hour. For
5
6 145 example, an individual with its valves open during a whole hour corresponds to a VOD of 100
7
8 146 %, while an individual with its valves closed during one hour corresponds to a VOD of 0 %.
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148 *Chronobiological analyses*

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

166 *Total RNA extraction and cDNA synthesis*

1
2 167 Total RNA was extracted from gills using Tri reagent (Invitrogen) and an SV Total RNA
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5 168 Isolation System kit (Promega). The total RNA quantity and quality were assessed by
6
7 169 spectrophotometry (OD230, OD260, OD280). RNA reverse transcription was realized using
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10 170 Goscript™ Reverse Transcription System kits (Promega).

13 171 *mRNA expression analysis by Real-Time PCR*

15 172 Real-Time qPCR was realized using GoTaq® qPCR Master Mix kit (Promega). Primers sets
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18 173 of clock and clock-associated genes (*CgClock*, *CgBmal*, *CgCry*, *CgPer*, *CgTim1*, *CgCry1*,
19
20 174 *CgRev-erb*, *CgRor*, *CgHiomt-like*, *CgOctβ2*, *CgRhodopsin-like 1*, *CgRhodopsin-like 2*,
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22
23 175 *CgRhodopsin-like 3*), and housekeeping genes (*CgEfl*, *Cg28S*, *CgGadph*) are listed in the
24
25 176 table S2. qPCR reactions were realized as follows: 95 °C for 2 min to activate the GoTaq
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27
28 177 polymerase, 40 cycles of 95 °C for 15 seconds (denaturation), and 60 °C for 1 minute
29
30 178 (annealing and extension) for amplification of target cDNA, followed by 2 minutes at 60 °C
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33 179 for the final elongation. In the end, melting curves were generated by gradually decreasing the
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35 180 temperature from 95 °C to 60 °C to control the primer specificity. The comparative Ct method
36
37 181 $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) was used to determine the relative transcript level of clock
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39
40 182 and clock-associated genes, where $\Delta Ct = Ct(\text{target gene}) - Ct(\text{housekeeping gene})$. Gene's
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42 183 expression was normalized with the geometric mean of the housekeeping genes *CgEfl* and
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44
45 184 *Cg28S*, based on stability values (Xie et al., 2012).

48 185 *Statistical analysis*

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51 186 All statistical analysis were performed using SigmaPlot software (version 13.0; Systat
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53 187 Software, USA). T-tests were performed for two groups' comparisons after checking
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56 188 assumptions (normality of data and equal variance) and if they were not validated the non-
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58 189 parametric Mann-Whitney rank sum test was performed. Two groups' comparisons were used
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60 190 for molecular analysis results. For multiple comparisons, one-way ANOVA were used after
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191 checking assumptions (normality of data and equal variance). When assumptions were not
192 validated, the non-parametric Kruskal-Wallis One-Way ANOVA on ranks test was
193 performed. In case of significant differences, a Tukey test was realized for all pairwise
194 multiple comparisons unless the groups did not have equal size, in which case the Dunn's test
195 was realized. Multiple comparisons were performed for behavioral analyses. On the whole
196 molecular data, a two-way ANOVA was also performed after checking assumptions
197 (normality of data and equal variance), and in case of significant differences, a Tuckey test
198 was used for all pairwise multiple comparisons. For all test results, a difference was
199 considered significant when $p < 0.05$.

200

201 **Results**

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

216 results in Table S3 and Table S4). At the group level, a significant daily rhythm persists in all
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2 217 conditions with no significant differences of period length between the conditions (Fig. 3A).
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4 218 However, ALAN affected several parameters of this rhythm. Indeed, there is a significant
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7 219 decrease ($p < 0.001$) of the percent rhythm (PR, %) of the daily rhythm when oysters are
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10 220 exposed to ALAN starting from 1 lux (Fig. 3B). Furthermore, the amplitude of the daily
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12 221 rhythm decreases significantly ($p < 0.001$) in all ALAN conditions, starting from 0.1 lux and
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14 222 without significant ALAN intensity-effect (Fig. 3C). At the individual level, results show an
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16 223 ALAN intensity-dependent decrease of the percentage of oysters having a significant daily
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18 224 rhythm (Fig. 3D). Figure 3E shows a decrease ($p < 0.001$) of the individual rhythmic period
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21 225 lengths, getting out the daily range for many individuals exposed to ALAN (Table S3). The
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23
24 226 mean period of oysters exposed to 1 lux and 25 lux are 17.9 ± 2.1 and 15.2 ± 1.7 h
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26 227 respectively, under the circadian range (20-28 h), explaining the global loss of rhythmicity at
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28
29 228 the daily scale shown in figure 3D. Furthermore, the PR of oysters having a daily rhythm
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31 229 tends to decrease starting from 0.1 lux, and it decreases significantly ($p = 0.015$) when they
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34 230 are exposed to 10 lux (Fig. 3F). Finally, the amplitude of oysters having a daily rhythm show
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36 231 no significant differences ($p = 0.169$) but a trend to decrease when oysters are exposed to
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38
39 232 ALAN (Fig 3G). Figures 4 and S4 shows the mRNA level during daytime and nighttime of
40
41 233 thirteen circadian clock (*CgClock*, *CgBmal*, *CgCry*, *CgPer*, *CgTim1*, *CgCry1*, *CgRev-erb*,
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43 234 *CgRor*) and clock-associated (*CgHiomt-like*, *CgOctβ2*, *CgRhodopsin-like 1*, *CgRhodopsin-*
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45 235 *like 2*, *CgRhodopsin-like 3*) genes for all of the tested ALAN intensities. No significant
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48 236 differences of gene expression are observed between the control and the ALAN conditions
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51 237 neither at daytime nor at nighttime. However, results show significant differences of mRNA
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53 238 expression levels between daytime and nighttime in the control condition for five genes (two
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55 239 circadian clock genes: *CgTim1* and *CgRev-erb*; and three clock-associated genes: *CgHiomt-*
56
57 240 *like*, *CgOctβ2* and *CgRhodopsin-like 1*), with an increase of expression during nighttime (Fig.

241 4). This daytime / nighttime difference of gene expression significantly disappears in all of the
1
2 242 five genes for all ALAN intensities tested. Finally, the table 1 shows the results of a two-way
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4 243 ANOVA, testing the effects of the period of the day (daytime and nighttime), ALAN
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7 244 intensities, and their interaction on the overall relative mRNA levels of the thirteen genes
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10 245 studied. These results reveal a significant difference ($p = 0.046$) of the overall mRNA level
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12 246 between daytime and nighttime only for the control condition ($p = 0.014$). In all ALAN
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14 247 conditions, the daytime / nighttime difference of gene expression is abolished.
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20 249 **Discussion**

23 250 Our study shows that ALAN affects the daily behavioral rhythm and the molecular clock of
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25 251 oysters starting from the lowest ALAN intensity tested, 0.1 lux, in the range of artificial
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27 252 skyglow. Multiples ALAN's effects on oyster's behavioral rhythm are observed: a decrease of
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29 253 the percentage of oysters having a daily rhythm, a decrease of the rhythm's robustness, a
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31 254 decrease of the rhythmic amplitude, an increase of the mesor (mean VOD), and a shift from a
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33 255 diurnal to a nocturnal peak activity. At the molecular level, the overall difference of clock and
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35 256 clock-associated gene expression observed in control condition between daytime and
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37 257 nighttime is abolished when oysters are exposed to ALAN. While a clear ALAN intensity-
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39 258 effect is observed at the individual level regarding the percentage of oysters having a daily
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41 259 behavioral rhythm, most of ALAN's effects at both behavioral and molecular levels occur
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43 260 from 0.1 to 25 lux without significant intensity-effect.
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49 261 We showed that ALAN exposure leads to an increase of oysters' VOD, explained by an
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51 262 increase of activity during nighttime leading to a decrease (at 0.1 lux) or a loss (at 1, 10, and
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53 263 25 lux) of daytime / nighttime differences of valve behavior and a shift of peak activity from
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55 264 daytime to nighttime. Activity increase during nighttime as an effect of ALAN has been also
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57 265 shown in other marine organisms, such as the diurnal fish *Girella laevis* exposed to 70 lux
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266 at night (Pulgar et al., 2019). On the contrary, the nocturnal crayfish *Pacifastacus leniusculus*
267 showed a decrease of activity during nighttime when exposed to ALAN at 12 lux using a HPS
268 streetlight bulb (Thomas et al., 2016). ALAN effects on organisms' behavior have also been
269 investigated in juvenile gastropods *Concholepas concholepas*, in which ALAN at an intensity
270 of 329.9 lux (LED technology) induces a less efficient search for prey during nighttime
271 (Manriquez et al., 2019). Moreover, some previous results of bivalve behavior disruption by
272 ALAN was shown by Christoforou (2022), which investigated the effects of several ALAN
273 wavelengths at an intensity of 19.86 lux using LED lightings on the behavior of the mussel
274 *Mytilus edulis*. The exposure of mussels to white ALAN induces a decrease during both
275 nighttime and daytime of the open/close frequency valve events. However, in this study, no
276 chronobiological analysis were performed and the relatively invasive experimental device
277 used to record mussels' behavior could have mask ALAN effects on mussels valve activity. In
278 our study, we went further in the investigation of ALAN's impacts on daily behavioral rhythm
279 using chronobiological analysis and lower ALAN intensities, using a non-invasive biosensor
280 (Andrade et al., 2016).

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

291 conducted in winter, when the food supply is very low in the environment. Moreover, the
1
2 292 rhythm at the daily scale could not be the only rhythm that can be disrupted by ALAN. It has
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4
5 293 been shown that oysters have other biological rhythms. For example, oysters showed a lunar
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7 294 rhythm i.e. their valve behavior is synchronized with lunar cycles by detecting moonlight
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10 295 illumination cycle (Payton and Tran, 2019). This lunar rhythm could be affected by ALAN
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12 296 since the maximal intensity of the moonlight ranges from 0.1 to 0.3 lux (Rich and Longcore,
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14 297 2006), thus the natural variations of moonlight intensity can be easily masked by ALAN
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17 298 intensities, such as the skyglow. The masking effect of lunar cycles by ALAN using LED has
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19 299 been investigated in the corals *Acropora millepora* and *Acropora digitifera*, in which
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21
22 300 exposition to ALAN delayed or masked their gametogenesis leading to a spawning
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24 301 desynchronization (Ayalon et al., 2021). Another example of ALAN effect on lunar rhythm is
25
26 302 the disruption of the monthly nocturnal foraging pattern of the gastropod *Nucella lapillus*,
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29 303 based on the lunar cycle, by ALAN produced by LED lighting at an intensity of 10 lux (Tidau
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31 304 et al., 2022). Moreover, oysters' seasonal rhythms could be disrupted by ALAN. Oysters use
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34 305 the variation of photoperiod through the year to anticipate seasonal changes and synchronize
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36 306 physiological processes such as behavior, growth, and spawning (Bernard et al., 2016; Payton
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38
39 307 et al., 2017b). However, ALAN being often present during dusk and dawn, it can mask these
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41 308 two natural phenomena, preventing oysters to detect the variation of photoperiod along the
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44 309 year, which could affect several seasonal physiological processes and decrease the fitness of
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46 310 organisms (Gaston et al., 2017). ALAN's effects on organisms' physiological processes and
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48
49 311 fitness have been shown for example in the amphipod *Orchestoidea tuberculata*, in which
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51 312 ALAN (halogen light) induces a decrease of growth (Luarte et al., 2016), and in barnacles, in
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53 313 which ALAN (LED lighting) affects the settlement process (Lynn et al., 2021; Manriquez et
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55
56 314 al., 2021). Moreover, ALAN's effects on organisms' fitness have also been observed in the
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58 315 juvenile crabs *Neohelice granulata* since it increases mortality, mainly by increasing
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316 cannibalistic interactions with adults at an intensity of approximately 40 lux (LED lighting)
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2 317 (Nunez et al., 2021).
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5 318 In addition to the effects on the oyster's behavioral daily rhythm, this study also showed that
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7 319 ALAN disturbs the circadian clockwork of *C. gigas* by annihilating differences between
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9 320 daytime and nighttime in clock and clock-associated genes expression starting from the
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11 321 intensity 0.1 lux. Genes for which their expression was mainly affected by ALAN are
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13 322 *CgTim1*, *CgRev-erb*, *CgHiomt-like*, *CgOctβ2*, and *CgRhodopsin-like 1*. *CgTim1* and *CgRev-*
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15 323 *erb* are both involved in the regulation's feedback loops of the oysters' molecular clock and
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17 324 thus are core clock genes (Perrigault and Tran, 2017). On the other hand, *CgHiomt-like*,
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19 325 *CgOctβ2*, and *CgRhodopsin-like 1* are clock-associated genes. The first one is a homologue of
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21 326 the mammal gene *Hiomt* involved in the final step of the synthesis of melatonin, known to be
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23 327 a key molecule of the day / night rhythm and a potent antioxidant with a proposed role in
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25 328 immune function (Jones et al., 2015; Pevet et al., 1980). *CgOctβ2* is the gene of the
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27 329 octopamine receptor β-2R. Octopamine is a biogenic amine well studied in arthropods and
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29 330 gastropods where it functions as a neurotransmitter and hormone, fulfilling the roles played
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31 331 by adrenalin in vertebrates. It is an extremely pleiotropic substance, participating in
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33 332 invertebrate development such as growth, maturation, and reproduction by activating their
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35 333 corresponding G protein-coupled receptors (GPCRs), and may interact with the circadian
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37 334 clock (Roeder, 2020). Finally, the last gene to show a disrupted expression in presence of
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39 335 ALAN is the gene of the rhodopsin (*CgRhodopsin-like 1*), a photoreceptor involved in
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41 336 oysters' light-sensitivity (Wu et al., 2018). Thus, ALAN affects five of the thirteen genes
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43 337 studied here. This relative low transcriptional response observed might be due to the fact that
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45 338 oysters have been sampled at only two sampling times over the daily cycle. Indeed, a stronger
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47 339 transcriptional disruption might possibly have been revealed by sampling at another time of
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49 340 the day. However, our results still show that ALAN can affect genes of the molecular clock of
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341 *C. gigas* (*CgTim1*, *CgRev-erb*), suggesting that the observed disruption of oyster's daily
342 behavioral rhythm would be a real consequence of the circadian clock disruption and not only
343 a direct reaction of light perception in presence of ALAN. ALAN also affects essential clock-
344 associated genes (*CgHiomt*, *CgOctβ2*, *CgRhodopsin-like 1*) involved in physiological
345 processes regulation. Thus, we can imagine that, by cascading effect, the expression of other
346 genes involved in several physiological functions can also be disrupted by ALAN. For
347 example, ALAN at 0.1–0.3 lux using a fluorescent bulbs induces the suppression of the
348 increase of melatonin during nighttime in *Salvelinus* (Liu et al., 2019). Moreover, in the
349 European perch *Perca fluviatilis* and the roach *Rutilus rutilus* the impairment of circadian
350 melatonin pattern by ALAN (HPS lamps) is associated with a decrease of the mRNA
351 expression of gonadotropins, which could disrupt their reproduction (Bruning et al., 2018).

352

353 **Conclusion**

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

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2 366 **Ethics:** All experiments complied with the laws in effect in France and they conformed to
3 international ethical standards.

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5 368 **Data accessibility:** The data underlying this study are available on Supplementary data.

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7
8 369 **Author contributions:** Study design and methodology, D.T., A.B., L.P.; experimentation,
9 A.B., D.T.; molecular analysis: A.B., L.P.; data treatment, A.B.; interpretation, A.B., D.T.,
10 370 L.P.; manuscript writing, A.B.; review and editing, all authors; funding, D.T. All authors
11 371 contributed critically to the drafts, and gave final approval for publication.
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21

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36 setup of the experiments.
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554 **Figures & legends**

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3 555 **Figure 1.** Behavioral daily pattern of oysters ($n = 15\text{--}16$ oysters) in control condition (0 lux)
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5 556 and exposed to different ALAN intensities (0.1 lux, 1 lux, 10 lux, and 25 lux). Mean hourly
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8 557 VOD data are shown as mean \pm SE ($n = 7$ days). White backgrounds indicate daytime and
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10 558 yellow and black backgrounds indicate nighttime. Arrows indicate the VOD peak during the
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16 560 **Figure 2.** Effect of ALAN intensity on (A) the mean daily valve opening duration (VOD),
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18 561 and (B) the mean VOD during the daytime and the nighttime. Data are expressed in mean \pm
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21 562 SE ($n = 7$ days). Different letters indicate significant differences between ALAN conditions
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23 563 and between daytime and nighttime ($p < 0.05$).

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26 564 **Figure 3.** Effect of ALAN intensity on oysters daily rhythm characteristics: (A) the period at
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29 565 group level, (B) the percent rhythm (PR) at group level, (C) amplitude of the rhythm at group
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31 566 level, (D) the percentage of oysters having a daily rhythm, (E), period of individual rhythmic
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34 567 oysters, dotted lines define the daily range, (F) the PR at individual level of oysters having a
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36 568 daily rhythm, (G) amplitude of the rhythm at the individual level of oysters having a daily
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39 569 rhythm. Data are expressed in mean \pm SE. For A-C, the SE shows the daily variability of the
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41 570 group's significant rhythm parameters ($n = 7$ days). For E-G the SE shows the individual
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43 571 variability ($n = 15\text{--}16$ oysters). Different letters indicate significant differences ($p < 0.05$).

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46 572 **Figure 4.** Effect of ALAN intensity on the difference in relative mRNA expression level
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49 573 (mean \pm SE, $n = 8$) on oyster gills tissues, between daytime and nighttime of five clock and
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51 574 clock-associated genes. White bars indicate the gene level during daytime and, colored bars
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54 575 during nighttime. Asterisks indicate significant differences between daytime and nighttime (p
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56 576 < 0.05).

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578 **Table 1.** Two-Way Analysis of Variance testing the effect of Daytime/Nighttime and the
 579 ALAN intensity (0 lux, 0.1 lux, 1 lux, 10 lux, 25 lux) on the relative mRNA expression level
 580 on gill tissues of 13 clock and clock-associated genes. In bold, 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		

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

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3 *Crassostrea gigas*

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21 **Abstract**

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3 22 Artificial Light At Night (ALAN) masks the natural light cycles and thus can disturb the
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5 23 synchronization of organisms' biological rhythms with their environment. Although
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8 24 coastlines are highly exposed to this growing threat, studies concerning the impacts of ALAN
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10 25 on coastal organisms remain scarce. In this study, we investigated the ALAN exposure effects
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13 26 at environmentally realistic intensities (0.1, 1, 10, 25 lux) on the oyster *Crassostrea gigas*, a
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15 27 sessile bivalve subject to light pollution on shores. We focused on the effects on oyster's daily
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18 28 rhythm at behavioral and molecular levels. Our results showed that ALAN disrupts the
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20 29 oyster's daily rhythm by increasing valve activity and annihilating day / night differences of
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23 30 expression of circadian clock and clock-associated genes. ALAN effects occur starting from
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25 31 0.1 lux, in the range of artificial skyglow illuminances. We concluded that realistic ALAN
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27 32 exposure affects oysters' biological rhythm, which could lead to severe physiological and
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30 33 ecological consequences.

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33 34 Keywords: ALAN, biological rhythm, circadian clock, *Crassostrea gigas*, behavior
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44 **Introduction**

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3 45 Natural light cycles play an important role in biological timings (Gaston et al., 2017). Indeed,
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5 46 predictable variations of light intensity over a day, a month, or a year are used as signals by
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7 47 organisms to synchronize their biological rhythms with their environment (Bradshaw and
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9 48 Holzapfel, 2010; Gaston et al., 2017). These biological rhythms are ubiquitous and find their
10
11 49 origin in each cell with an endogenous clock, which uses environmental cues, such as natural
12
13 50 light cycles, to synchronize organisms' physiological processes and behavior with their
14
15 51 environment. This synchronization enables organisms to be fully adapted to their environment
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17 52 and to anticipate its changes (Cermakian and Sassone-Corsi, 2000; Partch et al., 2014).
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19 53 However, Artificial Light At Night (ALAN) affects the natural nocturnal lighting levels,
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21 54 which can disrupt the organisms' perception of natural variations of light, and affect
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23 55 organisms' biological rhythms (daily, lunar, seasonal) and their physiology (Falchi et al.,
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25 56 2011; Gaston and Bennie, 2014). For example, it can disrupt migration, reproduction,
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27 57 foraging, or prey/predator interactions (Davies et al., 2014; Gaston et al., 2017; Longcore and
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29 58 Rich, 2004; Navara and Nelson, 2007). These ALAN's effects on organisms' physiology and
30
31 59 behavior occur in a large range of species, including diurnal ones, and at low intensities
32
33 60 (below 1 lux) (Sanders et al., 2021). Thus ALAN can have consequences at the individual,
34
35 61 population, and ecosystem scale. Nocturnal artificial lighting affects the whole world (Falchi
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37 62 et al., 2016) and spreads fast with a 6% increase in the sky luminosity per year (Hölker et al.,
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39 63 2010). The negative effects of ALAN on organism behavior or physiology become
40
41 64 increasingly studied over the years (Davies and Smyth, 2018) but they remain widely
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43 65 investigated for terrestrial organisms. However, ALAN effects on marine organisms, and
44
45 66 especially benthic organisms, recently received more attention. Studying the impacts of
46
47 67 ALAN on species living in coastal ecosystems is of great importance considering that already
48
49 68 22.2 % of the world's coasts are exposed to ALAN as well as 35% of marine protected areas
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69 (Davies et al., 2014, 2016), percentages that certainly will increase since the human
70 population living in coastal areas is predicted to grow (Kummu et al., 2016). Furthermore,
71 ALAN's negative effects could be worsened by the increasing use of LED (Light-Emitting
72 Diode) in public lighting (Gaston, 2018; Zisis and Bertoldi, 2018). Indeed, LED lights
73 spectrum contains more short-wavelength than spectra of other lighting types such as HPS
74 (High-Pressure Sodium) lighting (Bierman, 2012; Falchi et al., 2011; Longcore et al., 2018;
75 Luginbuhl et al., 2014). Considering that short-wavelength, such as blue wavelength, go
76 further into seawater (Davies et al., 2014; Grubisic et al., 2019) and that marine organisms
77 would be highly sensitive to blue wavelength (Grubisic, 2018), the negative effects of ALAN
78 could be amplified on the coastal fauna. Therefore, ALAN becomes over the years a growing
79 threat to marine life.

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

93 could make them vulnerable to disruption by ALAN, which could have consequences on their
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65
94 physiology and ecology.

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

107 **Materials and methods**

108 *General conditions*

109 The experiment was conducted from January to April 2021 in the Marine Station of Arcachon
110 on 160 oysters (85.8 ± 0.7 mm shell length; 48.4 ± 0.5 mm shell width; mean \pm SE) coming
111 from an oyster's farm source of the Arcachon bay (France). During the acclimation and
112 experimental duration, oysters were placed into tanks (L x W x H: 74.8 x 54.8 x 40.8 mm)
113 continuously supplied with natural seawater from the Arcachon bay, which was filtered ($<$
114 $1\mu\text{m}$), oxygenized, and the temperature was monitored ($T = 15.0 \pm 0.1$ °C). Tanks were
115 placed in an isolated room and equipped with an antivibration bench to minimize external
116 disturbances to animal behavior. The oysters were not fed during the experiment.

117 *Experimental protocol*

1
2 118 Oysters were maintained under a L:D 10:14 cycle with daytime from 7:00 to 17:00 h (all
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5 119 times noted in local time, UTC +1) (Fig. S1A). The light intensity during daytime varied
6
7 120 gradually to mimic the natural cycle of light using programmable white (413–688 nm, peak at
8
9 121 551 nm; Fig. S2A) LED light bars (MH3SP3 DSunY). The maximum intensity during
10
11 122 daytime was 1473.42 ± 106.21 lux (mean \pm SE) (Tab. S.1) between 11:30 to 12:30 h. After 7
12
13 123 to 9 days of acclimation to the experimental setup, the proper experiment started for a
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15 124 duration of 7 days. There were 5 conditions: a control condition and 4 conditions where
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17 125 oysters were exposed to several ALAN intensities (Tab. S1): 0.1, 1, 10, and 25 lux. In the 4
18
19 126 ALAN conditions, oysters were exposed to ALAN from 17:30 to 6:30 h (Fig. S1B) using
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21 127 white (411–687 nm, peak at 563 nm; Fig. S2B–S2E) LED strips (MiBoxer Mi-Light WL5).
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23 128 Illuminances underwater were measured at five positions in the tank (Fig. S3) using a
24
25 129 handheld spectroradiometer (Blue-Wave UVN-100, StellarNet Inc.). The control group was in
26
27 130 the complete dark at night, with a light intensity inferior to the detection limit of the
28
29 131 spectroradiometer (0.05 lux), mentioned in the study as 0 lux for convenience. For each of the
30
31 132 5 experimental conditions, 32 oysters were placed in the same tank, and the valve activity of
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33 133 16 of them was continuously measured using a High Frequency–Non Invasive (HFNI)
34
35 134 valvometer technology (Andrade et al., 2016). For each experimental conditions, 32 different
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37 135 oysters were used. On the 7th day of the experiment, the gill tissue of 8 oysters were sampled
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39 136 during daytime at 12:00 h and during nighttime at 19:00 h and stored in Tri Reagent
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41 137 (Invitrogen) at -80 °C for further molecular analysis.
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52 138 *Behavioral measurements*

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54 139 For valve activity measurement using HFNI valvometer technology, lightweight's
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56 140 electromagnets were glued on each valve of oysters and linked to a valvometer device using
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58 141 flexible wires (Andrade et al., 2016). The electromagnetic current generated between the two
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142 electromagnets allows to measure oyster valve activity. For each individual, the signal was
1 recorded every 4.8s. Data were processed using Labview 8.0 (National Instrument, Austin,
2 143 TX, USA). The study focused on the hourly Valve Opening Duration (VOD) meaning that we
3
4 144 determined the percentage of time each individual spent with its valve open for each hour. For
5
6 145 example, an individual with its valves open during a whole hour corresponds to a VOD of 100
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8 146 %, while an individual with its valves closed during one hour corresponds to a VOD of 0 %.
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148 *Chronobiological analyses*

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

166 *Total RNA extraction and cDNA synthesis*

1
2 167 Total RNA was extracted from gills using Tri reagent (Invitrogen) and an SV Total RNA
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5 168 Isolation System kit (Promega). The total RNA quantity and quality were assessed by
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7 169 spectrophotometry (OD230, OD260, OD280). RNA reverse transcription was realized using
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10 170 Goscript™ Reverse Transcription System kits (Promega).

13 171 *mRNA expression analysis by Real-Time PCR*

15 172 Real-Time qPCR was realized using GoTaq® qPCR Master Mix kit (Promega). Primers sets
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18 173 of clock and clock-associated genes (*CgClock*, *CgBmal*, *CgCry*, *CgPer*, *CgTim1*, *CgCry1*,
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20 174 *CgRev-erb*, *CgRor*, *CgHiomt-like*, *CgOctβ2*, *CgRhodopsin-like 1*, *CgRhodopsin-like 2*,
21
22 175 *CgRhodopsin-like 3*), and housekeeping genes (*CgEfl*, *Cg28S*, *CgGadph*) are listed in the
23
24
25 176 table S2. qPCR reactions were realized as follows: 95 °C for 2 min to activate the GoTaq
26
27 177 polymerase, 40 cycles of 95 °C for 15 seconds (denaturation), and 60 °C for 1 minute
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30 178 (annealing and extension) for amplification of target cDNA, followed by 2 minutes at 60 °C
31
32 179 for the final elongation. In the end, melting curves were generated by gradually decreasing the
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35 180 temperature from 95 °C to 60 °C to control the primer specificity. The comparative Ct method
36
37 181 $2^{-\Delta Ct}$ (Livak and Schmittgen, 2001) was used to determine the relative transcript level of clock
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40 182 and clock-associated genes, where $\Delta Ct = Ct(\text{target gene}) - Ct(\text{housekeeping gene})$. Gene's
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42 183 expression was normalized with the geometric mean of the housekeeping genes *CgEfl* and
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44 184 *Cg28S*, based on stability values (Xie et al., 2012).

48 185 *Statistical analysis*

50 186 All statistical analysis were performed using SigmaPlot software (version 13.0; Systat
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53 187 Software, USA). T-tests were performed for two groups' comparisons after checking
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55 188 assumptions (normality of data and equal variance) and if they were not validated the non-
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58 189 parametric Mann-Whitney rank sum test was performed. Two groups' comparisons were used
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60 190 for molecular analysis results. For multiple comparisons, one-way ANOVA were used after
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191 checking assumptions (normality of data and equal variance). When assumptions were not
192 validated, the non-parametric Kruskal-Wallis One-Way ANOVA on ranks test was
193 performed. In case of significant differences, a Tukey test was realized for all pairwise
194 multiple comparisons unless the groups did not have equal size, in which case the Dunn's test
195 was realized. Multiple comparisons were performed for behavioral analyses. On the whole
196 molecular data, a two-way ANOVA was also performed after checking assumptions
197 (normality of data and equal variance), and in case of significant differences, a Tuckey test
198 was used for all pairwise multiple comparisons. For all test results, a difference was
199 considered significant when $p < 0.05$.

200

201 **Results**

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

216 results in Table S3 and Table S4). At the group level, a significant daily rhythm persists in all
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2 217 conditions with no significant differences of period length between the conditions (Fig. 3A).
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4 218 However, ALAN affected several parameters of this rhythm. Indeed, there is a significant
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7 219 decrease ($p < 0.001$) of the percent rhythm (PR, %) of the daily rhythm when oysters are
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10 220 exposed to ALAN starting from 1 lux (Fig. 3B). Furthermore, the amplitude of the daily
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12 221 rhythm decreases significantly ($p < 0.001$) in all ALAN conditions, starting from 0.1 lux and
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14 222 without significant ALAN intensity-effect (Fig. 3C). At the individual level, results show an
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16 223 ALAN intensity-dependent decrease of the percentage of oysters having a significant daily
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19 224 rhythm (Fig. 3D). Figure 3E shows a decrease ($p < 0.001$) of the individual rhythmic period
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22 225 lengths, getting out the daily range for many individuals exposed to ALAN (Table S3). The
23
24 226 mean period of oysters exposed to 1 lux and 25 lux are 17.9 ± 2.1 and 15.2 ± 1.7 h
25
26 227 respectively, under the circadian range (20-28 h), explaining the global loss of rhythmicity at
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28
29 228 the daily scale shown in figure 3D. Furthermore, the PR of oysters having a daily rhythm
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31 229 tends to decrease starting from 0.1 lux, and it decreases significantly ($p = 0.015$) when they
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34 230 are exposed to 10 lux (Fig. 3F). Finally, the amplitude of oysters having a daily rhythm show
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36 231 no significant differences ($p = 0.169$) but a trend to decrease when oysters are exposed to
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39 232 ALAN (Fig 3G). Figures 4 and S4 shows the mRNA level during daytime and nighttime of
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41 233 thirteen circadian clock (*CgClock*, *CgBmal*, *CgCry*, *CgPer*, *CgTim1*, *CgCry1*, *CgRev-erb*,
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43 234 *CgRor*) and clock-associated (*CgHiomt-like*, *CgOctβ2*, *CgRhodopsin-like 1*, *CgRhodopsin-*
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45 235 *like 2*, *CgRhodopsin-like 3*) genes for all of the tested ALAN intensities. No significant
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48 236 differences of gene expression are observed between the control and the ALAN conditions
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51 237 neither at daytime nor at nighttime. However, results show significant differences of mRNA
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53 238 expression levels between daytime and nighttime in the control condition for five genes (two
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55 239 circadian clock genes: *CgTim1* and *CgRev-erb*; and three clock-associated genes: *CgHiomt-*
56
57 240 *like*, *CgOctβ2* and *CgRhodopsin-like 1*), with an increase of expression during nighttime (Fig.

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

249 Discussion

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

261 We showed that ALAN exposure leads to an increase of oysters' VOD, explained by an
262 increase of activity during nighttime leading to a decrease (at 0.1 lux) or a loss (at 1, 10, and
263 25 lux) of daytime / nighttime differences of valve behavior and a shift of peak activity from
264 daytime to nighttime. Activity increase during nighttime as an effect of ALAN has been also
265 shown in other marine organisms, such as the diurnal fish *Girella laevis* exposed to 70 lux

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

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

291 conducted in winter, when the food supply is very low in the environment. Moreover, the
1
2 292 rhythm at the daily scale could not be the only rhythm that can be disrupted by ALAN. It has
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4
5 293 been shown that oysters have other biological rhythms. For example, oysters showed a lunar
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7 294 rhythm i.e. their valve behavior is synchronized with lunar cycles by detecting moonlight
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10 295 illumination cycle (Payton and Tran, 2019). This lunar rhythm could be affected by ALAN
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12 296 since the maximal intensity of the moonlight ranges from 0.1 to 0.3 lux (Rich and Longcore,
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14 297 2006), thus the natural variations of moonlight intensity can be easily masked by ALAN
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17 298 intensities, such as the skyglow. The masking effect of lunar cycles by ALAN using LED has
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19 299 been investigated in the corals *Acropora millepora* and *Acropora digitifera*, in which
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21
22 300 exposition to ALAN delayed or masked their gametogenesis leading to a spawning
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24 301 desynchronization (Ayalon et al., 2021). Another example of ALAN effect on lunar rhythm is
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26 302 the disruption of the monthly nocturnal foraging pattern of the gastropod *Nucella lapillus*,
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29 303 based on the lunar cycle, by ALAN produced by LED lighting at an intensity of 10 lux (Tidau
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31 304 et al., 2022). Moreover, oysters' seasonal rhythms could be disrupted by ALAN. Oysters use
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34 305 the variation of photoperiod through the year to anticipate seasonal changes and synchronize
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36 306 physiological processes such as behavior, growth, and spawning (Bernard et al., 2016; Payton
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39 307 et al., 2017b). However, ALAN being often present during dusk and dawn, it can mask these
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41 308 two natural phenomena, preventing oysters to detect the variation of photoperiod along the
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44 309 year, which could affect several seasonal physiological processes and decrease the fitness of
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46 310 organisms (Gaston et al., 2017). ALAN's effects on organisms' physiological processes and
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48
49 311 fitness have been shown for example in the amphipod *Orchestoidea tuberculata*, in which
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51 312 ALAN (halogen light) induces a decrease of growth (Luarte et al., 2016), and in barnacles, in
52
53 313 which ALAN (LED lighting) affects the settlement process (Lynn et al., 2021; Manriquez et
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55
56 314 al., 2021). Moreover, ALAN's effects on organisms' fitness have also been observed in the
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58 315 juvenile crabs *Neohelice granulata* since it increases mortality, mainly by increasing
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316 cannibalistic interactions with adults at an intensity of approximately 40 lux (LED lighting)
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2 317 (Nunez et al., 2021).
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5 318 In addition to the effects on the oyster's behavioral daily rhythm, this study also showed that
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7 319 ALAN disturbs the circadian clockwork of *C. gigas* by annihilating differences between
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9 320 daytime and nighttime in clock and clock-associated genes expression starting from the
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11 321 intensity 0.1 lux. Genes for which their expression was mainly affected by ALAN are
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13 322 *CgTim1*, *CgRev-erb*, *CgHiomt-like*, *CgOctβ2*, and *CgRhodopsin-like 1*. *CgTim1* and *CgRev-*
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15 323 *erb* are both involved in the regulation's feedback loops of the oysters' molecular clock and
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17 324 thus are core clock genes (Perrigault and Tran, 2017). On the other hand, *CgHiomt-like*,
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19 325 *CgOctβ2*, and *CgRhodopsin-like 1* are clock-associated genes. The first one is a homologue of
20
21 326 the mammal gene *Hiomt* involved in the final step of the synthesis of melatonin, known to be
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23 327 a key molecule of the day / night rhythm and a potent antioxidant with a proposed role in
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25 328 immune function (Jones et al., 2015; Pevet et al., 1980). *CgOctβ2* is the gene of the
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27 329 octopamine receptor β-2R. Octopamine is a biogenic amine well studied in arthropods and
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29 330 gastropods where it functions as a neurotransmitter and hormone, fulfilling the roles played
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31 331 by adrenalin in vertebrates. It is an extremely pleiotropic substance, participating in
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33 332 invertebrate development such as growth, maturation, and reproduction by activating their
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35 333 corresponding G protein-coupled receptors (GPCRs), and may interact with the circadian
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37 334 clock (Roeder, 2020). Finally, the last gene to show a disrupted expression in presence of
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39 335 ALAN is the gene of the rhodopsin (*CgRhodopsin-like 1*), a photoreceptor involved in
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41 336 oysters' light-sensitivity (Wu et al., 2018). Thus, ALAN affects five of the thirteen genes
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43 337 studied here. This relative low transcriptional response observed might be due to the fact that
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45 338 oysters have been sampled at only two sampling times over the daily cycle. Indeed, a stronger
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47 339 transcriptional disruption might possibly have been revealed by sampling at another time of
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49 340 the day. However, our results still show that ALAN can affect genes of the molecular clock of
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341 *C. gigas* (*CgTim1*, *CgRev-erb*), suggesting that the observed disruption of oyster's daily
342 behavioral rhythm would be a real consequence of the circadian clock disruption and not only
343 a direct reaction of light perception in presence of ALAN. ALAN also affects essential clock-
344 associated genes (*CgHiomt*, *CgOctβ2*, *CgRhodopsin-like 1*) involved in physiological
345 processes regulation. Thus, we can imagine that, by cascading effect, the expression of other
346 genes involved in several physiological functions can also be disrupted by ALAN. For
347 example, ALAN at 0.1–0.3 lux using a fluorescent bulbs induces the suppression of the
348 increase of melatonin during nighttime in *Salvelinus* (Liu et al., 2019). Moreover, in the
349 European perch *Perca fluviatilis* and the roach *Rutilus rutilus* the impairment of circadian
350 melatonin pattern by ALAN (HPS lamps) is associated with a decrease of the mRNA
351 expression of gonadotropins, which could disrupt their reproduction (Bruning et al., 2018).

352

353 **Conclusion**

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

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2 366 **Ethics:** All experiments complied with the laws in effect in France and they conformed to
3 international ethical standards.

4
5 368 **Data accessibility:** The data underlying this study are available on Supplementary data.

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7
8 369 **Author contributions:** Study design and methodology, D.T., A.B., L.P.; experimentation,
9 A.B., D.T.; molecular analysis: A.B., L.P.; data treatment, A.B.; interpretation, A.B., D.T.,
10 370 L.P.; manuscript writing, A.B.; review and editing, all authors; funding, D.T. All authors
11 371 contributed critically to the drafts, and gave final approval for publication.
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21

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554 **Figures & legends**

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3 555 **Figure 1.** Behavioral daily pattern of oysters ($n = 15\text{--}16$ oysters) in control condition (0 lux)
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5 556 and exposed to different ALAN intensities (0.1 lux, 1 lux, 10 lux, and 25 lux). Mean hourly
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8 557 VOD data are shown as mean \pm SE ($n = 7$ days). White backgrounds indicate daytime and
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10 558 yellow and black backgrounds indicate nighttime. Arrows indicate the VOD peak during the
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16 560 **Figure 2.** Effect of ALAN intensity on (A) the mean daily valve opening duration (VOD),
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18 561 and (B) the mean VOD during the daytime and the nighttime. Data are expressed in mean \pm
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21 562 SE ($n = 7$ days). Different letters indicate significant differences between ALAN conditions
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23 563 and between daytime and nighttime ($p < 0.05$).

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26 564 **Figure 3.** Effect of ALAN intensity on oysters daily rhythm characteristics: (A) the period at
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29 565 group level, (B) the percent rhythm (PR) at group level, (C) amplitude of the rhythm at group
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31 566 level, (D) the percentage of oysters having a daily rhythm, (E), period of individual rhythmic
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34 567 oysters, dotted lines define the daily range, (F) the PR at individual level of oysters having a
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36 568 daily rhythm, (G) amplitude of the rhythm at the individual level of oysters having a daily
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39 569 rhythm. Data are expressed in mean \pm SE. For A-C, the SE shows the daily variability of the
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41 570 group's significant rhythm parameters ($n = 7$ days). For E-G the SE shows the individual
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43 571 variability ($n = 15\text{--}16$ oysters). Different letters indicate significant differences ($p < 0.05$).

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46 572 **Figure 4.** Effect of ALAN intensity on the difference in relative mRNA expression level
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49 573 (mean \pm SE, $n = 8$) on oyster gills tissues, between daytime and nighttime of five clock and
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51 574 clock-associated genes. White bars indicate the gene level during daytime and, colored bars
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54 575 during nighttime. Asterisks indicate significant differences between daytime and nighttime (p
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56 576 < 0.05).

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578 **Table 1.** Two-Way Analysis of Variance testing the effect of Daytime/Nighttime and the
 579 ALAN intensity (0 lux, 0.1 lux, 1 lux, 10 lux, 25 lux) on the relative mRNA expression level
 580 on gill tissues of 13 clock and clock-associated genes. In bold, 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		

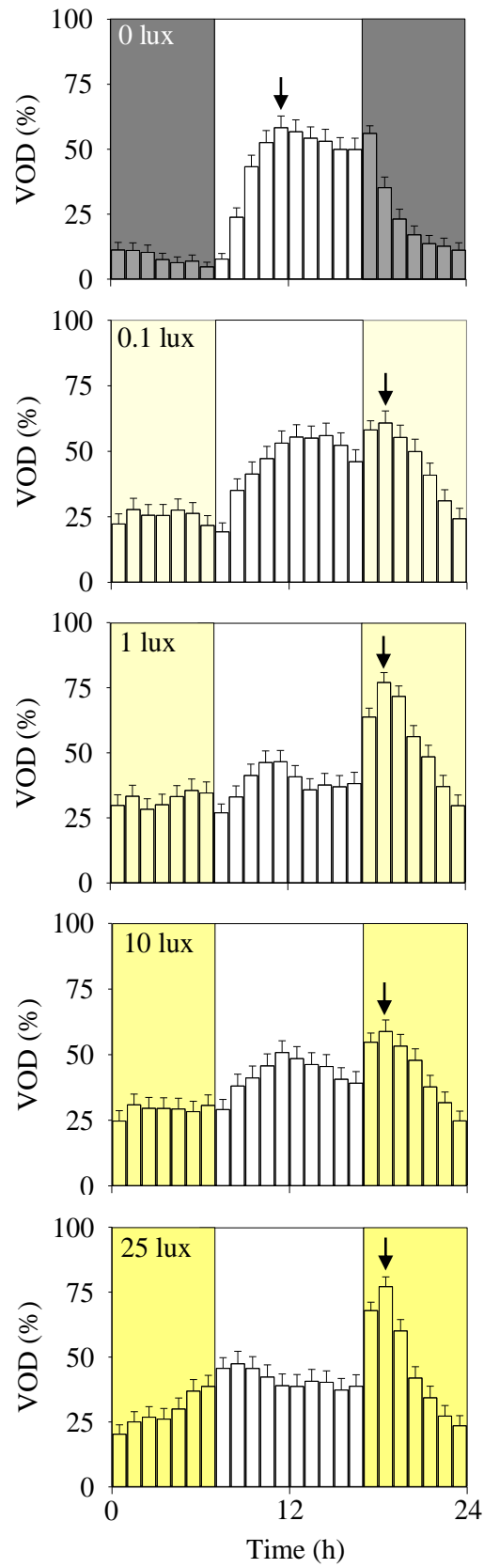


Figure 1. Botté et al.

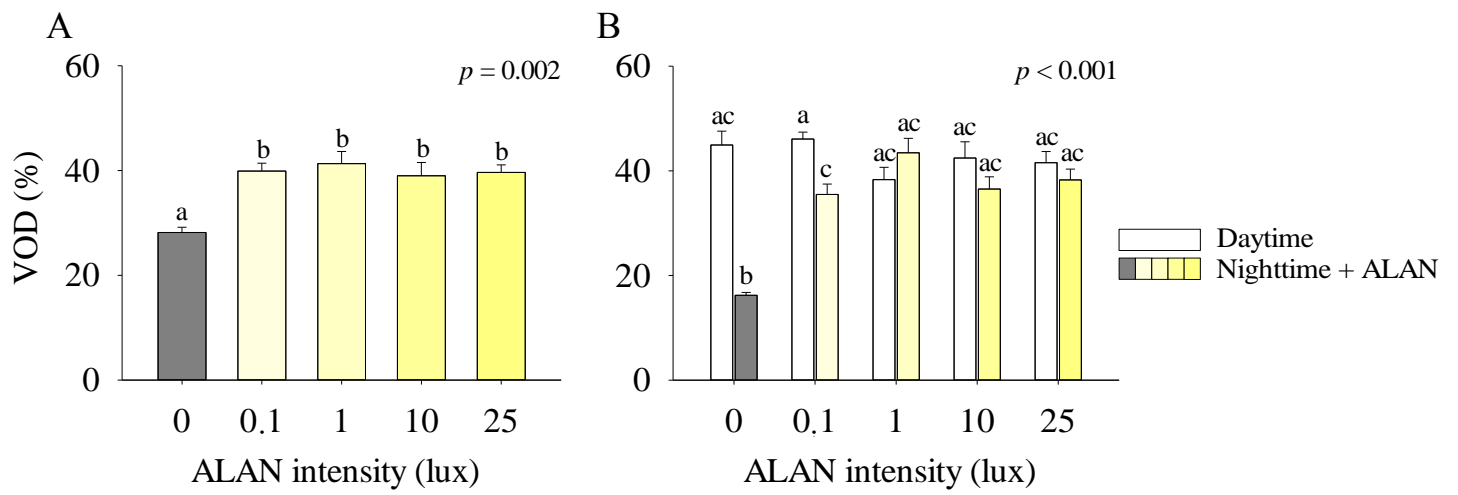


Figure 2. Botté et al.

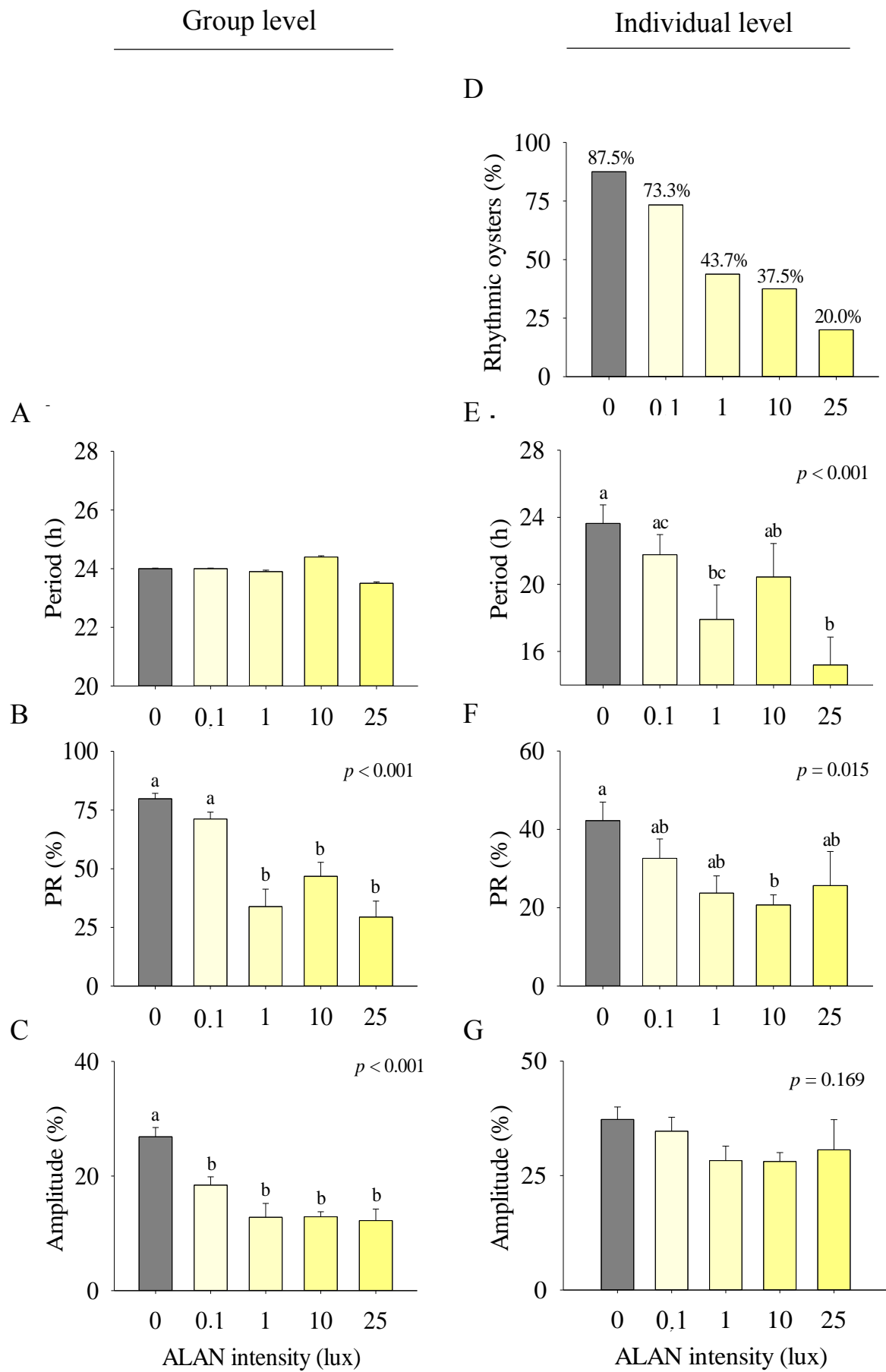


Figure 3. Botté et al.

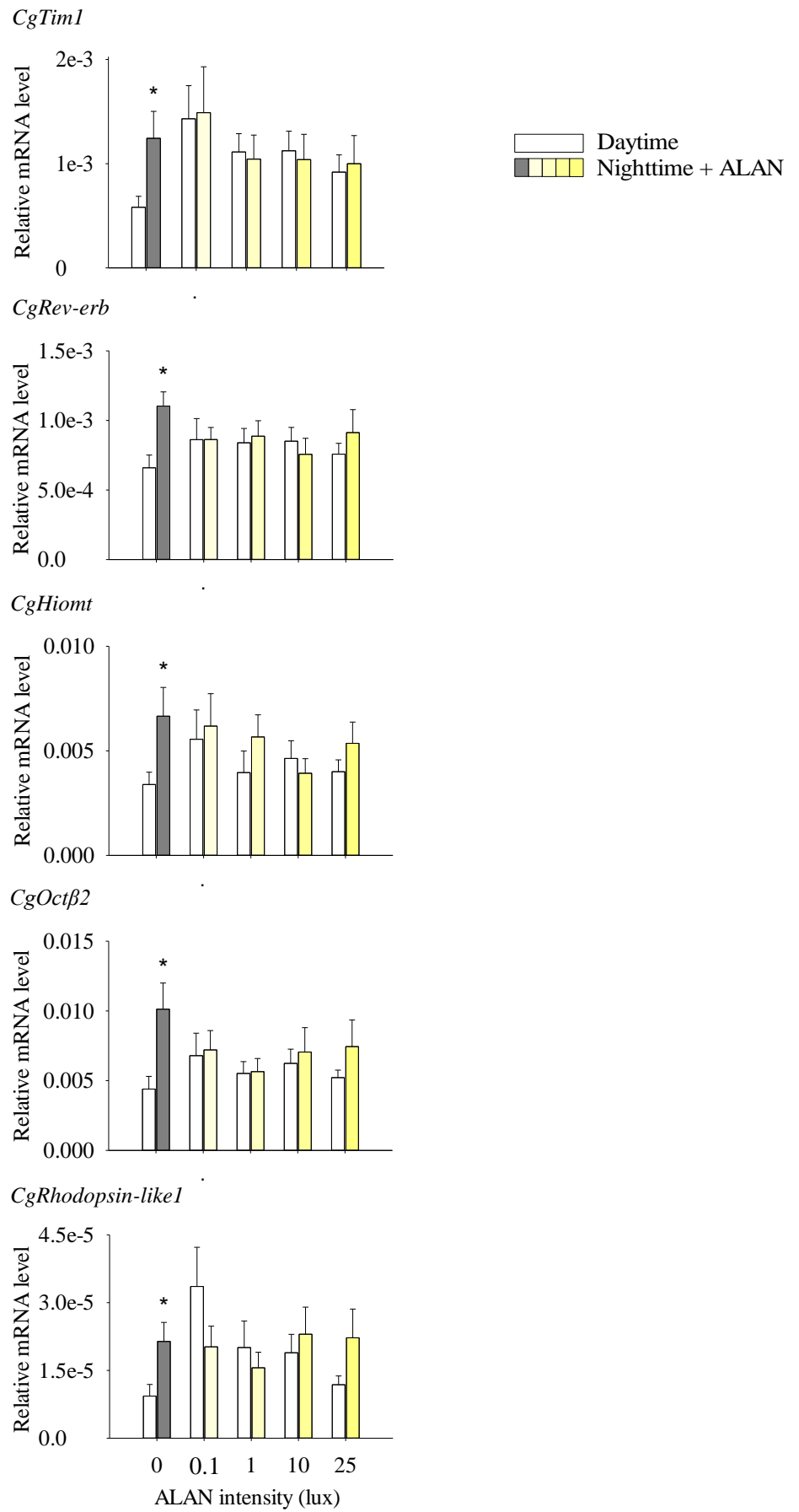


Figure 4. Botté et al.

Supplementary material

Artificial light at night disrupts daily rhythm of the oyster

Crassostrea gigas

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The following items are provided:

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 to 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.

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 \pm SE.

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

Table S3. Results of chronobiological analysis for the control condition and ALAN conditions at the group level. Rhythmic parameters are expressed as mean \pm 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 \pm 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 daytime and nighttime are significant for a p -value = 0.05.

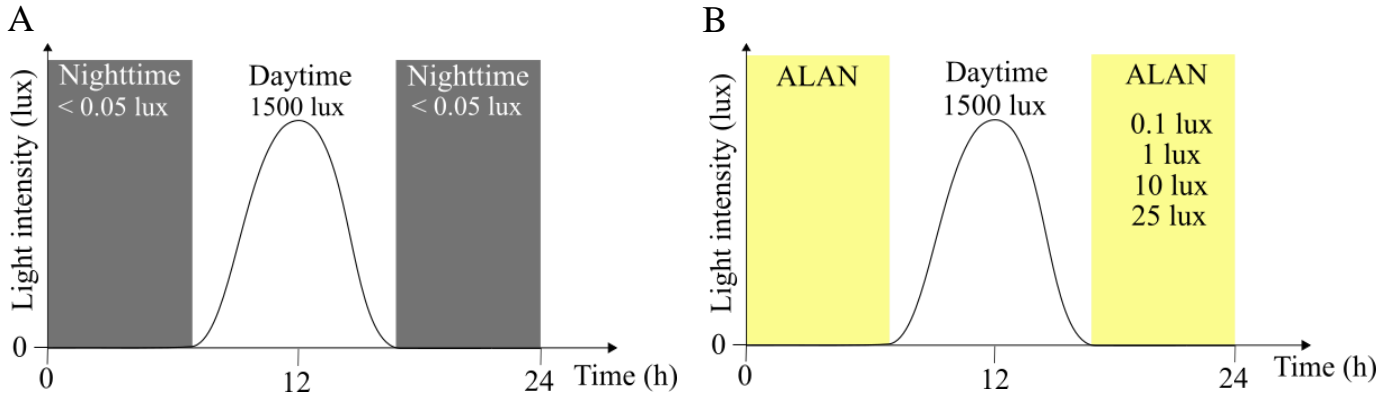


Figure S1. Daily light exposure of the control condition (A) and the ALAN conditions (B).

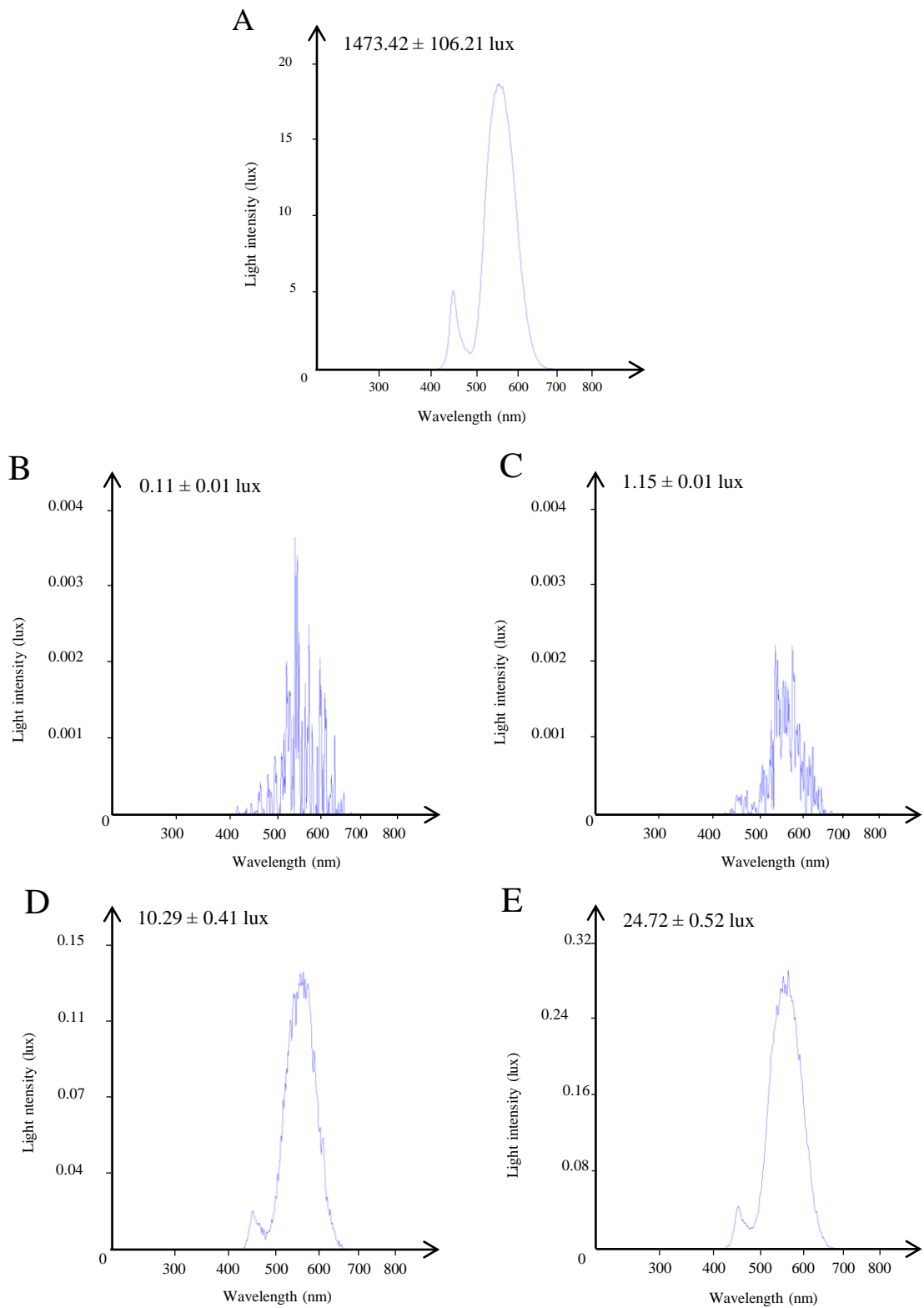


Figure S2. Spectrum of the maximum light intensity during daytime (A), and light spectra of ALAN intensities measured underwater near the oysters: 0.1 lux (B), 1 lux (C), 10 lux (D), and 25 lux (E) conditions.

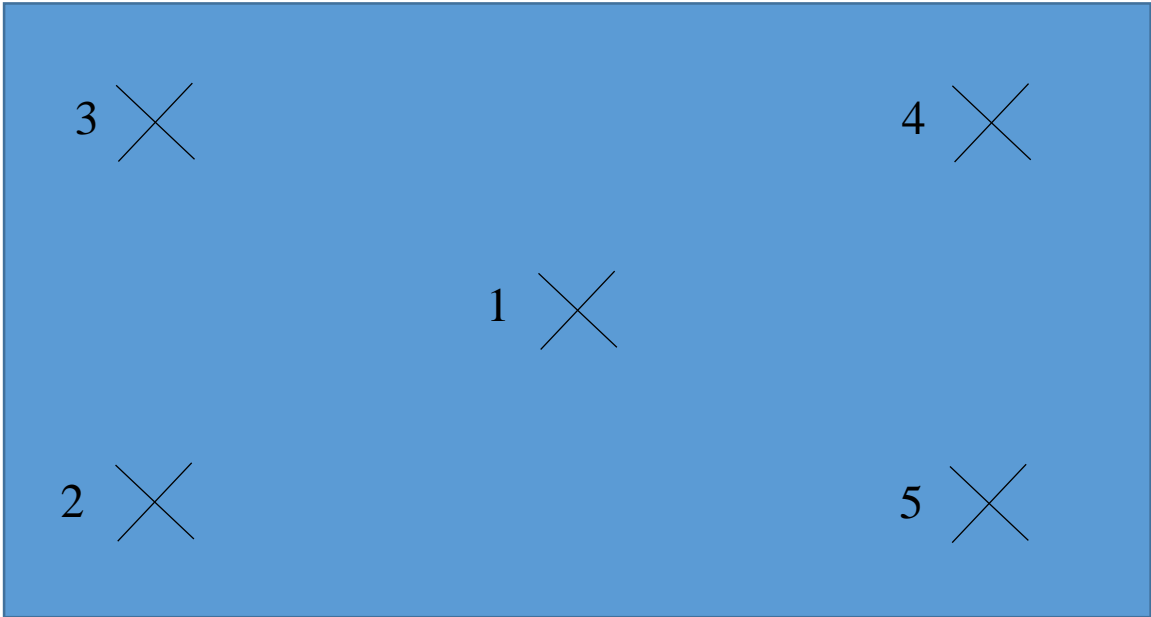


Figure S3. Five locations in the experimental tank used for underwater intensity measurements. The depth of the measure is done at the oyster level.

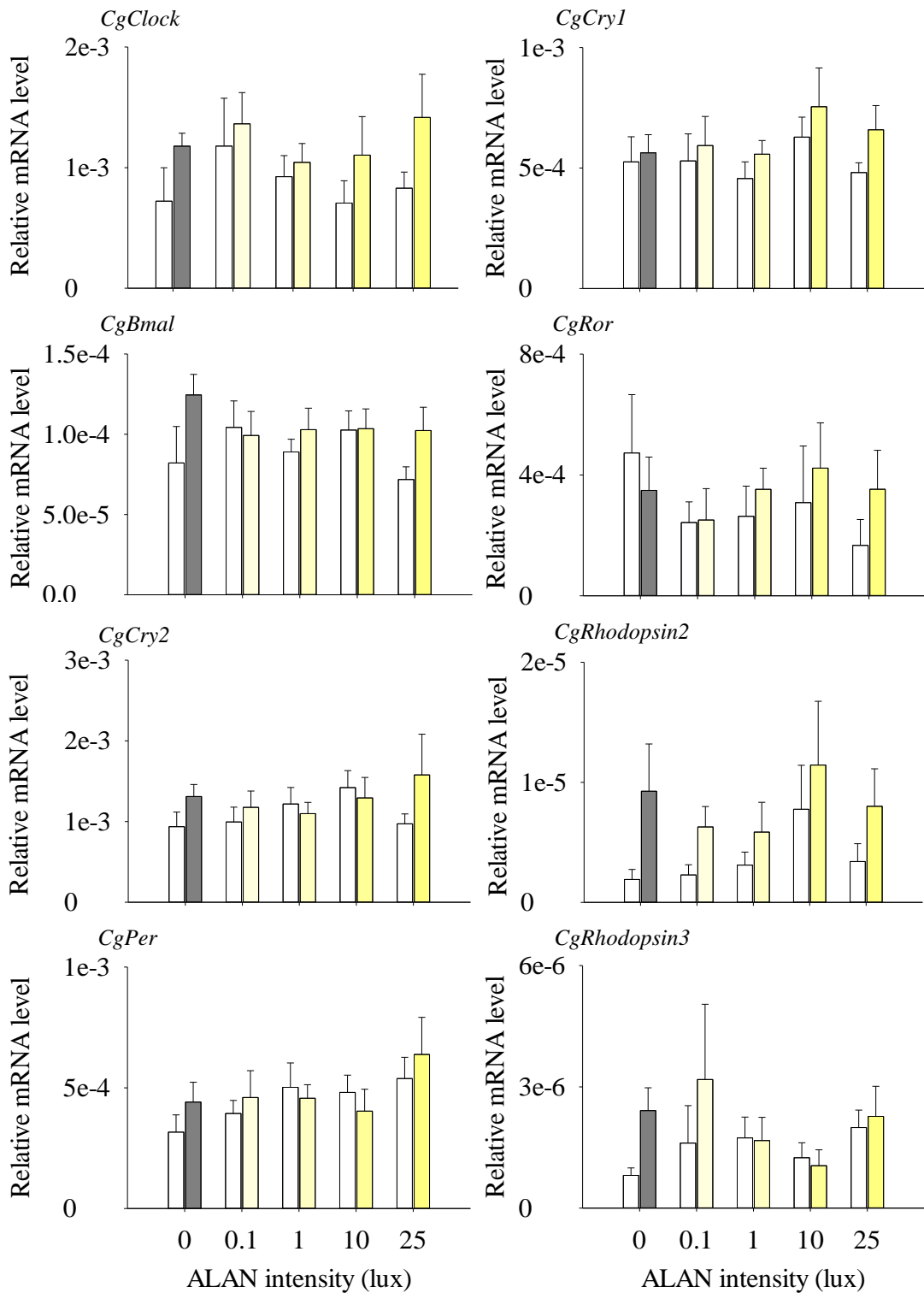


Figure S4. Relative mRNA expression levels (expressed as mean \pm SE) between daytime and nighttime of eight clock and clock-associated genes according to ALAN intensities exposure. White bars indicate the gene level during daytime and, colored bars during nighttime. Differences between daytime and nighttime 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
<i>Crassostrea gigas</i> core clock genes			
KX371073	<i>CgClock (Clock)</i>	5'-TGGGAATGATGTCCAACAGAG-3'	5'-GGTCCATCAATGACAGGAAGT-3'
KX371075	<i>CgBmal (Bmal)</i>	5'-CACAAAGTTCAGGTTCAGAGTGTAG-3'	5'-TCACCTGAGGTAGACTGGTTAT-3'
KX371074	<i>CgCryptochrome 2 (Cry2)</i>	5'-AACCTTACAGCAAGCACGAA-3'	5'-TGACATCTGGCTGTGGTTTC-3'
KX371076	<i>CgPeriod (Per)</i>	5'-CCGATGACAGAAATCCCAGTAG-3'	5'-CCATCCTATTCTCCTGCTCTTG-3'
KX371077	<i>CgTimless 1 (Tim1)</i>	5'-AAAGATCCCGGACACAGTATG-3'	5'-TGGAACTCGTTCCTGACTTG-3'
KT991835	<i>CgCryptochrome 1 (Cry1)</i>	5'-TCATGAAGCAGCTCAGATACG-3'	5'-ACCTCCCAGTCAACCAAAG-3'
KJ188106	<i>CgRev-erb (Rev-erb)</i>	5'-GACTTTGCTGATCGCTTCAAC-3'	5'-CTTTCCAAGTCTCCACATTTTC-3'
EKC18621	<i>CgRor (Ror)</i>	5'-CTACGTGAGCAGGTGTTTGA-3'	5'-CGTCCGCTATGTCCTTCAAT-3'
<i>Crassostrea gigas</i> clock-controlled genes			
EKC41768	<i>CgHiomt-like</i>	5'-CGGGTGGATCAGTGTTAGTAATG-3'	5'-TCTCTTGGCCCTGTGATAGA-3'
XM_011433587	<i>CgRhodopsin-like 1</i>	5'-TAGTTCGGCGTCGGAATTTATC-3'	5'-CTGTTTGAATCTCTGCTCTCAC-3'
XM_011448766	<i>CgRhodopsin-like 2</i>	5'-CCCTGAGTCATCCCAAATTCA-3'	5'-GATGTTCTCGGCGTAGCTTTA-3'
XM_020065754	<i>CgRhodopsin-like 3</i>	5'-TGACTTTGACGGCGATACTG-3'	5'-ATAGATCCGCCACCGAAATG-3'
XM_011427386	<i>CgOctβ2</i>	5'-AATCCAGCACACACTCCATAG-3'	5'-TCTGAGTCTCATCTGCGTTTG-3'
<i>Crassostrea gigas</i> housekeeping genes			
AB122066	<i>CgElongation Factor 1 (Ef1)</i>	5'-ACCACCCTGGTGAGATCAAG-3'	5'-ACGACGATCGATTTCTCTT-3'
CAD67717	<i>CgGadph</i>	5'-CGTACCAGTTCAGATGTTTCC-3'	5'-GCCTTGATGGCTGCCTTAATA-3'
Z29546	<i>Cg28S</i>	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	<i>12.3</i>	–	–
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	<i>10.1</i>	–	–
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	<i>11.8</i>	–	–	17.4	–	–	17.1	–	–
23.9	62.35	48.7	24	16.71	19.6	<i>7.96</i>	–	–	33.2	–	–	12.1	–	–
25.1	33.97	28.9	24.9	39.95	41.8	<i>9.69</i>	–	–	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	<i>16.5</i>	–	–	8.37	–	–	12.6	–	–	n.s.	–	–
24.3	46.16	37.8	<i>12.5</i>	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–
24	26.95	30.3	<i>12.1</i>	–	–	n.s.	–	–	n.s.	–	–	n.s.	–	–
<i>31.4</i>	–	–	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.