Circadian clock-controlled gene expression in co-cultured, mat-forming cyanobacteria

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Natural coastal microbial mat communities are multi-species assemblages that experience fluctuating environmental conditions and are shaped by resource competition as well as by cooperation. Laboratory studies rarely address the natural complexity of microbial communities but are usually limited to homogeneous mono-cultures of key species grown in liquid media. The mat-forming filamentous cyanobacteria *Lyngbya aestuarii* and *Coleofasciculus chthonoplastes* were cultured under different conditions to investigate the expression of circadian clock genes and genes that are under their control. The cyanobacteria were grown in liquid medium or on a solid substrate (glass beads) as mono- or as co-cultures under a light–dark regime and subsequently transferred to continuous light. TaqMan-probe based qPCR assays were used to quantify the expression of the circadian clock genes *kaiA*, *kaiB*, and *kaiC*, and of four genes that are under control of the circadian clock: *psbA*, *nifH*, *ftsZ*, and *prx*. Expression of *kaiABC* was influenced by co-culturing the cyanobacteria and whether grown in liquid media or on a solid substrate. Free-running (i.e. under continuous light) expression cycle of the circadian clock genes was observed in *L. aestuarii* but not in *C. chthonoplastes*. In the former organism, maximum expression of *psbA* and *nifH* occurred temporally separated and independent of the light regime, although the peak shifted in time when the culture was transferred to continuous illumination. Although functionally similar, both species of cyanobacteria displayed different 24-h transcriptional patterns in response to the experimental treatments, suggesting that their circadian clocks have adapted to different life strategies adopted by these mat-forming cyanobacteria.

The study of the ecology and evolution of natural microbial assemblages is often hindered by the complexity of these systems as well as by the fluctuations of environmental conditions. Laboratory experiments avoid these drawbacks and their outcomes are sometimes extrapolated in an attempt to understand the ecology of the microbial community in nature. Such laboratory experiments are usually carried out using pure cultures growing under well-defined conditions and high nutrient concentrations and under constant temperature and light. However, the results of these experiments often do not allow to predict the natural behaviour of the microorganisms. The emerging field of synthetic microbial ecology tackles these shortcomings by combining a lower complexity with mimicking conditions of natural ecosystems. This holds the promise of a better understanding of the ecology and evolution of microorganisms in natural ecosystems1,2. Currently, applications of synthetic microbial ecology address processes such as recycling of waste products3, industrial fermentation4, dairy industry5, production of chemical compounds6, and improvement of human health through fecal transplantation7.

Coastal microbial mats are amongst the most complex and diverse ecosystems8 and are exposed to microscale gradients (among others light, oxygen, salinity, and sulfide) fluctuating with the day-night and/or tidal cycles9–11. The dominant primary producers in these mats are cyanobacteria that form complex assemblages and maintain tight interactions with microorganisms belonging to different functional groups6. Cyanobacteria possess a circadian clock, a well-characterized molecular mechanism that produces approximately 24-h rhythms in gene expression and protein activity12. True circadian rhythms persist even under continuous illumination (free-run), and are able to shift their phases (timing of highest and lowest expression) according to environmental variations.

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KaiC autophosphorylation while excess of KaiB leads to KaiC dephosphorylation. Synchronization to the environment is regulated by KaiA and CikA through the indirect sensing of light. The bacteriophytochrome-like circadian input kinase CikA plays an important part in the timing of the circadian period by sensing changes in light intensity through the redox state of the plastoquinone pool. Overexpression of KaiA increases the phosphorylation of KaiC and the production of circadian rhythmicity. The nature of the cyanobacterial circadian clock has been vastly studied in planktonic mono-cultures of the unicellular cyanobacterium *Synechococcus elongatus* PCC7942. The attractiveness to study this model strain is its small genome size, simple cellular structure, accessibility to genetic modification and comprehensive standardization of high-throughput assays. However, little is known about how circadian clock-controlled gene expression is regulated in complex communities and whether the environmental complexity and presence of other species is of influence.

A potential external source of influence on rhythmic gene expression is the complexity of the environment, as was reported for *Staphylococcus aureus* and *Vibrio cholerae* in which gene regulation and metabolic activity depended on whether these bacteria were grown as a homogenized liquid culture or biofilm-grown. An additional source of influence on gene expression might be presented by another, independent circadian oscillator which is linked to the ubiquitous antioxidant enzyme peroxiredoxin. Peroxiredoxin follows a 24-h oxidation-reduction cycle and is highly conserved in all domains of life, however, the underlying mechanisms are not well understood.

To investigate whether growth condition and/or species interaction influences circadian gene expression patterns, co-cultures of two filamentous, non-heterocystous cyanobacteria *Lyngbya aestuarii* and *Coleofasciculus chthonoplastes* were subjected to quantitative PCR-based gene expression analysis. This technique was chosen over metatranscriptomics due to the large number of variables and replicates tested that add up to 648 samples to be analysed. In addition, alternative techniques like the use of reporter genes are not yet available for the over metatranscriptomics due to the large number of variables and replicates tested that add up to 648 samples to be analysed. In addition, alternative techniques like the use of reporter genes are not yet available for the identification of rhythmic gene expression patterns. Regardles the taxon and species, the clock reduces gene expression faster than from *L. aestuarii* (Table 1). Co-cultures yielded less gene expression than from *C. chthonoplastes*.

### Table 1. Average quantity (ng/µl) and standard deviations (SD) and the average quality (RIN) of total RNA extracted from *L. aestuarii* (LA) and *C. chthonoplastes* (CC). Average quantities and qualities are given for each treatment (L = liquid, B = immobilized, M = mono-culture, C = co-culture).

| Treatment | Species | ng/µl (± SD) | RIN |
|-----------|---------|--------------|-----|
| ML        | LA      | 24 (± 19)    | 9   |
|           | CC      | 33 (± 25)    | 7   |
| CL        | LA/CC   | 22 (± 16)    | 8   |
| MB        | LA      | 15 (± 18)    | 9   |
|           | CC      | 10 (± 10)    | 7   |
| CB        | LA/CC   | 16 (± 13)    | 6   |

The extracted total RNA was of good quality (average RIN: 7.7 ± 1) and yielded higher concentrations from *C. chthonoplastes* than from *L. aestuarii* (Table 1). Co-cultures yielded less total RNA than mono-cultures. Similarly, biofilm-grown cultures yielded less total RNA when compared to liquid cultures (Table 1).

The qPCR-derived Ct values of the biological replicates of the targeted circadian clock gene cluster *kaiABC*, nitrogenase gene *nifH*, cell division protein gene *ftsZ*, photosynthesis D1 protein gene *psbA*, peroxiredoxin gene *prx* and two commonly used cyanobacterial housekeeping genes, *rnpA* and *ppc* ranged from 23 to 39 in *L. aestuarii* and from 21 to 38 in *C. chthonoplastes*. The average *r* and amplification efficiencies of the qPCR assays were 0.95 and 100% in *L. aestuarii* and 0.92 and 92% in *C. chthonoplastes* (Table 2). Regardless the taxon and treatment, the highest absolute transcription was observed for the photosystem II protein D1 coding gene *psbA* with on average 153,425 gene-copies/µl in *L. aestuarii* and 78,592 gene-copies/µl in *C. chthonoplastes*.

### Validation of housekeeping genes.

Each qPCR run was accompanied by the quantitation of housekeeping genes for normalization. The genes *rnpA* (protein component of ribonuclease P) and *ppc* (phosphoenolpyruvate carboxylase).
| Species                  | Gene   | Function                                              | Primer/probe | Sequence (5′→3′)               | Amplicon size (bp) | Reporter | Amplification efficiency | $r^2$ |
|--------------------------|--------|-------------------------------------------------------|--------------|---------------------------------|--------------------|----------|--------------------------|-------|
|                          | cikA   | Circadian input kinase A                              | 2816P        | CGGCACGATGAATTAAACGGATGTG       | 123                | FAM      | 0.89                     | 0.95  |
|                          |        |                                                       | 2796F        | GTAAGCGTGGTTCAGTTCAACGGAC       |                    |          |                          |       |
|                          |        |                                                       | 2918R        | AGTGGACTTTATGGGCGCAA           |                    |          |                          |       |
|                          | ftsZ   | Filamenting temperature-sensitive mutant Z, cell division protein | 984P         | ACCCAAAACTCGATCCAGGGACTTCT     | 126                | FAM      | 0.90                     | 0.98  |
|                          |        |                                                       | 936F         | GTTAAAGGGGCGGATTTGGG           |                    |          |                          |       |
|                          |        |                                                       | 1061R        | TTGTTGTTGCTGGTGTTGGA           |                    |          |                          |       |
|                          | kaiA   | Circadian clock protein A                              | 330P         | GGAGGTTGACATATTGTGCTCAAGGGC    | 106                | FAM      | 0.80                     | 0.94  |
|                          |        |                                                       | 310F         | ACCGTTTGTTCCTACGTCCAGG         |                    |          |                          |       |
|                          |        |                                                       | 415R         | TTGCTTGTTCAGAGCATGT            |                    |          |                          |       |
|                          | kaiB   | Circadian clock protein B                              | 159P         | TGCACGAGGAAGATACCCAGCCGACGC    | 104                | HEX      | 0.84                     | 0.95  |
|                          |        |                                                       | 113F         | GGGTTACGGCACTGAAAGT             |                    |          |                          |       |
|                          |        |                                                       | 216R         | CGGAGGGGGTTAAAACCTTCGG         |                    |          |                          |       |
|                          | kaiC   | Circadian clock protein C                              | 539P         | TGATGACGGCCGAGCCGCGCGGGGA      | 135                | CY5      | 1.07                     | 0.89  |
|                          |        |                                                       | 517F         | AAGGAAATTTGTCTAGGG            |                    |          |                          |       |
|                          |        |                                                       | 651R         | TCGCGCGTTCCTCCTCTCAAAA         |                    |          |                          |       |
| C. chthonoplastes         | nifH   | Nitrogenase iron protein                              | 614P         | TCGTCGAGATCAACGGTCGCGCC       | 133                | HEX      | 1.22                     | 0.87  |
|                          |        |                                                       | 578F         | ACCCGAGGGTTTTGTCCATA           |                    |          |                          |       |
|                          |        |                                                       | 710R         | CCAAGCGATTCGCTACGGCCG         |                    |          |                          |       |
|                          | psbA   | Photosystem II protein D1                              | 458P         | GCCAGCGGCAATACGGCCGAGGC       | 109                | CY5      | 0.92                     | 0.90  |
|                          |        |                                                       | 399F         | CGGTGGTTTCACAGTAGCCAGA         |                    |          |                          |       |
|                          |        |                                                       | 507R         | CTGGATGACCCCTTCCAT             |                    |          |                          |       |
|                          | rnpA*  | Protein subunit of ribonuclease P                     | 139P         | GCCGCAAAGCGGGTCGCGTAAGGG       | 134                | CY5      | 0.85                     | 0.94  |
|                          |        |                                                       | 99F          | CTAATGACTGCGCAACCGGGTA         |                    |          |                          |       |
|                          |        |                                                       | 232R         | CCGCCACTGCATCGGGATT           |                    |          |                          |       |
|                          | ppC*   | Phosphoenolpyruvate carboxylase (PEPC)                | 2573R        | ACCCAAGGAATTGCGCGAGTAG        | 144                | ROX      | 0.89                     | 0.94  |
|                          |        |                                                       | 2513P        | CCGCTCCATCGTCGCGCTGTA         |                    |          |                          |       |
|                          |        |                                                       | 2424F        | TGCGGGTTTGTTGCTAAGGC          |                    |          |                          |       |
|                          | Prx    | Peroxiredoxin                                         | 76P          | AGGCGGAAACTTGGCTTGAGATGCGGA    | 108                | HEX      | 0.72                     | 0.93  |
|                          |        |                                                       | 24F          | TGGACTTAAACGGGATCGGGAG         |                    |          |                          |       |
|                          |        |                                                       | 131R         | TCGGGAGCTCAGAAAGCGATT         |                    |          |                          |       |

Continued
**Table 2.** RT-qPCR primer (forward (F), reverse (R)) and TaqMan-probe (P) information for *C. chthonoplastes* and *L. aestuarii*. Amplification efficiency and $r^2$ depict average values. All qPCR assays were run using an annealing temperature of 64 °C. *Housekeeping gene.

| Species | Gene | Function | Primer/probe Sequence (5’→3’) | Amplicon size (bp) | Reporter | Amplification efficiency | $r^2$ |
|---------|------|----------|--------------------------------|-------------------|----------|------------------------|-------|
| **L. aestuarii** | cikA | Circadian input kinase A | 2323P TCCCCGGACCTTGGG TACGGGT | 101 | FAM | 1.29 | 0.9 |
| | | | 2300F TGATCAATCAATAATCCCAT GACCA | | | |
| | | | 2400R AAAAACAGGCAGGAA GAAAGAAT | | | |
| | ftsZ | Filamenting temperature-sensitive mutant Z, cell division protein | 267P TGGGGCGGGCAAGTTG GGAA | 150 | FAM | 1.02 | 0.97 |
| | | | 240F TAACCAGGGAGAT TAAAGTCA | | | |
| | | | 389R AAAGGTTCAGTGCT TTTGACTG | | | |
| | kaiA | Circadian clock protein A | 290P TCTTGGGCTTGGGCA GGAAGCA | 119 | FAM | 1.00 | 0.98 |
| | | | 220F AAGGGGTIATCTCC GAAAATAA | | | |
| | | | 338R GCAGTTCTAAAAATTTCT TGGGGA | | | |
| | kaiB | Circadian clock protein B | 152P TGGCGGAGTTAGGA TTTTTCAAACGTCA | 145 | HEX | 1.07 | 0.97 |
| | | | 116F TTGCAATGGTATGTC GTGGC | | | |
| | | | 260R CGAACGTCGTCGAG CACGA | | | |
| | kaiC | Circadian clock protein C | 1081P CGGCTCAACCGGCA TGCCGG | 140 | CY5 | 0.98 | 0.99 |
| | | | 989F CGAGCAACCGGAAC TACCA | | | |
| | | | 1128R GTGACTCGGTTTTC CAACAC | | | |
| | nifH | Nitrogenase iron protein | 187P GCGATATTTCTGGGATTT GTGGTGTCG | 147 | HEX | 0.92 | 0.95 |
| | | | 127F GTTGTCAAATGGTGTG CGGAAG | | | |
| | | | 273R CGCTCAAAAGGATCA CAAAG | | | |
| | ppsA | Photosystem II protein D1 | 86P ACACCGGCTGGTGCG GCAGTTGG | 112 | CY5 | 1.01 | 0.90 |
| | | | 50F TCTGCAACTGGGTAAC GTCCAC | | | |
| | | | 161R GCGAGCAAGGCTAG ATCTGA | | | |
| | rnpA* | Protein subunit of ribonuclease P | 237P CGATCGGGGTCCGCTC GGTGCA | 131 | CY5,5 | 1.05 | 0.95 |
| | | | 199F CACACGGGCGTTGCT AAACCT | | | |
| | | | 329R CTCACCTTAAAGCTGA GGCGCA | | | |
| | ppc* | Phosphoenolpyruvate carboxylase (PEPC) | 2905P GCCATGGGCCCAGGA GTGATT | 118 | ROX | 1.11 | 0.91 |
| | | | 2882F AGCGTTTACAGTGCAG ATGGCA | | | |
| | | | 2999R CGACATCCAGGACGA ATACCC | | | |
| | prx | Peroxiredoxin | 55P ACGTGGGTCATATCCGGG TTTGACAAACCC | 106 | HEX | 1.02 | 0.98 |
| | | | 28F TTGAGGAGATTATCTTC TGGATCT | | | |
| | | | 133R ACCACATTCTGCAAC CTAATG | | | |
Private carboxylase) were selected as potential housekeeping genes (HKG) based on a previous qPCR study of *L. aestuarii* 26. Validation of the stability of expression of these genes under different growth conditions was tested using the housekeeping gene determination software package BestKeeper27 of which standard deviation (SD) and correlation coefficient ($r^2$) were used to verify HKG stability. According to the BestKeeper results for all treatments and light regimes, both HKGs revealed a stable expression in *L. aestuarii* ($rnpA$: SD = ±0.31–0.71, $r^2$ = 0.89–0.99; $ppc$: SD = ±0.30–0.71, $r^2$ = 0.90–0.99) and *C. chthonoplastes* ($rnpA$: SD = ±0.33–0.65, $r^2$ = 0.83–0.96; $ppc$: SD = ±0.31–0.67, $r^2$ = 0.87–0.95) and therefore both are suitable housekeeping genes for both species (Table 3).

### Table 3. BestKeeper (HKG) rnpA and pcc of *L. aestuarii* and *C. chthonoplastes* in each treatment. Standard deviations (SD) and correlation coefficients ($r^2$) are given.

| HKG  | Treatment | *L. aestuarii*  | *C. chthonoplastes* |
|------|-----------|-----------------|---------------------|
|      |           | SD  | $r^2$  | SD  | $r^2$  |
| rnpA | ML        | 0.31 | 0.99 | 0.45 | 0.87 |
|      | CL        | 0.71 | 0.92 | 0.33 | 0.96 |
|      | MB        | 0.37 | 0.89 | 0.65 | 0.85 |
|      | CB        | 0.38 | 0.89 | 0.51 | 0.83 |
| ppc  | ML        | 0.3  | 0.99 | 0.43 | 0.87 |
|      | CL        | 0.71 | 0.9  | 0.32 | 0.95 |
|      | MB        | 0.37 | 0.92 | 0.67 | 0.89 |
|      | CB        | 0.4  | 0.96 | 0.53 | 0.87 |

**Figure 1.** CCA plots of kaiABC, prx, psbA, nifH and ftsZ expression patterns in *L. aestuarii*. These genes were significantly influenced ($p<0.05$, 95% confidence ellipse) by one or more of the treatments (L = liquid culture, B = biofilm-grown culture, M = mono culture, C = co-culture). Only conditions that were significantly affecting gene expression patterns are shown.

**Effect of culturing on gene expression.** A constrained correspondence analysis (CCA) was carried out in order to investigate the influence of culturing on the expression patterns of the target genes (Figs. 1 and 2 for *L. aestuarii* and *C. chthonoplastes*, respectively.). Housekeeping gene-normalized transcript levels at the different time points served as input data. The results of the CCAs were subjected to an analysis of variance like permutation test provided by the ‘vegan: Adonis’ package in R28 providing Chi-square ($\chi^2$) and probability values ($p>F$) ($p<0.05$) for each treatment (culture condition and light regime) (Table 4). For visualization purposes CCA plots were used to display data relationships (Figs. 1 and 2). For each strain, comparisons were performed
relative to cells grown under “standard” laboratory conditions; i.e. liquid mono-cultures grown under LD light regime. Boxplots of the original data per gene per growth condition are presented in Figs. 3 and 4 for *L. aestuarii* and *C. chthonoplastes*, respectively. A direct comparison of the average gene expression levels between the different growth conditions is presented in supplementary figures S1A and B for *L. aestuarii* and *C. chthonoplastes*, respectively.

**kaiABC expression in L. aestuarii.** Comparison of the 24-h expression patterns of *kaiA*, *kaiB* and *kaiC* growing under different conditions (Fig. 1) revealed that their expression was significantly influenced by the way of culturing (liquid versus biofilm-grown growth; $X^2 = 0.033–0.074$, $p = 0.001–0.002$) as well as whether co-cultured or as mono-culture ($X^2 = 0.019–0.212$, $p = 0.001–0.003$) (Table 4). Potential rhythmic expression was deduced from comparing and fitting the expression patterns with a sine curve using the circadian cycle predictor program MetaCycle in accordance with a previous study in natural microbial mats (Supplementary Figure S2). Expression of *kaiA* followed a significant sinusoidal curve only in co-cultures under all growth conditions ($p = 0.0084–0.01$) and in biofilm-grown mono-culture under continuous illumination ($p = 0.005–0.007$) (Fig. 3a, b, Supplementary Figure S2). Highest expression of *kaiA* was observed in the light period, at point ZT9 in liquid mono- and co-cultures (ML & CL) and at ZT0 in biofilm-grown cultures (MB & CB) (Supplementary Figure S1A). A sinusoidal pattern for *kaiB* was only evident when biofilm-grown (LD-CB, LL-MB and LL-CB) ($p = 0.006–0.02$) (Fig. 3a, b, Supplementary Figure S2). The time point of maximum *kaiB* expression (ZT4.5) was largely unaffected whether co-cultured or not but changed from ZT4.5 in liquid cultures to ZT9/ZT14.5 in biofilm-grown cultures. Under continuous illumination, a similar pattern was observed for *kaiC* and differences in peak expression were also only observed between liquid (peak at CT4.5) and biofilm-grown (peak at CT9) cultures (Fig. 3b, Supplementary Figure S1A).

**prx expression in L. aestuarii.** Transcription of *prx* was overall low and was significantly affected when grown as liquid and biofilm-grown culture ($X^2 = 0.187$, $p = 0.005$) (Table 4). A sinusoidal pattern was observed for biofilm-grown mono-cultures (MB) under LD illumination ($p = 0.01$) and for CL and MB under continuous
illumination (p = 0.004–0.01) (Fig. 3a, b, Supplementary Figure S2). Highest expression of prx was observed in the late dark/early light period (ZT21, ZT0) in liquid mono-cultures and in biofilm-grown mono- and co-cultures. These expression peaks shifted in liquid cultures, grown under continuous light, to CT14.5 (ML) and CT17.45 (CL) and to CT4.5 (MB) and CT0 (CB) in biofilm-grown cultures (Supplementary Figure S1A).

**psbA expression in L. aestuarii.** The variation of expression of psbA in L. aestuarii during a 24-h period differs significantly between different light regimes (LD versus-LL; χ² = 0.307, p = 0.001) and the way of cultivation (biofilm-grown versus liquid; χ² = 0.094, p = 0.003) (Fig. 1, Table 4). Cultures growing under a light–dark regime revealed a sinusoidal pattern of psbA gene expression (p = 0.007–0.02) (Fig. 3a, b, Supplementary Figure S2) with the exception of CB cultures. Highest psbA expression was found during the beginning of the light period (ZT4.5–ZT9) followed by a sharp drop in expression in liquid cultures at ZT14.5 (Fig. 3a, b, Supplementary Figure S1A). In biofilm-grown cultures the overall expression level of psbA was lower than in the liquid

| Species          | Gene | Treatments          | ChiSquare | F    | Pr(> F) (p < 0.05) |
|------------------|------|---------------------|-----------|------|--------------------|
| L. aestuarii     | kaiA | mono_co             | 0.019847  | 3.3567 | 0.01               |
|                  |      | LD_LL               | 0.017184  | 2.9064 | 0.02               |
|                  |      | liquid_immobilized  | 0.074575  | 12.6132 | 0.001              |
|                  | kaiB | mono_co             | 0.048847  | 12.8941 | 0.001              |
|                  |      | LD_LL               | 0.001571  | 0.4147  | 0.744              |
|                  |      | liquid_immobilized  | 0.033414  | 8.8203  | 0.002              |
|                  | kaiC | mono_co             | 0.021277  | 4.6953  | 0.003              |
|                  |      | LD_LL               | 0.008953  | 1.9757  | 0.083              |
|                  |      | liquid_immobilized  | 0.03499   | 7.7215  | 0.001              |
|                  | psbA | mono_co             | 0.012166  | 0.8989  | 0.401              |
|                  |      | LD_LL               | 0.307779  | 22.7392 | 0.001              |
|                  |      | liquid_immobilized  | 0.094425  | 6.9763  | 0.003              |
|                  | ftsZ | mono_co             | 0.01304   | 4.07    | 0.012              |
|                  |      | LD_LL               | 0.020866  | 6.5129  | 0.004              |
|                  |      | liquid_immobilized  | 0.038839  | 12.1227 | 0.001              |
|                  | prx  | mono_co             | 0.19414   | 4.9258  | 0.057              |
|                  |      | LD_LL               | 0.12722   | 3.2278  | 0.062              |
|                  |      | liquid_immobilized  | 0.18735   | 4.7535  | 0.005              |
|                  | nifH | mono_co             | 0.019527  | 2.3505  | 0.108              |
|                  |      | LD_LL               | 0.296163  | 13.8281 | 0.001              |
|                  |      | liquid_immobilized  | 0.012233  | 1.3972  | 0.191              |
| C. chthonoplastes | kaiA | mono_co             | 0.077328  | 10.8547 | 0.001              |
|                  |      | LD_LL               | 0.058531  | 8.2161  | 0.001              |
|                  |      | liquid_immobilized  | 0.003109  | 0.4364  | 0.724              |
|                  | kaiB | mono_co             | 0.002273  | 0.261   | 0.924              |
|                  |      | LD_LL               | 0.0498    | 5.7195  | 0.002              |
|                  |      | liquid_immobilized  | 0.040236  | 4.6211  | 0.009              |
|                  | kaiC | mono_co             | 0.053274  | 4.4122  | 0.001              |
|                  |      | LD_LL               | 0.039746  | 3.2918  | 0.007              |
|                  |      | liquid_immobilized  | 0.083382  | 6.9058  | 0.001              |
|                  | psbA | mono_co             | 0.0294    | 2.8843  | 0.029              |
|                  |      | LD_LL               | 0.023192  | 2.2753  | 0.075              |
|                  |      | liquid_immobilized  | 0.02871   | 2.8166  | 0.031              |
|                  | ftsZ | mono_co             | 0.122411  | 18.9058 | 0.001              |
|                  |      | LD_LL               | 0.006125  | 0.946   | 0.442              |
|                  |      | liquid_immobilized  | 0.025181  | 3.8891  | 0.016              |
|                  | prx  | mono_co             | 0.263753  | 77.5317 | 0.001              |
|                  |      | LD_LL               | 0.022111  | 6.4996  | 0.007              |
|                  |      | liquid_immobilized  | 0.053278  | 15.6612 | 0.002              |
|                  | nifH | mono_co             | 0.09931   | 3.0874  | 0.029              |
|                  |      | LD_LL               | 0.21253   | 6.607   | 0.001              |
|                  |      | liquid_immobilized  | 0.26686   | 8.296   | 0.001              |

Table 4. Permutation (#999) test results for the constrained correspondence analysis. χ², F and p values are given for each cyanobacterial species, tested gene and way of cultivation. N = 24, degrees of freedom = 1.
cultures, while in CB cultures the highest expression was at the end of the light, beginning of the dark period (ZT14.5-ZT17.45). Under continuous illumination the overall expression levels in liquid cultures were slightly lower and a shift in expression pattern was observed with highest expression at ZT14.5-ZT17.45 (Fig. 3b, Supplementary Figure S2). The sinusoidal pattern was maintained under continuous illumination for CL and MB conditions biofilm-grown (p = 0.01–0.04) (Fig. 3a, b, Supplementary Figure S2).

Figure 3. Boxplots presenting the relative normalized expression patterns in liquid mono- (ML) and co-cultures (CL) and biofilm-grown mono- (MB) and co-cultures (CB) of L. aestuarii under (a) LD and (b) LL regime. X-axes show Zeitgeber time (ZT: light–dark regime) (a) and circadian time (CT: constant light regime) (b). Red boxplots display the on average highest expression. Expressions patterns which display a blue (dotted) line display significant (p < 0.05) circadian rhythmicity as estimated by MetaCycle29. The dotted lines indicated significantly rhythmicity under LD illumination, while continuous lines symbolize significant rhythmicity under both light regimes (a, b). Boxplots display the minimum (lower error bar), maximum (upper error bar), median (horizontal line) and the first (box below the median) and third quartile (box above the median) of the expression values of the biological replicates (n = 3) per sample. A bar on top of the first row of the boxplots indicates the dark (black) and light (white) period (a). Treatments are distributed into columns and each row represents a gene.
nifH expression in *L. aestuarii*. Under the standard dark–light regime, nifH expression was low during the light and was highest during the dark (ZT17.45-ZT21) (Fig. 3a, Supplementary Figure S1A). Moreover, nifH expression was significantly higher in liquid cultures compared to the biofilm-grown cultures. A significant sinusoidal curve under LD illumination was observed under all conditions ($p = 0.005–0.01$) except for the co-cultured biofilm-grown cultures (CB) (Fig. 3a, Supplementary Figure S2). The imposed light regime significantly affected the daily transcription pattern of nifH ($\chi^2 = 0.296$, $p = 0.001$) (Fig. 1, Table 4). Under continuous light, the pattern of expression in the liquid cultures peaked more often than in biofilm-grown cultures with high expression at CT0, CT4.5 and CT21. (Fig. 3b, Supplementary Figure S1A). In biofilm-grown mono-cultures nifH expression peaked at CT14.5 and CT21 and in co-cultures at CT17.45. No significant sinusoidal nifH gene expression was found under continuous illumination.

ftsz expression in *L. aestuarii*. The variation in expression of ftsZ in *L. aestuarii* during a 24-h period was large and only in co-cultured biofilm-grown cultures at LD illumination a sinusoidal like curve was observed with a maximum at ZT0 ($p = 0.03$) (Fig. 3a, Supplementary Figure S2). In contrast, under continuous illumination an opposite pattern is found with all the three other growth conditions providing significant sinusoidal ftsZ expression ($p = 0.008–0.01$) (Fig. 3b, Supplementary Figure S1A). Due to other more variable patterns, each of
the imposed cultivation conditions appeared to contribute significantly to the gene expression pattern (liquid vs. biofilm-grown: $X^2 = 0.038$, $p = 0.001$; LD/LL: $X^2 = 0.020$, $p = 0.004$ and mono-/co-culture: $X^2 = 0.013$, $p = 0.012$) (Fig. 1, Table 4).
kaiABc expression in C. chthonoplastes. The expression pattern of kaiA, kaiB and kaiC of C. chthonoplastes grown under a LD regime changed significantly when transferred to continuous light (\( \chi^2 = 0.049–0.058, p = 0.001–0.007 \)) (Fig. 2, Table 4). Furthermore, the expression of kaiA and kaiC differed when the organism was grown as a mono-culture or co-cultured with L. aestuarii (mono- vs. co-culture: \( \chi^2 = 0.053–0.077, p = 0.001 \)), while the way of culturing significantly impacted on the expression of kaiB and kaiC during a 24-h period (liquid- vs. biofilm-grown-culture: \( \chi^2 = 0.040–0.833, p = 0.001–0.009 \)) (Fig. 2, Table 4).

The expression of kaiA and kaiB did not seem to follow a particular pattern and only in liquid co-cultures a potential sinusoidal curve was discerned for kaiA expression (\( p = 0.005 \)) and in biofilm-grown mono-cultures for kaiB (\( p = 0.03 \)) (Fig. 4a, Supplementary Figure S1). Peak expression for kaiA in liquid co-cultures and biofilm-grown cultures was at the beginning of the light period (ZT0) and at the end of the dark period (ZT21) and peaked at ZT0 and ZT9 in liquid mono-cultures (Fig. 4a). A sinusoidal distribution was also observed for kaiC expression of cells grown in ML and CB conditions with respective highest expression at ZT0 and ZT4.5 (\( p = 0.01 \)) (Fig. 4a, Supplementary Figure S2).

Under continuous illumination, kaiA expression was sinusoidal at LL- MB (\( p = 0.01 \)) and kaiB at LL-CB (\( p = 0.003 \)) with peak expression at CT17.5 and CT14.5 (Fig. 4b, Supplementary Figure S2). Potential rhythmic expression of kaiC under LL conditions was not observed.
pxr expression in C. chthonoplastes. The expression pattern of prx in C. chthonoplastes differed significantly between a mono-culture and co-culture with L. aestuarii (χ² = 0.263, p = 0.001), and between a liquid medium and biofilm-grown culture (χ² = 0.053, p = 0.002), as well as upon transfer to continuous light (χ² = 0.022, p = 0.007) (Fig. 2, Table 4). Overall, the expression of prx was 5–10 fold higher in biofilm-grown than in liquid culture (Fig. 4a, b, Supplementary Figure S1B). Expression of prx under standard conditions (ML-LD) gradually increased from the beginning of light to the dark period while an almost opposite trend was observed in MB cultures with highest expression at time points ZT0 and ZT21. A sinusoidal pattern was observed in the co-cultures and in biofilm-grown mono-cultures independent of the light regimes tested (p = 0.006–0.04) (Fig. 4a, b, Supplementary Figure S2).

psbA expression in C. chthonoplastes. Expression of psbA in C. chthonoplastes was significantly influenced by two of the growth conditions (mono-/co-culture: χ² = 0.029, p = 0.029, liquid vs. biofilm-grown: χ² = 0.028, p = 0.031) (Fig. 2, Table 4). A sinusoidal pattern was observed for psbA under CL conditions at LD illumination (p = 0.01) and under CL and MB conditions under continuous illumination (p = 0.003–0.01) (Fig. 4a, b, Supplementary Figure S2). The expression of psbA in LD-grown cultures peaked during the early dark to early light period (ZT17.45–ZT0) (Fig. 4a, Supplementary Figure S1B). Under continuous illumination, co-cultures and MB cultures revealed peaks in the expression of psbA between CT14.5 and CT21, while the ML culture was characterized by high psbA expression at CT0 (Fig. 4b, Supplementary Figure S1B). No significant sinusoidal patterns were found for high psbA expression under any of the tested conditions.

nifH expression in C. chthonoplastes. The expression of nifH was significantly affected by the way of culturing (liquid vs. biofilm-grown: χ² = 0.266, p = 0.001, mono-/co-culture: χ² = 0.099, p = 0.029) and by transfer to constant light (χ² = 0.212, p = 0.001) (Fig. 2, Table 4). Relative nifH expression in biofilm-grown, co-culture-grown and LL-grown cultures displayed less fluctuation than displayed in liquid-grown, mono-culture-grown and LD-grown cultures. Expression of nifH in C. chthonoplastes was overall low with a single peak at the late dark/early light period in liquid cultures (ZT21 (ML), ZT0 (CL)) and during the light period in biofilm-grown cultures (ZT9 (MB), ZT4.5 (CB)) (Fig. 4a, Supplementary Figure S1B). In the liquid mono-culture at LL a single peak in the expression of nifH was observed at CT0 (Fig. 4b, Supplementary Figure S1B). No significant sinusoidal patterns were found for C. chthonoplastes nifH expression under any of the tested conditions.

ftsZ expression in C. chthonoplastes. Expression of the cell division protein coding gene ftsZ was significantly affected whether co-cultured or not (χ² = 0.122, p = 0.001) and liquid- or biofilm-grown culture (χ² = 0.025, p = 0.016) (Fig. 2, Table 4). The patterns of expression of ftsZ during a 24-h period varied. A sinusoidal curve was only observed in mono-cultures (p = 0.006–0.01) and at LL-CL and LL-MB (p = 0.008–0.04) (Fig. 4a, b, Supplementary Figure S2) with peak expression during the late dark and early light period in LD-grown cultures and at same time points in LL-grown cultures. (Fig. 4a, b, Supplementary Figure S1B).

Discussion
The circadian clock of L. aestuarii and C. chthonoplastes. Rhythmic gene expression in cyanobacteria involves the following three steps: (1) the external cues, mostly light, temperature and redox potential that (2) entrain the “clock” proteins that control the rhythmic expression of (3) the functional protein coding genes involved in processes that are separated in time.

The only controlled rhythmic cue in our experimental set-up was the illumination period while temperature was kept the same in all experiments. Given the large number of variables tested within one cycle and the need for at least 6 time points per cycle, we were not able to assay over a 48 h or longer period that would be needed to determine truly circadian control. However, the observed significant differences in expression patterns are sufficient to infer that circadian control may also be affected by the greater complexity of natural conditions.

Both the way of culturing (liquid or biofilm-grown) as well as whether the cyanobacteria were co- or mono-cultured affected the expression of the circadian clock genes kaiA, kaiB, and kaiC in both species, albeit in different ways.

The maximum expression of the circadian clock genes kaiABC of L. aestuarii shifted 4–8 h between liquid and biofilm-grown cultures. Shifting the cultures to continuous illumination for 2 days, from which samples were taken on the second day, had little influence on the variation of expression of the three kai genes over a 24-h period, which is indicative of the free-running cyanobacterial clock as has been previously shown for unicellular cyanobacteria. In contrast, the cultures of C. chthonoplastes did not display a free-running clock. Under continuous light, maximum gene expression of kaiABC in this organism shifted in regard to what has been found under a dark-light regime when grown in biofilm-grown cultures and co-culture.

Based on the aforementioned results we suggest that, despite being both members of the order of Oscillatoriales, the two species display major differences in how stringent the control over their circadian clocks is. These differences may be attributed to the life strategies of the two cyanobacteria regarding their response to high light. For example, C. chthonoplastes avoids light stress by migration, which may trigger several transcriptional responses in line with a metabolism that is not optimally adapted to continuous high light intensities. In contrast, Lyngbya only moves when differentiating into hormogonia: short, sheet-less motile trichomes, and therefore cannot avoid the imposed light stress. However, Lyngbya species evolved strategies to endure high light, desiccation and UV-stress. To avoid the latter, Lyngbya species synthesize UV-screening compounds such as mycosporine amino acid-like substances (MAAs) and carotenoids, in addition to coating of their trichomes.

These strategies may not require additional transcriptional regulation in response to continuous illumination and might explain why the expression of kaiABC in Lyngbya sp. does not differ between the two illumination set ups. The observed expression patterns in C. chthonoplastes are less well understood and perhaps appear more similar
to an ‘hourglass’ clock, which has been described for kaiA-lacking species such as Prochlorococcus marinus.37 However, since C. chthonoplastes does have, and expresses the kaiA gene, the differences in expression with L. aestuarii may reflect specific adaptation to their natural habitat. In support of this assumption is the low identity between kaiA nucleotide and protein sequences (<60% identity) of L. aestuarii and C. chthonoplastes (and also compared to the kaiA sequence of S. elongatus), which is also the lowest in comparison to nifH and kaiC (>75% identity), suggesting that kaiA is the least conserved gene and may experience the lowest selective pressure among the kai genes (Supplementary Table S1, Supplementary Figure S3). Even though the two species co-occur in many marine microbial mats, L. aestuarii and C. chthonoplastes may occupy different ecological niches that allow them to share light and micronutrients using differently tuned circadian clocks.

A kaiABC-independent circadian oscillator has been linked to the periodicity of peroxiredoxin which follows the generation of stress-induced reactive oxygen species.25,38 Overall, the expression patterns of prx reveal a significant sinusoidal curve over the 24-h sampling period for most conditions except the standard liquid mono-cultures in C. chthonoplastes and under some conditions in L. aestuarii. However, the expression levels of prx shifted significantly between mono- and co-cultures of liquid (L. aestuarii) and biofilm-grown (C. chthonoplastes) cultures. Highest prx expression was found in mono-cultures in the dark to early light period. This was also found in Crocosphaera watsonii strain WH8501 and it was attributed to the accumulation of reactive oxygen species (ROS) towards the end of the light period.35 In contrast to mono-cultures, prx expression in co-cultures of L. aestuarii and C. chthonoplastes displayed maxima towards the late light period. This would suggest that the accumulation of ROS occurs faster in co-cultures as the result of a higher rate of oxygen concentration due to the combined photosynthetic activity. Robust free-running prx expression25 without a peak shift was only found in co-cultures of liquid (C. chthonoplastes) and biofilm-grown (L. aestuarii) cultures under continuous illumination. Regardless of the illumination regime and treatment, average prx levels of C. chthonoplastes were more than a 100-fold higher than in cultures of L. aestuarii. The large transcriptional discrepancy between the species’ prx levels and between C. chthonoplastes’ liquid and biofilm-grown cultures suggests a higher sensitivity of C. chthonoplastes and especially of its biofilm-grown cultures, to oxidative stress and explains its migratory behaviour to avoid high light intensities.

The expression of circadian clock regulated genes in mono- and co-cultures grown in free-living or biofilm-grown mode. Depending on growth conditions, expression of psbA in C. chthonoplastes and L. aestuarii, and nifH in L. aestuarii followed a sinusoidal pattern during the 24-h period. In the unicellular cyanobacteria Cyanothecae sp., expression of psbA and nifH is under control of the kaiABC regulatory network,40 similar as for psbA expression in S. elongatus.11 The peak in expression of psbA in the early light period and of nifH in the dark is typical for non-heterocystous, circadian clock controlled cyanobacteria in which nitrogenase activity is confined to the light periods where photosynthetically produced oxygen is low.42,43 Remarkably, we did not find such distinction between the peaks of psbA and nifH expression in L. aestuarii grown as a biofilm-grown co-cultured with C. chthonoplastes. Instead, both psbA and nifH peaked during the dark. It is unclear why the expression of psbA shifted to the dark in biofilm-grown co-cultures. Moreover, under LD conditions psbA and nifH peak transcription levels in L. aestuarii were up to 11 times lower in biofilm-grown cultures compared to liquid cultures. Albeit less distinct, the same was seen in C. chthonoplastes. The lower expression of psbA in biofilm-grown cultures may have been caused by the lower exposure to light of the biofilm-grown trichomes or by a decreased rate of growth as has been observed in aggregated cells in biofilms of Escherichia coli.36 However, the latter explanation was not supported by lower transcription levels of the cell division gene ftsZ in biofilm-grown cultures. Alternatively, as is the case in S. elongatus, reactive oxygen species may target the de novo synthesis of psbA mRNA10 decreasing the rate of photosynthesis.25 This explanation is supported by the increase of prx expression in biofilm-grown cultures that is possibly a response to an increase in ROS.

When L. aestuarii was transferred to continuous illumination the expression peak of psbA shifted 4–8 h later during the 24-h cycle. In contrast, C. chthonoplastes, which does not display a free-running circadian rhythm, the peak of the expression of psbA did not shift when the culture was transferred to constant illumination. These results challenge the degree of control of the circadian clock on the expression of psbA in these cyanobacteria and call for another controlling mechanism.

The measurement of expression of nifH in C. chthonoplastes was hindered by technical difficulties in the qPCR reaction yielding in some runs false positive reactions above the threshold level in the non-template controls, which was attributed to a known reagent contamination problem.35 However, this problem is mostly neglectable when large amounts of species specific nifH containing DNA is added as template. With some caution we conclude that nifH expression in C. chthonoplastes revealed low transcript numbers and lack of temporal separation compared to psbA expression. This is unusual for non-heterocystous cyanobacteria. Moreover, it is still uncertain whether C. chthonoplastes actually fixes atmospheric dinitrogen since it has not yet been possible to grow the species diazotrophically in culture.49,50 It lacks the accessory genes nifOTWXZ, which are present in, for instance, L. aestuarii. The function of these accessory nif genes is not well understood and it is therefore not certain whether they are indispensable for N2 fixation. Daily patterns of nifH expression in biofilm-grown C. chthonoplastes cultures are similar to those observed in a coastal microbial mat13 as well as metatranscriptomes from the same mat.13 These studies report that nifH expression is high in the beginning and/or middle of the light period.

In conclusion, comparison of gene expression patterns between laboratory cultures and field samples are rare and may be contradictory. The daily variations of gene expression obtained from the metatranscriptome of oceanic surface water containing abundant picocyanobacteria (Synechococcus) were similar to those obtained from liquid cultures.53 The laboratory conditions for liquid cultures may better represent the natural conditions of
these planktonic species than would be the case for benthic microorganisms. The transcriptomes of Salinibacter ruber and Haloquadratum walsbyi reveal large differences in relative expression levels in a subset of genes while relative expression levels of other genes are well conserved in the metatranscriptomes from natural samples in which these organisms thrive (Bolhuis, unpublished data).

Our study shows that gene expression in two filamentous non-heterocystous cyanobacteria respond differently to external stimuli and potential zeitgebers. Phase shifts as well as changes in expression levels occurred as a function of the mode of growth (liquid medium versus biofilm-grown culture) and to the presence of the other competitor species. The observed species-specific expression patterns most likely reflect different life strategies, hinted at by differences in expression of prx, psbA, and nipH. In this study, neither co- or mono-cultivation nor liquid or biofilm-grown growth could be held as main responsible effectors for the observed differences in gene expression patterns. However, in combination these effectors are able to alter gene expression not only in the laboratory but also in nature. As a consequence, most laboratory derived cyanobacterial circadian clock (controlled) expression patterns are insufficient predictors for expression patterns in the field. This emphasizes the need to mimic the natural environment when testing bacteria in the laboratory.

Material and methods

Experimental setup. Stock cultures of the filamentous, non-heterocystous cyanobacteria Lyngbya aestuarii PCC8106 (synonym = Lyngbya sp. CCY9616) and Coleofasciculus (Microcoleus) chthonoplastes PCC7420 (CCY9604) served as inoculum for the experiments. These cyanobacteria were isolated from similar coastal microbial mats but at different times and at a large geographical distance (Mellum, Germany (L. aestuarii) and Woods Hole, USA (C. chthonoplastes)). Although the two species, both members of the order Oscillatoriales, are naturally co-occurring in coastal microbial mats and in many other similar habitats [54–57], the isolates tested

- sampling
- RNA extraction
- Gene quantification
- Material and methods

Gene quantification. Based on the genome sequences of L. aestuarii and C. chthonoplastes, primers and TaqMan probes of the targeted circadian clock gene cluster kaiABC, the circadian clock reporter gene cikA, nitrogenase gene nipH, cell division protein gene fisZ, photosynthesis D1 protein gene psbA, peroxiredoxin gene prx and two commonly used cyanobacterial housekeeping genes, rnap1 and ppc25, were designed using the genetic analysis software Geneious R 8.1.7 [28] (Table 2). The proper annealing temperatures of the primers were established by gradient PCR on a thermocycler (BioMetra) and specificity was confirmed by Sanger sequencing. Standard curves for RT-qPCR were prepared by using dilution series of PCR products and the primers were checked for species specificity by means of crossover PCRs and gel electrophoreses. Samples, standard curves and non-template controls were run in technical triplicates on a Rotor-Gene 6000® (Qiagen, USA).

RT-qPCR amplification of the targeted and housekeeping genes was performed in 15 µl volumes containing 2.25 µl MilliQ, 7.5 µl 2× Multiplex qPCR Perfecta Supermix (Quanta Biosciences), 0.38 µl (0.5 µM) of each target and housekeeping gene primer, 0.19 µl of each probe (0.25 µM) and 1.5 µl of template. RT-qPCR cycling
was performed at an initial activation step of 95 °C for 2 min followed by 40 cycles at 95 °C for 10 s and at 64 °C for 60 s. Gene amplification efficiencies (\(r^2\)), Ct values and transcript abundances (copies/µl) were retrieved from the program Rotor-Gen Q 2.1.0 (Qiagen) (Supplementary Table S2). Where needed, missing Ct values of biological replicates were surrogated by the median of the remaining biological replicates.19

**Statistics.** To evaluate the validity of the housekeeping genes across treatments, Ct values of genes of interest and housekeeping genes were used in the program BestKeeper27. T-tests were applied to examine the sample-wise expression within each genes’ expression profile and the influence of treatments on transcription was assessed using R-based CCA scripts and ANOVA (X², F- and p values (< 0.05)). In these tests normalized transcript abundances ((\(\frac{GMI}{\text{geomean(HKGs)}}\)) of each gene served as input data. CCAs were plotted and supplemented with confidence ellipses (95%) of the standard expression of the different genes. Potential significant rhythmic gene expression following a sinusoidal curve was predicted by the R based script Metacycle23 using the transcription levels at different time points as input matrix. The cycle analysis was performed with the meta2D function that integrates multiple cycle prediction methods and combines their p-values. Training of the cycling algorithm was performed by combining the datasets, thereby mimicking a 72 h sampling pattern and a p-value cut-off of 0.05 was used to determine potential sinusoidal patterns.

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**Author contributions**

Culturing was done by C.H. and M.G. followed by sampling which was executed by C.H., V.C.-G. and H.B. Molecular lab work was done by C.H. and V.C.-G. Gene expression data was analyzed by C.H. and the manuscript written by C.H., L.J.S. and H.B.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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