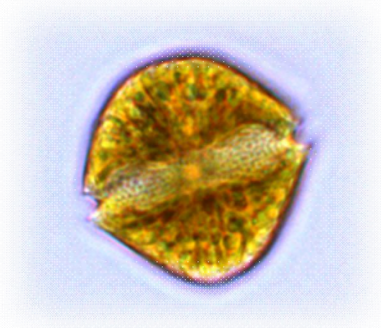

STIMULATED AND NATURAL PATTERNS OF BIOLUMINESCENCE IN THE DINOFLAGELLATE *ALEXANDRIUM OSTENFELDII*

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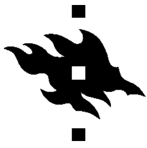

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The bioluminescent and toxin-producing dinoflagellate *Alexandrium ostenfeldii* forms dense blooms in coastal areas of the Baltic Sea. Bioluminescence, light production by an organism through a chemical reaction, is a nocturnal, rhythmic phenomenon in surface algae. In this study, the bioluminescence pattern and rhythm of *A. ostenfeldii* was under investigation. Procedures for continuous bioluminescence measurements, to support dedicated environmental monitoring of toxic dinoflagellate blooms, were developed.

The study consisted mainly of laboratory experiments. Semi-continuous field measurements were included for comparison. In the laboratory, the light production of monocultures of *A. ostenfeldii* was measured with a spectroluminometer or bathyphotometer, continuously during the night, or for several consecutive days. The method to stimulate bioluminescence was varied, as well as the recovery period of the cells after stimulation. Light regimes during growth and pre-measurement adaptation were also taken into account.

The experiments confirm that bioluminescence in *A. ostenfeldii* follows a circadian pattern and can be stimulated with the chosen methods. Bioluminescence could also be stimulated after culturing in continuous light. Measurement parameters for rhythm experiments (stimulation frequency and recovery period), were optimised. Multi-day experiments in complete darkness suggested that sufficient energy was available to maintain bioluminescent response during one night, although an endogenous rhythm remained present.

These experiments gave insight to the phenomenon of bioluminescence regulation in *A. ostenfeldii*, but also gave rise to new questions. Some repeated measurements resulted in very low bioluminescence intensity, without an obvious reason. The light regime is not the only factor controlling bioluminescence. The interplay between bioluminescence and the growth and condition of the cultures is of interest.

Avainsanat – Nyckelord – Keywords
dinoflagellate bioluminescence, stimulation of bioluminescence, diurnal rhythm, cyst formation, *Alexandrium ostenfeldii*

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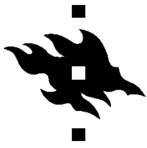

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Tiivistelmä – Referat – Abstract

Hohtava ja myrkyllinen panssarisiimalevä *Alexandrium ostenfeldii* on viime vuosina muodostanut tiheitä kukintoja Itämeren rannikkovesissä. Bioluminesenssi on eliöiden omaa valontuotantoa kemiallisen reaktion kautta. Pintalevien valontuotanto on rytmistä ja ajoittuu yöhön. Tässä opinnäytetyössä tutkittiin *A. ostenfeldii*n bioluminesenssin ominaispiirteitä ja rytmiikkaa. Pitkäaikaisia bioluminesenssimittauksia varten kehitettiin menetelmä.

Tutkimus koostui pääasiassa laboratoriokokeista. Lisäksi kenttätutkimuksen aineistoa käytettiin menetelmävertailuun. Laboratoriossa *A. ostenfeldii* -viljelmien valontuotanto mitattiin spektroluminometrillä tai batyfotometrillä yhtäjaksoisesti yön yli tai useiden päivien ajan. Kokeissa muutettiin bioluminesenssin käynnistysmenetelmää (eli kasvatusliuoksen sekoitusmenetelmää), aikaa ärsykkeiden välissä, sekä valo-olosuhteita kasvatuksen (mukaan lukien yhtäjaksoinen valo) ja mittausten aikana (mukaan lukien yhtäjaksoinen pimeys).

Kokeet osoittivat, että *A. ostenfeldii*n bioluminesenssi vaihtelee vuorokauden ajan mukaan ja se voidaan saada aikaan valittuja menetelmiä käyttäen. Bioluminesenssiä voitiin havaita myös levien kasvettua yhtäjaksoisesti valossa. Pitkäaikaismittausten menetelmää varten selvitettiin sopiva stimulaatiotiheys (sekoitustiheys). Useamman päivän kestäneet kokeet yhtäjaksoisessa pimeydessä osoittivat, että levien energiavarastot riittävät useimmiten vain yhden yön valontuotantoon, vaikka sisäinen rytmi pysyisikin pimeydessä.

Kokeet antoivat tietoa kyseessä olevasta ilmiöstä, mutta myös nostivat esille monia uusia kysymyksiä. Esimerkiksi jotkut rinnakkaisista mittauksista antoivat hyvin matalan bioluminesenssin voimakkuuden ilman ilmeistä syytä. Valo-olosuhteet eivät kokeiden perusteella ole ainoa bioluminesenssiä säätelevä tekijä. Bioluminesenssin vuorovaikutus kasvuolosuhteiden ja viljelmien kunnon kanssa on yksi mahdollisista lisätutkimuksen aiheista.

Avainsanat – Nyckelord – Keywords
panssarisiimalevien bioluminesenssi, bioluminesenssin tuottaminen, vuorokausirytmi, kystien muodostus, *Alexandrium ostenfeldii*

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Contributions

This Master's thesis was an individual part of the project "Bioluminescence in the Baltic Sea: exploring the potential of night-time optical markers for optical-taxonomical classification of harmful dinoflagellate blooms" in the Marine Research Centre of the Finnish Environment Institute SYKE, financed by the Academy of Finland (decision 132409). In this project PhD student Anniina Le Tortorec investigates the distribution of *Alexandrium ostenfeldii* and bioluminescence in the Baltic Sea, and the possibilities of optical sensors for monitoring of bioluminescent blooms. Specific contributions to the thesis by other team members are described in the table below.

Rhythm experiment cell counts:	Anniina Le Tortorec
Map of study area:	Stefan Simis
Processing raw rhythm experiment data:	Anniina Le Tortorec
Processing raw in situ data:	Stefan Simis
Construction of in situ data logger:	John Olsson

1 Introduction

1.1 Bioluminescence in the sea

A large variety of marine organisms bioluminesce, i.e. they are able to produce light via a natural chemical reaction. For example, many copepods, dinoflagellates, cnidarians, ctenophores and cephalopods are bioluminescent (Widder 2002; Haddock et al. 2010). Bioluminescence is a fundamental aspect of the visual environment in the oceans. In fact, bioluminescent organisms can be so abundant, that "-- any animal seeking darkness as a means of evading detection must cope with a potential bioluminescent minefield", as Widder (2002) appositely remarks.

Haddock et al. (2010) estimate that bioluminescence has evolved a minimum of 40 times and presumably more than 50 times among extant organisms. Several hypotheses have been formulated to explain the development of bioluminescent systems. Timmins et al. (2001) critically asses these and support the hypothesis that detoxificating molecular oxygen was the original benefit of ancient bioluminescence reactions. Instead of using antioxidant molecules for protection from oxygen toxicity, "futile" oxygen consumption is operated to reduce oxygen concentrations. These reactions are futile in the sense that no energy or useful end products are harnessed, but once the light emission achieved by these reactions became detectable (due to rising ambient oxygen pressure) by the light receptors present, it gained a biologically useful effect. From here on, evolution could act on the light production aspect of the reactions regardless of the oxygen consumption. With rising ambient oxygen concentrations, futile oxygen consumption by bioluminescent reactions would not suffice as a protective mechanism, and other systems evolved. However, this hypothesis doesn't extend to all bioluminescent groups, like the insects.

Bioluminescence in marine organisms serves a variety of purposes. These can be divided into three categories: 1) defence against predation, 2) finding prey, and 3)

interspecific communication (Haddock et al. 2010; Widder 2010). For dinoflagellates, defence against predation by alerting a secondary predator is hypothesized. By emitting light upon predation by zooplankton, the zooplankton becomes visible to secondary predators, e.g. small fish. Abrahams and Townsend (1993) observed that the mortality of copepods was greater in the presence of bioluminescing scotophase dinoflagellates. Experimental tanks contained three trophic levels: dinoflagellates, copepods, and a stickle-back. In the control tanks the dinoflagellates were in the photophase, i.e. their bioluminescence was greatly reduced, and copepod mortality was lower. The authors conclude that the bioluminescence of dinoflagellates contributed to predation on the copepods and thus aided survival of the dinoflagellates, supporting the 'burglar-alarm' hypothesis.

Fleisher and Case (1995) have similar results from studies with cephalopods. The night-active squids and cuttlefishes used in the experiment were utilizing the light from dinoflagellate bioluminescence for hunting nonluminous prey. In other experiments, midshipman fish predation on mysids was greater when bioluminescent dinoflagellates were present in apposite concentrations (Mensinger and Case 1992).

Studies also indicate that bioluminescence affects the copepods directly. The cell ingestion rates were lower on algae with high bioluminescence capacity. The authors presume that flashes, when produced at sufficiently long intervals, startle and confuse the predator (Esaias and Curl 1972). In the experiments of Buskey et al. (1983) copepods increased swimming speed and rapid movements, bursts of speed, in the presence of bioluminescent dinoflagellates. Thus, the copepods are probably startled by bioluminescence and swim away, reducing grazing. Further experiments with an artificial light source simulating dinoflagellate bioluminescence yielded consistent results for four other copepod species (out of six species tested) (Buskey and Swift 1985).

Other uses of bioluminescence among marine organisms include counterillumination (making the prey invisible to predators below it), warning

signals, luring, illuminating or confusing prey, distracting the predator by luminescing clouds or body parts, and finding mates (Haddock et al. 2010; Widder 2010).

1.2 Mechanism of bioluminescence

In the bioluminescence reaction, a light-emitting molecule, luciferin, is oxidized by the enzyme luciferace. Luciferaces are diverse and distinctive between groups of organisms, while only four types of luciferins represent most taxa. Most bioluminescence reactions release photons around a peak wavelength of 470 nm, in the blue part of the spectrum, but other colours occur (Haddock et al. 2010; Widder 2010).

Bioluminescence can also be emitted without addition of oxygen or substrate (luciferin), from proteins called Ca^{2+}-regulated photoproteins. These are found mainly in Ctenophora and Cnidaria. The substrate, coelenterazine (one of the four most common luciferins), is tightly bound to the protein, and is oxidatively decarboxylated in the reaction (Vysotski and Lee 2004).

The bioluminescence in dinoflagellates, among others, is internal, whereas for example some copepods and ostracods release bioluminescent chemicals into the water (Widder 2002).

The dinoflagellate luciferin is a tetrapyrrole and resembles the molecular structure of the plant pigment chlorophyll. It is speculated that the two molecules might be interconverted on a daily basis (Haddock et al. 2010). The same luciferin is found in euphausiids, which probably obtain it from their diet (Haddock et al. 2010).

Nicolas et al. (1987) labeled luciferace and recognized dense vesicles in the cells as light-emitting organelles in several dinoflagellate species. These vesicles, the scintillons, contain the reactive compounds of the bioluminescence reaction.

The basic knowledge of dinoflagellate bioluminescence comes through studies on the photosynthetic marine red-tide dinoflagellate *Lingulodinium polyedrum*

(formerly named *Gonyaulax polyedra*). In addition to luciferace and luciferin, the regulatory luciferin binding protein (LBP) has been identified. Luciferin binding protein binds luciferin in pH values above 7. Below 7, luciferin is freed, whereupon oxidation by luciferace and consequently light production occurs. Luciferace is also pH-sensitive, experiencing conformational changes (Morse et al. 1989a; Hastings et al. 2009). Bioluminescence is triggered by pressure on the cell membrane, which leads to increased fluidity of the cell membrane (Mallipattu et al. 2002), a signaling cascade and finally the pH change. Upon mechanical stimulation, Ca^{2+} enters the cytosol (possibly from both intra- and extracellular stores) and results in an action potential across the vacuole membrane, which leads to influx of protons into the cytoplasm (von Dassow and Latz 2002).

There are two types of light emission in *L. polyedrum*. Stimulation triggers short and intense light bursts (0.1 sec, flash peak intensity ~10^9 quanta s^{-1} $cell^{-1}$), visible to the human eye. Long-lasting (over a period of several hours) glow is weak (peak intensity ~10^4 quanta s^{-1} $cell^{-1}$) and probably without ecological significance (Fritz et al. 1990; Knaust et al. 1998; Hastings et al. 2009). Potentiation of light emission occurs at high stimulus intensities (Widder and Case 1981).

Pressure induced by water flow can be sufficient to stimulate bioluminescence. Bioluminescence response thresholds for water flows in four tested dinoflagellate species (*Ceratium fusus*, *Ceratocorys horrida*, *L. polyedrum* and *Pyrocystis fusiformis*) however proved to be several orders of magnitude higher than the flow patterns the algae typically encounter in the ocean, although differing between species. Exceptions like breaking waves produce strong enough cell wall shear stress levels to induce bioluminescence. Hence the bioluminescence capacity is not depleted in the ambient conditions and is operational for the antipredatory function. With increasing mechanical stimulation both the population response (number of organisms responding) and flash intensity increase. The bioluminescence response is similar for laminar and turbulent flows when the wall shear stress is similar (Latz and Rohr 1999; Latz et al. 2004).

1.3 Rhythmicity and regulation of bioluminescence

Organisms exhibit rhythms in their physiological processes, which enables to be prepared for the repeated changes in the environment. The rhythms are endogenous, and under constant conditions circadian rhythms run with a period of approximately 24 hours. The external zeitgeber, typically sunlight, entrains the rhythm to exactly 24 hours. For example, levels of hormones or the sleep-wake cycle are controlled by the endogenous circadian clock (Hill et al. 2008). The inner clock, an oscillating molecular pacemaker is in the level of a single cell (Roenneberg and Mittag 1996). In unicellular algae clear rhythms are observed in cell division cycle and photosynthesis (Sweeney 1982; Sweeney 1986; Roenneberg and Mittag 1996). The clock genes of dinoflagellates are different from those identified in other organisms and have not yet been revealed (Lin 2011). In *L. polyedrum* nitrate has been observed to work as a non-photic zeitgeber (Sweeney and Folli 1984).

Most, if not all, dinoflagellates show a circadian rhythm in bioluminescence. The night time mechanically stimulated bioluminescence can be 14–200 times higher than in the light phase (Hastings and Sweeney 1958; Biggley et al. 1969; Christianson and Sweeney 1972; Latz and Lee 1995). Among others, Knaust et al. (1998) reported a rhythmicity for *L. polyedrum, Pyrocystis noctiluca* and *Pyrocystis lunula* transferred to constant, dim light. Daily rhythms persisted for days. For the two first mentioned species, the rhythm involves both flashing and glow (terms explained in chapter 1.2), whereas in *P. lunula* only flashing is rhythmic. Hastings and Sweeney (1958) followed the rhythm of *L. polyedrum* for over two weeks. Kelly and Katona (1966) observed an endogenous rhythm for three days in natural populations from Massachusetts coast transferred into darkness. Most of the dinoflagellates indentified in these samples belonged to the genera *Peridinium* and *Gonylauax.*

The inner clock in *L. polyedrum* is observed to be temperature-independent, as in other organisms, with only relatively small changes in the period length in different temperatures (15 % over 10 °C) (Hastings and Sweeney 1957). Phase

shift in *L. polyedrum* can be induced by a single irradiation of the cultures (depending on the intensity and wave length of the light and the phase of the cultures) (Hastings and Sweeney 1960; Sweeney 1963), exposure to vanillic acid (by depolarizing the external membrane) and some other chemical compounds (Kiessig et al. 1979).

There are interspecific differences in the circadian regulation of bioluminescence. In *L. polyedrum*, the concentrations of the active compounds of the bioluminescence reaction fluctuate in a circadian cycle. Both the enzyme, luciferace, and the luciferin binding protein (LBP) are re-synthetized every night and almost completely degraded by morning (Johnson et al. 1984; Morse et al. 1989b; Knaust et al. 1998). However, in the *Pyrocystis* species LBP is not present and luciferace levels don't fluctuate (Knaust et al. 1998). The regulative mechanism for this taxon is suggested to be differences in the localization of the reaction components between photo and scotophases. Widder and Case (1982) detected diurnal fluctuations in the localization of the microsources (scintillons) responsible for light production. During the scotophase they are evenly distributed in the cytoplasm. During the photophase acid induced weak bioluminescence glow could only be observed around the nucleus. In constant dark conditions, however, the microsources did not disappear from the peripheral cytoplasm during the subjective photophase, but the perinuclear glow was visible, and the cell was mechanically inexcitable. In *L. polyedrum*, the amount of scintillons, along with the reaction compounds, is fluctuating in a circadian cycle (Fritz et al. 1990). The glow, occurring at the end of the scotophase, is believed to be caused by the breakdown of scintillons.

The circadian control of luciferin binding protein is on the translational and not transcriptional level: the messenger RNA (mRNA) levels of LBP do not fluctuate in *L. polyedrum* in a circadian rhythm (Morse et al. 1989b). Mittag et al. (1994) identified a protein in *L. polyedrum* that binds to the untranslated region of mRNA of LBP. The binding affinity shows a circadian rhythm, where the affinity decreases towards the night, when LBP synthesis begins. Thus translation is seemingly prevented during the day phase. Respectively, Knaust et al. (1998)

observed the luciferace mRNA levels to be constant throughout the daily cycle in *L. polyedrum, P. noctiluca* and *P. lunula.*

In addition to the biochemical rhythm, also the sensitivity to mechanical stimulation differs between scoto and photophases. In *L. polyedrum* the threshold flow rate needed to stimulate bioluminescence in a tube is lower for cells that are in the scotophase. Scotophase cells also emit twice as many flashes as photophase cells in response to the stimulation (Christianson and Sweeney 1972). *P. fusiformis* cells are mechanically excitable only in the scotophase. Acid stimulation induces bioluminescence also in the photophase, but it is about 10 times dimmer than in the scotophase (Widder and Case 1982).

Bioluminescence ability and its intensity in *Alexandrium ostenfeldii* appear to be strain-specific (personal observation). The circadian regulation mechanism for *A. ostenfeldii* is not reported, but Liu et al. (2004) refer to unpublished microscopy data of L. Liu and write that *Alexandrium affine* and *Alexandrium tamarense* have LBP. They are also believed to have scintillons. Their luciferaces are similar to those of *L. polyedrum* and the forementioned *Pyrocystis* species and have three catalytic domains. The corresponding individual domains are more similar between the investigated seven dinoflagellate species, than the three domains in the polypeptide (Liu et al. 2004).

The intensity of bioluminescence in the scotophase depends on the light intensity received on the previous photophase (Sweeney et al. 1959). Bioluminescence itself is inhibited by bright light. Together with the endogenous rhythm the photoinhibition of stimulable bioluminescence is responsible for the reduced light emission during daytime. This effect is not due to photolability of the components involved in the bioluminescence reaction, but the effect is mediated by pigments (Sweeney et al. 1959). The mechanism of photoinhibition is different between genera. Bioluminescence capacity or mechanical stimulability can be reduced, or it can be due to decoupling of the mechanical stimulation receptors from the bioluminescence system (Hamman et al. 1981). Kelly and Katona (1966) observed that also the sensitivity to light inhibition is controlled by an endogenous

rhythm: light was inhibiting more effectively during daytime in experiments with natural populations.

In addition to circadian rhythms and photoinhibition, the diurnal variations in bioluminescence in the oceans are affected by vertical migrations of the organisms (Utyushev et al. 1999; Widder 2002; Berge et al. 2012).

1.3 Dinoflagellates – a diverse group

Dinoflagellates are unicellular eukaryotes in the group Alveolata, together with ciliates and apicomplexans. Dinoflagellates contain both marine and freshwater taxa, but marine species dominate (90 %). Most dinoflagellates are free-living, but the group also includes parasites, and symbionts of reef-building corals (zooxanthellae). Bioluminescence and toxicity is widely distributed in the group. The red tides, harmful monospecific blooms, are produced by dinoflagellates (Taylor et al. 2008; Lin 2011).

The number of living dinoflagellate species is estimated around 2000 (morphospecies), in addition to 2500 identified fossil species. The first undoubted fossil records are from Middle Triassic sediments, 240 million years old. The number of taxa is constantly growing as new ones are described (Taylor et al. 2008).

Dinoflagellate diversity is similar in the northern and southern hemispheres, separated by a belt of circumtropical species. Coastal and oceanic assemblages differ, coastal species often including a benthic resting (cyst) stage to overcome unfavourable environmental conditions (Taylor et al. 2008).

Dinoflagellates contain equal numbers of phototrophic and heterotrophic taxa. Mixotrophy is widespread. Feeding habits vary: apart from ingesting whole cells also peduncules (a straw-like tubing) are used (Taylor et al. 2008; Lin 2011). B-vitamin auxotrophy is common (Tang et al. 2010).

The main morphological difference between dinoflagellate taxa is the cell cover. Some dinoflagellates are covered by cellulosic plates (thecate), while others are naked (athecate). There are colonial forms among dinoflagellates and even a few multicellular ones. Movement is gained by two flagellas; one rooted in the longitudinal groove and one in the transverse groove (Taylor et al. 2008; Lin 2011). Plate pattern of the cover, theca, is used in species indentification (Tomas 1996).

Most species have a unique nucleus called dinokaryon (Taylor et al. 2008). The chromosomes are always condensed. Nuclear genomes are very large (up to over 100 chromosomes containing 3–245 giga basepairs of DNA, 1 to 80 times the human haploid genome) and have undergone massive duplications. Abundant gene transfer from the plastid to the nuclear genome occurs, and also the plastids are diverse in origin. Horizontal gene transfer from varied algae and bacteria make the genomes highly diverse (Lin 2011).

Many dinoflagellates are toxic and can produce harmful algal blooms. A wide variety of animals connected to the marine food web are affected during dense blooms. The toxins may influence humans through seafood, and various poisoning syndromes are known. Dinoflagellates produce neurotoxins and hepatoxins. The neurotoxins interact with the neurotransmitter receptors (voltage-gated ion channels) in the nervous cells leading to various symptoms, e.g. paralysis and breathing problems (Wang 2008). Global climate change is expected to change abundance patterns and timing of harmful algal blooms, and better monitoring of these species is required to cope with the danger caused for example to seafood production (Hallegraeff 2010).

1.4.1 *Alexandrium ostenfeldii* in the Baltic Sea

Species of the genus *Alexandrium* are coastal, and many produce toxins that accumulate in shellfish. The species division based on morphological identification is under discussion after several species were found to belong to the same genetical complexes (Taylor et al. 2008).

A. ostenfeldii is a marine, thecate dinoflagellate capable of mixotrophy (Gribble et al. 2005). During the past decade, dense bioluminescent blooms of this species have occurred in the Baltic Sea (Hajdu et al. 2006; Kremp et al. 2009; Hakanen et al. 2012). Isolates from the northern Baltic Sea are genetically distinct from most other *A. ostenfeldii* strains but are close related to *Alexandrium peruvianum* strains from Spain and *A. ostenfeldii* strains from southern England. Variation in morphological features makes the differentiation from *A. peruvianum* somewhat vague – both species might belong to the same complex. The Baltic Sea strains tolerate low salinities and grow optimally in 6–10 ‰ brackish water (Kremp et al. 2009).

Blooms in the Åland archipelago are not monospecific, but consist of several dinoflagellate and other species. The investigated blooms are restricted to a narrow and shallow sound in the Föglö municipality (see Figure 9), and high cell concentrations are tightly linked to the occurrence of resting cysts in the sediments. Blooms are typically formed in late summer in warm water (19–23 °C). In these blooms, paralytic shellfish poisoning (PSP) toxins are produced (Hakanen et al. 2012). Toxins accumulate to potentially harmful concentrations in bivalves in bloom areas (O. Setälä, personal communication). Contradictory to other areas, in the Baltic Sea *A. ostenfeldii* is present in high (up to 1.0×10^6 cells l^{-1}) cell concentrations (Gribble et al. 2005; Kremp et al. 2009; Hakanen et al. 2012). Because blooms of *A. ostenfeldii* have become an annual phenomenon in distinct locations in the Baltic Sea and are potentially harming the ecosystem, effective monitoring techniques are needed to prepare for and assess the risk caused by the blooms.

1.5 Study aims

The purpose of this study was to develop a method for continuous bioluminescence measurements allowing experimental studies of the circadian rhythm of bioluminescence in *A. ostenfeldii*. The experiments would also help to deepen the knowledge of bioluminescence in this species which had not yet been

studied in detail. The main study aim was to identify an effective and reproducible method to mechanically stimulate bioluminescence in artificially grown cells without depleting the capacity to bioluminescence throughout the night by overstimulation. Diminished light emission at the end of the night should reflect the natural rhythm.

Mechanical stimulation can be achieved by a diversity of means. Growth media have to be brought into motion, so that the cells experience pressure on the cell wall. For example stirring of the water, bubbling with air, and a thin capillary or a tube with water flow have been used (Kelly and Katona 1966; Biggley et al. 1969; Christianson and Sweeney 1972; Latz et al. 2004). Bioluminescence can also be induced by acid addition (for example Widder and Case 1982), as well as with a variety of other chemicals (Hamman and Seliger 1972), although such intrusive methods are not favoured for semi-continuous experiments.

Individual experiments were set up to address the following questions:

- Comparing bubbling and stirring of the water as a stimulation mechanism: which is more effective? Do they induce bioluminescence in the same way?
- How long should the recovery period between stimulation events be to not exhaust the cells, yet optimizing data density? Is this different for stirring and bubbling?
- Is spontaneous bioluminescence detectable? How does it compare to the induced bioluminescence (intensity, nightly pattern)?
- Is bioluminescence inducible in cells grown in continuous light? How is the pattern of the light emission curve?
- Is a rhythm detectable in continuous darkness? How long does it persist without energy supplement (photosynthesis)?

These experiments are referred to as methodological experiments (see Tables 1 and 2 for summary of the experiments). Additionally, an experiment to test the plasticity of daily rhythms of these dinoflagellates was started. Cultures of *A. ostenfeldii* were moved to different light-dark rhythms and their bioluminescence

patterns were followed. This mimics a geographical displacement, a change of latitude during the bloom period. Cells at higher latitudes experience shorter nights during the late summer bloom peak. The aim was to find out whether a rhythm change can be detected, indicating acclimation to changing night length. For environmental monitoring it is important to know the circadian rhythm of bioluminescence, because short bioluminescent periods during short summer nights would restrict the spatial coverage of optical monitoring done from moving platforms (e.g. ferry lines). Problems with culture growth delayed the start of these experiments and they only contribute to reflection on methods comparison in this thesis. Many of the experiments presented here were intended as pilot experiments for the rhythm studies, so variety of experimental conditions is presented.

Measurements were also conducted in the field. Data were collected during the bloom peak in Föglö in August–September 2011. The relevant activity for this thesis was to compare results obtained under a natural light regime with the laboratory experiments.

Generic hypotheses on method validity are challenging to form, as bioluminescence is variable between species. For example, (Biggley et al. 1969) found interspecific differences in the effectiveness of stirring and bubbling. For the continuous light experiment I hypothesized, that bioluminescence is measurable as soon as inhibition by light ceases. In continuous darkness some bioluminescence was expected to be visible also in the following nights without intermediate light entrainment.

Widder and Case (1981) concluded that for not fully exhausted *P. fusiformis*, a large photosynthetic dinoflagellate, 30–60 minutes were required to restore the bioluminescence kinetics, including the bright and rapid first flash characteristic for unfatigued cells. However, total restoration of fully exhausted cells required over 4 days. In our experiments, the time between stimulations (recovery period) was in the range of 10–90 minutes, where the middle values were expected to be sufficient.

2 Methods

2.1 Culturing of dinoflagellates

The *A. ostenfeldii* strain investigated was AOF-0930, isolated from the Föglö bloom site (N60°56′17, E20°32′44) in Åland, Finland. In some methodological experiments, other strains (AOKAL-25 from Sweden, N56°42′40, E16°21′45, and AOPL-61 from Poland, N54°45′31, E18°30′32) were used as indicated in the respective results. All cultured dinoflagellates were obtained from the culture collection of Dr. Anke Kremp, Marine Research Centre, Finnish Environment Institute.

The dinoflagellates were cultured in 6-‰ F/2-Si medium (Guillard and Ryther 1962) in temperature controlled climate rooms at 16 °C. Most cultures were grown at a light intensity of 70 µmol m^{-2} s^{-1}. In the rhythm experiments, culturing compartments were separated with black plastic sheets to allow isolated light-dark regimes, and light intensity was 50 µmol m^{-2} s^{-1}. The isolated chambers experienced stronger fluctuation in temperatures due to installed lamps, partly mitigated by fans. The air temperature varied between 16–22 °C. The algae grown in continuous light (40 µmol m^{-2} s^{-1}) were kept at room temperature (22.1–23.6 °C). For the cultures of the methodological experiments the light-dark rhythm was 14:10, where lights went on at 9:00h and off at 23:00h. Selected cultures were taken from 16:8 rhythm (light period 10:00h–2:00h), grown in the rhythm experiment conditions (indicated in the results). The growth conditions in each experiment are described in Table 1.

Table 1 Growth conditions in the methodological experiments. Growth light rhythm, light intensity and temperature alternate respectively, i.e. for a culture in the growth rhythm 14:10 light intensity was 70 μmol m^{-2} s^{-1} and growth temperature 16 °C.

Experiment	Aim	Stimulation method	Strain	Growth light rhythm (L:D)	Light intensity (μmol m^{-2} s^{-1})	Growth temperature (°C)
Stimulation mechanism	Determine most suitable mechanical stimulation principle to observe bioluminescence kinetics	alternating stirring and bubbling	AOF-0930 and AOKAL-25	14:10 and 16:8	70 and 50	16 and 16–22
Recovery period / bubbling	Optimise recovery period between stimulation events	bubbling	AOF-0930	14:10	70	16
Recovery period / stirring	Optimise recovery period between stimulation events	stirring	AOF-0930	14:10	70	16
Spontaneous bioluminescence	Determine significance of spontaneous bioluminescence	stirring or without stimulation	AOF-0930 and AOPL-61	14:10	70	16
Persistence - continuous light	Determine effect of light period on persistence of bioluminescence patterns	stirring	AOF-0930	continuous light	40	23
Persistence - continuous darkness	Determine persistence of endogenous rhythm and exhaustion of bioluminescence potential in prolonged darkness	stirring or bubbling	AOF-0930	14:10 and 16:8	70 and 50	16 and 16–22

Algae were cultured either in 250-mL VWR Cell Star tissue culture flasks, or 2-L Duran bottles with a loose cap (Figure 1). Large bottles were used to have sufficient volume of homogenous culture for repeated measurements. From the large Duran bottles, 150 mL was transferred to the measurement bottle, and mixed back to the culturing bottle after each measuring period. The measurement volume of the small culture flasks was discarded after the measurement period (Figure 2). Measurements were made from Nalgene 250-mL square plastic bottles. The caps were replaced with rubber stoppers that contained air exchange systems (Figure 1, see also Figures 6 and 8).

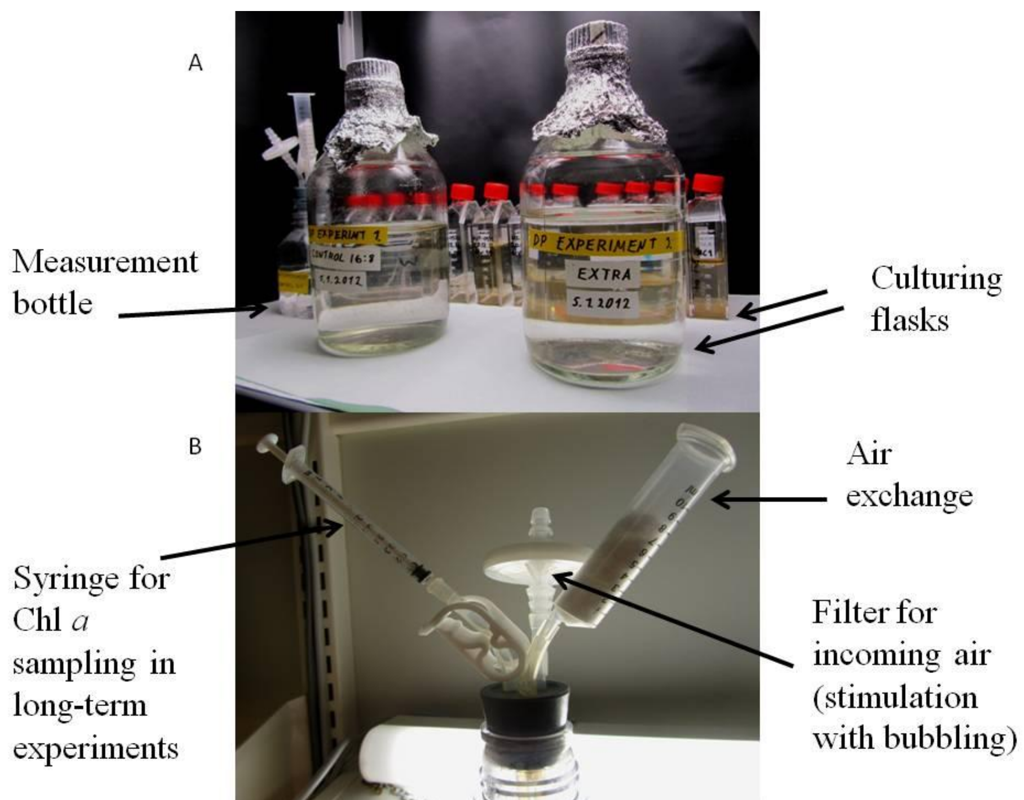


Figure 1 Culturing and measurement flasks. A: in the front 2-L Duran bottles. In the back red capped culturing flasks and (far left) a measurement bottle. B: detailed view of the cap of the measurement bottle.

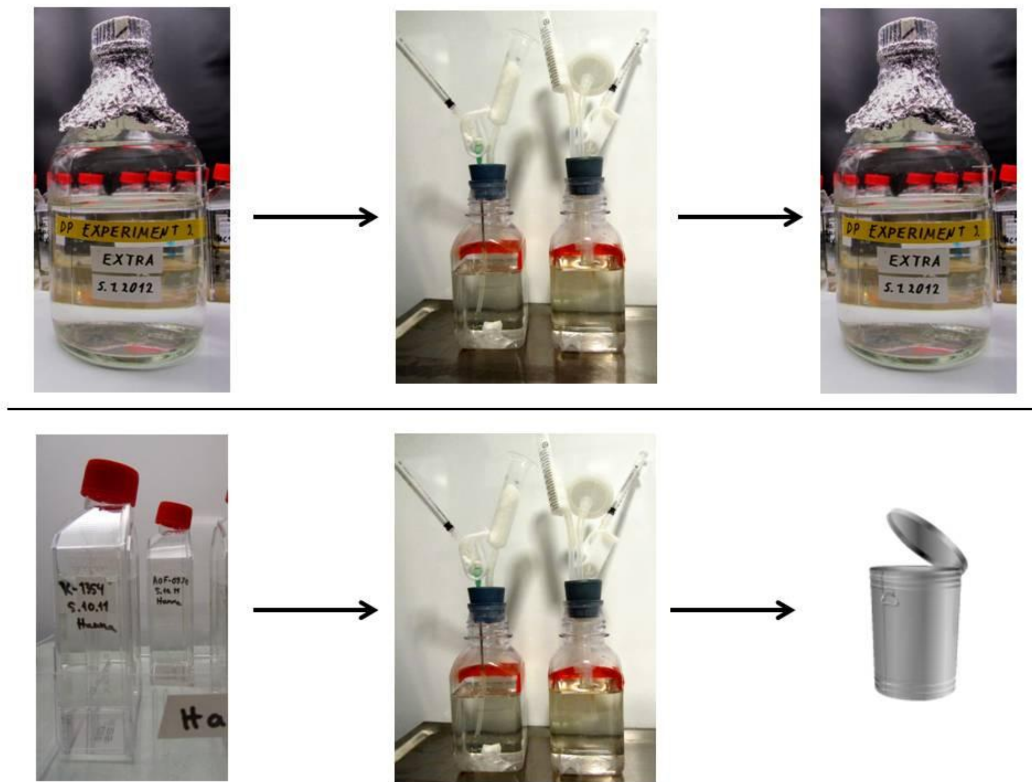


Figure 2 The sampling and measurement procedure. For repeated measurements from the same experimental culture, a large volume bottle was used and subsamples were returned after measurement (upper row). For single measurement runs, 250-mL flasks were used for culturing and subsamples were not returned after measurements.

2.2 Sampling of the cultures

Cultures were sampled during their exponential growth phase to avoid physiological biases between measurements. To monitor the growth stage of the cultures, chlorophyll *a* was extracted and measured fluorometrically following the HELCOM protocol (HELCOM 2005). The pigment samples were either (depending on the experiment) pipetted into a syringe attached to a filter holder (Swinnex) or sampled directly with a syringe from the culture, and subsequently filtered onto a Whatman GF/F glass fiber filter (Ø 25 mm), or concentrated using a filtration manifold. The sample volume (1–5 mL) was kept small to not deplete the culture volume in repeated sampling, but was increased for larger culturing flasks to reduce possible sampling error. The samples were extracted with 96-%

ethanol, overnight, at room temperature, re-filtered, and analyzed with a Cary Eclipse spectrofluorometer. The added ethanol volume was 1.65–20 mL, optimized based on sample volume and culture density. The obtained extract was either centrifuged for 10 minutes in 12.7 × *g*, or filtered through a GF/F filter prior to the measurement, depending on the sample vial. The excitation wavelength was 430 nm and the emission wavelength 670 nm.

Microscope cell counts were carried out on samples fixed with Lugol's medium, using Leica DMIL and Leica DMI 3000 B microscopes. Cell damage was inspected by taking samples during different bioluminescence stimulation treatments and counting minimum of 400 cells. Damage was then expressed as percentage of damaged cells (Figure 3).

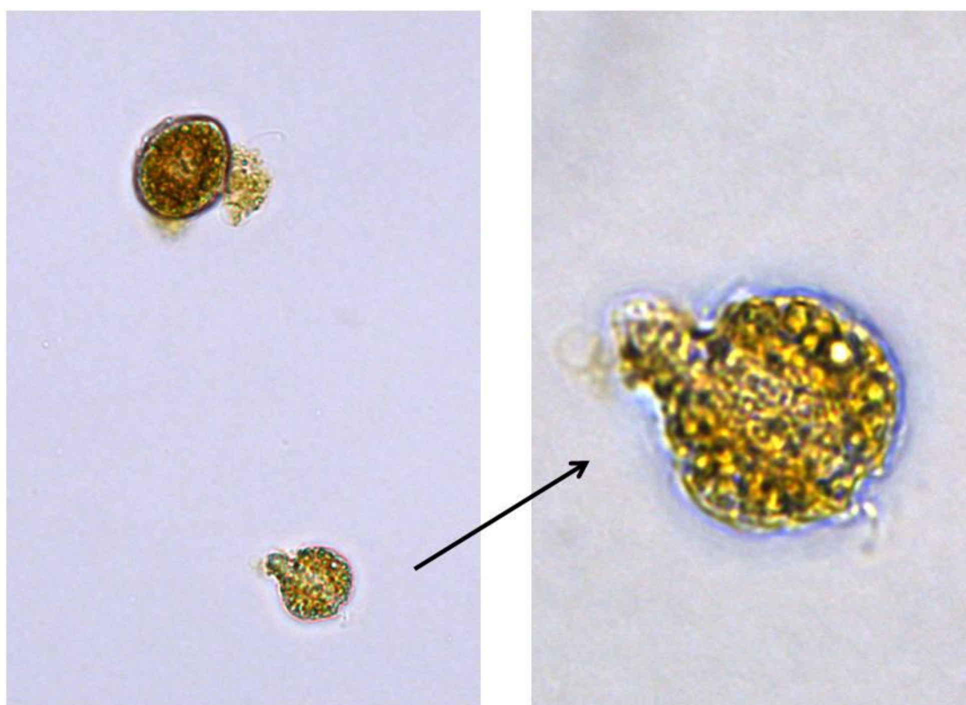


Figure 3 Examples of cells that were counted as damaged in the microscope comparison of the stimulation mechanisms.

2.3 Methodological experiments

The methodological experiments were carried out with a Cary Eclipse spectroluminometer that was set to measure continuously with a frequency of 2–20 Hz (frequency depending on the experiment and emission intensity of the cultures). The detection window was set to 470 nm and a bandwidth of 20 nm.

To stimulate bioluminescence, the culture was disturbed either by stirring or bubbling the medium. For the stirring treatment a magnetic stirrer platform was placed on a rack in the measurement chamber of the instrument (Figure 6). The bottles contained a 20–30 mm wide stirring cross. Stirring speeds were 180 rounds (turns) per minute for the 30 mm wide cross ("large cross"), and 105 rpm for the 20 mm wide cross ("small cross"). The speed was set to the highest possible without inducing a vortex. Bubbling treatments used an aquarium pump, the air flow distributed through the pump end of a sterile Pasteur pipette, manually fixed with holes (Figure 5). Air flow was either 1.1 L min^{-1} ("moderate flow") or 2.6 L min^{-1} ("high flow", Figure 4).

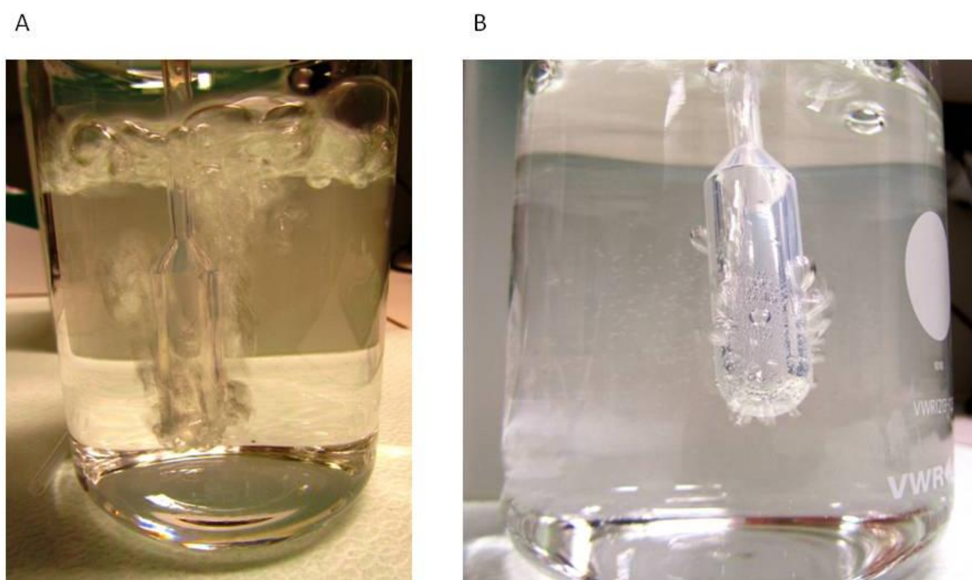


Figure 4 Difference between bubbling with (A) very high and (B) low air flow. High flow resulted in a vigorous stream of bubbles, whereas at the low setting only a few of the holes in the pipette released air bubbles. Experimental treatments used air flow between these extremes.

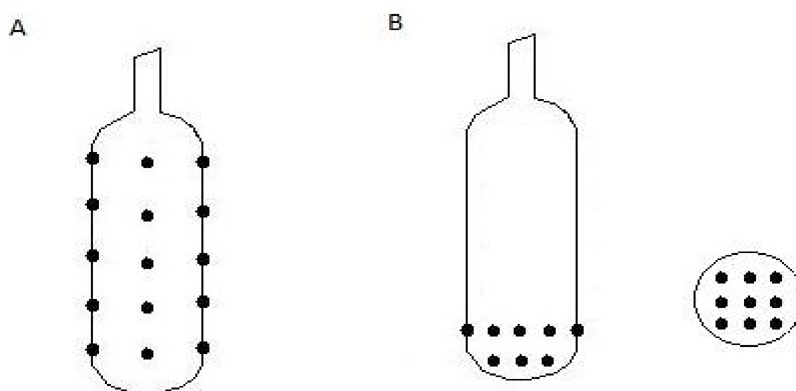


Figure 5 Location of the holes (black dots in the figure) in the air distributing pipettes. (A) configuration used in the initial stimulation mechanism comparison (indicated in results) and in the recovery period experiment with bubbling. (B) configuration used in all other stimulation mechanism experiments (on the right bottom view).

In the first tests, timing of stimulation was controlled with a timer electric socket with 15-min resolution. Subsequently, the stirrer and aquarium pump were controlled through a Phidgets USB relay interface and a program developed in Labview 2010, with 1-s resolution.

The bottles were fixed in the instrument to prevent movement. The instrument itself was covered with darkening fabric to prevent ambient light from disturbing the measurements at the sensitive instrument settings used to capture weak bioluminescence (Figure 7). The room was kept dark when possible, and most experiments were carried out overnight.

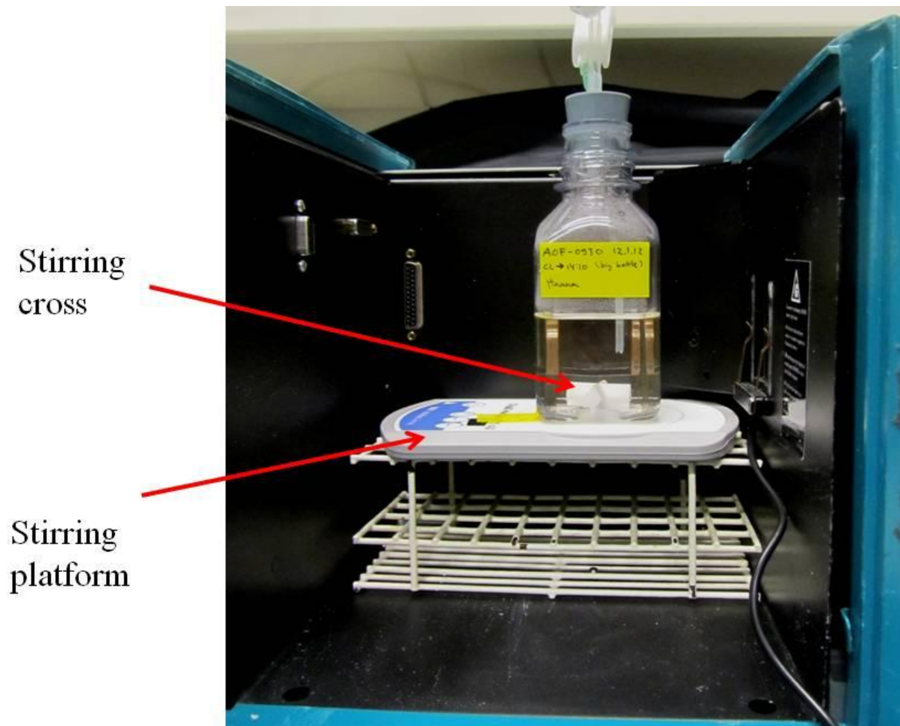


Figure 6 A measurement bottle inside the spectroluminometer setup for bioluminescence stimulation by stirring.

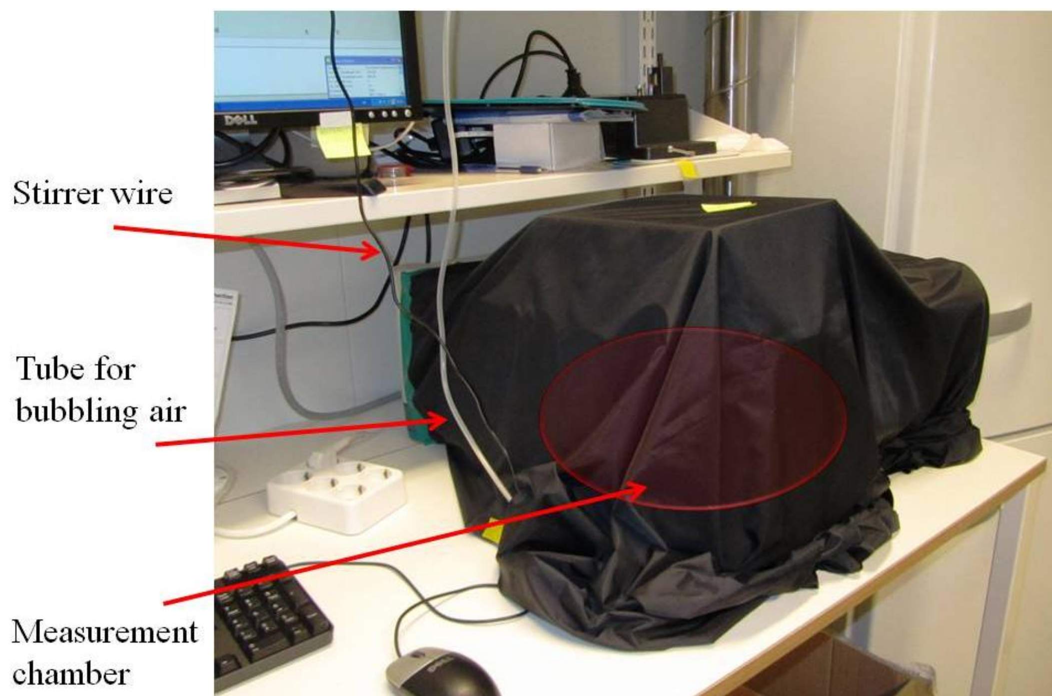


Figure 7 The spectroluminometer covered with a black cloth to exclude the ambient light during the measurement.

2.3.1 Experiment procedures

The experiments are summarised in Table 2.

Stimulation mechanism. To compare the stimulation effect of bubbling and stirring, a culture was disturbed by alternating bubbling and stirring in the same night. The measurement bottle contained both the stirring cross and the bubbling pipette. The different stirring and bubbling settings are summarised in Table 2 and listed in the results.

Recovery period. To test the effect of recovery period between stimulation events, 150-mL samples from a large culturing bottle were followed during subsequent nights, using different recovery periods. After the measurement the sample was mixed back to the culturing bottle. Recovery period ranged 1–40 minutes and stimulation was 5 minutes in the experiment with bubbling. The setting was high bubbling with pipette type A (Figure 5). For stirring, recovery period ranged 10–90 min and stimulation was 1 minute. The large stirring cross was used. An additional measurement set with recovery periods 10, 40 and 90 minutes was carried out using stirring. These measurements were done from separate bottles that were created prior to the experiment and were not mixed between the measurements. A table of the experiment procedure of the stirring experiment is in Appendix 1.

Spontaneous bioluminescence. To compare the induced bioluminescence with spontaneous bioluminescence, the culture (AOF-0930) or identical cultures (AOPL-61) were tested in subsequent nights. For AOF-0930, stimulation occurred only in the second measurement night. For AOPL-61, only one of the two identical cultures was stimulated. The experimental setup was kept the same in the night without stimulation (the bottle contained the stirring cross etc), but the timer was not started. When stimulation was used, recovery period was 29 minutes and stimulation 1 minute. Small stirring cross was used.

Persistence - continuous light. One culture was placed to grow in continuous light and was inoculated again for 3 times in approximately 10 days intervals to gain

cells grown only in constant light. Before the measurements, 3 culture flasks were mixed and divided again into 3 measurement bottles, containing 200 mL of culture each. The bottles were measured in following nights, 2 of the bottles twice. The second measurements were after the first set of measurements of all three bottles. The recovery period was 44 minutes, stimulation 1 minute and the large stirring cross was used.

Persistence - continuous darkness. Measurements reaching over several days were used to test the persistence of the bioluminescence rhythm in continuous darkness. The cultures were not illuminated between the nights, and stimulation occurred also during the day. The different stirring and bubbling settings are summarised in Table 2 and listed in the results.

Table 2 Experiment conditions in the methodological experiments.

Experiment	Aim	Stimulation method	Bubbling speed	Stirring cross	Stimulation time (min)	Recovery period (min)
Stimulation mechanism	Determine most suitable mechanical stimulation principle to observe bioluminescence kinetics	alternating stirring and bubbling	moderate or high	small, medium or large	1-10	10-59
Recovery period / bubbling	Optimise recovery period between stimulation events	bubbling	high	-	5	1-40
Recovery period / stirring	Optimise recovery period between stimulation events	stirring	-	large	1	10-90
Spontaneous bioluminescence	Determine significance of spontaneous bioluminescence	stirring or without stimulation	-	small	1	29
Persistence - continuous light	Determine effect of light period on persistence of bioluminescence patterns	stirring	-	large	1	44
Persistence - continuous darkness	Determine persistence of endogenous rhythm and exhaustion of bioluminescence potential in prolonged darkness	stirring or bubbling	high	medium or large	1-15	40-90

2.4 Rhythm experiments

The rhythm experiments were carried out with a Glowtracka bathyphotometer (Chelsea Technologies Group, UK; see Figure 8). The culture was stirred 1 minute every 30 (experiment I) or 45 minutes (experiment II). The speed of the stirrer used in these experiments was 225 rpm. In contrast to the methodological experiments, the cultures were illuminated during their day phase. The light was switched off for each period of mechanical stimulation, while the light meter was temporarily switched on. The timer and the lamp were controlled using a computer program developed in Labview 2010 and a Phidgets USB relay board. Chlorophyll *a* sampling was prior to each measurement.

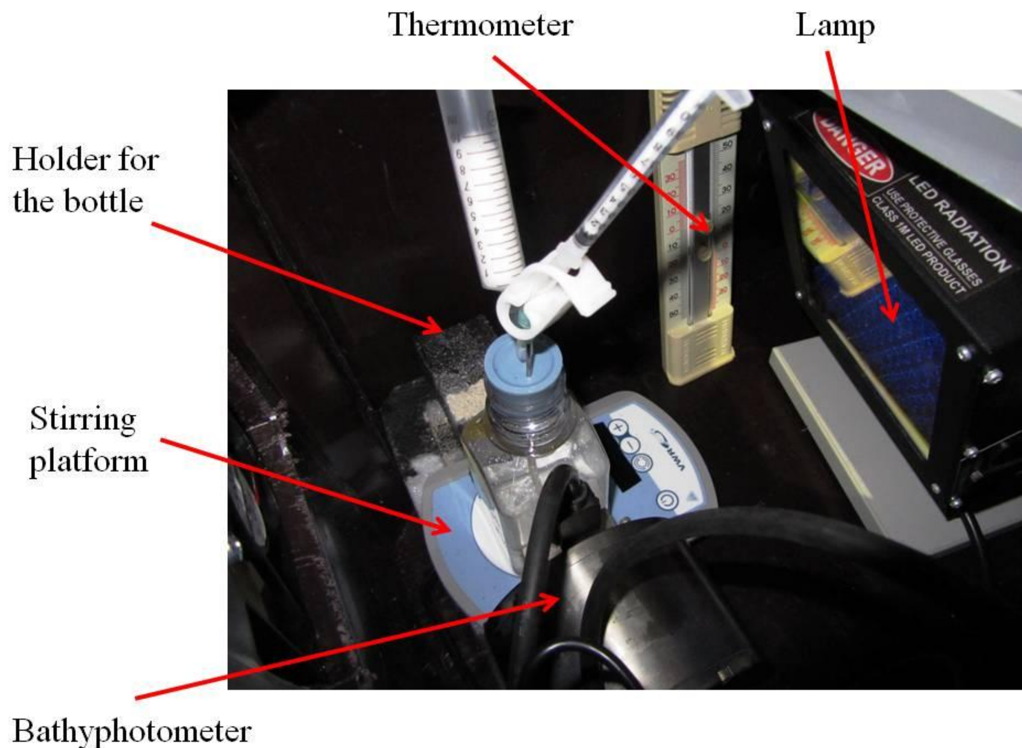


Figure 8 A measurement bottle inside the Glowtracka measurement box.

In experiment I, cultures were inoculated into measurement bottles prior to the experiment and these were measured by turns for approximately 24 hours. The

treatment bottles were moved to a light rhythm of 19:5 L:D. The controls were kept in 16:8.

In experiment II, large 2-L culturing bottles were set up. For measuring a 150-mL sample was taken, and later returned to the culturing bottle. The light rhythm for the treatment bottle was 12:12 L:D. The control was kept in 16:8.

In the beginning of each experiment the cell density was approximately 500 cells mL^{-1}. Every three weeks culturing media were replenished to account for the loss due to sampling and to maintain optimal growth conditions.

2.5 Field measurements

Bioluminescence in the field was monitored in Föglö, a group of islands in the Åland archipelago (Figures 9 and 10). Dense bioluminescent blooms of *A. ostenfeldii* have been observed in the past years at this site. Bioluminescence was recorded at the bloom site (N60°5′33.11, E20°32′12.8) throughout the growth season (July-September). The device, a Glowtracka bathyphotometer, was in place from 14.7.2012, but due to day light reaching the measurement device in the beginning, proper data was obtained from end of July. A pump brought sea water through the measuring chamber with the speed of 6 L min^{-1}. The device was switched on for 1 minute every 10 minutes. Besides bioluminescence, fluorescence (a SCUFA fluoroprobe, Turner Designs) was measured continuously to have an indication of the biomass in the bloom site. The device was cleaned weekly, and approximately every two weeks the data was collected (the data logger was located in a box with a solar panel). I participated in the installing trip mid July and in a multiday field work in the beginning of August. The above mentioned parameters are used in this thesis. Additionally, connected to the PhD project sampling of phytoplankton and optical parameters was carried out in the vicinity of the device, and temperature was monitored.

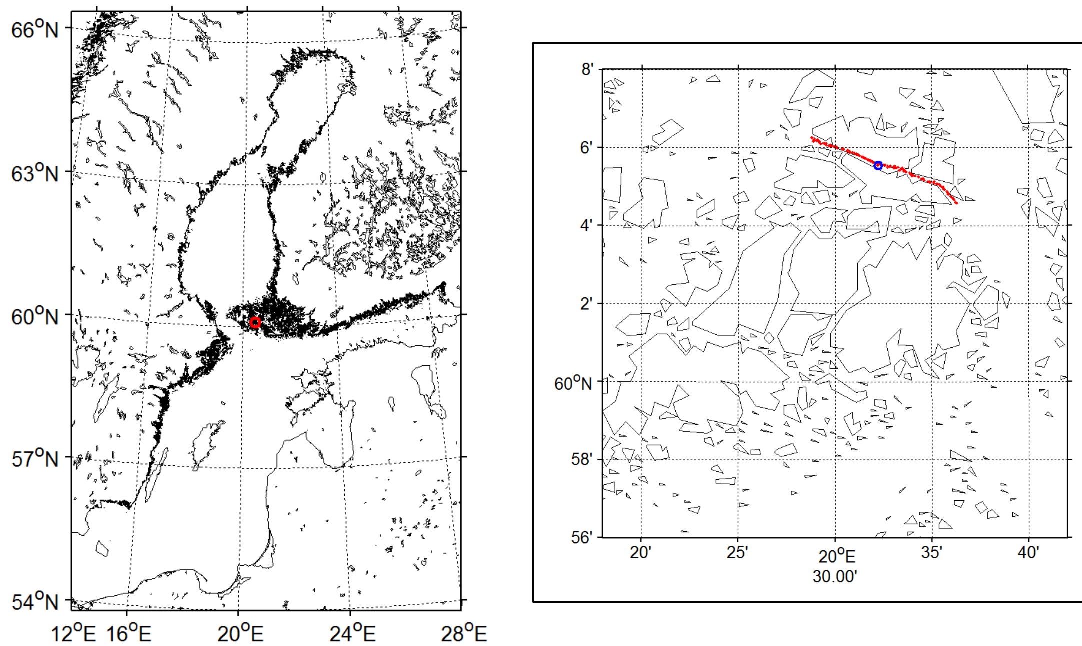


Figure 9 The measurement site in the Åland archipelago. The red circle in the map of the Baltic Sea (on the left) points to the area of Föglö, which is detailed in the map on the right. The blue circle in the enlarged map shows the site of the continuous measurements, and the red line the sound in which visible bioluminescence occurs.

Figure 10 View of the sound in the Åland archipelago where bioluminescent blooms were recorded.

2.6 Data analysis

For mean bioluminescence intensity, the light emission over the stimulation period was integrated and then divided by the elapsed time in seconds.

For a single measurement point of the instrument the integration time in the methodological experiments was 250–500 ms to have a sufficient signal-to-noise ratio. A well developed single flash of a dinoflagellate is shorter (*P. fusiformis* examined by Widder and Case 1981). Moreover, the light emission of the whole culture was measured in all experiments. Thus in our results flashes of individual organisms overlap and a population response is seen in the recording file. Bioluminescence intensity is presented in arbitrary raw instrument units, as the dynamics between the treatments rather than the absolute light emission is of interest.

In the methodological experiments with the spectroluminometer a baseline was subtracted from each recording. A blank measurement with purified water characterises instrument drift over the course of several hours (Figure 11).

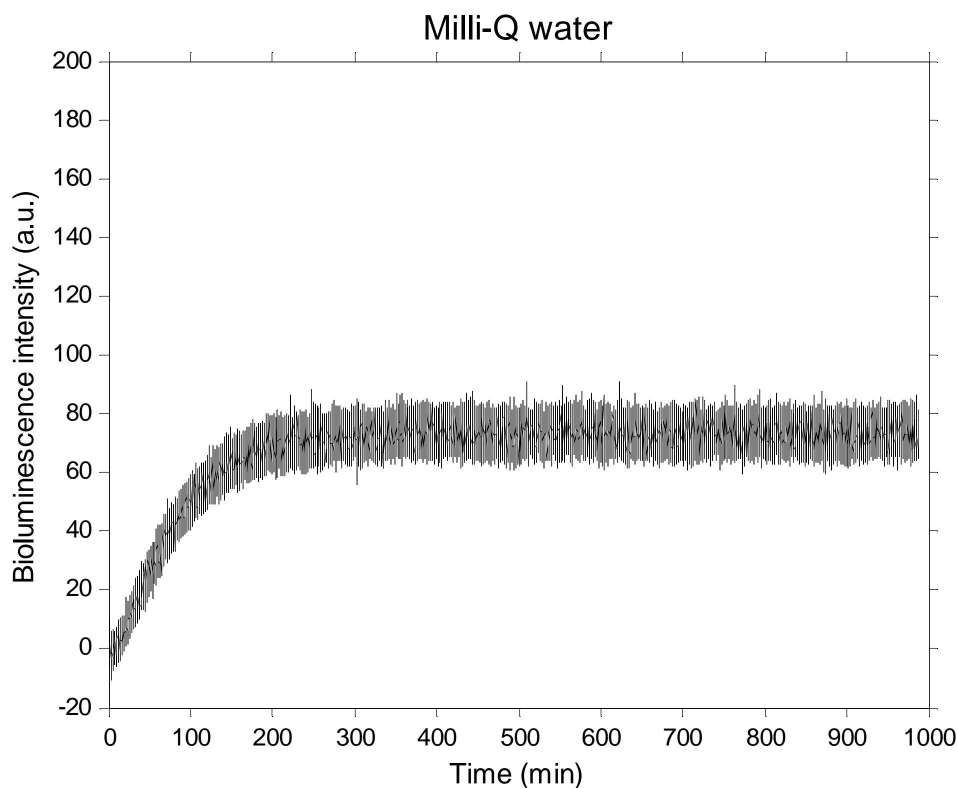


Figure 11 A blank measurement with purified water shows the rising baseline in the light intensity recordings. The measurement frequency was 2 Hz.

The initial slope corresponding to instrument drift was not constant between measurement sequences. To solve this, a 5-minute period preceding each stimulation was averaged to give a corresponding zero (noise) level for each stimulation (Figure 12). This value was subtracted from each value in the stirring or bubbling event. The corrected values of the single stimulation event were then summed to give the integrated bioluminescence. When the interval between stimulation events was 5 minutes or shorter, 0.5–2 minute periods preceding each stimulation event were used for baseline corrections.

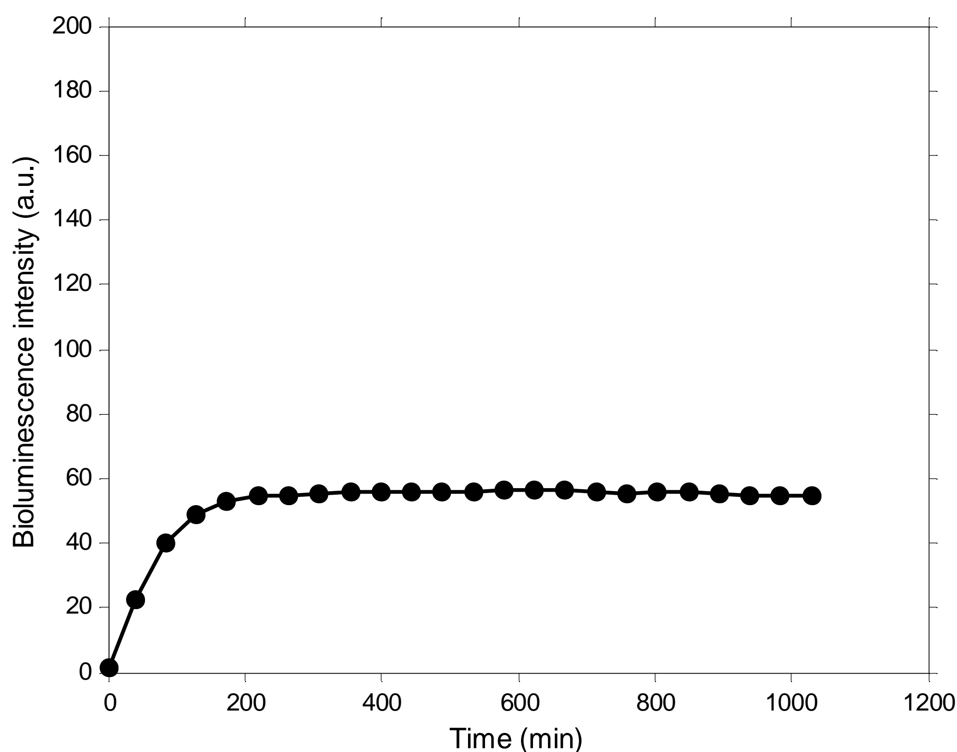


Figure 12 A typical baseline. The calculated baseline values from non-stimulated periods were used to correct the values of subsequent stimulation events.

For spontaneous bioluminescence measurements the above mentioned procedure could not be applied, as there were no distinct stimulation and non-stimulation periods. Instead, one single average for the whole measurement period was calculated to give the baseline. This average was subtracted from the values integrated for each data point. To obtain bioluminescence throughout the night, the corrected values were integrated in 10-minute periods. The values of the first two hours were excluded, since the baseline changes most in the beginning and one single average is most unreliable there. When one single average over the whole measurement period was used for baseline construction also for the comparison measurements with stimulation, intensity showed essentially the same curve as in the data treatment with the baseline consisting of separate values from non-stirring periods.

In a few initial stimulation mechanism comparison experiments the used timer had 15 minute intervals. The light emission, however, occurs mostly during the first minute. Bioluminescence was integrated for 5 first minutes of the stimulation event, even though it lasted for 15 minutes, to reduce biases resulting from the artificial baseline when no light emission occurs, and to be in line with the other measurements. These results are presented in chapter 3.5 Persistence - continuous darkness.

Detector sensitivity of the instrument was optimized for each experiment according to the response of the culture or other similar cultures. This and varying growth stages imply that the intensity level of light emission cannot in this thesis directly be compared between different experiments. This can be overcome by scaling the results to each other by measuring the response of the instrument with a known light source. For the results presented here, such corrections were not highly relevant and are not included.

Selected results are normalised against their mean to allow comparison between samples and to exclude differences resulting from e.g. uneven distribution of the cells or other artifacts. Normalising against chlorophyll *a* values removes the effect of differing biomass, but it was not used for the laboratory results because of the variation between the parallel samples (possibly due to flocculation of cells, that might become considerable in the small sample volumes used, or difficulties in extraction because of the robust cell wall). Field measurements are shown normalised against the chlorophyll *a* concentration obtained from the calibrated fluorometer. An average of the 10-minute period corresponding to the bioluminescence measurement period is used.

For the rhythm experiments first half of the stimulation minute was used for integrating – the integrated time period was checked to contain the essential information, and not interference from illuminating the culture. For field measurements, an average of the light emission was calculated over the stimulation period (1 minute).

To study the effect of the experimental light regimes and the regulation of bioluminescence, selected measurements of methodological, rhythm and field data were compared. The question was, how fast the bioluminescence reaches its peak level after the initiated light emission. For this the slopes from start to peak level (beginning of constant light emission) were calculated. The data were first normalised to maximum value to exclude the effect of differences in peak intensities. For measurements from the methodological experiments, the start point was the start of the measurement, since bioluminescence could be observed soon after placement into the dark instrument. For measurements from rhythm experiments and field data, the start point was at the end of the fairly constant day minimum level. For the field measurements, the start point is not steady, as day length is changing and for example cloud cover affects the intensity of illumination in the water. The beginning of the peak level of bioluminescence light emission is also somewhat ambiguous. In the figures, it is marked with red lines where the start points and peak levels were defined. Statistical analyses were performed using PASW (Predictive Analytics SoftWare) Statistics 18 (SPSS Science Software). The variable in question, the slope of the rise of bioluminescence emission in the beginning of night, was not normally distributed, so the Kruskal-Wallis test was used.

3 Results

In total, 54 measurement sequences were recorded in the methodological experiments. Those presented in this thesis form ensembles and exclude the most basic methodological trials. The rhythm experiments were carried out over a period of ca 3 months (and continued after this thesis), but in the first half of the period data gathering was hindered by problems in culture growth. Only some results suitable for method comparison are selected. In the field recording took place from 14th of July to 7th of September, but proper data was obtained from the end of July. Again, those periods suitable for methods comparison are selected.

In a typical light emission a rapid rise of the intensity is seen at the onset of stimulation, followed by a period of low light emission even though the stimulation is continuing: most of the light is emitted within a few seconds.

In all graphs that follow, a grey rectangle marks the dark period experienced during culture growth. In the methodological experiments, the conditions were dark throughout the whole measurement (also during the "day"), but the rectangle shows the time of the scotophase according to the growth rhythm. Negative light intensities are caused by baseline subtraction and instrument noise. The first values are most susceptible to errors rising from the baseline, since the baseline changes rapidly in the beginning of each measurement sequence. The instrument is widely used in the laboratory and could not be reserved for acclimatisation prior to measurements.

3.1 Stimulation mechanism

Cultures were stimulated by alternating stirring and bubbling in the same night. Bubbling induced 9–99 times stronger bioluminescence than stirring, when expressed as the relation of the peak intensities of the bioluminescent light emission. Two bubbling speeds and two pipette types were used. The setting pairs for moderate bubbling were a) moderate bubbling with pipette type B (Figure 5) and large stirring cross (Figure 13), and b) moderate bubbling with pipette type B

and small stirring cross (Figure 14). This resulted in the smallest differences: bubbling gave 9–24 times higher peak heights than stirring. For c) high bubbling with pipette type A and medium stirring cross (Figure 15) the peak intensity of bubbling was up to 99 times the stirring peak intensity. Unfortunately, the stirring speed setting for the cross of this medium size (25 mm) was lost. The strain used in the c) setting was AOKAL-25. The culture of the b) setting had a light rhythm of 16:8.

Of course, more measurements are needed to reliably quantify the difference for each pair, but the experiment was mainly set to demonstrate the overall difference of the two stimulation mechanisms. In summary, in all cases bubbling induced stronger bioluminescence than stirring.

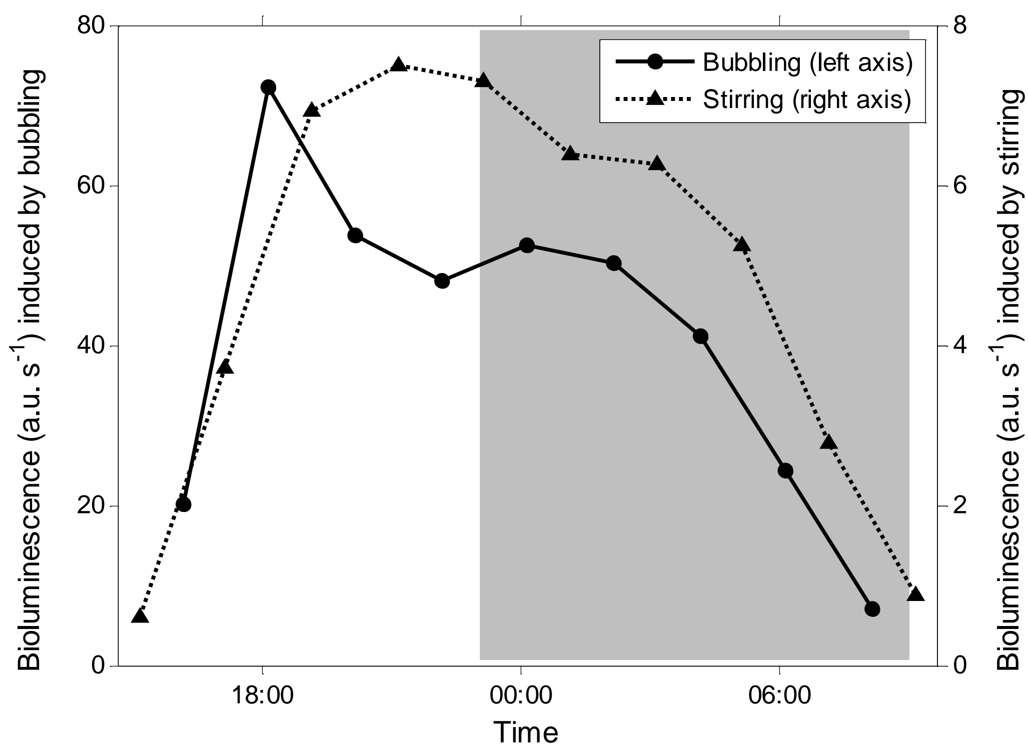


Figure 13 Bioluminescence stimulated for 1 minute alternating between stirring (large cross) and bubbling (moderate). The interval between stimulations was 59 min.

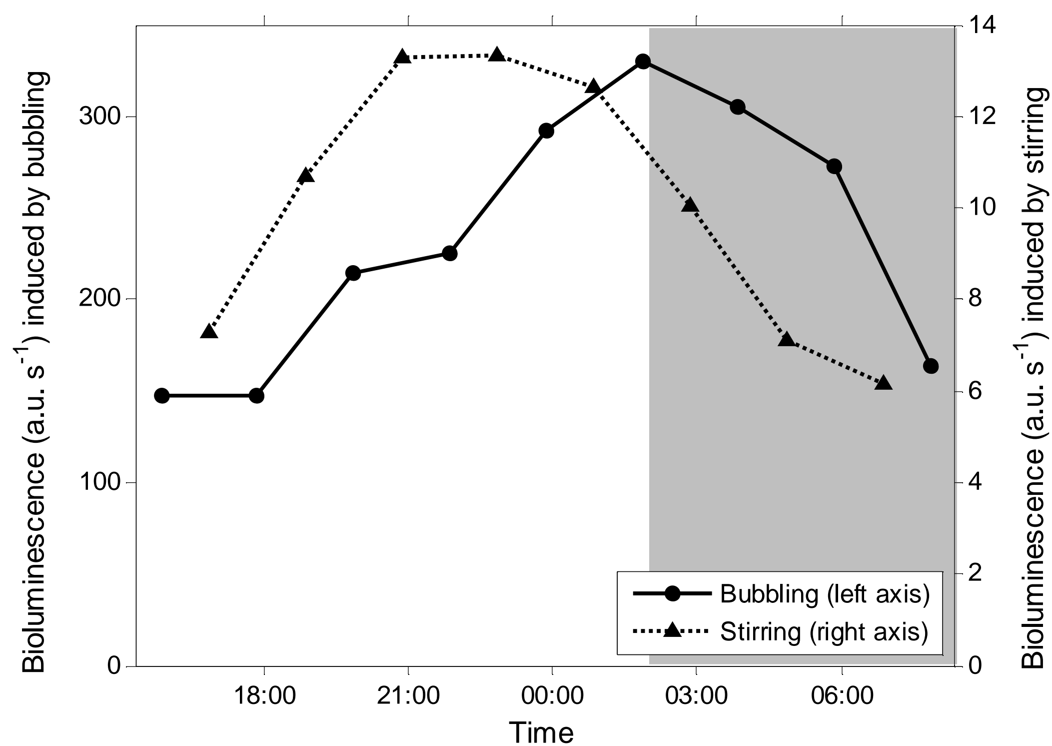
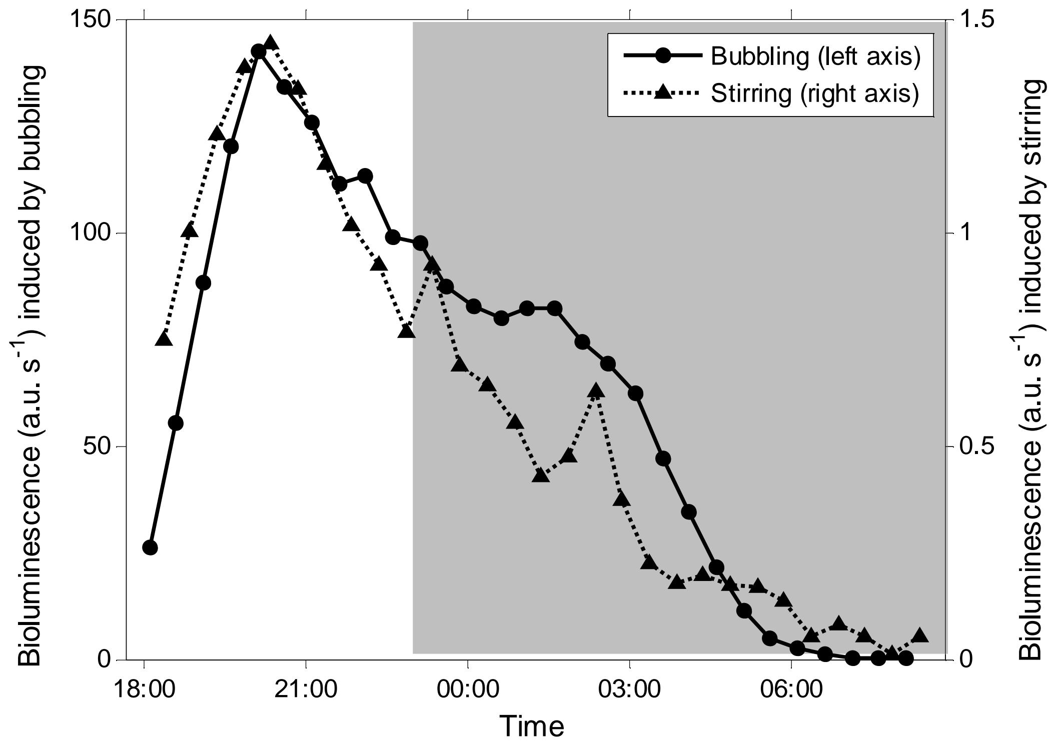


Figure 14 Bioluminescence stimulated for 1 minute alternating between stirring (small cross) and bubbling (moderate). The interval between stimulations was 59 min.

A.



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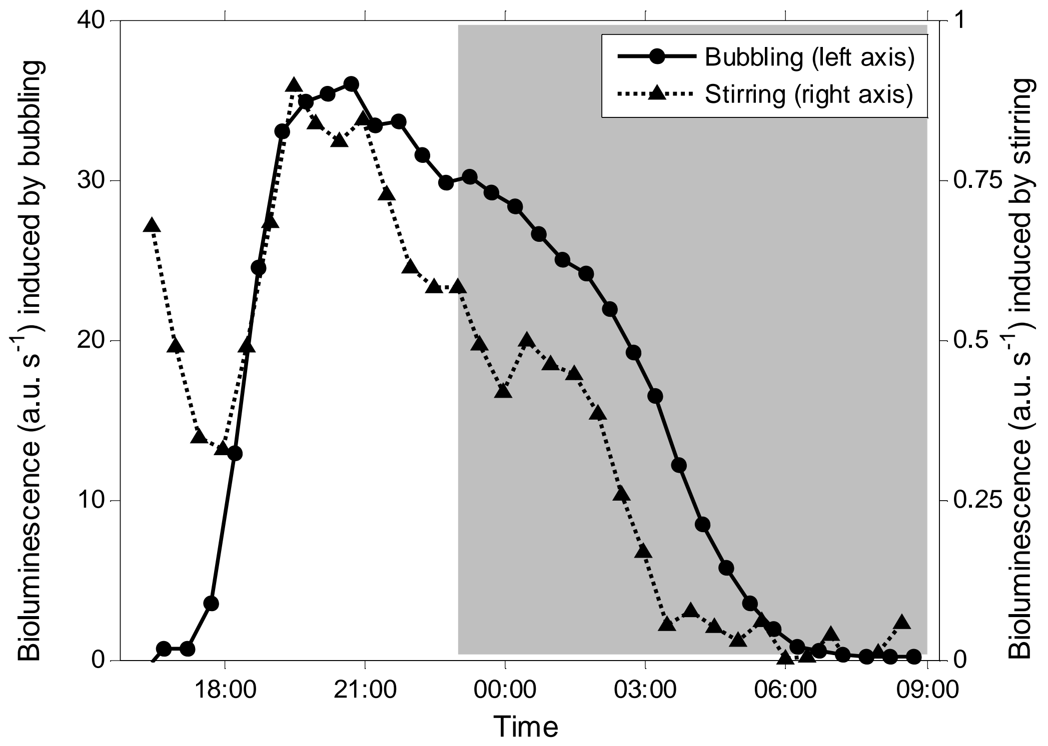

C.

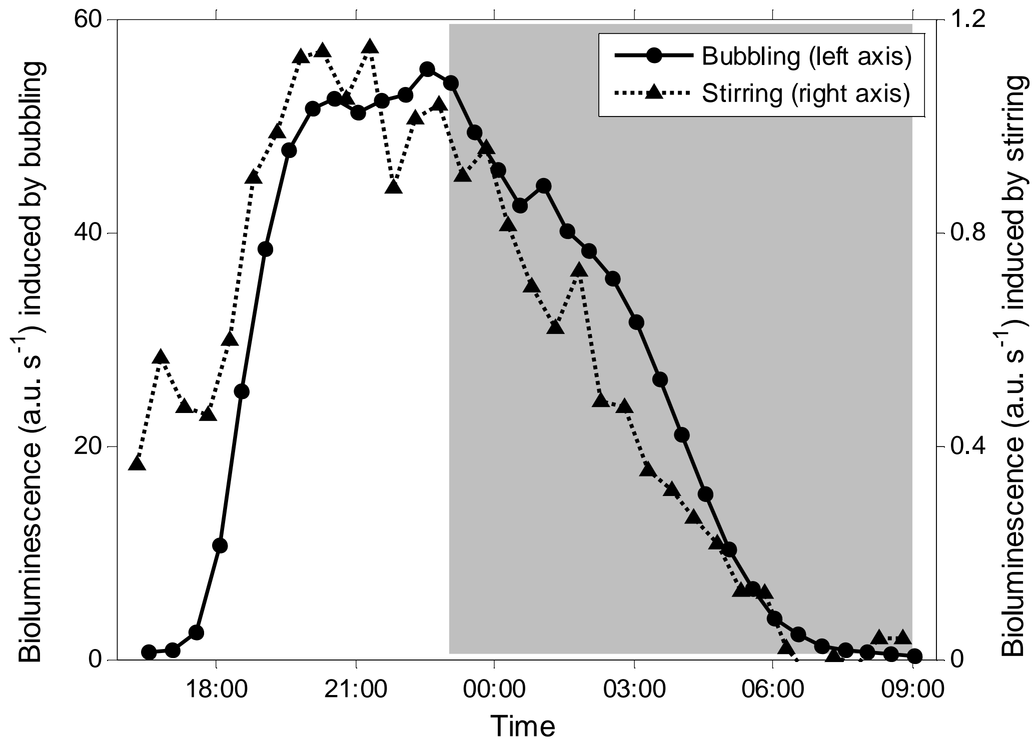

Figure 15 Bioluminescence stimulated by alternating between stirring (medium cross) and bubbling (high).). A. Stimulation time 2 minutes, interval between stimulation 13 minutes. B. Stimulation time 5 minutes, interval between stimulation 10 minutes. C. Stimulation time 2 minutes, interval between stimulation 13 minutes. In these early-stage measurements, the same culture bottle was measured in subsequent nights. The stimulation likely damaged the cells, as later concluded, which may account for the decreasing intensity of the light emission in subsequent experiments. The repeated measurements also resulted in a short day-length between the experiments with this culture.

3.2 Recovery period

In the recovery period experiments subsamples of a large culture bottle were tested for different intervals between stimulations. Between stirring treatments biomass increased continuously up until three days before the experiment ended. At this time chl *a* values were over 200 µg L^{-1}. In contrast, chlorophyll *a* concentrations varied from 30 to 42 and 37 µg L^{-1} (4. and 5. measurement day, respectively) between bubbling experiments. After three days without

measurements the concentration increased to 66 μg L^{-1}. Damage caused by bubbling was suspected as the cause of this growth pattern.

Instrument sensitivity and cultures differed between the stirring and bubbling experiments. For stirring, an extra set of measurements was made with the same settings, but a different culture.

Stimulation by stirring was repeated for 13 nights (in two sets) using varying recovery periods. The bioluminescence intensity differed between subsamples. The shape of the bioluminescence patterns, however, appeared to be strongly influenced by the recovery period in the first set of measurements, which are first discussed. Recovery periods of 20 minutes or longer led to typical bioluminescence patterns, increasing from the moment the culture was transferred to darkness and rising close to the start of the scotophase in their growth light regime. The emission intensity then remained constant for up to 6 hours after which it gradually decreased down to initial conditions (Figure 16). Shortening the recovery period to 10 minutes resulted in lower intensities, earlier peaking, and lack of a plateau.

In the second set of measurements, intensities were highly variable. A recovery period of 90 minutes resulted in the same typical pattern as observed with long recovery periods in the first set of measurements. A measurement with a recovery period of 10 minutes again showed low intensity and early peaking, whereas no pattern could be discerned from the samples subjected to a stimulation rhythm with 40-min recovery periods (Figure 17). These results are included here to illustrate that the cultures were highly sensitive to manipulation, and many repeated observations are necessary to draw conclusions of their intrinsic bioluminescence rhythms.

In the bubbling treatment bioluminescence intensities increased with increasing recovery period (Figure 18). Even with the longest recovery period, 40 minutes, the intensity curve shows the atypical sharp shape, lacking a plateau light emission for the duration of the night. The peak is also reached early before the scotophase of the growth light rhythm.

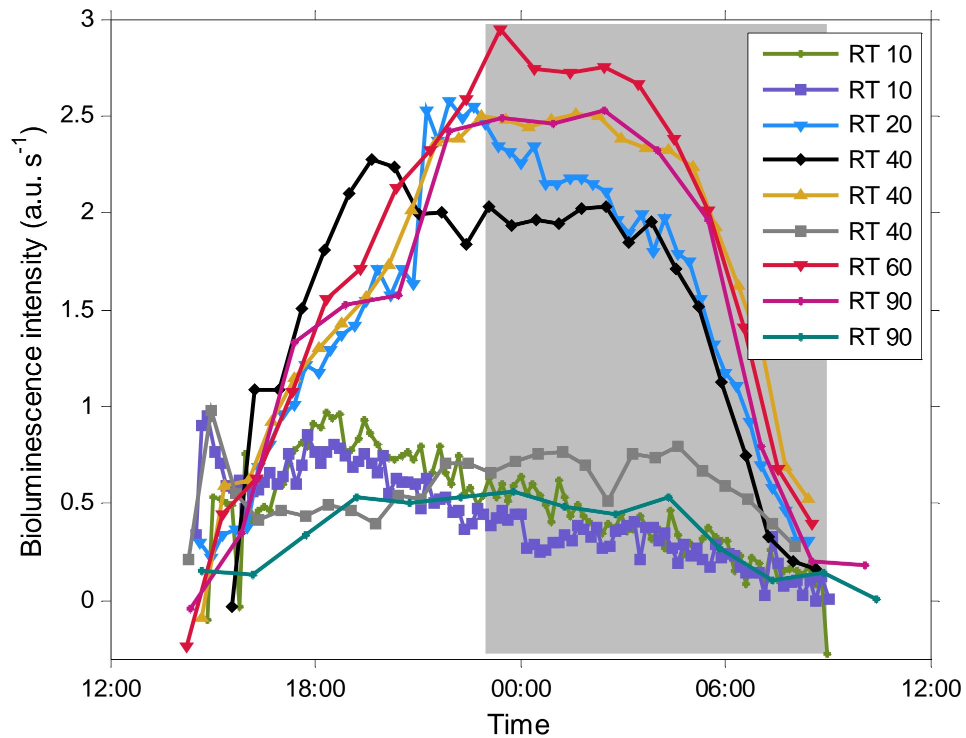

Figure 16 Recovery period effect on bioluminescence with stirring stimulation. RT10 – RT90 refer to the recovery periods 10–90 minutes. With multiple entries there has been several subsamples tested with the same recovery period. The recovery periods with which high intensity levels were reached are 20–90 minutes (upper group of lines). RT10 measurements resulted in low intensity (violet and green lines), as well as the last 40 and 90 minutes measurements (grey and turquoise). The peaks of the RT10, however, are around 18:00, whereas RT40 peaks close to the scotophase. RT90 shows a plateau in the light intensity. Values normalised to chlorophyll *a*, accounting for the growth of the culture, showed the same pattern.

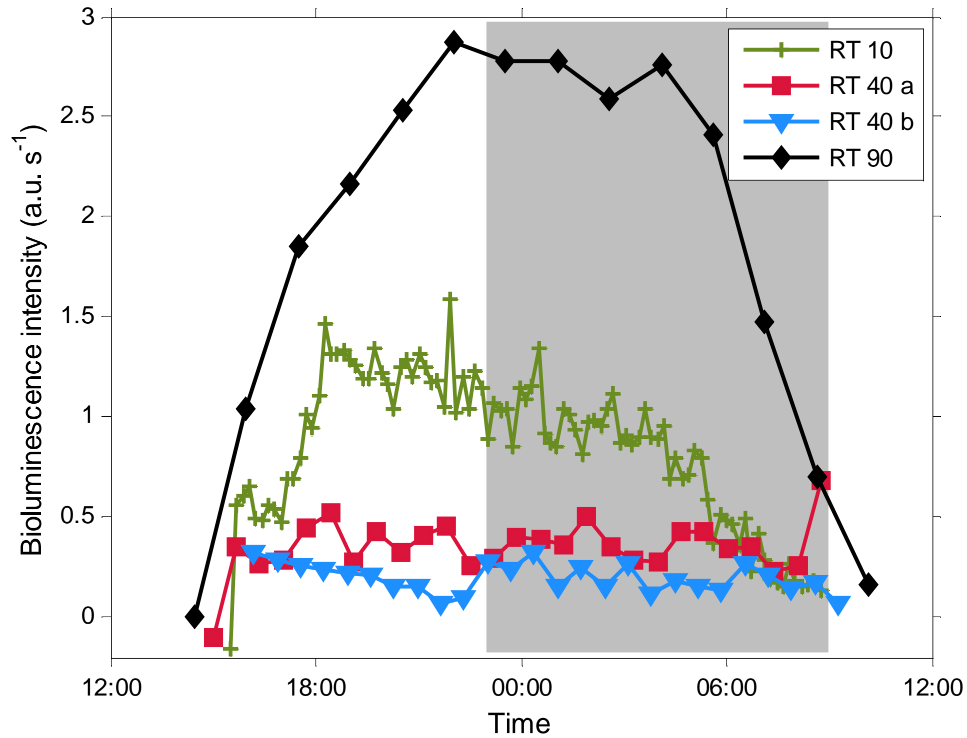

Figure 17 Recovery period effect on bioluminescence with stirring stimulation, second set of measurements. RT10 – RT90 refer to the recovery periods 10–90 minutes, a and b to different measurements of the same bottle. 90 minutes of recovery period results in higher light emission level than 10 minutes of recovery period.

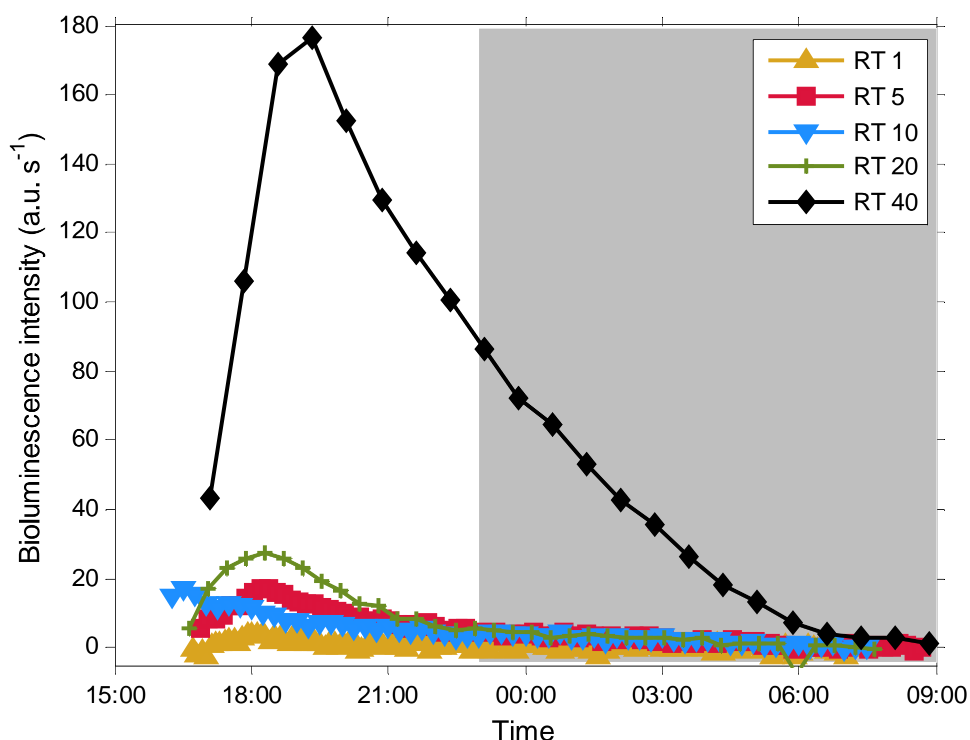

Figure 18 Recovery period effect on bioluminescence with bubbling stimulation. RT1 – RT40 refer to the recovery periods 1–40 minutes. With longer recovery periods higher bioluminescence intensities are recorded, but the intensity declines sharply after the peak without having a plateau phase. Values normalised to chlorophyll *a*, accounting for the growth of the culture, showed the same pattern.

3.3 Spontaneous bioluminescence

For determining the significance of spontaneous bioluminescence some cultures were not stimulated during the measurement. Nevertheless, light emission is recorded (lower graphs in Figures 19 and 21). The intensity of spontaneous emission is however lower than in the measurements with mechanical stimulation (upper graphs in Figures 19 and 21). Also the original recording files (Figures 20 and 22) show that light emission above the baseline occurred (compare to Figure 11). The patterns differ from the regular light emission obtained by stimulation (compare to Figure 23).

When the results were analysed with the help of a constant baseline for the whole period, the values of the first two hours are not included because of the instrument

drift (see methods). These values are disproportionally low and hinder scaling the axes. In the second experiment (Figures 21–23) the sensitivity of the instrument was lower than in the first experiment.

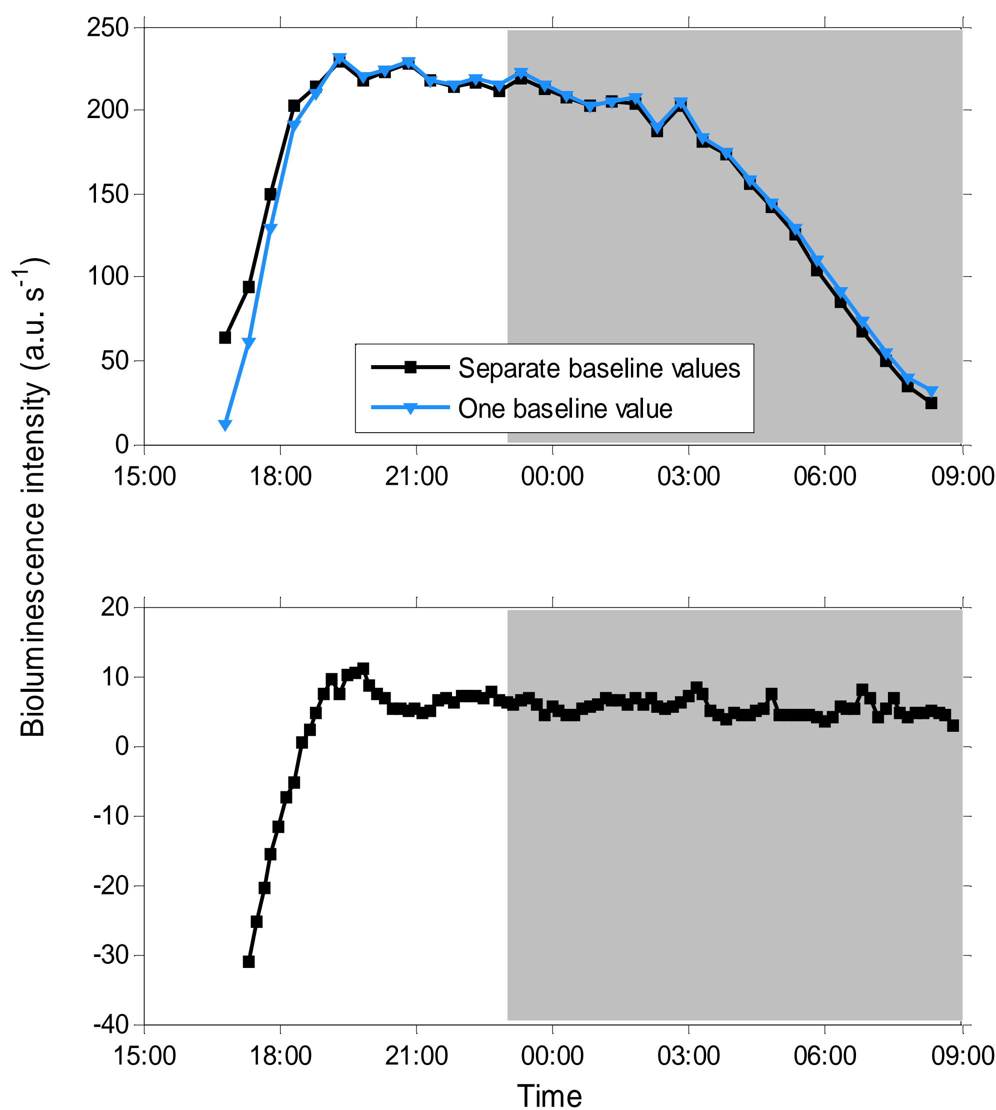

Figure 19 Stimulated (upper graph) and spontaneous (lower graph) bioluminescence. For the stimulated bioluminescence, a baseline was subtracted either by using the average signal in the preceding non-stimulating period (separate baseline values), or by using the average signal of the whole measurement period (one baseline value). For spontaneous emission, one baseline value is used and recordings are integrated for 10-minute periods. The culture used in these measurements was AOF-0930. Stimulated and spontaneous measurements were made from the same culture flask in subsequent nights.

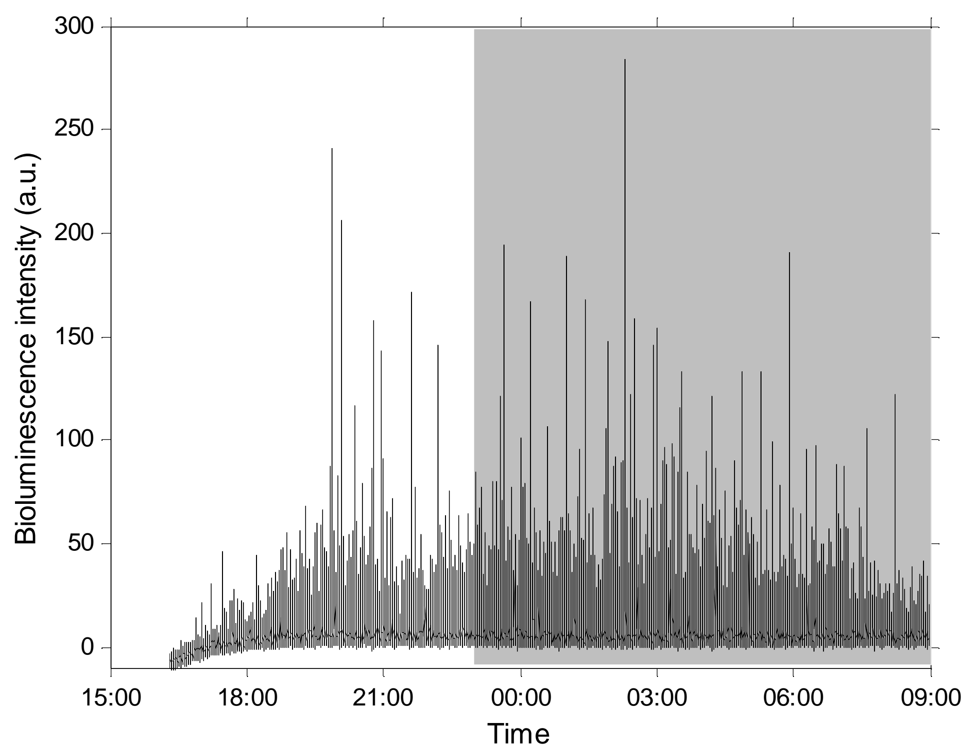

Figure 20 Spontaneous bioluminescence. The original recording file for the measurement analysed in the lower graph of Figure 19.

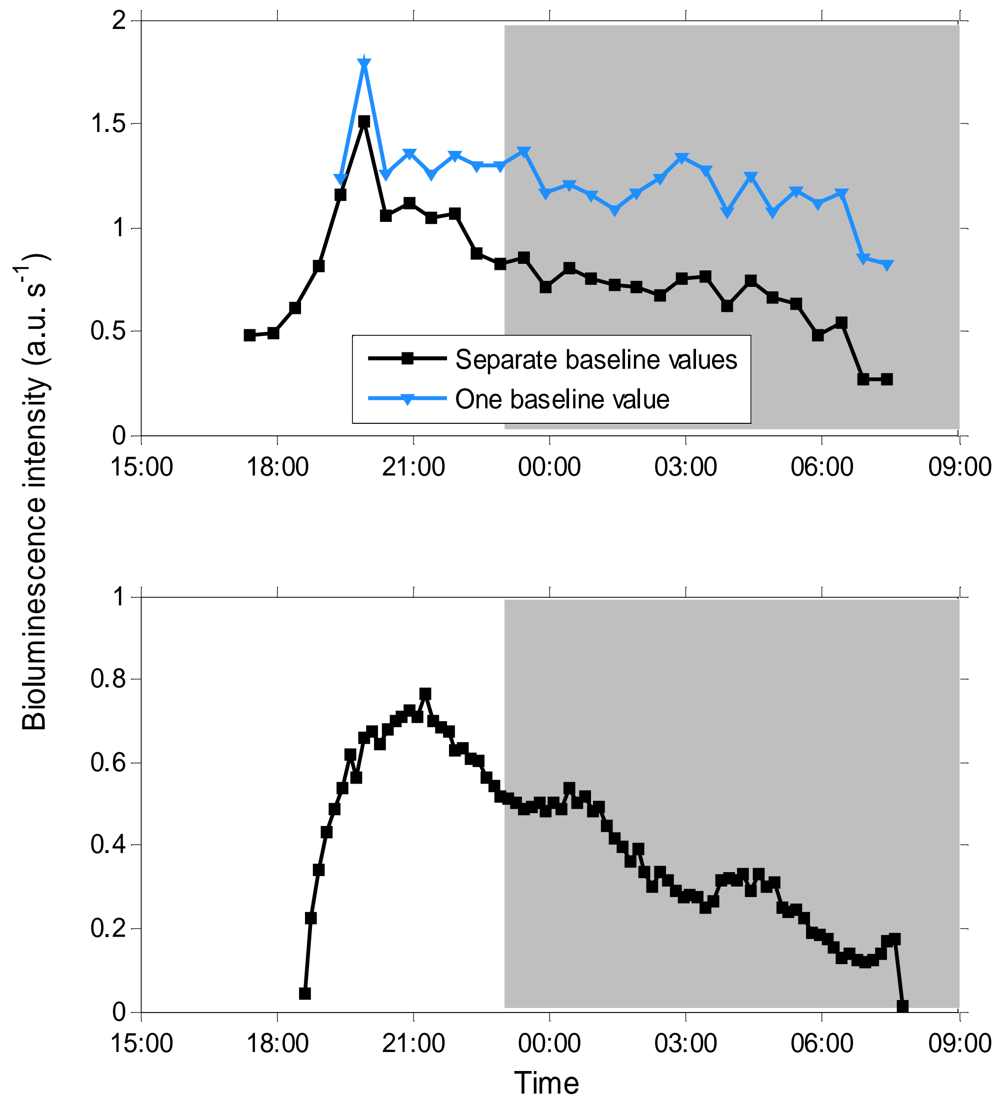

Figure 21 Stimulated (upper graph) and spontaneous (lower graph) bioluminescence. For the stimulated bioluminescence, a baseline was subtracted either by using the average signal in the preceding non-stimulating period (separate baseline values), or by using the average signal of the whole measurement period (one baseline value). For spontaneous emission, one baseline value is used and recordings are integrated for 10-minute periods. The culture used in these measurements was AOPL-61. Stimulated and spontaneous measurements were made from two separate but identical culture flasks. The growth in these cultures was poor.

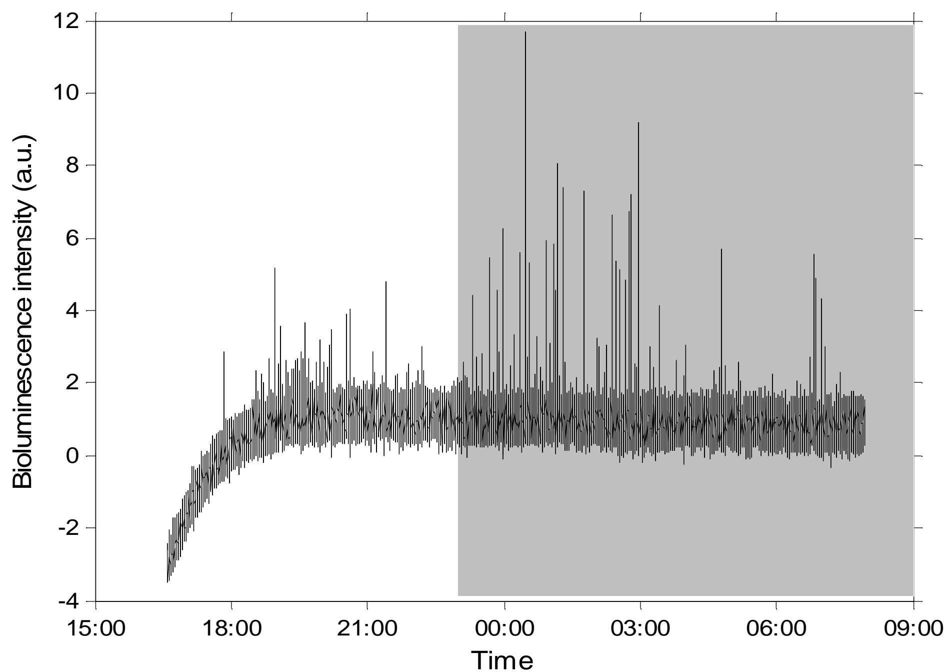

Figure 22 Spontaneous bioluminescence. The original recording file for the measurement analysed in the lower graph of Figure 21.

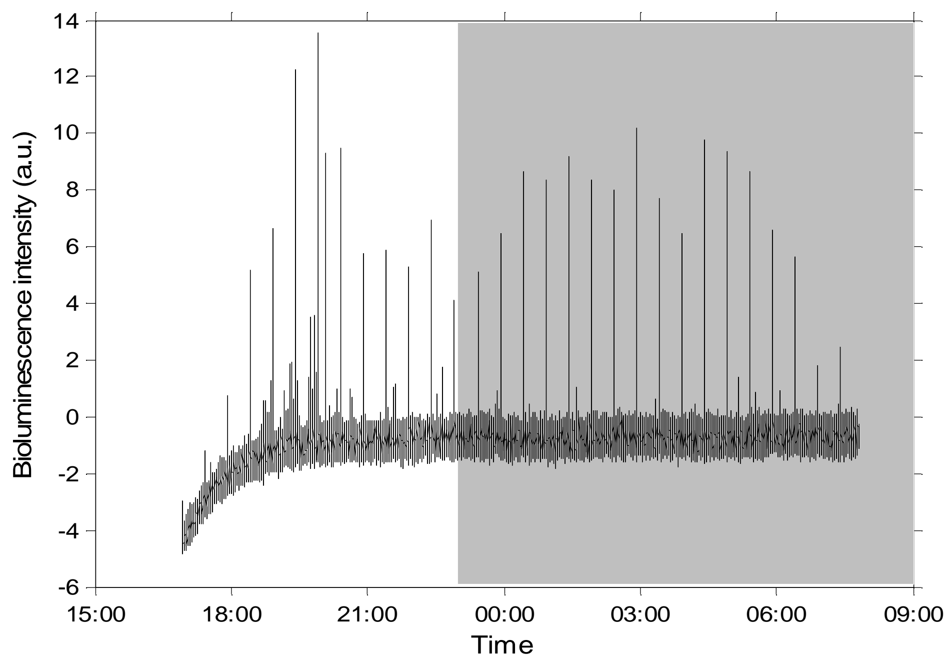

Figure 23 Stimulated bioluminescence. For comparison, the recording file of the measurement with stimulation, that is showed analysed in Figure 21 upper graph. The light emission peaks occur regularly when stimulation is switched on.

3.4 Persistence - continuous light

The measured bioluminescence in cultures grown in continuous light, and then transferred into dark measuring conditions, is shown in Figure 24. In three of the five measurements (CL1 a and b, CL2 a), a notable light emission curve is visible, thus bioluminescence occurred and rose to a peak value during the measurement. In two of the measurements, the curve is rather flat. The dynamics in measurements CL1 a, b and CL2 a are highly similar when normalized to their mean (Figure 25).

The cultures were growing continuously and chlorophyll *a* increased from 24 to 45 µg L^{-1} (bottle 1, sampling ranging over four days), from 24 to 62 µg L^{-1} (bottle 2, range 5 days) or from 27 to 45 µg L^{-1} (bottle 3, range 3 days).

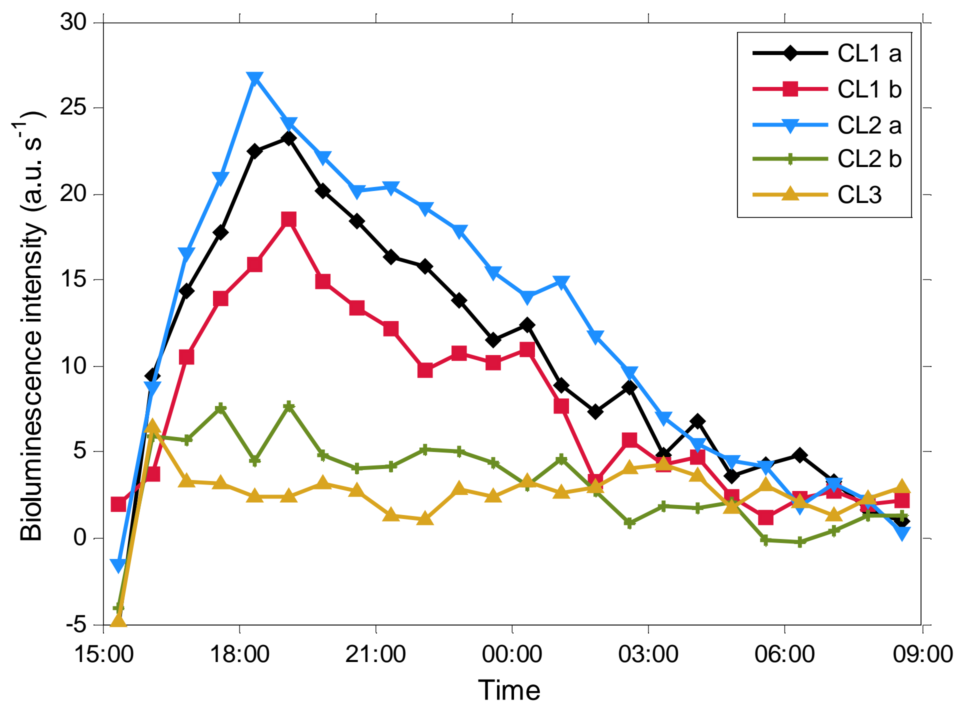

Figure 24 Bioluminescence after continuous light growth conditions. Light emission occurred after placement into the dark measuring conditions. CL1–3 refer to different culture bottles, a and b to replicate measurements.

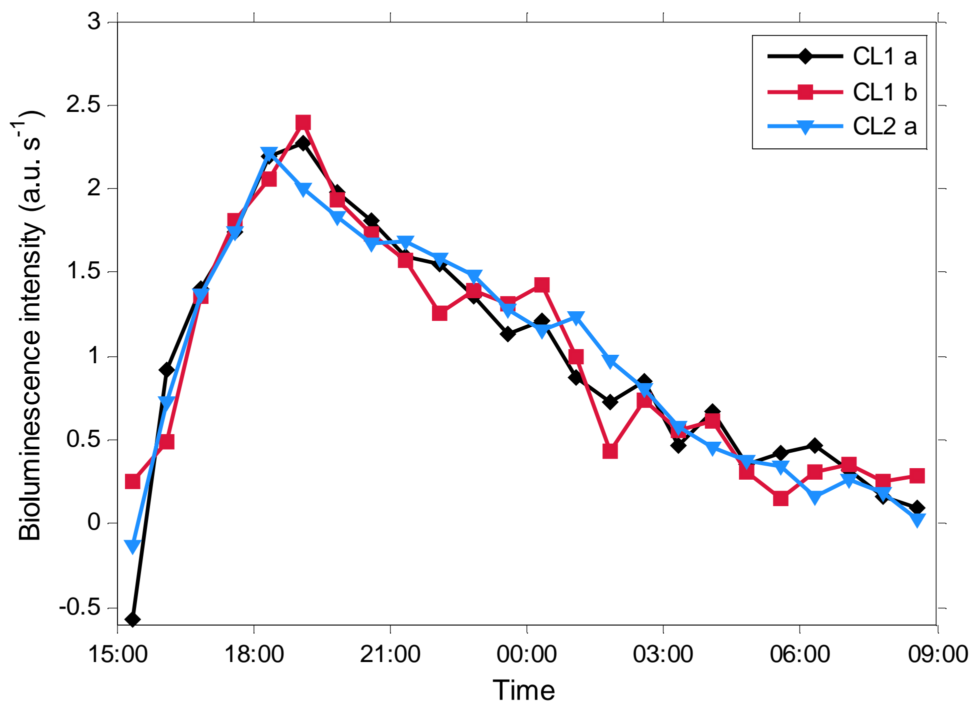

Figure 25 Bioluminescence patterns normalized to their mean. The responses of the cultures to placement from continuous light into darkness are very similar. CL1–2 refer to different culture bottles, a and b to replicate measurements.

3.5 Persistence - continuous darkness

To study the persistence of the bioluminescence rhythm without exogenous clues, cultures were kept in continuous dark conditions during multiday measurements. The results presented here are collected from different experimental setups and pilot experiments, so the recovery periods ranged from 40 to 90 minutes and stimulation times from 1 to 15 minutes, cultures had different densities, and the sensitivity of the instrument was higher in the measurement from recovery period experiment with bubbling (Figure 27 bottom). Differences are indicated in the captions.

Bioluminescence did not occur after the first night in four out of six cultures (Figures 26 and 27). In two measurements a rise in the light emission level occurs also at the time corresponding to the second scotophase of the growth rhythm.

Additionally, reversing photoinhibition results in high initial emission levels even before the rhythm determined night.

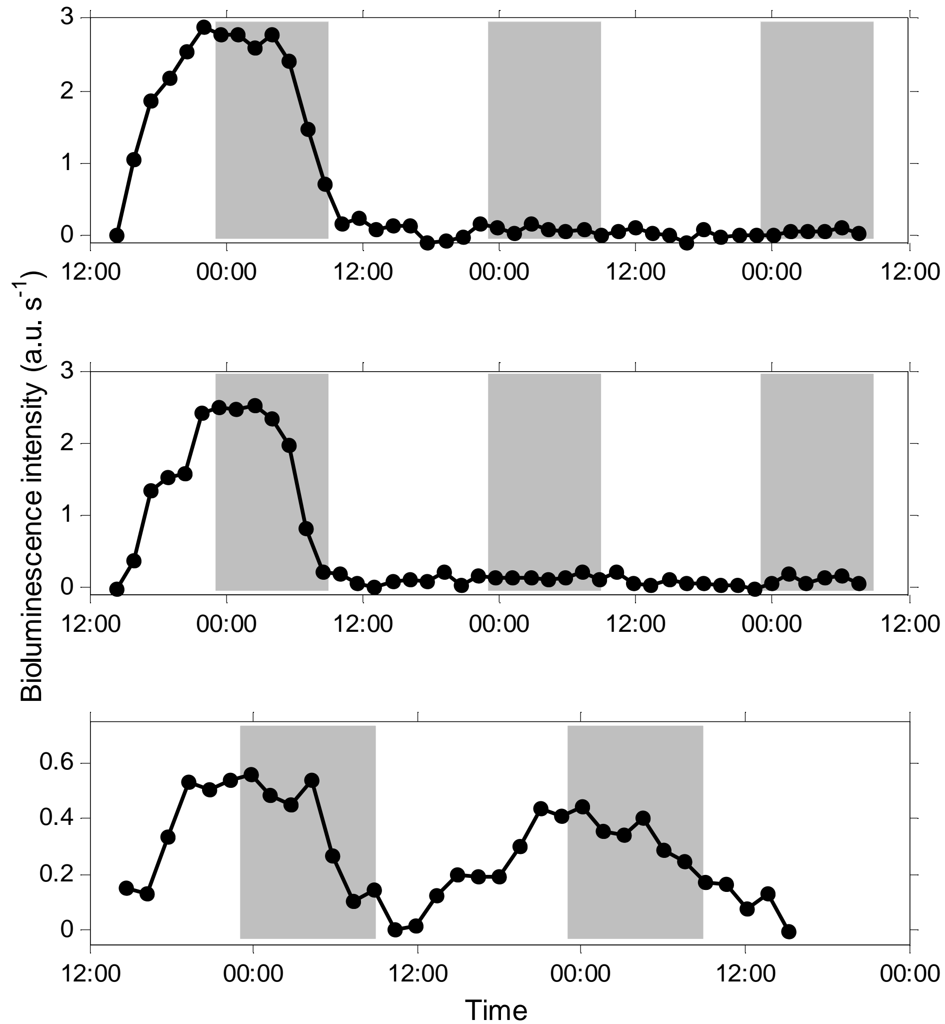

Figure 26 Bioluminescence in continuous darkness. Multiday measurements with 90 minutes recovery period and 1 minute stimulation duration, and large stirring cross (data from the recovery period experiment using stirring). In one of the measurements (bottom) bioluminescence is visible also in the second night, although the intensity is low in general (note different axis scale). The middle and bottom graphs are from subsamples of the same culture, the uppermost one is a different culture.

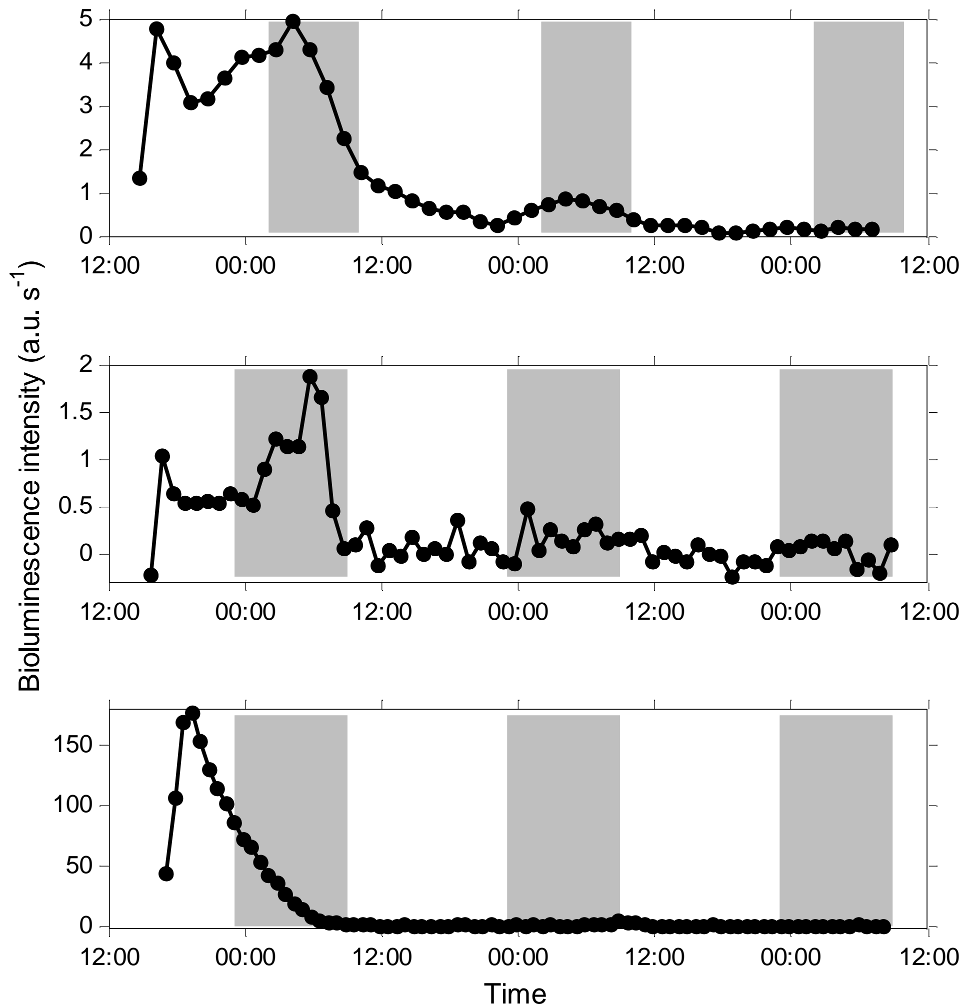

Figure 27 Bioluminescence in continuous darkness. *Top:* Measurement with 89 minutes recovery period, 1 minute stimulation duration, and large stirring cross. The growth rhythm for this culture was 16:8. The initial rapid rise is presumed to be due to reversing photoinhibition. After that the intensity declines, and rises again to the peak value according to the entrained light rhythm of the culture prior to the experiment. A second rise, although weaker, is visible corresponding to the second scotophase. *Middle:* Measurement with 45 minutes recovery period, 15 minutes stimulation duration, and medium stirring cross. Bioluminescence is clearly visible only during the first day and night. *Bottom:* Measurement with 40 minutes recovery period, 5 minutes stimulation duration, and high bubbling with pipette type A (data from recovery period experiment using bubbling). Bioluminescence is visible only during the first day and night. Note different axes scaling.

3.6 In situ vs laboratory rhythms of bioluminescence

To study bioluminescence rhythms under different light regimes, in situ data was compared with laboratory measurements of methodological and rhythm experiments. For estimation of how fast bioluminescence reaches its peak level after the initiated light emission, the slopes from start to peak level (beginning of constant light emission) were calculated. The field measurements (Figure 30) were from the end of August when daylength (from sunrise to sunset) in the period in question was 14.5–15 hours. The methodological experiment data used is from the recovery period experiment with stirring (Figure 28). The growth rhythm for this culture was 14:10, thus resembling the in situ conditions. Measurements with recovery periods 20–60 minutes are used (except the RT40 measurement that resulted in unusually low light emission). The data from the rhythm experiments (Figure 29) is drawn from the control measurements where the rhythm was 16:8, being as close as possible to the light regime of the other comparison datasets. Before the comparison calculations, the light intensity values of a night were normalised to their maximum value, to concentrate on the effect of the elapsed time (time needed to reach the peak level) and not on the different peak intensities. The intensity was expressed as percentage of the peak value.

Culture growth was poor in the first attempts to set up batch volumes for rhythm experiments, and the results are not included. In the rhythm experiment II larger culturing volumes allowed better growth with chlorophyll *a* increasing up to 562–602 µg L^{-1}, after which culturing media were replenished and the culture diluted. In the period of the measurements (24.2.–6.3.2012) and culture (control 16:8 L:D) used for this comparison, growth from 315 to 622 µg L^{-1} took place. In the field, chlorophyll *a* values ranged from 7.5 to 30.4 µg L^{-1} in the period used for the comparison (22.–29.8.2011). Field chlorophyll *a* values were used for normalising.

Data used for the mean slope calculation comprised 4 (methodological experiments) or 7 (rhythm experiment and field data) measurement nights (see Figures 28–30). The mean slopes of bioluminescence rise (with standard

deviations in brackets) from the beginning of the bioluminescent light emission to the peak level were 14.2 (5.4), 33.2 (2.2) and 30.6 (7.1) % hr^{-1} for data from methodological experiments, rhythm experiments and field, respectively. The individual slope values with exact time boundaries are listed in Appendix 2. A Kruskal-Wallis test of independent samples showed significant differences in rate of bioluminescence rise between experiments (p=0.016). Pairwise Mann-Whitney tests showed that the significant difference was between methodological and rhythm experiments (Bonferroni corrected p=0.018), and between methodological experiments and field (Bonferroni corrected p=0.036). Rhythm experiment and field data have similar slopes and the bioluminescence rise in the measurements is steeper. Thus, cultures placed in darkness before their entrained night show a slower bioluminescence rise and reach their peak later compared to observed in situ rhythms and measurements where the light-dark rhythm is maintained during the measurement.

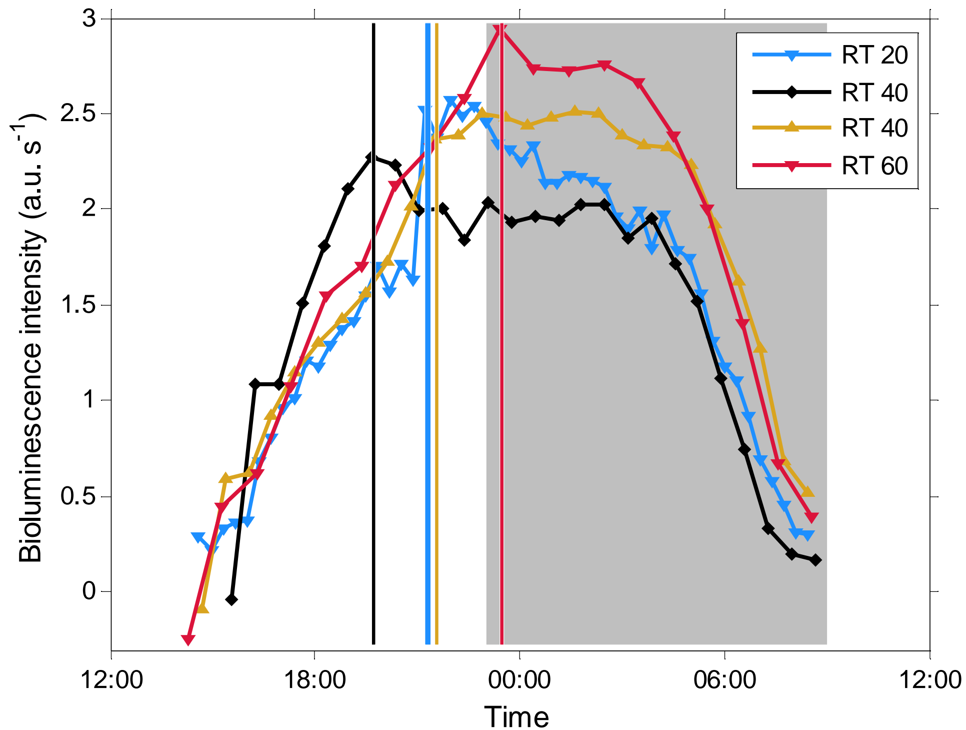

Figure 28 Bioluminescence in dark measurement conditions (data from recovery period experiment using stirring). The lines mark the end points for the slope calculation (colours respectively). Bioluminescence rises soon after the culture is placed in the dark measurement chamber at the start of the measurement.

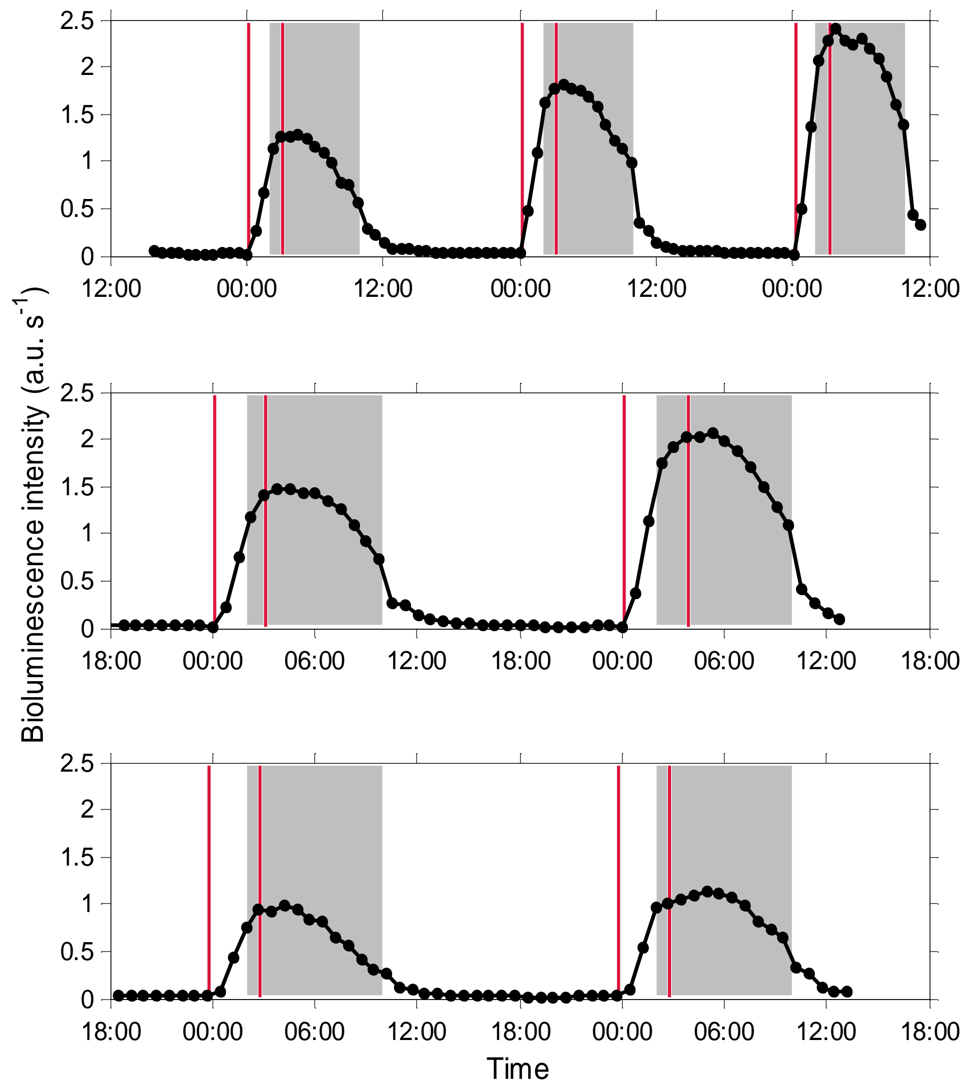

Figure 29 Bioluminescence under constant light-dark rhythm (data from rhythm experiments with 16:8 L:D). Red vertical lines mark the start and end points for calculation of the slope of the bioluminescence rise. The bioluminescence pattern is constant and light emission reaches its maximum at the beginning of the night. Bioluminescence ceases towards the end of the night but is not completely finished.

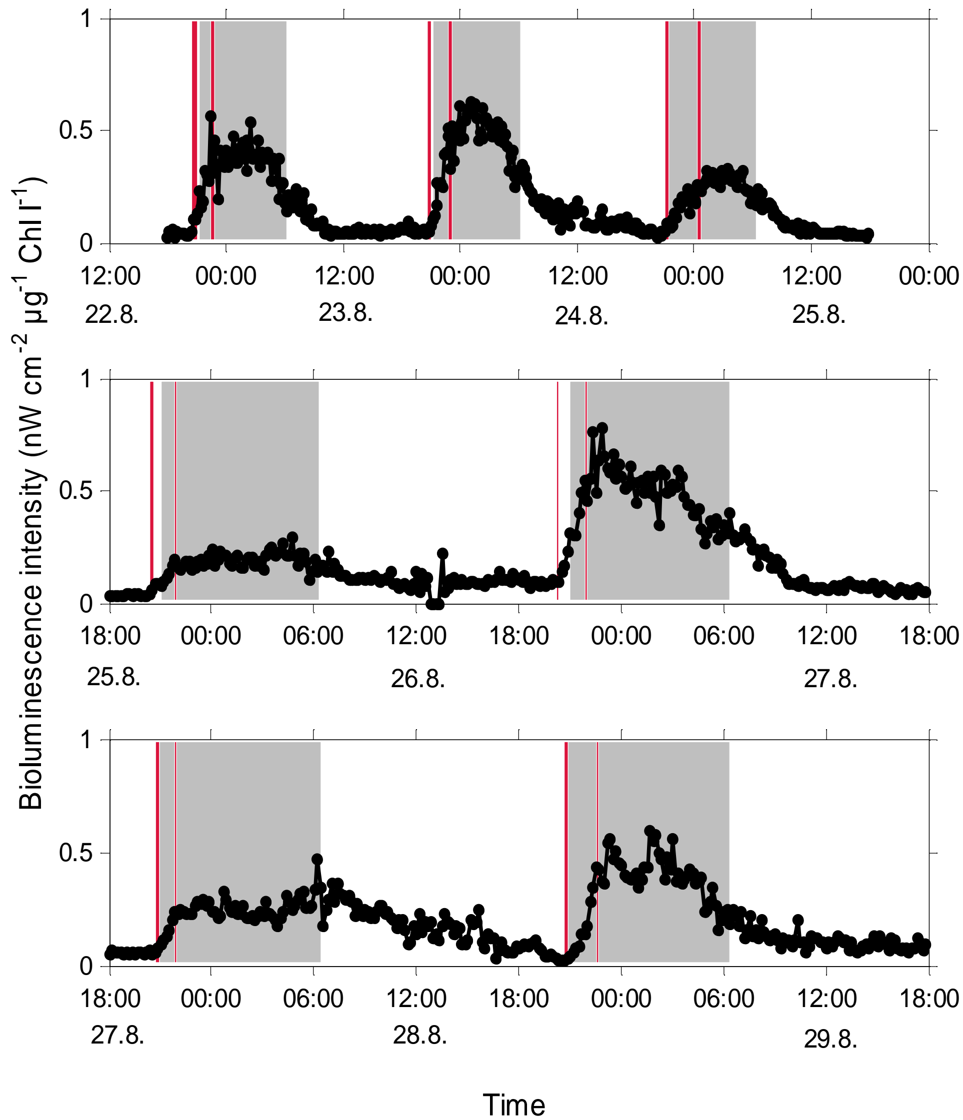

Figure 30 Bioluminescence in nature 22.-29.8.2011. Bioluminescence patterns are more variable than in the laboratory, but a clear diurnal pattern can be seen. Although very weak, light emission is not completely zero during the day. The graphs present a continuous sequence of measurements. *Uppermost graph*: 22.-25.8. *Middle graph*: 25.-27.8. An interruption for cleaning of the device is visible as zero-values on 26 Aug. *Lowermost graph*: 27.-29.8. Red vertical lines mark the start and end points for calculation of the slope of the bioluminescence rise. The night (grey rectangles) is marked from sunset to sunrise.

4 Discussion

4.1 Method of measuring bioluminescence

4.1.1 Stimulation mechanism and recovery period

Bubbling induced more intensive bioluminescence than stirring. In the stimulation mechanism experiments the difference was 9–99 fold, seemingly depending on the bubbling intensity. Bubbling is a stronger stimulus and disturbs the water in a more random and continuous way than stirring. Once the stirrer is on, the flow pattern does not change much. Cussatlegras and Le Gal (2007) report on their studies, and refer to other investigations, that for massive emissions the cells should experience temporal changes in the flow pattern. In their experiments acceleration was necessary to trigger the main bioluminescent response of *P. lunula*. In the experiments of Biggley et al (1969), higher response to stirring than to bubbling was observed for *L. polyedrum*, but they describe the stirring as “extremely turbulent”, whereas the bubbling air flow was in the range of our experiments.

Differences in the stimulation pattern could partly be caused by accumulation of the cells in the air distributing pipette. After every bubbling period the pipette gradually filled with culturing media until the water level was reached, and was emptied again when the air flow started. When the bottle was observed after the measurement, i.e. no bubbling was taking place, the media inside the pipette seemed denser than elsewhere. If this represented a true difference in concentration, dinoflagellates preferred to swim into the pipette in the resting periods, and the initial bioluminescence at emptying the pipette was disproportionally high.

There are differences in the bioluminescence pattern throughout the night that the two mechanisms induce. No clear trend can be detected, and more experiments are needed to find out whether it is random variation. In some measurements, stirring induced relatively stronger bioluminescence than bubbling in the beginning of the measurement. Changes in sensitivity to mechanical stimulation

caused by circadian regulation or reversing photoinhibition can occur, and result in different patterns if the two methods cause a different flow pattern in the medium. At the same time, stirring values are more susceptible to errors rising from the calculated baseline because of the lower signal to noise ratio.

Of course, varying intensities of bubbling and more different types of stirring bars and speeds could be tested for their stimulation properties. In experiments with low air flow rates, air distribution became uneven; air escaped through a fraction of holes in the pipette, which could have lead to differences between measurement bottles. One possibility could have been to bubble only through the tube end, without the distributing pipette. This would also have reduced the possible clustering of the cells in the device.

Stirring was chosen as the more useful stimulation mechanism because it was suspected that bubbling damaged cells. Bubbling exerts a great force in the water and can break cells. Bursting of bubbles causes cell death in insect cells (Hua et al. 1993), while studies on the effect of bursting bubbles on algae are lacking. In our experiments, a culture was bubbled during two consecutive nights and exhibited greatly reduced bioluminescence intensity during the second night (data not shown). Culture AOKAL-25 was studied for several consecutive nights in the bubbling/stirring experiment and showed the same pattern of decreasing bioluminescence. Other aspects of the experiment, like extended darkness, cannot be discounted as causes of stress. Bubbling was suspected to be the reason for growth problems in the rhythm experiments, however the problems with poor growth continued with stirring and untreated cultures. Generally speaking, small-scale turbulence in water can inhibit population growth in dinoflagellates, but the response varies depending on the experimental conditions and species, and no unambiguous conclusions can be drawn on physiological effects of turbulence (Sullivan et al. 2003). Bubbling could also cause continuous low-level light emission (glow) by damaging cells (see discussion in chapter 4.1.2 Spontaneous bioluminescence).

Based on microscopy observations, bubbling does not visibly harm the cells. The proportion of damaged cells (Figure 3) did not differ between samples that were bubbled, stirred or not stimulated. More sophisticated methods could be used to differentiate dead and degraded cells and cysts. Haberkorn et al. (2011) used dying methods to enumerate dead cells and estimated the number of different cell states in samples after exposure to environmental stresses.

A clear problem with bubbling is the long recovery period needed. Recovery period is the time between stimulation events required for full recovery from stimulation. When recovering, light emission capacity returns to the preceding level, involving e.g. synthesis of new reaction compounds or release from storage. After bubbling stimulation, 40 minutes of recovery is not sufficient. With recovery period ranging from 1 to 40 minutes, peak bioluminescence intensity increased; increasing recovery time allowed the culture to produce more flashes. The peak in the measurement with 40 minutes recovery period was reached later than in the other measurements, but already before the night. This suggests that insufficient recovery time causes depletion of the bioluminescence capacity early or before the night so that the 'natural' pattern cannot be observed. The experiment was not extended beyond 40 minutes recovery period, so it is unsure whether the intensity would continue increasing. Based on the shape of the curve, even 40 minutes is insufficient to recover from the bubbling stimulation. The typical curve of stirred cultures has a plateau phase, whereas all curves in the bubble experiments were sharp with a steady decline after the peak. Further experiments would reveal the sufficient recovery period for stimulation with bubbling. However, very long recovery periods would result in insufficient data density and loss of information by limited resolution.

In contrast, in the corresponding experiment with stirring only the measurements with 10 minutes recovery period showed the sharp shape of the curve and peaked early, suggesting depletion of the bioluminescence substrate in these stimulation frequencies. With 20 minutes recovery period the intensity was already at the level of the measurements with longer (40–90 minutes) recovery periods. On the other hand, the plateau was not as clear as in the measurements with longer

recovery periods: the intensity gradually dropped, while preserving the quadrangle shape. Thus, 40 minutes recovery (possibly as short as 20 min) is sufficient between stimulation events using a stirrer bar. The shorter recovery period of stirring compared to bubbling is reasonable, as stirring stimulates a smaller portion of the bioluminescence capacity than bubbling (results from experiments with alternating stirring and bubbling discussed in this chapter).

Two measurements of the first set of the stirring experiment, with recovery periods of 90 and 40 minutes, showed very low bioluminescence intensity. The intensity was comparable to the 10-minute recovery period measurements. The shape of the curve and the timing of the peak resembled the expected. This happened after the measurements with 10-minute recovery periods, i.e. after part of the culture had experienced extensive disturbance. It also coincided with the culture reaching declining chl *a* values. The role of encystment in the observed drop in intensity is discussed in chapter 4.1.4 Culturing conditions and experimental setup. Why the last measurements of the second set with a different culture showed the same feature, is more puzzling (see also discussion in 4.2.1 Bioluminescence after growth in continuous light).

4.1.2 Spontaneous bioluminescence

Spontaneous bioluminescence was detectable in the experiments. The pattern of the bioluminescence throughout the night has elements similar to the curves obtained by mechanical stimulation. The intensity is lower but rises to a peak and declines in similar fashion as in stimulated bioluminescence. The data analysis, however, is susceptible to error. Determining a baseline in the presence of instrument drift is particularly challenging. Baseline-corrected values are uncertain even beyond the excluded first two hours, when instrument drift is most rapid. Especially in the first experiment (Figures 19 and 20) the calculated baseline is probably too high due to strong light emission and dampens the bioluminescence pattern.

In the ocean, no spontaneous bioluminescence could be detected when the measuring device was neutrally buoyant, causing no mechanical stimuli (Widder

2002). Nevertheless, spontaneous bioluminescence is mentioned in literature (for example Biggley et al. 1969; Esaias and Curl 1972). Detailed studies exist as well: in *C. horrida*, spontaneous bioluminescence was observable (Latz and Lee 1995). Both flashing and glow were under circadian control, while spontaneous flashes were weaker and shorter in duration that stimulated ones. The rhythm of spontaneous bioluminescence persisted in continuous darkness for three cycles. The authors also refer to studies of 5 other species displaying spontaneous bioluminescence.

It is possible that although no apparent stimulation was present in the experiments, conditions in the measuring instrument triggered bioluminescence. Slight shaking of the instrument could act as a mechanical stimulus. However, the irregularity of flash peaks (Figure 22) does not support this theory. It is also unlikely that swimming currents or collisions of dinoflagellates would be sufficient to trigger light emission, as even the feeding current of a copepod is usually not strong enough. Latz et al. (2004) tested four dinoflagellate species, and in only one (in a sensitive subpopulation of it) the bioluminescence threshold value was low enough to theoretically permit stimulation by feeding currents. Bioluminescence thus needs contact with predator to occur. Furthermore, Latz and Lee (1995) observed the spontaneous bioluminescence to be independent of cell concentration, hence presumably not resulting from interaction with other specimen. Obviously, by being triggered by other dinoflagellates bioluminescence would lose its meaning as an antipredatory mechanism. What then triggers the spontaneous bioluminescence, and particularly what is its ecological significance, remains to be discussed.

From the results the question rises whether there is always spontaneous bioluminescence present, also in the measurements with mechanical stimulation. To check this, measurements that had an unusual shape of baseline (declining after an initial rise instead of the typical steady rising one) were checked for deviations of the minimum level between stimulations. Intensity values from which a moving 1-min minimum was subtracted were used to construct a baseline. This could better reveal spontaneous flashes than the usual baseline

construction. No diurnal shape in the spontaneous bioluminescence/ instrumental noise could be observed. The intensity of the signal could be high, but it means that instrumental noise is high in relation to induced bioluminescence level. Very sensitive instrument settings had to be used to capture the weak bioluminescence light. Even though noise would, and probably does, overlap with spontaneous bioluminescence it does not affect the diurnal shape in these measurements, or it is negligible compared to the stimulated light emission. On the other hand, a significant proportion of spontaneous flashes could occur outside the periods chosen for this analysis. The time periods used were the same than in the usual baseline construction and preceded each stimulation. In the unstimulated spontaneous bioluminescence measurements a similar resolution (10 minutes integrated every hour) however suggested a diurnal pattern in the spontaneous bioluminescence.

The atypical baseline shapes discussed above could have been the result of continuous glow. It would elevate the minimum level of intensity recordings and would therefore not be visible in the above mentioned analysis of spontaneous bioluminescence. The glow can be emitted by broken cells. Biggley et al (1969) presumed that due to mechanical stimulation used in their experiments, cells of *P. lunula* were damaged and emitted a low-level continuous glow. In our measurements in question the atypical baseline is highest at the start, when also stimulated light emission is high, so the glow could be linked to the total bioluminescence capacity of the cells. However, these baseline shapes were also observed in some measurements with stirring, which was not considered as damaging as bubbling. In one pilot experiment done with a different species (*Protoceratum reticulatum*, data not shown), the rise and descent in the baseline took place towards the end of night, which theoretically could be caused by the breakdown of scintillons (Fritz et al. 1990).

In conclusion, the measurements suggest a circadian pattern in the recorded spontaneous bioluminescence. In the stimulated cultures changes in mechanical stimulation sensitivity can account for rhythms in bioluminescence. In the absence of mechanical stimulation the circadian regulation must be operating in the

reaction level. Both mechanisms of circadian regulation described in the introduction, fluctuations in the reaction compound levels and localization of the compounds, remain as possibilities.

4.1.3 In situ vs laboratory rhythms of bioluminescence

Comparison of field data with two different laboratory experiments gave insight into three different experimental light regimes: constant darkness in the spectroluminometer, light rhythm with abrupt change between day and night with the bathyphotometer, and a natural light rhythm with a gradual transition from light to dark.

Bioluminescence, after the emission levels start rising, reaches its maximum level faster in measurements where a light-dark rhythm is present. In darkness, the rise is slowed down when not occurring at the onset of the entrained scotophase. Due to the endogenous rhythm there is no capacity to reach the peak earlier than usual although the light emission has started. However, darkness reverses the inhibition of bioluminescence emission by light and bioluminescence can be measured already many hours before the actual scotophase.

In the rhythm experiments with pronounced light-dark rhythm, rising bioluminescence levels can be sheen shortly before the night. Peak level is reached just in time for the night. These cultures have been entrained to the daily rhythm they receive, and their endogenous rhythm allows them to “prepare” for the night by increasing the bioluminescence capacity (e.g. synthesising reaction compounds), even when still in the photophase. Also the sensitivity to photoinhibition can be under circadian regulation (Kelly and Katona 1966), which would aid this preparation under photophase.

In field, light regime comprises a natural day-night cycle, although more variable than in laboratory. The natural patterns of bioluminescence are similar to what is observed in the rhythm experiments.

The differences in bioluminescence patterns between the field measurement nights can reflect the composition of the sample: the material pumped into the sensor

chamber cannot be controlled, and fluorescence measurements do not respond only to dinoflagellates. The relatively high bioluminescence intensities recorded during some days could be related to heavy cloud cover or other meteorological or hydrographical factors that weaken the underwater light field and thus photoinhibition. Coupling of meteorological data to these results might help to interpret the diurnal dynamics of the light emission. In general, the low sun angle before sunset and after sunrise causes the light period to be actually shorter under water than above the surface, because at low angles the sunlight is reflected from the surface.

Biggley et al (1969) observed a rapid increase in light emission levels at the beginning of the experimental night, maximum values within 60–90 minutes, and constant light emission at the plateau, resulting in a square shape of the emission curve. The rate of decrease of the light emission at the onset of the photophase showed interspecies differences. Our results are in many aspects similar. They also experimented with dark measurement conditions and observed a very gradual decrease in the light emission after the scotophase, which was not seen in our experiments. Also the stepwise rise of light emission in natural samples placed into the darkness early ahead of their subjective night was not as clear in our cultures, but similar trends are observed (e.g. Figures 26 and 27). They recorded for these samples a pronounced initial rise in intensity, followed by a plateau until the time of the subjective night, when the intensity started rising again reaching its peak in the night. In general, our measurements in darkness (methodological experiments) resulted in irregular patterns: the diurnal pattern was visible, but the measurements that preserved the light rhythm (rhythm experiments) showed very uniform shapes in comparison.

Unfortunately, methodological and rhythm experiment data from cultures in equivalent growth rhythm were not available and the comparison included the closest possibilities (growth rhythms 14:10 and 16:8). The difference in rhythm could result in different dynamics in the cultures. However, the rhythm experiment cultures were inoculated consistently in the same growth rhythm and thus should be adjusted to it. Additional measurements and experiments

concentrating on this matter (thus having identical conditions in each group) and having more replicates would strengthen the conclusion.

Latz and Rohr (1999) observed that bioluminescence response to different flow fields is similar in laboratory cultures of *L. polyedrum* and field samples (dominated by *L. polyedrum*). Our experiments yield similar values for laboratory and field measurements concerning the slope of the initial increase of bioluminescence after darkness. Settings of the bathyphotometer allowed illuminating the cultures during subjective day in the experiments and the rhythm experiment setup was thus the laboratory equivalent for the field measurements.

Roenneberg and Mittag (1996) point out that even culturing conditions that are constant from human perspective might bear hints of circadian time: for example the metabolism of the cells can change the chemical composition of the volume restricted growth media in a circadian manner and thus complicate the research on endogenous rhythm. Laboratory experiments with small volumes of cultures also require a sufficient time interval between the bioluminescence stimulation events to not exhaust the cells. In our field experimental design this is irrelevant: the pump takes in new material from some distance from the instrument and the chance of drawing in the same cells within a short time is small. At the same time, the concentration or composition of the sample cannot be regulated. Next to the instrument was a fluorometer, but as mentioned the blooms are not monospecific (Hakanen et al. 2012) and changes in chlorophyll *a* concentration do not necessarily reflect changes in bioluminescent dinoflagellate concentration.

4.1.4 Culturing conditions and experimental setup

Apart from the light regime there were other factors affecting the bioluminescence pattern of the cultures. Certain results suggested that the condition of the cultures plays a role and handling of the cultures has to be carried out with care.

The effect of the growth phase on the bioluminescence production was not directly tested, but the unusually low intensities recorded in the recovery period experiment with stirring imply that cultures in their stationary phase are weaker in

their bioluminescence capacity (possibly due to cyst formation, discussed later). At the same time, from chlorophyll data it was visible that extended periods in the dark, like in the multiday measurements, affected growth negatively. To a certain extent, though, bioluminescence might still be intact despite of restricted growth: Latz and Jeong (1996) observed in the heterothropic dinoflagellates *Protoperidinium* cf. *divergens* and *P. crassipes* that insufficient nutrition affected growth rate before bioluminescence, i.e. with some energy limitation bioluminescence was still high but growth rate decreased.

Challenges with culturing algae and subsequent measurements with negligible bioluminescence recorded likewise showed that it is important to maintain to cultures in the best growth stage for the experiments. Interestingly, Sweeney and Folli (1984) used cultures in the stationary phase for their experiments on the nitrate effect on the circadian rhythmicity, and Kiessig et al. (1979) in phase shifting experiments. They used acid as the bioluminescence stimulation mechanism, which can bypass changes in mechanical stimulability and possibly is less dependent on the physiological state of the cultures.

With dinoflagellates not just the culturing but also the experiment conditions should be kept as stable as possible. Disturbances should also be avoided when transferring cultures to measuring conditions. Dinoflagellates form cysts, non-motile stages, under stressful environmental conditions. There are two types of cysts: the resting cysts are double-walled and require a dormancy period to germinate. These are formed to overcome unfavourable environmental conditions. Pellicle cysts (or ecdysal or temporal cysts) are single-walled and can germinate without a dormancy period, or the period is significantly shorter than that of the resting cyst. They are formed in response to short-term adverse conditions. Additionally, thecate cysts are observed; they are non-motile cells that have a theca (Bravo et al. 2010). Pellicle cyst formation occurs in *A. ostenfeldii* in connection with ageing of cultures. At the same time, germination of the cysts is common (Jensen and Moestrup 1997).

Balzer and Hardeland (1991) observed that *L. polyedrum* forms ample cysts when exposed to lowered temperatures (15 °C) and short day-lengths. Practically all cells encysted at day-lengths of 10 hours and less. In higher temperatures (20 °C) encystment did not occur in response to day-length. The combined effect of temperature and light was thus the environmental cue for encystment. It was also found out that added melatonin and an analogue (5-methoxytryptamine, both occurring in the cells) could induce cyst formation and thus imitate the effects of darkness treatments – a similar dark-mediator effect to vertebrates.

In our methodological experiments the conditions changed somewhat: the spectroluminometer was continuously darkened, not temperature controlled, and cultures had to be carried from the 16 °C culture room. In this kind of experiments the measuring instrument is fairly determining for the experimental setup and it depends on the instrument how well the measuring environment can be adapted. The transfer to measurement bottles might also have disturbed the cultures. The dinoflagellates seemed to grow poorly in the measurement bottles, so those were not used to grow stock cultures. Moreover, with repeated measurements of the same culture it is useful to have a larger volume, from which a sample for the measurement is taken. This minimises the effect of the measuring procedure itself. Chlorophyll *a* sampling prior to measurements likely was harmless, as in the below described stress experiments (Haberkorn et al. 2011) careful pipette handling did not cause cyst formation.

Von Dassow and Latz (2002) mention that even mild centrifugation caused pellicle cyst formation in *L. polyedrum*. Haberkorn et al. (2011) exposed *Alexandrium minutum* cells to mechanical (centrifuging), thermal (cooling and freezing) and chemical (saponine and H_2O_2) stress. These cultures were monitored for 14 days and by different dyeing techniques the different cell states (vegetative cells, cysts, degraded cells, empty theca, dead cells) were indentified. Pellicle cyst formation occurred after mechanical stress, but the excystment was fast (75 % after 24 h). Chemical stress caused increasing cyst formation depending on the dose. After 7 and 14 days, the proportion of cysts was elevated in the centrifuging treatment (53 and 58 % compared to 36 and <19 % in the control culture,

respectively). The fraction of degraded cells was higher in the thermal and mechanical stress treatments than in the control culture. Dead cells were not found in the cultures after mechanical stress, but after thermal stress. The stressed cultures also reached a plateau in the growth earlier than the control culture. In general, the authors note the occurrence of pellicle cysts to be connected to the ageing of the cultures and compare it to other *Alexandrium* species.

Judging from the above described experiment, mechanical (and other) stress can affect the physiology of a culture for days afterwards. In the recovery period experiment with stirring, occurrence of cysts because of the experimental conditions, together with the culture having passed the exponential growth stage, could explain the last results. Bioluminescence intensity in the last two samples dropped markedly, although recovery period was sufficient to follow the pattern of bioluminescence during the night. It can be speculated that a large proportion of the cells had experienced sufficient stress – stirring in the measurement – as well as shortened day-lengths, to induce encystment. The recovery period experiment with bubbling lasted for a shorter time and started with lower cell densities, which could explain why the same phenomenon did not occur. Degrading of the cells because of the mechanical stress cannot be excluded either. However, the light microscopy of cultures after different treatments (stirring, bubbling, control) did not indicate this.

In summary, dinoflagellates are a challenging group of algae to keep in culture. Repeated inoculations in exponential growth stage are often required for successful experimental cultures (A. Kremp, personal communication). Some of the problems in the growth of the cultures could have been avoided with a stricter culture maintenance schedule and close monitoring of the culture health. The cause for problematic growth in measurement bottles remained unknown. The same cultures grew normally in other flasks placed in the same conditions.

The length of the chosen measurement period and number of replicates is connected to the culture growth and maintenance. In the measurements we used 250-mL Nalgene bottles, containing 150–200 mL culture. One bottle was

followed for a whole night or 24-hour period, i.e. one night's results are based on one sample. This means there has to be enough volume to not exhaust the culture at any point during the night. Stirring with the magnetic stirrer also required a sufficient size of the bottle and volume of the experiment culture. In contrast, Sweeney and colleagues (for example described in Hastings and Sweeney 1958) used 2-mL aliquots of the stock cultures of dinoflagellates. Prior to their experiment the culture was divided into aliquots which were placed into the experimental conditions. At each measurement point, two of the aliquots were used and then discarded. Bioluminescence in their experiments was induced either by bubbling of the water or acid addition.

If the same culture is to be followed for a whole cycle like we did, the instrument is occupied for that time. This means only one replicate can be measured at a time, and there is at least a day difference between measurements. It limits the length of the experiment and the number of replicates – otherwise there are big temporal gaps between follow-up measurements. The growth stage and condition of cultures is in continuous change. Thus it is challenging to carry out experiments with only one instrument – but also with several, due to possible differences between instruments.

4.2 Regulation of bioluminescence

4.2.1 Bioluminescence after growth in continuous light

Cultures for this experiment were grown in continuous light. After transferring a measurement bottle into the spectroluminometer and hence to dark conditions, a gradual rise in the bioluminescence intensity was observed in two out of three cultures. In the absence of photoinhibition, light emission could occur. Only a phase shift in the rhythm was thus achieved with continuous light conditions, not a stop in the bioluminescence machinery.

Cultures transferred from continuous light into darkness exhibited a similar bioluminescence pattern as cultures where recovery period between stimuli was too short. With sufficient recovery time, there is a plateau in light emission during

the night and a decline towards morning. In the continuous light experiment recovery period was 44 minutes, which was concluded to be adequate. Intensity decreased right after the peak and the sharp shape of the intensity curve was observed. It can be that despite the maintained bioluminescence capacity the storages are insufficient and the cultures are obviously not entrained for the length of the night. It appears that for each time of the entrained night phase there is a certain targeted amount of reaction compounds to be present in the cell – provided that synthesising and degrading of the reaction compounds is the circadian control mechanism. When a sharply shaped intensity curve occurs, there has not been enough energy or storage bioluminescence material to reach the target point after the peak intensity.

Hastings and Sweeney (1958) mention that cells grown in continuous bright light for months or even years exhibit a diurnal rhythm when transferred to darkness. In experiments about photoinhibition caused by bright light (Sweeney 1979), circadian rhythms dampened in four weeks in continuous bright light. For at least two weeks the rhythm in bioluminescence was detectable by acid induction, although not seen by mechanical stimulation. Continuous light thus affects the bioluminescence stimulability directly. In these experiments, much brighter light intensities were used compared to our experiments; 110–360 µmol m^{-2} s^{-1} (exact value depending on the conversion factor; original values 2.4–4.5 mW cm^{-2} or 11 000–20 000 lux) compared to our 40 µmol m^{-2} s^{-1} in the continuous light experiment.

The kinetics in two out of three continuous light experiment bottles is remarkably similar. A peak is reached around the same time and the slope of the curve is the same. The third culture behaved differently. A second measurement of the second bottle also showed weak bioluminescence. Intensity remained low and at least for the third culture no distinct peak could be observed. In the second culture the intensity was somewhat higher in the beginning, albeit low in general.

In earlier chapters, encystment was suspected to be the reason for low intensities. In this experiment the cultures were in good exponential growth, when the low

intensity curves occurred. One of the cultures had not been measured before, hence it had not experienced mechanical stress. However, it stayed in the measurement bottle for two days after sampling from the stock culture and prior to the measurement, which could have disturbed the culture. For these differences in sample handling to cause the low intensity, the encystment would have had to be severe and act much faster than in the recovery period experiment.

One can speculate if experienced mechanical stimulation or other stress can, apart from encystment, lead to lower bioluminescence intensity. Is there a possibility to downregulate or modify the light emission in the case of high external stimulation, such as caused by waves? Is “decisionmaking” in the short term possible, or is the intensity of light emission merely controlled by rhythm, light and growth conditions? Widder and Case (1981) speculate, based on experiments with *P. fusiformis*, that upon exhausting mechanical stimulation not just the bioluminescent material is depleted, but also excitability.

Low bioluminescence intensities without preceding measurements (i.e. mechanical disturbance) were encountered also in the second set of the recovery period experiment using stirring. One of the three cultures did not show typical response curves despite sufficient recovery period. The cultures were not mixed between measurements, unlike during the first set of measurements when samples were returned to the large culturing bottle. The cultures were transferred to the measuring bottle just before the measurement, unlike the continuous light experiment cultures discussed above. Similar to continuous light experiment was that these cultures were combined by mixing two culture flasks to achieve a sufficient homogenous volume prior to the experiment. The mixing and new culturing bottle might have disturbed the physiology of the cultures, which only became apparent after some days in the culture that was measured last.

As mentioned, the cultures in the two above discussed experiments were mixed and then divided into the measurement bottles. Dinoflagellates of this species are good swimmers, but mixing was done carefully, so it is unlikely that the distribution of cells in the three different bottles would be so heterogeneous to be

the cause for the low intensities described above. Also the culture that first showed a typical response, and low intensities in the second measurement, implies that it was not the reason.

4.2.2 Persistence of endogenous rhythm in darkness

For investigating endogenous rhythm of bioluminescence and depletion of bioluminescence potential the cultures were kept in continuous darkness. In 4 out of 6 experiments presented here, no bioluminescence was detectable after the first night. The recovery periods were 40–90 minutes, thus sufficient for experiments with stirring as the stimulation mechanism. One of the six multiday measurements used bubbling, hence recovery period most probably was insufficient. As exhaustion of bioluminescence potential by bubbling seems to take place already during the first night, emission during subsequent nights is not likely. In addition to the results presented here, two other measurements with bubbling showed no bioluminescence in the second night.

In two of the six continuous darkness experiments, a bioluminescence pattern was visible during the second night. In the first case, where the intensity during the first night was high and the culture seemed healthy, the intensity during the second night was markedly weaker. It is noteworthy that the peaks are 24 hours apart. Thus, an endogenous rhythm seems to be present, but energy supplies are in most cases insufficient to produce bioluminescence for more than one night.

In one measurement of the recovery period experiment with stirring, bioluminescence was recorded during the second night. The intensity was very low already during the first night, and the second night reached almost the same intensity. If the low intensity was due to the formation of cysts, the dynamics of encystment and excystment could have facilitated the emission on the second night. During the second night there possibly was a pool of excysted cells that had not used up their reserves. If some other mechanism caused the low intensity during the first night and allowed the population to downregulate bioluminescence and save on resources, light emission during the second night would likewise be possible. In both cases the time between stimulation events (recovery period) was

long (89 and 90 minutes, respectively), which results in less bioluminescence and energy usage compared to higher stimulation frequency (shorter recovery periods). Experiments where a culture is first kept in darkness but not stimulated, and then measured with stimulation in the following night, would give information on the energy usage and availability for bioluminescence.

P. fusiformis has storages to allow partial recovery from exhausting bioluminescence even after being in darkness for 24 hours (Widder and Case 1981). The rhythm in *L. polyedrum* persists in continuous darkness, but the amplitude decreases due to depleting energy resources. In dim light, however, the rhythm was observed to stay unchanged for over two weeks (Hastings and Sweeney 1958; Hastings and Sweeney 1960). According to Biggley et al (1969), 72 hours of extended darkness will kill the cultures, but a dampened rhythm could be observed for *Pyrodinium bahamense*, *L. polyedrum* and *P. lunula*.

From intensity curves in dark measurement conditions it can be seen that bioluminescence intensity begins to rise soon after placement into the dark measurement chamber, even though it would happen in the middle of the subjective day of the cultures. Reversing photoinhibition explains this observation. However, the peak is reached close to the onset of or in the scotophase, following the entrained rhythm. Thus both mechanisms operate in the regulation of bioluminescence. Comparing the dark measurement conditions with other light regimes, discussed in chapter 4.1.3 In situ vs laboratory rhythms of bioluminescence, strengthened the conclusion. At the end of the night, bioluminescence declined and ceased by the time the photophase would start in a normal growth rhythm. As recovery periods were concluded to be sufficient, this is presumed to happen according to the endogenous rhythm and not due to exhaustion of cells.

4.3 Summary

The experiments showed that bioluminescence in *A. ostenfeldii* follows a circadian pattern and can be stimulated with the chosen methods.

Bioluminescence could also be induced after a period lacking entrainment (continuous light or darkness). From experiments in prolonged darkness it became clear that energy supplies in most cases support bioluminescence for only one night. With sufficient energy, the endogenous rhythm times the further bioluminescence activity to the accurate moment. Measurement parameters for rhythm experiments, like appropriate stimulation frequency, were clarified. Experiments with two different instruments and measurements both in the laboratory and in the field allowed comparison between natural and artificial light rhythms.

Clearly, more replicate samples are needed to grasp the variation and have more solid results. Mostly it was possible to run only one set of measurements in each experiment. Additionally, the replicates used here are actually pseudo replicates, because they were inoculated from the same culture. This rules out differences originating from different growth history, but allows testing of only one genotype response. It was limited how many cultures could be tested at a certain time because only one instrument was in use. The steady change in biomass and physiology of the cultures sets a tight time window where the measurements and comparison of different cultures can be done.

The experiments presented in this thesis gave insight to the phenomenon, but also many new questions rose. For example the atypical low intensity curves recorded in part of the experiments are an offset for further experiments. Light regime is not the only mechanism controlling bioluminescence. The interplay between bioluminescence and the growth and condition of the cultures is important. Understanding the molecular basis of the regulation of bioluminescence in this, or at least in closely related, species would also aid understanding of the phenomenon. One aspect for future experiments is further development of stimulation mechanisms that are harmless to the cultures and not inducing changes in their physiology or behaviour.

Recommendations for a method

When experimenting with dinoflagellates, one should take notice of the stability of the culturing and measuring conditions. Also the growth and inoculation history of the experiment cultures has to be planned carefully. Due to possible encystment the re-use of cultures can be limited. With long-term experiments culture media should be regularly renewed.

Measurement bottles used in our experiments were suitable for measuring bioluminescence but not for growing the cultures. Thus it is important to ensure the usability of the flasks. Naturally, the instrument and stimulation mechanism used are determining the borders for example for flask size.

When stimulating bioluminescence by stirring the cultures with the intensities used in this thesis, it is advisable to have at least 20–30 minutes break between stimulations. While bubbling excites stronger bioluminescence the possibility of cell damage has to be considered, and sufficient recovery period determined.

The amount and use of replicates is important. Measurements with samples originating from the same source culture and identical settings varied in bioluminescence pattern.

Light regime in the measurement depends on the aim of the experiment. For investigating the persistence of endogenous rhythm in constant conditions, continuous darkness is needed. In other experiments the cultures have to be illuminated during the measurement to have the light rhythm continuing. Constant dim light conditions can be the solution for prolonged endogenous rhythm experiments (provided the light intensity is not sufficient to entrain the rhythm), as the energy supplies are insufficient in total darkness, and photoinhibition prevents bioluminescence in constant bright light conditions.

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Appendix 1. Experimental procedure in the recovery period experiment with stirring.

	Tu	Th	Fr	Mo	Tu	We	Th	Fr	Tu
date	24.1.	26.1.	27.1.	30.1.	31.1.	1.2.	2.2.	3.2.	7.2.
recovery time (min)	40	20	90	40	60	10	10	90	40
subsample ID	a	b	c	d	e	f	g	h	i

	Th	Fr	Mo	We
date	9.2.	10.2.	13.2.	15.2.
recovery time (min)	10	90	40	40
subsample ID	A	B	C	C

strain AOF-0930 12.1.12
total volume 1500 mL, for each measurement 150 mL sampled (a-i)

AOF-0930 16.1.12 & 27.1.12 mixture
2 culturing bottles (16.1.12 and 27.1.12) were mixed (with some additional culture media) and divided into 3 (A-C)

stimulation 1 min of stirring, bigger stirring cross
chlorophyll sampling from measurement bottle, 2 x 1 mL + 1,65 mL ethanol or 2 x 2 mL + 5 mL ethanol
growth rhythm 14:10

Appendix 2. Details of slope calculation in field and laboratory comparison.

Slope values of bioluminescence rise and time boundaries (start and end time of rise) in the calculations for the different datasets.

Dataset	Mean slope (% hr^{-1})	Standard deviation (pp hr^{-1})	All slopes (% hr^{-1})	Start time of rise	End time of rise
Methodological experiments (dark conditions)	14.2	5.4			
			22.3	15:34	19:40
			12.1	14:41	21:31
			11.2	14:16	23:25
			11.0	14:36	21:15
Rhythm experiments (light rhythm)	33.2	2.2			
			34.8	0:04	3:04
			34.2	0:04	3:04
			33.5	0:04	3:04
			34.0	0:02	3:02
			28.4	0:03	3:48
			33.8	23:43	2:43
			33.7	23:43	2:43
Field measurements (natural light rhythm)	30.6	7.1			
			34.9	20:32	22:22
			33.3	20:42	22:42
			15.1	21:02	0:22
			30.6	20:22	21:52
			34.4	20:12	21:52
			30.8	20:42	21:52
			35.0	20:42	22:32