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The Ecology and Photobiology of Mixotrophic Alveolates in the Baltic Sea

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Declaration:

Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

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Miksotroofsete alveolaatide ökoloogia ja fotobioloogia Läänemeres

KARIN OJAMÄE

TTÜ KIRJASTUS

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II Hansen, P.J., Ojamäe, K., Berge, T., Trampe, E.C.L., Nielsen L.T., Lips, I., Kühl, M. (2016). Photoregulation in a kleptoplastidic dinoflagellate, *Dinophysis acuta*. Frontiers in Microbiology 7:785.
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Author's contribution

I – The author was responsible for planning and carrying out the experiments, analysing data and writing of the manuscript.

II – The author participated in planning the experiment, carried out variable fluorescence measurements, and contributed to the writing of the manuscript.

III – The author was responsible for planning and carrying out the experiment, analysing data and writing the manuscript.

INTRODUCTION

The mixotrophic way of life

Planktonic eukaryotic organisms are often seen as two mutually exclusive guilds: the autotrophic phytoplankton, which use inorganic carbon to build up their biomass and the heterotrophic zooplankton, which are dependent upon organic carbon sources. However, confusion arises when both nutritional strategies co-occur in a single unicellular eukaryote, i.e. in a mixotroph.

Living organisms need energy to sustain their metabolic activity. Energy may originate from the sunlight (phototrophy), from organic carbon sources (organotrophy) or inorganic substances (lithotrophy). Mixotrophy usually refers to the combination of photoautotrophy and organoheterotrophy, and this is what the term "mixotrophy" will be used for in this thesis. The heterotrophic component in mixotrophs may involve uptake of dissolved organic substrates (osmotrophy) and/or ingestion of particulate matter (phagotrophy; Jones 1997). The balance between phototrophy and heterotrophy on the nutritional spectrum depends on the mixotrophic organism, its physiological state and environmental conditions (Jones 1997; Stoecker 1998). As the same single-celled mixotrophic organism may be a net producer or a net consumer, it is difficult to quantify mixotrophs' contribution to primary or secondary production and to the marine biogeochemical cycles.

Mixotrophy among planktonic organisms has been known for a long time, but particularly during the past decades, it has been acknowledged that mixotrophy is by far the most common life form in illuminated water layers (Jones 1997; Stoecker 1998; Stoecker et al. 2009). Knowing this, it is about time to change our default expectation of photosynthetic protists being regarded as phototrophs and not as mixotrophs (Flynn et al. 2012).

Mixotrophy is not a new "invention", but an arduous journey. A single endosymbiotic event, involving a non-photosynthetic eukaryote that engulfed a cyanobacterium, which escaped digestion and was maintained as a photosynthetic endosymbiont, is thought to have given rise to the three basal groups of algae: the chlorophyta, the glaucocystophyta, and the rhodophyta (Keeling 2010). The endosymbiont was turned into a chloroplast controlled by the host (Archibald & Keeling 2002; Keeling 2010). Subsequently, the plastids were passed on to other eukaryotes via secondary and tertiary endosymbiosis events (Archibald & Keeling 2002; Keeling 2010; McFadden 2001). Thus, the ability to ingest particulate food was crucial in the evolution of photosynthetic eukaryotes (Raven 1997). Hence, phagotrophy in the phototrophic eukaryotes is a fundamental character and its absence is a derived character (Raven 1997). Mixotrophs are found in all aquatic environments (Stoecker 1998) and discovered in many planktonic organisms (flagellates, ciliates, radiolarians) as well as in sponges, corals, rotifers, and even in higher plants (Burkholder et al. 2008; Tittel et al. 2003).

Motivation and objective

Most animals obtain nutrients by scavenging through their refrigerator or by consuming live prey. Plants, in general, manufacture their own food through the process of photosynthesis using carbon dioxide, water, nutrients and sun's energy. Mixotrophic organisms, whether terrestrial or aquatic inhabitants, can do both: their flexible way of life allows them to photosynthesize and consume prey. The way organisms gather food, has an impact on the food web dynamics and elemental fluxes in the Earth's biosphere. In general, we have gained a fairly adequate understanding of how farming crops and livestock or hunting wildlife for dinner will affect the ecosystems that the human populations exploit and inhabit. Also, our knowledge of the biogeochemical cycles and transfer of biomass within the oceans, terrestrial ecosystems, and the atmosphere is sufficiently extensive. What remains unclear is the contribution of mixotrophic protists to the net community production and their impact on the global carbon cycle.

The aim of this PhD study was to investigate predator-prey interactions and the regulation of photophysiological and nutritional processes in mixotrophs. This was done to improve our understanding of the potential leadership of mixotrophy in the marine food web. Mixotrophic species, selected for the studies **I** and **II** have lately received acute attention from several research groups around the world. The discovery of the trophic link between kleptochloroplastidic protists, the ciliate *Mesodinium rubrum* Lohmann, 1908 and the dinoflagellate *Dinophysis acuminata* Claparède & Lachmann 1859 (Park et al. 2006) opened up opportunities to study the incorporation of fresh kleptochloroplasts (Kim et al. 2012; Raho et al. 2014), toxin production (Kamiyama et al. 2010; Nielsen et al. 2012; Riobó et al. 2013), and the role of food uptake for photosynthesis and growth (Kim et al. 2008; Riisgaard & Hansen 2009) in *Dinophysis* spp. The interest towards *Heterocapsa triquetra* (which shaped into study **III**) was fuelled by the investigations carried out in the Gulf of Finland that showed the domination of *H. triquetra* in the layers of deep chlorophyll maxima during seasonal water column stratification in summer (Hällfors et al. 2011; Kononen et al. 2003; Lips & Lips 2014).

In the framework of this thesis laboratory experiments were conducted to test three main hypotheses:

1. The proportion of mucus-entrapped ciliates, *Mesodiniun rubrum* is influenced by the cell concentration of *Dinophysis* spp. and by the ratio of the two species (**I**).
2. The regulation of kleptochloroplasts in *Dinophysis* spp. works through behavioural regulation: the number of chloroplasts may be increased, and the chloroplasts may fuse, which leads to an increase in plastid size and cellular chlorophyll *a* content when grown under low irradiance (**II**).
3. The ecophysiology of *Heterocapsa triquetra* encompasses nutrient uptake in cold and dark environmental conditions and the following

improvement of cell growth and photosynthetic capacity in the light field, potentially supported by internal nutrient storages of the cells (**III**).

The specific objectives were:

- to describe the feeding behaviour of *Dinophysis* spp. (**I**);
- to quantify the interactions between *Dinophysis* and *Mesodinium* (**I**);
- to reveal to what extent *Dinophysis acuta* regulates it's chloroplasts (plastid size and pigment synthesis) in the absence of prey when exposed to high and low light intensities (**II**);
- to demonstrate that *Heterocapsa triquetra* takes up inorganic nitrogen and phosphorus in a dark and cold environment (**III**).

ABBREVIATIONS

Chl *a* – chlorophyll *a*
DCM – deep chlorophyll maxima
DIC – dissolved inorganic carbon
DSP – Diarrhoetic Shellfish Poisoning
DTX – Dinophysis toxins
DVM – diel vertical migration
E – irradiance
E_k – light saturation parameter
F_v/F_m – Maximum photochemical quantum yield of PSII
GLM - generalised linear models
I_D – light field: dark
I_{LD} – light field: light-dark
N – nitrogen
OA – Okadaic acid
O_2 - oxygen
P – phosphorus
PAM – Pulse Amplitude Modulation
PSI – Photosystem I
PSII – Photosystem II
PTX – Pectenotoxins
qP – photochemical fluorescence quenching
rETR – relative electron transport rate
rETRmax – maximum relative electron transport rate
RuBisCO - Ribulose-1,5-bisphosphate carboxylase/oxygenase
TPG – Teleaulax-Plagioselmis-Geminigera
φ_{II} – effective photochemical quantum yield of PSII
φ_{NO} – effective photochemical quantum yield of PSII
φ_{NO} – quantum yield of non-regulated heat dissipation and fluorescence emission
φ_{NPQ} – quantum yield of light-induced non-photochemical fluorescence quenching
^{14}C – radiocarbon

1. THE ECOLOGY AND PHOTOBIOLOGY OF MIXOTROPHS

1.1 Mixotrophic protists in the Baltic Sea

Mixotrophs tend to dominate over specialist phototrophs and heterotrophs only under limitation by various resources (light, nutrients, and prey; Crane 2010; Tittel et al. 2003). In the Baltic Sea, the proportion of mixotrophs in the plankton community increases with the decrease of inorganic nutrients in the upper layer in late April early May. The first high abundant/high biomass forming mixotroph, which appears in the plankton community after the decline of the spring phytoplankton bloom (formed by diatoms and dinoflagellates) is the photosynthetic ciliate *Mesodinium rubrum*.

The marine ciliate *M. rubrum* is an important microscopic primary producer in the stratified Baltic Sea (Sjöqvist et al. 2011). This species performs diurnal vertical migration (DVM) to exploit available resources in different depths (Crawford & Lindholm 1997; Lindholm & Mörk 1990). It is well known for its trophic link with *Teleaulax* and *Dinophysis* species (Gustafson et al. 2000), phototrophic capacity, and for its role in forming productive red tides in coastal and upwelling zones worldwide (Crawford 1989; Johnson et al. 2013; Kang et al. 2013; ref.-s in Moeller et al. 2011; Wilkerson & Grunseich 1990). In the Baltic Sea, the highest abundances/biomasses and the largest size distribution of *M. rubrum* are observed after the spring bloom, usually in May-June (Lindholm 1985; Passow 1991; Rychert 2004; Thamm et al. 2004). More or less uniform size distribution and significant contribution to the phytoplankton communities below 10 m from the surface has been measured in late summer (Lips & Lips 2014). The bloom cell concentrations may reach to the order 10^5–10^6 cells L^{-1} (Crawford & Lindholm 1997; Lips & Lips 2014; Sjöqvist & Lindholm 2011).

A diverse and an abundant group of eukaryotic organisms in the Baltic Sea are the dinoflagellates. The dinoflagellate blooms are annually occurring phenomena in spring and summer. The representatives belonging to the genus *Dinophysis* are particularly present during summer months and may occasionally occur in abundances that cause Diarrhoetic Shellfish Poisoning (DSP; Hällfors et al. 2011). The most widely distributed *Dinophysis* species in the brackish Baltic Sea is *Dinopysis acuminata* (Edler et al. 1996). *Dinophysis* spp. have found to form population maxima either in the mixed surface waters or in sub-surface thin layers near the thermocline (Carpenter et al. 1995; Moita et al. 2006; Pitcher et al. 2011; Setälä et al. 2005). Regradless of these findings, *Dinophysis* spp. are not known to perform vertical migrations, hence the biomass peaks found at the sub-surface layers may be related to unusually deeper mixed surface layer (e.g. Hällfors et al. 2011) or an avoidance of warm surface layers (Carpenter et al. 1995).

The potentially mixotrophic dinoflagellate *Heterocapsa triquetra* Ehrenberg (Stein) have been found to form high biomasses in very different conditions in the Baltic Sea. In some years (e.g. 1996, 2003, 2004 and 2008) the biomass of

H. triquetra reached up to 70–90% of the total phytoplankton biomass in the Gulf of Finland (Jaanus et al. 2011; Jaanus & Pellikka 2003). The surface bloom in 1996 was connected to a heavy rainfall and the renewal process of the Baltic deep waters (Kononen et al. 1999). The high biomass in 1998 was stimulated by a coastal upwelling event (Kanoshina et al. 2003). The elevated biomass values of *H. triquetra* have often been registered in July during the period of strongest stratification and inorganic nutrient deficiency in the surface layer in the Baltic Sea. Over the same period, layers of sub-surface and deep chlorophyll *a* maxima (DCM) are reported in different areas of the Baltic Sea (Hällfors et al. 2011; Kononen et al. 2003; Lips & Lips 2014; Lips et al. 2011; Lips et al. 2010; Pavelson et al. 1999). These phytoplankton biomass maxima have mostly been detected at the depth of the nitracline (15–35 m; Lips et al. 2010) and were primarily dominated by the dinoflagellate *H. triquetra* (Lips et al. 2011; Lips & Lips 2014). Very few studies are available on the phagotrophic properties of this dinoflagellate. In the laboratory conditions, the species has been shown to reject bacteria as food but ingest small phytoplankton cells, e.g. *Thalassiosira pseudonana*, under nutrient-depleted conditions (Legrand et al. 1998).

Other very abundant mixotrophs in the Baltic Sea are haptophytes, such as *Prymnesium parvum*, *P. polylepis* and *Chrysochromulina* Lackey, 1939 spp. which occur regularly and in significant abundance in the plankton mostly in late spring and summer (Hajdu et al. 1996; Hällfors 2004). In the stratified Baltic Sea, *Chrysochromulina* spp. abundances may reach to the order of 10^6 cells L^{-1} (Hajdu 1997; Hajdu et al. 2007; Hajdu et al. 1996; Kononen et al. 1998). The increase in *Chrysochromulina* spp. abundance may coincide with the period of phosphorus limitation in the surface layer and blooms of diazotrophic cyanobacteria. In these conditions, phagotrophy and toxin production of *Chrysochromulina* spp. may be induced (e.g. Hajdu et al. 2007; Johansson & Graneli 1999; Legrand et al. 1996). *Chrysochromulina* spp. have been shown to ingest detritus, bacteria and nanoplankton (ref.-s in Hajdu et al. 2007).

1.2 Acquired phototrophy in *Mesodinium* and *Dinophysis*

Acquired phototrophy i.e. the capability of hosting an algal endosymbiont (Rumpho et al. 2011) or harbouring algal organelles from ingested prey to employ them for the light utilization process of photosynthesis, can be found in many species of marine ciliates and dinoflagellates (ref.-s in Hansen et al. 2013). *Mesodinium rubrum* and *Dinophysis* spp. are well-known examples of "plastid farmers". Both genera sequester chloroplasts of cryptophyte origin. It has only recently been established that the success of *M. rubrum* relies on the feeding of cryptophyte prey (Gustafson et al. 2000) and the photosynthetic *Dinophysis* spp. are dependent on *M. rubrum* as prey (Park et al. 2006). The chloroplast gene sequences of the cryptophytes belonging to the *Teleaulax, Plagioselmis* or *Geminigera* (TPG) clade are identical to the sequences of those that *M. rubrum* and *Dinophysis* spp. have obtained into their cells. This suggests

continuous chloroplast sequestration from the prey (Hackett et al. 2003; Janson 2004; Kim et al. 2012; Minnhagen & Janson 2006; Minnhagen et al. 2011; Nagai et al. 2008).

M. rubrum digests the periplast and the membranes of the cryptophyte prey and maintains not only the chloroplasts of its prey, but also a number of other prey cell organelles and one or more cryptophyte nuclei (Kim et al. 2016). Cryptophyte plastid-bearing *Mesodinium* spp. can keep the ingested prey organelles functionally active for several months providing enough photosynthetically fixed carbon for their survival in periods of low prey availability (Hansen & Fenchel 2006; Johnson et al. 2007; Johnson & Stoecker 2005; Johnson et al. 2006; Smith & Hansen 2007).

The maximum growth rate of *M. rubrum* is achieved by the ingestion of single cryptophyte cell per day, which accounts for 1-2% of its daily carbon needs (Smith & Hansen 2007). Acquired photosynthesis may last for several months, allowing a population to grow over 100-fold in absence of prey (Kim et al. 2016; Myung et al. 2013; Yih et al. 2004). Kim et al. (2016) have shown that *M. rubrum* may acquire stable photosynthesis over 13 weeks by keeping the chloroplast division rate in pace with the cell division of the ciliate. This is achieved by the continuous transcriptional activity of the cryptophyte nuclei inside the ciliate. In other kleptoplastidic ciliates, only prey chloroplasts are sequestered and typically remain functional in the predator cell for hours up to a few days (Stoecker et al. 2009). However, if the sequestered chloroplasts cannot be replicated with sufficient speed, the host will soon have an insufficient amount of chloroplasts and the growth of the ciliate will eventually cease (Johnson et al. 2007). A limiting growth factor for *M. rubrum* is the dilution of sequestered chloroplasts resulting from host cell division (Hansen et al. 2012). *M. rubrum* may divide 5-6 times without additional food (Myung et al. 2013) and has been shown to control the replication of its new chloroplasts and to be able to synthesise and replicate new chloroplasts 3-5 times (Hansen & Fenchel 2006). Some of the research have documented that this ability is lost already after a few cell divisions when starved of prey (Johnson et al. 2007; Moeller et al. 2011). *M. rubrum* displays photoacclimation, i.e. the regulation of the amount of pigmentation in response to light intensity (Hansen et al. 2013).

Dinophysis spp. employ "third-hand" chloroplasts supplied from the ciliate prey, but the rest of the ingested material, including the cryptophyte nuclei and nucleomorphs, are digested (ref.-s in Hansen et al. 2013). The newly ingested chloroplasts go through significant structural changes during the first 24 hours and are transferred to the pre-existing clusters of chloroplasts (Kim et al. 2012). The regulatory functions over the chloroplasts are taken over by the host. Five proteins complete with plastid-targeting peptides have been detected in the nuclear genome of *D. acuminata* that function in photosystem stabilization and metabolite transport (Wisecaver & Hackett 2010). Only one out of five, the photosystem II subunit M (psbM) that is involved in photosystem dimer formation (Ferreira et al. 2004), is of cryptophyte origin. The rest have been derived over evolutionary time from ancestral peridinin-containing

dinoflagellates and from dinoflagellates that have replaced the peridinin plastid with one derived from haptophytes containing the photosynthetic pigment fucoxanthin. Since *M. rubrum* can feed on a variety of cryptophytes (Myung et al. 2011; Nishitani et al. 2010; Park et al. 2007), other types of plastids from the members of *Teleaulax*/*Geminigera* genus complex in addition to *T. amphioxeia* type plastids are temporarily retained at the same time in a single *Dinophysis* cell (Kim et al. 2012).

Unlike *M. rubrum*, *D. acuminata* appears to be unable to divide the plastids, thus it is dependent upon a continuous supply of chloroplasts (Hansen et al. 2013). However, *Dinophysis* cells grown at low irradiances contain more and larger chloroplasts (Nielsen et al. 2013). All *Dinophysis* species require light to grow, even when given an ample food supply (ref.-s in Hansen et al. 2013). First results on changes in pigmentation in response to different light fields in *Dinophysis* have been published as part of this thesis (section 3.2, **II**).

1.3 Thin layers of phytoplankton as trophic hotspots

The spatial distribution of phytoplankton in the sea is highly heterogeneous. At large scale, patchiness in the Baltic Sea is primarily driven by locally enhanced growth rates, favored by the formation and development of different hydrophysical processes like eddies and jet currents (Kononen et al. 1996; Lips et al. 2010), river inflow, upwelling events (Talpsepp et al. 1994) and seasonal vertical stratification (Kononen & Niemi 1986). Vertical stratification plays a key role in determining the chemical and hydrodynamic gradients in the water column and hence, shaping the spatial distribution and temporal dynamics of planktonic organisms.

Photosynthetic microorganisms may occur in high biomasses within a small depth interval. These congregations are known as thin layers of phytoplankton (Durham & Stocker 2012). Thin layers are typically characterised by the thickness of several centimetres to a few meters and often extend horizontally for many kilometres (Dekshenieks et al. 2001; Durham & Stocker 2012; Moline et al. 2010). Thin layers and deep chlorophyll (Chl) *a* maxima are essentially different phenomena. To qualify as a thin layer, the Chl *a* fluorescence level must exceed the background fluorescence value by at least three times (Dekshenieks et al., 2001).

Dinophysis spp. background cell concentrations are usually found to be below 1000 cells L^{-1} (Hällfors et al. 2011) but in bloom conditions the abundances may reach up to 10^4–10^5 cells L^{-1} (Sjöqvist & Lindholm 2011). *Dinophysis* species are frequently found in thin sub-surface layers (e.g. Carpenter et al. 1995; Moita et al. 2006; Pitcher et al. 2011; Setälä et al. 2005). In the Baltic Sea, they are often present at the thermocline at 15-25 m depth (Carpenter et al. 1995; Gisselson et al. 2002; Hällfors et al. 2011). *M. rubrum* cell concentrations in bloom conditions are of the order 10^5-10^6 cells l^{-1} (Crawford & Lindholm 1997; Lips & Lips 2014; Sjöqvist & Lindholm 2011). As *M. rubrum* perform DVM, deep biomass maxima of these ciliates have been recorded (Lips & Lips 2014; Sjöqvist & Lindholm 2011) and in some cases,

they may form two vertical depth maxima (Sjöqvist & Lindholm 2011). Thin layers of the prey cells have the potential to induce predator accumulation and thus substantially increase trophic transfer rates compared with more homogeneous phytoplankton distributions (Cowles et al. 1998; Durham & Stocker 2012; Tiselius et al. 1993). Thus, if the predator and prey biomass maxima co-occur in the same vertical and horizontal space, the populations of *M. rubrum* could be easily controlled by *Dinophysis* (Lips & Lips 2014; Mouritsen & Richardson 2003; Sjöqvist & Lindholm 2011; Velo-Suarez et al. 2014).

Zooplankters tend to avoid phytoplankton layers that are formed by toxic and mucus-rich species but thin phytoplankton layers also correlate with grazer abundances (ref.-s in Durham & Stocker 2012). Herbivorous zooplankters, but also *Dinophysis* spp. likely use chemical and/or physical cues to locate thin layers of prey cells (Hansen et al. 2013; Menden-Deuer & Grünbaum 2006; Woodson et al. 2007). Grazers aggregate at pycnoclines/thermoclines, which are retention areas for thin layer populations. Grazer aggregations might be, in turn, exposed to higher mortality rates due to predation by higher-level consumers (e.g., larger zooplankton or fish; Durham & Stocker 2012).

2. MATERIAL AND METHODS

2.1 Experiments

For the first study (**I**) single cells of *Dinophysis* spp. were isolated onto a 24-well tissue culture plate using a drawn-out Pasteur micropipette and were thereafter allowed to acclimate 2-4 hours before the start of the experiments. All cultures were maintained on a glass table in autoclaved sterile-filtered f/2 medium (Guillard 1975) with a salinity of 35 g kg^{-1} at a temperature of 15 °C. Illumination was provided from beneath by cool white fluorescent lights of 100 μmol photons m^{-2} s^{-1} (PAR, 400–700 nm) on a 14:10 hour light:dark cycle.

Three experiments were conducted. The aim of the first experiment was to obtain a description of the feeding behaviour of *Dinophysis* spp. For this, starved cultures of *Dinophysis* spp. were pipetted onto 24-well tissue culture plates and were mixed with *M. rubrum* (final concentrations were: 29, 88 and 206 cells ml^{-1} and 290, 880 and 2060 cells ml^{-1} for *Dinophysis* spp. and *M. rubrum,* respectively). The experiment was carried out over 8 days. Observations were documented as videos and pictures.

The aim of the second and third experiment in the first study (**I**) was to determine the effect of concentration and exposure time of *D. acuminata* on the motility of *M. rubrum* and the effect of *D. acuminata* bloom on the small populations of ciliates. For this, cells of *D. acuminata* were isolated into 2 ml of f/2 medium in a 24-well tissue culture plate. The cultures were mixed with *M. rubrum* (final concentration of 588 cells ml^{-1} in experiment 2 and an array of concentrations in experiment 3). The low *M. rubrum* concentration levels (0.3–11 cell ml^{-1}) were chosen to investigate if low amounts of prey can be detected or captured by the predators when mixed with a bloom concentration of *Dinophysis*. The final concentrations of *D. acuminata* and predator–prey ratios in experiment 2 and 3 are shown in study **III**. The number of single *M. rubrum* cells that displayed abnormal swimming behaviour, immobilised cells, aggregates and cells in each aggregate were counted at specific time steps during the experiements (Fig. 2,3). Single cells with altered motility were counted as an aggregate of the size of one cell.

For the second study (**II**) the cultures of *M. rubrum* were fed *T. amphioxeia* as described by Nielsen et al. (2013). All three species (*T. amphioxeia*, *M. rubrum*, *D. acuta*) were grown in f/2 medium (Guillard 1975) based on autoclaved seawater with a salinity of 32 g kg^{-1}, a dissolved inorganic carbon (DIC) concentration of 2.3 ± 0.1 mmol l^{-1} and a pH of 8.0 ± 0.05. All organisms were grown at 15.0 °C. For each sample collection, *Dinophysis* cells were picked individually from a sub-sample using a drawn-out Pasteur micropipette and transferred to clean growth medium twice to remove all *M. rubrum* cells before measurements. Fresh growth medium was added at each sampling to replace the water volume removed.

Two experiments were conducted for this study (**II**). The first experiment was designed to study photoregulation in a recently prey starved culture of *D. acuta*. A culture of *D. acuta* was maintained in 750 ml tissue culture flasks filled with 500 ml culture medium under a photon irradiance of 100 µmol photons m^{-2} s^{-1} with prey for at least 2 weeks before the experiment and was eventually allowed to deplete its ciliate prey. At the initiation of the experiment, subsamples of the culture were split in two and poured into 270 ml tissue culture flasks to capacity at a *D. acuta* cell density of 200 cells ml^{-1} (in triplicate). One set of flasks were maintained at 100 µmol photons m^{-2} s^{-1} (I_{100}), while the other set of flasks was shifted to a photon irradiance of 15 µmol photons m^{-2} s^{-1} (I_{15}). Subsamples were withdrawn for measurements of cell concentration, ^{14}C fixation, algal pigment concentration, photosynthetic capacity using variable chlorophyll fluorimetry, and O_2 optode-based respiration measurements.

The second experiment was carried out to measure Chl *a* and physiological rates in well-fed cultures of *D. acuta* at an irradiance of 100 µmol photons m^{-1} s^{-1}. Cultures were maintained in 750 ml tissue culture flasks filled with 500 ml culture medium before the experiment. At the initiation of the experiment, 270 ml tissue culture flasks were filled to capacity with an initial concentration of 400 *D. acuta* cells ml^{-1} and 1000 *M. rubrum* cells ml^{-1} (in triplicate). Samples were withdrawn for measurements of cell concentration, ^{14}C fixation, photophysiological information using variable fluorescence, and O_2 optode-based respiration measurements.

In the third study (**III**), 10 days prior the experiment, a small amount of culture (1-2 ml) was inoculated into two 250 ml Erlenmeyer flasks prefilled with 120 ml filtered and autoclaved seawater. Nutrients were added directly into the cultures according to Spilling et al. (2011), with a final concentration of nitrate 24 µmol l^{-1} and phosphate 6 µmol l^{-1}. Phosphate was added in excess (N:P = 4) to stimulate the natural environment of N-limitation in the Gulf of Finland.

The aim of the experiment was to measure inorganic N and P uptake in cold, dark conditions and in the optimal growth conditions to study the usage of internal nutrient pool for cell growth and photoregulation. At the initiation of the experiment, subsamples of the cultures were poured into 500 ml Erlenmeyer flasks (in triplicate) to full capacity (500 ml). On day 6 of the experiment, *H. triquetra* cells had reached the stationary growth phase. Thereafter, the cultures were split and poured into six 500 ml Erlenmeyer flasks up to 230 ± 2.5 ml. One set of flasks was maintained at light and temperature conditions described above until the termination of the experiment and additional nutrients were never added. The other set of flasks was wrapped in foil and after that two-times diluted T2 medium was added. Nutrient concentration increased to a final concentration of nitrate 4.53 µM and phosphate 0.91 µM (N:P = 5) in the cultures. The flasks were incubated in the dark at 4 °C for 48 hours (I_D) and brought back to the previous culturing conditions (I_{LD}) described above after

that. Samples for measurements of cell concentration and photosynthetic capacity were withdrawn in every 1-2 days over 2 weeks.

2.2 Establishment of phytoplankton cultures

The cultures of the cryptophyte *Teleaulax amphioxeia* (K-1837; SCCAP) and the ciliate *Mesodinium rubrum* (MBL-DK2009) were established from single cells isolated from water samples collected from Helsingør Harbour in 2009 (Nielsen et al. 2013). The culture of *Dinophysis acuta* (DANA-2010) was established in June 2010 from the North Sea (Nielsen et al. 2013) and *Dinophysis acuminata* (strain FR101009) was isolated from Little Belt, Denmark in October 2009 (Nielsen et al. 2012). Cells were transferred to and grown in 24-well tissue culture plates. Cultures of *M. rubrum* were fed with *T. amphioxeia* at a predator-prey ratio of 1:5 once in every two weeks and cultures of *Dinophysis* spp. were fed with *M. rubrum* at a predator-prey ratio of 1:10 to enable mixotrophic growth (**I**, **II**). All three species were grown in f/2 medium (Guillard 1975) based on autoclaved seawater with a salinity of 32-35 g kg^{-1} (salinities differed slightly between studies), a dissolved inorganic carbon (DIC) concentration of 2.3 ± 0.1 mmol l^{-1} and a pH of 8.0 ± 0.05. Temperature and light conditions were set to 15 °C with a 14:10-hour ligh-dark cycle (100 µmol m^{-2} s^{-1}).

For the third study (**III**) a non-axenic monoculture of *Heterocapsa triquetra* was established from single cells isolated from water samples collected from the Gulf of Finland in 2012. The culture was grown in T2 medium (Spilling et al. 2011), with a salinity of 6 g kg^{-1}, at 15 °C and under a light irradiance of 200 µmol photons m^{-2} s^{-1} with a 16:8-hour light-dark cycle.

2.3 Variable fluorescence

The variable fluorescence measurements in studies **II** and **III** were conducted with the MULTI-COLOR-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) using the saturation pulse method (Schreiber et al. 1986). All fluorescence parameters were calculated after subtraction of the blank fluorescence, measured in filtered and autoclaved seawater. Before fluorescence measurements, cells were incubated at *in situ* temperature in the quartz cuvettes for ~20 minutes in the dark to allow full oxidation of the primary electron acceptor, quinone A (Q_A).

To obtain the maximum quantum yield of PSII (F_v/F_m), samples were pre-illuminated with the weak pulsating light of 1 µmol photons m^{-2} s^{-1} at 725 nm for 5 seconds (study **II**) or at 440 nm for 30 seconds (study **III**) prior to measurement of F_0. Pre-illumination excites Photosystem I (PSI) and thus oxidises the plastoquinone and Q_A pools associated with PSII. Subsequently, the cultures were exposed to a saturating blue light pulse of 5710 µmol photons m^{-2} s^{-1} at 440 nm for 300 ms to allow the Q_A pool to be reduced and to measure F_m. Fluorescence parameters, F_0 and F_m were measured three times for each

experiment flask with 30-second intervals to allow re-oxidation of the Q_A pool between saturation pulses.

The effective photochemical quantum yield of PSII, ϕ_{II}, was obtained from light adapted samples where the steady-state chlorophyll fluorescence yield (F) was measured after exposing samples to actinic light of 115 and 18 μmol m^{-2} s^{-1} for high and low light cultures respectively (study **II**) or 200 μmol m^{-2} s^{-1} (study **III**) at 440 nm for 30 seconds. During this illumination time, the fluorescence yield stabilised at a constant level. Subsequently, a saturating light pulse was applied, and F_m' was determined. Similarly to the parameters for dark-adapted samples, F and F_m' were measured three times for each experiment flask and thereafter, parameters for the evaluation of the photosynthetic apparatus were calculated.

For relative electron transport rate (rETR) measurements (**II**), samples were sequentially exposed to 14 irradiances (E) from 0 to 512 μmol m^{-2} s^{-1} for 30 seconds at each step. The obtained light response curves were fitted using the model of Eilers & Peeters (1988) in order to derive fitting parameters for the initial slope (α), inhibition term (β), light saturation parameter (Ek) and for the maximum relative electron transport rate (rETRmax). All parameters were measured after subtraction of the blank fluorescence, measured on sterile-filtered f/2 medium. Cells were stirred in the cuvette during all measurements.

2.4 Statistical analyses

Generalised linear models (GLMs) with a negative binomial distribution and a logit link were used (**I**) to test the associations between proportions of immobilised *M. rubrum* cells in relation to the treatments. A two-column response vector was created. This contained counts of successes (number of immobile ciliates) and failures (number of immobile ciliates subtracted from the total number of ciliates). A likelihood-ratio test was performed to check for a significant treatment effect and a Tukey post hoc test from the multkomp package in R (R Development Core Team 2008) was applied (**I**) to inspect the differences among treatments. One-way ANOVA was used to test Chl *a*-specific carbon uptake and respiration rates as a function of prey starvation after one day of starvation (**II**). Two-way ANOVA was used to test for differences in these parameters at the two irradiances (**II**). Paired Student's t-tests were used to compare the nutrient concentrations prior and after the treatment effect and independent t-test were used to compare photosynthetic parameters between the treatments (**III**).

3. RESULTS AND DISCUSSION

3.1 Interactions between *Dinophysis* and *Mesodinium*

The predator-prey encounters in cultures of *Dinophysis* spp. and *Mesodinium rubrum* visually appear quite melodramatic. *Dinophysis acuta* and *Dinophysis acuminata*, as specialized feeders on *M. rubrum*, display a strategy for prey capture (**I**), which has been overlooked in the previous studies. Moreover, the interactions taking place, when the predator and prey encounter each other, lead to a devastating effect on the population of *M. rubrum* as the mass mortality of the ciliates becomes inevitable.

The central role in regulating the ciliate population size is played by the sticky elongated mucus threads, to which ciliates jump into and get easily stuck with shortly (10-20 minutes) after prey is added to *Dinophysis* spp. cultures. As a result, the normal swimming pattern of *M. rubrum* becomes immediately disturbed. The translucent mucus threads being expelled or being trailed behind any cells were not observed in the present study, but they were surrounding *Dinophysis* cells in the culture. A large mucus matrix developed approximately one hour after *Dinophysis* cells were mixed with the ciliates. Ciliate cells trapped in the mucus performed frequent attempts of escape jumps and swam or rotated helically in the same position during the first 10-20 minutes after contact with the mucus secretions. Accidental encounters between free *M. rubrum* cells and trapped cells resulted in the formation and enlargement of aggregates of *M. rubrum* (Fig. 1A). As the ciliates were mostly unable to break free from the mucus connection, cells became completely immobile and eventually lysed. Even if some cells were occasionally able to break free, cell lysis seemed to be inevitable after contact with the mucus. A seemingly allelopathic response of *M. rubrum* occured within 30-60 minutes after the entrapment: cells (either in aggregates or single cells trapped in mucus) lost all their cirri and the cell shape became rounded, after which the cells lysed.

Dinophysis was witnessed to attack freshly trapped *M. rubrum* cells only. *Dinophysis* seem to have an advantage over their prey as they can detect *Mesodinium* cells from a greater distance than is needed by the ciliates to escape from *Dinophysis* cells. This is thought to be possible via hydromechanical and/or chemical sensing (Hansen et al. 2013). Before the attack, *Dinophysis* cells commonly displayed a changed swimming behaviour: cells circulated around a ciliate or an entire aggregate of prey cells and frequently stopped near immobile but alive cells, often with an extended peduncle. The change in the swimming pattern indicated that *Dinophysis* cells have detected *Mesodinium*. The extended peduncle was used to attack a trapped ciliate. After the contact with the ciliate, the peduncle contracted, and the captured ciliate was drawn closer to the *Dinophysis* cell.

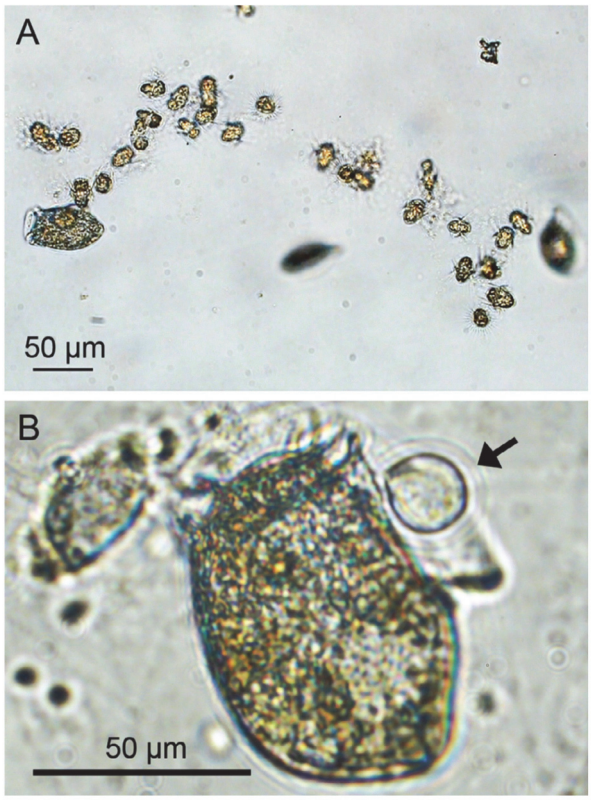

Figure 1. A stretched out aggregate of Mesodinium rubrum *in the presence of* Dinophysis acuta *(A). The feeding position of* D. acuta *with the ciliate that has been sucked out of chloroplasts. The arrow indicates the remains of the prey (B).*

It has been suggested earlier that *Dinophysis* uses a capture filament to initially immobilise the *Mesodinium* cells (Hansen et al. 2013), but this was not confirmed in the present study. Instead, the ciliates were immobilised in the mucus and the first contact between the *Dinophysis* cells and the ciliates was made by the peduncle. Since the formation of mucus in *Dinophysis* cultures was a new finding, the observer in Hansen et al. (2013) may have misinterpreted the observations, which were made at low magnification.

A few minutes after the start of the ingestion, the cirri of the ciliates were shed, and the cell shape became swollen. Ingestion time was about 15 minutes until the contents of the prey cell disappeared. During this stage, the prey cell was attached to the predator cell by its peduncle. After that, the remains of the prey cell were carried along for some time by *Dinophysis* (Fig. 1B) but were eventually left behind. Normal swimming behaviour of *Dinophysis* resumed after a few minutes. The actual time depended on how fast *Dinophysis* could release the ciliate from the mucus connection.

The proportion of mucus-entrapped prey cells was influenced by the concentration of *Dinophysis* and *Mesodinium* and by the cell concentration ratio

of the two species. Cell immobilisation did not occur at very high predator-prey ratios (300 to 9; fixed *D. acuminata* concentrations of 100 cells ml^{-1}), and less than 0.2% of the ciliate population was immobilised at very low ratios (0.04 to 0.0005; *D. acuminata* concentrations of 0.3–20.6 cells ml^{-1}). The number of aggregates was highest at predator-prey ratios of 0.3 to 0.04 (Figure 2A, see also Figure 3C in study **I**) and the aggregates were largest at ratios 0.1 to 0.7, reaching 4 to 7 cells per aggregate (Figure 2B, 3A). At high cell concentrations of prey and predator (predator-prey ratio of 0.7), a maximum of 17% of *Mesodinium* cells became immobile and went through cell lysis (Figure 3B).

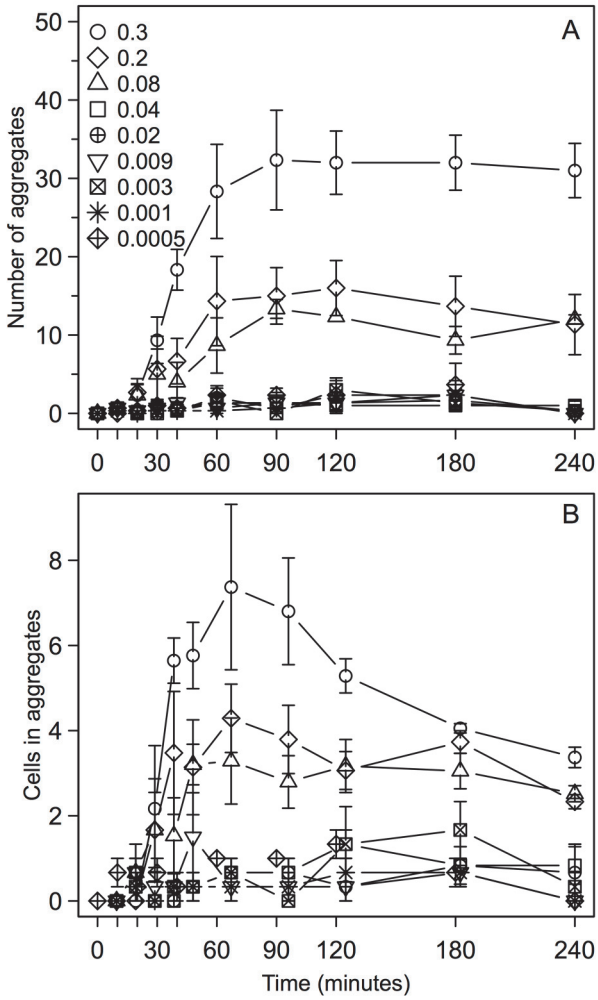

*Figure 2. Results from the experiment 2 (Paper **I**) with different concentrations of* Dinophysis *and a fixed concentration of* Mesodinium*. The number of aggregates of* M. rubrum *cells (A) and the number of* M. rubrum *cells per aggregate at each time step (B). Figure legend (A and B share a common legend shown in A) denotes predator–prey ratios in the 3.4 mL cultures. Error bars represent standard error of the mean (n = 3).*

All prey cells trapped in the mucus eventually lysed. Large aggregates of *M. rubrum* turned into a field of chloroplasts scattered over the bottom of the culture vessel. As far as followed by the author, *Dinophysis* only fed on live and immobile or semi-immobile *M. rubrum*. Some findings from the present study have shown cells circulating the remains of the ciliates scattered over the bottom of the culture vessel. Still, no feeding on the dead material has been apparent. Chemical sensing may explain the rotation of *D. acuminata* around the cell remains of the ciliates. Previous studies have shown the swimming response of *D. acuminata* towards copepod faecal pellets and interpreted the finding as the reaction to the leakage of dissolved organic matter of algal origin (Poulsen et al. 2011).

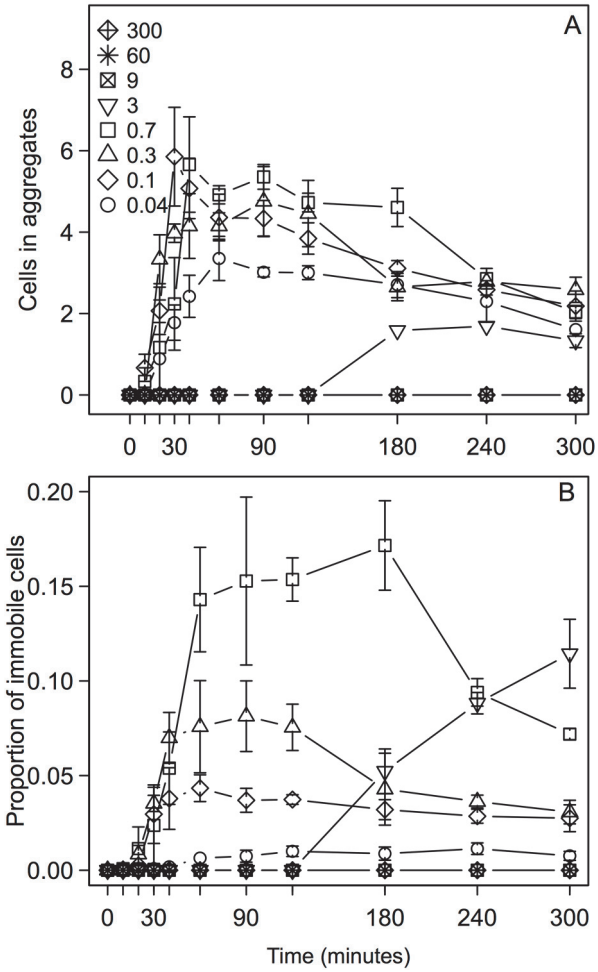

Figure 3. Results from experiment 3 (Study ***I****) with fixed concentrations of* Dinophysis *and different concentrations of* Mesodinium*. The number of* M. rubrum *cells per aggregate at each time step (A) and the proportion of immobilised* M. rubrum *as a function of exposure time (min) at different treatment concentrations to* D. acuminata *(B),. Figure legend (A and B share a common legend shown in A) denotes predator–prey ratios in the 3 mL cultures. Error bars represent standard error of the mean (n = 3).*

On some occasions, the ciliates were observed being attacked and fed upon simultaneously by two or more cells of *Dinophysis*. After attacks, dinoflagellates made attempts to swim in different directions during ingestion, which resulted in stretching the prey cell between them. Eventually, only one of the predators swam away with the prey while other(s) lost the connection via peduncle before feeding was completed. In overall, prey attacks and ingestions were relatively rarely observed during the experiments, in less than 10 occasions per experiment.

As described above, the entrapment, immobilisation, and formation of aggregates of *Mesodinium* cells in the mucus threads seemed to be an important aspect of *Dinophysis* feeding behaviour. However, when it comes to pointing fingers over who was responsible for mucus production, the evidence in hand does not provide any explicit proof. At least two possible interpretations can be delivered: (1) *Dinophysis* cells exude mucus in which the *Mesodinium* cells get entrapped, or (2) *Dinophysis* cells exude toxic compounds (allelochemicals) that make the *Mesodinium* cells leaky and after that excrete a lot of mucus, which then entrap other *Mesodinium* cells. Mucus secretions have been reported on the cell surface of *Dinophysis caudata* (Nishitani et al. 2008) and *Dinophysis fortii* (Nagai et al. 2008), which indicates that mucus production may be characteristic to *Dinophysis*. While the cell organelles responsible for the mucus production remain to be clarified, it is known that members of Dinophysiales possess numerous unique organelles called rhabdosomes (Vesk & Lucas 1986), which potentially may be involved. The discharge of the mucus could be possible via mucocysts, which are located below the thecal pores of the cell (Lucas & Vesk 1990).

The finding that the entrapped ciliates became immobile, lost all their cilia, became swollen and lysed, indicated that the mucus might have contained some kind of toxic compound(s), which caused these seemingly allelopathic effects on the ciliates. These symptoms were typical responses of the target cells when exposed to their predator also in earlier studies (e.g. Hansen, 1989; Tillmann, 1998; Skovgaard and Hansen, 2003). Noteworthy is that the cells attacked by *Dinophysis* went through identical changes instantly after the peduncle was inserted into *M. rubrum*, which indicates the possible effect of the same compound(s) directly released into the prey. The *D. acuta* strain used in the present study is known to produce all three types of DSP toxins: PTX, OA and DTX (Nielsen et al. 2013), whereas *D. acuminata* strain used, only produces PTX (Nielsen et al. 2012). Quantitative studies on *Dinophysis* spp. toxicity have found the majority of DSP toxins extracellularly excreted to the surrounding medium (Pizarro et al. 2009; Nagai et al. 2011; Nielsen et al. 2013). However, the role of these toxins as allelochemicals with lytic effects on other protists has not been demonstrated so far. Thus, the use of toxins as allelochemicals and the importance to the feeding process remains to be studied further.

The obtained data suggest that the negative impact of *Dinophysis* on the *Mesodinium* population is not solely by the means of direct feeding, but also due to the cell lysis of *Mesodinium*. This lytic response must be taken into

account in the prey uptake rate calculations of *Dinophysis*. Ingestion rates of *D. acuminata* have been reported to be 3.2–11 prey cells day^{-1} at high prey concentrations (<1000 cells ml-1; Kim et al. 2008; Riisgaard & Hansen 2009). These rates were determined as the decrease in prey concentration in treatment bottles was compared to the concentrations of a monoculture of *M. rubrum* in control flasks. The reported ingestion rates would be equivalent to 160–440% of body carbon day^{-1}. The latter is much higher compared to mixotrophic dinoflagellates, which have been reported to ingest a maximum of 12–36% of its body carbon day^{-1} when fed with ciliates or cryptophytes (e.g. Bockstahler & Coats 1993; Li et al. 2000; Smalley & Coats 2002). Therefore, the grazing rates of *M. rubrum* reported so far may be overestimated due to the overlooked impact of *M. rubrum* cell lysis.

3.2 Photoregulation in *Dinophysis* spp.

Paper **II** is a pioneer in reporting photoregulation in a kleptoplastidic dinoflagellate. *Dinophysis acuta* cultures subjected to prey starvation at low irradiance (I_{15} = 15 µmol photons m^{-2} s^{-1}) went through cell division 2.3 times during one-month long incubation and were still able to maintain their cellular Chl *a* and phycoerythrin contents (Figure 4, Figure 5). An increase of Chl *a* content in these incubations from 11 to 46 ng Chl *a* ml^{-1} by day 30 took place. Cultures at high photon irradiance (I_{100} = 100 µmol photons m^{-2} s^{-1}) went through on average 2.8 cell divisions (Figure 4A) and the cellular Chl *a* decreased from ~53 to 16 pg Chl *a* $cell^{-1}$ (Figure 4B), leading to an overall increase in Chl *a* concentration from 11 to a maximum of 26 ng Chl *a* ml^{-1} at Day 16. These results indicate a higher net production of Chl *a* in prey-starved *D. acuta* cells grown in low light than at high light.

Cells exposed to high irradiance initially lost Chl *a* at a faster rate than alloxanthin (Figure 4C), a pigment which has photoprotective properties in cryptophytes (Laviale & Neveux 2011; Schlüter et al. 2000) and thus may play a similar role in *D. acuta*. At high irradiance the alloxanthin:Chl *a* ratio increased from 0.86 to ~1.2 over the first 10 days while this pigment was initially reduced in cells incubated under low irradiance, a decrease in the ratio from 0.86 to 0.7 was measured during the first two weeks. The latter was followed by an increase of the alloxanthin:Chl *a* ratio reaching ~1 at the end of the experiment. *In vivo* measurements of Chl *a* and the light harvesting pigment phycoerythrin showed that the changes in phycoerythrin content under the two experimental irradiance regimes matched those found in Chl *a* (Figure 4D).

In well-fed cultures of *D. acuta*, cellular Chl *a* had a broad distribution with hotspots clearly defining 4-5 chloroplast centers with maximum PSII quantum yields (F_v/F_m) of ~0.5 (Figure 5A). These results indicate that the photosynthetic apparatus in those cultures was well functioning. During the starvation experiment, the Chl *a* coverage in cells at high irradiance declined continuously with incubation time and after the first two days also with F_v/F_m (Figure 5 B-D, also see figure Figure S1 in the online version of the study **II**).

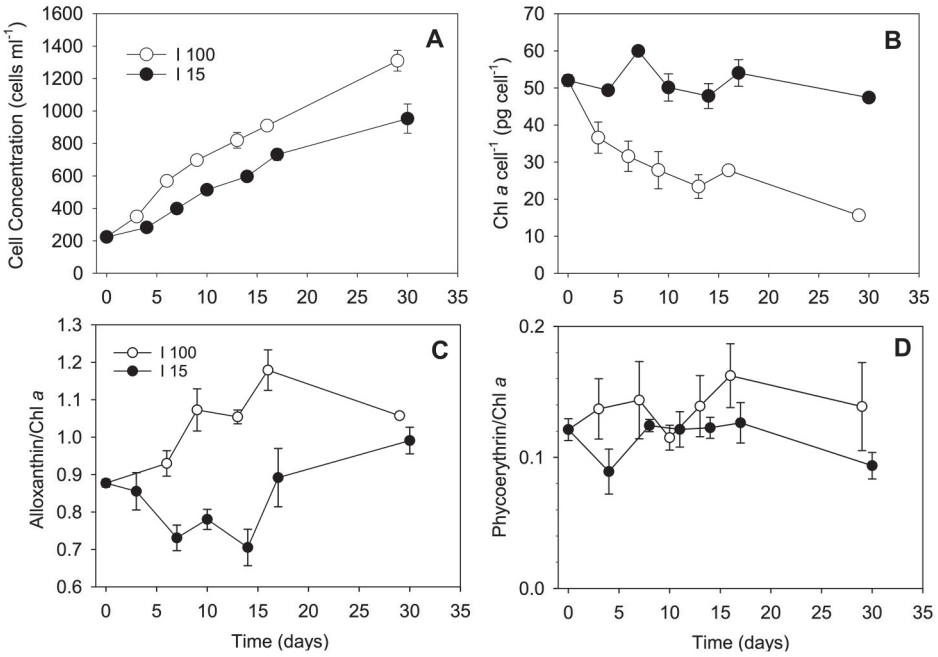

Figure 4. The developments in cell concentrations (A; cells ml^{-1}), cellular Chl a *(B; pg Chl* a *cell^{-1}), the changes in the alloxanthin:Chl* a *ratio (C), and phycoerythrine:Chl* a *ratio in* D. acuta *cells (D) when subjected to prey starvation at the start of the experiment and incubated for 1 month under an irradiance of (o) 100 µmol photons m^{-2} s^{-1} (I_{100}) and (•) 15 µmol photons m^{-2} s^{-1} (I_{15}), respectively.*

Moreover, the use of excitation energy for photosynthesis (φ_{II}) in these cultures was lowered to 38% due to partial closure of PSII reaction centers and non-photochemical energy losses (see Figure S2 A and B in the online version of the study **II**). Despite the decreases in the chloroplast coverage, Chl *a* content and in F_v/F_m the cells were unlikely to incur photodamage, since no changes in non-photochemical quenching (φ_{NPQ}) or in the proportion of functional reaction centers (qP) occurred. φ_{NPQ} is one of the most important safety valves for the regulation of light harvest (Müller et al. 2001) and will decrease if absorbed light energy can no longer be safely driven away. qP gives information if photosynthetic efficiency has been altered by a changed proportion of functional reaction centers, but in this case, both parameters were relatively stable throughout the experiment.

Cells at low irradiance exhibited a rapid increase in F_v/F_m to ~0.6, which remained to a high level even after 30 days. The Chl *a* coverage in these cells was nearly similar to the well-fed cells with similar condensation in four chloroplast centers (Figures 5E- G; Figure S1). In addition, the photochemical efficiency was high – 70% of the absorbed light was used for photosynthesis (Figure S2, panel C in the online version of the study **II**).

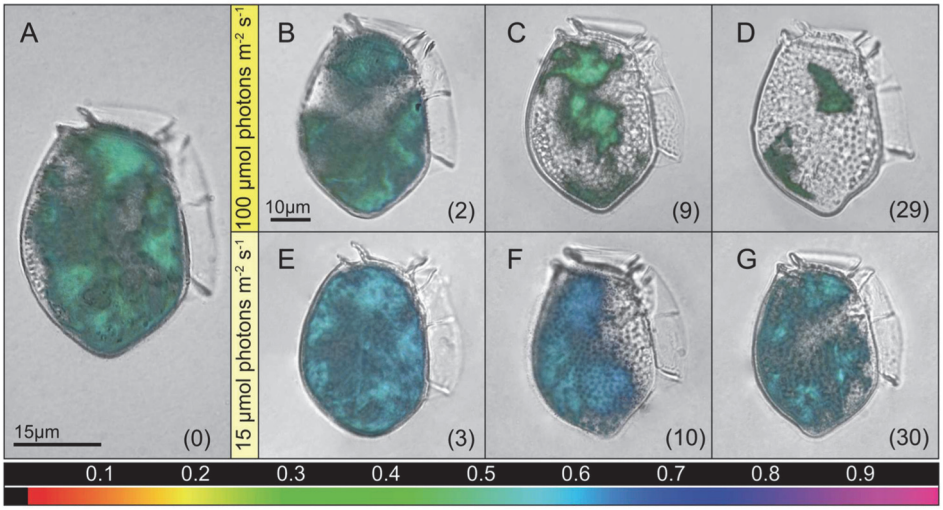

Figure 5. Single cell variable chlorophyll fluorescence imaging. Maximum PSII quantum yield (F_v/F_m) of single D. acuta *cell during the starvation experiment. Images display cell at different time points with the quantum yield as an overlay in false colour (values corresponding to the color scalebar). A culture representative image for fed cells growing at I_{100} prior to starvation (A), culture representative images for starved cells after transfer to irradiances of I_{100} (B–D) and I_{15} (E–G). Numbers in brackets present days of starvation, and time maintained under the given irradiance.*

Photosynthesis *vs*. irradiance parameters like α rETR or rETRmax showed no changes between the light treatments during one month of starvation that would characterise algae displaying photoacclimation (MacIntyre et al. 2002). *D. acuta* is able to keep its chloroplasts functional and maintain Chl *a* and other photosynthetic pigment quota despite growing as a population, but the conspicuous absence of photoacclimation was revealed as *D. acuta* did not respond to a light limitation with an increase in cellular pigment content. Rather, chloroplast regulation worked with limited photoregulation. It is also possible that *Dinophysis* cells may ingest chloroplasts, which photosynthetic apparatus is already fine-tuned to the prevailing light field, aiming at maximal photosynthetic efficiency, by the prey cell. Chloroplast division, however, was not apparent, since these were diluted out. The literature provides no evidence for division of kleptochloroplasts in *Dinophysis* spp., suggesting that the cellular number of chloroplasts will decrease during cell division (Minnhagen et al. 2008). Hence, the ability of *Dinophysis* to maintain cellular photosynthetic pigment concentrations may potentially lead to enlargement (rather than division) of individual chloroplasts (Nielsen et al. 2013).

The capacity for inorganic carbon fixation was dropped dramatically and was eventually lost in cells subjected to starvation at high irradiance, an exponential decrease during the first 16 days (from 2.83 ± 0.23 ng C cell^{-1} d^{-1} to 0.38 ng C cell^{-1}d^{-1}; see Figure 5A in the published study **II**) was measured. While it dropped even further during the following two weeks, the Chl *a*-specific carbon uptake stayed constant after day 16 (1.07 pg C pg Chl *a*$^{-1}$ h^{-1}),

indicating that, although *D. acuta* was able to produce Chl *a*, this did not translate into increased inorganic carbon uptake. Under low irradiance, inorganic carbon uptake remained constant for the first 17 days and dropped only marginally (to 0.33 ± 0.03 ng C $cell^{-1}$ d^{-1}) after 1 month of incubation. Thus, the Chl *a*-specific inorganic carbon uptake rates of the starved *D. acuta* at low irradiance stayed constant at ~0.6-0.7 pg C µg Chl a^{-1} h^{-1} during the entire experiment.

The reason behind the dramatic drop in inorganic carbon fixation may be strongly affected by the loss of genes involved in the regulation of RuBisCO that are located in the cryptophyte nuclei material. The sequestered prey chloroplasts in *Dinophysis* spp. are retained without prey nuclei and nucleomorphs (Kim et al. 2012) where such genes may come from. The RuBisCo regulatory protein, CbbX, for instance, is encoded by the cryptophyte nucleomorph in cryptophytes (Maier et al. 2000). Another explanation for this is that as *D. acuta* cannot divide the acquired chloroplasts, it will also be unable to divide its pyrenoids, where RuBisCo is located in most algae (Garcia-Cuetos et al. 2012; Giordano et al. 2005; Holdsworth 1971), hence this missing control factor may be the key to regulate fully and divide sequestered "third hand" chloroplasts.

The results described above suggest that the cryptophyte chloroplasts are not independent entities, which will function on their own inside the *Dinophysis* cell. The photosynthetic performance of kleptochloroplasts may depend on genes, which in the past have been transferred from prey nuclei and nucleomorphs to the dinoflagellate genome. However, not much is known about the gene transfer from the cryptophyte genome to the genome of *Dinophysis* spp., and only one species, *D. acuminata*, has been investigated so far (Wisecaver & Hackett 2010). In the nuclear genome of *D. acuminata,* five nuclear-encoded plastid proteins that function in photosystem stabilisation and metabolite transport have been found (Wisecaver & Hackett 2010). However, it seems unlikely that those genes alone allow for the extensive regulation of photosynthetic and photoprotective pigments that were observed in *D. acuta*. Results on chloroplast function indicate that many more genes than those reported by Wisecaver & Hackett (2010) may have been transferred to the nucleus of *D. acuta,* which remain to be identified.

Upon initiation of prey starvation, the excess inorganic carbon uptake during the first ~9 days in cultures under high irradiance was found. The population size continued to increase even after the respiration rates started to exceed rates of inorganic carbon uptake. Microscopic observations showed that *D. acuta* cells became full of storage material (probably lipids and starch; Clement et al. 1988). Similar observations have been noted earlier in *D. caudata* (Park et al. 2008). The build-up of carbon storage upon the onset of prey starvation and maintenance of fully active kleptochloroplasts thus allows *D. acuta* to survive for extended periods of time (months; Nielsen et al. 2012; Nielsen et al. 2013; Park et al. 2008). This is an important trait of these kleptochloroplastidic

dinoflagellates and explains how they can survive in a fluctuating environment and still depend on *M. rubrum* as a single type of prey.

3.3 Dark nutrient acquisition and photoregulation in *Heterocapsa triquetra*

For some time now, it has been hypothesised that in the Gulf of Finland the populations of *Heterocapsa triquetra* migrate vertically from warm surface layers to the deeper cold layers to acquire inorganic nutrients and thereafter photosynthesise and assimilate nutrients into the biomass in the euphotic layer (Lips et al. 2011; Lips et al. 2010). The goal of the study was to gain information on how the *H. triquetra* populations overcome nutrient-limitation in the surface water layer in summer and reach blooming concentrations. For this, some of the main environmental factors met in the Gulf of Finland below the seasonal thermocline and in the euphotic water layer, that influence nutrient uptake and photosynthetic efficiency in phytoplankton were simulated. These factors were: light availability, temperature and nutrient pre-conditioning (**III**).

The results reveal that *H. triquetra* can take up inorganic nitrogen and phosphorus in a dark, cold environment. This documented ability for dark nutrient uptake supports the hypothesis that *H. triquetra* migrations to the thermocline could be aimed to fill the requirements for inorganic nutrients that are at that time lacking in the surface layers. The observed N:P uptake ratios by *H. triquetra* were similar in the dark, cold (4 °C) and illuminated, warm (15 °C) environments – 14:1 and 12:1, respectively. This is noteworthy since significantly lower N:P uptake ratios in the dark have been reported in the previous studies (e.g. Müller et al, 2008; Riegman et al. 2000). The average consumption rates of NO_3^- during the dark, cold incubation (I_D) was 1.04 μM d^{-1} = 0.04 μM h^{-1} and in the following warm light:dark cycle (I_{LD}) it was 1.69 μM d^{-1} = 0.07 μM h^{-1}. The consumption rates of PO_4^{3-} in I_D was 0.075 μM d^{-1} = 0.003 μM h^{-1} and in following I_{LD} 0.14 μM d^{-1} = 0.006 μM h^{-1}. The growth rate increased to 0.22 day^{-1} in two days after relocating the nutrient-amended cultures from I_D to I_{LD}. A 34% higher cell concentration was yielded by day 11 (1390 cells mL^{-1}) when compared with the non-amended cultures at the same day (1040 cells mL^{-1}; Figure 6A).

While the influence of single environmental factors (light, temperature, nutrient pre-conditioning) on nutrient uptake have been previously studied in dinoflagellates (Cullen 1985; Paasche et al. 1984; Reay et al. 1999; Riegman et al. 2000; Smayda 1997), the significance of the present study is in investigating the effect of combined environmental factors on the nutrient uptake. Dark nutrient uptake rates at different temperatures or dark nutrient uptake by cells previously grown in an illuminated but nutrient-poor environment have received very little attention. Some earlier laboratory studies have shown that N-deprived

phytoplankton cells have greater dark uptake rates of inorganic nitrogen compared with N-replete cells (Cochlan et al. 1991; Dortch & Maske 1982).

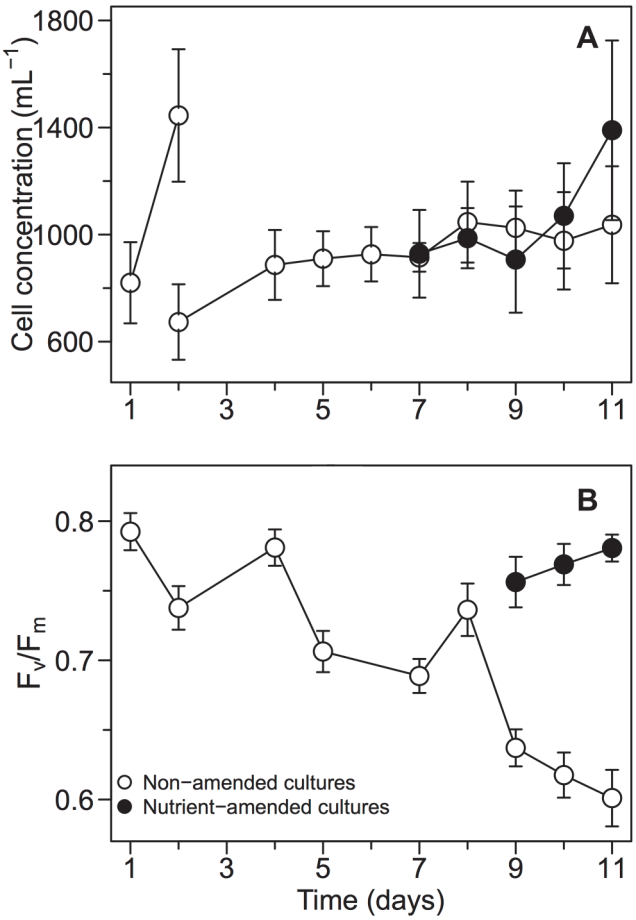

Figure 6. Cell abundance (cells mL^{-1}; A) and maximum photochemical quantum yield of PSII (F_v/F_m; B) in Heterocapsa triquetra *cultures subjected to nutrient limitation (non-amended cultures; open symbols) and nutrient supply (nutrient-amended cultures; closed symbols). Nutrient-amended cultures were dark-incubated from day 6 to day 8 (48 hours), F_v/F_m was not measured in the nutrient-amended cultures at that time. Data points represent treatment means with error bars (n=3).*

The maximum PSII quantum yield (F_v/F_m) for the non-amended *H. triquetra* cultures declined as a function of time from 0.79 to 0.60 during the experiment (Figure 6B). The mean F_v/F_m in the nutrient-amended cultures after the incubation at I_D was 0.77 ± 0.01, being on average higher than the mean F_v/F_m in the non-amended cultures (0.66 ± 0.03) during the same period (*t*-test, $p < 0.01$). F_v/F_m value over 0.6, however, is still a relatively high value, commonly measured for cultures with a well-functioning photosynthetic apparatus (López-Rosales et al. 2014; Suggett et al. 2009). The high F_v/F_m together with the long duration of the stationary growth phase in the non-amended *H. triquetra*

cultures (~9 days) may indicate that this species can acclimate to low nutrient conditions and survive relatively long periods in a nutrient-depleted surface layer in the sea. Toleration of low-nutrient conditions may be potentially achieved by the use of intracellular nutrient storages, as e.g. dinoflagellates store significant amounts of inorganic and organic nitrogen forms (e.g. Müller et al, 2008; Dagenais-Bellefeuille & Morse 2013). It is thought, that in stationary growth phase nutrients are reallocated to the functions where they are most needed, and the balance between the light harvest and electron transport are adjusted in such a way as to maximise efficiency (Parkhill et al. 2001).

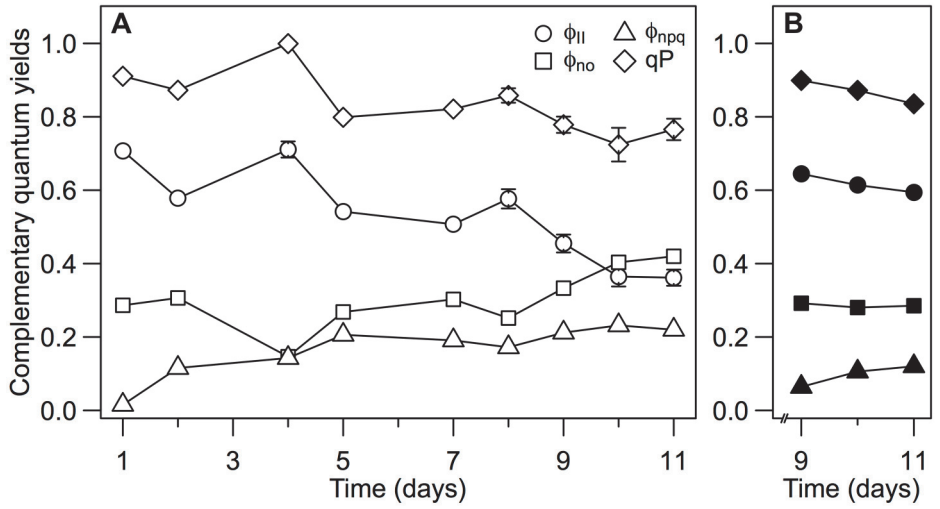

Figure 7. The dynamics of the variable chlorophyll fluorescence in Heterocapsa triquetra *cultures during the experiment when (a) subjected to nutrient limitation (non-amended cultures), and (b) after nutrient supply and the following 48-hour dark incubation period (nutrient-amended cultures). Data points represent treatment means with error bars (n=3).*

The effective photochemical yield (φ_{II}) for the non-amended cultures declined with time from 0.71 to 0.36. During the first 5 days, the decline mainly resulted from the compensatory changes in antenna downregulation, φ_{NPQ} (the latter showed an increase of ten times, from 0.02 to 0.21; Figure 7A). Under these conditions, the PSII reaction centers were essentially completely open (high qP) and photosynthetic yield was primarily determined by changes in non-photochemical quenching. Onwards from day 5, the capacity of φ_{NPQ} to regulate light capture became saturated, leading to the gradual decline of the proportion of open reaction centers, qP. From day 8 until the end of the experiment, φ_{II} was mainly influenced by non-light induced (basal or dark) quenching processes, φ_{NO} (which increased from by 0.25 – 0.42 during that period; Figure 7A). Increased energy dissipation as φ_{NPQ} and φ_{NO} caused a photoregulative change in the non-amended *H. triquetra* cultures on days 10 – 11, i.e. φ_{II} was lowered

down to 0.36. In the nutrient-amended cultures φ_{II} recovered up to 0.64 and was on average higher when compared to the non-amended cultures (Figure 7B). Energy dissipation as φ_{NO} was stable (ranged from 0.29 – 0.31; Figure 7B). Enrichments with limiting nutrients often restore the values of φII, implying that nutrient limitation on photosynthetic energy conversion is common in the sea (Falkowski 1992; Falkowski et al. 1991).

The results of the study **III** provide evidence that the dark nutrient uptake was followed by an improved performance of the photosynthetic apparatus of *H. triquetra* later in the illuminated conditions. Potentially, the use of intracellular nutrient reserves could have been used to improve the efficiency of PSII reaction centers. Thus, *H. triquetra* populations indeed win from compromises between nutrient acquisition in dark deep layers and photosynthesis in the euphotic layer during the day.

CONCLUSIONS

This thesis aims to unravel seemingly complicated links between autotrophy, heterotrophy and species behavioural ecology to explain why mixotrophs gain an advantage over competitive species and occasionally dominate phytoplankton communities in many aquatic habitats. Understanding the species ecophysiology and factors affecting population dynamics is essential to evaluate the contribution of mixotrophs to the net community production. Specifically, studies covered by the current thesis help to shed new light on why *Dinophysis* species are successful and often form massive toxic blooms around the globe while depending on the availability of a single type of prey – *Mesodinium rubrum*. The ciliate *M. rubrum* jumps at an extraordinary speed, it can reach ~11 mm s^{-1}, and still, this must not be enough to escape its most specialised predator, *Dinophsyis. M. rubrum* and *Heterocapsa triquetra* both perform DVM in the stratified Baltic Sea, most likely to acquire inorganic nutrients that are lacking in the surface layers at that time. Hence, mixotrophs may have an important part also in the upward vertical flux of inorganic nutrients induced by migrations in comparison to the contribution of hydrophysical processes (e.g. transport by upwelling events).

The main results of the present thesis can be summarised as follows:

- Populations of *Mesodinium rubrum* are vulnerable in cultures with *Dinophysis acuminata* and *D. acuta* species. Ciliates swim into mucus threads that are most probably excreted by *Dinophysis* and thereafter become immobilised and form aggregations. The proportion of mucus-entrapped prey cells was influenced by the concentration of *Dinophysis* and *M. rubrum* and by the ratio of these two species.
- Immobilised prey seemed to be an important aspect of *Dinophysis* feeding behaviour because only live cells that were incapable of normal swimming were attacked by *Dinophysis*. The prey cells were detected by *Dinophysis* from the distance, and a peduncle was used to create contact with the cells and to suck out the cell contents. The collected data suggest that the negative impact of *Dinophysis* on the *M. rubrum* population is not solely by the means of direct feeding, but also due to the cell lysis of *M. rubrum*.
- The regulation of kleptochloroplasts in prey-starved cultures of *Dinophysis acuta* works through photoregulation. *D. acuta* cells starved of prey started to produce photosynthetic and photoprotective pigments when incubated at different irradiances. Our data point to a hitherto unstudied role of gene transfer from prey to *D. acuta* that may enable it to regulate the function of its

kleptochloroplast, although the exact genetic and biochemical mechanisms remain to be identified.

- In the natural environment, *D. acuta* and other *Dinophysis* species may also indirectly photoregulate via increased retention of kleptochloroplasts, when prey cells are available, or they may indirectly achieve "photoacclimation" by ingestion of photoacclimated prey cells and maintaining a higher number of chloroplasts.
- Inorganic phosphate and nitrate uptake by *H. triquetra* in dark, cold (4 °C) environment, characteristic to the water layers below the seasonal thermocline in summer in the Baltic Sea, was found. Signs of improved photophysiology after nutrient uptake in the dark and followed growth in the illuminated field were apparent.
- Nearly constant cell concentration and relatively high effective photochemical quantum yield in the potentially nutrient-limited cultures indicated the ability of the *H. triquetra* population to survive for extended periods in the low nutrient conditions.

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Mass entrapment and lysis of *Mesodinium rubrum* cells in mucus threads observed in cultures with *Dinophysis*

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ABSTRACT

The entrapment and death of the ciliate *Mesodinium rubrum* in the mucus threads in cultures with *Dinophysis* is described and quantified. Feeding experiments with different concentrations and predator–prey ratios of *Dinophysis acuta*, *Dinophysis acuminata* and *M. rubrum* to study the motility loss and aggregate formation of the ciliates and the feeding behaviour of *Dinophysis* were carried out. In cultures of either *Dinophysis* species, the ciliates became entrapped in the mucus, which led to the formation of immobile aggregates of *M. rubrum* and subsequent cell lysis. The proportion of entrapped ciliates was influenced by the concentration of *Dinophysis* and the ratio of predator and prey in the cultures. At high cell concentrations of prey (136 cells mL^{-1}) and predator (100 cells mL^{-1}), a maximum of 17% of *M. rubrum* cells became immobile and went through cell lysis. Ciliates were observed trapped in the mucus even when a single *D. acuminata* cell was present in a 3.4 mL growth medium. Both *Dinophysis* species were able to detect immobile or partly immobile ciliates at a distance and circled around the prey prior to the capture with a stretched out peduncle. Relatively high entrapment and lysis of *M. rubrum* cells in the mucus threads indicates that under certain conditions *Dinophysis* might have a considerable impact on the population of *M. rubrum*.

1. Introduction

Species of the genus *Dinophysis* have a worldwide distribution and are well known producers of Diarrhetic Shellfish Toxins (DSTs), thereby causing a global health risk to shellfish consumers (Reguera et al., 2014). The toxins produced by *Dinophysis* include okadaic acid (OA), dinophysistoxin (DTX), and pectenotoxins (PTX), which may accumulate in mussels that feed on toxin-containing *Dinophysis* cells. The genus is globally distributed in marine environments over a broad range of salinities. In temperate waters, members of the "*Dinophysis acuminata* species complex", which also include the morphospecies *Dinophysis sacculus* and *Dinophysis ovum*, are the most widespread members of genus (Reguera et al., 2012). These species peak in abundance from late spring to early autumn, but can be found throughout the year and extend into estuaries with quite low salinity (Reguera et al., 2012).

Dinophysis acuta Ehrenberg 1839 is another common species in temperate waters which tend to be common during summer and autumn periods (Escalera et al., 2006; Hällfors et al., 2011; Farrell et al., 2012).

Conventional peridinin-containing chloroplasts, so typical of phototrophic dinoflagellates, are lacking in *Dinophysis* species (*sensu stricto*). Instead they contain phycoerythrin-rich cryptophyte chloroplasts (Schnepf and Elbrächter, 1988; Meyer-Harms and Pollehne, 1998; Janson and Granéli, 2003). The eight species of the genus cultured so far all depend upon red *Mesodinium* spp. as their prey for long term growth; they are obligate mixotrophs (Kim et al., 2008; Riisgaard and Hansen, 2009; Hansen et al., 2013; Reguera et al., 2014). Recently, it has been documented that the chloroplasts of *Dinophysis acuta* and *Dinophysis caudata* Saville-Kent 1881 are indeed kleptochloroplasts, which are sequestered from the ciliate prey (e.g. Kim et al., 2012; Raho et al., 2014). It is presently unknown to what extent this is true for the remaining ~90 species of *Dinophysis*, and more research on this topic is required.

The ciliate *Mesodinium rubrum* (Lohmann, 1908) and other red forms of the genus are known for their characteristic backward jumps interrupted by periods where the cells remain non-motile (e.g. Fenchel and Hansen, 2006; Garcia-Cuetos et al., 2012). In this

Abbreviations: DTX, dinophysistoxin; OA, okadaic acid; PTX, pectenotoxins.

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non-motile stage, cirri are stretched out from the cell in all directions sensing changes in shear caused by approaching predators. This allows the ciliate to escape from being caught by predators (Jonsson and Tiselius, 1990). In comparison with *M. rubrum*, *Dinophysis* swims fairly slowly (Smayda, 2010), and very few studies are available that describe and document how *Dinophysis* spp. manage to catch the ciliates. Direct observations at low magnification indicate that *Dinophysis* cells can detect their prey from a certain distance and start to circle around the prey prior to capture. The exact capture mechanism is still unknown, but it has been reported that after circling around the prey for some time, the *Dinophysis* cells become connected with the prey. It has been proposed that similarly to many other phagotrophic dinoflagellates (e.g. Hansen and Calado, 1999) a capture filament may be involved (Hansen et al., 2013). It is important to emphasize that the previous observations have been carried out at low magnification with limited resolution and no photographic or video documentation of a capture filament has been published.

Nagai et al. (2008) and Nishitani et al. (2008) have observed *Mesodinium* cells forming aggregates with significantly altered mobility when mixed with *Dinophysis* spp. and have witnessed *Dinophysis* to feed on them. These authors have not tried to quantify this interaction, neither have they described the fate of the *Mesodinium* aggregates. In the present study the feeding behaviour of *Dinophysis acuminata* (Claparède & Lachmann, 1859) and *Dinophysis acuta* were looked into and the immobilization and aggregate formation of *Mesodinium rubrum* when exposed to its predator were described and quantified. It is hypothesized that the proportion of immobilized ciliates and the formation of aggregates are related to the cell concentrations of *Dinophysis* and *Mesodinium* and to the predator–prey ratios. To investigate this phenomenon, a mixture of short-term experiments and microscopic observations of these species using a variety of predator–prey ratios and cell concentrations were designed. Cell concentrations will most likely impact the amount of chemical substances released into the surroundings by either the predator or the prey. The amount of chemical substances may play a role in cell swimming or in prey capture efficiency.

2. Materials and methods

2.1. Cultures and culturing conditions

The cultures of the cryptophyte *Teleaulax amphioxeia* (K-1837; SCCAP) and the ciliate *Mesodinium rubrum* (MBL-DK2009) were established from single cells isolated from water samples collected from Helsingør Harbour in 2009. Cultures of *M. rubrum* were fed with *T. amphioxeia* at a predator–prey ratio of 1:5 once in every two weeks to enable mixotrophic growth. The culture of *Dinophysis acuta* (DANA-2010) was established in June 2010 from the North Sea (Nielsen et al., 2013) and *Dinophysis acuminata* (strain FR101009) was isolated from Little Belt, Denmark in October 2009 (Nielsen et al., 2012). All cultures were maintained on a glass table in autoclaved sterile-filtered f/2 medium (Guillard and Ryther, 1962) with a salinity of 35 at temperature of 15 °C. Illumination was provided from beneath by cool white fluorescent lights of 100 μmol photons m^{-2} s^{-1} on a 14:10 h light:dark cycle. All cultures were non-axenic.

Prior each experiment a new culture of *Dinophysis acuminata* and/or *Dinophysis acuta* was established onto a 24-well tissue culture plate by transferring a fraction from a culture starved for 5–7 days into newly filtered f/2 medium and subsequently fed with *Mesodinium rubrum* at a predator–prey ratio of ≈1:10. Prey was allowed to disappear from the wells during the next few days. Cell isolations of *D. acuminata* (experiments 2 and 3) were carried out instantly after pooling cells into a 65 mL tissue culture flask for homogenization and subsequent pipetting on a new multi-dish plate. Single cells were isolated using a drawn-out Pasteur micropipette and were thereafter allowed to acclimate 2–4 h before the start of experiments (also in experiment 1 after bulk pipetting both *D. acuminata* and *D. acuta*). To ensure that the cells were as healthy as possible, only swimming cells were selected; dividing cells have not been observed to feed on prey and were therefore not selected. In experiment 1 the two species of *Dinophysis* were used to see possible interspecies behavioural differences. In experiments 2 and 3 the focus was set on *D. acuminata* only.

2.2. Experiment 1. Behaviour of Dinophysis in absence and presence of M. rubrum

To give a general description of the changes in the behaviour of *Dinophysis acuminata* and *Dinophysis acuta* after addition of prey, cultures of cells starved before for 2–3 days were pooled into 65 mL (true capacity) tissue culture flasks (TPP, Switzerland) and cell suspensions of *D. acuminata* and *D. acuta* containing ca. 100, 300 and 700 cells (respectively for both species) were pipetted in triplicates into 1 mL of f/2 medium onto 24-well tissue culture plates. The cultures of *D. acuminata* at concentrations of 84, 194 and 307 cells mL^{-1} and the cultures of *D. acuta* at concentrations of 89, 217 and 368 cells mL^{-1} were allowed to acclimate for 2–3 h. After acclimatization, microscopic observations were carried out to describe the swimming behaviour of dinoflagellates in absence of *Mesodinium rubrum*. For further observations in the presence of ciliates, each *Dinophysis* cell suspension was mixed with *M. rubrum* cell suspension of 0.06, 0.180 and 0.42 mL (ca. 1000, 3000, and 7000 cells, respectively). Culture mixtures in each well were raised to the full capacity (to the rim) by adding f/2 medium and covered with a cover glass. Final concentrations for *D. acuminata* and *D. acuta* were 29, 88 and 206 cells mL^{-1} and for *M. rubrum* 290, 880 and 2060 cells mL^{-1}. The experiment was carried out over 8 days. Observations were documented as videos and pictures using an inverted microscope (Nikon Diaphot-TMD, Nikon Corporation, Japan) equipped with a digital camera (Canon EOS 5D Mark III, Canon, Japan).

2.3. Experiment 2. Effect of exposure time of D. acuminata on M. rubrum motility: different concentrations of Dinophysis and fixed concentrations of Mesodinium

A timed experiment was carried out to determine the effect of concentration and exposure time of *Dinophysis acuminata* on the motility of *Mesodinium rubrum*. The cells of *D. acuminata* were isolated with a drawn out Pasteur pipette in triplicates into 2 mL of f/2 medium in a 24-well tissue culture plate and were allowed to acclimate for 2–3 h. Thereafter the cultures were mixed with 0.28 mL *M. rubrum* cell suspension (2000 cells, final concentration 588 cells mL^{-1}), which marked the starting point of the experiment. Each culture mix was raised to 3.4 mL by adding f/2 medium and was covered with a cover glass for microscopic observations. The initial number of cells for *D. acuminata* treatments in 3.4 mL culture were 1, 2, 5, 17, 34, 70, 153, 306, 510, resulting in concentrations of 0.3, 0.6, 1.5, 5, 10, 20.6, 45, 90, 150 cells mL^{-1} and in predator–prey ratios of 0.0005, 0.001, 0.003, 0.009, 0.02, 0.04, 0.08, 0.2 and 0.3. The concentration levels chosen for *D. acuminata* corrrespond to common natural abundances of *Dinophysis*. For controls, three replicates of *M. rubrum* without *D. acuminata* were established at the same concentrations as in the treatment wells. The number of single *M. rubrum* cells that displayed abnormal swimming behaviour, immobilized cells, aggregates and cells in each aggregate were counted at the time steps of 0, 10, 20, 30, 40, 60, 90, 120, 180, 240 min. Single cells with altered motility were

counted as an aggregate of the size of one cell. Cells laying at the bottom of the wells were enumerated using an inverted microscope (Nikon Diaphot-TMD, Nikon Corporation, Japan) and cells in the water column using a stereomicroscope (Olympus SZ61).

2.4. Experiment 3: Effect of exposure time of D. acuminata on M. rubrum motility: fixed concentrations of Dinophysis and different concentrations of Mesodinium

This experiment was performed to study the effect of *Dinophysis acuminata* bloom on the small populations of ciliates. For this, a series of cultures with high predator–prey ratios were established. The experiment followed the same protocol as described for experiment 2, but fixed cell concentrations of *D. acuminata* (300 cells, final concentration 100 cells mL^{-1}) and a suite of cell densities for *Mesodinium rubrum* were used. It was concluded from experiment 2, that the motility of the ciliates was strongly influenced by a *Dinophysis* concentration of ~100 cells mL^{-1}. Thus, initial cell density of 100 cells mL^{-1} was used in experiment 3. The cells of *D. acuminata* were isolated in triplicates into 2 mL of f/2 medium in a 24-well culture plate and were allowed to acclimate for ca. 2 h. The initial number of cells for different *M. rubrum* treatments in 3 mL culture were: 1, 5, 34, 102, 408, 1020, 2500, 6800 resulting in concentrations of 0.3, 1.7, 11, 34, 136, 340, 833, 2267 cells mL^{-1} and in predator–prey ratios of 300, 60, 9, 3, 0.7, 0.3, 0.1 and 0.04. The low *M. rubrum* concentration levels (0.3–11 cell mL^{-1}) were chosen to investigate if low amounts of prey can be detected or captured by the predators when mixed with a bloom concentration of *Dinophysis*. High *M. rubrum* concentrations roughly correspond to the bloom conditions of the ciliates. Culture mixtures were raised to 3 mL and cells were counted at the same time steps as in experiment 2 with one additional time step of 300 min. For controls, three replicates of *M. rubrum* were established at the same concentrations as in the treatment wells. Cells were counted using an inverted microscope (Olympus IX51, Olympus Corporation, Japan).

2.5. Statistical analysis

Generalized linear models (GLMs) with a negative binomial distribution and a logit link were used to test the associations between proportions of immobilized *Mesodinium rubrum* cells in relation to the treatments. This method was chosen to overcome the poor performance of excess zero-count data transformation that is used to meet the assumptions of parametric statistics, i.e. ANOVA (O'hara and Kotze, 2010). For the proportion data analysis, a two-column response vector was created. This contained counts of successes (number of immobile ciliates) and failures (number of immobile ciliates subtracted from the total number of ciliates). A likelihood-ratio test was performed to check for a significant treatment effect and a Tukey post hoc test from the *multkomp* package in R (R Development Core Team, 2008) was applied to inspect the differences among treatments. Time was not included to the analysis. An alpha level of 0.05 was used for all statistical tests.

3. Results

3.1. Visual observations of the behaviour of Dinophysis in the absence and presence of prey

In the absence of prey, *Dinophysis acuminata* and *Dinophysis acuta* displayed a helical swimming path typical for dinoflagellates, interrupted by changes in swimming direction or cell rotation. The mucus threads were not seen by means of direct microscopic observations in the freshly picked *Dinophysis* cultures prior to adding the prey. About 10–20 min after the addition of *Mesodinium rubrum* the ciliates were observed to swim into the elongated mucus threads, and as a result, their normal swimming pattern was immediately disturbed. Freshly trapped ciliates performed frequent attempts of escape jumps but were mostly unable to break free. Even if some cells were occasionally able to break free from the mucus connection, cell lysis seemed to be inevitable after contact with the mucus. Entrapped cells became completely immobile in the mucus during 10–20 min. Accidental encounters between free *M. rubrum* cells and trapped cells resulted in the formation and enlargement of aggregates of *M. rubrum* (Fig. 1A). A seemingly allelopathic response of *M. rubrum* occurred within 30–60 min after the entrapment: cells (either in aggregates or single cells trapped in mucus) were observed to lose all their cirri and the cell shape became rounded, after which the cells lysed. Mucus threads were not observed being expelled nor being trailed behind any cells, but were surrounding *Dinophysis* cells in the culture and were attached to the *M. rubrum* cells as they were swimming into them. A large mucus matrix had developed approximately 1 h after the start of the experiment. Mucus production and aggregate formation was not observed in the *M. rubrum* control cultures.

Freshly trapped *Mesodinium rubrum* cells were observed to be attacked by *Dinophysis*. Prior to the attack *Dinophysis* displayed a changed swimming behaviour characterized by decreased swimming speed, circulations around the aggregates of *M. rubrum* and frequent stops near immobile but alive prey cells, often with an extended peduncle. *Dinophysis* then attacked a trapped ciliate with an extended peduncle (Video S1), which contracted after contact with *M. rubrum* and the captured ciliate was drawn closer to the *Dinophysis* cell. A capture filament was not observed during attacks. The cirri were shed and cell shape became swollen in the next few minutes after the start of the ingestion. Ingestion time was about 15 min, until the contents of the prey cell had disappeared. During this stage the prey cell was attached to the

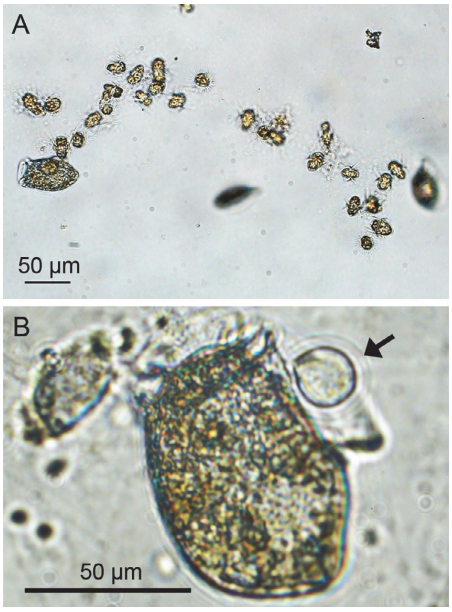

Fig. 1. A stretched out aggregate of *Mesodinium rubrum* in the presence of *Dinophysis acuta* (A). Feeding position of *D. acuta* with the ciliate that has been sucked out of chloroplasts. The arrow indicates the remains of the prey (B).

predator cell by its peduncle. After that, the remains of the prey cell were carried along for some time (Fig. 1B), but eventually left behind. Normal swimming behaviour of *Dinophysis* resumed after a few minutes. The actual time depended on how fast *Dinophysis* was able to release the ciliate from the mucus connection. Ciliates that had lost their normal cell shape (probably dead cells) while being imprisoned in the mucus secretions were not observed to be attacked.

On some occasions, ciliates were attacked and fed upon simultaneously by two or more cells of *Dinophysis*. As dinoflagellates were observed to make attempts to swim in different directions during ingestion (Video S2) the prey cells were stretched between them. Eventually only one of the predators swam away with the prey while other(s) lost the connection via peduncle before feeding was completed.

Most of the *Dinophysis acuta* cells in high cell concentration treatments (88 and 206 cells mL^{-1}) had changed their typical swimming pattern approximately 24 h after feeding. Instead of swimming helically, cells were only revolving around their cell axis. These cells formed distinctive groups near the bottom of the vessel. At a lower *D. acuta* concentration (29 cells mL^{-1}) the number of dinoflagellate cells with decreased motility was smaller (1–10 cells). In the mixed cultures of *Dinophysis acuminata* and *Mesodinium rubrum*, neither formation of such groups nor changes in swimming patterns were observed. Frequent vegetative cell divisions of *D. acuta* were observed starting from the fourth day of the experiment. Lately divided cells, which in *Dinophysis* spp. differ from older cells by having only one part of the left sulcal list, were found to have reduced motility, mostly laying on the bottom of the vessel.

All prey cells trapped in the mucus lysed by the end of the experiment. Large aggregates of *Mesodinium rubrum* turned into a field of chloroplasts scattered over the bottom of the culture vessel. This aspect was observed to be especially pronounced in very high predator–prey concentrations (206 and 2060 cells mL^{-1}, respectively, predator–prey ratio 0.1). Occasionally, the cells of *Dinophysis acuminata* were observed to stop near such chloroplast aggregations, and to display a swimming behaviour characteristic for cells that were about to feed.

3.2. *Effect of exposure time and concentration of* D. acuminata *on* M. rubrum *motility*

Observations of the treatment cultures revealed the formation of distinct aggregates of *Mesodinium rubrum* at the presence of *Dinophysis acuminata*. The first negative effects on the motility of the ciliates were apparent after 10 min of exposure to the *D. acuminata* culture. The concentration of the *Dinophysis* cells had a significant effect on the proportions of immobilized ciliate cells ($p < 0.001$). Immobile cells were found in all treatments, but at high predator–prey ratios (0.08, 0.2 and 0.3; *D. acuminata* ≥45 cells mL^{-1}) the proportion of immobile cells was significantly higher compared to all the other treatments (Tukey post hoc test, $p < 0.001$) (Fig. 2A). From the initial *M. rubrum* concentration, 2% of ciliate cells were immobilized at the predator–prey ratio of 0.08 (45 *D. acuminata* cells mL^{-1}), a maximum of 4% of cells at the ratio

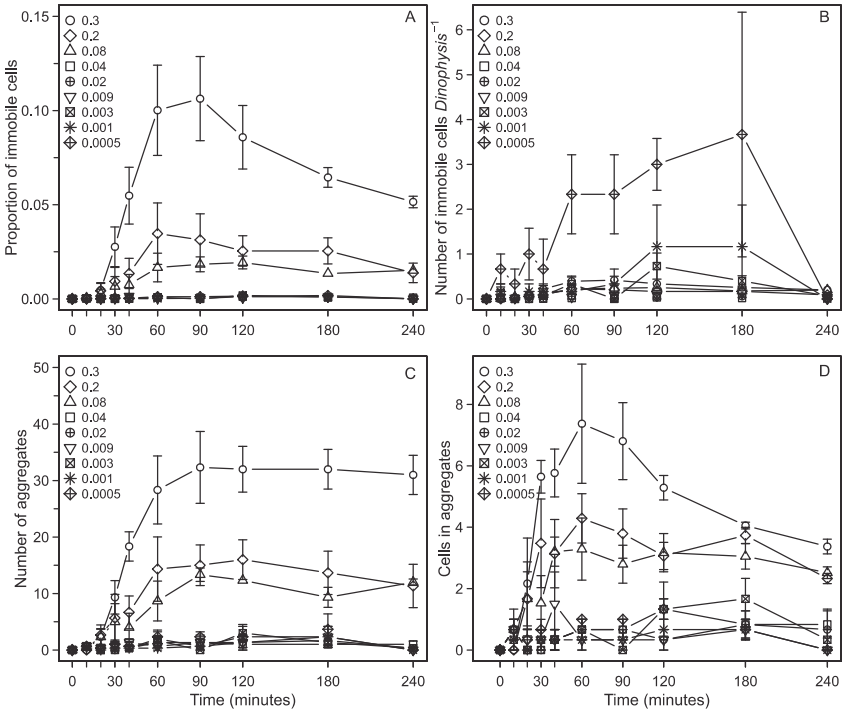

Fig. 2. Results from experiment 2 with different concentrations of *Dinophysis* and a fixed concentration of *Mesodinium*. Proportion of immobilized *Mesodinium rubrum* as a function of exposure time (min) to different treatment concentrations of *Dinophysis acuminata* (A). Number of immobile cells per one *Dinophysis* cell (B). Number of aggregates of *M. rubrum* cells (C) and the number of *M. rubrum* cells per aggregate at each time step (D). Figure legend denotes predator–prey ratios in the 3.4 mL cultures. Error bars represent standard error of the mean ($n = 3$).

of 0.2 (90 *D. acuminata* cells mL^{-1}) and 11% at the ratio of 0.3 (150 *D. acuminata* cells mL^{-1}) (Fig. 2A). Less than 0.2% of *M. rubrum* cells were immobilized at lower predator–prey ratios (0.0005 to 0.04; *D. acuminata* concentrations of 0.3–20.6 cells mL^{-1}). At all the latter ratios the proportion of immobilized cells was similar between them (Tukey post hoc test, $p > 0.05$). Due to the start of the cell lysis after 1 h of the incubation time, the count of immobile cells decreased or reached a plateau (Fig. 2A). The number of immobile *M. rubrum* cells per *Dinophysis* cell was highest at the lowest predator–prey ratios (Fig. 2B).

The number of aggregates at predator–prey ratios of 0.08, 0.2 and 0.3 increased in time during the first hour and reached 12, 11 and 31, respectively, by the end of the incubation time (Fig. 2C). Occasionally, the number of aggregates was affected by aggregate to aggregate encounters, which resulted in fewer but larger aggregates. Aggregate sizes at high predator–prey ratios (0.3–0.8) mostly increased until the start of cell lysis (Fig. 2D) and were thereafter driven by the decreasing proportion of aggregates with six or more intact cells and increasing proportion of aggregates with only one intact cell (data not shown). In the control wells *Mesodinium rubrum* cells were evenly distributed in the culture.

Prey attacks and ingestions were relatively rarely observed during this experiment, only on nine occasions (data not shown). Screening of the experimental wells after prey depletion from the cultures showed net population growth of *Dinophysis*. Assuming, that only recently fed cells went through cell division, prey attacks could have been more frequent than recorded by the observer.

3.3. *Effect of exposure time of* D. acuminata *on immobilization of* M. rubrum *at various concentrations*

The first immobile cells of *Mesodinium rubrum* were observed after 10 min of exposure to *Dinophysis* at predator–prey ratios of 0.7 and 0.1. The proportion of immobilized ciliates was highly dependent on the concentration of the ciliates ($p < 0.001$). At high predator–prey ratios of 300, 60 and 9, cell immobilization did not occur (Fig. 3A). At the predator–prey ratio of 3, a time lag of 3 h was observed until the start of cell immobilization. A maximum of 11% of cells from the initial *Mesodinium* population were immobilized in this treatment by the end of the experiment. The highest proportion (17%) of immobile *M. rubrum* cells were found at the predator–prey ratio of 0.7. Significantly smaller proportions of immobilized cells (Tukey post hoc test, $p < 0.001$) were detected at ratios of 0.1–0.3 (4–8%), and 1% at the ratio of 0.04 (Fig. 3A).

The maximum number of prey immobilized per one *Dinophysis* cell was approximately 0.4 at a predator–prey ratio of 0.1, 0.3 at the ratios of 0.3 and 0.04, and 0.2 at the ratio of 0.7 (Fig. 3B). Similarly to experiment 2, cell lysis of immobile cells started after 60 min of incubation, which slowed down the increase of the number of aggregates and aggregate sizes (Fig. 3C, D). The number of aggregates at ratios of 3, 0.7, 0.3, 0.1 and 0.04 reached 9, 15, 12, 30 and 31, respectively, by the end of the incubation time (Fig. 3C). Aggregates were largest at the ratios of 3, 0.7 and 0.3 and started to decrease in size after 2–3 h due to cell lysis (Fig. 3D).

As in the previous experiment, prey attacks and ingestions were relatively rare events, observed only on eight occasions in this

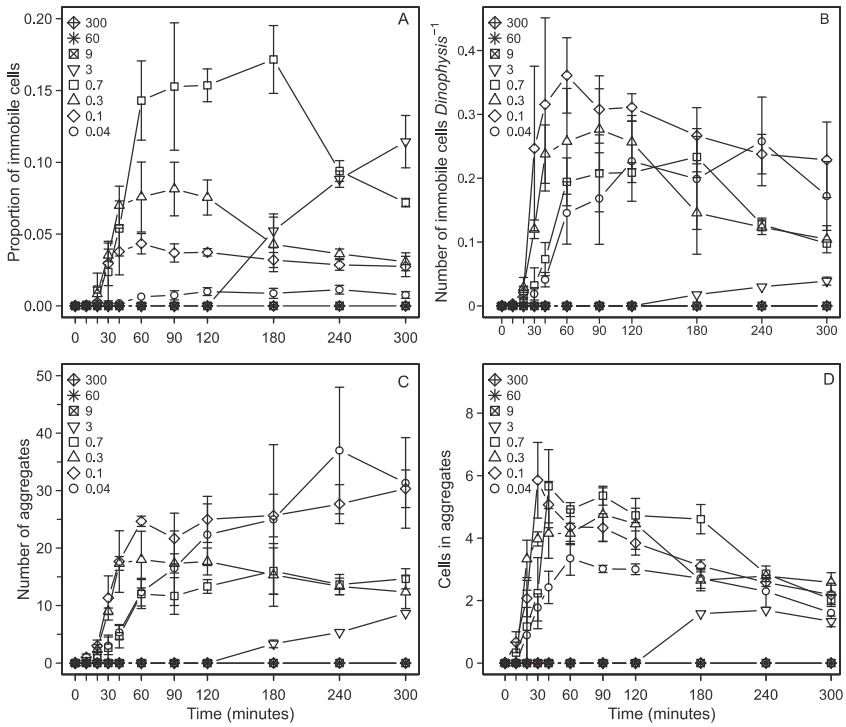

Fig. 3. Results from experiment 3 with fixed concentrations of *Dinophysis* and different concentrations of *Mesodinium*. Proportion of immobilized *Mesodinium rubrum* as a function of exposure time (min) at different treatment concentrations to *Dinophysis acuminata* (A). Number of immobile cells per one *Dinophysis* cell (B). Number of aggregates of *M. rubrum* cells (C) and the number of *M. rubrum* cells per aggregate at each time step (D). Figure legend denotes predator–prey ratios in the 3 mL cultures. Error bars represent standard error of the mean ($n = 3$).

experiment (data not shown). In the control *Mesodinium rubrum* cells were evenly distributed in the wells and aggregate formation was not observed.

4. Discussion

4.1. Immobilization, aggregation and lysis of M. rubrum cells when mixed with D. acuta and D. acuminata

Several species of *Dinophysis* are obligate mixotrophs that rely on red *Mesodinium* spp. as a food for long term growth (Kim et al., 2012; Reguera et al., 2012; Hansen et al., 2013). Yet, mass disappearances of *Mesodinium rubrum* cells due to lysis in presence of *Dinophysis* were observed in the present study. This raises the question as to the nature of the interactions taking place when the predators and prey encounter each other. Observations demonstrating ciliate entrapment in mucus, and ciliate helical swimming or rotation in the same position before becoming completely immobile have been noted earlier for *M. rubrum* in mixed cultures of *Dinophysis caudata* (Nishitani et al., 2008) and *Dinophysis fortii* (Nagai et al., 2008), but exclusively at high *Dinophysis* cell concentrations.

In the present experiments mucus seemed to play a central role in regulating the population size of *Mesodinium rubrum*. The swimming cells of *M. rubrum* were directly observed to jump into elongated mucus threads and getting trapped there. Encounters between normally swimming cells and trapped cells led to the formation of aggregates of *M. rubrum* cells stuck in the mucus. The question that arises is where does the mucus come from? At least two possible interpretations can be delivered: (1) *Dinophysis* cells exude mucus in which the *Mesodinium* cells get entrapped, or (2) *Dinophysis* cells exude toxic compounds (allelochemicals) that make the *Mesodinium* cells leaky and thereafter excrete a lot of mucus, which then entrap other *Mesodinium* cells.

The mucus secretions have been reported on the cell surface of *Dinophysis caudata* (Nishitani et al., 2008) and *Dinophysis fortii* (Nagai et al., 2008), which indicates that mucus production may be characteristic to *Dinophysis*. The cell organelles responsible for the mucus production still remain to be clarified. Members of Dinophysiales possess numerous unique organelles called rhabdosomes (Vesk and Lucas, 1986), which potentially may be involved. The discharge of the mucus could be possible via mucocysts, which are located below the thecal pores of the cell (Lucas and Vesk, 1990). The current data do not provide any explicit proof where the mucus comes from.

Another question raised from present study is why *Mesodinium* immobilization and cell lysis takes place after the cells have come into contact with the mucus? The finding, that the entrapped ciliates became immobile, lost all their cilia, became swollen and lysed indicates that the mucus may contain some kind of toxic compounds which cause these seemingly allelopathic effects on the ciliates. These symptoms are typical responses of the target cells when exposed to their predator (e.g. Hansen, 1989; Tillmann, 1998; Skovgaard and Hansen, 2003). Noteworthy is, that the cells attacked by *Dinophysis* went through identical changes instantly after the peduncle was inserted into *Mesodinium rubrum*, which indicates the possible effect of the same compounds directly released into the prey.

There is little information available on the cellular dynamics of *Dinophysis* toxin production. The *Dinophysis acuta* strain used in the present study, is known to produce all three types of DSP toxins: PTX, OA and DTX (Nielsen et al., 2013), whereas *Dinophysis acuminata* strain used, only produces PTX (Nielsen et al., 2012). Quantitative studies on *Dinophysis* spp. toxicity have found the majority of DSP toxins extracellularly excreted to the surrounding medium (Pizarro et al., 2009; Nagai et al., 2011; Nielsen et al., 2013), however the role of these toxins as allelochemicals with lytic effects on other protists has not been demonstrated so far. Thus, the use of toxins as allelochemicals and the importance to the feeding process remains to be studied further.

4.2. Dinophysis attacks on Mesodinium cells

The *Dinophysis* cells seem to have an advantage over their prey in our experiments as they are able to detect *Mesodinium* cells from a greater distance than is needed by the ciliates to escape from *Dinophysis* cells. This is thought to be possible via hydromechanical and/or chemical sensing (Hansen et al., 2013). In the present experiments, the *Dinophysis* cells changed their helical swimming pattern to a circular movement with an extended peduncle around a ciliate and/or an entire aggregate of prey cells. The change in the swimming pattern indicates that *Dinophysis* cells detected *Mesodinium*. It has been suggested that *Dinophysis* uses a capture filament to initially immobilize the *Mesodinium* cells (Hansen et al., 2013), but it has not been possible in the present study to confirm the use of a capture filament in either of the species studied. Instead, the ciliates were immobilized in the mucus and the first contact between the *Dinophysis* cells and the ciliates was made by the peduncle (Video S1). Since the formation of mucus in *Dinophysis* cultures is a new discovery, the observer in Hansen et al. (2013) may have misinterpreted the observations, which were made at a low magnification.

In the present experiments, *Dinophysis* only fed on live and immobile or semi-immobile prey cells that were already trapped in the mucus. Cell circulations around the cell remains of the ciliates were noticed but no feeding on dead material was observed. Chemical sensing may explain the rotation of *Dinophysis acuminata* around the cell remains of the ciliates scattered over the bottom of the culture vessel. Previous studies have shown the swimming response of *D. acuminata* towards copepod faecal pellets and explained the finding as the response to the leakage of dissolved organic matter of algal origin (e.g. Poulsen et al., 2011). In the present study the *Mesodinium* cell lysis took place about 30–60 min after the entrapment into mucus. As *Dinophysis* only fed on live prey the attack by *Dinophysis* and prey separation from the mucus needs to be well timed. The prey lysis has previously been documented in cultures of *Alexandrium pseudogonyaulax* – a mixotrophic dinoflagellate that secretes a toxic mucus trap, where prey items get stuck prior to ingestion. The lysis of prey items was species specific, fragile cells were lysed within a shorter period compared with other species trapped in the mucus (Blossom et al., 2012).

4.3. Implications of mucus excretion for reported ingestion rates

The results of the present study raise some concerns about published grazing rates. In experiments 2 and 3 the number of *Mesodinium rubrum* cells ingested by *Dinophysis acuminata*, as well as the number of immobile ciliates, was directly counted at each time step through microscopic observation. The decrease of *M. rubrum* abundance due to the cell lysis was apparent as cells with leaking content were observed approximately after 1 h of incubation. Due to lysis of ciliate cells the decrease in average aggregate sizes at high *D. acuminata* and *M. rubrum* cell concentrations (Figs. 2D and 3D) was observed. This lytic response has not been taken into account in published grazing studies on *Dinophysis*. Ingestion rates of *D. acuminata* have been reported to be 3.2–11 prey cells d^{-1} at high prey concentrations (<1000 cells mL^{-1}; Kim et al., 2008; Riisgaard and Hansen, 2009). These rates were determined as the decrease in prey concentration in treatment bottles compared to the concentrations of a monoculture of *M. rubrum* in control bottles. The reported

ingestion rates would be equivalent to 160–440% of body carbon d^{-1}. This is much higher compared to other mixotrophic dinoflagellates, which have been reported to ingest a maximum of 12–36% of its body carbon d^{-1} when fed with ciliates or cryptophytes (e.g. Bockstahler and Coats, 1993; Li et al., 2000; Smalley and Coats, 2002). Therefore, the grazing rates of *M. rubrum* reported so far may be overestimates due to the overlooked impact of *M. rubrum* cell lysis. The magnitude of cells immobilized and lysed in the mucus was relatively high in the course of the present experiments.

4.4. Ecological implications

In the natural environment the *Dinophysis* cell concentrations are usually found to be below 1000 cells L^{-1} but in bloom conditions the abundances reach up to 10^4–10^5 cells L^{-1} (Reguera et al., 2012). Species of *Dinophysis* are frequently found in subsurface thin layers (e.g. Carpenter et al., 1995; Setälä et al., 2005; Moita et al., 2006; Pitcher et al., 2011). These layers have often been associated with temperature- and salinity-driven density gradients in the water column (Koukaras and Nikolaidis, 2004; Moita et al., 2006; Pitcher et al., 2011), with in situ cell division and accumulation at a certain depth (Velo-Suarez et al., 2008; Escalera et al., 2010), or with vertical migration patterns (Reguera et al., 2003, 2012; and references therein). In addition, the vertical location of the cells in the water column and the dynamics of the *Dinophysis* population, may be influenced by the biological processes, such as availability of prey.

In bloom conditions, in the Baltic Sea, the concentrations of *Mesodinium rubrum* are found of the order 10^5–10^6 cells L^{-1} (Sjöqvist and Lindholm, 2011; Crawford and Lindholm, 1997; Lips and Lips, 2014). The populations of *M. rubrum* could be controlled by *Dinophysis* in nature, provided that predator and prey biomass maxima co-occur in the same vertical and horizontal space. Several studies report that even if these species co-occur in the same water mass, they do not always share the same vertical distributions (Gonzalez-Gil et al., 2010; Lips and Lips, 2014; Velo-Suarez et al., 2014; and references therein). The peak densities of *Dinophysis* spp. have been frequently observed to be preceded by or coincide with high densities of *M. rubrum* in space and time (e.g. Mouritsen and Richardson, 2003; Sjöqvist and Lindholm, 2011; Lips and Lips, 2014; Velo-Suarez et al., 2014).

To understand the dynamics of *Dinophysis* and *Mesodinium* populations in nature, the present study demonstrated the role of mucus in trapping the prey cells prior to *Dinophysis* attacks. The proportion of trapped *Mesodinium* cells was dependent on the concentration of *Dinophysis* and *Mesodinium*. The maximum proportion of cells immobilized was as high as 17% at high *Dinophysis acuminata* (100 cells mL^{-1}) and *Mesodinium rubrum* (408 cells mL^{-1}) abundances. Thus, the present data indicate that the negative impact of the predators on the prey population is not only expressed with the direct feeding impact, but the cell lysis of *M. rubrum* after swimming into mucus threads plays a major role in *Mesodinium* population dynamics.

5. Conclusions

The present study investigated the fate of the ciliates in cultures with *Dinophysis* species. The entrapment, immobilization and formation of aggregates of *Mesodinium* cells in the mucus threads seemed to be an important aspect in *Dinophysis* feeding behaviour. Only live prey entangled in the mucus threads were attacked by *Dinophysis*. The prey cells were detected from a distance and a peduncle was used to create contact with the cells and to suck out the cell contents. The proportion of mucus-entrapped prey cells was influenced by the concentration of *Dinophysis* and *Mesodinium* and by the ratio of the two species. Lysis of *Mesodinium* cells in about 1 h after the entrapment or contact with mucus was observed. At high cell concentrations of prey and predator, a maximum of 17% of *Mesodinium* cells became immobile and went through cell lysis. This phenomenon must be taken into account in the prey uptake rate calculations of *Dinophysis*.

The collected data suggest that the negative impact of *Dinophysis* on the *Mesodinium* population is not solely by the means of direct feeding, but also due to the cell lysis of *Mesodinium*. The suggestion that the impact of *Dinophysis* species on *Mesodinium* population in the nature could be direct (through mucus secretion by *Dinophysis*) or indirect (potential exudation of allelochemicals by *Dinophysis*) was made. The feeding activity seems to be a complex behavioural trait, which we do not yet fully understand.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2016.02.001.

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Paper 2

Hansen, P.J., Ojamäe, K., Berge, T., Trampe, E.C.L., Nielsen L.T., Lips, I., Kühl, M. (2016). Photoregulation in a kleptoplastidic dinoflagellate, *Dinophysis acuta*. Frontiers in Microbiology 7:785.

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Photoregulation in a Kleptochloroplastidic Dinoflagellate, *Dinophysis acuta*

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Some phagotrophic organisms can retain chloroplasts of their photosynthetic prey as so-called kleptochloroplasts and maintain their function for shorter or longer periods of time. Here we show for the first time that the dinoflagellate *Dinophysis acuta* takes control over "third-hand" chloroplasts obtained from its ciliate prey *Mesodinium* spp. that originally ingested the cryptophyte chloroplasts. With its kleptochloroplasts, *D. acuta* can synthesize photosynthetic as well as photoprotective pigments under long-term starvation in the light. Variable chlorophyll fluorescence measurements showed that the kleptochloroplasts were fully functional during 1 month of prey starvation, while the chlorophyll *a*-specific inorganic carbon uptake decreased within days of prey starvation under an irradiance of 100 μmol photons m^{-2} s^{-1}. While *D. acuta* cells can regulate their pigmentation and function of kleptochloroplasts they apparently lose the ability to maintain high inorganic carbon fixation rates.

Keywords: acquired phototrophy, *Dinophysis*, kleptochloroplasts, photoregulation, photosynthesis

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INTRODUCTION

Some free-living phagotrophic protists sequester chloroplasts from their algal prey and utilize them for shorter or longer time; a life style that is common among ciliates, dinoflagellates and radiolarians (e.g., Stoecker et al., 2009; Johnson, 2011). In many such species, however, other prey cell organelles are retained as well. Red tide ciliates, *Mesodinium* spp., ingest certain cryptophyte prey species and sequester not only the chloroplasts (Johnson et al., 2006; Moeller et al., 2011; Hansen et al., 2012), but also a number of other prey cell organelles, such as mitochondria, the prey nucleus, and the nucleomorph (a reduced former nucleus of an earlier endosymbiont found in cryptophytes). These red-pigmented *Mesodinium* spp. can keep the ingested prey organelles functionally active for several months gaining enough photosynthate for survival in periods of low prey abundance (Johnson and Stoecker, 2005; Hansen and Fenchel, 2006; Johnson et al., 2006, 2007; Smith and Hansen, 2007). In fact, the ingestion of only a single cryptophyte prey cell per day (∼1% of daily carbon needs) is sufficient to maintain maximum growth rate (Smith and Hansen, 2007). The red-pigmented *Mesodinium* spp. can control the division of its ingested prey organelles (Hansen and Fenchel, 2006), but this ability seems to get lost after a few cell divisions in prey-starved cultures probably due to loss of prey nuclei material (Johnson et al., 2007; Moeller et al., 2011). Red-pigmented *Mesodinium* spp. display photoacclimation, i.e., increases in cellular photosynthetic pigments at low irradiance and a change in photosynthesis vs. irradiance response curves (Johnson et al., 2006; Moeller et al., 2011).

In other protist species, only prey chloroplasts are sequestered and typically only remain functional in the predator cell for hours up to a few days. However, in some cases such kleptochloroplasts are kept active over several weeks, allowing kleptochloroplastidic predators to survive periods of prey starvation a lot better than purely heterotrophic protists (Stoecker et al., 2009). One conspicuous case is found among the dinoflagellate genus *Dinophysis*, which are associated with diarrheic shellfish poisoning worldwide. Species within this genus have chloroplasts of cryptophyte origin and the first culture of a *Dinophysis* spp. was established by feeding it a red-pigmented *Mesodinium* spp. suggesting that its cryptophyte chloroplasts were supplied from the ciliate prey (Park et al., 2006). This was later confirmed by Kim et al. (2012) and *Dinophysis* spp. can thus employ "third-hand" chloroplasts. A total of eight species have now been cultured all relying on red-pigmented *Mesodinium* spp. as prey (Hansen et al., 2013). *Dinophysis* spp. use a peduncle to suck out the contents of the ciliate prey. *Dinophysis* spp. do not ingest cryptophytes directly and thus rely entirely on *Mesodinium* spp. for the supply of chloroplasts and food. There are no direct evidence suggesting that *Dinophysis* spp. may feed other protists, detritus, or bacteria (Poulsen et al., 2011; Hansen et al., 2013).

It has recently been shown that *Dinophysis acuta* and *D. caudata*, grown together with red *Mesodinium* spp. under low irradiance have an elevated cellular Chl *a* content compared to cells grown at high irradiance (Rial et al., 2013). *Dinophysis* cells grown under low irradiance contain more and larger kleptochloroplasts as compared to cells grown at high irradiance (Nielsen et al., 2012, 2013). This suggests that *Dinophysis* spp. may have the capacity to photoregulate, but how is this possible when neither prey nuclei nor nucleomorphs are retained by *Dinophysis* spp. remains unknown.

The apparent regulation of kleptochloroplasts in *Dinophysis* spp. could work in three ways, alone or in combination: (i) Behavioral regulation: *Dinophysis* spp. may increase the number of chloroplasts incorporated via ingestion when grown under low irradiance and may fuse ingested chloroplasts leading to the observed increase in chloroplast size and cellular Chl *a* content; (ii) Photoregulation: *Dinophysis* spp. may be able to produce Chl *a* and other photosynthetic pigments, while the photosynthetic light response curves are unaffected; (iii) Photoacclimation: If *Dinophysis* spp. perform photoacclimation, an increase in cellular Chl *a* concentration as well as a change in the photosynthetic light response curve is expected. This would indicate full control over the acquired chloroplasts. In the latter two cases, the genes involved in either of such regulations must have been transferred to the host cell. A few chloroplast housekeeping genes have been found in the genome of *Dinophysis acuminata*, but it is unlikely that they alone allow for either photoregulation or photoacclimation (Wisecaver and Hackett, 2010).

In the present study, we investigated the photoregulation potential of *D. acuta* cells deprived of prey while being subjected to low irradiance to test whether photoregulation or photoacclimation occurs in sequestered chloroplasts of cryptophyte origin. For this, we employed a suite of different experimental techniques quantifying cell devisions, photosynthetic performance, inorganic carbon (C_i) uptake, and respiration of *D. acuta* in cultures starved at the initiation of the experiments and incubated at different irradiance levels. We hypothesize that *D. acuta* is only capable of the first of the three options listed above, behavioral regulation, and thus will display no, or limited, control over its kleptochloroplasts during starvation. This would include the lack of ability to synthesize Chl *a* and limited adaptation of photosynthetic light curve response parameters. Thus, we expect to find an exponential decline in cellular Chl *a* content as well as declining values of C_i assimilation rate and photosynthetic performance, as cells divide during prey starvation.

MATERIALS AND METHODS

Organisms and Culture Conditions

Cultures of the cryptophyte *Teleaulax amphioxeia* (K-1837; SCCAP) and the ciliate *Mesodinium rubrum* (MBL-DK2009) were established from water samples from Helsingør Harbor in 2009. Cultures of *M. rubrum* were fed *T. amphioxeia* at a predator:prey ratio of 1:10 twice a week. Cultures of *D. acuta* were established in June 2010 from the North Sea (Nielsen et al., 2013). *M. rubrum* was added as prey organism twice per week at a predator:prey ratio of ∼1:10 to allow mixotrophic growth. Only *M. rubrum* cultures that had completely removed their cryptophyte prey were used to feed *D. acuta*. All three species were grown in f/2 medium (Guillard, 1983) based on autoclaved seawater with a salinity of 32, a dissolved inorganic carbon (DIC) concentration of 2.3 ± 0.1 mmol l^{-1} and a pH of 8.0 ± 0.05. pH was monitored directly in the flasks with a SenTix41® pH electrode (WTW, Germany) connected to a pH meter (WTW, pH 3210, Germany), and calibrated by measurements in pH 7 and pH 10 standard buffers (WTW, Technischer, NIST, buffers). All organisms were grown at 15.0 ± 1.0°C in a temperature regulated room, under a photon irradiance of 100 μmol photons m^{-2} s^{-1} (PAR, 400–700 nm), as controlled by a timer to a light:dark cycle of 16:8 h, unless otherwise stated. Culture flasks were placed on a glass table with light coming from below. Light was provided by cool white fluorescent tubes (OSRAM 58W, 840) and photon irradiance was measured (in air) at the level of incubation flasks using a light meter equipped with a spherical quantum sensor (ULM & US-SQS/L, Walz GmbH, Germany). All cultures were xenic.

Experimental Design

Experiment 1

This experiment was designed to study photoregulation in a recently prey starved culture of *D. acuta*. A culture of *D. acuta* was maintained in 750 ml tissue culture flasks filled with 500 ml culture medium under a photon irradiance of 100 μmol photons m^{-2} s^{-1} with prey for at least 2 weeks prior to the experiment and was eventually allowed to deplete its ciliate prey (below detection limit which was a few cells ml^{-1}). At the initiation of the experiment, subsamples of the culture were split in two and poured into 270 ml tissue culture flasks to capacity at a *D. acuta* cell density of 200 cells ml^{-1} (in triplicate). One set

of flasks were maintained at 100 μmol photons m^{-2} s^{-1} (I_{100}), while the other set of flasks was shifted to a photon irradiance of 15 μmol photons m^{-2} s^{-1} (I_{15}). Subsamples were withdrawn on day 3, 6, 9, 13, 16, and 29 (I_{100}), and day 4, 7, 10, 14, 17, and 30 (I_{15}) for measurements of cell concentration, ^{14}C fixation, algal pigment concentration using both fluorometry and HPLC techniques, photosynthetic capacity using variable chlorophyll fluorimetry, and O_2 optode-based respiration measurements (see descriptions of these techniques below).

pH was checked on each sampling occasion to avoid physiological effects of elevated pH in laboratory cultures (Hansen, 2002). Any cryptophytes left in the incubation flasks at the start of the experiment grew to concentrations where they could affect pH, especially at I_{100}. As the *Dinophysis* culture increased in cell concentration they also affected the pH of the culture medium. Therefore, we removed most of the culture medium once each week during the experiment, using gentle inverse filtration, and applied fresh f/2 culture medium to ensure optimal growth conditions (i.e., nutrients, pH, etc.). A plastic tube with plankton gauze (mesh size 20 μm) attached to the tip was lowered directly into the experimental flasks and ∼90% "old" growth medium was removed gently using a pipette and replaced with fresh growth medium. This was repeated three times each time.

Experiment 2

This experiment was carried out to measure Chl *a*, and physiological rates in well fed cultures of *D. acuta* at an irradiance of 100 μmol photons m^{-2} s^{-1}. Cultures were maintained in 750 ml tissue culture flasks filled with 500 ml culture medium prior to the experiment. At the initiation of the experiment, 270 ml tissue culture flasks were filled to capacity with an initial concentration of 400 *D. acuta* cells ml^{-1} and 1000 *M. rubrum* cells ml^{-1} (in triplicate). Samples were withdrawn three times over 6 days for measurements of cell concentration, ^{14}C fixation, photosynthetic capacity using variable chlorophyll fluorimetry, and O_2 optode-based respiration measurements (see descriptions of these techniques below). Fresh growth medium was added at each sampling to replace the water volume removed. *Dinophysis* cells were picked individually using a drawn Pasteur glass pipette and transferred to clean growth medium twice to remove all *M. rubrum* cells before measurements.

Cell Enumeration

Cells were fixed in Lugol's solution (1% final conc.) and were enumerated using a Sedgewick Rafter cell and an inverted microscope (Olympus®, CK-40). A minimum of 200 *Dinophysis* cells were counted each time and all samples were checked for possible lefteover of ciliate prey cells.

Photosynthetic Pigments

Fluorimetric Measurements of Algal Pigments

Cellular chlorophyll concentration was measured by extracting chlorophyll of single cells according to Skovgaard et al. (2000). One ml of 96% ethanol was added to the borosilicate measuring vials. A total of 80 individual *Dinophysis* cells were pipetted directly into the ethanol and placed in the dark. This process took place in a temperature-regulated room kept at experimental temperature. The chlorophyll *a* concentration (μg Chl *a* l^{-1}) was measured in the extract after 20 min using a bench-top fluorometer (Trilogy, Turner designs, USA) equipped with the manufacture's module for Chl *a* determination. The fluorometer was calibrated against a pure Chl *a* standard (2.13 mg Chl *a* l^{-1}) of cyanobacterial origin (DHI, Hørsholm, Denmark).

Additionally, we measured the *in vivo* chlorophyll, and phycoerythrin fluorescence using the appropriate filter set modules for the fluorimeter. For this, 80 individual *Dinophysis* cells were isolated and transferred into plastic measuring cuvettes containing 1 ml of fresh medium. The cells were kept in suspension by carefully blowing air with a pipette immediately before the measurements. Due to high variation between single measurements, the *in vivo* fluorescence was measured every second and averaged over a period of 10 s.

Photopigment Analysis

We analyzed the pigment composition of *D. acuta* with high-pressure liquid chromatography (HPLC) using a slightly modified method from Frigaard et al. (1996) measuring the major light harvesting pigment Chl *a*, the photoprotective pigment alloxanthin, and the ratio between the two in *D. acuta* throughout the starvation experiments. For pigment conservation prior to HPLC analysis, 200 *D. acuta* cells were picked with a micropipette and transferred twice into fresh growth medium, before they were spun down at 10 g for 5 min. The supernatant was removed, and the pellet was frozen at –80°C. Before HPLC analysis, each pellet containing 200 *D. acuta* cells was resuspended for pigment extraction in 125 μl of an acetone-methanol (7:2, vol/vol) mixture and sonicated using an ultrasonicator (Misonix 4000, Qsonica LLC., Newtown, CT, USA) under dim light for 20 s. The sonicated cells were extracted in darkness on ice for 2 min. The extracts were briefly centrifuged to pellet cell debris, and the supernatants were mixed with 15 μL ammoniumacetate (1 M) in 0.3 ml HPLC vials. A 100 μL sample of the mixture was then immediately injected into the HPLC.

Pigment extracts were separated and analyzed by a diode array detector connected to the HPLC system (HPLC-DAD & Agilent 1260 Infinity, Agilent Technologies, Santa Clara, CA, USA) fitted with a Nova-pak C18 column (dimensions: 3.9 × 300 mm), detecting specific absorption wavelengths of compounds. The extracts were run at a column temperature of 30°C for 69 min. Injected extracts moved with a flow-rate of 1.0 ml min^{-1} in solvent A (methanol:acetonitrile:water, 42:33:25, vol/vol/vol), and solvent B (methanol:acetonitrile:ethyl acetate, 50:20:30, vol/vol/vol). In the separation process, the mobile phase changed linearly from 30% solvent B at the time of injection to 100% at 52 min, staying at 100% for 15 min and then falling back to 30% within 2 min. Chl *a* and alloxanthin were identified manually from HPLC chromatograms, and pigment ratios were calculated from the derived integrated peak areas using the manufacturers software (OpenLAB CDS ChemStation Edition, Agilent Technologies, Santa Clara, CA, USA).

Variable Chlorophyll Fluorescence Analysis

We assessed the photosynthetic capacity of *D. acuta*, mainly by means of evaluating the dark adapted maximum PSII quantum

yield, F_v/F_m as measured by the pulse saturation method with pulse-amplitude modulated (PAM) fluorimeters (Schreiber et al., 1994). Individual cells were imaged with a variable chlorophyll fluorescence imaging system (RGB Microscopy PAM, Walz GmbH, Germany; Trampe et al., 2011), while bulk culture samples were investigated in a cuvette-based chlorophyll fluorimeter (MULTI-COLOR-PAM; Walz GmbH, Germany). Detailed descriptions of the two types of PAM measurements, and definitions of the parameters used, are provided in the Supplementary Material, Section Material and Methods.

Sample preparation – Imaging PAM

1.5 ml subsamples of *D. acuta* culture were transferred to 1.5 ml tubes and centrifuged at 43 g for 2 min. The 1.4 ml supernatant was removed, and the pellet was resuspended in the remaining 0.1 ml yielding a concentrated sample facilitating a low search time for cells of interest at high magnification under the microscope. Control experiments showed no significant effect on cell structure or photosynthetic capacity by the treatment (data not shown). A droplet (16.5 μL) of concentrated sample was transferred to a special treated microscope slide displaying a permanently positive charge (Superfrost Ultra Plus, Thermo Scientific, Gerhard Menzel GmbH, Braunschweig, Germany) resulting in good cell adhesion by electrostatic attraction, limiting motion of cells during imaging. Variable chlorophyll fluorescence imaging measurements rely on recording of consecutive images when calculating photosynthetic parameters, and it is thus crucial that there is no cell movement during the saturation pulse. The sample was sealed with a cover glass using petroleum jelly (Vaseline™) at the periphery to avoid evaporation, and the slide was placed at 12°C in a thermostated slide holder (see details in Trampe et al., 2011).

Sample prepation – MULTI-COLOR PAM

For each sample, 1000 (Experiment 1) or 300 (Experiment 2) cells were isolated with a drawn out Pasteur pipette and transferred several times through sterile-filtered seawater before final transfer into quartz cuvettes and the volume was adjusted to 1 ml using 30 psu sterile-filtered f/2 medium. Samples were stirred with a magnetic stirrer during measurements.

Inorganic Carbon Uptake and Respiration

^{14}C Incorporation

Subsamples (1–2 ml) were removed from each culture flask for photosynthesis measurements. A total of 80 *D. acuta* cells were picked from the subsamples and washed in f/2 medium by micromanipulation using a stereoscope to remove all prey. A total of 40 *D. acuta* cells were transferred to each of two 20 ml glass scintillation vials containing 2 ml f/2 medium, and 20 μl of a $NaH^{14}CO_3$ stock solution (specific activity = 100 μCi ml^{-1}; Carbon-14 Centralen, Denmark) was added. One vial was incubated for ∼3 h under the treatment irradiance, while the other was kept in complete darkness (by wrapping in several layers of aluminum foil). After incubation, a 100 μL sub-sample was transferred to a new vial containing 200 μl phenylethylamine for determination of specific activity (see Skovgaard et al., 2000 for a detailed method description). The remaining 1.9 ml were acidified with 2 ml 10% glacial acetic acid in methanol, and evaporated overnight at 60°C to remove all inorganic carbon. The residue was then re-dissolved in 2 ml Milli-Q water. All vials were treated with 10 ml Packard Insta-Gel Plus scintillation cocktail, and disintegrations per minute were measured using a Packard 1500 Tri-Carb liquid scintillation analyzer with automatic quench correction. The photosynthetic activity (PA, pg C $cell^{-1}$ h^{-1}) per cell was calculated as follows:

$$PA = \frac{DPM \times [DIC]}{^{14}C \times h \times N}$$

where DPM is disintegrations min^{-1} (in 1.9ml) in the light corrected for dark values, DIC is the concentration of DIC (pg C ml^{-1}), ^{14}C is the specific activity (disintegrations min^{-1} ml^{-1}), h is the incubation time, and N is the number of cells in the vial (1.9 ml). DIC concentrations were measured on 1 ml subsamples using an infrared gas analyzer (ADC 225 Mk3 Gas analyzer, Analytic Development Co. Ltd., Hoddesdon, England) as described in detail elsewhere (Nielsen et al., 2007). Glass vials with screw caps were used for DIC samples allowing no headspace, and the samples were analyzed within a few hours.

Respiration Measurements

Respiration rates of *D. acuta* were measured in 1.8 ml glass vials ($n = 4$) equipped with calibrated optical O_2 sensor spots with optical isolation (Pyroscience GmbH, Germany). The O_2-dependent luminescence of each sensor spot was monitored contactless across the vial wall using an optical fiber cable fixed at one end to the glass vial using a solid plexi-glass adaptor and connected at the other end to a fiber-optic O_2 meter (FireSting, PyroScience, Germany). The sensor spot readout was calibrated from readings in anoxic and fully aerated medium. A total of 400 *Dinophysis* cells were isolated by micropipetting and washed 3 times in sterile filtered (pore size 0.22 μm) f/2 culture medium prior to addition to each of the 4 glass vials containing 1 ml sterile f/2 medium. A glass bead was added to each of the vials that were filled to capacity with sterile filtered medium and carefully sealed avoiding any air bubbles inside the closed vials. The vials were mounted on a modified whirley mixer run at low speed to homogenize internal O_2 gradients in the sample (Trampe et al., 2015). The measurements were done in darkness in a temperature regulated room (15°C), and O_2 was measured with the built-in temperature correction of the O_2 meter. The O_2 concentration in the glass vials was measured for 1–3 h, and the linear decrease in O_2-concentration (μmol l^{-1} s^{-1}) was converted into cellular respiration rate (pg C $cell^{-1}$ day^{-1}) by assuming a respiratory quotient of 1.

RESULTS

Changes in Photosynthetic Pigments in *D. acuta* Starved of Prey

No ciliate prey cells were observed during enumeration of *Dinophysis* cells for the entire duration the prey starvation experiment. *D. acuta* cultures subjected to prey starvation at high photon irradiance (I_{100} = 100 μmol photons m^{-2} s^{-1}) increased

from 200 to 1400 cells ml^{-1} during 1 month of incubation, equivalent to an average growth of 2.8 cell divisions in total (Experiment 1, **Figure 1A**). The subculture that was exposed to low photon irradiance (I_{15} = 15 μmol photons m^{-2} s^{-1}) increased in cell concentration from 200 to 1000 cells ml^{-1} and thus had <2.3 cell divisions during the 1 month of incubation (**Figure 1A**). The cellular Chl *a* concentration in *D. acuta* incubated at I_{100} decreased exponentially from the initial ∼53–16 pg Chl *a* cell^{-1} (**Figure 1B**), leading to an overall increase in Chl *a* concentration from 11 to a maximum of 26 ng Chl *a* ml^{-1} at Day 16 (**Figure 1C**). Cells incubated at I_{15}, however, maintained their cellular Chl *a* concentration during the 1 month long incubation, even though cells divided 2.3 times. This led to an increase in the amount of Chl *a* in these incubations from 11 to 46 ng Chl *a* ml^{-1} at Day 30, indicating a higher net production of Chl *a* production in prey-starved *Dinophysis* cells grown at I_{15}, compared to those at I_{100} (**Figure 1C**). For comparison, well fed cultures of *D. acuta* grown at I_{100} had a cellular Chl *a* concentration of ∼40 pg Chl *a* cell^{-1} (Experiment 2).

Cultures exposed to I_{100} exhibited a slight increase in the alloxanthin:Chl *a* ratio from 0.86 to ∼1.2 over the first 10 days. Hereafter, the ratio remained constant indicating that Chl *a* was initially lost at a faster rate than alloxanthin (**Figure 2A**). With cultures exposed to I_{15}, a significant decrease in the alloxanthin:Chl *a* ratio from 0.86 to 0.7 was observed over the first 2 weeks indicating dilution due to growth, which was followed by an increase of the alloxanthin:Chl *a* ratio reaching ∼1 at the end of the experiment. *In vivo* measurements of Chl *a* and phycoerythrin showed that the changes in phycoerythrin content under the two experimental irradiance regimes matched those found in Chl *a* (**Figure 2B**).

Single-Cell Variable Chlorophyll Fluorescence Imaging

In well fed cultures of *D. acuta,* incubated at an irridiance of 100 μmol photons m^{-2} s^{-1}, microscopy revealed a broad distribution of cellular Chl *a* with hotspots clearly defining 4–5 chloroplast centers with high fluorescence. Maximum PSII quantum yields (F_v/F_m) of ∼0.5 in these centers indicated a healthy and functioning photosynthetic apparatus (**Figure 3A**). During the starvation experiment at I_{100}, we found a continuously declining Chl *a* coverage in the cells with incubation time (**Figures 3B–D**). After a slight increase in F_v/F_m over the first 2 days, F_v/F_m decreased with the declining chlorophyll coverage (**Figure 3B**; Supplementary Figure S1). At I_{15} we observed a rapid increase in F_v/F_m to ∼0.6 over the first 3 days, which remained high even after 10 days, while the Chl *a* coverage had decreased a bit (**Figures 3E,F**; Supplementary Figure S1). After 30 days, we still observed an even coverage, almost similar to the well-fed cells, with similar condensation in four chloroplast centers, and with a sustained high level of F_v/F_m (**Figure 3G**).

Bulk Measurements of Photochemical and Non-photochemical Quenching

Measurements on bulk cell samples yielded more insight into the photochemical, and non-photochemical quenching processes

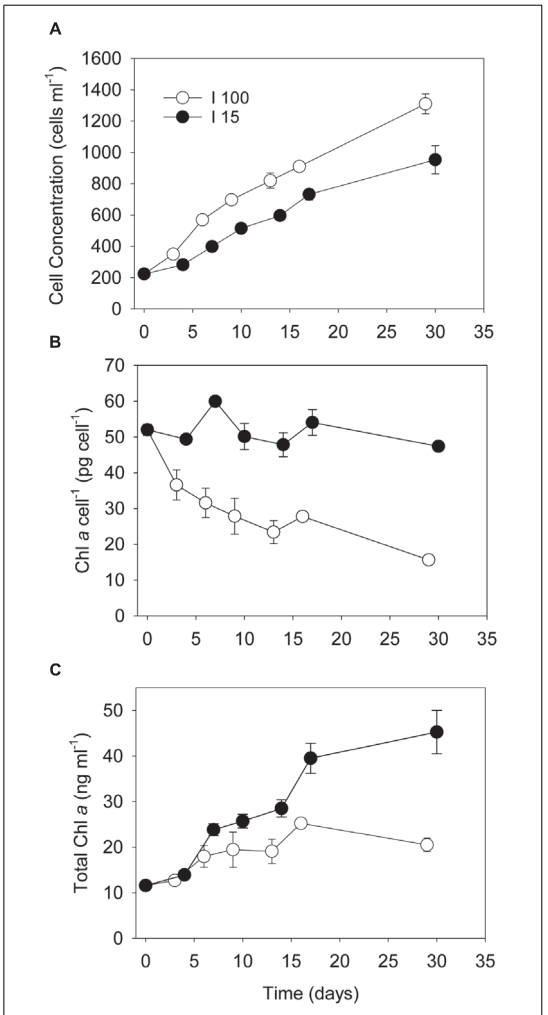

FIGURE 1 | Developments in *Dinophysis acuta* cell concentrations, cellular Chl *a* content and total Chl *a* concentrations when subjected to prey starvation at the start of the experiment and incubated for 1 month under an irradiance of (o) 100 μmol photons m^{-2} s^{-1} (I_{100}) and (•) 15 μmol photons m^{-2} s^{-1} (I_{15}), respectively. (A) cell concentration (cells ml^{-1}), **(B)** cellular Chl *a* concentration (pg Chl *a* cell^{-1}), **(C)** total Chl *a* concentration (ng Chl *a* ml^{-1}). Data points represent treatment means and error bars indicate standard errors (n = 3).

in starved *D. acuta* cultures. The maximum PSII quantum yield (F_v/F_m) was on average higher for cells at I_{15} (0.74 ± 0.04; average ± SE, n = 18) than I_{100} (0.65 ± 0.05, n = 21; **Figure 4A**). In both treatments, F_v/F_m showed an initial increase during the 1 week, but only cells at I_{15} were able to maintain a high F_v/F_m of ∼0.76, indicating better photosynthetic capacity than cells at I_{100}. The change in F_v/F_m resulted primarily from the change in

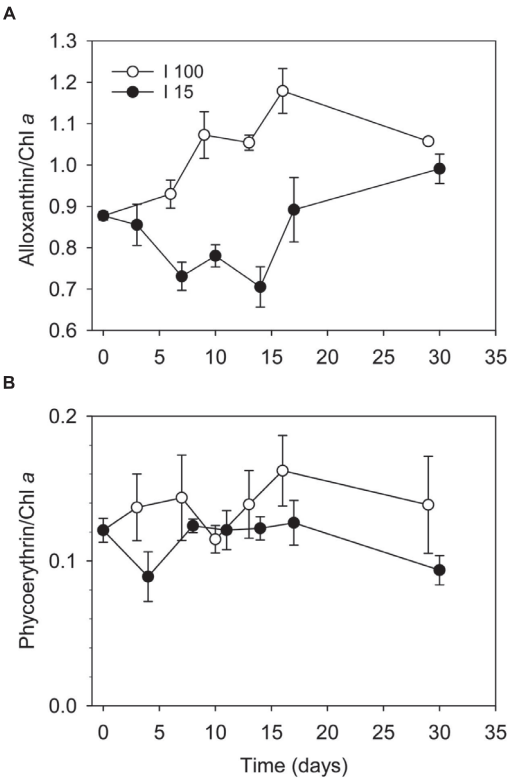

FIGURE 2 | Changes in the ratio of (A) alloxanthin:Chl *a* ratio, and (B) phycoerythrine:Chl *a* ratio in *D. acuta* cells during the starvation experiment under irradiances of I_{100} (o) and I_{15} (•), respectively. Data points represent treatment means, and error bars indicate standard errors ($n = 3$).

the fraction of "open" PSII reaction centers, qP (Supplementary Figure S2A). An increase in qP to 0.94 ± 0.02 ($n = 15$) at I_{15} indicates a decrease in the proportion of "closed" PSII centers due to lower excitation pressure, than compared to cells at I_{100}, which had a lower qP of 0.67 ± 0.02 ($n = 21$) throughout the starvation experiment.

Absorbed light energy partitioning profiles for *D. acuta* (Supplement Figures S2A,B) show a photoregulative change in response to changes in photon irradiance. The use of excitation energy for photosynthesis (ϕ_{II}) in cultures kept at I_{100} was lowered with respect to F_v/F_m by partial closure of PSII centers (Supplementary Figure S2A), and non-photochemical energy losses (ϕ_{NPQ} and ϕ_{NO}) induced by illumination. Only 38% of the absorbed light energy was used for photosynthetic reactions (Supplementary Figure S2B), whereas cells kept at I_{15} used 70% of the absorbed light for photosynthesis (Supplementary Figure S2C).

The maximum relative electron transport rate ($rETR_{max}$), and the initial slope of the light response curves (α rETR) showed no changes between the treatments (**Figures 4B,C**). For cultures incubated at I_{100}, the average $rETR_{max}$ and α rETR values were 76.3 ± 8.14 and 0.25 ± 0.01 ($n = 21$), respectively. Cultures at I_{15} showed average $rETR_{max}$ and α rETR values of 88.2 ± 2.58 and 0.31 ± 0.02 ($n = 18$), respectively, (**Figures 4B,C**).

Inorganic Carbon Uptake and Respiration before and during Starvation

Inorganic carbon uptake was 138 ± 6 pg C $cell^{-1}$ h^{-1} (average ± SE) in well fed cultures of *D. acuta* at I_{100}, equivalent to 2.21 ± 0.94 ng C $cell^{-1}$ day^{-1} (light:dark cycle of 16:8 h), leading to a Chl *a* specific rate of 3.5 ± 0.5 pg C pg Chl a^{-1}. The inorganic carbon uptake of *D. acuta* cells subjected to starvation at I_{100} decreased exponentially from 177 ± 14 to 23.7 ± 4.2 pg C $cell^{-1}$ h^{-1} at Day 16 (equivalent to 2.83 ± 0.23 and 0.38 ng C $cell^{-1}day^{-1}$, respectively, **Figure 5A**), i.e., a 6.1-fold decrease in photosynthetic carbon fixation. This translated into a significant decline in the Chl *a*-specific carbon uptake from the initial 3.4 to 1.0 pg C pg Chl a^{-1} h^{-1} at Day 16 (one-way ANOVA, $P < 0.05$; **Figure 5B**). The following 2 weeks, the inorganic carbon uptake rate dropped further to 16.8 ± 1.1 pg C $cell^{-1}$ h^{-1} (=0.27 ± 0.02 ng C $cell^{-1}$ day^{-1}), but the Chl *a*-specific carbon uptake stayed constant (1.07 pg C pg Chl a^{-1} h^{-1}).

Under low irradiance (I_{15}), inorganic carbon uptake remained constant for the first 17 days at 33.1 ± 2.8 pg C $cell^{-1}$ h^{-1} (=0.53 ± 0.03 ng C $cell^{-1}$ day^{-1}), and dropped only marginally to 20.7 ± 1.9 pg C $cell^{-1}$ h^{-1} (=0.33 ± 0.03 ng C $cell^{-1}$ day^{-1}) after 1 month of incubation. Thus, the Chl *a*-specific inorganic carbon uptake rates of the starved low irradiance *D. acuta* stayed constant at ∼0.6–0.7 pg C μg Chl a^{-1} h^{-1} during the entire experiment.

Well-fed cells of *D. acuta* had a respiration rate of 86.5 ± 0.16 pg C $cell^{-1}$ h^{-1} at I_{100}, equivalent to 2.08 ± 0.38 ng C $cell^{-1}$ day^{-1} (average ± SE, $n = 9$). No difference in respiration rate was observed as a function of prey starvation after 1 day of starvation in either of the light treatments, thus data were merged for each irradiance (One way-ANOVA, $P > 0.05$). Data on respiration of prey starved cells are only available until day 16 and 17 for the high and low irradiances, respectively. Starved cells had approximately halved their respiration rates [42.9 ± 7.1 ($n = 11$) and 36.2 ± 6.6 ($n = 12$) pg C $cell^{-1}$ h^{-1} at I_{100} and I_{15}, respectively]; respiration rates at the two irradiances were not statistically different (Two way-ANOVA, $P > 0.05$).

DISCUSSION

The present study documents for the first time that kleptochloroplasts (without prey nuclear material) taken up by *D. acuta* exhibit photoregulation, where photosynthetic pigments are produced to improve growth under both high and low irradiances. However, we saw no changes in photosynthesis vs. irradiance parameters like α rETR or max rETR during 1 month of starvation, similar to what is usually found in regular

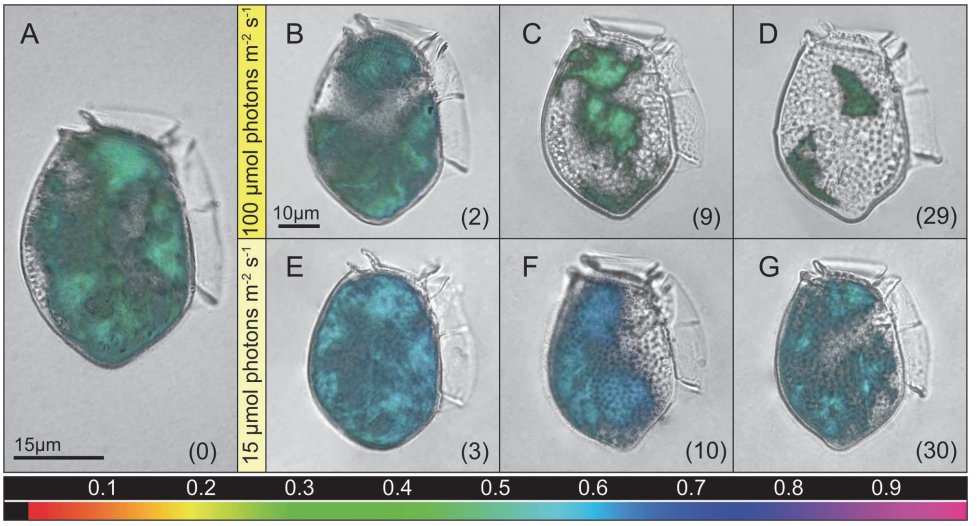

FIGURE 3 | Single cell variable chlorophyll fluorescence imaging. Maximum PSII quantum yield (F_v/F_m) of single *D. acuta* cells during the starvation experiment. Images display cells at different time points with the quantum yield as an overlay in false color (values corresponding to the color scalebar). A culture representative image for fed cells growing at I_{100} prior to starvation **(A)**, and culture representative images for starved cells after transfer to irradiances of I_{100} **(B–D)** and I_{15} **(E–G)**. Numbers in brackets presents days of starvation, and time maintained under the given irradiance.

algae displaying photoacclimation (e.g., MacIntyre et al., 2001). Thus, we found photoregulation but not true photoacclimation in *D. acuta*.

Under low irradiance, photoregulation enabled *D. acuta* to maintain their cellular Chl *a* and phycoerythrin contents even though cells divided several times. We also found that the concentration of the alloxanthin increased relative to Chl *a* in cells exposed to high irradiance, while this pigment was initially reduced in cells incubated under low irradiance. While the role of alloxanthin as a light harvesting or photoprotective pigment in cryptophytes is not well established, the few available reports suggest that the alloxanthin:Chl *a* ratio in cryptophytes is elevated at high irradiances indicating that it may have photoprotective properties (Schlüter et al., 2000; Laviale and Neveux, 2011) and alloxanthin may thus play a similar role in *D. acuta*.

The literature provides no evidence for division of kleptochloroplasts in *Dinophysis* spp., suggesting that the cellular number of chloroplasts will decrease during cell division (Minnhagen et al., 2008). Hence, the ability of *Dinophysis* to maintain cellular photosynthetic pigment concentrations may potentially lead to enlargement (rather than division) of individual chloroplasts (Nielsen et al., 2013). Enlargement of kleptochloroplasts has recently been shown in other dinoflagellates such as *Nusuttodinium aeruginosum* and *N. myriopyrenoides,* where a 20-fold enlargement of the chloroplasts was observed within 120 min after ingestion in *N. aeruginosum* (Yamaguchi et al., 2011; Onuma and Horiguchi, 2015). Up to 10-fold enlargement of ingested chloroplasts has also been observed in the katablepharid *Hatena arenicola* (Yamaguchi et al., 2011). However, in all these cases, prey nuclei and nucleomorphs were retained by the host cell, which is not the case in *Dinophysis* spp.

Variable chlorophyll fluorescence data suggested that chloroplasts in *D. acuta* were competent with maximum PSII quantum yields (F_v/F_m) between 0.5 and 0.8 throughout the duration of the experiment (30 days). These values are similar to what is usually found in well-functioning microalgae (Falkowski and Raven, 2007). Similar observations have been obtained on *D. caudata* cells starved for up to 85 days (Park et al., 2008), where F_v/F_m began to decline after 24 days of starvation, and reached 0.4 after 45 days of starvation, i.e., at the same time as cell divisions stopped. Thereafter, F_v/F_m decreased steadily over the next days and reached zero after 85 days of starvation. We saw no indications of photodamage. Photodamage would be characterized by a decrease in ϕ_{NPQ}, which is one of the most important safety valves for the regulation of light harvest (Müller et al., 2001). Also, if the cells were photodamaged we would have expected to see a consecutive decrease of qP (Mackey et al., 2008), which gives information if photosynthetic efficiency has been altered by a changed proportion of functional reaction centers. In our case, ϕ_{NPQ} was relatively stable (∼20%) and qP was constant in high light (60–70%) throughout the experiment, so the cells were unlikely to experience photodamage.

The prey species of *Dinophysis* spp., i.e., the red *Mesodinium* spp. retain ingested cryptophyte nuclei for up to 100 days and these have been shown to be transcriptionally active (Johnson et al., 2007; Lasek-Nesselquist et al., 2015). However, it has also been shown that the expression of a cryptophyte nuclear-encoded

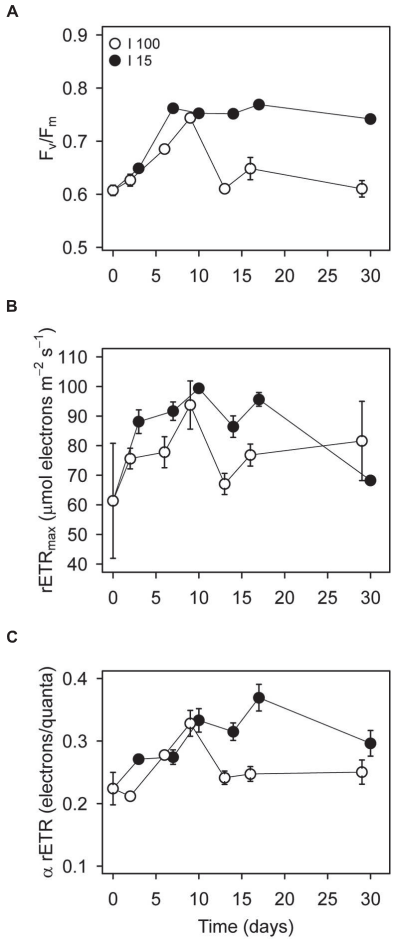

FIGURE 4 | Variable chlorophyll fluorescence measurements on *D. acuta* culture samples incubated at I_{100} (o) and I_{15} (•) as a function of time after initiation of prey starvation. The culture had been growing at I_{100} prior to the initiation of the experiment. **(A)** Maximum PSII quantum yield (F_v/F_m). **(B)** Maximum relative electron transport rates (rETRmax) and **(C)** initial slopes of the light response curves (α rETR), i.e., rETR vs irradiance. Data points represent the mean value parameter estimates obtained from the rapid light response curves. Error bars show standard error of the mean ($n = 3$).

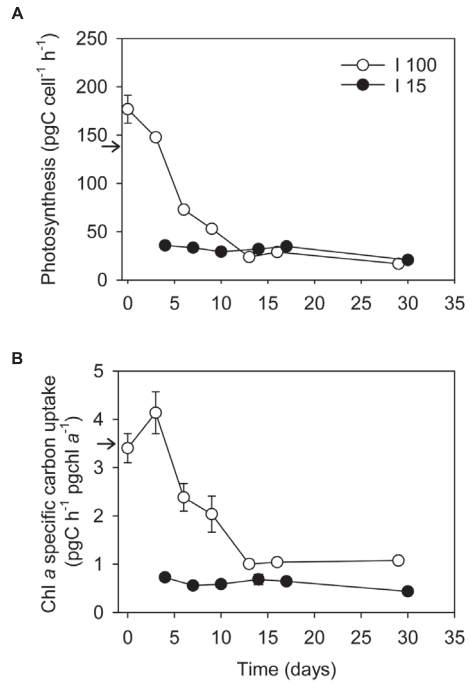

FIGURE 5 | Changes in (A) Cellular inorganic carbon uptake (pg C $cell^{-1}$ h^{-1}), and (B) Chl *a*-specific inorganic carbon uptake (pg C pg Chl a^{-1} h^{-1}) in *D. acuta* during the prey starvation experiment at two irradiances, I_{100} (o) and I_{15} (•), respectively. Data points represent treatment means and error bars indicate standard errors ($n = 3$). Arrows indicate values from well fed cultures for comparison.

gene for the plastid-targeted protein, *LHCC10*, involved in plastid function, declined in *Mesodinium* cells as the sequestered nuclei disappeared from the population (Johnson et al., 2007). In the red *Mesodinium* spp., F_v/F_m values of 0.54–0.66 have been observed when grown for up to a month without prey (Johnson et al., 2006; Kim et al., 2014).

Another kleptochloroplastidic dinoflagellate, *Amylax triacantha,* also preys on red *Mesodinium* spp. like *Dinophysis* spp. (Kim et al., 2014). Besides the cryptophyte chloroplasts, *A. triacantha* also retains cryptophyte mitochondria, cryptophyte nuclei and nucleomorphs. However, unlike in *M. rubrum*, the ingested cryptophytes are kept as individual packages. In *A. triacantha* cultures subjected to prey starvation, F_v/F_m remains at 0.6 for the first 5–8 days, whereafter it declines over time to reach F_v/F_m values of ∼0.05 after 25 days of prey starvation in conjunction with the loss of cryptophyte nuclear material, i.e., a much faster decline in photosynthetic capacity compared to the red *Mesodinium* spp.

Available evidence thus suggests that the cryptophyte chloroplasts are not independent entities, which will function on their own inside a host cell. In the red *Mesodinium* spp. and in *A. triacantha* the photosynthetic performance of kleptochloroplasts is coupled to the sequestration of prey nuclei and nucleomorph material. This is not the case in *Dinophysis* spp. Thus, it seems that the function of chloroplasts in *Dinophysis* may depend upon genes, which in the past have been transferred from prey nuclei and nucleomorphs to the dinoflagellate genome.

Very little is known about the transfer of genes from the cryptophyte genome to the genome of *Dinophysis* spp., and only one species, *D. acuminata*, has been investigated (Wisecaver and

Hackett, 2010). Five proteins, complete with plastid-targeting peptides that may function in photosystem stabilization and metabolite transport, have been found encoded in the nuclear genome of *D. acuminata* (Wisecaver and Hackett, 2010). It seems unlikely that those genes alone allow for the extensive regulation of photosynthetic and photoprotective pigments that we observed in *D. acuta,* and more genes controlling photoregulation in *D. acuta* and other *Dinophysis* spp. remain to be identified.

The Chl *a*-specific inorganic carbon uptake dropped dramatically in cells grown at high irradiance indicating that, although *D. acuta* was able to produce Chl *a*, this did not translate into increased inorganic carbon uptake. There may be several reasons for this observation: First, since *Dinophysis* spp. only sequester the chloroplasts from their prey, any loss of genes involved in the regulation of RuBisCO that are located in the cryptophyte nuclei material are lost and this will strongly affect inorganic carbon fixation. Such genes may come from the prey nucleus, but they may also come from the nucleomorph. The Rubisco regulatory protein, CbbX, for instance, is encoded by the cryptophyte nucleomorph in cryptophytes (Maier et al., 2000).

Second, RuBisCo is located in the single terminally placed pyrenoid, contained by every chloroplast in most algae (Holdsworth, 1971; Giordano et al., 2005; Garcia-Cuetos et al., 2010). As *D. acuta* cannot divide the aquired chloroplasts, it will also be unable to divide its pyrenoids, which seems to be a key missing control factor, lacked by these protists to fully regulate and divide the sequestered "third hand" chloroplasts.

In purely phototrophic dinoflagellates, respiration rates typically account for ∼20 and ∼40% of gross photosynthesis in the exponential and stationary growth phase, respectively (Geider and Osborne, 1989; Geider, 1992; López-Sandoval et al., 2014). In the kleptochloroplastidic *D. acuta*, respiration rates accounted for ∼50% of gross photosynthesis in well fed cells, and that percentage increased when the cells were subjected to prey starvation. Under high irradiance, *D. acuta* cells became full of storage material (unpublished observations, probably lipids and starch; Durand-Clement et al., 1988) upon initiation of prey starvation, in accordance with earlier observations in *D. caudata* (Park et al., 2008). Accordingly, we found excess inorganic carbon uptake during the first ∼9 days under high irradiance, whereafter respiration rates started to slightly exceed rates of inorganic carbon uptake. Under low irradiance, respiration rates slightly exceeded the inorganic carbon uptake at all times. The build up of carbon storage upon onset of prey starvation and maintenance of fully active kleptochloroplasts thus allows *D. acuta* to survive for extended periods of time (months; Park et al., 2008; Nielsen et al., 2012, 2013). This is an important trait of these kleptochloroplastidic dinoflagellates, and explains how they can survive in a fluctuating environment and still depend upon *M. rubrum* as a single type of prey.

CONCLUSION

We found strong evidence of photoregulation via production of photosynthetic and photoprotective pigments in the kleptochloroplastidic dinoflagellate, *D. acuta*, which only retains the chloroplasts from its prey. No direct evidence of changes in photosynthetis vs. irradiance parameters and thus no signs of photoacclimation were observed in *D. acuta* incubated at different irradiances, and thus no signs of photoacclimation were found. A decrease in Chl *a*-specific inorganic carbon uptake in prey-starved cells indicated a dilution of carbon fixing units (Rubisco) among daughter cells in combination with the production of Chl *a*. However, in their natural environment *D. acuta* and the >100 species that belong to the genus *Dinophysis* may also indirectly photoregulate via increased retention of kleptochloroplasts, when prey cells are available. It is also possible that *Dinophysis* cells may indirectly achieve "photoacclimation" by ingestion of photoacclimated prey cells and maintaining a higher number of chloroplasts. Our data point to a hitherto unstudied role of gene transfer from prey to *D. acuta* that may enable it to regulate the function of its kleptochloroplast, although the exact genetic and biochemical mechanisms remain to be identified.

AUTHOR CONTRIBUTIONS

All authors were involved in the design and planning of the experiments. PH prepared the cultures for experimentation and was in charge of sampling scheme, carried out all ^{14}C measurements and data analysis, except for the last sampling where TB carried out the measurements. TB did all respiration measurements and data analysis, isolation of cells for all pigment analyses and carried out fluorometric measurements and connected data analysis of phytopigments. ET carried out all Imaging PAM and HPLC measurements of phytopigments and the connected data analysis. KO carried out all MultiColorPAM measurements and the connected data analysis. PH wrote the manuscript with inputs from all of the co-authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00785

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Paper 3

Ojamäe, K., Peterson, A., Lips, I. Dark nutrient uptake at low temperature and subsequent light use efficiency by *Heterocapsa triquetra*. Marine Biology Research. DOI 10.1080/17451000.2016.1216570

Dark nutrient uptake at low temperature and subsequent light use efficiency by the Dinoflagellate *Heterocapsa triquetra*

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Abstract

For population growth in stratified environments, flagellates are known to migrate to the nutrient-rich water layers below the thermocline to take up inorganic nutrients and ascend to the nutrient-deficient euphotic water layer, to photosynthesize. The present study investigated dark nutrient uptake at 4 °C (characteristic water temperature below the thermocline in the Baltic Sea) by the dinoflagellate *Heterocapsa triquetra*. The recovery of photosynthetic performance and the improvement of cell growth after dark nutrient acquisition and meeting suitable light conditions were also studied. On average, the consumption of NO_3^- and PO_4^{3-} in the absence of light at 4 °C was 0.04 and 0.003 μM h^{-1}, respectively. N:P uptake ratios were similar during dark, cold incubation and a following light-dark cycle. In the nutrient deficient cultures, the effective photochemical yield was lowered to 36% while it recovered to 64% after simultaneous dark incubation and nutrient addition. Increased photosynthetic efficiency yielded a 34% higher cell concentration after incubation in dark, cold nutrient enriched conditions in comparison to the parallel N-deficient cultures that received no nutrients. The results suggest that *H. triquetra* can successfully compromise between dark nutrient acquisition and the use of the internal nutrient storage for photosynthesis later in the light field.

Keywords: dark nutrient uptake, dinoflagellates, *Heterocapsa*, photoregulation, Pulse-Amplitude-Modulation (PAM)

Introduction

During spring and summer the development of temperature stratification is common in temperate seas. In April-May, a seasonal thermocline starts developing in the Baltic Sea. The upper mixed layer with a typical depth of 10-20 m and temperature of 15-20 °C becomes separated from the cold intermediate layer of 2-4 °C by mid-June. After the spring bloom, the inorganic nutrients are depleted in the euphotic layer, and a density gradient prevents mixing between the nutrient poor and nutrient rich water layers. In late July, during the period of strongest stratification and inorganic nutrient deficiency in the surface layer, high biomass concentrations of the dinoflagellate *Heterocapsa triquetra* (Ehrenberg) Stein are often found. Over the same period, layers of sub-surface and deep

chlorophyll *a* maxima (DCM) are reported in different areas of the Baltic Sea (Hällfors et al. 2011; Kononen et al. 2003; Lips & Lips 2014; Lips et al. 2010, 2011; Pavelson et al. 1999). These phytoplankton biomass maxima have mostly been detected at the depth of the nitracline (15–35 m), which usually coincides with the base of the seasonal thermocline (e.g. Lips et al. 2010). In the Gulf of Finland in July/August, DCM are mostly dominated by the dinoflagellate *H. triquetra*, although *Dinophysis* spp. and photosynthetic ciliate *Mesodinium rubrum* (Lohmann) may also contribute to these maxima on some occasions (Hällfors et al. 2011; Kononen et al. 2003; Lips & Lips 2014).

The DCM can often be an indication of phytoplankton migration patterns. Migrations to the deep nutrient-rich layers to harvest inorganic nutrients have been shown in flagellates to overcome nutrient limitation (Cullen & Horrigan 1981; Doblin et al. 2006; Heaney & Eppley 1981; Jephson & Carlsson 2009). At night, cells may descend to the dark and deep nutrient-replete layers below the thermocline to take up inorganic nutrients. Cells ascend to the nutrient-deficient euphotic layer during the day, where they use the incident solar radiation for photosynthesis. Vertical sampling and high-resolution autonomous vertical profiling have demonstrated diel and bi-diurnal migration patterns for a phytoplankton community dominated by *H. triquetra* (Lips & Lips 2014). Migrations to the deeper nutrient-rich layers have been hypothesised to explain the high biomass values of this species in the nutrient-depleted surface layer in July in the Gulf of Finland (e.g. Lips et al. 2011; Lips & Lips, 2014). In fact, a cost-benefit analysis by Raven & Richardson (1984) hypothesises that dinoflagellates win energetically from the vertical migration behaviour even when the energy and nitrogen costs (synthesis and operation of the flagellar apparatus, transport and storage capacity for nitrate) have been taken into account. Moreover, nutrient assimilation in the dark has been shown to support the growth of many dinoflagellates (Cullen & Horrigan 1981; MacIntyre et al. 1997; Olsson & Granéli 1991). The question arises – does *H. triquetra* perform vertical migrations for nutrient acquisition, and does it take up sufficient nutrients in the deep, dark, cold waters to allow it to survive and bloom in the nutrient deficient Baltic Sea surface layer in late summer?

In laboratory conditions, the dark nutrient uptake by *H. triquetra* has only been documented in a warm growth environment of 20 °C (Paasche et al. 1984). At the same time, the maximum nutrient uptake rate of phytoplankton is known as a function of molecular diffusivity, which is a linear function of temperature – slower molecular diffusivity at lower temperatures (Baird et al. 2001). The literature also does not provide any direct evidence indicating the extent to which *H. triquetra* benefits from dark nutrient uptake in a way that would manifest in either higher growth rate, biomass increase or improved photosynthetic performance later in the light field. In the present study, our experimental

conditions were aimed to simulate some of the main environmental factors, met in the Gulf of Finland that influence nutrient uptake and photosynthetic efficiency in phytoplankton: (i) light availability, (ii) temperature, and (iii) nutrient pre-conditioning. The primary objective of this study was to indicate, in a laboratory experiment, the ability of *H. triquetra* to take up inorganic nutrients (nitrate and phosphate) in cold and dark conditions and to demonstrate that nutrients previously taken up in dark improve cell growth and photosynthetic capacity later in the light field.

Materials and methods

Culturing conditions and experimental design

A non-axenic monoculture of *Heterocapsa triquetra* was established from single cells isolated from the Gulf of Finland in 2012. The culture was maintained in T2 medium (Spilling et al. 2011), which is modified f/2 medium (Guillard 1975), where the molar nutrient ratio, N:P, is adjusted to 16:1. The culture, with salinity around 6, was grown at 15 °C and an irradiance of 200 μmol m^{-2} s^{-1} with the light-dark cycle of 16:8 hours. Ten days prior the experiment, a small amount of culture (1-2 mL) was inoculated into two 250 mL Erlenmeyer flasks prefilled with 120 mL filtered and autoclaved seawater. Nutrients were added directly into the cultures according to Spilling et al. (2011), with a final concentration of nitrate 24 μmol L^{-1} and phosphate 6 μmol L^{-1}. Phosphate was added in excess (N:P = 4) to stimulate the natural environment of N-limitation in the Gulf of Finland.

To initiate the experiment 500 mL flasks (n=3; Fig. 1) were filled with the above cultures at 820 cells mL^{-1} and maintained at the light and temperature conditions above. Once cell density was >1000 cells mL^{-1} (day 2), cultures were diluted with sterile seawater. The dilution was necessary to avoid CO_2 limitation and consecutive pH rise as a result of high cell numbers in the late exponential phase. By day 6 of the experiment, when *H. triquetra* cells had reached the stationary growth phase, the cultures were split in two and poured in triplicate into 500 mL Erlenmeyer flasks up to 230 ± 2.5 mL. One set of flasks was maintained at the light and temperature conditions described above until the termination of the experiment, additional nutrients were never added. The other set of flasks was wrapped in foil, and after that nutrient-rich medium was added. Nutrient concentration increased to a final concentration of nitrate 4.53 μM and phosphate 0.91 μM (N:P = 5) in the nutrient amended cultures. Similar nutrient concentration levels are found in the surface layer of the Gulf of Finland before the formation of the thermal stratification in the water column in spring. Nutrient concentrations at the upper part of the nutriclines (15–35 m) in the cold intermediate layer are usually lower. However, we aimed to avoid nitrate being depleted from the dark incubated cultures since maximum growth yield would not be possible to show in that case. The flasks were incubated in the dark at 4 °C

for 48 hours (I_D) and after that brought back to the previous culturing conditions (I_{LD}) described above. Samples for measurements of cell concentration and photosynthetic capacity were withdrawn in every 1-2 days over 11 days. For cell enumeration samples were fixed with acid Lugol's solution and cells counted using a Sedgewick-Rafter counting chamber and an inverted microscope. The specific growth rates (μ), between consecutive time points, were calculated during exponential growth as:

$$\mu = \frac{\ln(N_2) - \ln(N_1)}{t_2 - t_1} \qquad (1)$$

where N_1 and N_2 denote the abundances at time steps t_1 and t_2, respectively. Culture pH was monitored daily directly in the flasks and was regulated with a 1M HCL solution during the first days of the experiment (pH meter Mettler Toledo SevenEasy).

Variable chlorophyll fluorescence analysis

Standard variable chlorophyll fluorescence measurements were done using the MULTI-COLOR-PAM chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) following the saturation pulse method (Schreiber 2004). Parameters used for the assessment of the photosynthetic capacity of *Heterocapsa triquetra*, obtained with the saturation pulses were: effective photochemical quantum yield (ϕ_{II}), regulated non-photochemical energy conversion (ϕ_{NPQ}), non-regulated heat dissipation and fluorescence emission (ϕ_{NO}), maximum photochemical quantum yield (F_v/F_m) and photochemical fluorescence quenching (qP; Table 1). These parameters provide basic information on the fate of absorbed light energy (Klughammer & Schreiber 2008; Kramer et al. 2004). Parameters for the light regimes used were: measuring light = 1 μmol photons m^{-2} s^{-1} at 440 nm (30 sec), saturating light = 5 710 μmol photons m^{-2} s^{-1} at 440 nm (300 ms), actinic light = 200 μmol m^{-2} s^{-1} at 440 nm (30 sec). All fluorescence parameters were calculated after subtraction of the blank fluorescence, measured in filtered (GF/F glass-fibre filter) and autoclaved seawater. Before fluorescence measurements, cells were incubated at *in situ* temperature in the quartz cuvettes for 10 to 20 minutes in the dark to allow full oxidation of the primary electron acceptor, quinone A (Q_A). Cells were stirred in the cuvette during measurements.

Inorganic nutrient measurements

Nutrient analysis was carried out from filtered and autoclaved seawater prior use in the experiment. During the experiment, samples for nutrient analysis were collected at five different time steps: on day 2 after culture dilutions, on day 6 before and after nutrient additions prior dark incubation period, on day 8 from nutrient-enriched cultures after the dark incubation period, and on day 9 from

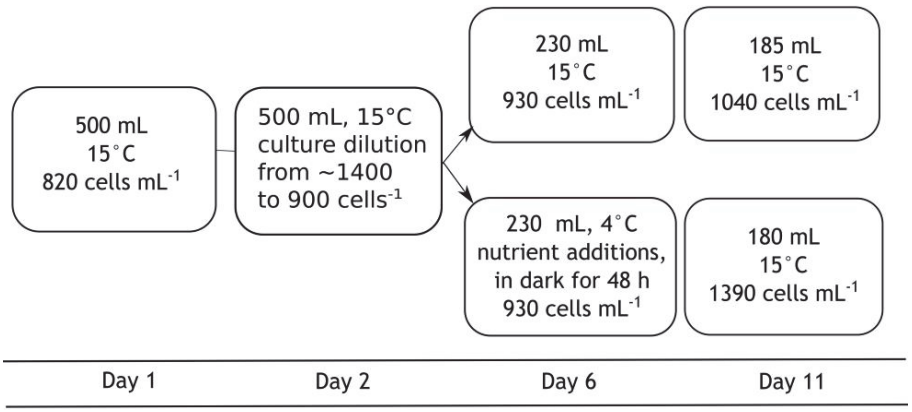

Figure 1. The timeline of the experiment with information on subculture volume, growth conditions and cell densities on different experimental phases. On day 6 cultures were split into two sets of subcultures: one set was left unmodified until the end of the experiment (arrow pointing up), and the second set received nutrients and went through the 48-hour dark (ID) incubation period at 4 °C (arrow pointing down) before brought back to the initial light and temperature conditions.

Table I. Chlorophyll fluorescence parameters used in this study.

PSII fluorescence parameter		Calculation	References
F_v/F_m	Maximum photochemical quantum yield of PSII	$(F_m-F_0)/F_m$	Kitajima & Butler (1975)
ϕ_{II}	Effective photochemical quantum yield of PSII	$(F_m'-F)/F_m'$	Genty et al. (1989)
ϕ_{NO}	Quantum yield of non-regulated heat dissipation and fluorescence emission	F/F_m	Hendrickson et al. (2004)
ϕ_{NPQ}	Quantum yield of light-induced non-photochemical fluorescence quenching	$F/F_m'-F/F_m$	Hendrickson et al. (2004)
qP	Photochemical fluorescence quenching	$(F_m'-F)/(F_m'-F_0')$	Schreiber et al. (1986)
F_0'	Minimal fluorescence in the light-adapted state	$(F_m'-F)/(F_m'-F_0')$	Oxborough & Baker (1997)

nutrient-enriched cultures. Samples were analysed using the Lachat QuikChem 8500 Series 2 (Lachat Instruments, Hach Company) for NO_3^- and PO_4^{3-} according to the ISO 15681-1 method for PO_4^{3-} and ISO 13395 method for NO_3^-. The lower detection range for PO_4^{3-} and NO_3^- were 0.06 and 0.07 µM,

respectively.

The nutrient uptake rates were calculated from the decreases of the respective nutrient concentrations. To compare the rates in units of μM h^{-1}, nutrient uptake was assumed to be constant over the measured periods, which differed in length. The dark incubation period at 4 °C (I_D) of 48 hours was compared to the period of a light-dark cycle (I_{LD}) of 24 hours. Hence, the amount of nutrients taken up during I_D or I_{LD} was divided by the respective hours of the measured period.

Results

Nutrient and cell concentrations

The dynamics of the nutrient levels in the cultures and the amounts of nutrients taken up in different experimental phases by *Heterocapsa triquetra* are shown in Figure 2a. The concentration of NO_3^- in the non-amended cultures was stable at 0.44 ± 0.02 μM (open circles; last day of measurement in non-amended cultures was day 6, no measurements were made on day 9). Whereas the level of PO_4^{3-} decreased by 26% down to the concentration of 0.67 ± 0.1 μM by day 6 (open rectangulars).

In the nutrient-amended cultures, the concentrations of both, NO_3^- and PO_4^{3-}, decreased during dark, cold (4 °C) incubation (I_D) (group means before and after I_D were significantly different for both nutrients; paired *t*-test, $p < 0.05$). The average consumption rates of NO_3^- during I_D was 1.04 μM d^{-1} = 0.04 μM h^{-1} and in following warm (15 °C) light:dark cycle (I_{LD}) it was 1.69 μM d^{-1} = 0.07 μM h^{-1}. The consumption rates of PO_4^{3-} in I_D was 0.075 μM d^{-1} = 0.003 μM h^{-1} and in following I_{LD} 0.14 μM d^{-1} = 0.006 μM h^{-1}. However, the differences between the consumption rates of both nutrients in I_D and I_{LD} were not statistically significant (t-test, $p > 0.05$) most probably due to the small data set. Nutrients were consumed in similar N:P ratios of 14:1 and 12:1 during I_D and I_{LD}, respectively.

The cell concentration in the non-amended cultures was mostly stable around 1000 cells mL^{-1} during the whole experiment (Figure 2b). Cell abundance decreased slightly after relocating the nutrient-amended cultures from I_D to I_{LD} (negative growth rate of -0.11 d^{-1} by day 9). After that the growth rate in these cultures increased to 0.22 d^{-1} in two days yielding a 34% higher cell concentration (1390 cells mL^{-1}) by day 11, when compared with the non-amended cultures at the same day (1040 cells mL^{-1}).

Variable fluorescence dynamics

The maximum PSII quantum yield (F_v/F_m) for the non-amended *Heterocapsa triquetra* cultures declined as a function of time from 0.79 to 0.60 during the experiment (Figure 2c). The mean F_v/F_m in the nutrient-amended cultures after the I_D (the last three days of the experiment) was 0.77 ± 0.01; mean ± SE, $n = 9$), being on average significantly higher than the mean in the non-amended cultures

(0.66 ± 0.03) during the same period (*t*-test, $p < 0.01$).

Measurements of photophysiological variables yielded more insight into the photochemical and non-photochemical quenching processes in the non-amended *H. triquetra* cultures. The effective photochemical yield (φ_{II}) for the non-amended cultures declined with time from 0.71 to 0.36. During the first 5 days, the decline mainly resulted from compensatory changes in antenna downregulation, φ_{NPQ} (the latter increased from 0.02 – 0.21; Figure 3a). Under these conditions, the PSII reaction centres were essentially completely open (high qP), and photosynthetic yield was primarily determined by changes in non-photochemical quenching. Onwards from day 5, the capacity of φ_{NPQ} to regulate light capture was saturated, leading to the gradual decline of open reaction centres (qP). However, from day 8 until the end of the experiment, φ_{II} was mainly influenced by non-light induced (basal or dark) quenching processes (φ_{NO} increased from 0.25 – 0.42; Figure 3a). Increased energy dissipation as φ_{NPQ} and φ_{NO} caused a photoregulative change in the non-amended *H. triquetra* cultures on days 10 – 11, i.e. φ_{II} was lowered down to 0.36.

In the nutrient-amended cultures φ_{II} recovered up to 0.64 and was on average higher when compared to the non-amended cultures (*t*-test, $p = 0.01$). The decline of φ_{II} resulted mainly from the increasing φ_{NPQ} (0.06 – 0.17) but also from a slight decrease in qP (0.90 – 0.83; Figure 3b). Energy dissipation as φ_{NO} was stable (ranged from 0.29 – 0.31; Figure 3b).

Discussion

The present study documents that the dinoflagellate *Heterocapsa triquetra* can take up both inorganic nitrogen and phosphorus in a dark and cold (4 °C) environment. Similar physical conditions are characteristic of the water layers below the seasonal thermocline in summer in the Baltic Sea. The documented ability for dark nutrient uptake supports our hypothesis that *H. triquetra* migrations from warm surface layers to the deeper cold layers could be aimed to acquire available inorganic nutrients that are at that time lacking in the surface layer. The described migration pattern (Lips et al. 2011) together with dark nutrient uptake, presented in this study, explains the competitive advantage of *H. triquetra* populations in the nutrient-limited surface layer and the species' ability to reach bloom concentrations in the summer when the Gulf of Finland is strongly stratified. As the dark uptake of NO_3^- was demonstrated to be accompanied with the uptake of PO_4^{3-}, the importance of nutrient-retrieval migrations to the nutrient limited surface layer are even more emphasised by the present study. The observed N:P uptake ratios by *H. triquetra* were similar in the dark, cold (4 °C) and illuminated, warm (15 °C) environments –14:1 and 12:1, respectively. This is noteworthy since significantly lower N:P uptake ratio in the dark or no

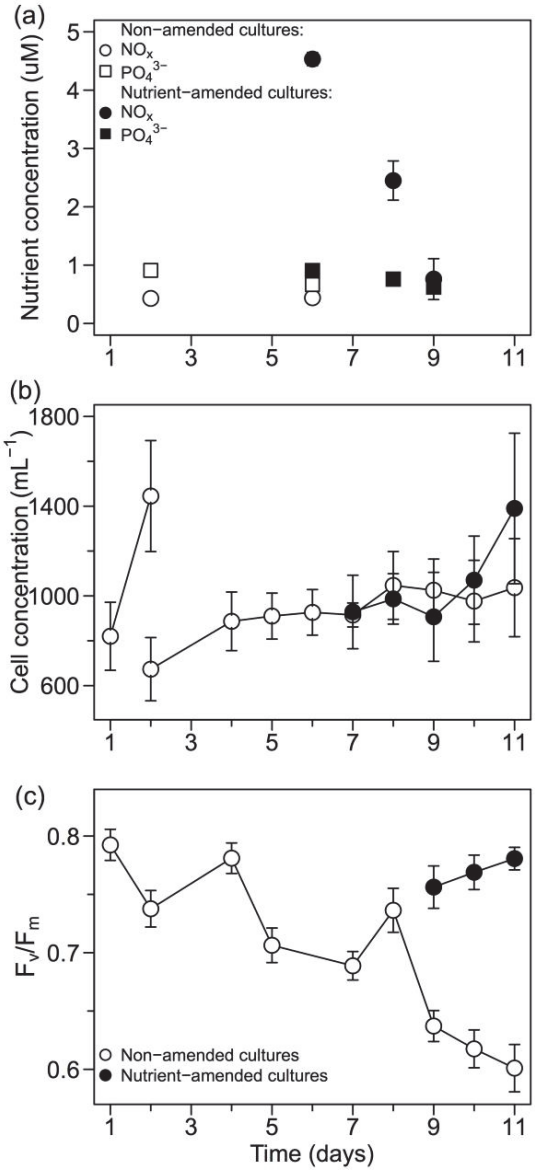

Figure 2. The dynamics in NO_3^- and PO_4^{3-} concentration (μM; a), cell abundance (cells mL^{-1}; b) and maximum photochemical quantum yield of PSII (F_v/F_m; c) in Heterocapsa triquetra cultures subjected to nutrient limitation (non-amended cultures; open symbols) and nutrient supply (nutrient-amended cultures; closed symbols). Nutrient-amended cultures were dark-incubated from day 6 to day 8 (48 hours), F_v/F_m was not measured in the nutrient-amended cultures at that time. Data points represent treatment means with error bars (n=3).

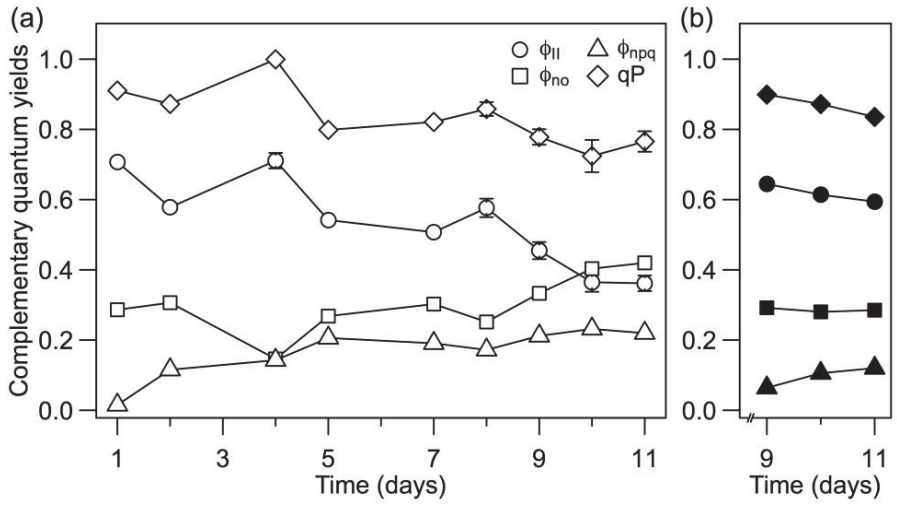

Figure 3. The dynamics of the variable chlorophyll fluorescence in Heterocapsa triquetra cultures during the experiment when (a) subjected to nutrient limitation (non-amended cultures), and (b) after nutrient supply and the following 48-hour dark incubation period (nutrient-amended cultures). Data points represent treatment means with error bars (n=3).

difference in light-dark uptake of P is reported in previous studies (e.g. Müller et al. 2008; Riegman et al. 2000; Rivkin & Swift 1985).

While the influence of single environmental factors on nutrient uptake have rigorously been studied in dinoflagellates, studies with combined environmental factors on the nutrient uptake are lacking. In fact, little attention has been paid to the dark uptake rates of nutrients in different temperatures or dark nutrient uptake by cells previously grown in an illuminated but nutrient-poor environment. Often higher uptake rates of nitrates in the light compared with dark uptake rates are reported (e.g. Dortch & Maske, 1982; Riegman et al. 2000; Jauzein et al. 2011). *Heterocapsa niei* (Loeblich III) Morrill & Loeblich III takes up 60% less nitrate nocturnally as compared to the uptake during the day (Cullen 1985), whereas *H. triquetra* is able to take up nitrate more or less equally in light and dark conditions at 20 °C (Paasche et al. 1984). In general, nutrient uptake decreases with temperature (Reay et al. 1999; Smayda 1997). In the present study, potentially half the nutrients were taken up in the dark, cold conditions compared with the uptake in the warm, illuminated conditions. However, as the uptake correlations were found not to be statistically significant, most probably due to the small data set, this issue needs to be studied further. Aditionally, the cells' history of the nutrient supply can play an important role. Dinoflagellates differ widely in their

ability to take up nitrogen when grown in N-sufficiency (Paasche et al. 1984). Nitrate uptake and reduction relies on carbohydrates that are used up immediately in the light if nitrate is available, but are stored in N-starved cells and could be used for dark nitrate assimilation (Cullen 1985). Some earlier laboratory studies have shown that N-deprived phytoplankton cells have greater dark uptake rates of inorganic nitrogen compared with N-replete cells (Dortch & Maske, 1982; Cochlan et al. 1991).

The present data also provide evidence that the nutrient uptake in the dark was followed by improved performance of the photosynthetic apparatus of the cells later in the illuminated conditions. The maximum PSII quantum yield (F_v/F_m) was higher after nutrient additions (up to 0.76), and the majority of the absorbed light energy was channelled into photochemical processes (φ_{II}). In dinoflagellate cultures, F_v/F_m of 0.6 has been considered as an indication of cells with a well-functioning photosynthetic apparatus (López-Rosales et al. 2014; Suggett et al. 2009). In the present experiment, F_v/F_m declined in the non-amended cultures but was never lower than 0.6. Relatively high values of F_v/F_m together with the long duration of the stationary growth phase in the non-amended *H. triquetra* cultures (~9 days), may indicate that *H. triquetra* cells are able to acclimate to low nutrient conditions and survive for relatively long periods in a nutrient-depleted surface layer in the sea. The described ability may be potentially achieved by the use of intracellular nutrient storages, as dinoflagellates can store significant amounts of inorganic and organic nitrogen forms (e.g. review by Dagenais-Bellefeuille & Morse 2013). It is thought, that in the stationary growth phase nutrients are reallocated to the functions where they are most needed, and the balance between the light harvest and electron transport are adjusted in such a way as to maximise efficiency (Parkhill et al. 2001).

The decrease in maximum photochemical efficiency (φ_{II}) in the nutrient-limited cells has been reported (Lippemeier et al. 1999; Roberts et al. 2008), as well as the recovery following nutrient re-addition after starvation (e.g. Greene et al. 1992; Lippemeier et al. 2001; Young & Beardall 2003). Fluorescence, as opposed to the cell division, responded faster to the nutrient additions in our experiment, which is in agreement with nutrient spiking found in other algae (Lippemeier et al. 2001, 2003). Decreasing φ_{II} and complementarily increasing φ_{NPQ} and φ_{NO} in the non-amended *H. triquetra* cultures, to a great part, was related to the damage in the reaction centres. The operating efficiency of PSII (φ_{II}) during the final days in the non-amended cultures was sufficient (0.36 – 0.45) to maintain the population size at a steady level. The sum of the φ_{NPQ} and φ_{NO} contributed to a substantial fraction (0.55 – 0.64) of the absorbed light energy lost non-photochemically. Although, the concentration of the light-harvesting pigments in *H. triquetra* have been shown to decrease heavily under nutrient starvation and only slight changes in their relative concentration to chlorophyll *a* take place

(Latasa & Berdalet 1994). The latter suggests that the general structure of the photosynthetic apparatus does not change and in the case of nutrient re-supply, a faster recovery of the cells may be expected. In the present study, the φ_{II} recovered up to 64% after nutrient addition. Enrichments with limiting nutrients often restore the values of φ_{II}, implying that nutrient limitation on photosynthetic energy conversion is common in the sea (Falkowski 1992; Falkowski et al. 1991).

In conclusion, evidence of phosphate and nitrate uptake by the migratory dinoflagellate *H. triquetra* in a cold environment in the absence of light was found. Some signs of improved photophysiology (after nutrient uptake in the dark) and cell growth was also apparent. A recovery of the effective photochemical efficiency after the end of the dark incubation period indicated the potential use of intracellular nutrient reserves to improve the efficiency of PSII reaction centres. Nearly constant cell concentration and relatively high effective photochemical quantum yield in the potentially nitrogen-limited cultures indicated the ability of the *H. triquetra* population to survive for extended periods in low nutrient conditions. The presented data show that *H. triquetra* populations indeed win from compromises between nutrient acquisition in deep, dark, cold layers at night and subsequent photosynthesis in the warm euphotic layer during the day in the Baltic Sea. In order to estimate the importance of upward vertical flux of inorganic nutrients induced by migration of *H. triquetra* in comparison to the contribution of hydrophysical processes (as turbulent mixing, transport by upwelling events etc.) further laboratory and field studies are needed.

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ABSTRACT

The ecology and photobiology of mixotrophic alveolates in the Baltic Sea

Mixotrophic protists live a flexible lifestyle by combining autotrophic and heterotrophic nutritional strategies. During the past decades, it has been acknowledged that mixotrophic behaviour is widespread among protists. However, the contribution of mixotrophs to net community production still remains unclear. In the Baltic Sea, during the period of seasonal water column stratification and nutrient deficiency in the upper mixed layer, a significant proportion of the biomass of the microplankton community is formed by the mixotrophic species. The objective of this PhD thesis was to understand better the ecophysiology of mixotrophs; how different environmental factors and predator-prey relationships influence the population dynamics of common summertime species in the Baltic Sea.

Laboratory experiments were conducted to study predator-prey interactions, feeding behaviour, regulation of kleptochloroplasts at different light fields in the absence of prey, capacity for inorganic nutrient uptake in cold and dark environmental conditions, improvement of the population photophysiology after dark nutrient uptake, and population survival in nutrient deficient conditions. The influence of mixotrophic predators on the prey population was quantified. Direct impact via the trophic link and a major indirect impact (prey lysis) on the prey population by the toxic predators (*Dinophysis* spp.) were revealed. Synthesis of photosynthetic and photoprotective pigments and photoregulation of kleptochloroplasts in different light fields by *Dinophysis* in the absence of prey were discovered. Measurements of nutrient uptake in dark and cold conditions support the hypothesis of nutrient-retrieval migrations to deep layers by *Heterocapsa triquetra*. These results contribute to a more explicit understanding of the success of the mixotrophic species in optimal and non-optimal environmental conditions.

RESÜMEE

Miksotroofsete alveolaatide ökoloogia ja fotobioloogia Läänemeres

Miksotroofsetele protistidele on omane paindlik toitumisstrateegia, mis ühendab endas auto- ja heterotroofsuse. Viimase paarikümne aasta jooksul on leitud, et miksotroofne eluviis on protistide seas väga levinud. Küll aga ei ole veel selge, milline on miksotroofsete organismide panus mikroplanktoni koosluse netoproduktsiooni. Läänemeres, sesoonse temperatuuri kihistumise perioodil, mil ülemine segunenud veekiht on toitainetevaene, moodustavad miksotroofsed liigid olulise osa suvisest mikroplanktoni biomassist. Käesoleva doktoritöö eesmärk oli paremini mõista miksotroofide ökofüsioloogiat – kuidas mõjutavad varieeruvad keskkonnatingimused ning kiskja ja saakorganismi omavahelised suhted suvel esinevate miksotroofsete liikide populatsioonidünaamikat.

Doktoritöö raames viidi läbi laboratoorsed eksperimendid uurimaks kiskja ning saagi omavahelisi interaktsioone ja toitumiskäitumist, kleptotkloroplastide fotoregulatsiooni saagi puudumisel, anorgaaniliste toitainete omastamist jahedas vees valguse puudumisel, populatsiooni fotofüsioloogilise seisundi paranemist pärast toitainete pimedas omastamist ja populatsiooni füsioloogiat toitainetevaestes tingimustes. Kirjeldati kvantitatiivselt miksotroofsete kiskjate mõju saakorganismi populatsioonile ning leiti, et toksilise dinoflagellaadi *Dinophysis* mõju saakorganismidele võib olla nii otsene (toitumine saagist) kui ka kaudne (saagi lüüsumine). Dokumenteeriti valgust püüdvate ja valguse eest kaitsvate pigmentide süntees ning kleptokloroplastide fotoregulatsioon *Dinophysis* rakkudes saakorganismide puudumisel. Dinoflagellaat *Heterocapsa triquetra* poolt toitainete, pimedas ja külmas keskkonnas, omastamise registreerimine toetab eelnevat hüpoteesi, et antud liik migreerub vertikaalselt veesamba sügavamatesse kihtidesse pinnakihis puuduvate toitainete omastamiseks. Doktoritöö tulemused aitavad paremini mõista miksotroofsete liikide edukust (biomassi maksimumide moodustamist) optimaalsetes ja mitteoptimaalsetes keskkonnatingimustes.

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Hansen, P.J., Ojamäe, K., Berge, T., Trampe, E.C.L., Nielsen L.T., Lips, I., Kühl, M. (2016). Photoregulation in a kleptoplastidic dinoflagellate, *Dinophysis acuta*. Frontiers in Microbiology 7:785.

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Anneliis Peterson, magistritöö "Miksotroofse dinoflagellaadi *Heterocapsa triquetra* kasvudünaamika ja toitainete omastamine". Tallinna Tehnikaülikool, Meresüsteemide Instituut, 2015.

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