Acquisition of Photoautotrophy in Kleptoplastic Dinoflagellates – *Nusuttodinium aeruginosum*/*acidotum* as a case study

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Kurzzusammenfassung in deutscher Sprache

Die Integrierung stabiler Plastiden durch Endosymbiosen führte zu einer enormen Vielfalt an photosynthetischen eukaryotischen Organismen auf der Erde. Allerdings sind die Schritte während der Etablierung stabiler Endosymbiosen nur schlecht verstanden. Um Licht auf diesen frühen Schritt der Evolution zu werfen, wurden viele Studien an Organismen mit vorübergehenden Plastiden durchgeführt. Ursprünglich heterotroph, sind diese Organismen in der Lage, Photoautotrophie durch die Aufnahme photosynthetischer Beute zu erwerben. Anstatt verdaut zu werden, behält die Beute ihre Fähigkeit zur Photosynthese bei und versorgt den Wirt mit Photosyntheseprodukten. Der Beuteorganismus kann entweder zu einem Endosymbiont oder sogar zu einem Plastid reduziert werden. Im letzteren Fall werden die Plastiden der photosynthetischen Beute gestohlen und daher ‚Kleptoplastiden' genannt. Kleptoplastiden sind innerhalb der Eukaryoten weit verbreitet und kommen in vielzelligen, wie auch in einzelligen Organismen vor. Eine der bekanntesten Gruppen, welche Kleptoplastiden beherbergt, sind die Dinoflagellaten. Diese Studie fokussiert auf Süßwasserisolate der Kleptoplastiden beherbergenden Gattung *Nusuttodinium* mit einem besonderen Fokus auf die Art *N. aeruginosum/acidotum*, welche ihre Kleptoplastiden von der blau-grünen cryptophytischen Gattung *Chroomonas* erlangt. Es wurden von *N. aeruginosum/acidotum*, sowie einer weiteren Art, *N. amphidinoides* stabile, bakterienfreie Co-Kulturen etabliert und ihr Lebenszyklus wurde untersucht. Im Gegensatz zu allen anderen Systemen, welche bis heute beschrieben wurden, ist *N. aeruginosum/acidotum* in der Lage, Kleptoplastiden unterschiedlicher Morphologie und Stabilität zu beherbergen, wodurch diese Art ein ideales Modellsystem für die Untersuchung möglicher Schritte während der Erlangung stabiler, permanenter Plastiden ist. Abhängig von der phylogenetischen Stellung der Beute können entweder sogenannte ‚granuläre' oder U-förmige' Kleptoplastiden gebildet werden. Morphologisch sind granuläre Kleptoplastiden durch ihre erhöhte Anzahl und geringe Größe charakterisiert. Jeder Kleptoplast ist mit einem kleinen cryptophytischen Zellkern, dem sogenannten ‚Kleptokaryon' assoziiert und ähnelt frisch aufgenommener Beute. Kleptokarya sind für bis zu 10 - und die Kleptoplastiden für bis zu 14 Tage stabil. Im granulären Zustand nehmen die Zellen permanent Beute auf, ganz besonders kurz nach der Zellteilung, die während der Dunkelphase stattfindet. Im Gegensatz dazu ist die U-förmige Situation durch einen einzigen, großen U-förmigen Plastiden gekennzeichnet, welcher die Zelle ausfüllt, sowie durch einen einzigen großen Kleptokaryon, welcher sich antapikal in dem Bogen des U befindet. Verglichen zur freilebenden Beute und granulären Situation nimmt die Größe des Kleptoplasten um das bis zu 20 fache - und die des Kleptokaryon um das bis zu 16 fache zu.

Während der Kleptoplast bei der Zellteilung gleichermaßen an beide Tochterzellen vererbt wird, erhält nur eine den Kleptokaryon. Für den Kleptokaryon wurde eine Stabilität von bis zu 50 Tagen- und für den Kleptoplasten von bis zu 60 Tagen beobachtet. DNA-Fluoreszenzmessungen, sowie eine auf PCR basierende Herangehensweise mit Primern, spezifisch für den Cryptophyten-Zellkern deuten auf eine Polyploidisierung des cryptophytischen Zellkerns nach Aufnahme durch den Dinoflagellaten hin. Zusätzlich erhöhen die vergrößerten Kleptokarya die Stabilität der Kleptoplasten und beeinflussen das Verhalten von *N. aeruginosum/acidotum*. Die Aufnahme von Beute wird gehemmt und die Dinoflagellaten zeigen größtenteils ein gerades Schwimmverhalten, welches sich deutlich von dem in der granulären Situation unterscheidet. Herrscht eine sehr hohe Beuteverfügbarkeit, formt *N. aeruginosum/acidotum* anstelle der U-förmigen Kleptoplasten granuläre, welche verdaut werden, falls die Beuteverügbarkeit hoch bleibt oder, falls die Beuteverfügbarkeit abnimmt, gleichermaßen auf die Tochterzellen verteilt werden, in welchen sie sich letztendlich zu U-förmigen, stabilen Kleptoplasten entwickeln. Im Gegensatz zu *N. aeruginosum/acidotum* repräsentiert *N. amphidinoides* ein primitiveres Stadium während der Erlangung von Plastiden, da die Kleptokarya kurz nach der Beuteaufnahme verdaut werden und die Kleptoplasten weniger modifiziert und unstabiler, als die U-Plastiden sind. Der Vergleich der ‚pimitiven' granulären Situation mit der ‚fortgeschritteneren' U-förmigen Situation unter Rücksichtnahme der Literatur erlaubt die Aufstellung einer neue Hypothese, nach der Beutemangel ein Auslöser- und Polyploidisierung einer der wichtigsten Schritte zur Erlangung stabiler Plastiden sind.

Abstract in englischer Sprache

The integration of stable plastids by endosymbiosis led to an enormous diversity of photosynthetic eukaryotic organisms on earth. However, the steps during the establishment of a stable endosymbiosis are poorly understood. To address this early step of evolution, many studies have been performed on organisms with transient plastids. Originally heterotrophic, are these organisms able to acquire photoautotrophy through the uptake of photosynthetic prey. In contrast to being digested, the prey preserves its photosynthetic ability and supplies the host with photosynthetic products. The prey can either be reduced to an endosymbiont or even to a single plastid. In the latter case the plastids are stolen from photosynthetic prey and therefore called ‘kleptoplasts’. Kleptoplasts are widespread among eukaryotes and occur in multicellular as well as in unicellular organisms. One of the most prominent groups harboring kleptoplasts is the dinoflagellates. This study focuses on freshwater isolates of the kleptoplastic genus *Nusuttodinium* with a special focus on the species *N. aeruginosum*/*acidotum* that acquires its kleptoplasts from the blue-green cryptophyte genus *Chroomonas*. Stable axenic co-cultures from different locations were established for *N. aeruginosum/acidotum* and for another species *N. amphidinoides* and their live cycles were studied. In contrast to all other systems described until now, *N. aeruginosum/acidotum* is able to harbor kleptoplasts of different morphology and stability making it an ideal model system to study possible steps during the acquisition of stable permanent plastids. Depending on the phylogenetic position of the prey either ‘granular’ or ‘U-shaped’ kleptoplasts are formed. Granular kleptoplasts are characterized morphologically by their multiple number and small size. Each kleptoplast is associated with a small cryptophyte nucleus, the ‘kleptokaryon’ and resembles recently ingested prey. Kleptokarya are stable for up to 10 days and kleptoplasts for up to 14 days. In the granular stage cells took up prey permanently, especially just after cell division that takes place during the dark phase. In contrast, the U-shaped situation is characterized by just one large U-shaped kleptoplast filling out the cell and a single large kleptokaryon located antapically in the curve of the U. Compared to the free living prey and the granular situation, the kleptoplast increases up to 20 fold in size and the kleptokaryon up to 16 fold. Whereas the kleptoplast is distributed equally to both daughter cells during cell division, just one inherits the kleptokaryon. A stability of up to 50 days was recorded for the kleptokaryon and up to 60 days for the kleptoplast. DNA fluorescence measurements and a PCR based approach with primers specific to the cryptophyte nucleus indicate polyploidization of the cryptophyte nucleus after ingestion by the dinoflagellate. In addition enlarged kleptokarya increase kleptoplast stability and influence the behavior of *N.*

acidotum/aeruginosum. Prey uptake is inhibited by intact kleptokarya and dinoflagellates mostly show straight swimming behavior that clearly differs from the behavior in the granular situation. If prey availability is very high *N. aeruginosum/acidotum* does not form U-shaped kleptoplasts but instead forms granular kleptoplasts that are digested if prey availability remains high or, if prey availability decreases, are distributed equally to the daughter cells in which they develop into stable U-shaped kleptoplasts. In contrast to *N. aeruginosum/acidotum, N. amphidinoides* represents a more primitive stage during plastid acquisition as kleptokarya are digested shortly after ingestion and kleptoplasts are less modified and stable than the U-shaped plastids. Comparing the 'primitive' granular with the 'advanced' U-shaped situation with respect to literature allows the formulation of a new hypothesis after which prey scarcity is a trigger and polyploidization one of the most important steps towards the acquisition of stable plastids.

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1. Introduction

1.1 Acquisition of photosynthesis within Eukaryotes

The acquisition of photosynthesis was undoubtably one of the most important evolutionary steps on earth. The capability to produce energy-rich sugars from light, carbon dioxide and water proved to be very successful until today. It is generally accepted that oxygenic photosynthesis first evolved in the ancestors of recent cyanobacteria more than two billion years ago (Kaufmann et al., 2007). In a second process called 'primary endosymbiosis' a free living cyanobacterium was ingested by a heterotrophic eukaryote. Instead of being digested, the cyanobacterium was retained by the host and transformed into an organelle called 'plastid' that allowed the host to acquire photosynthesis. This transformation included a reduction of the original genome after Endosymbiotic Gene Transfer (EGT) to the host cell nucleus, the development of a protein import machinery and as a consequence the total dependency on the host (Cavalier-Smith and Lee, 1985, Theissen and Martin, 2006, Burki et al., 2014). Three eukaryotic lineages, the Glaucophyta, Rhodophyta and Viridiplantae all possess double-membrane-bound plastids derived from primary endosymbiosis (Moreira et al., 2000, Keeling, 2010) that is supposed to have occurred just once (Rodriguez-Ezpeleta et al., 2005). Subsequently, other eukaryotic lineages acquired plastids through endosymbioses with primary algae, a process called 'secondary endosymbiosis' (Keeling, 2013). Secondary endosymbiosis occurred multiple times as organisms with plastids of red- and green algal origin exist (Rogers et al., 2007). Secondary plastids are usually surrounded by three or four envelope membranes. The lineages Euglenophyta and Dinophyta contain secondary plastids with three envelope membranes whereas the plastids within the lineages Stramenopiles, Haptophyta, Cryptophyta and Chlorarachniophyta are surrounded by four envelope membranes. The Apicomplexa, a group of unicellular eukaryotes, mostly living as parasites within multicellular organisms contain a specialized plastid of red algal origin called 'apicoplast'. The apicoplast is surrounded by four envelope membranes and has lost the ability to perform photosynthesis (Delwiche and Palmer, 1997, Archibald, 2009). The complex plastid history is particularly visible in the dinoflagellates. About 50% of all described species lack visible plastids although their ancestor is supposed to already have a plastid of red algal origin (Janouskovec et al., 2010, Shalchian-Tabrizi et al., 2006). Some dinoflagellates even went one step further and replaced their original red algal plastid by plastids derived through primary endosymbiosis in a process called 'serial secondary endosymbiosis' (Watanabe et al., 1990) or by plastids themselves derived through secondary

endosymbiosis , a process called ‘tertiary endosymbiosis’ (Keeling, 2010, Wisecaver and Hackett, 2011). Serial secondary endosymbiosis has been described for the green-algal-plastid containing species *Lepidodinium chlorophorum* and *L. viride.* Tertiary endosymbiosis occurred multiple times with diatoms, haptophytes and cryptophytes. Whereas the plastids derived from diatoms and haptophytes are stable, cryptophyte plastids are temporary and have to be replaced regularly in a process called ‘kleptoplasty’. Kleptoplasts occur in many different groups of organisms ranging from unicellular foraminifers, katablepharids, dinoflagellates and ciliates to multicellular organisms like sea slugs. To get an answer how a taken up prey is transformed into a plastid many studies have been performed on such organisms that are on the way to establish permanent plastids. Another key organism is the filose amoeba *Paulinella chromatophora* that has been known for more than 100 years (Lauterborn, 1895). *P. chromatophora* represents an independent primary endosymbiosis with a *Synechococcus*-like cyanobacterium that occurred much more recently (Marin et al., 2005). Despite the amount of research that has been performed on such organisms, little is known about the dynamics during plastid establishment as all organisms studied until now just represent one evolutionary stage and many studies were performed on natural samples. One of the most studied examples for the acquisition of photosynthesis in eukaryotes is the coral-*Symbiodinium* symbiosis. The symbiotic dinoflagellate *Symbiodinium* maintains its full genome and is able to divide in its coral host during its whole life. In contrast to all other examples mentioned above, *Symbiodinium* keeps its autonomy and can even continue its life after the death of its host (Cavalier-Smith and Lee, 1985, DeSalvo et al., 2010).

1.2 Dinoflagellates – A group with an exceptional plastid history

Dinoflagellates are a widespread group of unicellular or colony forming protists in marine and freshwater habitats. As one of the major groups of phytoplankton they play an important role in primary production. They are popular for their symbiosis with corals but also for their potential to form dangerous red tides (LaJeunesse et al., 2009). Free-living as well as parasitic forms occur. Motile cells are biflagellate with one flagellum inserted in a transverse furrow, the cingulum and the other flagellum inserted in a ventral longitudinal furrow, the sulcus. Movement of both flagella causes their typical rotating swimming behavior (Graham and Wilcox., 2009). The nucleus is known to contain large amounts of DNA organized in chromosomes that remain condensed throughout the cell cycle. Phylogenetically dinoflagellates belong to the superphylum Alveolata that also includes the ciliates and

apicomplexans (Stoecker et al., 2009). A major characteristic of this group are the alveoli, flattened vesicles that form a continous layer under the plasma membrane (Cavalier-Smith, 1991). Depending on the content of these vesicles, naked dinoflagellates are distinguished from armoured. The alveoli of naked dinoflagellates appear empty whereas those of armoured dinoflagellates contain cellulosic plates. Dinoflagellates represent an exceptional group within the eukaryotes concerning their plastid diversity and trophic strategies. Originally dinoflagellates are supposed to have acquired their plastids via secondary endosymbiosis with red algae. However about 50% of all described species lack visible plastids. Such species are supposed to have lost their plastids secondarily. The majority of described photosynthetic dinoflagellates contain peridinin plastids that are surrounded by three membranes and derived via secondary endosymbiosis with red algae. They are characterized by thylacoids in stacks of three and pyrenoids (Schnepf and Elbrächter, 1999). A remarkable feature is their DNA-organization in small minicircles of 2-3 kb (Zhang et al., 1999). As major pigments they contain the chlorophylls a and c and the carotinoid peridinin. Some dinoflagellates however lost their peridinin plastid and replaced it by another plastid. The two species *Lepidodinium chlorophorum* and *L. viride* got their plastids from a prasinophyte green alga and therefore appear green in color. Their plastids are enveloped by two membranes and both species grow in unialgal cultures showing that their plastids are permanent. As both species retained their plastids from a green alga, the term ‘serial secondary endosymbiosis’ is used (Schnepf and Elbrächter, 1999, Minge et al., 2010). In both cases the plastids are permanent and depend on protein import from the host which makes them to true organelles. Other dinoflagellates acquired their plastids via tertiary endosymbiosis. The genera *Karenia* and *Karlodinium* both contain permanent plastids of haptophyte origin (Tengs et al., 2000, Bergholtz et al., 2006). Their plastids are surrounded by three membranes (Steidinger et al., 1978). Both genera are naked and known to feed mixotrophically via a peduncle (Steidinger et al., 1998, Berge et al., 2008, Adolf et al., 2006) . Some dinoflagellates harbor permanent plastids of diatom origin. In contrast to *Karenia* and *Karlodinium*, these so called ‘dinotoms’ still contain the diatoms mitochondria, nucleus and the cytoplasm with ribosomes, the Golgi apparatus and the endoplasmic reticulum that are separated by a single membrane from the dinophyte cytoplasm (Tomas and Cox, 1973, Imanian and Keeling, 2007, Takano et al., 2008).The plastids are enveloped by two membranes (Schnepf and Elbrächter, 1999). Examples for Dinotoms are the species *Kryptoperidinium foliaceum, Durinskia baltica* and *Peridinium quinquecorne* (Inagaki et al., 2000, Horiguchi and Takano, 2006). It has been shown that the endosymbiont is closely related to the pennate diatom genus *Nitzschia* in *K. foliaceum* and *D. baltica* (Chesnick et al.,

1996, 1997, McEwan and Keeling, 2004) and to the centric diatom genus *Chaetoceros* in *P. quinquecorne* (Horiguchi and Takano, 2006). As both situations are permanent but the prey is less reduced compared to *Karenia,* they resemble more permanent endosymbionts than plastids. Some dinoflagellates harbor plastids that are 'stolen' from taken up prey and used to supply the host with photosynthetic products. These so called 'kleptoplasts' are just stable for a limited time and have to be replaced regularly (Nowack and Melkonian, 2010). A known genus with kleptoplasts of cryptophyte origin is *Nusuttodinium* (Takano et al., 2014). Dinoflagellates show an extraordinary variety of nutritional modes including photoautotrophy, mixotrophy as well as heterotrophy. Whereas all species lacking a visible plastid live heterotrophically, some plastid containing species live autotrophically and others mixotrophically (Stoecker, 1999; Hansen, 2011). Mixotrophic species supplement their nutrition by the uptake of bacteria or eukaryotic prey (Jeong et al., 2010; Hansen, 2011). Examples for mixotrophic dinoflagellates are the genera *Polykrikos* and *Esoptrodinium* (Hoppenrath and Leander, 2006, Calado et al., 2006, Fawcett and Parrow, 2012, Fawcett and Parrow, 2013). Whereas obligate mixotrophs depend on the uptake of prey, facultative mixotrophs can also live without additional prey. The uptake mechanisms include phagocytosis, myzocytosis and pallium feeding. Phagocytosis describes the engulfment of whole cells and often occurs in athecate dinoflagellates (Hansen and Calado, 1999). Myzocytosis describes the ingestion of cellular contents like the cytoplasm and whole organelles via a feeding tube called peduncle that penetrates the prey cell (Calado et al., 1998). The third type of feeding called pallium feeding is just described for a few species of heterotrophic thecate dinoflagellates. A pseudopodium-like structure emerging from the sulcal region is used to capture and envelope the prey that is digested outside the main cell body. Digested material is ingested whereas non-digestible material is released to the environment (Hansen and Calado, 1999).

1.3 Kleptoplasty – A potential key step during the acquisition of photoautotrophy

Kleptoplasty is the temporary retention of plastids obtained from ingested algal prey that supply the host with photosynthetic products and have to be replaced regularly (Nowack and Melkonian, 2010). The term 'cleptochloroplast' was first introduced by Schnepf et al. (1989) to describe the plastid of cryptophyte origin in the blue-green freshwater dinoflagellate *Gymnodinium aeruginosum* (today *Nusuttodinium aeruginosum*) that should be analogous to

'cleptocnides' of some mollusks. Kleptoplasts are widespread and found in dinoflagellates, ciliates, foraminifers, katablepharids and molluscs. Sources for kleptoplasts are cryptophytes, diatoms, golden algae, green algae, red algae and in some cases dinoflagellates (Lee, 2006). The uptake can be either direct through phagocytosis or indirect via an intermediate host. Direct uptake has been described for all groups mentioned above. The prey is ingested through a specialized feeding apparatus and directly incorporated into the cytoplasm or tissue. Indirect uptake has been described for the armored dinoflagellate genera *Dinophysis* and *Amylax*. Species of these two genera acquire their plastids through the intermediate host *Mesodinium* that itself acquires its plastids through direct uptake of the cryptophyte genus *Teleaulax* (Park et al., 2006). Both genera use a specialized feeding tube called 'peduncle' to suck out the cell contents including the kleptoplasts from *Mesodinium* (Park et al., 2006, Koike and Takishita, 2008). The degree to which the prey is reduced strongly varies between the predator species. Marine sea slugs of the genera *Plakobranchus* and *Elysia* just retain the plastids (Christa et al., 2013) whereas the ciliate *Mesodinium rubrum* nearly retains the whole prey. The ciliate digests the periplasts and membranes of its cryptophyte prey directly after ingestion and retains the preys plastids, mitochondria and nuclei (Oakley and Taylor, 1978) making the prey more an endosymbiont than a kleptoplast. A special feature making these organisms to intermediates between animals and plants is the ability to retain endosymbionts or kleptoplasts for several weeks up to several months. Kleptoplasts within the dinoflagellate *Dinophysis* and the ciliate *Mesodinium* have been reported to be stable for 2-3 months (Park et al., 2008). The deep-sea foraminiferan *Nonionella stella* is even able to retain its diatom derived kleptoplasts for up to 12 months (Grzymski et al., 2002, Bernhard, 2003) and a dinoflagellate species described from the Ross Sea (Antarctica) is able to maintain kleptoplasts from the haptophyte species *Phaeocystis antarctica* for 5-8 months (Gast et al., 2007). In other cases like the dinoflagellate *Nusuttodinium myriopyrenoides* nothing is known about the kleptoplast stability due to lacking cultures (Yamaguchi et al., 2011). One of the major aims concerning such organisms is to find out the mechanisms that are responsible for the longevity of the kleptoplasts. Studies on *Mesodinium rubrum* show that its kleptoplasts remain functionally active up to 80 days (Myung et al., 2013). A recent study shows that the cryptophycean nuclei within *Mesodinium rubrum* are transcriptional active over 13 weeks. Genes associated with light and dark reactions of photosynthesis and chlorophyll assimilation are up regulated after ingestion. Although cryptophyte plastids and nuclei are able to divide in the ciliate the lacking synchronization with host cell division finally leads to a cessation of the kleptoplasty (Kim et al., 2016). In *M. rubrum* as well as the dinoflagellate from the Ross Sea

(Antarctica) the authors hypothesize that kleptoplast stability is supported by the presence of the prey nucleus. However, some organisms are able to harbor kleptoplasts in the absence of prey algal nuclei. Dinoflagellates of the genus *Dinophysis* ingest the cell contents of the ciliate *Mesodinium rubrum* via myzocytosis and just maintain the cryptophyte plastids. However, these kleptoplasts are stable for up to two months (Park et al., 2008). Other examples are found in marine sea slugs of the genera *Elysia* and *Plakobranchus* that feed on specific algal prey during their juvenile stages and maintain their kleptoplasts for several months (Rumpho et al., 2000, Händeler et al., 2009, Wägele et al., 2011). It was suggested that in these cases lateral gene transfer from the prey to the host nucleus occurred. Pierce et al (2012) found 100 genes of algal origin in the transcriptome of *Elysia chlorotica*. However, these 100 genes were interpreted as algal contaminations in the transcriptome Christa et al. (2013). To exclude the possibility of contamination with prey algal DNA, a FISH-labeling approach was done on chromosomes extracted from chloroplast-lacking eggs of *Elysia chlorotica*. Although this approach seemed to confirm gene transfer between alga and slug (Schwartz et al. 2014), no genes of algal origin have been found in the sea slug nuclear genome (Bhattacharya et al, 2013). However due to the big differences in plastid maintenance seen in the systems studied, no conclusive hypothesis could be created yet.

1.4 The kleptoplastic genus *Nusuttodinium*

The genus *Nusuttodinium* Takano et Horiguchi has been created in 2014 and includes unarmoured dinoflagellates with plastids derived from endosymbiotic, periodically acquired and temporarily retained cryptomonads. The term ‘nusutto’ is Japanese and means ‘robbery’ indicating that the members have the ability to steal plastids from their prey. Currently, seven species of both freshwater and marine habitats are known. Marine species include *N. desymbiontum*, *N. latum*, *N. myriopyrenoides* and *N. poecilochroum.* Freshwater species are represented by *N. acidotum*, *N. aeruginosum* and *N. amphidinoides*. All species are characterized by an apical groove in an anticlockwise direction also found within the genus *Gymnodinium* and a lack of vesicular chambers in the nuclear envelope. When the genus was erected in 2014, it was supposed to be monophyletic (Takano et al., 2014). Already one year later the phagotrophic plastid lacking species *Pellucidodinium psammophilum* was described that falls into the genus *Nusuttodinium* phylogenetically making it paraphyletic (Onuma et al., 2015). In contrast to members of the genus *Nusuttodinium,* members of *Pelludidodinium* do not harbor kleptoplasts but digest their prey directly. Therefore the authors hypothesize that

Pellucidodinium represents an 'intermediate' between phagotrophic and kleptoplastidic dinoflagellates. In addition, Onuma et al. (2015) described the nonkleptoplastic species *N. desymbiontum* that 'never ingests cryptomonads' and showed that it also belongs to the genus *Nusuttodinium* phylogenetically. The authors made the hypothesis that *N. desymbiontum* lost its ability to keep kleptoplasts secondarily. However this study with its hypotheses should be viewed carefully as all observations were just made on natural samples due to difficulties in the establishment of cultures. Phylogenetically, *Nusuttodinium* is closely related to the marine photoautotrophic species *Spiniferodinium galeiforme* and the freshwater species *Gymnodinium palustre* (Takano et al., 2014). Until now no kleptoplastic species of *Nusuttodinium* is available in public culture collections showing the difficulties in establishing cultures of such dinoflagellates. The ability to harbor kleptoplasts makes the genus *Nusuttodinium* interesting to study the evolutionary question of how taken up prey organisms are transformed into plastids. All species except for *N. desymbiontum* were described to harbor kleptoplasts of cryptomonad origin. Interestingly, the species show big differences between prey selection, modification after ingestion and kleptoplast stability. The marine sand-dwelling species *N. poecilochroum* (originally *Amphidinium poecilochroum*) was described 1985 by Larsen as having 4-8 plastids of blue-green or brown color derived from *Chroomonas* or *Rhodomonas*. The cryptomonad plastids are just slightly modified in shape after ingestion and stable for a few days (no exact information is available concerning kleptoplast stability). Digestion of the kleptokaryon already starts 3 hours after ingestion. *N. poecilochroum* was established in culture but not deposited into any public culture collection (Onuma and Horiguchi, 2013). Another marine sand-dwelling species is *N. latum* (originally *Amphidinium latum*). It was described in 1992 by Horiguchi and Pienaar to contain 1-4 plastids of blue-green, bright-green or yellowish-brown color. It is able to survive for 2 weeks in seawater medium but does not divide in the absence of prey. Attempts to establish a culture failed (Horiguchi and Pienaar, 1992, Takano et al. 2014). *N. myriopyrenoides* (originally *Gymnodinium myriopyrenoides*) is the last marine member and was described 2011 by Yamaguchi et al. In contrast to the members described above, it just contains a single large blue-geen U-shaped kleptoplast probably derived from the genera *Chroomona*s or *Hemiselmis*. Organelles including the nucleus, nucleomorph, mitochondria, Golgi bodies and plastids are retained. No information about prey specifity and kleptoplast stability are available as no cultures could be established (Yamaguchi et al., 2011). Actually three freshwater species are included in the genus *Nusuttodinium*. *N. amphidinoides* (originally *Amphidinium amphidinoides*) was originally described in 1924 by Geitler. Already on year

later Woloszynska described a similar species *Amphidinium vigrense* (Woloszynska, 1925) that is currently seen as a synonym to *N. amphidinoides* due to its similarities and the high variability of the cell shape found (Takano et al., 2014). *N. amphidinoides* contains blue-green or yellow-brown kleptoplasts of cryptomonad origin. The most detailed study on this species was published in 1985 by Wilcox and Wedemeyer. The number of kleptoplasts was found to differ from 2-7. Serial TEM-sections revealed neither an association with cryptomonad nuclei nor with nucleomorphs. Interestingly, the cryptomonad kleptoplasts were enveloped by three membranes, a feature characteristic for permanent plastids of the peridinin type. Therefore the authors hypothesized that the plastids were permanent. However, also this study is just based on natural material. In 2014 Takano et al. disproved this hypothesis and showed a colorless cell of *N. amphidinoides* that was induced after 21 days of starvation from a blue-green cell. However, they confirmed the number of envelope membranes to be three.

1.5 *Nusuttodinium acidotum* and *Nusuttodinium aeruginosum*

The two kleptoplastic freshwater species *N. acidotum* and *N. aeruginosum* are of particular interest in evolutionary biology as both species strongly modify their cryptomonad prey after ingestion. *N. acidotum* (originally *Gymnodinium acidotum*) was described in 1950 by Nygaard as having a pointed antapex whereas *N. aeruginosum* (originally *Gymnodinium aeruginosum*) was described by Stein in 1883 as having a round antapex. In addition, a third species, *Gymnodinium eucyaneum* was originally described in China (Hu et al., 1980). However, this species is highly similar to *N. acidotum* except for two differences: The size of the hypocone is 1.3 times longer than the epicone in *G. eucyaneum* whereas it is 1:1 in *N. acidotum*. Moreover 1 bp difference to *N. acidotum* was observed in the partial LSUrDNA (Xia et al., 2013). Due to these minor differences it can be regarded as a synonym of *N. acidotum* (Takano et al., 2014). Both species, *N. acidotum* and *N. aeruginosum* are known to harbor an endosymbiont derived from the genus *Chroomonas*. The endosymbiont consists of the cryptomonad cytoplasm, plastid, nucleus and nucleomorph and is separated by a single membrane from the dinophyte cytoplasm. The plastid is strongly enlarged, highly lobed and fills most of the periphery of the cell after ingestion by the dinoflagellate. It is surrounded by four envelope membranes (Wilcox and Wedemayer, 1984, Schnepf et al., 1989, Farmer and Roberts, 1990). A cryptomonad nucleus can be present or absent. The frequency of binucleate cells in natural populations varied from 10 % (Schnepf et al., 1989) to 33 % (Farmer and Roberts, 1990). The first successful culture of *N. acidotum* was established by Fields and

Rhodes (1991) by adding cells of the genus *Chroomonas* to the dinoflagellates. The frequency of binucleate cells in this culture was 57%. The genus *Cryptomonas* was not accepted as prey. However, the culture was just maintained for 9 months. Most studies on *N. acidotum* and *N. aeruginosum* showed the presence of just one cryptomonad nucleus. Farmer and Roberts (1990) showed cells with two cryptomonad nuclei and Shi et al. (1983) even reported that the number of cryptomonad nuclei can vary from 0-10 (information taken from Xia et al., 2013). However, this study is not widely known as it is written in Chinese. It was suggested that the host and endosymbiont division cycles are not synchronized (Farmed and Roberts, 1990). The number of kleptoplasts observed also varied. Whereas most studies reported the presence of just one U-shaped kleptoplast, Walker (1973) reported that the number of kleptoplasts can vary depending on the season. The stability of the kleptoplast varies between 2 and 4 weeks (Shi et al., 1983, Fields and Rhodes, 1991). Many attempts have been made to identify the endosymbiont of *N. aeruginosum* and *N.acidotum*. Microspectrophotometry did not prove to be precise enough to identify the endosymbiont on the species level (Barsanti et al., 2009). Therefore attempts were made to identify the endosymbiont molecularly. Xia et al. (2013) found by sequencing of the plastid 23SrDNA that the endosymbiont is closely related to *Chroomonas coerulea*. However, their sequences did not match with any known sequence. Onuma and Horiguchi (2016) sequenced the 16S rDNA of multiple cells of *N. aeruginosum* and found 10 different genotypes of *Chroomonas* all belonging to subclade 4. Fields and Rhodes (1991) offered three different strains of *Chroomonas* to *N. acidotum* all with the same results. These results indicate that *N. aeruginosum* and *N.acidotum* are able to maintain kleptoplasts of genetically diverse *Chroomonas* strains. Phylogenetically, *N. aeruginosum* and *N. acidotum* are closely related to the naked kleptoplastic species mentioned in 1.4 and belong to the order Gymnodiniales. Comparison of the ITS region revealed four different genotypes of *N. aeruginosum* and one genotype of *N. acidotum* (Takano et al., 2014). However, no studies have been done concerning the morphological variability of both species under culture conditions and therefore no clear identification is available yet. In addition sequences of the ITS-region of cells with and without a pointed antapex from the same lake were identical. Takano et al. (2014) redescribed the species *N. aeruginosum* as having a pointed or round antapex that contradicts to the original description and also to personal observations made on stable axenic cultures during this study. Therefore, the dinoflagellate presented in this study is referred to as *N. aeruginosum/acidotum*.

1.6 Aims of this study

The major aim of this study was to address the question about the evolutionary steps that are necessary to establish permanent plastids from taken up prey. Many studies have been performed on organisms with transient plastids to get an idea about this important early step of evolution that led to the development of all photosynthetic eukaryotes on earth. However, all organisms studied until now are able to just form one type of plastid making it difficult to draw conclusions about the dynamic processes. In addition many studies were performed on natural samples due to problems in establishing cultures of such organisms. To get an idea about the necessary steps during plastid establishment, the life cycle and endosymbionts of the dinoflagellate freshwater species *Nusuttodinium aeruginosum/acidotum* should be studied. In contrast to all other systems studied until now, this species is able to harbor endosymbionts of different morphology and stability. The first step was to establish a collection of genetically diverse strains of the genus *Chroomonas* from different habitats, particularly where also *N. aeruginosum/acidotum* was found. This collection formed the basis for the establishment of a stable axenic co-culture of *N. aeruginosum/acidotum*. Feeding experiments under controlled conditions should be performed to determine the food spectrum and the variety of kleptoplasts formed. A phylogenetic tree for all taken up *Chroomonas* strains should be established to determine if the type of kleptoplast formed depends on the phylogenetic position of the prey. The stability and morphology of all kleptoplast types and the life cycle of N. *aeruginosum/acidotum* should be studied. Moreover a comparative study should be performed on *N. amphidinoides* that seems to harbor more primitive kleptoplasts compared to *N. aeruginosum/acidotum.*

2. Material and Methods

2.1 Material

Table 1. Instruments and aids.

Name	Product name	Source
Autoclave	VX-150	Systec
Binocular	SZX 16	Olympus
Centrifuge small	Pico 17	HERAEUS
Centrifuge large	MULTIFUGE 35R+	Thermo Scientific
Clark electrode		Hansatech
Clean bench sterile	Biowizard Silver SL-130 class ll	Kojair Tech Oy
Confocal Laser Scanning Microscope	DM5500Q	Leica
Critical-Point-Drying (CPD)-machine	Polaron CPD	Polaron
Culture room		Johnsons Controls
Elektrophoresis chamber	Vari-gel	Roth
Diamond knife 35°		Diatome
Fine balance	Satorius Portable	Satorius
Geldocumentation mashine		Intas®
Gelform	Vari-gel	Roth
Fluorescent lamp, cold white light	OSRAM	Biolux
Fluorescent lamp, warm white light	OSRAM	Lumilux
Incubator small	INCUBAT	MELAG
Incubator large	WTB Binder Wärmeschrank	BINDER
Knife maker	Knife Maker Type 7801A	LKB
Light measurer	Li-250A Light Meter	LI-COR
Microscope everse	ECLIPSE E800	Nikon
Microscope inverse	CXK41	OLYMPUS
Microscope inverse	IM	ZEISS
Microwave	Micromaxx®	MTC Medion
Mortar		VWR
Objektmikrometer		PYSER-SGI LTD
One channel pipettes (0,5 – 10 µl, 10 – 100 µl, 20 – 200 µl, 100 – 1000µl)	Ergonomic High Performance	VWR
Reflex camera	EOS 1000D	Canon
Scanning Electron Microscope	LEO 430	ZEISS
Sequencer	LI-COR 4200	LI-COR
Sludge lifter		Werkstatt Uni-Köln
Sonicator	Soniprep 150	MSE
Sputter mashine	Sputter coater 108 auto	Cressington
Thermocycler	Primus 96^{plus}	AVISO
Transmission Electron Microscope	CM10	Philips
Ultramicrotome	Ultra Cut	Leica
Vortexer	Vortex Genie 2^{TM}	Bender & Hobein

Table 2. Software.

Name	Source
AlignIR	LI-COR
Basic local alignment search tool (BLAST)	NCBI
Cell F	Olympus
EOS Utility	Canon
e-Seq	LI-COR
Excel 2007	Microsoft
Illustrator	Adobe
Image J	Freeware (National Institutes of Health)
LAS AF Lite	Leica (Germany)
Metamorph	Visitron Systems
Photoshop	Adobe
Seaview 4.2	Freeware (Galtier et al., 1996)
Statiscica 13.0	StatSoft
Gatan Digital Micrograph	Philips
Word 2007	Microsoft
REM-Software	Olympus

Table 3. Chemicals and kits.

Name	Formula	Source
Agarose		Biozym
Ammoniumperoxodisulfate	$(NH_4)_2S_2O_8$	Roth
Biotin		Sigma
Boric acid	H_3BO_3	Roth
Calciumchlorid dihydrate	$CaCl_2*2H_2O$	Merck
Calciumnitrate tetrahydrate	$CA(NO_3)_2*4H_2O$	Merck
Cetyl-Trimethyl-Ammoniumbromide (CTAB)		Sigma Aldrich
Chloroform	$CHCl_3$	VWR
Cobaltchloride Hexahydrate	$COCl_2*6H_2O$	Merck
4,6-Diamidino-2-phenylindole (DAPI)	$C_{16}H_{15}N_5$	
Desoxyribonucleotid-Triphosphate (dNTP's)		Fermentas
Di-Ammonium-hydrogenphosphate	$(NH_4)_2HPO_4$	Merck
di-potassiumhydrogenphosphat Trihydrate	$K_2HPO_4*3H_2O$	Merck
Dimethylsulfoxide		Roth
DNA ladder (1 kb)		Fermentas
Plant DNA Kit		OMEGA
Dodecenyl succinic anhydride (DDSA)		Agar Scientific
Dynabeads		Invitrogen
Ethylendiamintetraacetic acid (EDTA)	$C_{10}H_{14}N_2Na_2O_82H_2O$	Roth
Ethanol	C_2H_6O	Roth
Ethidiumbromide		Applichem
Glucose	$C_6H_{12}O_6$	Merck
Glutaraldehyde (EM-grade), 25%		Serva

Table 3 continued. Chemicals and kits.

Name	Formula	Source
Glycidether 100		Roth
5x green GoTaq Reacion Buffer		Promega
N-2-Hydroxyethylpiperazin-N‘-2-ethan-sulfonsäure (HEPES)	$C_8H_{18}N_2O_4S$	Roth
Isoamylalcohol	$C_5H_{12}O$	Merck
Lithiumchloride	LiCl	Applichem
Magnesiumsulfate heptahydrate	$MgSO_4*7H_2O$	Merck
Manganchloride heptahydrate	$MgCl_2*4H_2O$	Merck
Meat extrakt		Merck
ß-Mercaptoethanol	C_2H_6OS	Sigma Aldrich
Methylnadic anhydride	$C_{10}H_{10}O_3$	SERVA
Molecular sieve		Sigma
Niacinamide		Sigma
Nitrilotri acetic acid (NTA, Titriplex1)		Merck
N,N,N‘,N‘-Tetramethylethyldiamin (TEMED)	$C_6H_{16}N_2$	Roth
Nonenyl succinic anhydride pure	$C_{13}H_{20}O_3$	SERVA
Osmiumtetroxide		Plano
Peptone		Merck
Phenol acidic	C_6H_6O	Sigma Aldrich
Pioloform in chloroform (0,5%)		unknown
Poly-L-Lysine		Sigma
Polyvinylpyrrolidone (PVP)		Sigma Aldrich
Potassium chloride	KCl	Merck
Potassium nitrate	KNO_3	Merck
± Propylene oxide	C_3H_6O	Sigma Aldrich
Sequagel XR Acrylamid		Biozym
Sodiumcarbonate	Na_2CO_3	Merck
Sodiumchloride	NaCl	Merck
Sodiumdodecylsulfate (SDS)	$C_{15}H_{25}NaO_4S$	Serva
Sodiumhydroxide	NaOH	Applichem
Sodiumnitrate	$NaNO_3$	Merck
Soil for soilextract		CCAC
Spermidine		Sigma Aldrich
Thermo Sequenase^{TM}Dye Primer Manual Cycle Sequencing Kit		Affymetrix
20x Glycerol tolerant Gel buffer ultrapure		USB
Thiamine-HCL		Serva
Tris		Roth
2,4,6,Tris(dimethylaminomethyl) phenol	$C_{15}H_{27}N_3O$	Merck
urea	$CO(NH_2)_2$	Merck
Vitamin B12		Serva
Water for molecular biology	H_2O	AppliChem
Yeast extract		Merck
Zinksulfate heptahydrate	$ZnSO_4*7H_2O$	Merck

Table 4. Commodities.

Name	Source
Aluminiumcap	Schott
Carbon band	Plano
Cellulose plugs	VWR
Cotton (100%)	Hartmann
Cover slip large (24x50 mm)	Paul Marienfeld
Cover slip round (Ø = 10 mm)	Menzel
DNeasy Plant Mini Kit	Quiagen
Erlenmeyer flask 50 ml	VWR
Falcon tubes	BD Falcon
Glass stripes for knife making (6 mm)	Agar
Fernbach flask 1l	VWR
Immersion oil	Merck
Marburg flask	VWR
Mikrotiter plates 96 Well	BD Falcon
Mikrotiter plates 24 Well	BD Falcon
Object carrier	Paul Marienfeld
Parafilm	Pechiney, Plastik Packaging
Pasteur pipets (230 mm)	VWR
Petri dishes large (Ø ca. 9 cm)	SARSTEDT
Petri dishes small (Ø ca. 5 cm)	SARSTEDT
Pipet tips (0,1-10 µl, 200 µl, 1000 µl)	SARSTEDT
Reaction tubes 0,2 ml	Biostep
Reaction tubes 1,5 ml	SARSTEDT
Reaction tubes 2ml	SARSTEDT
Schott bottles	SCHOTT
Slot grids (copper)	Agar

2.2 Used strains of algae and culture conditions

Used strains of algae

Algal strains named with ACOI, CCAC, CCAP, CCMP, M, NIES, SAG and UTEX were obtained from culture collections. The other strains were established by Sebastian Wittek.

Table 5. Abbreviations and addresses of culture collections.

Abbreviation	Culture collection and adress
ACOI	Coimbra Collection of Algae, Coimbra, Portugal
CCAC	Culture collection of algae at the University of Cologne
CCAP	Culture Collection of Algae and Protozoa, Oban, Schottland
CCMP	National Center for Marine Algae and Microbiota, Maine, USA
M	Culture collection of Prof. Dr. M. Melkonian (University of Cologne)
NIES	National Institute for Environmental Studies, Ibaraki, Japan
SAG	Collection of algal cultures at the University Göttingen (Sammlung für Algenkulturen der Universität Göttingen)
UTEX	Culture Collection of Algae at the University of Texas Austin, Austin, Texas

Table 6. Dinoflagellate strains investigated in this study.

Strain (prey)	Species	Origin	Coordinates	Time of isolation	Culture medium	Isolated by
CCAC[ax] 4851 (CCAC 4832)	*Nusuttodinium aeruginosum/ acidotum*	Heidemoor, Dahlem Eifel G.	50°23'21.81''N, 6°34'8.35'' E	09.05.2011	W/2 Sph + 1% B	Wittek, S
N.a. WH[ax] (CCAC 4832)	*Nusuttodinium aeruginosum/ acidotum*	Wahner Heide Fuchskaule G.	50°52'49.45'' N, 7°10'56.86'' O	16.09.2012	W/2 Sph + 1% B	Wittek, S
N.a. Mar-magen[ax] (CCAC 4832)	*Nusuttodinium aeruginosum/ acidotum*	Oberer Stauteich Marmagen G.[1]	50°48'62.85" N, 6°56'91.69" O	05.06.2012	W/2 Sph + 1% B	Wittek, S
N.a. Haitz[ax] (CCAC 4832)	*Nusuttodinium aeruginosum/ acidotum*	Haitz Gelnhausen[2]	-	25.05.2012	W/2 Sph + 1% B	Wittek, S
N.a. HLM[ax] (CCAC 4832)	*Nusuttodinium aeruginosum/ acidotum*	Hopsten Steinfurt, Großes Heiliges Meer	52° 21′ 7″ N, 7° 38′ 1″ O	19.04.2012	W/2 Sph + 1% B	Wittek, S
N.a. Krefeld[ax] (CCAC 4832)	*Nusuttodinium aeruginosum/ acidotum*	Krefeld	51°33'34.35"N, 6°63'2.46"O	05.06.2013	W/2 Sph + 1% B	Wittek, S
N. amph. Römerwei her[ax] (CCAC 0060)	*Nusuttodinium amphidinoides*	Nettersheim Römerweiher	50°48'21.57"N, 6°62'3.11"O	25.03.2012	W/2 Sph + 1% B	Wittek, S

Table 6 continued. Dinoflagellate strains investigated in this study.

Strain (prey)	**Species**	**Origin**	**Coordinates**	**Time of isolation**	**Culture medium**	**Isolated by**
N.amph. Kolk[ax] (M0874)	*Nusuttodinium amphidinoides*	Rees nahe Grietherbusch Kolk	51°76'31.15"N, 6°41'6.48"O	28.04.2012	W/2 Sph + 1% B	Wittek, S
N.amph. Fluss[ax] (CCAC 0060)	*Nusuttodinium amphidinoides*	Grietherbusch Fluss	51°79'04.88"N, 6°33'7.00"O	28.04.2012	W/2 Sph + 1% B	Wittek, S
N. amph. HLM[x] (CCAC 0060)	*Nusuttodinium amphidinoides*	Fischteich nahe großes Heiliges Meer	52°35'50.76"N, 7°63'8.60"O	19.04.2012	W/2 Sph	Wittek, S
N.amph. Marma-gen[ax] (CCAC 0060)	*Nusuttodinium amphidinoides*	Oberer Stauteich Marmagen G.	50°48'62.85" N, 6°56'91.69" O	15.06.2013	W/2 Sph + 1% B	Wittek, S
N.amph. WH[ax] (CCAC 0060)	*Nusuttodinium amphidinoides*	Wahner Heide Fuchskaule	50°88'02.30"N, 7°18'1.79"O	14.06.2012	W/2 Sph + 1% B	Wittek, S
N.amph. Dahlem[ax] (CCAC 0060)	*Nusuttodinium amphidinoides*	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	26.02.2013	W/2 Sph + 1% B	Wittek, S
N.amph. Krefeld Burg Linn[ax] (CCAC 0060)	*Nusuttodinium amphidinoides*	Krefeld Burg Linn	51°33'34.35"N, 6°63'2.46"O	04.05.2012	W/2 Sph + 1% B	Wittek, S
N. amph. Krefeld Bockum[ax] (CCAC 0060)	*Nusuttodinium amphidinoides*	Krefeld Bockum	51°34'40.06"N, 6°60'9.95"O	28.06.2013	W/2 Sph + 1% B	Wittek, S
CCAC 5709 N. amph. kl. Epicone[ax] (CCAC 4832)	*Nusuttodinium amphidinoides* with small epicone	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	26.02.2013	W/2 Sph + 1% B	Wittek, S
M 4765	*Kryptoperidi-nium* sp.	Aegean Sea, Greece	37°58’33.33’’N, 22°75’O	2010	ASP-12	Kirsch, M.

[ax] axenic; [x] xenic;1 the sample was collected by Dr. Karl-Heinz Linne von Berg, [2]The sample was collected by Dr Max Klingberg; (x) = food strain used to maintain the dinoflagellate

culture; W = Waris-H; Sph = Sphagnumextract; B = Bacteria Standart Medium; G = Germany; - = data not known; strains shaded in blue were in the focus of this study and used to perform the majority of experiments.

Table 7. Strains of *Chroomonas* used for maintaining the dinoflagellate cultures and for feeding experiments.

Strain	Species	Origin	Coordinates	Time of isolation	Culture Medium	Isolated by
Chro afr-^{ax}	*Chroomonas africana*	Cape Province, South Africa	-	1984	W + 1% B	Meyer and Pienaar
CCAC 0060^{ax}	*Chroomonas* sp.	Griether Ort, G.	-	1991	W	Schilke, A.
CCAC 1074^{ax}	*Komma caudata*	Köln, Universitäts-bibliothek G.	-	1993	W + 1% B	Hoef-Emden,K.
CCAC 1481^{ax}	*Chroomonas coerulea*	Spessart G.	-	1994	W + 1% B	Leukart, P.
CCAC 1624^{ax}	*Chroomonas* sp.	Dänemark, Sjaelland	-	1998	W + 1% B	Hoef-Emden, K
CCAC 1953^{ax}	*Chroomonas* sp.	Schloss Hülshoff nahe Münster G.	-	03.2002	W + 1% B	Feja, N.
CCAC 2067^{ax}	*Chroomonas* sp.	Heidemoor nahe Dahlem G.	-	08.2012	W + 1% B	Melko-nian, M.
CCAC 3453 (SAG 980-1)^{ax}	*Chroomonas* sp.	UK, Wales.	-	1945	W + 1% B	Pringshei m, E.G.
CCAC 4826^{ax}	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	22.03.2011	W + 1% B	Wittek, S
CCAC 4827^{ax}	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	22.03.2011	W + 1% B	Wittek, S
CCAC 4828^{ax}	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	22.03.2011	W + 1% B	Wittek, S
CCAC 4829^{ax}	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	22.03.2011	W + 1% B	Wittek, S
CCAC 4830^{ax}	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	22.03.2011	W + 1% B	Wittek, S
CCAC 4831^{ax}	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	22.03.2011	W + 1% B	Wittek, S
CCAC 4832^{ax}	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23’21.81‘‘N, 6°34‘8.35‘‘ E	22.03.2011	W + 1% B	Wittek, S

Table 7 continued. Strains of *Chroomonas* used for maintaining the dinoflagellate cultures and for feeding experiments.

Strain	Species	Origin	Coordinates	Time of isolation	Culture Medium	Isola-ted by
CCAC 4833[ax]	*Chroomonas* sp.	Heidemoor, Dahlem Eifel G.	50°23'21.81''N, 6°34'8.35'' E	22.03.2011	W + 1% B	Wittek, S.
CCAC 4834[ax]	*Chroomonas* sp.	Hopsten Steinfurt, Großes Heiliges Meer, G	52° 21′ 7″ N, 7° 38′ 1″ O	19.04.2011	W + 1% B	Wittek, S.
CCAC 4835[ax]	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°52'49.45'' N, 7°10'56.86'' O	02.11.2010	W + 1% B	Wittek, S.
CCAC 4836[ax]	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°52'49.45'' N, 7°10'56.86'' O	02.11.2010	W + 1% B	Wittek, S.
CCAC 4837[ax]	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°52'49.45'' N, 7°10'56.86'' O	02.11.2010	W + 1% B	Wittek, S.
CCAC 4838B	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°52'49.45'' N, 7°10'56.86'' O	02.11.2010	W	Wittek, S.
CCAC 4839[ax]	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°52'48.94'' N, 7°10'54.28'' O	02.11.2010	W + 1% B	Wittek, S.
CCAC 4840[ax]	*Komma caudata*	Wahner Heide Fuchskaule, G.	50°52'46.38'' N, 7°10'53.81'' O	05.01.2011	W + 1% B	Wittek, S.
CCAC 4841B	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°87'42.74" N, 7°17'18.31" O	06.04.2011	W	Wittek, S.
CCAC 4842[ax]	*Chroomonas* sp.	Hopsten Steinfurt, Großes Heiliges Meer, G.	52° 21′ 7″ N, 7° 38′ 1″ O	19.04.2012	W + 1% B	Wittek, S.
CCAC 4843[ax]	*Chroomonas* sp.	Fischteich near großes Heiliges Meer, G.	52°35'50.76"N, 7°63'8.60" O	19.04.2012	W + 1% B	Wittek, S.
CCAC 4844[ax]	*Chroomonas* sp.	Haitz Geln-hausen, G.	-	25.5.2012	W + 1% B	Wittek, S.
CCAC 4846[ax]	*Chroomonas* sp	Rees nahe Griether-busch, Kolk	51°76'31.15"N, 6°41'6.48"O	28.04.2012	W + 1% B	Wittek, S.
CCAC 4847[ax]	*Chroomonas* sp.	Fischteich near großes Heiliges Meer, G.	52°35'50.76"N, 7°63'8.60" O	19.04.2012	W + 1% B	Wittek, S.
CCAC 4848[ax]	*Chroomonas* sp.	Krefeld Burg Linn, G.	51°33'34.35"N, 6°63'2.46"O	04.05.2012	W + 1% B	Wittek, S.
CCAC 4849B	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°52'49.45'' N, 7°10'56.86'' O	16.09.2012	W	Wittek, S.
CCAC 4850[ax]	*Chroomonas* sp.	Wahner Heide Fuchskaule, G.	50°87'38.39" N, 7°17'22.74" O	16.09.2012	W + 1% B	Wittek, S.
CCAC 4852[ax]	*Chroomonas* sp.	Nettersheim Römerweiher, G	50°48'21.57"N, 6°62'3.11"O	25.03.2012	W + 1% B	Wittek, S.
ACOI 1366[x]	*Chroomonas coerulea*	Coimbra, Portugal	-	2001	W	Carval-ho, G.
CCAP 978/8 [ax]	*Chroomonas placoidea*	-	-	-	W + 1% B	-

Table 7 continued. Strains of *Chroomonas* used for maintaining the dinoflagellate cultures and for feeding experiments.

Strain	Species	Origin	Coordinates	Time of isolation	Culture Medium	Isolated by
CCMP 269[x]	*Chroomonas* sp.	USA Maryland,	-	-	W	Guillard,R.
CCMP 1168[x]	*Chroomonas mesostigmatica*	-	-	-	W	-
NIES 712[x]	*Chroomonas caudata*	Funada-Ike-Pond, Chiba Chiba Japan	-	13.09.1985	W	Erata, M.
NIES 713[x]	*Chroomonas coerulea*	Nagano, Japan	-	10.11.1985	W	Erata, M.
NIES 714[x]	*Chroomonas coerulea*	Nagano, Japan	-	12.11.1985	W	Erata, M.
UTEX 2779[x]	*Chroomonas pochmannii*	Colorado, USA	-	2000	W	Kugrens, P.
UTEX 2780[ax]	*Chroomonas coerulea*	Colorado, USA	-	2000	W + 1% B	Kugrens, P.
CCAC 0006[ax]	*Cryptomonas curvata*	Cormwall, Trenant, England	-	1978	W + 1% B	Melkonian, M.
CCAC 0018[ax]	*Cryptomonas erosa*	Senckenberg, G.	-	1991	W + 1% B	Schilke, A.
CCAC 0031[ax]	*Cryptomonas obovoidea*	Bergisches Land, G.	-	1994	W + 1% B	Hoef-Emden, K.
CCAC 0032[ax]	*Cryptomonas pyrenoidifera*	Wahner Heide, G.	-	1994	W + 1% B	Hoef-Emden, K.
CCAC 0064[ax]	*Cryptomonas ovata*	Senckenberg, G.	-	1990	W + 1% B	Reize, I.B.
CCAC 0104[ax]	*Cryptomonas massonii*	Helgoland	-	1996	W + 1% B	Hoef-Emden, K.
CCAC 0107[ax]	*Cryptomonas lundii*	Köln	-	1991	W + 1% B	Reize, I.B.
CCAC 0108[ax]	*Cryptomonas gyropyrenoidosa*	Dörpeltal-sperre, G.	-	1993	W + 1% B	Hoef-Emden, K.
CCAC 0113[ax]	*Cryptomonas borealis*	Köln	-	1993	W + 1% B	Hoef-Emden, K.
CCAC 0172[ax]	*Cryptomonas tetrapyrenoidosa*	Jylland, Dänemark	-	1998	W + 1% B	Hoef-Emden, K.
CCAC 0187[ax]	*Cryptomonas phaseolus*	Wahner Heide, Agger, G.	-	2003	W + 1% B	Melkonian, M.
CCAC 0191[ax]	*Cryptomonas loricata*	Harz, G.	-	2002	W + 1% B	Melkonian, M.
CCAC 0192[ax]	*Cryptomonas erosa*	Harz, G.	-	2002	W + 1% B	Melkonian, M.
CCAC 2195[B]	*Cryptomonas* sp.	Eifel, G.	-	2003	W	Melkonian, M.
CCAC 2290[B]	*Cryptomonas* sp.	Wahner Heide, G.	-	2003	W	Melkonian, M.

Table 7 continued. Strains of *Chroomonas* used for maintaining the dinoflagellate cultures and for feeding experiments.

Strain	Species	Origin	Coordinates	Time of isolation	Culture Medium	Isolated by
CCAC 2504[B]	*Cryptomonas* sp.	Wahner Heide, G.	-	2003	W	Melkonian, M.
CCAC 2807[B]	*Cryptomonas* sp.	Spessart, G.	-	1998	W	Klingberg, M.
M 2922	*Rhodomonas* sp.	Köln,Lindenthal, G.	-	2006	W	Melkonian, M.
CCAC 1479[B]	*Rhodomonas* sp.	Lewadia, Greece	-	1997	W	Hoef-Emden, K.

[ax] axenic; [x] xenic; W = Waris-H;; B = Bacteria Standart Medium; G = Germany; - = data not known

2.3 Culture Media

Culture media

Freshwater strains were cultivated in modifications of the medium Waris-H (Mc. Fadden and Melkonian, 1986)

Table 8. Composition of the used modification of Waris-H (McFadden and Melkonian, 1986).

Ingredients	Final concentration
$CaNO_3$	0,42 mM
Fe-EDTA	18,0 µM
HEPES	1,0 mM
KNO_3	1,0 mM
$MGSO_4$	81,1 µM
$(NH_4)_2HPO_4$	0,15 mM
Soil extrakt 1 ml[1]	-
Trace metals	
$COCl_2$	16,8 nM
H_3BO_3	18,43 µM
$MnCl_2$	0,58 µM
Na_2-EDTA	8,06 µM
$ZnSO_4$	73,04 nM
Vitamins	
Biotin	0,82 nM
Nicotinacidamid	4,1 nM
Thiamin-HCl	0,3 µM
Vitamin B_{12}	0,15 nM

The pH was adjusted to 7 with NaOH/HCL. [1] For the production of soil extract 100 g of dried soil (CCAC) was grind with morter and pestill and autoclaved in 1l of H_2O. Coarse particles were removed via sedimentation for 1 week. Afterwards fine particles were removed via centrifugation at 4000 g for 30 min. The cleared supernatant was stored at -20°C until application.

Waris-H/2 + Sphagnum extract (W/2 Sph)

For the preparation of W/2 Sph Waris-H was mixed in a relationship of 1:1 with H_2O and 6 ml/l of Sphagnum-extract[1] were added. The pH was adjusted to 7.

[1]25 g of dried *Sphagnum* moss was boiled 1 h in 750 ml H_2O and the extract was filtered through glass fiber, 1.2 µm, 0.8 µm, 0.4 µm, 0.2 µm. The extract was stored at -20°C until application.

ASP-12

This medium was prepared according to (Provasoli et al., 1957, McFadden & Melkonian, 1986)

Bacterial Standard Medium (BSM)

Bacterial Standard Medium (BSM) was added to the culture medium of axenic cultures to detect bacterial contaminations rapidly. This component was always added in a total concentration of 1% (v/v) to the culture medium. It contains 0.8% (w/v) peptone, 0.1% (w/v) glucose, 0.1% (w/v) meat extract and 0.1% (w/v) yeast extract. BSM was autoclaved and stored at -20°C until application.

Culture conditions

Cultivation and experiments were done in culture chambers. The following culture conditions were used:

16°C and a light intensity of 3.22 ± 0.94 µmol*m^{-2}*s^{-1} with a light/dark cycle of 14/10 h.

23°C and a light intensity of 7.6 ± 1.16 µmol*m^{-2}*s^{-1} with a light/dark cycle of 14/10 h.

4°C and a light intensity of 5.95 ± 1.01 µmol*m^{-2}*s^{-1} with a light/dark cycle of 14.5/9.5 h.

The light intensities are average values with standard deviation, measured at 5 different points within the cultivation area.

2.4 Establishment of an axenic collection of kleptoplastic dinoflagellates and maintenance of dinoflagellate-cryptophyte co-cultures

The establishment of a stable culture is one of the most important steps for the study of kleptoplastic organisms. It is known since long time that members of the kleptoplastic genus *Nusuttodinium* obtain their kleptoplasts from cryptomonad prey. Therefore the first step was to establish a collection of potential prey organisms with a focus on the genus *Chroomonas*. Water bodies from different locations in Germany were sampled. From previous experience it is known that the two species *N. aeruginosum/acidotum* and *N. amphidinoides* as well as the genus *Chroomonas* live close to the sediment. Therefore the upper layers of the sediment were sampled with a sludge lifter. Samples were stored in 50ml falcon tubes or plastic bottles and brought to the lab as fast as possible. They were filled in Petri dishes and directly screened with an inverted microscope (CXK 41, Olympus). The samples were maintained at 16°C. The main focus was on samples that contained both kleptoplastic dinoflagellates and *Chroomonas* but *Chroomonas* was also isolated from samples where no kleptoplastic dinoflagellates were found. Isolation was done with micro capillaries that were produced from glass Pasteur pipettes. Single cells of *Chroomonas* were isolated without previous washing into 250µl of Waris-H medium into 96- well microtiterplates and incubated at 16°C. After successful growth, the established cultures were purified and made axenic. Therefore approx. 5ml of culture was centrifuged at 500G for 5min at room temperature.The supernatant was removed and substituted by sterile medium. This step was repeated eight times to remove most of the bacteria. Afterwards the cell pellet was resuspended in 20ml of sterile medium and single cells were isolated with micro capillaries into 250µl of sterile Waris-H (W) with the addition of 1% Bacteria Standard Medium (BSM) to detect bacterial contaminations rapidly. The reisolation step ensured that each culture is single-cell derived. After successful growth, the cryptomonad cultures were maintained in W+1% BSM. Dinoflagellate cells were also isolated with micro capillaries but pre-washed in drops of sterile W/2 +Sph before being isolated into 250µl of sterile medium into 96-well microtiterplates. A drop of *Chroomonas* culture was added and dinoflagellates were observed daily. Cells where uptake of cryptomonad prey has been observed were transferred every two weeks into fresh medium. Especially at the beginning it was very important to regularly separate the dinoflagellates from the cryptophytes as the cryptophytes overgrew the dinoflagellates rapidly. This was done via micro capillary isolation at the beginning. Once the cultures were established *N. aeruginosum/acidotum* had to be treated in a different way than *N. amphidinoides*. The

cultures were transferred into fresh medium once per month in both cases. As *N. aeruginosum/acidotum* rapidly tends to be overgrown by the cryptomonad prey, the dinoflagellates had to be concentrated each time before inoculation. This was done by filling the culture into 50ml Falcon tubes. The dinoflagellates settled to the bottom whereas the cryptomonad prey stayed in the water column making it possible to separate both organisms. Inoculation was done in an approx. 1:1 ratio. The treatment of the *N. amphidinoides* cultures differed as overgrowth did not occur often. Approx. 3ml of old culture was transferred without previous concentration step into 50ml of fresh medium and approx. 3ml of dense *Chroomonas* culture were added. For the establishment of axenic co-cultures, axenic cultures of the fed *Chroomonas* strains were a prerequisite. Dinflagellate cells were isolated via microcapillaries and washed 8 times in sterile medium. Single cells were isolated into 250µl of sterile medium and fed with a drop of axenic *Chroomonas* culture. The following procedure was as described above.

2.5 Determination of food spectra

The determination of the food spectrum is essential to understand the life cycle of kleptoplastic dinoflagellates. Nearly no information concerning the variability of potential prey organisms is available as nearly no cultures exist. For both species *N. aeruginosum/acidotum* and *N. amphidinoides* the food spectrum was determined. The food spectrum of *N. amphidinoides* small epicone CCAC 5709 was determined in cooperation with Isabell Luther during her Bachelor Thesis (Luther, 2015). Strains within the genus *Chroomonas* (Table 7) and within the two genera *Cryptomonas* and *Rhodomonas* were tested. Moreover, a few species of algae from the cyanobacteria, glaucophytes, gold algae, haptophytes, euglenophytes, diatoms, chlorophytic and streptophytic green algae were tested (list available in the Bachelor thesis of Isabell Luther, 2015). Single cells of *N. aeruginosum/acidotum* were isolated into 250µl W/2 + Sph into 96-well microtiter plates and starved in the absence of prey until just a rest of the original kleptoplast was visible. Depending on the density of the culture, 1-3 drops of prey culture were added. The exact number of prey cells was not determined but it was important to not add to many prey cells. Successful ingestion was determined by an increase in dinoflagellate cell size, visible plastids and cell division. For *N. amphidinoides* five cells were isolated and treated in the same way like *N. aeruginosum/acidotum*.

2.6 Microscopy

2.6.1 Light and fluorescence Microscopy

The status of all cultures was determined regularly using a CXK 41 inverted microscope (Olympus, Japan). Light micrographs were taken under 1000 fold magnification with an immersion oil objective (Plan 100/1.25 Oel, ZEISS) under an inverted microscope IM 35 (Zeiss IM) equipped with DIC optics. All dinoflagellate cultures except for those that were starved were in exponential growth phase when photographs were taken. The dinoflagellate cells were always concentrated in 50ml Falcon tubes via sedimentation for 5min prior to microscopy. Plastid autofluorescence as well as the DAPI signal were always photographed in combination from fixed cell material. Therefore the cells were fixed in a final concentration of 2.5% Glutaraldehyde, 25% Dimethylsulfoxide, stained with 1µg/ml DAPI and incubated for 5 min at room temperature. Fluorescence images were taken with an upright microscope Eclipse E800 (Nikon, Germany) equipped with immersion oil objectives, DIC optics and fluorescence technology. For plastid autofluorescence an excitation wavelength of 568 nm and for DAPI a wavelength of 340-380 nm were used. High resolution fluorescence images were taken with a DM5500Q confocal laser scanning microscope (Leica, Germany) equipped with a CTR6500 laser (Leica, Germany). For the determination of the division rates of *N. aeruginosum/acidotum*, single cells were isolated into 250µl of W/2 +Sph into 96-well microtiterplates and a drop of the *Chroomonas* cultures CCAC 3453 or CCAC 4832 was added. The plates were incubated at 16°C and the number of dinoflagellates was determined daily for 12 days. Light- and fluorescence micrographs of dividing cells were taken at the beginning of the dark phase. Therefore two-celled chains of dividing dinoflagellates were isolated with microcapillaries and fixed and stained in the same way as described above. The sizes of the cells as well as the stained nuclei were measured with the program Image J. Calculation of nuclear volume was done after nuclear staining based on the formula for an ellipsoid in the case of nuclei in free living cryptophytes and for a sphaeroid in the case of enlarged kleptokarya in *N. aeruginosum/acidotum* ($4/3*\pi*r^3$ in both cases). The contrast as well as the background of light micrographs was adjusted with Photoshop. Videos of the dinoflagellate behavior were taken by Gert Günter with a DMIRB inverted microscope (Leica) and a Fluotar objective using the camera Canon EOS 5D MK2. The behavior was recorded 1-2h after the dark phase.

2.6.2 Scanning Electron Microscopy

For Scanning Electron Microscopy, cell material was concentrated via sedimentation as described above. 100µl of concentrated cell material were added to 200µl of fixation mixture (100µl 2% OsO_4 in H_2O + 100µl Waris-H) and incubated at room temperature for 1min. Afterwards the fixed cell material was incubated for additional 5min on ice. The cells were centrifuged for 1 min at 50G and the supernatant was substituted by ice cold W/2 to wash the cells. This step was repeated twice. 250µl of fixed cell material were pipetted onto round glass cover slips treated with L-polyL-lysin[1] and cells were allowed to settle to the cover slip for 30min. The cells were washed once with W/2 and dehydrated in an increasing series of Ethanol concentrations that were diluted in W/2. The following concentrations were applied each for 15 min at 4°C: 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95%, 98%, 100% (treated with molecular sieve) 3x. Afterwards the ethanol was substituted by liquid CO_2 in a Polaron Critical Point Dryer at 10°C. During this step it was important to make sure that the ethanol is substituted completely by liquid CO_2. The pressure chamber was filled approx. 2/3 with liquid CO_2 and the temperature was increased slowly in 5°C steps to 40°C. When no CO_2 was visible any more, the pressure was released very carefully. Finally the cells were sputtered with gold (Cressington) and observed with a LEO Scanning Electron Microscope (Zeiss, Germany).

[1]Preparation of L-polyL-lysin coated round glass coverslips

Round glass cover slips were washed for 24 h in 1 M HCL at 60°C. Afterwards they were rinsed in deionized water and incubated in 0.05% L-polyL-lysin solution at room temperature for 6h on a shaker. Finally the coverslips were again washed several times in deionized water and dried on tissue paper. The coverslips were stored in Petri dishes until application.

2.6.3 Transmission Electron Microscopy

The ultrastructure of both species *N. aeruginosum/acidotum* and *N. amphidinoides* was investigated using a Transmission Electron Microscope. Therefore approx. 50ml of cell culture were concentrated via sedimentation. The cells were fixed simultaneously with Glutaraldehyde (GA) and Osmiumtetroxide (OSO_4) in Hepes-buffered culture medium. 1ml of concentrated cell material was mixed with 1 ml of fixation buffer resulting in the final concentrations of 0.91% OSO_4, 2.27% GA and 1.5mM Hepes. The cells were fixed for 1 min at room temperature and incubated for 1 h on ice. The cells were centrifuged at 100 G for 5min and washed twice with culture medium. Afterwards the cells were concentrated to a

volume of 50µl and mixed with 50µl of 20% Bovine Serum Albumine (BSA) dissolved in water. The cell-BSA mixture was filled into Beem capsules and incubated at 4°C for 30min. The Beem capsules were centrifuged at 100G for 5min and the supernatant was removed. The pellet was overlayed with 1.25% GA and incubated at 4°C for 1h to link the protein molecules. This was followed by a dehydration series in ethanol diluted with culture medium ((30%, 50% at 4°C), (70%, 90%, 100%, 100% at -20°C)) for 15min each step. This was followed by incubation in a 1:1 mixture of ethanol-propyleneoxide for 15min at -20°C. The cells were incubated in a 1:1 mixture of propyleneoxide:epon over night. The next day the samples were warmed to room temperature and the tubes were opened to let the propyleneoxide evaporate for approx. 12h. The cells were transferred to fresh epon two times and incubated for one day. Finally the cells were placed into embedding mats and incubated at 60°C for 36h. Samples were trimmed with glass knifes on an ultramicrotome (Ultra cut, Leica). Cuttings of 60nm thickness were done with a diamond knife (Diatome) and collected on slot grids coated with 0.5% pioloform (in chloroform). The slides were stained in 2% aqueous uranylacetate in the dark for 10min and washed thoroughly in water. A second staining with lead citrate was done in a CO2 free chamber for exact 3 min 15s followed by thorough washing in water. The slides were dried and observed with a CM10 Transmission Electron Microscope (Philips).

2.7 Determination of the DNA content of kleptokarya

The DNA content within the kleptokarya of *N. aeruginosum/acidotum* and within the free living cryptophyte prey was determined and compared using two independent approaches. The first approach included an intensity measurement of the DAPI signal of stained nuclei. The dinoflagellate as well as the cryptophyte cells within a co-culture were concentrated using the sedimentation method for the dinoflagellates and a subsequent centrifugation step at 500G for 5min to also collect the cryptophytes. The cells were fixed and stained as described in 2.6.1. Fluorescence images were taken with a DM5500Q confocal laser scanning microscope (Leica, Germany). During the acquisition of the photographs it was important to adjust the laser in a way that no saturation of the DAPI signal is observed. The signal intensities of 10 cryptophyte cells and 10 dinoflagellate cells were measured using the program LAS AF Lite and the relative dinoflagellate:cryptophyte ratio was determined. Data were tested for statistical significance using a T-test. The second approach included Polymerase Chain Reaction (PCR) with primers specific for the cryptomonad nucleus. The principle of this

approach was to amplify a small DNA fragment within the cryptomonad nucleus in the free living state and after ingestion and modification by the dinoflagellate. In theory, polyploidization of the cryptomonad DNA should go hand in hand with an increase in copy number that should be visible after PCR amplification on an ethidiumbromide agarose gel. To ensure a constant number of templates (whose number is not known), the PCR was done on single cells that were isolated with microcapillaries. Single cells of *N. aeruginosum/acidotum* were directly isolated into PCR tubes containing 10µl ultra pure water and frozen in liquid nitrogen. The same was done for 5, 10 and 20 cells of the *Chroomonas* strain CCAC 4832 that induced enlarged kleptokarya. The isolation was done directly after the dark phase to make sure that no cells were in division states. To degrade potential DNAses, all PCR-tubes were heated at 80°C for 5min. The tubes were stored at -20°C until use. Accuracy of PCR amplification was ensured by amplifying a very small product of just 160bp. To exclude the amplification of the dinoflagellate DNA, the two primers LSU BancF02 and LSU BancR02 (Table 9) were designed on variable areas within the partial nuclear LSU rDNA specific for the genus *Chroomonas*. A part of the B domain and the almost entire C domain were chosen as tags. The primers were thoroughfully checked for cross priming, hairpin formation and self-dimerization by using the Multiple Primer Analyzer (Thermo Fisher Scientific). Moreover their specificity for the genus *Chroomonas* was tested with blasr searches at NCBI. Both primers were designed by PD Dr. Kerstin Hoef-Emden.

Table 9. Primers specific for the cryptomonad nucleus used for the semi-quantitative PCR.

Primer name	**Sequence (5'→3')**
LSUBancF02	GAGTGAACCGGGAAGAGCTCAAGC
LSUBancR02	TTGRCCTGCATTCCCAAGCAARCCGAC

The specificity of the primers was tested on colorless cells of *N. aeruginosum/acidotum* lacking a kleptokaryon. PCR's were done in a total volume of 25µl containing 2.5mM dNTP's, 10µM forward/reverse primer and 1.25U of DNA Polymerase. The PCR program contained an initial degradation step at 95°C for 2min and 35 cycles with the following steps: 95°C for 1min (degradation), 55°C for 1min (annealing), 68°C for 30s (elongation) and after the cycles a final elongation step at 68°C for 3min. 5µl of PCR product were loaded onto a 1% agarose gel containing 6 µl ethidiumbromide /100 ml. The intensities of the PCR-products were measured for 20 replicates of 5, 10 and 20 cryptophyte cells and 58 replicates of single dinoflagellate cells and compared to a standard sample that always remained identical (a PCR

product with 1µl of extracted DNA from 3ml of culture of the strain CCAC 4832 as template using the same primers) using Image J.

2.8 Molecular Biological Techniques

2.8.1 DNA extraction

Total DNA was extracted from all cultures of *Chroomonas* that were isolated and established by Sebastian Wittek (Table 7). All cultures were clonal but not all were axenic. About 1.5ml of well grown culture was centrifuged at 17000G for 5min in ultra-pure Tubes (Biosphere) and the cell pellet was frozen in liquid nitrogen. DNA was extracted with the DNeasy Plant Mini Kit (Quiagen) according to the manual. The extracted DNA was dissolved in ultra pure water and stored at -20°C. For the genus *Nusuttodinium* single cells were starved until they appeared colorless. Single cells were isolated into 10µl ultra pure water in PCR tubes and directly frozen in liquid nitrogen. PCR tubes were heated afterwards at 80°C for 5min and stored at -20°C until application.

2.8.2 RNA extraction

Total RNA of *Chroomonas*, *N. aeruginosum/acidotum* and *N. amphidinoides* were extracted for future analyses of their transcriptomes. Only axenic material was used for RNA extraction. To get insight into transcriptomic changes of *N. aeruginosum/acidotum*, in total 4 different culture stages were prepared. Table 10 gives an overview of all samples prepared.

Table 10. Samples and stages for RNA extraction.

Species / Strain	Stage
Chroomonas sp. CCAC 3453	Normal culture
Chroomonas sp. CCAC 4832	Normal culture
Chroomonas placoidea CCAP 978/8	Normal culture
Nusuttodinium amphidinoides CCAC 5709 small epicone	Culture fed with *Chroomonas* sp. CCAC 4832 (granular kleptoplasts)
Nusuttodinium aeruginosum/acidotum CCAC 4851	Culture fed with *Chroomonas* sp. CCAC 3453 (granular kleptoplasts)
Nusuttodinium aeruginosum/acidotum CCAC 4851	Culture fed with low concentration of *Chroomonas* sp. CCAC 4832 (U-shaped kleptoplast)
Nusuttodinium aeruginosum/acidotum CCAC 4851	Culture fed with low concentration of *Chroomonas placoidea* CCAP 978/8 (U-shaped kleptoplast)
Nusuttodinium aeruginosum/acidotum CCAC 4851	Culture fed with high concentration of *Chroomonas placoidea* CCAP 978/8 (granular kleptoplasts)
Nusuttodinium aeruginosum/acidotum CCAC 4851	Starved culture (colorless cells)

All mass cultures except for *N. amphidinoides* small epicone CCAC 5709 (16°C) were grown at 23°C. A total volume of 3l of each *Chroomonas* strain was grown in Marburg flasks under permanent aeration. For the dinoflagellates a total volume of 20 l of each condition was grown in multiple Fernbach flasks, 2l culture flasks or large Erlenmeyer flasks. The dinoflagellate cultures were not aerated but it was important to ensure a large surface to volume ratio. The cultures were harvested via centrifugation at 500G (*Croomonas*) or via sedimentation (dinoflagellates). As the dinoflagellates were grown in co-cultures with *Chroomonas,* it was important to separate both organisms prior to extraction. Therefore the dinoflagellate cells were sedimented and washed in sterile culture medium via sedimentation as often as no cryptomonad cells were visible any more under the microscope. To prepare 20l of colorless *N. aeruginosum/acidotum* 20l of cells with granular kleptoplasts derived from the strain CCAC 3453 were grown and used as starting material. The granular kleptoplast stage derived from this strain was chosen because it allowed inducing a uniform quality of colorless cells within just 14 days. During the starvation time of 14 days the dinoflagellates had to be transferred once to fresh medium. Harvested cells were directly frozen in liquid nitrogen, homogenized with a mortar and frozen cell material was stored at -80°C until extraction. The RNA extraction was done following the CTAB-PVP method (BGI, China). Extracted RNA

was dissolved in RNAse-free water (Applichem) and stored at -80°C. The transcriptomes were sequenced by BGI.

2.8.3 Determination of the phylogenetic position of the established *Chroomonas* strains

All *Chroomonas* strains isolated during this study were analyzed phylogenetically. In total three different phylogenetic markers were used: 1. The Internal Transcribed Spacer 2 (ITS2) of the ribosomal operon within the nucleus; 2. The partial large subunit (partial LSU) of the ribosomal operon within the nucleus; 3. The small subunit of the ribosomal operon within the nucleomorph.

Table 11. PCR primers used for the amplification of the nuclear and nucleomorph ribosomal operon (source: Hoef-Emden, 2007).

Primer name	Molecular Marker	Sequence (5' → 3')
CrN1F	partial nuclear LSU rDNA	CTG CCA GTA GTC ATA TGC TTG TCT C
CrN3F*	partial nuclear LSU rDNA	TGG TTA ATT CCG TTA ACG AAC GAG ACC TCA G
LSU1433R$_{biot}$	partial nuclear LSU rDNA	AAT ATT TGC TAC TAC CAC CAA GAT C
AFK$_{biot}$	Nucleomorph SSU rDNA	CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT
NmITS066R	Nucleomorph SSU rDNA	CTT GGT CCG TGT TTC AAG ACG GGT C
NmITS044R*	Nucleomorph SSU rDNA	GAG CTG CAC TCC CAA GCA AC

*primers were used for reamplification; $_{biot}$ primer is biotinylated at the 5‘ position

A sheme with the approximate binding positions is shown in Figure 1.

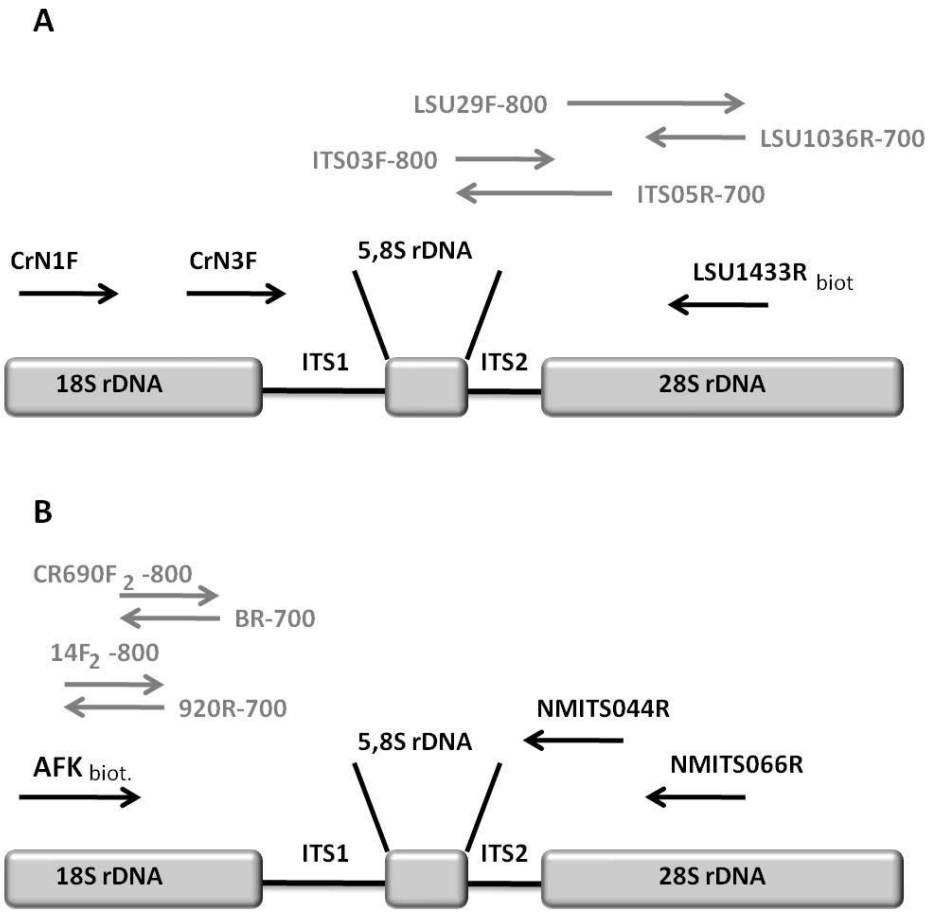

Figure 1. Sheme showing the DNA regions amplified and the approximate binding positions of the primers used to amplify the ribosomal operon of the cryptophyte nucleus (**A**) and the nucleomorph (**B**). All primers written in grey were used for sequencing reactions. -700/800 = primers were labeled with a fluorescence dye (MWG Biotech). The figure is adopted from the Bachelor Thesis of Sebastian Wittek (2011).

PCR's were prepared in a total volume of 50µl containing 2.5mM dNTP's, 10µM primer and 1.25U of Taq DNA Polymerase. The following PCR program was used: Predenaturation at 95°C for 3min, 35 cycles with denaturation at 95°C for 1min, annealing at 60°C for 1min, synthesis at 68°C for 5min and after the cycles a final synthesis step at 68°C for 10min. The PCR products were stored at 4°C. All PCR's were checked for the presence of products on 1% Agarose gels containing 6µl/100 ml Ethidiumbromide. In the case of very weak bands the PCR product was reamplified. Therefore the PCR product was diluted 1:10 with water and 1µl was used as template. The primers used for reamplification are shown in Figure 1. The PCR program used was the the same as for the primary PCR. Successfully amplified PCR products were sequenced using the Sanger method (Sanger et al., 1977). Sequencing reactions were prepared with the Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit (Affymetrix) according to the manual. Primers used for sequencing reactions were labeled

with a fluorescent dye that could be excited using a laser. Forward primers had an emission wavelength of 800nm and reverse primers of 700nm.

Table 12. Sequencing primers (source: Hoef-Emden, 2007).

Combi-nation	Primer name	Sequence (5' → 3')
1	ITS03F-800	CGA TGA AGA ACA CG YAG CGA
	ITS05R-700	TAC TTG TTC GCT ATC GGT CTC T
2	LSU29F-800	TGA ACT TAA GCA TAT CAA TAA GCG G
	LSU1036R-700	ATT TAA ATG TTG AGA ATA GGT
3	$14F_2$-800	GAT CCT GCC AGT AGT CAT ATG CTY G
	920R-700	CAA TTC CTT TAA GTT TC
4	$CR60F_2$-800	GAG GTG AAA TTC TTA G
	BR-700	TTG ATC CTT CTG CAG GTT CAC CTA C

The following PCR program was used for sequencing reactions: Predenaturation at 95°C for 2min and 30 cycles with denaturation at 95°C for 30s, annealing at 40°C for 30s and synthesis at 70°C for 1min. The sequencing reactions were stored in the dark at -20°C until use. Sequencing was done using the plate sequencer LICor 4200L. Therefore a polyacrylamide gel with the following composition was prepared: 4.8g urea, 6.4ml H_2O, 800µl 10 xTBE buffer (1l contains: 162g Tris, 9.3g Na_2-EDTA x $2H_2O$, 27.5g H_3BO_3), 400µl DMSO, 32ml of Mono-Bisacrylamide SequaGelXR, 6.4ml SequaGelXR buffer, 12µl TEMED and 230µl Ammoniumperoxodisulfate (APS). Sequencing reactions were loaded onto the gel and run for 18h at the following conditions: 2000V tension, 35mA current, 50W power at a temperature of 45°C. Sequence data were read with the program e-Seq 3.08 (LICor, Bad-Homburg, Germany). The quality check and assemble of each sequence was done using the program Align IR V.2 (LiCor Bad-Homburg, Germany). The affiliation of each sequence was checked with BLAST search (NCBI). Two separate alignments were built with the obtained sequences, one for partial LSU rDNA of the nucleus and one for the SSU rDNA of the nucleomorph using the program Seaview 4.2. The alignments were supplemented with already published sequence data. The phylogenetic position of the *Chroomonas* sp. strain CCAC 4832 was determined using its nuclear LSU rDNA (ca. 900nt of 5' terminus) and nucleomorph SSU rDNA. The sequences were added to the corresponding alignments of PD Dr. Kerstin Hoef-Emden (Hoef-Emden, 2014) using the multiple alignment editor SeaView 4.3.0 (Gouy et al., 2010). Inspection by eye, exclusion of non-alignable positions and concatenation of the three alignments resulted in an analysis data set comprising 49 OTUs (incl. 6 outgroup OTUs) and 4088 positions (nuclear SSU rDNA: 1596, but gapped, since the

sequence of strain CCAC 4832 was incomplete with only 965nt of 5' terminus; partial nuclear LSU rDNA: 915nt; nucleomorph SSU rDNA: 1577nt). To determine the phylogenetic positon of the *Chroomonas* strains established during this study (Table 7), their partial nuclear LSU rDNA was added to the partial nuclear LSU rDNA alignment, which already contained the sequence of strain CCAC 4832. Also the nuclear SSU rDNA sequences of Xia et al. (2013) were added to the nuclear SSU rDNA alignment. After exclusion of non-alignable positions, the resulting analysis data sets were combined with the nucleomorph SSU rDNA data set to a gapped supermatrix yielding 4079 positions (partial LSU rDNA reduced to 906nt.). The partial LSU rDNA sequences were sufficiently variable to be positioned across the tree, whereas the concatenation with nucleomorph and nuclear SSU rDNA increased the resolution at internal branches of the tree. Only character-based methods, i.e. maximum likelihood and Bayesian inference, were used to infer phylogenetic trees and support values. RAxML 7.7.2 was used for maximum likelihood analysis (evolutionary model: GTR + I + Γ; heuristic search: 50 starting trees; bootstrap analyses: 1000 replicates). For Bayesian analysis MrBayes 3.2.1 was used (Ronquist et al., 2012). Settings were as follows: 3 partitions (corresponding to the 3 ribosomal genes), evolutionary model: GTR + I + Γ, all parameters unlinked except for tree topology; 6 mio. generations, 2 runs with 4 chains, sampling every 100th generation, no relative burn-in. Convergence of the chains was checked with the sump command, which was also used to determine the burn-in phase. The phylogenetic analyses were done in co-operation with PD Dr. Kerstin Hoef-Emden.

2.8.4 Identification of the kleptoplast of *N. aeruginosum/acidotum* in natural populations

To get an idea about the identity and diversity of the kleptoplast of *N. aeruginosum/acidotum* within natural populations, single cells were isolated from the bogpond Heidemoor near Dahlem Eifel G. on 09.05.2011 and from the Großes Heiliges Meer near Hopsten, Steinfurt on 19.04.2011. The cells were isolated from sludge samples with microcapillaries and washed twice in sterile culture medium. Single cells were isolated into 10µl of ultra pure water into PCR tubes and directly frozen in liquid nitrogen. These cells were used as template for the PCR. To exclude an amplification of the dinoflagellate DNA, two primers specific for the nuclear LSU rDNA was designed (Table 13) with the help of PD Dr. Kerstin Hoef-Emden and Dr. Nicole Sausen using an alignment containing dinoflagellates of the *Gymnodinium* clade as well as the genus *Chroomonas*. The primer AGCrNu523F (**A**nti ***G****ymnodinium* pro

***Ch**roomonas* **Nu**cleus binding position **523 F**orward) was combined with the universal eukaryotic backward primer LSU1433R$_{biot}$. For reamplification the primer AGCrNu523F was substituted by the primer AGCrNu452F.

Table. 2. PCR primer specific for the *Chroomonas* nuclear partial LSU rDNA.

Primer name	Sequence (5' → 3')
AGCrNu523F	AGC AGC CGC GGT AAT TCC AGC TCT
AGCrNu452F	TCT TGT TAT TGG AAT GAG AAC

The PCR as well as sequencing reactions were prepared as described in 2.8.3. The sequences obtained were added to the partial LSU rDNA alignment described above that also contained the newly established *Chroomonas* strains. Phylogenetic analysis was the same as described in 2.8.3.

2.9 Density experiments

The effect of different prey densities on the formation of kleptoplasts in *N. aeruginosum/acidotum* was investigated. Density experiments were carried out with two genetically different *Chroomonas* strains CCAP 978/8 and NIES 714 both belonging to subclade 4 (the strain CCAC 4832 that is in the focus of this study was not chosen for density experiments as it tends to form strong palmellae making the cryptophytes hardly available for the dinoflagellates). Cells derived from a co-culture with *Chroomonas* sp. CCAC 3453 were starved until they became colorless. 5 colorless cells of *N. aeruginosum/acidotum* were isolated into 250µl W/2+Sph into 96-well Microtiter plates with microcapillaries. Cryptophyte cells were added in the following ratios (number of cryptophyte cells per 1 dinoflagellate cell): 1; 2; 4; 8; 10; 20; 50; 70; 100; 200; 500; 1000. For ratios from 1-10 cryptophytes were isolated with microcapillaries and added to the dinoflagellates whereas for higher ratios the number of cryptophytes within a culture was counted with a Neubauer counting chamber (Marienfeld, Germany) and the calculated volume was added to the dinoflagellates to reach the ratio. Density experiments were carried out at 23°C for 8 days. The number of dinoflagellate cells was counted and the type of kleptoplast formed was determined for each cell via light microscopy.

2.10 Determination of the biovolume of *N. aeruginosum/acidotum* before and after starvation

The biovolume of *N. aeruginosum/acidotum* was determined before starvation (permanent prey availability) and after starvation. Cells derived from co-cultures with the *Choomonas* strains CCAC 3453 (granular kleptoplasts) and CCAC 4832 (U-shaped kleptoplast) were investigated. Cell length, width and depth were measured from 100 cells per co-culture before starvation and the approximate biovolume was calculated using the formula for an elliptic sphere ($4/3*\pi*r^3$). To immobilize the cells for photographs, an embedding protocol in low gelling agarose was chosen according to Reize and Melkonian (1989). This initial biovolume was compared to the biovolume formed after starvation. To starve the cells, single dinoflagellates were separated from cryptophyte prey via isolation with microcapillaries and isolated into 250 µl culture medium into 96 well-microtiterplates. From previous experiments it is known that the maximum cell number of *N. aeruginosum/acidotum* with granular kleptoplasts (derived from CCAC3453) is reached after 14 days of starvation whereas with U-shaped kleptoplasts (derived from CCAC 4832) it is reached after 60 days of starvation. These two time points were used for the determination of the formed biovolume. As the total number of formed daughter cells was too high to calculate the biovolume of each cell separately, another approach was chosen. The average biovolume of 12 colorless cells was determined as described above and this value was multiplied with the number of colorless cells formed.

2.11 Oxygen measurements

To get an idea whether the kleptoplasts formed by *N. aeruginosum/acidotum* are photosynthetically active, oxygen production of the dinoflagellate as well as the cryptophyte cultures was measured. A polarographic measurement using a Clark electrode was chosen (Fig. 2). The cell suspension was separated from a concentrated KCl solution via an oxygen permeable Teflon membrane of 12µm thickness. The amount of oxygen production was proportional to the electron flow that was measured. To be able to quantify the amount of oxygen, a calibration was done before with culture medium saturated with oxygen (pure oxygen was bubbled into the medium) and culture medium depleted of oxygen (pure nitrogen was bubbled into the medium). The values measured for the cell suspensions were compared with the calibration curve and the amount of oxygen was calculated. *N. aeruginosum/acidotum* with granular kleptoplasts (derived from *Chroomonas* sp. CCAC

3453) and with U-shaped kleptoplasts (derived from *Chroomonas* sp. CCAC 4832) as well as their cryptophyte prey strains were chosen for the measurements. The dinoflagellates were separated from the cryptophyte prey via sedimentation and washed twice with sterile culture medium. 1 ml cell suspension with a density of 10000cells/ml was used for experiments. The cells were illuminated with a photon flux density of 8µmol*m^{-2}*s^{-1} at a temperature of 22°C that resembled their culture conditions. The cell suspension was stirred permanently during measurements. To make sure that the cells were not damaged they were microscoped before and after measurements. 5 replicates were measured for each culture. Data were tested for statistical significance using the program Statistica 13.0 (StatSoft).

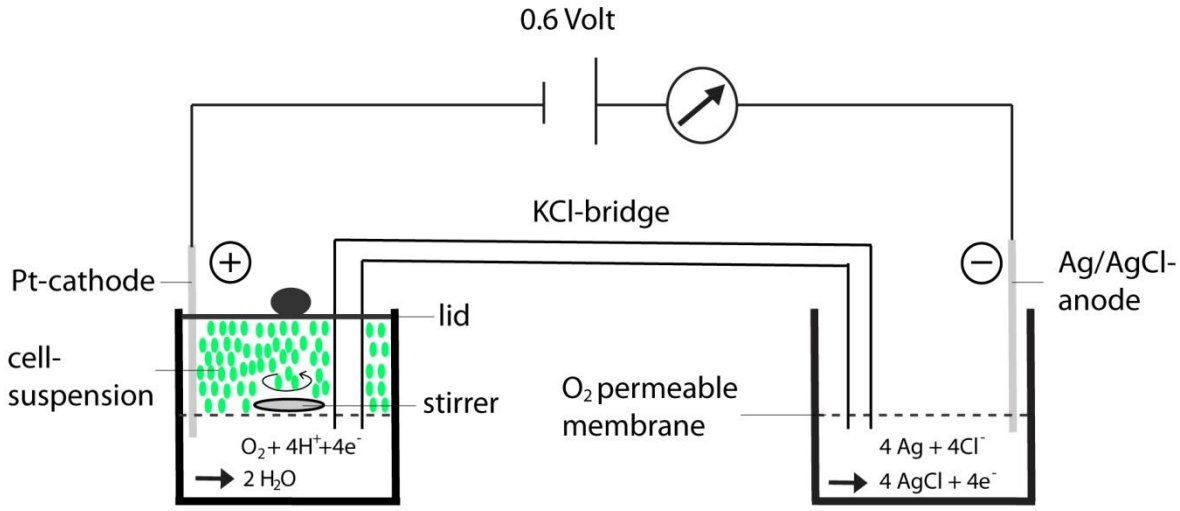

Figure 2. Experimental setup of the oxygen measurement using a Clark electrode.

3. Results

3.1 Occurrence of kleptoplastic dinoflagellates in natural samples

A variety of different waterbodies was sampled including puddles, ponds, bog bonds as well as rivers. The samples were screened for the presence of kleptoplastic dinoflagellates and the genus *Chroomonas*. Kleptoplastic dinoflagellates were just found to co-occur with members of the genus *Chroomonas* whereas this genus was also found in water bodies where no kleptoplastic dinoflagellates were found. *Chroomonas* was found in each type of water body sampled. It also occurred in puddles that dry out during the summer. In contrast, kleptoplastic dinoflagellates were just found in water bodies that do not dry out regularly. Kleptoplastic dinoflagellates as well as *Chroomonas* were just found in samples taken close to or from the sediment. In total three different types of kleptoplastic dinoflagellates were found. The first type included the two species *Nusuttodinium aeruginosum* and *Nusuttodinium acidotum* that were originally distinguished by the shape of their hypocone. As this study shows that it is not possible to distinguish between both species without a culture, they are combined to *N. aeruginosum/acidotum*. *N. aeruginosum/acidotum* was found in 7 different water bodies but cultures could just be established from 6 different locations. As the parameters concerning the water quality were just recorded for the bog pond 'Heidemoor an der Wasserdell' near Dahlem Germany, just personal impressions about the water quality of the other water bodies can be given. *N. aeruginosum/acidotum* was found throughout the whole year in very clear, unpolluted waters like the Heidemoor an der Wasserdell that can be classified as oligotrophic (conductivity: 38.7 ± 4.19µS/cm; pH: 6.22 ± 0.12; temperature: 11.55 ± 0.16°C, data adopted from the Bachelor thesis of Sebastian Wittek (2011)) but also in the 'Großes Heiliges Meer' that was classified personally as mesotrophic as well as a highly polluted duck pond in Krefeld giving the impression of being eutrophic. Interstingly, this dinoflagellate was just observed in sediment samples. Until now no studies were done investigating the water quality in or close to the sediment of the sampled waterbodies. *N. aeruginosum/acidotum* was found as either immobile or free swimming vegetative stages. It always contained blue-green kleptoplasts but the size of the cells as well as the amount of kleptoplast per cell could differ. The distribution of the cells within a water body varied strongly but the reason for this phenomenon could not be clarified yet (Wittek, 2011). The second type of kleptoplastic dinoflagellates found was identified as the species *N. amphidinoides*. This dinoflagellate was found in nine different water bodies that also differed strongly in their water quality. It often co-occurred with *N. aeruginosum/acidotum*. In all 9 locations *N. amphidinoides* contained

multiple small blue-green kleptoplasts that varied between 4 and 7 in number. Just in the bogpond Heidemoor an der Wasserdell it was also found with brown kleptoplasts. Colorless cells were not found in natural samples. *N. amphidinoides* was always in motile stage when it was found. Cultures of this dinoflagellate could be established from all 9 locations. The third type of kleptoplastic dinoflagellates could not be identified by literature research but the investigation of its phylogenetic position suggests close affiliation to the species *N. amphidinoides* (Isabell Luther, Bachelor Thesis 2015, supervised by Sebastian Wittek). As this dinoflagellate is characterized by a very small epicone the term '*N. amphidinoides* small epicone' is used in the following. The dinoflagellate was just found in the bog pond Heidemoor an der Wasserdell. A high number of cells were present in a sample taken on 26.2.2013 showing both, blue-green and brown kleptoplasts. Interestingly a high number of *Chroomonas* and *Cryptomonas* cells were also present in the sample. *N. amphidinoides* small epicone sticked to sludge particles with its hypocone and was just rarely seen to be swimming freely. This dinoflagellate was not found in samples taken in autumn or winter. In spring 2015 a single cell of this dinoflagellate could be found again in a sample from this pond but mass-occurrences like in 2013 were not observed any more. The dinoflagellate could be established in culture.

3.2 Cultures of kleptoplastic dinoflagellates

The establishment of cultures from kleptoplastic dinoflagellates is one of the most important prerequisites to study this group. As these dinoflagellates are not able to grow alone, a collection of potential prey organisms had to be established first. In total 111 strains of *Chroomonas* were tested in this study. 17 strains were obtained from various culture collections and 94 new strains were established. Whereas all strains were tested in feeding experiments, 42 were also studied phylogenetically. These *Chroomonas* strains served as the basis for this study. Another important factor for a successful cultivation of these dinoflagellates is the right culture medium. The freshwated medium Waris-H served as a basis and was modified with different additives such as organic substances. However, just the combination of half strength Waris-H with the addition of 6ml/l Sphagnumextract proved to be successful to cultivate the species *N. aeruginosum/acidotum*. *N. amphidinoides* in contrast was less demanding and grew in W/2+Sph as well as in normal Waris-H. The second prerequisite that had to be met was the choice of a suitable *Chroomonas* strain. As nearly no information is available concerning the food spectrum of kleptoplastic dinoflagellates, the

focus was on strains isolated from the same location where also the dinoflagellates occurred but also other strains were tested. A *Chroomonas* strain that proved to be suitable for the cultivation of *N. aeruginosum/acidotum* is CCAC 4832. This strain was isolated from the Heidemoor an der Wasserdell near Dahlem, Germany on 22.03.2011. Concerning *N. amphidinoides* the strain CCAC 0060 was suitable for the establishment of cultures. This strain was isolated by Schilke from Griether Ort, Germany 1991. Especially the initial stages during the establishment of cultures were critical for both species due to permanent overgrowth by cryptophyte cells. Isolated cells were observed every two days. When overgrowth by cryptophytes was observed, the dinoflagellates had to be reisolated and concentrated. This procedure had to be done every two weeks during the first three months. After being transferred into 100 ml Erlenmeyer flasks containing 50 ml of medium the dinoflagellates were concentrated via sedimentation as described above. This procedure was done once per month after the establishment of cultures for both species to maintain the cultures. All cultures except for the strain *N. amphidinoides* HLM could be made axenic. In total 7 attempts to make *N. amphidinoides* HLM axenic failed as the dinoflagellates did not divide under axenic conditions. All other strains were established in axenic culture by washing the cells 8 times in sterile culture medium via microcapillaries and incubating them with the same cryptophytes strains as described above. No differences were observed concerning dinoflagellate and cryptophytes behavior between axenic and xenic cultures. Six axenic strains from different locations were established for *N. aeruginosum/acidotum* and 10 strains of *N. amphidinoides* from different locations of which 9 are axenic (Table 6).

3.3 *Nusuttodinium aeruginosum/acidotum*

3.3.1 Feeding experiments and observed types of kleptoplasts in *Nusuttodinium aeruginosum/acidotum*

The successufull establishment of a stable axenic culture of *N. aeruginosum/acidotum* allowed a detailed investigation of of its food spectrum (Fig. 3A). In the absence of prey *N. aeruginosum/acidotum* becomes colorless showing the transient nature of its kleptoplasts (Fig. 3B). Feeding experiments were carried out with colorless or nearly colorless dinoflagellates to be able to to recognize newly ingested prey. All 111 strains of *Chroomonas* were offered as prey (but not all were sequenced) as well as the cryptophyte genera *Cryptomonas* and *Rhodomonas*. Just members within the genus *Chroomonas* were accepted as prey. The ingestion of prey could already be detected 1-2 days after feeding. Prey was exclusively

ingested via phagocytosis at the antapex. Successful ingestion resulted in an increase of cell size as well as cell number whereas no ingestion resulted in the death of the dinoflagellate. Two different types of kleptoplasts were observed after ingestion.

The first type is characterized by multiple small blue-green kleptoplasts distributed throughout the dinoflagellate cell declared here as ‘granular’. Detailed studies concerning the granular kleptoplasts were performed on a co-culture with the strain CCAC 3453 as prey. In a permanent co-culture with a cryptophyte : dinoflagellate ratio of 101:1 about 12 ± 3 kleptoplasts with a maximum of 21 were present (n=100). Each kleptoplast was associated with a small cryptophyte nucleus, a kleptokaryon. Both, kleptoplast and kleptokaryon resembled free living cryptophyte prey in size and morphology (Fig. 3C). This morphotype was established 3-4 days after reinfection with the cryptophyte prey.

The second type is characterized by a single blue-green, large, U-shaped kleptoplast filling most of the periphery of the cell. Due to its shape, this type is declared here as ‘U-shaped’. Detailed studies concerning the U-shaped kleptoplast were performed on a co-culture with the strain CCAC 4832 as prey. In a permanent co-culture with a cryptophyte : dinoflagellate ratio of 77 : 1 all cells exclusively contained one kleptoplast (n=100). The kleptoplast was associated with one large round kleptokaryon located antapically in the curve of the U. However 23% of the cells investigated within the co-culture lacked a kleptokaryon (n=100). Both, kleptoplast and kleptokaryon were highly enlarged compared to the free living cryptophyte (Fig. 3D). This morphotype was established 7-10 days after reinfection of colorless cells and resembles the situation usually found in nature.

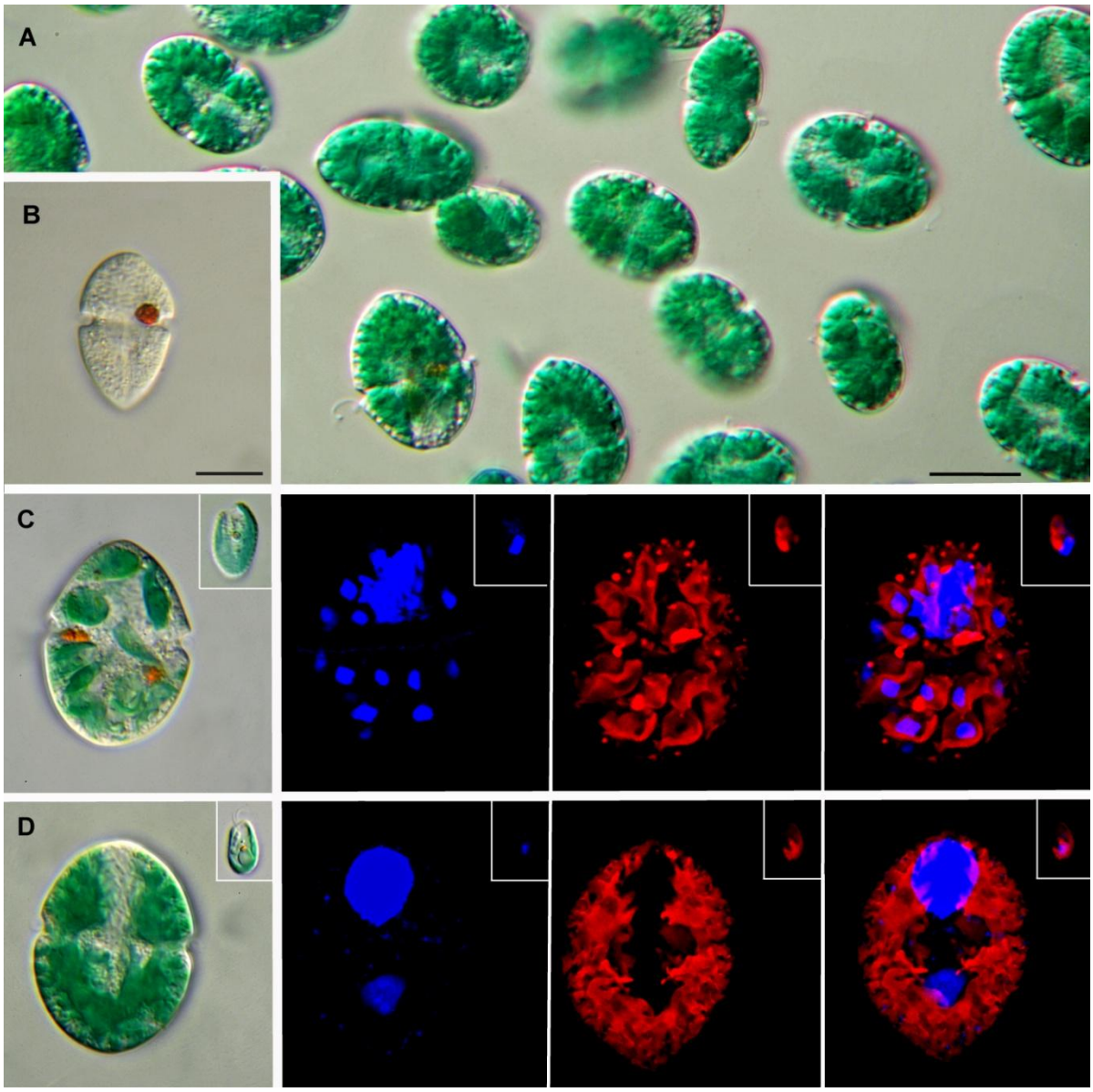

Figure 3. *Nusuttodinium aeruginosum/acidotum* is able to make different types of kleptoplasts. **A** Harvested cells showing successful cultivation of *N. aeruginosum/acidotum.* **B** Colorless cell after starvation. The red-brown body represents a digested kleptoplast. **C** Granular kleptoplasts derived from *Chroomonas* sp. CCAC 3453. **D** U-shaped kleptoplast derived from *Chroomonas* sp. CCAC 4832. Figures **C** and **D** show DIC images, DAPI fluorescence, chlorophyll fluorescence and overlay of DAPI and chlorophyll fluorescence (from left to right) from a *dinoflagellate* cell with kleptoplasts and the free living cryptophyte prey. The cells shown in the DIC images do not correspond to the fluorescence images (bar in **B** also applies to **C** and **D** = 10µm).

The results obtained from feeding experiments were compared with the phylogenetic position of the prey. Currently, the genus *Chroomonas* is paraphyletic and splitted into three subclades (1,3,4). Subclade 2 is formed by the marine genus *Hemiselmis* that resembles *Chroomonas.* Sequencing of the nuclear SSUrDNA, the partial nuclear LSUrDNA and the nucleomorph SSUrDNA showed a clear separation of strains inducing granular and U-shaped kleptoplasts.

All tested strains belonging to subclades 3 and 4 were accepted as prey. Strains belonging to subclade 1 were not accepted and strains of subclade 2 were not tested as all of them are marine. All strains offered within subclade 3 (arrowheads Figs. 4+5) just induced granular kleptoplasts. U-shaped kleptoplasts in contrast were induced when prey belonging to subclade 4 was offered. However, also two additional kleptoplast types, the intermediate and the granular within subclade 4 were observed that are described in 3.3.8 – 3.3.11 (Figs. 4+5). To get an idea about the identity of the kleptoplast within natural populations of *N. aeruginosum/acidotum*, cells from two different locations, the Großes Heiliges Meer and the Heidemoor an der Wasserdell were isolated and the partial nuclear LSU rDNA was used as a marker for identification. The combination of the two primers AGCrNu523F and LSU1433R (reamplification: AGCrNu452F + LSU1433R) proved to be successful to specifically amplify the kleptokaryon. Concerning the population in the Heidemoor Dahlem, in total 4 genetically different kleptoplasts were identified. Three kleptoplasts belonged to subclade 3 and one to subclade 4. Within subclade 3 no sequence was completely identical to a culture strain. However, the kleptoplasts of two cells were closely related to the three *Chroomonas strains* CCAC 4826, CCAC 4829, CCAC 4830 that also originated from Dahlem. The third sequence belonging to subclade 3 was closely related to the three strains CCAC 4835, CCAC 4836, CCAC 4837 that were established from a fishpond near Wahner Heide. The kleptoplast sequence that belonged to subclade 4 was completely identical to the established strain CCAC 4842 that comes from the Großes Heiliges Meer. It was very closely related to 5 additional strains CCAC 4832, CCAC 4833, CCAC 4844, CCAC 4848, CCAC 4850 that originated from Dahlem, Wahner Heide, Krefeld and Gelnhausen. As some sequences were in low quality, it cannot be excluded that these small differences in sequences are due to sequencing errors. Concerning the kleptoplast identity within the population of *N. aeruginosum/acidotum* from the großes Heiliges Meer, just one readable sequence was obtained. The sequence showed close relationship to the strains CCAC 4826, CCAC 4829, CCAC 4830 that all come from Dahlem (Fig. 5).

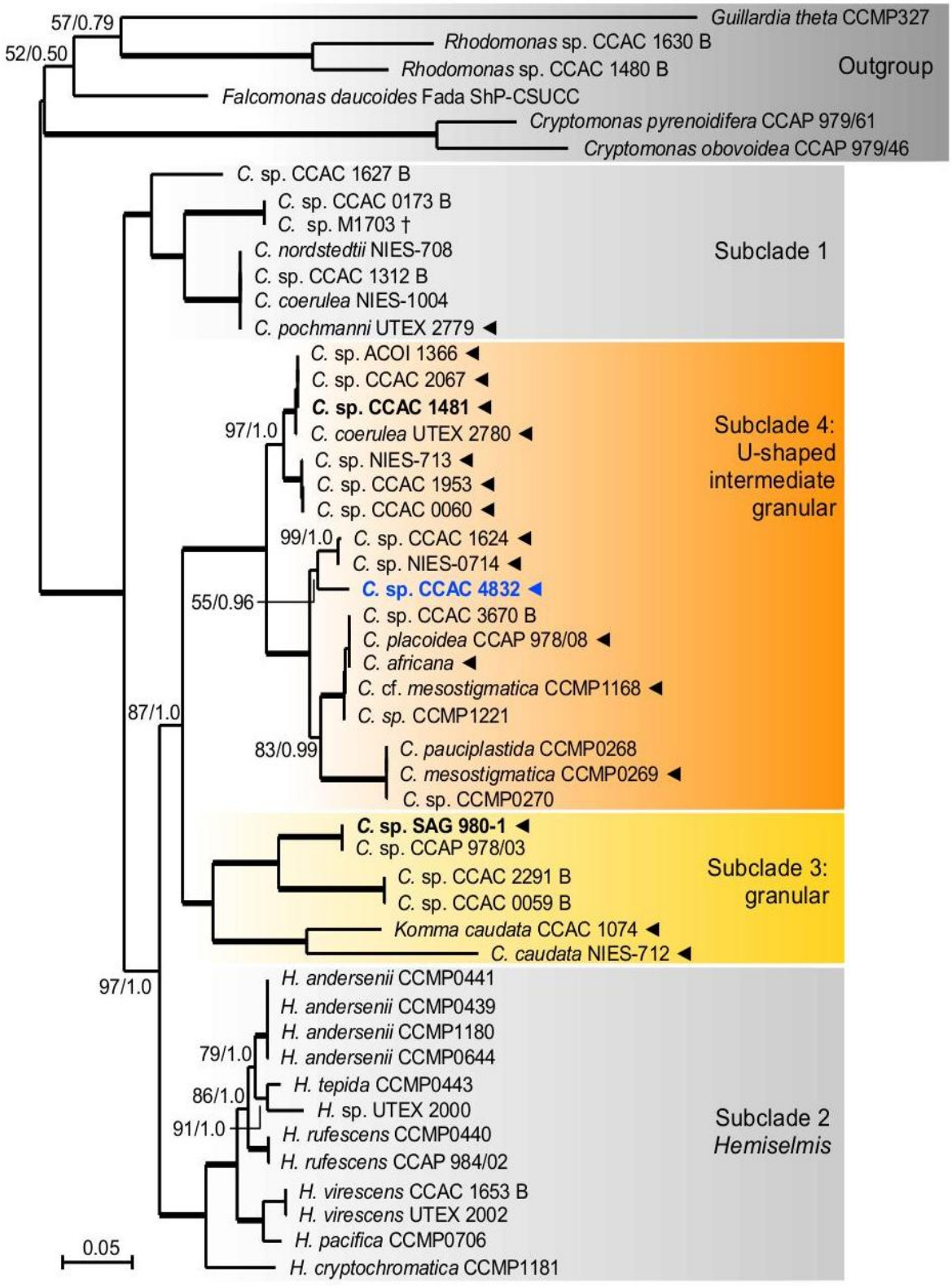

Figure 4. Rooted maximum likelihood tree of combined nuclear SSU rDNA, partial nuclear LSU rDNA and nucleomorph SSU rDNA sequences (46 OTUs, 4088 positions). Blue labels: new OTU. Arrowheads: strains tested for feeding experiments, bold: strains used for the establishment of permanent co-cultures. Subclades shaded in gray were not accepted as prey. Support values: maximum likelihood bootstrap/posterior probabilities; bold branches: 100 % bootstrap support and PP = 1.0. Scale bar: substitutions per site.

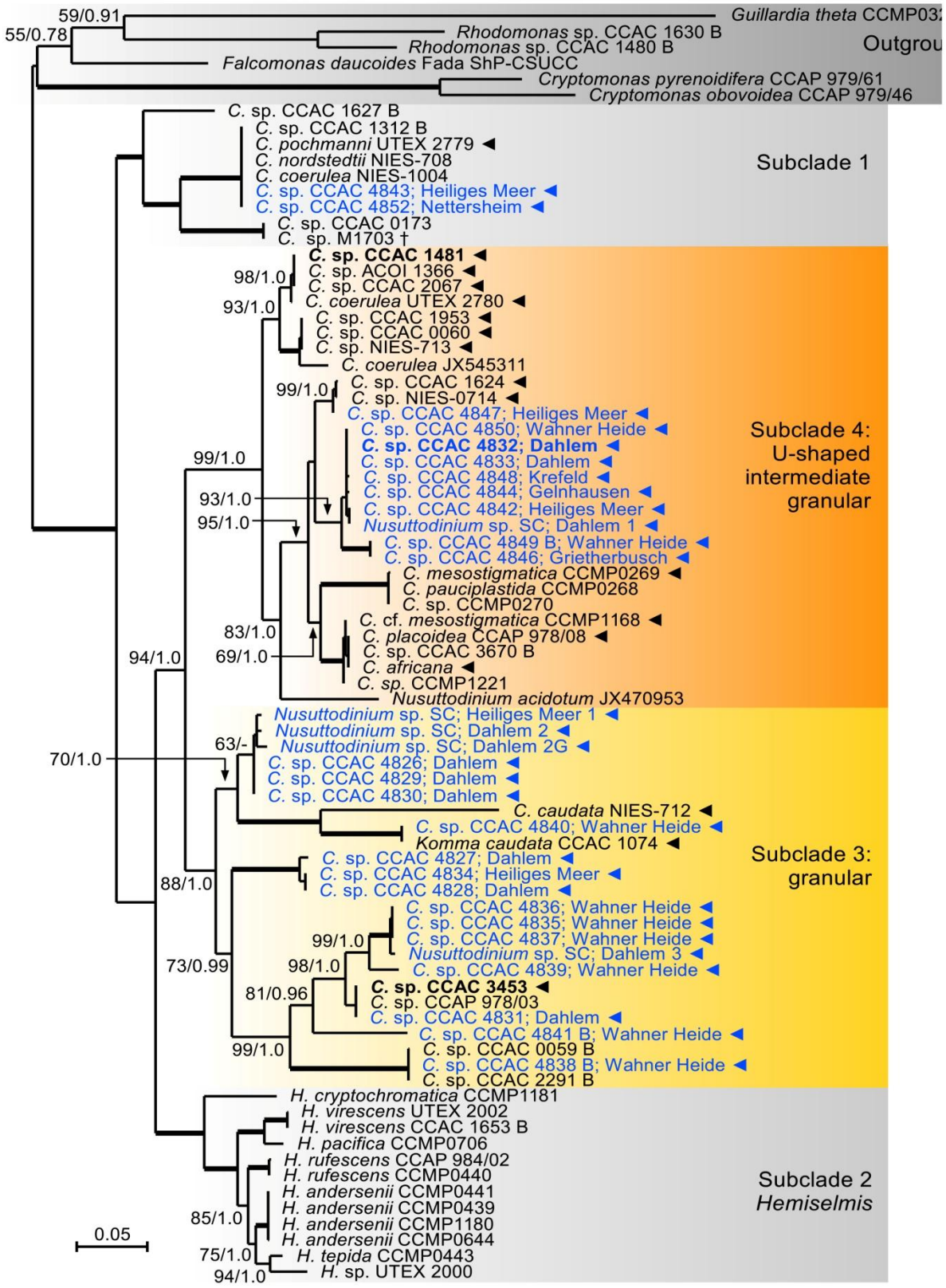

Figure 5. Rooted maximum likelihood tree inferred from a combined gapped alignment (80 OTUs, 4079 positions) with combined partial nuclear LSU rDNA (gaps: *Nusuttodinium acidotum* JX470953 and *C. coerulea* JX54311), nuclear SSU rDNA (gaps: all blue labeled OTUs, except for *C.* sp. CCAC 4832) and nucleomorph SSU rDNA sequences (gaps: *Nusuttodinium acidotum* JX470953, *C. coerulea* JX54311 and all blue labeled OTUs, except

for *C.* sp. CCAC 4832). Blue labels: new sequences/OTUs. Arrowheads: strains tested in feeding experiments. SC: Single cell. *Nusuttodinium* sp.: *Nusuttodinium aeruginosum/acidotum*. Subclades shaded in gray were not accepted as prey. Support values: maximum likelihood bootstrap/posterior probabilities; bold branches: 100 % bootstrap support and PP = 1.0. Scale bar: substitutions per site.

3.3.2 DNA content of kleptokarya

The granular and U-shaped kleptoplasts obtained by feeding experiments showed strong morphological differences (Fig. 3). The first difference was the shape and size of the kleptoplasts. Whereas both resemble the free living condition in the granular situation, the kleptoplast is highly branched and enlarged up to 20 fold in the U-shaped condition (Onuma and Horiguchi, 2013). The second difference is the shape and size of the kleptokarya. Cells with granular kleptoplasts contained 12 ± 3 kleptokarya with a maximum of 21 that was identical to the number of kleptoplasts (n=100). The elliptic shape of the kleptokarya was identical to the nuclei in free living cryptophytes. An approximate calculation of the nuclear volume revealed about 30 μm^3 for both situations and no significant difference ($p= 0.79 > 0.05$) (n=100). In contrast, shape and size of the kleptokaryon in the U-shaped situation strongly differed from the situation in the free living cryptophyte. Whereas the cryptophyte nucleus is elliptic and small, it becomes round and highly enlarged as a kleptokaryon. The average volume of the nucleus is 14.4 ± 2.1μm^3 in the cryptophytes and increased to 123.4 ± 59μm^3 as a kleptokaryon in the dinoflagellate. Volumes of cryptophyte nuclei derived from the strain *Chroomonas* sp. CCAC 4832 before and after starvation were significantly different ($p= 0.00 < 0.05$). The extremely high standard deviation measured for the enlarged kleptokarya was due to different developmental stages in the enlargement process (Fig. 6).

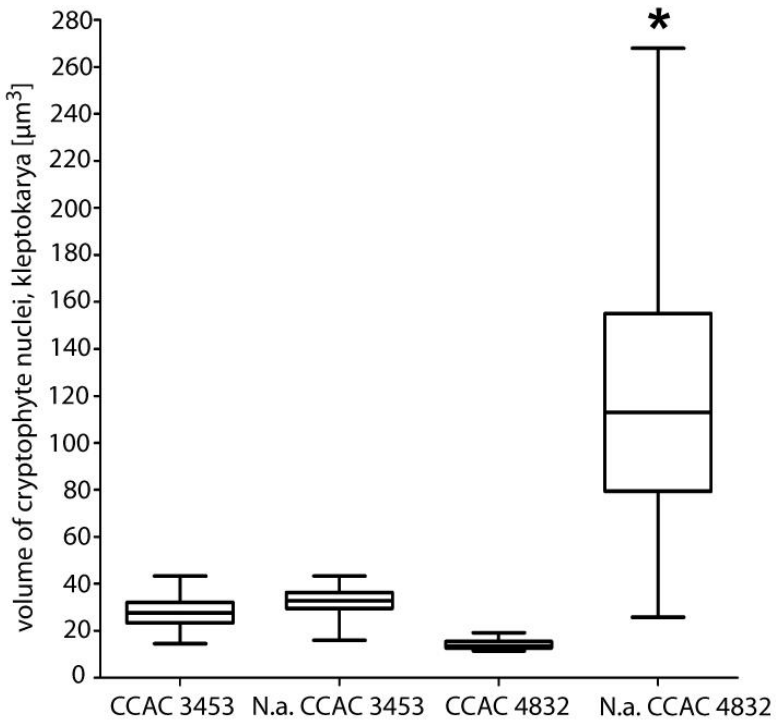

Figure 6. Volumes of the cryptophyte nuclei and kleptokarya in the granular (CCAC 3453) and U-shaped (CCAC 4832) situation. N.a. = Kleptokarya in the dinoflagellate *N. aeruginosum/acidotum*.* Significance between CCAC 4832 and N.a. CCAC 4832 ($p = 0.00 < 0.05$, Kruskal-Wallis test).

In addition to the measurements of the nuclear volumes, the DNA contents of both cryptophyte nuclei and kleptokarya were estimated by measuring fluorescence intensities after DAPI staining. DAPI staining resulted in clear signals of the nuclei in both, dinoflagellate and cryptophyte cells. However, additional weak signals could be observed in the periphery of the dinoflagellate that probably represent nucleomorphs or mitochondrial DNA. A big challenge during the quantification of the signals in the U-shaped situation was the enormously strong fluorescence of the dinoflagellate nucleus due to its large size and high DNA content and the extremely weak signal obtained from the nucleus of the free living cryptophytes that had to be recorded using the same settings (Fig. 3D). The ratio of nuclear DNA content (cryptophyte nucleus/kleptokaryon) was compared between the granular and U-shaped kleptoplasts. Within the granular situation no differences in the strength of the DAPI signal could be detected between the cryptophyte nuclei and the kleptokarya resulting in a ratio of about 1 (n= 10). In contrast an enormous increase in signal strength was measured for the kleptokaryon in the U-shaped situation. A maximum of 16 fold increase was measured compared to the free living cryptophyte with an average ratio of 7.1 ± 3.9 (n= 15). As for the measurements of the nuclear volumes, the high standard deviation is due to the differences in the developmental stages of the kleptokarya (Fig. 7).

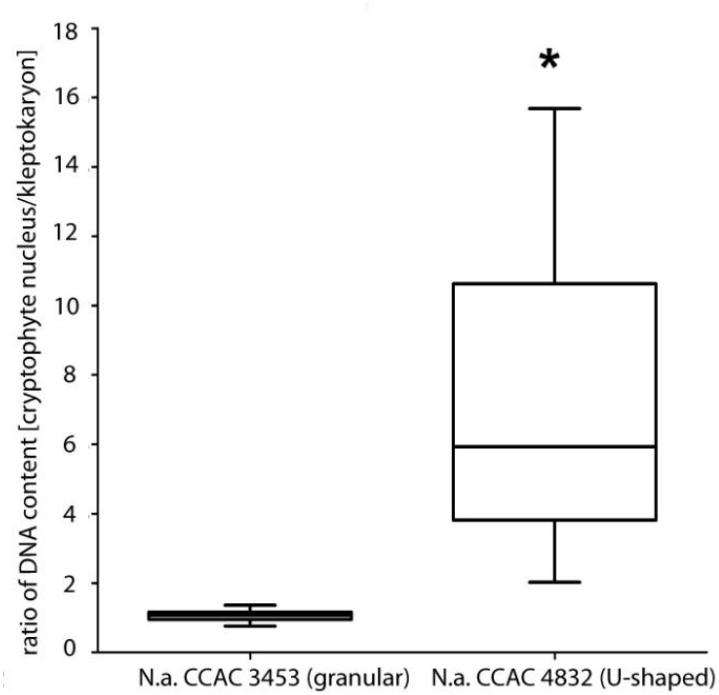

Figure 7. Ratio of DNA content of kleptokarya and cryptophyte nuclei in the granular (CCAC 3453) and U-shaped (CCAC 4832) situation calculated from quantitative measurements of DAPI signals. N.a. = Kleptokarya in the dinoflagellate *N. aeruginosum/acidotum,* * = significance (p= 0.000074 < 0.005, T-test).

As the measurements of the volumes as well as DAPI signals gave no information about a potential polyploidization of the cryptophyte nuclear DNA after ingestion by the dinoflagellate, a PCR based approach was chosen to estimate the relative number of copies of the multi copy gene partial LSU rDNA. Therefore a standard PCR was applied on single dinoflagellate and cryptophyte cells and the fluorescence intensities of a defined volume of PCR product were measured and compared. As the results described above suggest an up to 16 fold increase in DNA content and an up to 15 fold increase in volume, a spectrum ranging from 5 to 20 cryptophyte cells was chosen and compared to single enlarged kleptokarya. The signal obtained from less than 5 cryptophyte cells was too weak to be measured accurately and therefore the spectrum starts with 5 cells. Extracted DNA of the strain CCAC 4832 always resulted in a positive amplification (positive control) whereas no product was amplified for colorless dinoflagellates (negative control) using the two primers LSUBancF02 and LSUBancR02. As expected an approximate doubling of the relative fluorescence was observed between 5 and 10 cells and 10 and 20 cells (n = 20). Amplification success for dinoflagellates containing U-shaped kleptoplasts confirmed the number of kleptokarya observed after DAPI staining of the permanent co-culture with the strain CCAC 4832 (21% of the cells resulted in a PCR product whereas 23% showed a positive DAPI signal). The relative fluorescence measured for the single dinoflagellate cells matched the intensities measured for

5-20 cryptophyte cells indicating an up to 20 fold increase in copy number compared to the initial situation (n = 58). Also here enormous variations were observed in fluorescence that can be traced back to the developmental stages of the kleptokarya (Fig. 8).

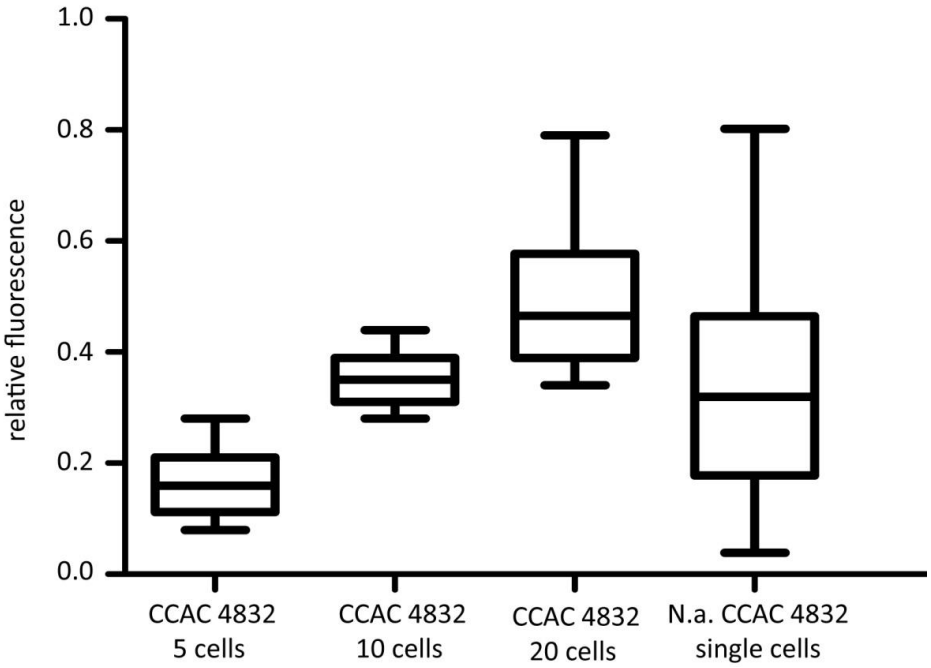

Figure 8. Relative fluorescences of PCR products obtained from amplification of a defined number of cryptophyte cells and single dinoflagellates with *Chroomonas* nucleus/kleptokaryon specific primers. CCAC 4832 = free living cryptophytes. N.a. = Kleptokarya in the dinoflagellate *N. aeruginosum/acidotum.*

3.3.3 Ultrastructure

To get an impression of the detailed structure of the granular and U-shaped kleptoplasts, the ultrastructure of *N. aeruginosum/acidotum* was investigated using the TEM. Cells in the granular state showed that each kleptoplast is associated with a kleptokaryon that resembles in structure the situation in the free living cryptophyte cell (Fig. 9A, B). The position of the kleptokaryon was always close to the kleptoplast. The kleptokaryon could be recognized by the presence of heteochromatin and a small electron dense structure, the nucleolus. The thylacoids were typical for cryptophyte plastids organized in stacks of two. Intraplastidal starch was observed within the kleptoplasts but it is not clear whether this starch was produced before or after ingestion of the cryptophyte cell by the dinoflagellate. The cytoplasm of the ingested prey was still present (Fig. 9C) and just separated by a single membrane from the dinoflagellate cytoplasm. Dinoflagellate mitochondria showed the typical tubular cristae whereas those of the cryptophyte mitochondria were flattened (Fig. 9D).

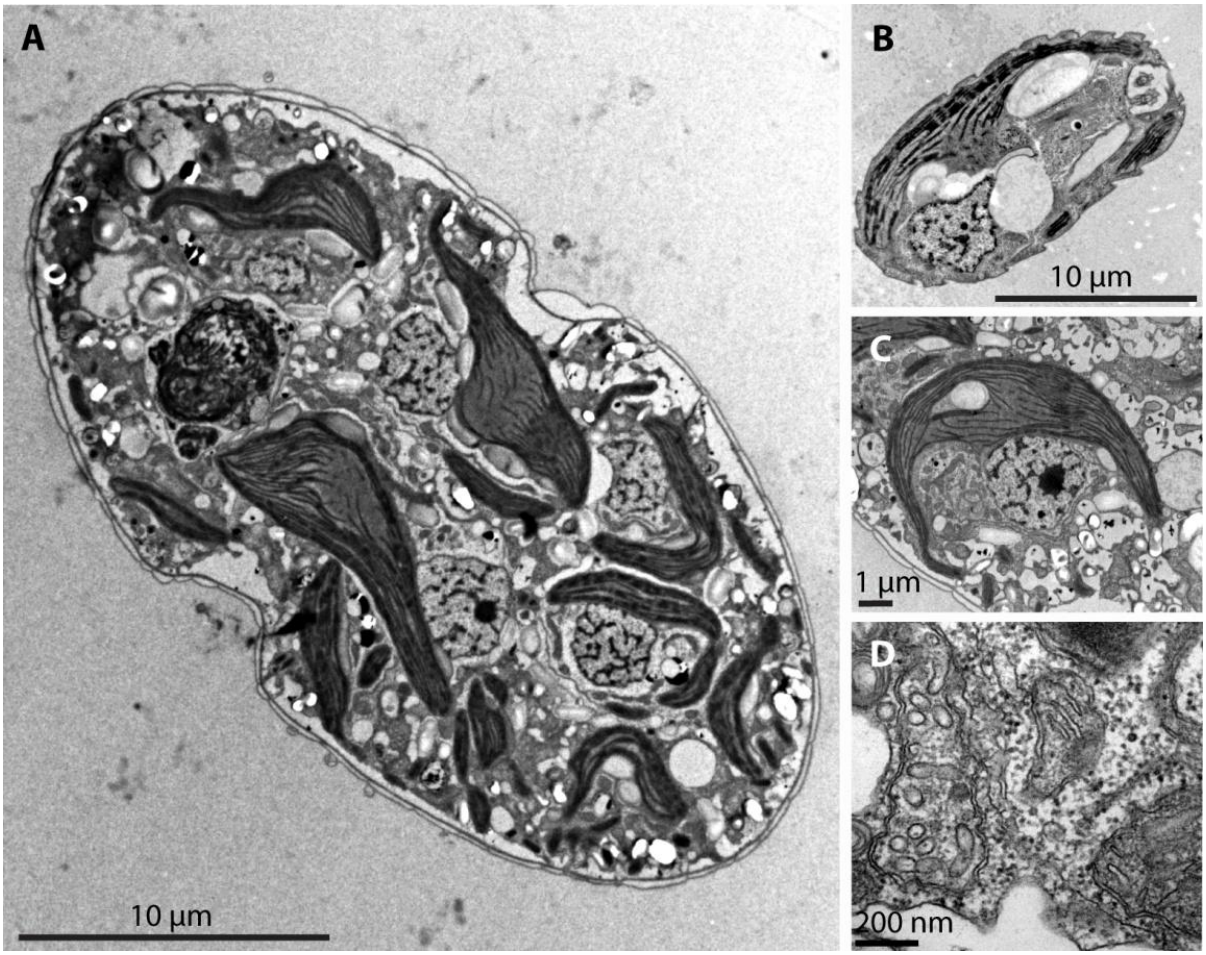

Figure 9. Ultrastructure of *N. aeruginosum/acidotum* with granular kleptoplasts derived from *Chroomonas* sp. CCAC 3453. **A** Longitudinal section through a cell showing the presence of multiple small kleptoplasts and kleptokarya. **B** Longitudinal section through a *Chroomonas* cell. **C** Granular kleptoplast associated with a kleptokaryon. **D** A single membrane separates the dinoflagellate cytoplasm (recognizable by the mitochondrion with tubular cristae, left) from the cytoplasm of the ingested cryptomonad (recognizable by the mitochondrion with flattened cristae, right).

Including the membrane that separates the cryptophyte from the dinoflagellate cytoplasm, cryptophyte plastids were surrounded by five membranes. Cells with U-shaped kleptoplasts just showed one highly enlarged kleptoplast filling most of the periphery of the cell. A large kleptokaryon similar in size to the dinokaryon was present in many but not all cells. The kleptokaryon can be recognized by the presence of heterochomatin and a nucleolus whereas the dinokaryon is characterized by the permanently condensed chromosomes (Fig. 10A). Cells without a kleptokaryon however often showed higher numbers of vacuoles filled with mucilage in the cell periphery (Fig. 10B). As in the granular situation, the kleptokaryon is closely associated with the kleptoplast and also the cryptophyte cytoplasm is still present but not as clearly visible as in the granular situation (Fig. 9C). As in the granular kleptoplast, also

the U-shaped kleptoplast shows intraplastidal starch. Thylacoids were typical for cyptophytes in stacks of two. A nucleomorph was still present (Fig. 9D).

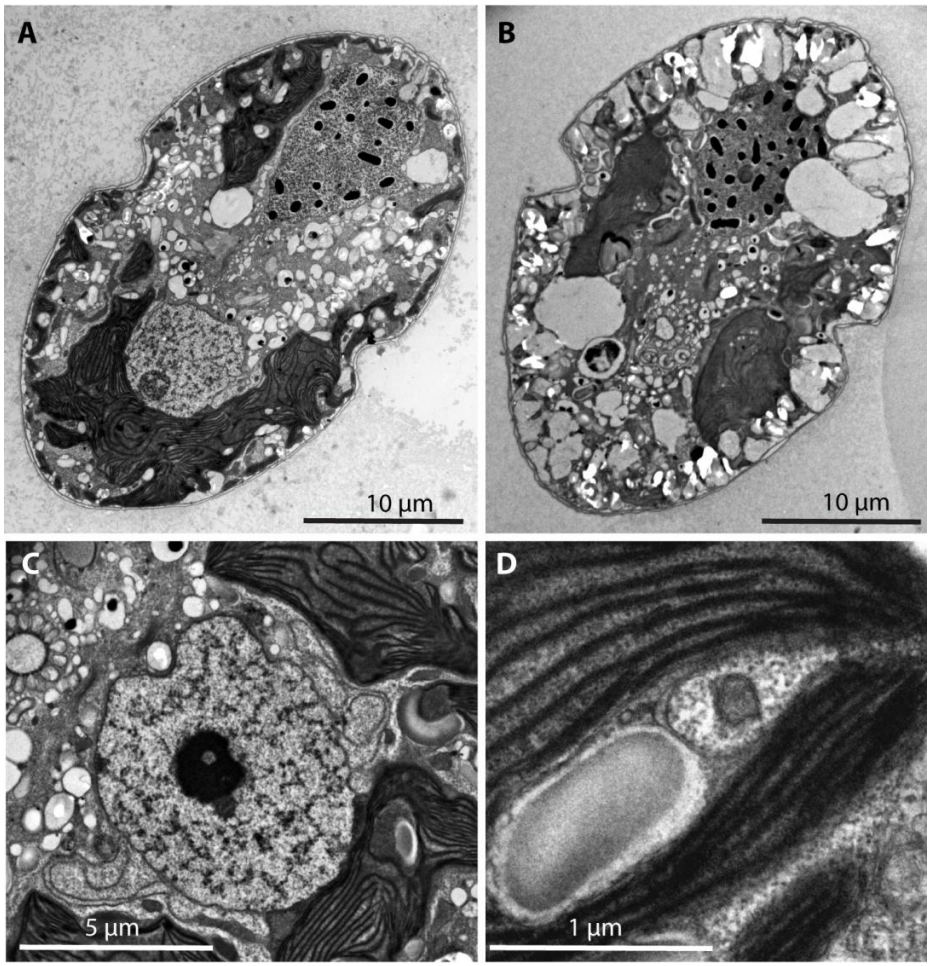

Figure 10. Ultrastructure of *N. aeruginosum/acidotum* with U-shaped kleptoplast derived from *Chroomonas* sp. CCAC 4832. **A** Longitudinal section through a cell showing the large U-shaped kleptoplast and the enlarged kleptokaryon. **B** Longitudinal section through a cell without kleptokaryon. **C** Detailed image of the enlarged kleptokaryon. **D** Starch grain (left homogenous structure) and nucleomorph (right structure) within a U-shaped kleptoplast.

3.3.4 Stability of the observed kleptoplast types

The stability of the granular and U-shaped kleptoplasts was investigated by keeping dinoflagellate cells in the absence of prey and waiting until they became colorless. The granular kleptoplasts were stable for 12-14 days in average. Dinoflagellate cells were able to divide up to three times resulting in an average number of eight daughter cells. Each cell division resulted in a bisection of the number of kleptoplasts (Fig. 18). Small modifications in the shape of the kleptoplasts were observed (Fig. 11A2). Moreover the cell size decreased during starvation (Fig. 11A1-A3). Usually all daughter cells were colorless after 14 days and

showed the presence of multiple small red-brown bodies probably representing digested kleptoplasts (Fig. 11A3). The stability of kleptokarya was determined via DAPI staining. Signals of the kleptokarya became weaker during starvation and the last signals were detected after 10 days (data not shown). The U-shaped kleptoplasts were stable for up to 60 days. Cells were able to divide up to seven times. The kleptoplast was distributed equally to the daughter cells during cell division whereas the kleptokaryon was just inherited to one daughter cell and did not divide (Fig. 18). Whereas starvation of cells with granular kleptoplasts usually resulted in daughter cells of uniform quality, starvation of cells with U-shaped kleptoplasts resulted in highly unequal daughter cells. DAPI staining of daughter cells derived from one cell that was starved for 50 showed that the kleptokaryon was still present but the signal was very weak probably due to degradation. Interestingly, the cell with the kleptokaryon had the biggest size and also the biggest kleptoplast (data not shown). Cells with U-shaped kleptoplasts were able to divide up to seven times resulting in a total number of 128 cells. Colorless cells usually just contained one red brown body probably representing the digested kleptoplast (Fig. 11B4).

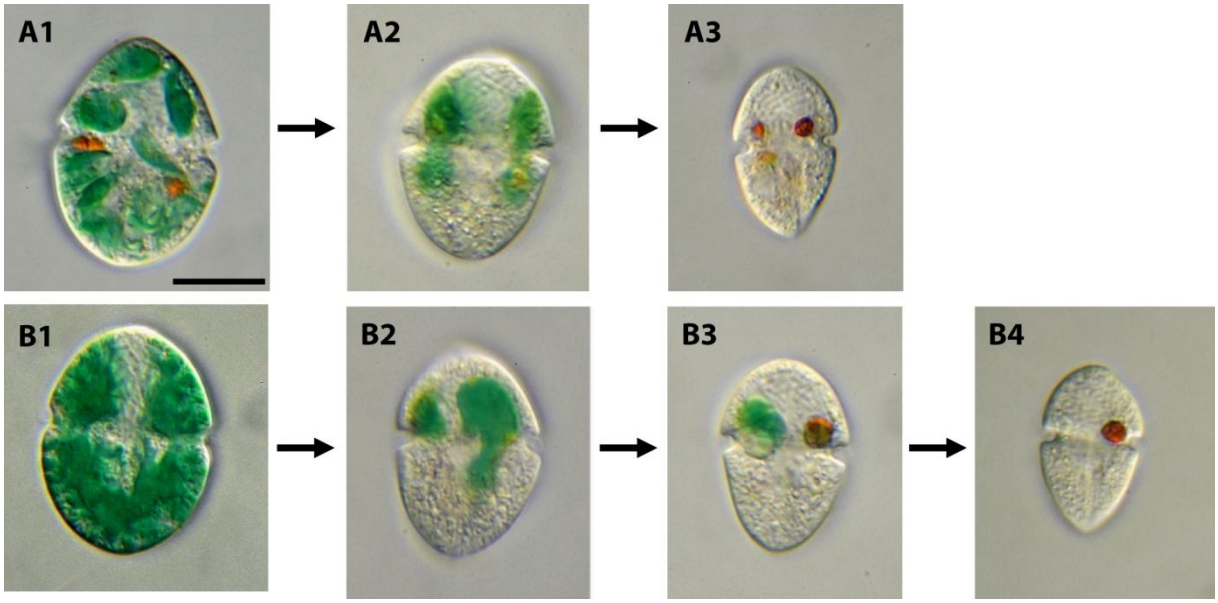

Figure 11. Loss of kleptoplasts during starvation. **A** Cells with granular kleptoplasts usually result in colorless cells containing multiple red-brown bodies. **A1** Cell under permanent prey availability. **A2** Cell after starvation of 10 days. **A3** Cell after starvation of 14 days. **B** Cells containing a U-shaped kleptoplast usually result in colorless cells containing only one red-brown body. **B1**Cell under permanent prey availability. **B2** Cell after starvation of 4 weeks. **B3** Cell after starvation of 6 weeks. **B4** Cell after starvation of 8 weeks (bar applies to **A** and **B** = 10µm).

Starvation experiments on cells isolated from the bog pond Heidemoor an der Wasserdell, Dahlem Germany, in spring 2011showed that many cells already lost their kleptoplasts after

12-16 days whereas a few were able to keep their kleptoplasts for more than 42 days (Wittek, 2011). This result suggests that many cells contained granular and just a few contained U-shaped kleptoplasts. It also fits to the sequences obtained concerning the kleptoplast identity. Three of the four sequences obtained were positioned in subclade 3 and just one in subclade 4 (Fig. 5). No information concerning kleptoplast morphology of the cells sequenced is present.

3.3.5 Biovolume production of cells with U-shaped and granular kleptoplasts

The biovolume of single dinoflagellate cells with granular and U-shaped kleptoplasts before and after starvation should give an impression about the productivity of the kleptoplasts. Cells with granular kleptoplasts showed a biovolume of about 7341 ± 2077µm^3 (n=100). The average maximum number of 8 cells was reached after 14 days and the biovolume increased approximately 4 fold to 30155 ± 10846µm^3 (n= 58). Cells with U-shaped kleptoplasts were slightly larger than cells with granular kleptoplasts showing an average biovolume of 8087 ± 2029µm^3 (n= 100). After starvation of 60 days, the biovolume increased up to 25 fold to a value of 203637 ± 29134µm^3 (n= 5). Biovolumes of cells with granular and U-shaped kleptoplasts before starvation did not differ significantly. In contrast, a significant difference between the biovolumes after starvation was observed (p= 0.0002 < 0.05, Mann-Whitney U-test).

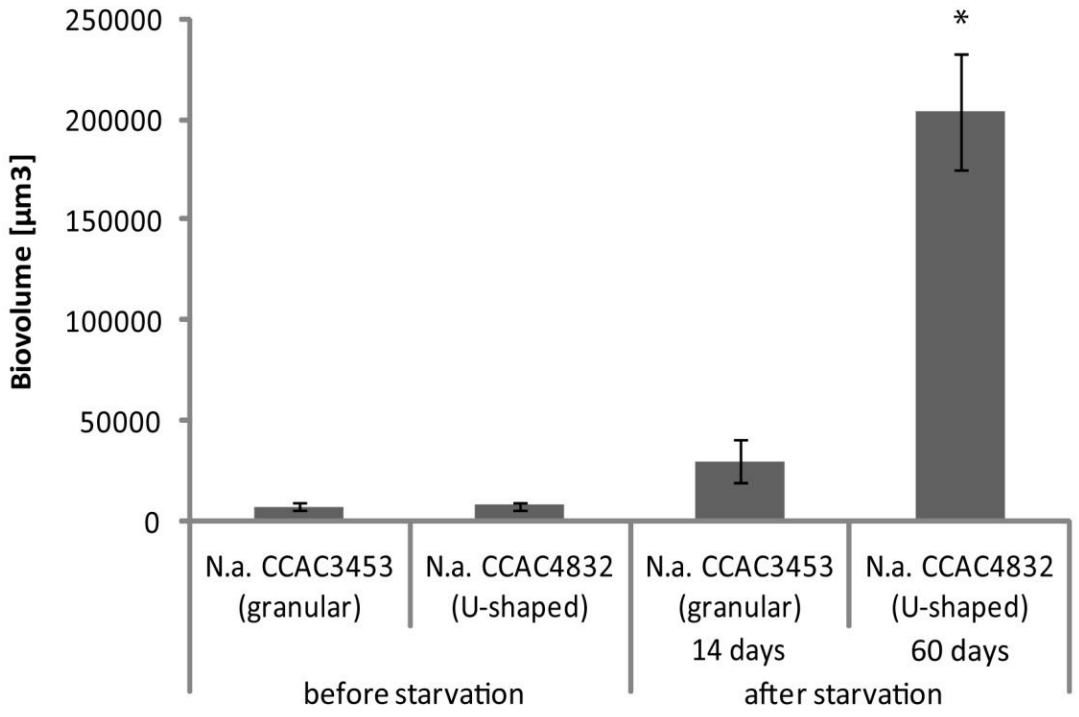

Figure 12. Biovolume of *N. aeruginosum/acidotum* before and after starvation with granular kleptoplasts derived from *Chroomonas* sp. CCAC 3453 and U-shaped kleptoplasts derived from *Chroomonas* sp. CCAC 4832. Maximum cell numbers were observed after 14 days for the granular situation and after 60 days for the U-shaped situation. * Significant difference between the biovolumes after starvation ($p= 0.0002 < 0.05$, Mann-Whitney U-test).

3.3.6 Oxygen measurements

The measurement of produced oxygen should give additional information about the functionality and productivity of granular and U-shaped kleptoplasts. Measurements were carried out on pure dinoflagellates separated from their cryptophyte prey and on their corresponding prey cultures. Cells with granular kleptoplasts showed a lower oxygen production than cells with U-shaped kleptoplasts. Dinoflagellates with granular kleptoplasts showed an oxygen production of 0.77 ± 0.02nmol O_2*min^{-1}*10000 cells*ml^{-1} whereas oxygen production of dinoflagellates with U-shaped kleptoplasts was more than double as high with a value of 1.88 ± 0.18nmol O_2*min^{-1}*10000 cells*ml^{-1} ($n = 5$) (Fig. 13A). A comparison of the oxygen production of the cryptophyte prey cultures also showed that the strain *Chroomonas* sp. CCAC 3453 that induces granular kleptoplasts had a lower oxygen production compared to the strain *Chroomonas* sp. CCAC 4832 (Fig. 13B). One dinoflagellate cell with a U-shaped kleptoplast had a 78 fold higher oxygen production than one cell of the corresponding cryptophyte. In contrast one cell with granular kleptoplasts just showed a 22 fold increase in oxygen production compared to one free living cryptophytes cell. The fact that one cell can contain up to 21 kleptoplasts in the co-culture tested indicates that the kleptoplasts do not increase in productivity.

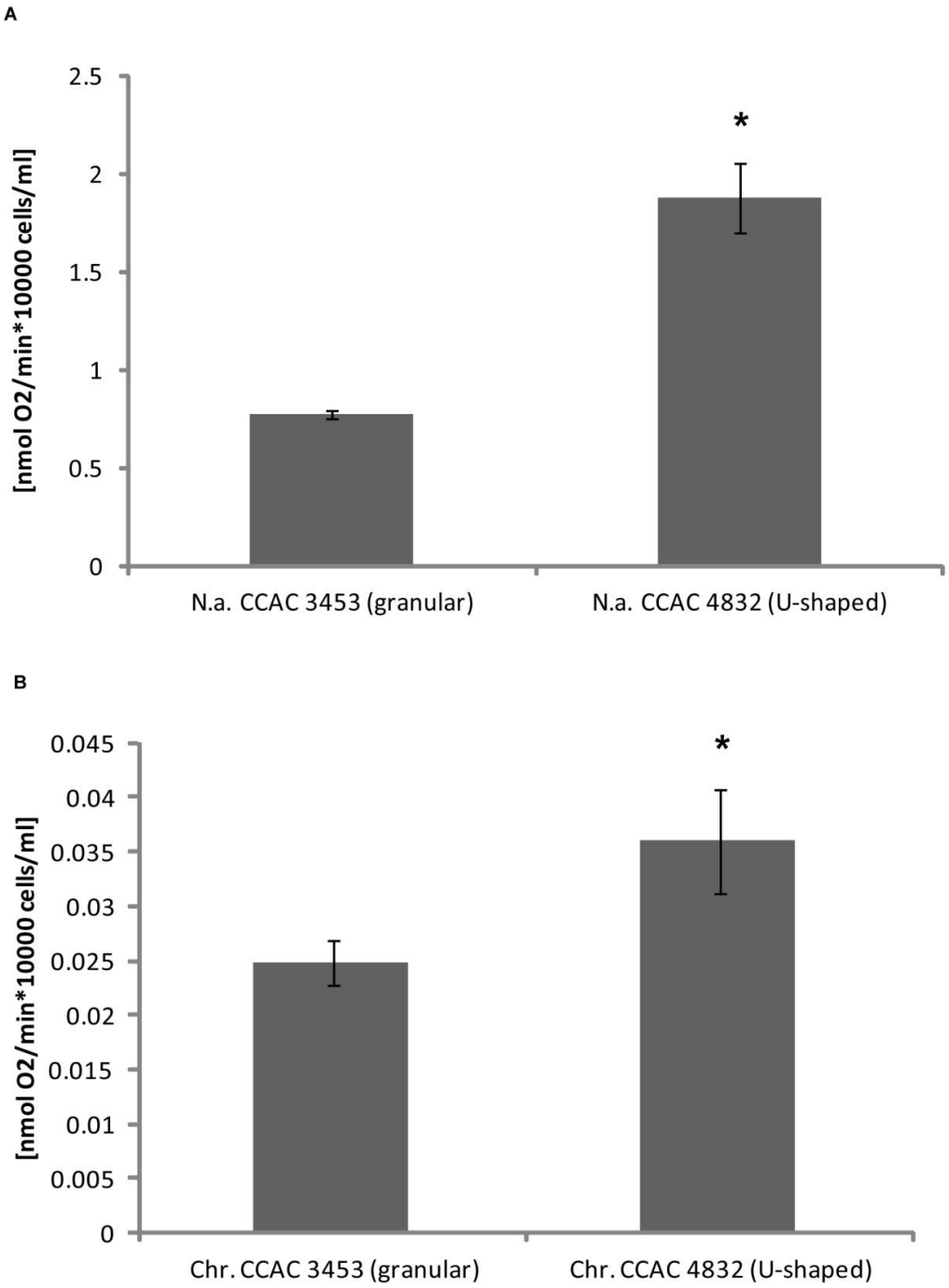

Figure 13. Oxygen production by *N. aeruginosum/acidotum* with granular and U-shaped kleptoplasts (**A**) and the corresponding cryptophyte strains (**B**). A * = significance ($p = 0.0121 < 0.05$, Mann-Whitney U-Test). B * = significance ($p = 0.0014 < 0.005$, T-test).

3.3.7 Behavior of *N. aeruginosum/acidotum* with granular and U-shaped kleptoplasts

One of the most striking differences between *N. aeruginosum/acidotum* with granular and U-shaped kleptoplasts is the behavior of the dinoflagellates. Dinoflagellate cells with granular kleptoplasts showed a continuous uptake of prey whereas cells with U-shaped kleptoplasts took up prey just very rarely. The swimming behavior of cells with granular kleptoplasts was characterized by circular movements and a permanent search for prey at the bottom of the culture vessel. Such a behavior could be observed especially during the first hours after the dark phase. Uptake of prey took place via phagocytosis at the antapex of the dinoflagellate. In contrast, dinoflagellates with a U-shaped kleptoplast swam in long straight lines and just sometimes touched the bottom of the culture vessel (supplementary videos 1+2). Also the division rate of *N. aeruginosum/acidotum* with granular and U-shaped kleptoplasts differed under permanent prey availability. Cells with granular kleptoplasts derived from *Chroomonas* sp. CCAC 3453 showed an average division rate of 0.33 ± 0.04 *day^{-1} (n= 58) whereas cells with U-shaped kleptoplasts however showed a division rate of just 0.22 ± 0.06 *day^{-1} (n= 57) that was significantly lower (Fig. 14).

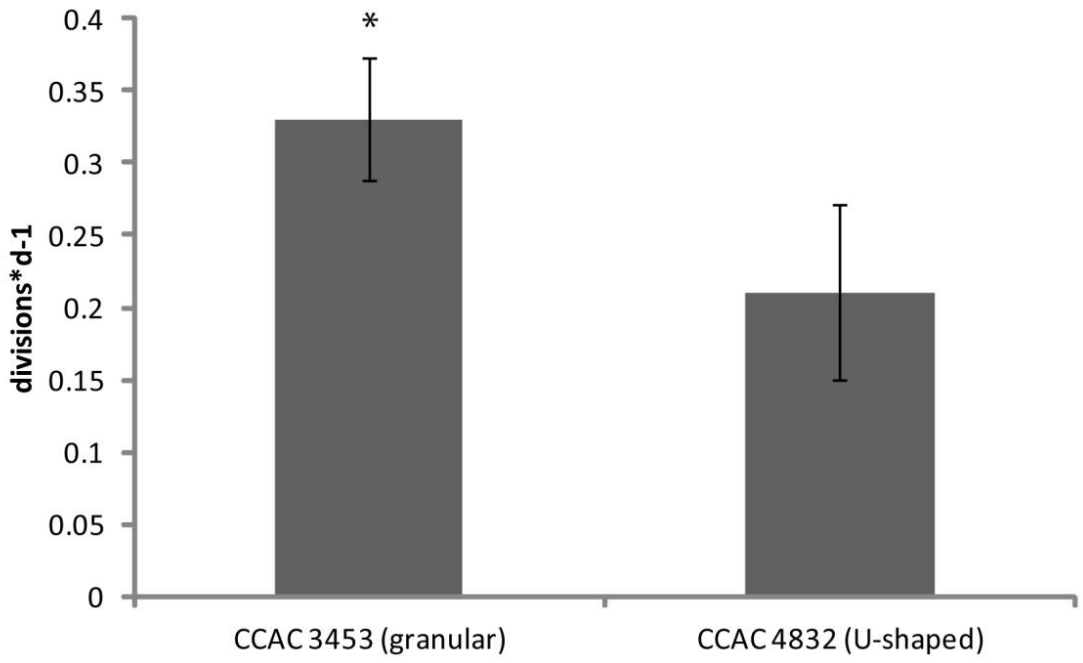

Figure 14. Division rates of *N. aeruginosum/acidotum* with granular kleptoplasts derived from *Chroomonas* sp. CCAC 3453 and U-shaped kleptoplasts derived from *Chroomonas* sp. CCAC 4832 in the presence of prey. * Significance (p= 0.000 < 0.05, Mann-Whitney U-test).

Another difference was observed in old cultures (2 months). Whereas dinoflagellates with granular kleptoplasts tended to consume the whole prey within a culture vessel, cells with U-shaped kleptoplasts often showed the problem of being overgrown by their prey. Old cultures of cells with granular kleptoplasts contained a large number of resting cysts whereas cultures of cells with U-shaped kleptoplasts just showed very few resting cysts and mostly vegetative cells (numbers were not quantified). To test whether *N. aeruginosum/acidotum* is able to survive the winter with both types of kleptoplasts, freshly inoculated co-cultures were transferred to 4°C. In both cases most dinoflagellates became immobile and just the cryptophyte cells continued moving. As a consequence of the immobility the cells with granular kleptoplasts stopped the uptake of new prey. Moreover, no division was observed for the dinoflagellates whereas the cryptophytes cells continued to divide. After two months at 4°C all dinoflagellates disappeared. In contrast cells with U-shaped kleptoplasts survived for more than six months without any problems at 4°C and even increased in cell number.

3.3.8 Density experiments with prey

The establishment of permanent stable cultures of *N. aeruginosum/acidotum* allowed a variety of different experiments under controlled conditions. Little is known about the behavior of kleptoplastic organisms under different prey concentrations. To test this, starved dinoflagellate cells were fed with different prey densities and the type of kleptoplast formed as well as their behavior were documented. This experiment was carried out with the two *Chroomonas* strains *Chroomonas coerulea* NIES 714 and *Chroomonas placoidea* CCAP 978/8 that belong to subclade 4 phylogenetically. A control was also done with *Chroomonas* sp. CCAC 3453 belonging to subclade 3. The strain *Chroomonas* sp. CCAC 4832 that is in the focus of this study was not chosen for this experiment as it strongly tends to form cell aggregates (palmellae) that do not make the prey available for the predator. The other strains chosen do not show this behavior so distinct. Cryptophyte to dinoflagellate ratios from 1 to 1000 were tested. For both strains, NIES 714 and CCAP 978/8, *N. aeruginosum/acidotum* formed U-shaped kleptoplasts under low cryptophyte to dinoflagellate ratios ranging from 1-50. However, just at a ratio of 1 all dinoflagellates showed U-shaped kleptoplasts when fed with both strains. Already at a ratio of 2 cells with more than one kleptoplast were visible. These cells resembled cells with granular kleptoplasts derived from subclade 3 with the only difference that the granular kleptoplasts derived from subclade 4 were smaller. The number of granular kleptoplasts ranged from 5 to 22 with an average of 8. Cryptophyte to dinoflagellate

ratios higher that 2 resulted in a decline of the number of cells with U-shaped kleptoplasts and an increase of cells with granular kleptoplasts. At a ratio of 20 most dinoflagellates contained granular kleptoplasts and at ratios higher than 50 just granular kleptoplasts were observed (Fig. 15). In contrast to feeding experiments with strains of subclade 4, no differences in the type of kleptoplast formed were observed under the ratios tested with *Chroomonas* sp. CCAC3453 belonging to subclade 3.

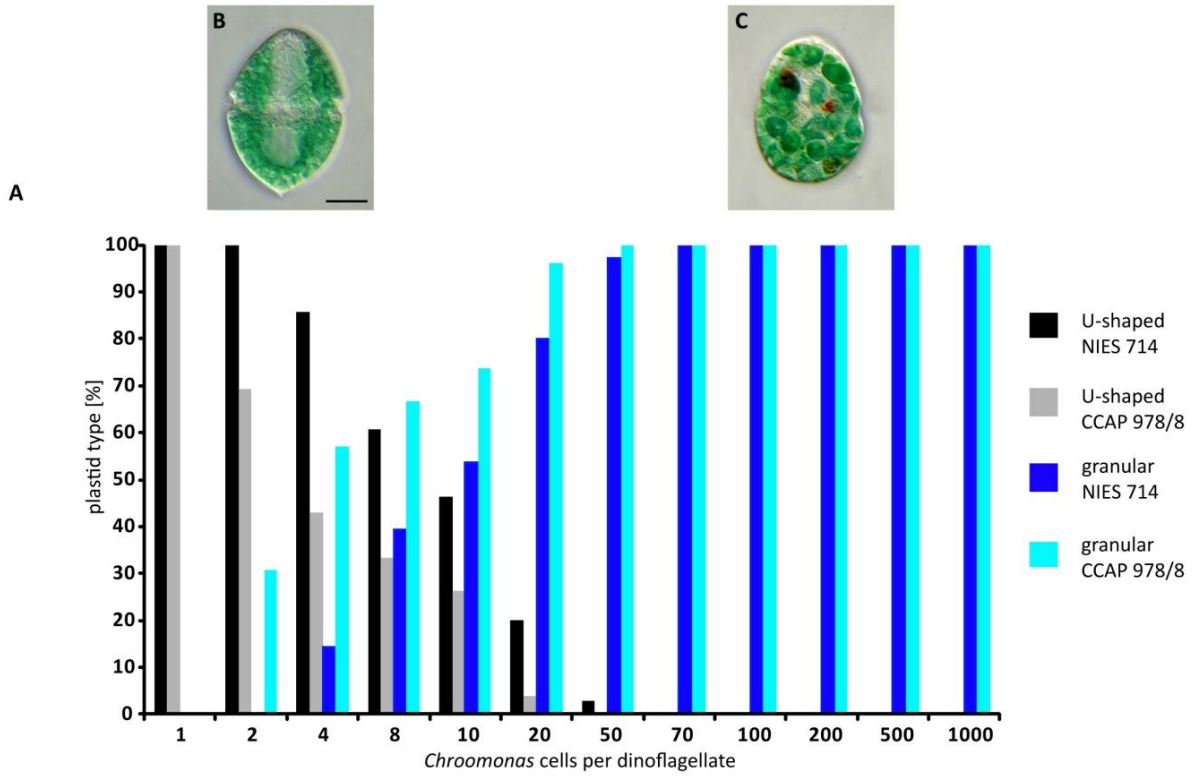

Figure 15. The type of kleptoplast formed by *N. aeruginosum/acidotum* in the presence of *Chroomonas* strains from subclade 4 depends on the prey density. **A** Plastid type formed in the presence of *Chroomonas coerulea* NIES 714 and *Chroomonas placoidea* CCAP978/8 under different initial densities. **B** DIC image of *N. acidotum* fed with *Chroomonas coerulea* NIES 714 with the U-shaped kleptoplast observed under low prey density. **C** DIC image of *N. acidotum* fed with *Chroomonas coerulea* NIES 714 with granular plastids observed under high prey density. The results were obtained 8 days after incubation at 23°C (bar in **B** also applies to **C** = 10µm).

3.3.9 Transformation of the U-shaped kleptoplasts into granular kleptoplasts (subclade 4) and vice versa

The observation of granular kleptoplasts derived from *Chroomonas* strains of subclade 4 gave rise to two questions: 1. What causes the switch from U-shaped kleptoplasts to granular kleptoplasts? 2. What is the fate of the granular kleptoplasts after reisolation? To answer the first question, a co-culture with the strain *Chroomonas coerulea* NIES 714 was established with a ratio of 1-2 cryptophyte cells per dinoflagellate to get all dinoflagellates into the U-shaped condition. DAPI staining of this culture revealed that 28% (n= 100) of the cells contained an enlarged kleptokaryon. To find out whether the kleptokaryon is able to divide, single dinoflagellate cells were isolated into 250µl of culture medium and their two daughter cells were again stained with DAPI. Just two cases were observed: 1. Both daughter cells lacked a kleptokaryon. 2. Just one daughter cell contained a kleptokaryon indicating that the kleptokaryon is inherited to just one daughter cell. Subsequently, the behavior of *N. aeruginosum/acidotum* with and without the enlarged kleptokaryon was observed in the presence of high prey density. Therefore cryptophyte cells were added to the established culture containing 23% of binucleate cells in a ratio of 1000 cryptophytes per dinoflagellate. The culture was incubated for two days and then stained with DAPI. Only cells lacking an enlarged kleptokaryon ingested new prey indicating that the kleptokaryon has some inhibitory effect on prey uptake. After two months of incubation all dinoflagellates ingested new prey and contained granular kleptoplasts (Fig. 16). The swimming and ingestion behavior of dinoflagellates with granular kleptoplasts was identical with cells containing granular kleptoplasts derived from *Chroomonas* strains of subclade 3.

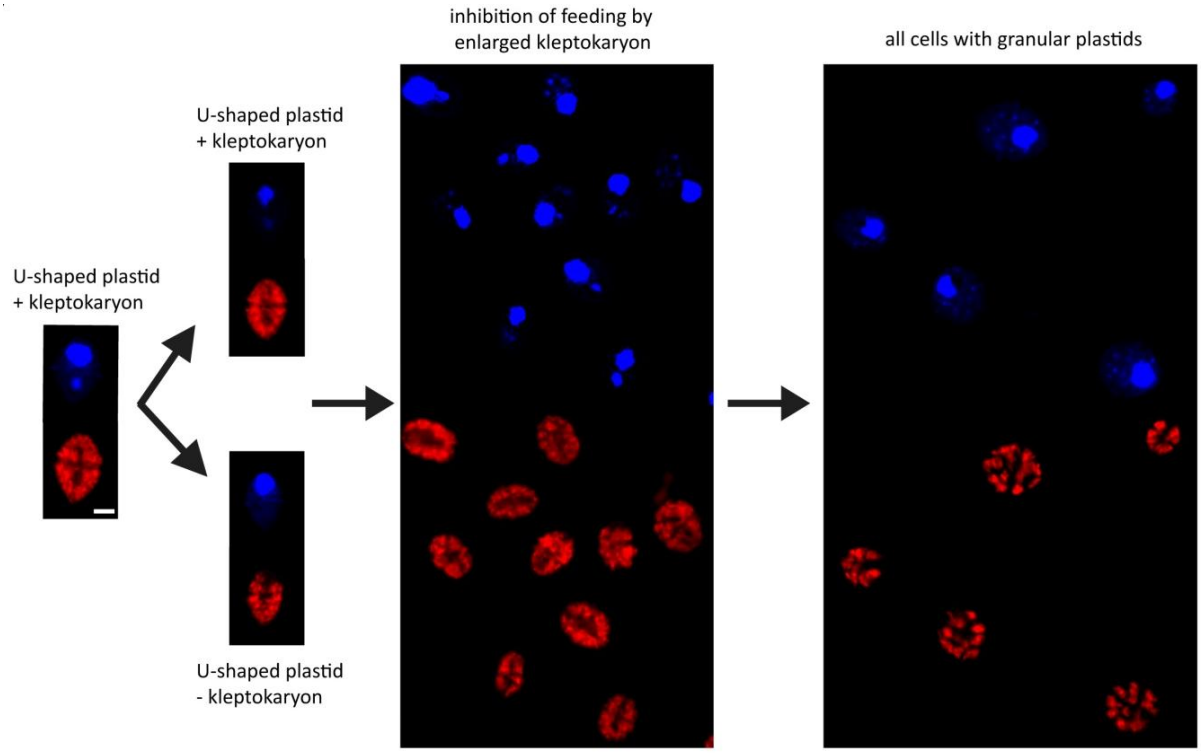

Figure 16. The uptake of new prey is inhibited by the presence of an enlarged kleptokaryon in the U-shaped situation. From left to right: DAPI image (above) and kleptoplast autofluorescence (below) of a cell with a U-shaped kleptoplast and kleptokaryon derived from *Chroomonas coerulea* NIES 714. Cell division results in one cell with and one cell without a kleptokaryon. Just cells without an enlarged kleptokaryon took up new prey. After two months of incubation all cells took up new prey and contained granular kleptoplasts (bar applies to all images = 10µm).

To address the second question about the fate of granular kleptoplasts derived from *Chroomonas* strains of subclade 4, single dinoflagellate cells with granular kleptoplasts were reisolated and starved in the absence of prey. The average number of kleptoplasts per dinoflagellate cell was 8 in the tested culture. Each granular kleptoplast was associated with a small kleptokaryon at the beginning. Both, kleptoplast and kleptokaryon were not enlarged compared to the free living prey. All eight cells tested had a division rate of 1 per day. DAPI staining as well as chlorophyll autofluorescence revealed that kleptoplasts and kleptokarya were distributed equally to both daughter cells (Fig. 18). After two divisions, four daughter cells were present each containing two kleptoplasts and kleptokarya. However, an enlargement of both in size was observed at this stage. After three divisions eight daughter cells each containing a large U-shaped kleptoplast and a large kleptokaryon were observed (Fig. 17). Cells with granular kleptoplasts derived from *Chroomona*s strains of subclade 3 in contrast never showed an enlargement of their kleptokarya. The subsequend behavior of the

cells with the U-shaped kleptoplast and enlarged kleptokaryon was the typical for cells with U-shaped kleptoplasts.

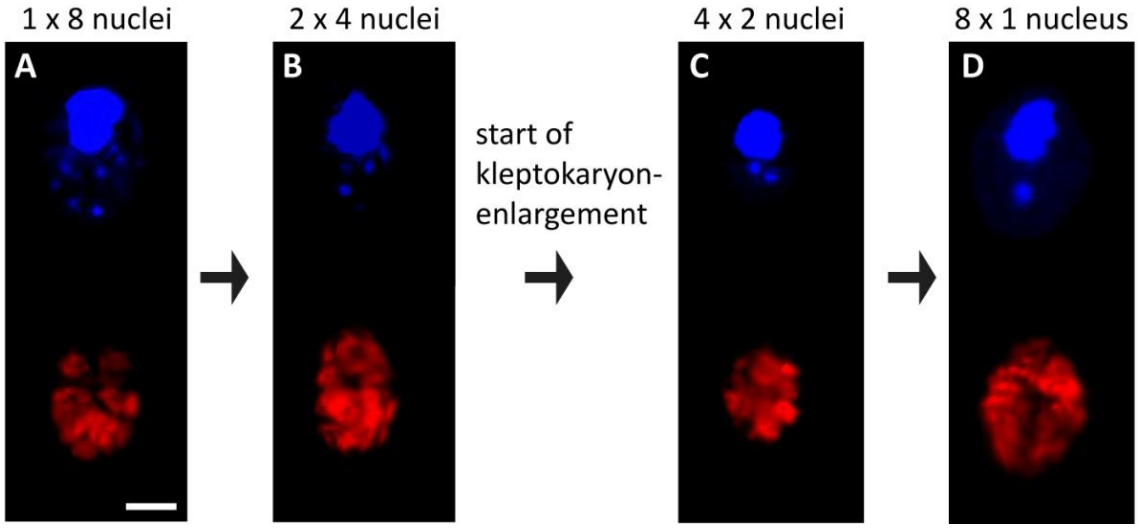

Figure 17. Fate of granular kleptoplasts formed under high availability of prey from subclade 4. **A** Starting cell with an average number of 8 kleptoplasts kleptokarya. **B** One division results in two cells with four kleptoplasts and kleptokarya. **C** Two divisions result in 4 cells with 2 kleptoplasts and kleptokarya. Kleptokarya and kleptoplasts are already enlarged. **D** After three divisions all eight daughter cells contained a large U-shaped kleptoplast and an enlarged kleptokaryon (bar in **A** also applies to **B**-**D** = 10µm).

3.3.10 Inheritance of cryptomonad organelles

The knowledge about the inheritance of cryptomonad organelles during cell division of the host is essential to understand the life history of *N. aeruginosum/acidotum*. Division of this dinoflagellate always occurred during the dark phase. Typical was the formation of a two celled chain in which the antapex of the anterior cell was connected to the apex of the posterior cell. Cells with granular kleptoplasts derived from *Chroomonas* strains of subclade 3 always showed an equal distribution of kleptokarya and kleptoplasts to both daughter cells except for uneven starting numbers of kleptoplasts and kleptokarya (e. g. 13). In this case one cell got one more kleptoplast and kleptokaryon than the other cell (e. g. 6 and 7). Cells with a U-shaped kleptoplast and an enlarged kleptokaryon always inherited the kleptoplast to both daughter cells equally whereas the kleptokaryon was just inherited to one daughter cell. However, the kleptokaryon was always inherited to the anterior cell of the chain. Another type of kleptoplast is the so called ‘intermediate’ kleptoplast that is introduced in 3.3.11 and characterized by cells containing more than one enlarged kleptoplasts and kleptokarya derived from *Chroomonas* strains of subclade 4. In the intermediate situation both, kleptoplasts and kleptokarya were inherited equally to the daughter cells except for uneven numbers. In such a

case one cell got one more kleptoplast and kleptokaryon than the other cell. The inheritance of granular kleptoplasts and kleptokarya derived from *Chroomonas* of subclade 4 always occured in a 1:1 ratio to both daughter cells (Fig. 18).

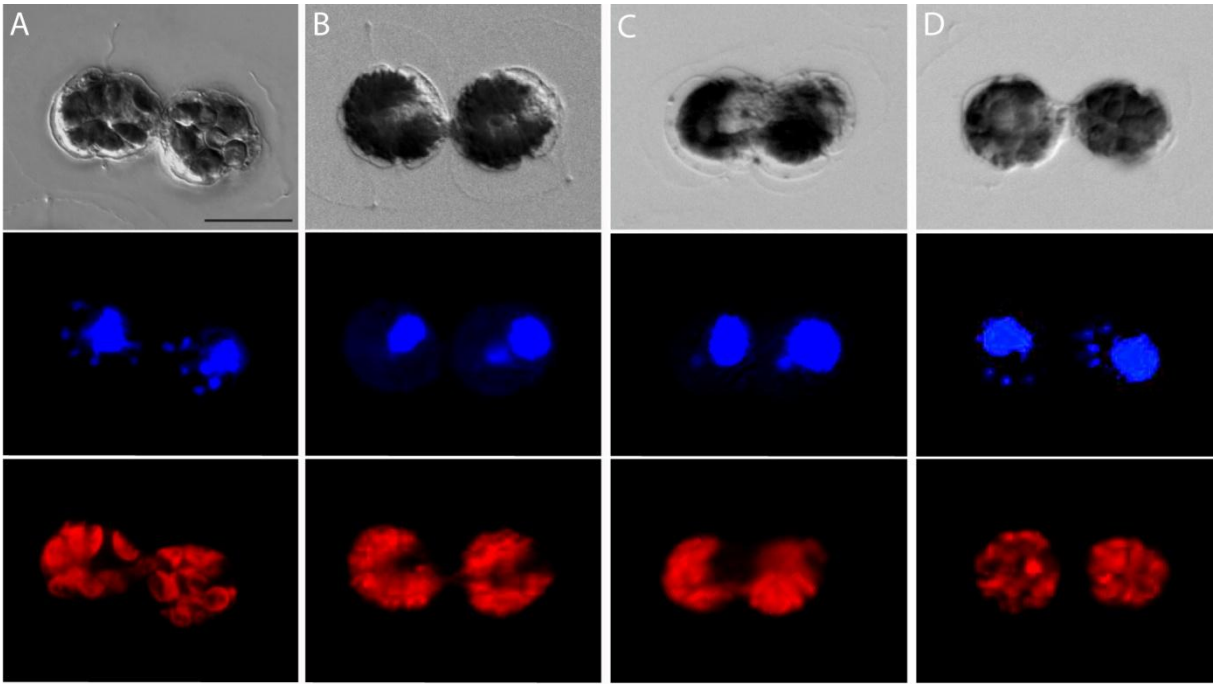

Figure 18. Inheritance of kleptoplasts and kleptokarya during cell division of *N. aeruginosum/acidotum*. **A** Granular kleptoplasts derived from *Chroomonas* sp. CCAC 3453 (subclade 3). **B** U-shaped kleptoplast derived from *Chroomonas* sp. CCAC 4832 (subclade 4). **C** Intermediate kleptoplasts derived from *Chroomonas* sp. CCAC 1481 (subclade 4). **D** Granular kleptoplasts derived from *Chroomonas placoidea* CCAP 978/8 (subclade 4). Upper images show DIC, middle images show DAPI-fluorescence and lower images show chlorophyll autofluorescence (bar in **A** also applies to **B**-**D** = 20µm).

3.3.11 The intermediate kleptoplast

In addition to the U-shaped and granular kleptoplasts, a third type was observed in co-cultures of *N. aeruginosum/acidotum* with *Chroomonas* strains of subclade 4. This type of kleptoplast differed in its morphological appearance from both situations described before as it seems to combine characters typical for the U-shaped situation with characters typical for the granular situation resembling an intermediate between both. Therefore this type of kleptoplast is called ‘intermediate’ in this study. The intermediate situation is characterized by 2-4 enlarged kleptoplasts each associated with an enlarged kleptokaryon. The kleptoplasts are highly irregular in size, often branched and their position within the dinoflagellate cell can vary between different cells. The higher the number of intermediate kleptoplasts within a cell the smaller is the size of the size of both, kleptoplasts and kleptokarya (Fig. 19). Intermediate

kleptoplasts were observed most frequently in co-cultures with *Chroomonas* sp. CCAC 1481 as prey.

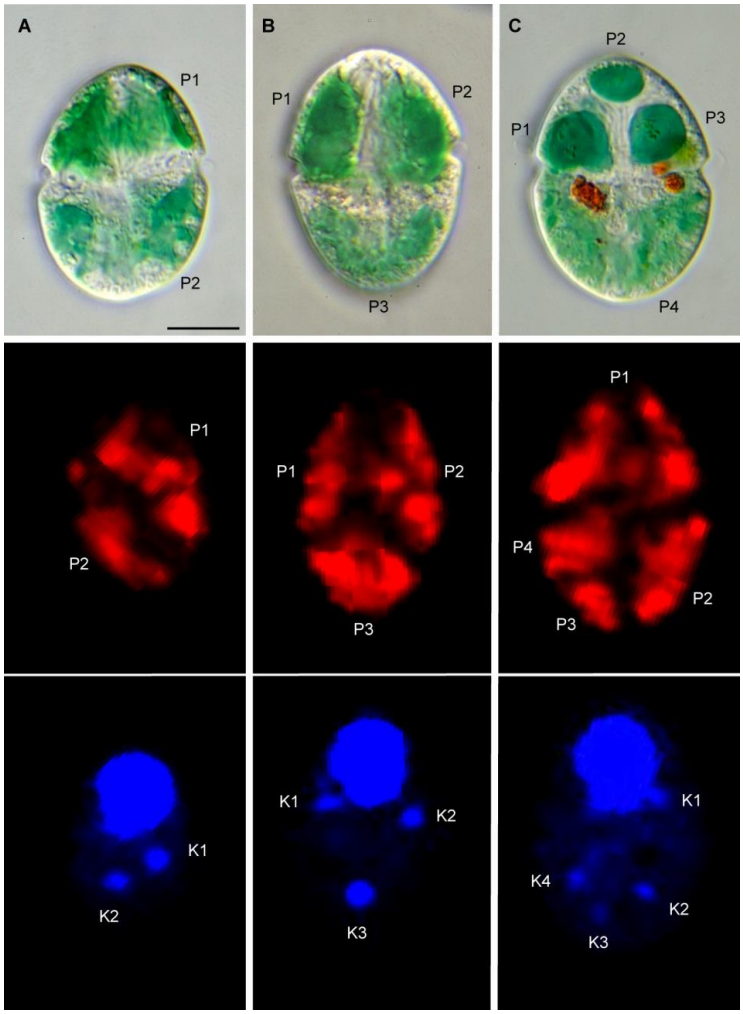

Figure 19. Intermediate kleptoplasts rarely observed in co-cultures with *Chroomonas* strains of subclade 4.The images show *N. aeruginosum/acidotum* from a co-culture with *Chroomonas* sp. CCAC 1481. **A** Cell with two enlarged kleptoplasts and two enlarged kleptokarya. **B** Cell with three enlarged kleptoplasts and three enlarged kleptokarya. **C** Cell with four enlarged kleptoplasts and four enlarged kleptokarya. Middle images represent chlorophyll autofluorescence, lower images represent DAPI staining of the DNA. DIC images were obtained from different cells than chlorophyll and DAPI images. P = kleptoplast, K = kleptokaryon, bar in **A** also applies to **B**-**C** = 10µm).

Intermediate kleptoplasts were just formed if two conditions were fulfilled. The first condition was that the dinoflagellate had to take up 2-4 prey cells within a short time period (the same day). The uptake of more prey cells resulted in granular kleptoplasts described above. The second condition was that the dinoflagellate should not divide within 6-7 days after prey uptake. This condition was just fulfilled for starved cells. Cells that contained a U-shaped kleptoplast and lacked a kleptokaryon continued dividing after the ingestion of new prey and distributed additional kleptoplasts and kleptokarya to the daughter cells equally. Due to this

fact, intermediate kleptoplasts were seen very rarely in permanent cultures. The study of the ultrastructure of intermediate kleptoplasts and kleptokarya showed that the kleptokarya were closely associated with the kleptoplasts (Fig. 20A). As for the granular and U-shaped situation, the cryptophyte cytoplasm was also present in the intermediate situation. The kleptokarya could be easily distinguished from the dinoflagellate nucleus by the presence of heterochromatin (Fig. 20B).

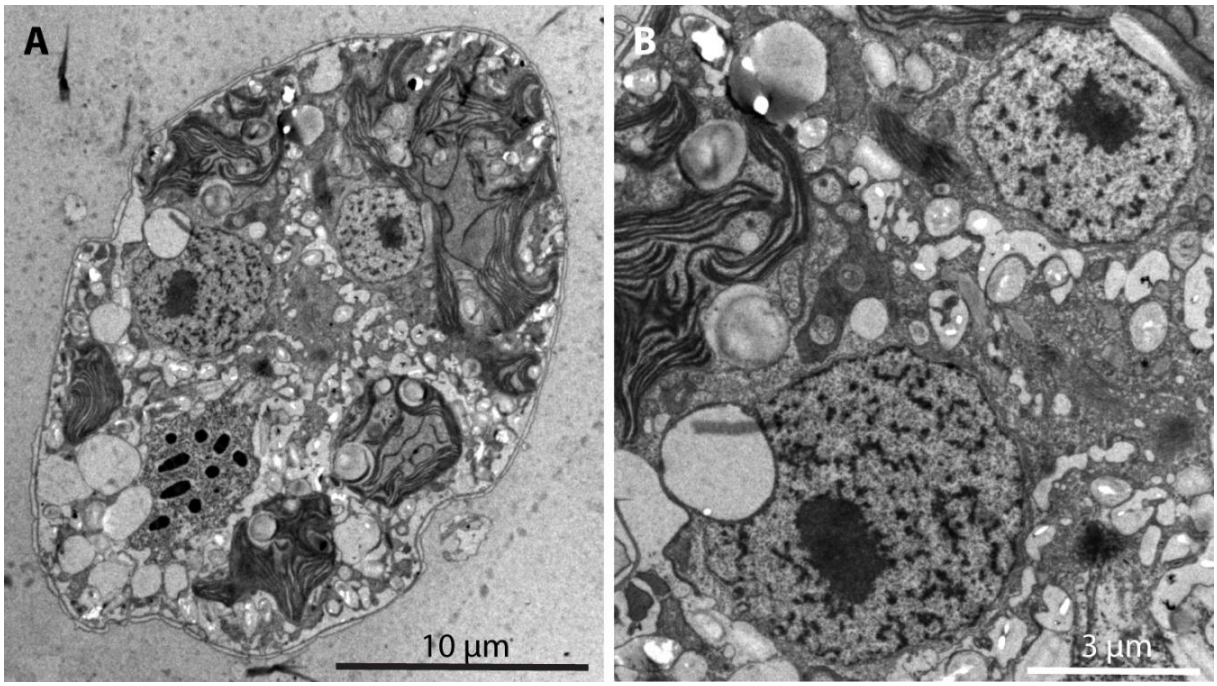

Figure 20. Ultrastructure of *N. aeruginosum/acidotum* with intermediate kleptoplasts derived from *Chroomonas* sp. CCAC 1481. **A** Longitudinal section through a cell showing two enlarged kleptokarya. **B** Detailed image of the two kleptokarya shown in **A**.

Intermediate kleptoplasts were stable for 3-4 weeks. Inheritance of intermediate kleptoplasts and kleptokarya occured equally to both daughter cells (Fig. 18C). No further enlargement of intermediate kleptoplasts was observed after distribution to daughter cells. No additional prey was ingested by cells with enlarged kleptokarya. The stability of the kleptokarya in the intermediate condition was not studied.

3.3.12 Variability of the cell shape within the established strains of N. *aeruginosum/acidotum*

Two species, *N. aeruginosum* and *N. acidotum* were distinguished in the past based on the shape of their hypocone. *N. aeruginosum* was described as having a round antapex whereas *N. acidotum* was described as having a pointed antapex (1.5). However, the establishment of stable cultures showed that this feature alone cannot be used for a differentiation of both species. To get an idea about the diversity of these species, in total six cultures were established from six different locations within Germany. All cultures were made axenic and compared under the same conditions. The locations comprised the regions Dahlem, Haitz Gelnhausen, Wahner Heide, Marmagen, Recke Steinfurt (Großes Heiliges Meer) and Krefeld. As the strain from Dahlem was already presented above, just the additional 5 strains are presented here. Each strain was observed in the U-shaped condition fed with the *Chroomonas* sp. strain CCAC4832 and in the granular condition fed with the *Chroomonas* sp. strain CCAC 3453. All strains investigated were able to form both types of kleptoplasts. However, small morphological differences were observed between the strains when grown with the two *Chroomonas* strains. The strain N.a. Haitz always contained a pointed antapex with U-shaped as well as granular kleptoplasts. Cells with U-shaped kleptoplasts gave the impression to be slightly larger than cells with granular kleptoplasts (no measurements were done) as also observed for the strain from Dahlem. The pointed shape of cells with U-shaped kleptoplasts was more distinct than of cells with granular kleptoplasts that appeared to have a more roundish hypocone. This strain showed the typical morphology described for *N. acidotum* (formerly *Gymnodinium acidotum*) (Fig. 21A). The two strains from Wahner Heide and Marmagen resembled the strain from Dahlem that is in the focus of this study. The shape of the hypocone with U-shaped kleptoplast is just slightly pointed, much less distinct compared to the strain N.a. Haitz and in addition, also cells with round antapex were observed (Fig. 3D). Also these three strains became more roundish in their shape in the granular state (Figs. 3C, 21B+C). All three strains resembled in the U-shaped situation an intermediate between *N. aeruginosum* and *N. acidotum*. In contrast, the two strains N.a. HLM and N.a. Krefeld never showed a pointed antapex. They appeared more roundish in both situations than the other strains and resembled the typical morphology for *N. aeruginosum* (formerly *Gymnodinium aeruginosum*) (Fig. 21D+E).

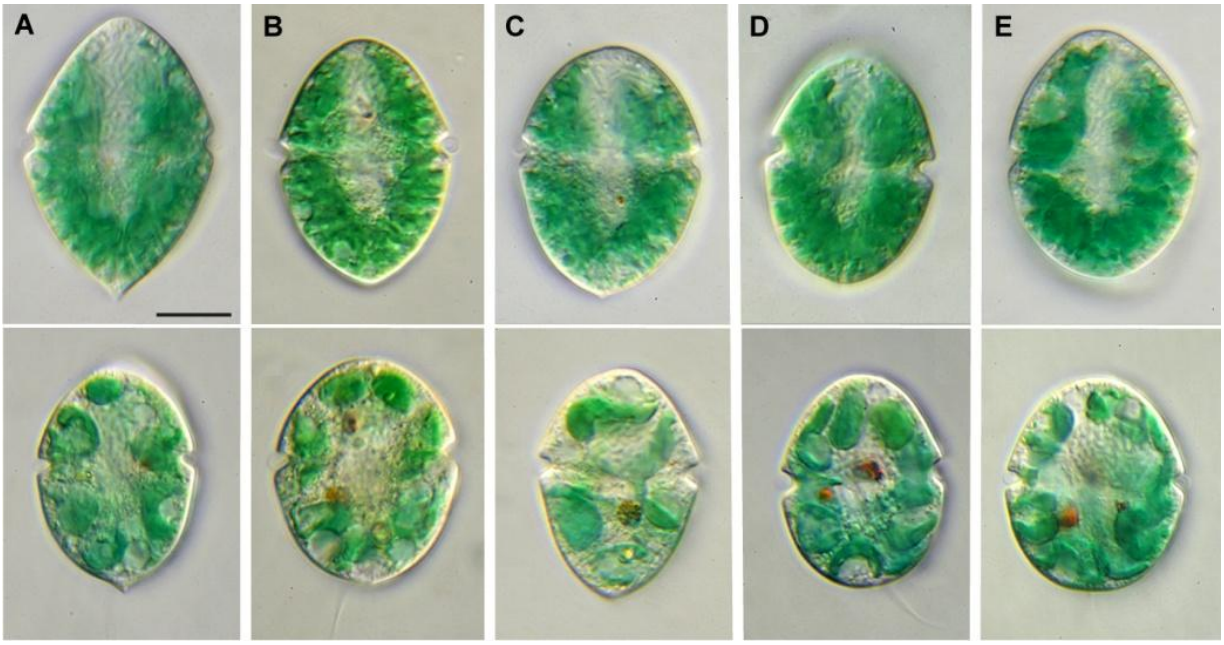

Figure 21. Additional strains of *N. aeruginosum/acidotum* established during this study from different locations within Germany with U-shaped kleptoplast (upper images) derived from *Chroomonas* sp. CCAC 4832 and granular kleptoplasts (lower images) derived from *Chroomonas* sp. CCAC 3453. **A** Strain from Haitz Gelnhausen (N.a. Haitz). **B** Strain from Wahner Heide Fuchskaule (N.a. WH). **C** Strain from Marmagen (N.a. Marmagen). **D** Strain from the Großes Heiliges Meer in Hopsten Steinfurt (N.a. HLM). **E** Strain from Krefeld (N.a. Krefeld) (bar in **A** also applies to **B**-**E** = 10µm).

As the strain N.a. Dahlem that is referred to as *N. aeruginosum/acidotum* in this study could not be clearly classified neither as *N. aeruginosum* nor as *N. acidotum* its ultrastructure was further investigated using the SEM. Cells with U-shaped kleptoplasts showed a slightly pointed antapex (Fig. 22A+C) whereas cells with granular kleptoplasts always showed a round antapex (Fig. 22B). Other differences were not observed between dinoflagellates with U-shaped and granular kleptoplasts. However small granular structures were present on the surface of the dinoflagellate cells (Fig. 22D). All cells investigated contained an apical groove in anticlockwise direction typical for members within the genus *Nusuttodinium* (Fig. 22E). The ultrastructure of the cryptophyte strains offered as prey revealed that the strain CCAC 4832 contained rectangular periplast plates whereas the strain CCAC 3453 contained hexangular periplast plates (Fig. 22A+B small insertions).

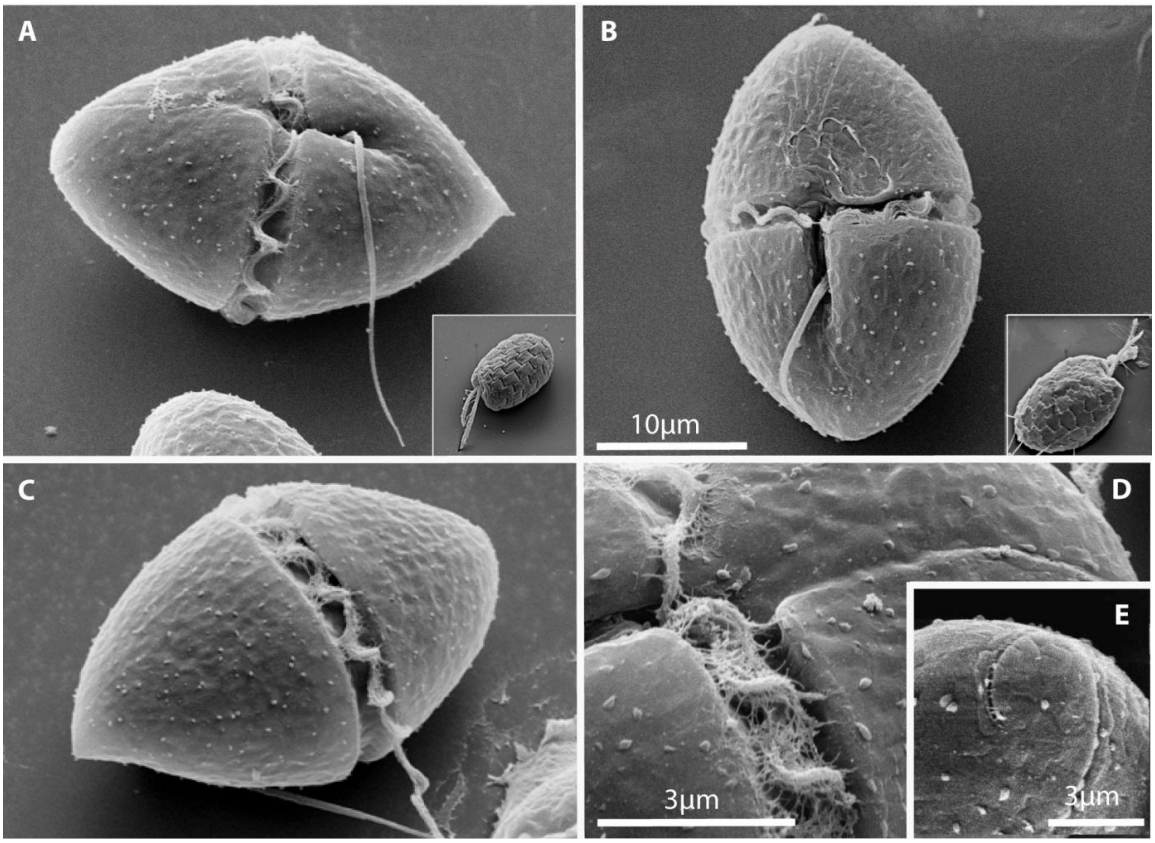

Figure 22. SEM micrographs of *N. aeruginosum/acidotum* strain N.a. Dahlem. **A** Cell with U-shaped kleptoplast derived from *Chroomonas* sp. CCAC 4832 often showing a pointed antapex. Small image: *Chroomonas* sp. CCAC 4832. **B** Cell with granular kleptoplasts derived from *Chroomonas* sp. CCAC 3453 usually showing a round antapex. Small image: *Chroomonas* sp. CCAC 3453. **C** Dorsal view of a cell with a pointed antapex. **D** Detailed image of the cingulum and the cell surface. **E** Apical groove typical for the genus *Nusuttodinium*. Images **D** and **E** were taken from a cell with a pointed antapex (bar in **B** also applies for **A** and **C**).

3.3.13 Life cycle of *N. aeruginosum/acidotum*

N. aeruginosum/acidotum showed a very complex life history that strongly depended on the prey availability and the type of prey ingested. In total four different ways were possible whereas three ways were linked to *Chroomonas* strains of subclade 4 and one was linked to strains of subclade 3 (Fig. 23).

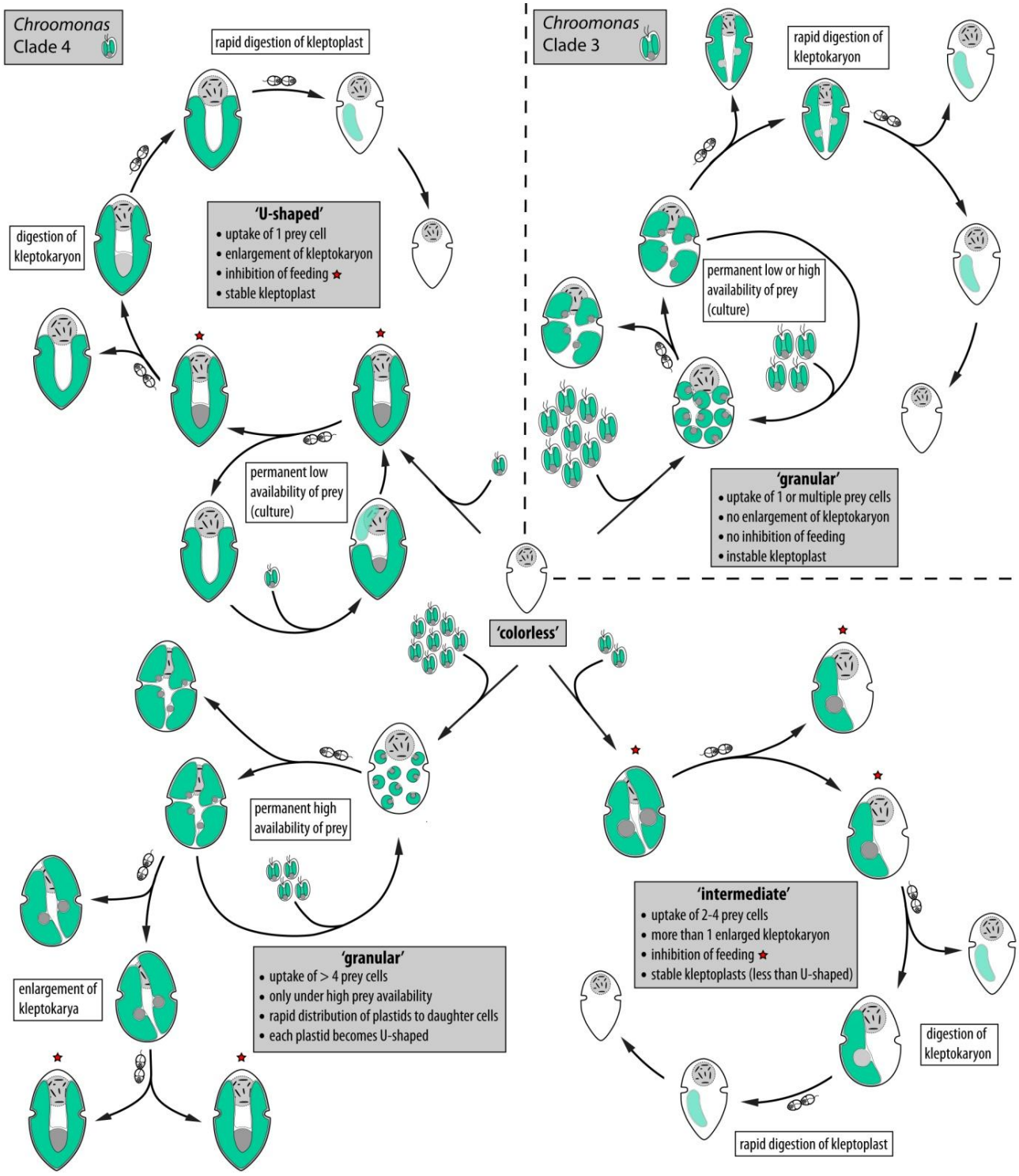

Figure 23. Life cycle of *N. aeruginosum/acidotum*. Two celled chains represent division.

3.4 *Nusuttodinium amphidinoides* (formerly *Amphidinium amphidinoides* Schiller, 1932)

Stable axenic cultures of the freshwater species *Nusuttodinium amphidinoides* were established during this study to compare its kleptoplastic situation to *N. aeruginosum/acidotum*. The only detailed study performed on this organism was published in 1985 by Wilcox and Wedemeyer. Light microscopical as well as ultrastructural studies were performed on natural samples and revealed that the kleptoplasts were associated neither with a kleptokaryon nor with a nucleomorph. Moreover the number of enveloping membranes was observed to be three whereby the authors hypothesized that the plastids are permanent. In total 10 cultures could be established from different locations from which nine also grew in axenic culture (see Table 6 for the strain list). However, the strain N. amph. HLM from a small fishpond near the Großes Heiliges Meer, Recke Steinfurt, Germany did not divide under axenic conditions. Another strain, N. amph. small epicone CCAC 5709 differs so much from the original description by its morphology as well as behavior that it is treated in a separate chapter (3.5). Phylogenetically, both types belong to the genus *Nusuttodinium* and form the sister group of *N. aeruginosum/acidotum*. Both types are very closely related despite the big differences in behavior and morphology (Luther, 2015).

3.4.1 Feeding experiments and observed types of kleptoplasts in *Nusuttodinium amphidinoides*

In natural samples *N. amphidinoides* was always motile and usually contained 4-7 small kleptoplasts of blue-green color. The kleptoplasts were round or slightly elongated and distributed throughout the cell (Fig. 24A). Cells were 15 – 26 µm long and 11 – 21 µm wide. Starvation of this dinoflagellate for 14-21 days resulted in a loss of kleptoplasts and a decrease in cell size (Fig. 24 B+C). Resting cysts with multiple brown inclusions were formed in old cultures (Fig. 24 D).

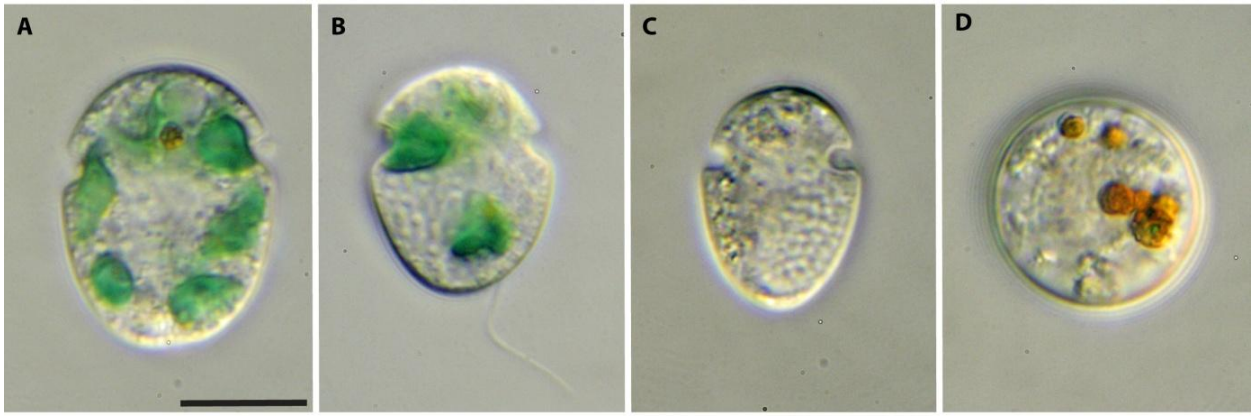

Figure 24. Loss of kleptoplasts in the dinoflagellate *Nusuttodinium amphidinoides* strain N. amph. Römerweiher. **A** Cell permanently well fed with the strain *Chroomonas* sp. CCAC 0060. **B** Cell starved for 14 days. **C** Cell starved for 21 days. **D** Resting cyst observed in old cultures (bar in **A** also applies to **B**-**D** = 10µm).

The motility of *N. amphidinoides* within a culture was observed to strongly depend on the availability of prey. High prey availability resulted in cells densely packed with ingested prey that were mostly immobile at the bottom of the culture vessel whereas low prey availability resulted in high motility. Feeding experiments showed that *N. amphidinoides* was able to acquire its kleptoplasts from a broader spectrum of prey organisms compared to *N. aeruginosum/acidotum*. *N. amphidinoides* accepted all *Chroomonas* strains tested (Tab. 7). Also strains belonging to subclade 1 that were not ingested by *N. aeruginosum/acidotum* were accepted as prey (Fig. 25B). In addition to *Chroomonas*, also the genera *Cryptomonas* and *Rhodomonas* were accepted (Fig. 25 A+C). Prey was always ingested through the antapex of the dinoflagellate sometimes resulting in cells with two different colors depending on the previously ingested prey (Fig. 25C). However, the range of potential prey organisms was limited for the three cryptophyte genera *Chroomonas*, *Cryptomonas* and *Rhodomonas* (Tab. 14).

Table 14. Strains of the cryptophyte genera *Cryptomonas* and *Rhodomonas* tested as potential source of kleptoplasts in *N. amphidinoides* strain N. amph. Römerweiher (+ = ingested, - = not ingested).

Strain	Species	Ingested
CCAC 0006	*Cryptomonas curvata*	+
CCAC 0018	*Cryptomonas erosa*	-
CCAC 0031	*Cryptomonas obovoidea*	+
CCAC 0032	*Cryptomonas pyrenoidifera*	+
CCAC 0064	*Cryptomonas ovata*	-
CCAC 0104	*Cryptomonas massonii*	-
CCAC 0107	*Cryptomonas lundii*	-
CCAC 0108	*Cryptomonas gyropyrenoidosa*	-
CCAC 0113	*Cryptomonas borealis*	-
CCAC0172	*Cryptomonas tetrapyrenoidosa*	+
CCAC 0187	*Cryptomonas phaseolus*	+
CCAC 0191	*Cryptomonas loricata*	+
CCAC 0192	*Cryptomonas erosa*	-
CCAC 2195B	*Cryptomonas* sp.	+
CCAC 2290B	*Cryptomonas* sp.	+
CCAC 2504B	*Cryptomonas* sp.	+
CCAC 2807B	*Cryptomonas* sp.	+
M 2922	*Rhodomonas* sp.	+
CCAC 1479B	*Rhodomonas* sp.	+

Algae belonging to other groups were not ingested (data not shown). Moreover the uptake of prey was size limited. Just prey smaller than the dinoflagellate was ingested.

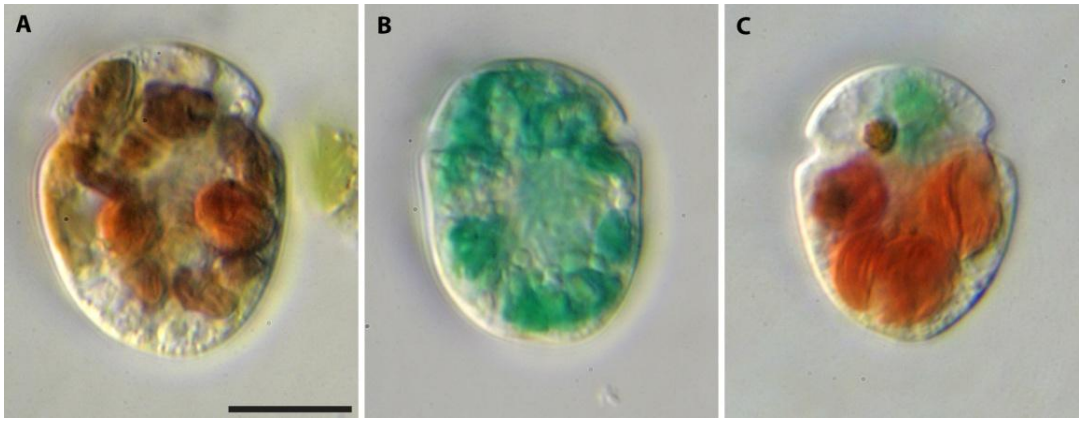

Figure 25. Variety of kleptoplasts in *N. amphidinoides* strain N. amph. Römerweiher. **A** Brown kleptoplasts derived from *Cryptomonas* sp. CCAC 2195B. **B** Blue-green kleptoplasts derived from *Chroomonas pochmannii* UTEX 2779. **C** Red kleptoplasts derived from *Rhodomonas* sp. M0194. The prey is ingested through the antapex. The blue-green kleptoplast in the apical part of the cell is a rest of the former kleptoplasts derived from *Chroomonas* sp. CCAC 0060 (bar in **A** also applies to **B**-**C** = 10µm).

3.4.2 Kleptoplast stability in *Nusuttodinium amphidinoides*

The stability of kleptoplasts in *N. amphidinoides* strain *N. amph*. Römerweiher was tested as it was also done for *N. aeruginosum/acidotum*. As *N. amphidinoides* ingested all strains of *Chroomonas* tested, kleptoplasts derived from three different subclades were tested (subclades 1, 3 and 4). For each subclade one strain was chosen (*Chroomonas pochmannii* UTEX 2779 for subclade 1, *Chroomonas* sp. CCAC 3453 for subclade 3 and *Chroomonas* sp. CCAC 4832 for subclade 4). Moreover the stability of kleptoplasts derived from three different species of *Cryptomonas* was tested (*Cryptomonas pyrenoidifera* CCAC 0032, *Cryptomonas loricata* CCAC 0191 and *Cryptomonas* sp. CCAC 2504B). Kleptoplasts derived from the genus *Rhodomonas* were not tested as no stable co-culture could be established. In total 10 cells derived from permanent co-cultures were starved until they lost their kleptoplasts. In all cases cells lost their kleptoplasts within 14 days. No differences in stability were observed between kleptoplast derived from *Chroomonas* or *Cryptomonas*. Cells just divided once during starvation. This experiment showed that kleptoplasts in *N. amphidinoides* were less stable than U-shaped kleptoplasts in *N. aeruginosum/acidotum* but had a similar stability like the granular kleptoplasts. Wilcox and Wedemeyer (1985) did not find any kleptokaryon within the cells they examined. The presence of a kleptokaryon was therefore tested by staining cells from a permanent co-culture with DAPI. No kleptokaryon could be found using this method (Fig. 26A+B). However, staining of the strain N. amph. HLM that just survived in the

presence of bacteria revealed the presence of bacteria-like structures within the epicone indicating uptake (Fig. 26C+D).

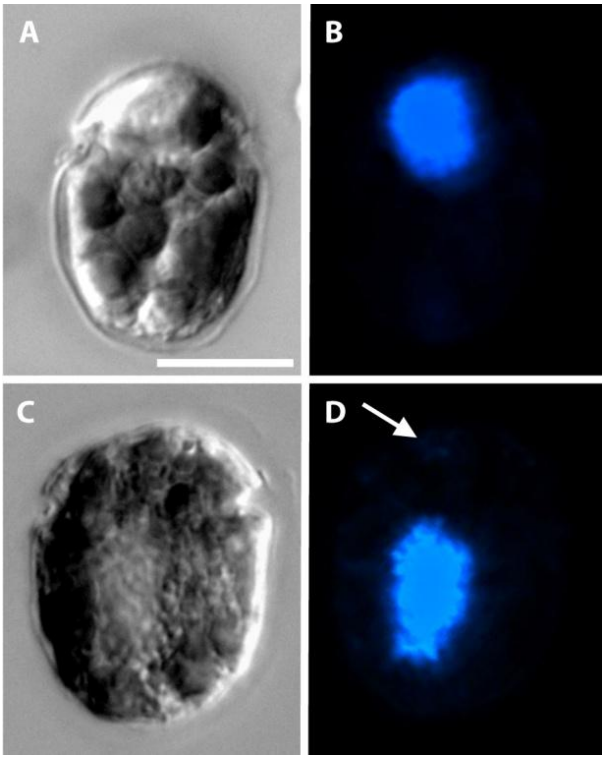

Figure 26. DAPI staining of *N. amphidinoides* well fed with *Chroomonas* sp. CCAC 0060. **A-B** *N. amphidinoides* strain N. amph Römerweiher. No kleptokarya are visible in **B**. **C-D** *N. amphidinoides* strain HLM. No kleptokarya are visible in **D**. White arrow: Bacteria-like structures within the epicone (bar in **A** also applies to **B-D** = 10µm).

3.4.3 Scanning Electron Microscopy (SEM) of *Nusuttodinium amphidinoides*

The ultrastructure of *N. amphidinoides* was investigated using the SEM. As no differences in the shape of the cells were observed in the presence of different prey strains, an already established co-culture with *Chroomonas* sp. CCAC 0060 was investigated. *N. amphidinoides* showed a smooth cell surface that contained small blister-like structures that were also observed for N. *aeruginosum/acidotum* (Fig. 27 A-E). A deep furrow was present on the ventral side of the epicone (Fig. 27 A-C). An apical groove in anticlockwise direction typical for the genus *Nusuttodinium* was observed (Fig. 27 C, E).

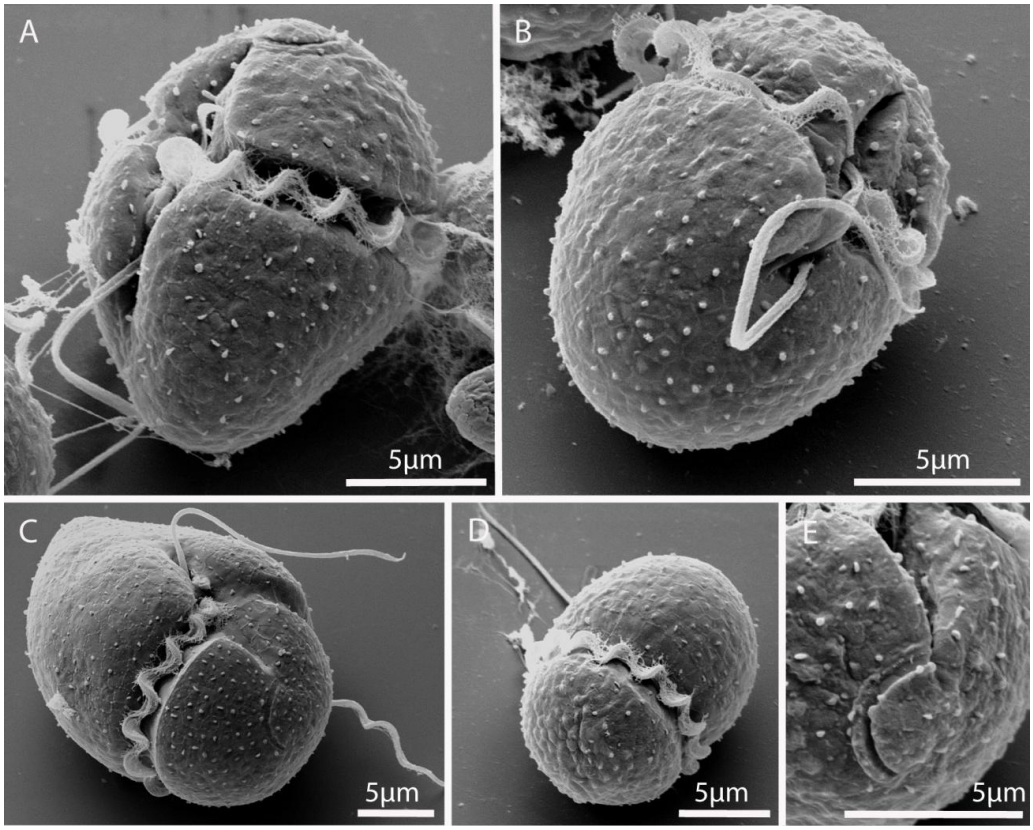

Figure 27. Scanning electron micrographs (SEM) showing the ultrastructure of *N. amphidinoides* strain N. amph. Römerweiher. **A-C** Ventral view of cells. **D** Dorsal view. **E** Detailed image of the apex of the cell showing the presence of an apical groove in an anticlockwise direction.

3.5 *Nusuttodinium amphidinoides* small epicone CCAC 5709

As already mentioned in 3.4, two different types of *N. amphidinoides* were established in culture during this study. Whereas one type could be clearly identified as *N. amphidinoides* by its morphology as well as phylogenetic position, the other type was not found in literature. Due to its very close phylogenetic relationship to *N. amphidinoides* and its small epicone it is called '*Nusuttodinium amphidinoides* small epicone' in this study although its cell shape and behavior differ from *N. amphidinoides*. Studies on this dinoflagellate were partially done in co-operation with Isabell Luther during her Bachelor Thesis. Data abstracted from her Bachelor Thesis are mentioned as those.

3.5.1 Feeding experiments and observed types of kleptoplasts in *Nusuttodinium amphidinoides* small epicone

N. amphidinoides small epicone is characterized by multiple small kleptoplasts distributed within the hypocone of the cell. The epicone is comparatively small compared to the hypocone and just rarely contains kleptoplasts (Fig. 28A). Cells within a permanent co-culture with *Chroomonas* sp. CCAC 4832 as prey were 17.93 ± 1.72µm long, 17.50 ± 2.60µm wide and 15.18 ± 1.30µm deep (Luther, 2015).

Unter permanent prey availability cell division took place during the dark phase and was characterized by formation of two celled chains in which the epicone of the posterior cell was connected to the hypocone of the anterior cell (Fig. 28B). Capture of prey strongly differed from *N. amphidinoides* and is therefore treated in a separate chapter also due to its complexity (3.5.2). Ingestion of prey occurred through the hypocone (Fig. 28C). Kleptoplasts derived from the strain *Chroomonas* sp. CCAC 4832 were usually stable for up to 14 days but did not change in their morphology during starvation (Fig. 28D+E). Old cultures were characterized by the presence of resting cysts (Fig. 28F).

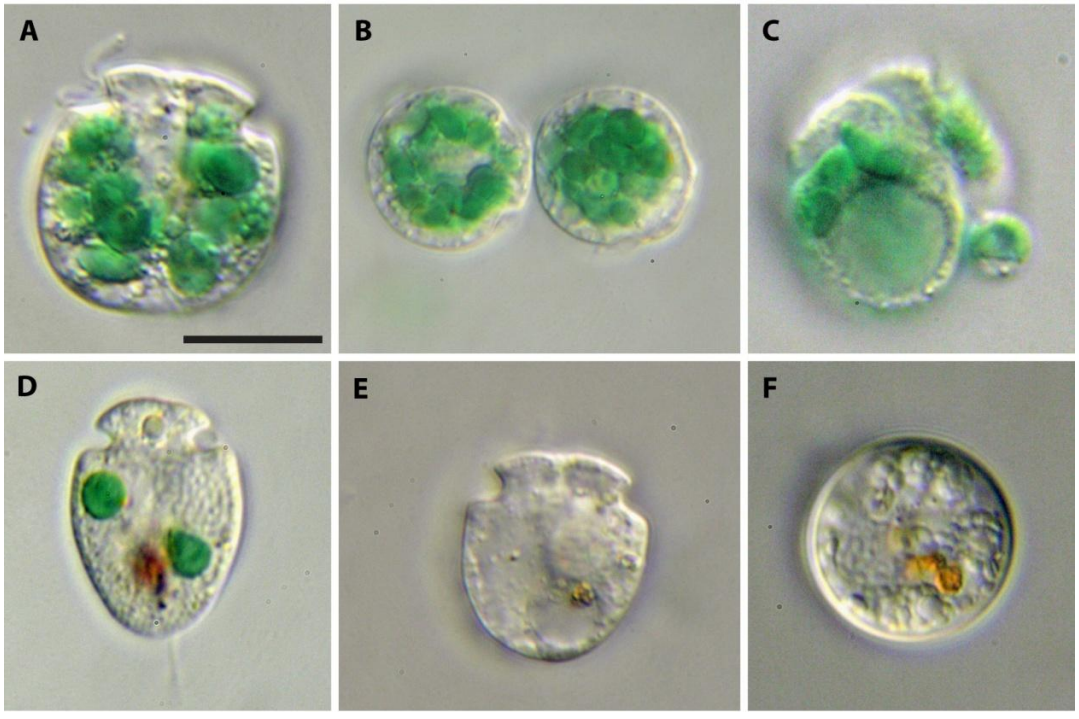

Figure 28. Loss of kleptoplasts in the dinoflagellate *N. amphidinoides* small epicone strain CCAC 5709. **A** Cell well fed with *Chroomonas* sp. CCAC 4832. **B** Two celled chain during cell division. The apex of the posterior cell is connected to the antapex of the anterior cell. **C** Ingestion of *Chroomonas* sp. CCAC 4832 through the antapex. **D** Cell starved for 10 days. **E** Cell starved for 14 days. **F** Resting cyst usually observed in old cultures (bar in A also applies for **B-F** = 10µm). The images **C** and **F** were taken in co-operation with Isabell Luther.

Feeding experiments showed that the same cryptophyte strains were ingested as also observed for *N. amphidinoides*. In addition, the colorless cryptophyte species *Cryptomonas paramecium* CCAC 1634B was tested. It was also ingested and resulted in colorless cells containing colorless kleptoplasts (Fig. 29D). However, *N. amphidinoides* small epicone showed a broader food spectrum compared to *N. amphidinoides* as it also ingested prey belonging to other algal groups than cryptophytes. It also ingested members of gold algae, haptophytes and a cell wall deficient mutant of *Chlamydomonas* (green algae) (Tab. 15). Just prey without solid cell wall was ingested. Moreover, ingestion was limited to prey smaller or equal in size to the dinoflagellate.

Table 15. Strains of algae belonging to diverse groups tested as potential source for kleptoplasts in *N. amphidinoides* small epicone strain CCAC 5709 (+ = ingested, - = not ingested). The data were acquired in co-operation with Isabell Luther. The table is adopted from (Luther, 2015).

Strain	Species	Group	Ingested
CCAC 2944B	*Synechococcus sp.*	Cyanobacteria	-
M 1781	*Synechocystis sp.*		-
SAG 45.84	*Cyanophora paradoxa*	Glaucophyta	-
CCAC 2322B	*Cyanoptyche sp.*		-
SAG 933-7	*Ochromonas danica*	Gold algae	-
CCAP 909/9	*Chromulina chionophila*		+
CCAC 1889B	*Chrysochromulina parva*	Haptophyta	+
CCAC 0933B	*Prymnesium sp.*		-
CCAC 2682B	*Gomphonema sp.*	Diatoms	-
CCAC 1772	*Navicula sp.*		-
CCAC 0128	*Lepocinclis tripteris*	Euglenophyta	-
CCAC 0003	*Euglena mutabilis*		-
CCAC 3520B	*Kirchneriella sp.*	Chlorophyta	-
SAG 2.88	*Spermatozopsis exsultans*		-
SAG 73.72	*Chlamydomonas reinhardtii*		-
SAG 83.81	*Chlamydomonas* cell wall deficient mutant		+
CCAC 0105	*Nephroselmis olivaceae*		-
CCAC 0207	*Mesostigma sp.*	Streptophyta	-

As *N. amphidinoides* is originally colorless, it adapts the color of its taken up prey (Fig. 29).

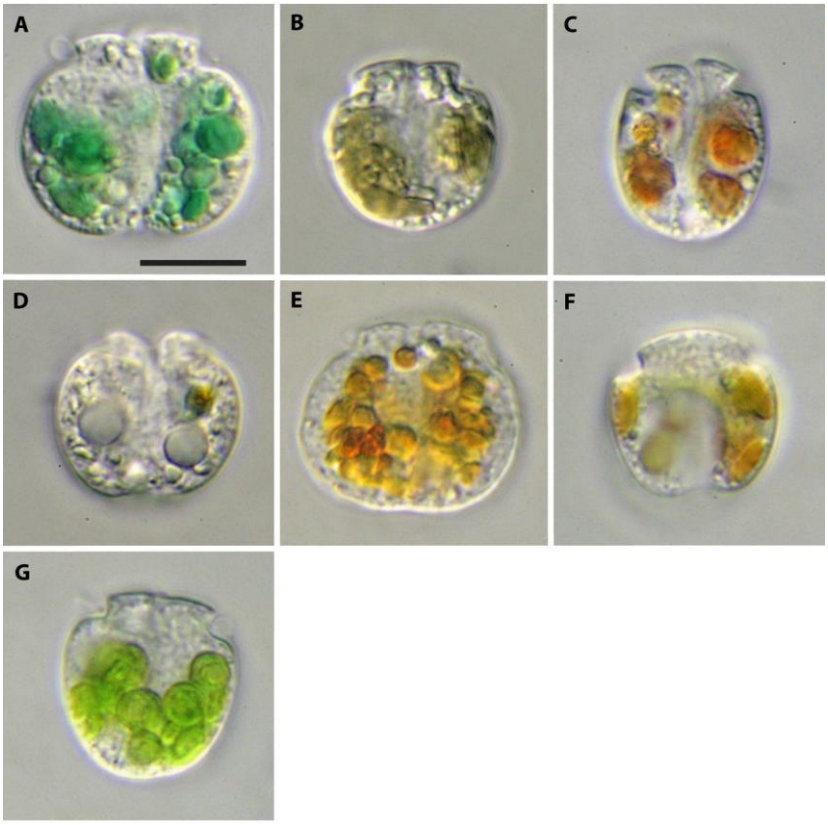

Figure 29. Variety of kleptoplasts observed in *N. amphidinoides* small epicone strain CCAC 5709. **A** Blue-green kleptoplasts derived from *Chroomonas* sp. CCAC 3453. **B** Brown kleptoplasts derived from *Cryptomonas* sp. CCAC 0104. **C** Red kleptoplasts derived from *Rhodomonas* sp. CCAC 1480B. **D** Colorless kleptoplasts derived from *Cryptomonas paramecium* CCAC 1634B. **E** Gold kleptoplasts derived from *Chrysochromulina* sp. CCAC 1889B. **F** Gold kleptoplasts derived from *Chromulina chionophila* CCAP 909/9. **G** Green kleptoplasts derived from the cell wall deficient *Chlamydomonas* mutant SAG 38.81 (bar in **A** also applies for **B-G** = 10µm). The images **A-G** were taken in co-operation with Isabell Luther.

3.5.2 Prey capture and behavior of *N. amphidinoides* small epicone

The behavior of *N. amphidinoides* small epicone within a culture strongly differed from that of *N. amphidinoides*. A specific behavior for this species was the attachment of the longitudinal flagellum to the bottom of the culture vessel or another substrate and a rotation around the own axis, sometimes with a change in direction. However, also free swimming cells were observed, especially under low prey availability. Swimming cells showed the typical rotation caused by the movement of both flagella. The uptake of prey occurred during the attached state. The rotation behavior observed seemed to increase the probability to find prey organisms. As soon as a prey organism came in contact with the surface of the epicone, it became immobilized. At this stage the rotation behavior of the dinoflagellate stopped. In the

next step the prey was transported via an unknown mechanism through the longitudinal furrow to the antapex of the dinoflagellate and ingested through phagocytosis (Fig. 30).

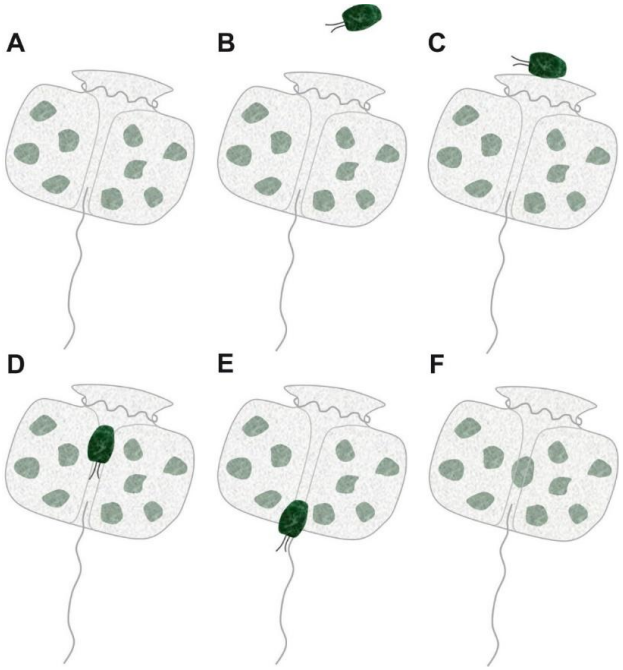

Figure 30. Schematic drawing of the prey uptake mechanism observed for *N. amphidinoides* small epicone strain CCAC 5709. **A** The dinoflagellate is attached to the substrate via the longitudinal flagellum. **B-C** A prey cell touches the epicone of the dinoflagellate and becomes immobilized. **D-E** The prey is transported through the longitudinal furrow to the antapex of the dinoflagellate and ingested (**F**). The drawing is adopted from Luther (2015).

3.5.3 Kleptoplast stability in *Nusuttodinium amphidinoides* small epicone

N. amphidinoides small epicone was able to ingest prey of diverse algal groups ranging from the cryptophyte genera *Chroomonas*, *Cryptomonas* and *Rhodomonas* to gold algae, haptophytes and green algae. Accordingly, the question about the stability of such diverse kleptoplasts arises. Kleptoplasts were not or just little modified (Fig. 29). Kleptoplasts derived from *Chroomonas* sp. CCAC 4832, a strain that is also used to maintain the culture of this dinoflagellate were usually stable for up to 14 days. Dinoflagellates divided just once in average during this time. Cell division always resulted in a 1:1 distribution of the kleptoplasts (Luther, 2015). No change of the kleptoplasts in their shape was observed during the starvation process (Fig. 28D). To get an impression whether the cryptophyte nuclei are retained by the dinoflagellate, DAPI staining was performed on cells that were permanently well fed with prey. No signal except for the dinoflagellate nucleus was observed by applying this method (Fig. 31).

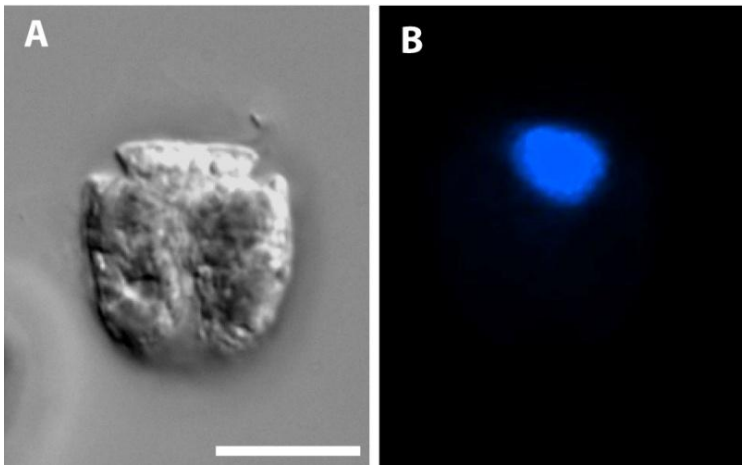

Figure 31. DAPI staining of *N. amphidinoides* small epicone strain CCAC 5709 well fed with *Chroomonas* sp. CCAC 4832 (**A-B**). No kleptokarya are visible in **B** (bar in **A** also applies to **B** = 10µm).

Starvation of cells fed with *Chrysochromulina parva* CCAC 1889B, *Chromulina chionophila* CCAP 909/9 and *Chlamydomonas* cell wall deficient mutant SAG 83.81 resulted in a complete loss of kleptoplasts already after 10 days. Stability of kleptoplasts derived from *Cryptomonas* and *Rhodomonas* was not tested.

3.5.4 Ultrastructure of *Nusuttodinium amphidinoides* small epicone

The ultrastructure of *N. amphidinoides* small epicone was investigated using both, SEM and TEM. *N. amphidinoides* small epicone was much more sensitive compared to *N. amphidinoides* during SEM preparations shown by some indentations of the cell surface as well as secretion of mucilage visible as net-like structures in the preparation. In general, the surface of this dinoflagellate was smooth except for a few blister-like structures that were also present in *N. amphidinoides* and *N. aeruginosum/acidotum* (Fig. 32 A-E). The ventral furrow was very distinct and proceeded deeply into the epicone (Fig. 32 A, B, C, E). An apical groove in anticlockwise direction was present (Fig. 32E). One dinoflagellate cell could be observed during the uptake of cryptophyte prey showing that the uptake occurs through the antapex. The cryptophyte cell sticked with its ventral side to the sulcus of the dinoflagellate (Fig. 32 F). As already indicated by Figure 28B, two celled chains were formed during cell division in which the apex of the posterior cell sticks to the antapex of the anterior cell (Fig. 32G). Resting cysts were spherical and showed a smooth surface (Fig. 32H).

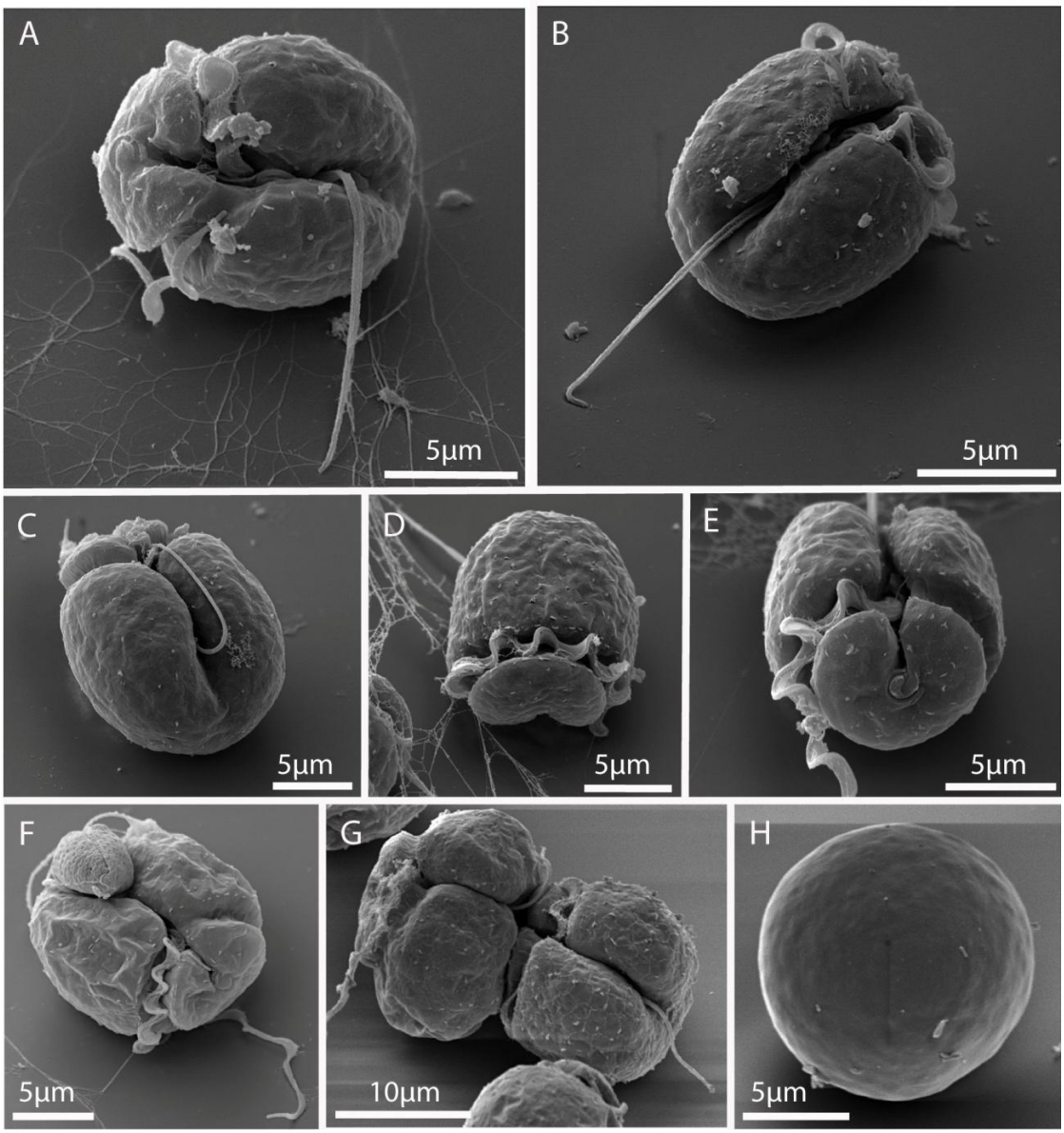

Figure 32. Scanning electron micrographs (SEM) showing the ultrastructure of *N. amphidinoides* small epicone strain CCAC 5709. **A-C** Ventral view of cells. **D** Dorsal view. **E** Apical view showing the presence of an apical groove in an anticlockwise direction. **F** Ingestion of prey through the antapex. **G** Two celled chain during cell division. **H** Resting cyst. The images **F-H** were taken in co-operation with Isabell Luther.

To get an idea about the structure of the kleptoplasts as well as the principle of the very characteristic prey immobilization- and uptake mechanism, *N. amphidinoides* small epicone was investigated using the TEM. Therefore a co-culture established with the prey strain *Chroomonas* sp. CCAC 4832 was used. The culture used was 4 days old (after inoculation) and all dinoflagellates were filled with blue-green kleptoplasts. The general ultrastructure was typical for naked dinoflagellates showing the absence of thecal cellulosic plates within the alveoli and a pusulum probably involved in osmotic regulation. The cell periphery was characterized by the presence of multiple electron permeable vacuoles filled with mucilage

and probably causing the problems during SEM preparation. Numerous kleptoplasts as well as a digestion vacuole were observed within the hypocone of the cell (Fig. 33A). The kleptoplasts were spherical and their ultrastructure was the typical of cryptophyte plastids with thylacoids in stacks of 2. Neither additional organelles nor cytoplasm of cryptophyte origin was found to be associated with the kleptoplasts (Fig. 33B). The epicone of *N. amphidinoides* small epicone was examined for the presence of structures that might be responsible for the immobilization of the prey after contact. Small electron dense structures directly under the surface of the epicone were found. Most of them were positioned between alveoli. Their structure resembled those of trichocysts (Fig. 33 C-E). However, these structures were not found at the periphery of the hypocone.

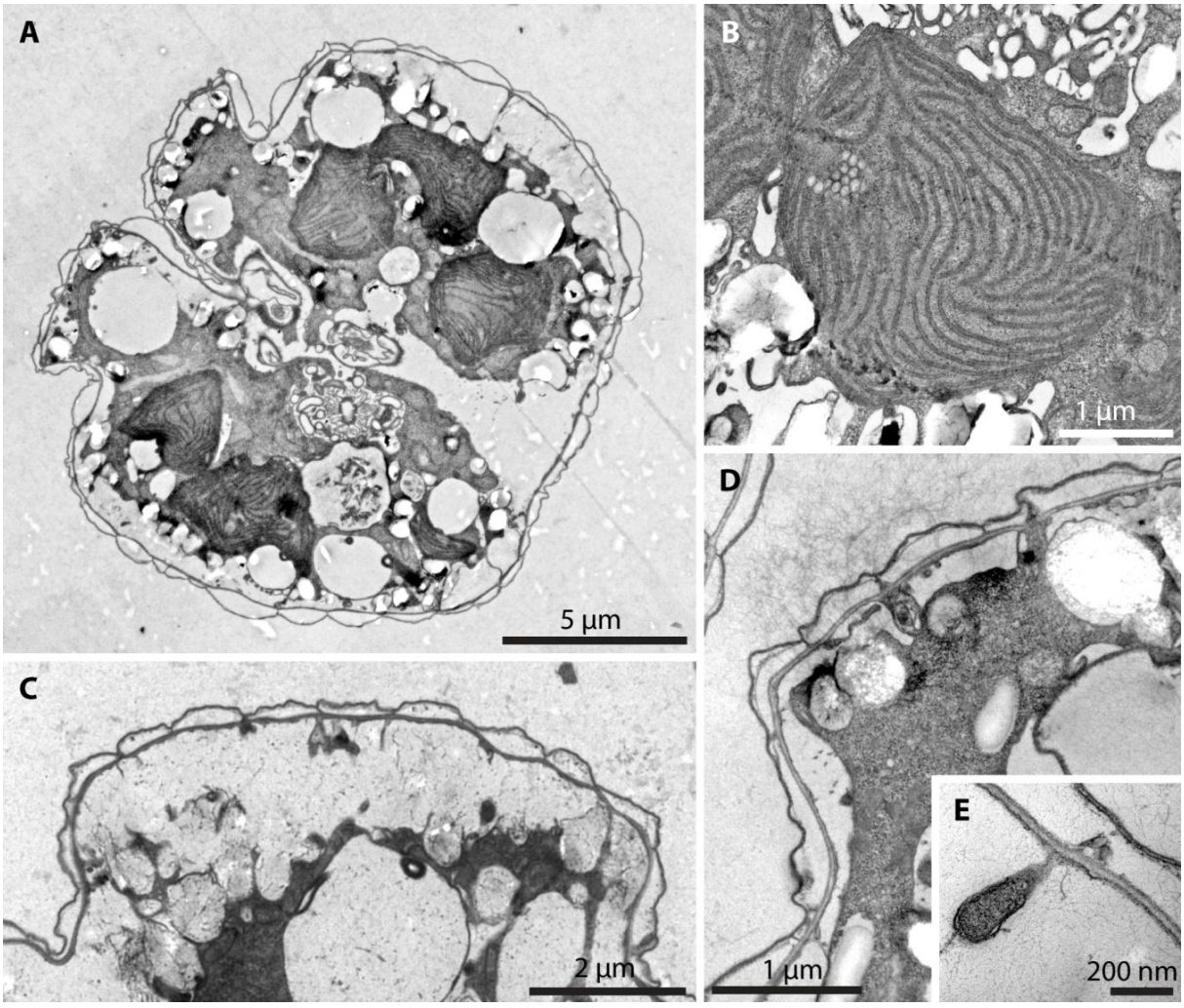

Figure 33. Ultrastructure of *N. amphidinoides* small epicone strain CCAC 5709 with granular kleptoplasts derived from *Chroomonas* sp. CCAC 4832. **A** Longitudinal section of a cell showing the granular kleptoplasts distributed throughout the hypocone. **B** Detailed image of a granular kleptoplast showing the typical organization of cryptophyte thylacoids in stacks of two. **C** Longitudinal section through the epicone showing the presence of associated electron

dense structures probably involved in prey capture. **D** Detailed image of a part of the epicone. **E** Trichocyst-like structure within the epicone.

3.6 Extraction of total RNA for future transcriptomic research

To understand the molecular basis for the different stabilities and morphologies of the kleptoplasts observed during this study, RNA should be extracted and transcriptomes should be sequenced from all stages observed for *N. aeruginosum/acidotum* and also from *N. amphidinoides* small epicone. Mass cultures of dinoflagellate-cryptophyte co-cultures as well as the pure cryptophytes cultures were grown for extraction of total RNA (Fig. 34A). A prerequisite that had to be fulfilled for this project was a successful separation of huge numbers of dinoflagellates from their cryptophyte prey. Personal observations showed that most of the dinoflagellates were always located close to the bottom of the culture vessel whereas the cryptophytes were also located within the water column. This observation was used as a basis for the separation of both organisms. Filling of the dinoflagellate-cryptophyte co-culture into cylindric vessels resulted in a sedimentation of the dinoflagellates to the bottom within a few minutes depending on the height of the vessel whereas most of the cryptophyte cells stayed within the column. The dinoflagellate fraction was taken and washed again in the same way in sterile culture medium until no cryptophyte cells were found any more (Fig. 34B). Successfully washed dinoflagellates are shown in Figure 3A.

Figure 34. Preparations for RNA extraction of kleptoplastic dinoflagellates. **A** Mass cultures in non aerated culture vessels with a high surface to volume ratio. **B** Separation of dinoflagellates from cryptophyte prey via the sedimentation method.

Total RNA was extracted successfully from all cultures grown. However, the pattern of the RNA samples after gel electrophoresis differed between the samples (Fig. 35). Sequencing of the samples was done by the company BGI (China). The sequenced transcriptomes were not analyzed during this study.

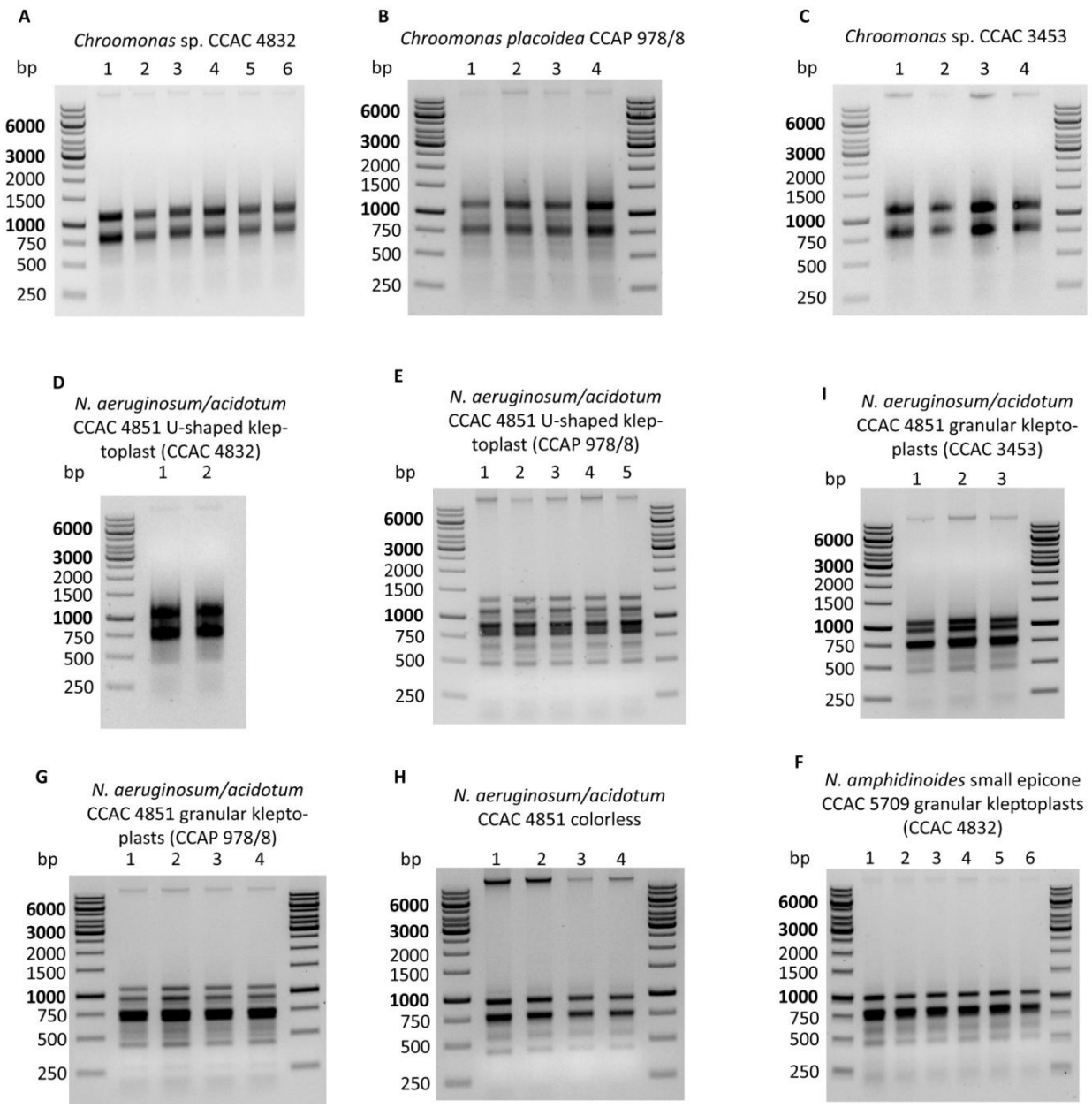

Figure 35. Images of Agarose gel electrophoresis of the extracted RNA samples of the cryptophyte prey strains (**A-C**) and the kleptoplastic dinoflagellates grown under different conditions (**D-F**).

4. Discussion

4.1 The identity of *Nusuttodinium aeruginosum* and *Nussuttodinium acidotum*

Many studies related to the two dinoflagellate species *Nusuttodinium aeruginosum* and *Nusuttodinium acidotum* have been published in the past. The genus *Nusuttodinium* was created in 2014 for a monophyletic group of naked dinoflagellates that acquires photoautotrophy through the ingestion of photosynthetic prey (Takano et al., 2014). Formerly the two species were named '*Gymnodinium aeruginosum'* according to the original description by Stein (1883) and '*Gymnodinium acidotum*' according to the original description by Nygaard (1950). Whereas *G. aeruginosum* was characterized by a round antapex, *G. acidotum* was characterized by a pointed antapex. Most published studies abided to this rule (Schnepf et al., 1989, Farmer and Roberts, 1990, Fields and Rhodes, 1991, Onuma and Horiguchi, 2013, Onuma et al., 2015, Onuma and Horiguchi, 2016). However, in 2009 Barsanti et al. published photographs of dinoflagellate cells clearly showing a round antapex and named it *Gymnodinium acidotum* breaking this rule. In addition a third species, *Gymnodinium eucyaneum* was originally described from China by Hu et al. (1980). It is characterized by a pointed antapex as also *G. acidotum* but has a hypocone that is more than 1.3 times as long as the epicone whereas it is ½ in *G. acidotum*. Moreover just 1 bp substitution to *G. acidotum* was found in the partial LSU rDNA (Xia et al., 2013). Due to its high similarities to *G. acidotum, G. eucyaneum* is currently regarded as a synonym (Takano et al., 2014). When the genus *Nusuttodinium* was created in 2014, Takano et al. redescribed the species *G. aeruginosum* as having a round or pointed antapex due to their finding that cells with both morphologies isolated from the same pond were identical in their sequence. They clearly distinguished it from *G. acidotum* that always contained a pointed antapex except during cell division. Studies on stable cultures under defined conditions indicate that both species cannot be distinguished morphologically just by observing natural populations. Cells strongly modified their shape depending on the prey ingested. All strains were more elliptic with U-shaped kleptoplasts whereas they became more roundish with granular kleptoplasts. Moreover the strains N. a. Dahlem CCAC 4851, N. a WH and N. a. Marmagen completely lost their pointed shape in the granular state. The only strain that contained a pointed antapex under both conditions was N. a. Haitz. The two strains N.a. HLM and N.a. Krefeld never formed a pointed antapex and were in general more roundish than the other strains. Based on these findings, in total three types have to be distinguished. The first type always contains a

pointed antapex (except during cell division and in very old cultures) and showed the typical morphology originally described for the 'true' *G. acidotum*. The second type never forms a pointed antapex and shows the typical morphology originally described for the 'true' *G. aeruginosum*. The third type however seems to be an intermediate between both species as it combines their character traits. It just showed the presence of a pointed antapex in the U-shaped condition but it was much less distinct compared to the strain N.a. Haitz. Phylogenetic studies on the ITS regions sequenced from single cells that were isolated from nature showed that at least five different genotypes exist. One genotype was defined as *N. acidotum* and the other four genotypes were defined as *N. aeruginosum* (Takano et al., 2014). No detailed phylogenetic studies were done yet on the six strains established during this study but morphological observations clearly indicate the necessity of a redefinition of the species *N. aeruginosum* due to the intermediate morphotype found.

4.2 Endosymbiont, kleptoplast or food vacuole? – Confusions in terminology

Many different terms were used in the past to describe taken up prey that is not digested directly and supplies the host with photosynthetic products for a limited time. The most common terms used are 'endosymbiont' (Farmer and Roberts, 1990) or 'kleptoplast' (Gast et al., 2007) whereas the latter term varies from 'kleptoplastid' (Kim and Archibald, 2010), 'kleptoplast' to 'kleptochloroplast' (Onuma and Horiguchi, 2015).The term 'cleptochloroplast' was first introduced by Schnepf (1989) to describe the cryptophyte derived plastid in *G. aeruginosum*. However it remains unclear whether this term just described the stolen plastid or also included the organelles associated with the plastid. Already one year later a similar situation in the related species *Gymnodinium acidotum* was called endosymbiont (Farmer and Roberts, 1990). In 2014 the genus *Nusuttodinium* was created and its definition included the retention of endosymbiotic cyptomonads. However, species within this genus strongly differ in the degree of prey reduction after ingestion. *N. amphidinoides* (formerly *Amphidinium wigrense*) for example just retained the cryptophyte plastids (Wilcox and Wedemeyer, 1985) whereas *N. acidotum* nearly retained the whole cryptophyte cell (Farmer and Roberts, 1990). Moreover the term endosymbiont is also used to describe the *Symbiodinium* coral-endosymbiosis (Sorek and Levy, 2012). Whereas these dinoflagellates are able to continue their life after death of the host, the endosymbiont of *N. acidotum* dies within its host. Also this comparison shows that the same term is used to describe two

different situations. The study presented here uses the term kleptoplast as an analog to chloroplast.This term is just used to describe the stolen plastids and does not include associated organelles. The addition of words like ‘incomplete’ or ‘complete’ to the term endosymbiont could help to avoid confusions. Moreover a new term like ‘kleptoendosymbiont’ could help to distinguish between ‘modified’ endosymbionts that have to be replaced over time like in *N. aeruginosum/acidotum* and permanent endosymbionts like in *Kryptoperidinium foliaceum* or within the corals. The dinoflagellate *N. amphidinoides* small epicone CCAC 5709 was shown to ingest algae of diverse groups. As prey plastids were often digested within 10 days after ingestion the term ‘food vacuole’ would be more appropriate. Little is known yet about the differences between a simple digestion system and an instable kleptoplast. Molecular approaches like a comparative investigation of transcriptomes of such organisms could help to define a border. Moreover comparative studies during the digestion process of the ingested prey could help to answer this question.

4.3 Prey selectivity of kleptoplastic organisms

Many kleptoplastic systems have been described until today. The identification of the source of kleptoplasts is essential to establish co-cultures of these organisms that are the necessary basis to understand the biology behind. However, many researchers still describe results just based on cells from natural samples and often introduce hypotheses that are not based on solid scientific results (Okamoto and Inouye, 2006, Yamaguchi et al., 2011, Xia et al., 2013, Onuma and Horiguchi, 2015). The results shown in this study indicate that kleptoplastic organisms are much more complex than previously thought. Most of the results shown were just observed in culture. Although many studies have been published about *N. aeruginosum* (that is already known for more than a decade) and *N. acidotum* (that is known for more than half a decade) in the past, none of them was able to demonstrate the complexity of the kleptoplast biology of both species due to lacking cultures. Therefore the first step should always be the establishment of a stable culture. The identification of the prey spectrum of kleptoplastic organisms not only helps to bring these organisms into culture but is also necessary for a comparison of the kleptoplast with the free living prey. Kleptoplastic organisms strongly differ in their prey spectra despite close relationship. The marine sea slug *Elysia chlorotica* is highly specialized on the xantophyte species *Vaucheria litorea* (Rumpho et al., 2008) whereas a closely related species *Plakobranchus ocellatus* accepts at least 8 different algal sources of kleptoplasts (Maeda et al., 2012). A phylogenetic approach to

identify the diversity of endosymbiotic *Nephroselmis* in the katablepharid flagellate *Hatena arenicola* suggested at least three distinct strains within the *Nephroselmis rotunda* clade. However, this result could not be verified by feeding experiments due to lacking cultures (Yamaguchi et al., 2014). Sequencing of natural populations of the ciliate *Mesodinium rubrum* revealed that at least three different species, *Teleaulax amphioxeia*, *Teleaulax acuta* and *Geminigera cryophila* were ingested. However, the ciliate seemed to show preference for the species *Teleaulax amphioxeia* (Nishitani et al., 2010). The preference for specific prey strains was confirmed by feeding experiments in which the ciliate showed up to five fold differences in division rates when grown on different strains (Myung et al., 2011). Also Park et al. (2007) showed in feeding experiments with six different cryptophyte strains that division rates of *M. rubrum* strongly varied from 0.04 $*d^{-1}$ – $0.4*d^{-1}$. Prey related to *Teleaulax amphioxeia* resulted in the highest division rate whereas prey related to *Pyrenomonas salina* resulted in the lowest division rate. Differences in division rate were also observed for *N. aeruginosum/acidotum* when fed with *Chroomonas* sp. CCAC 3453 and *Chroomonas* sp. CCAC 4832. Cells with granular kleptoplasts showed a significantly higher division rate than cells with U-shaped kleptoplasts. A possible explanation could be that the energy derived from digestion processes is higher than the energy derived via photosynthesis. This is further supported by the fact that dinoflagellates with granular kleptoplasts showed a lower oxygen production than dinoflagellates with U-shaped kleptoplasts. However the observation of oxygen production in the granular state indicates that *N. aeruginosum/acidotum* profits from both, photosynthesis as well as digeston of the kleptoplasts. The acceptance of a broad spectrum of potential prey organisms seems to be widespread among kleptoplastic organisms. *N. aeruginosum/acidotum* ingested all strains of *Chroomonas* belonging to subclades 3 and 4. This strategy might increase the probability to settle new habitats as the presence of acceptable prey is a prerequisite. The acceptance of cells belonging to subclade 3 might help in nature to survive periods until a cell belonging to subclade 4 is ingested that induces the more stable kleptoplast. No studies were done whether *N. aeruginosum* shows a preference for strains belonging to subclades 3 or 4 but the double sequences obtained during sequencing of cells from natural habitats indicate that the same cell can contain kleptoplasts of two genetically different *Chroomonas* cells the same time. However it remains unclear why strains belonging to subclade 1 were not accepted as prey and how the dinoflagellate recognizes acceptable prey. Personal observations showed that strains belonging to subclade 1 had the biggest cells (no measurements were done) and the cells were mostly immobile located within palmellae. The blister-like structures observed in

the SEM might be involved in prey recognition. If the prey is covered by a layer of mucilage it might not be recognized as prey. The prey size also seems to play an important role. As members of the genus *Nusuttodinium* ingest the whole prey, they might be limited by its size. This was observed in particular for *N. amphidinoides* that just ingested prey that was smaller or equally in size. Moreover just prey lacking a solid cell wall was ingested. A possible explanation might be that the cell wall prevents flexibility of the shape of the prey that is necessary during the ingestion process. The comparison of the food spectra of *N. aeruginosum/acidotum* with *N. amphidinoides* indicates a possible correlation with the stability of the derived kleptoplasts. *N. amphidinoides* showed a broader food spectrum also ingesting *Cryptomonas* and *Rhodomonas* species and all offered *Chroomonas* strains but its kleptoplasts were less stable compared to *N. aeruginosum/acidotum*. *N. amphidinoides* small epicone even ingested algae belonging to other groups than cryptophytes but the derived kleptoplasts (or food vacuoles) were again less stable than those of *N. amphidinoides*. The acceptance of a broad food spectrum seems to be necessary if the plastids can not be retained for extended time. However, it also prevents longer retention times of the kleptoplasts. It remains unclear how important bacteria are for the nutrition of kleptoplastic dinoflagellates. Wilcox and Wedemeyer showed the presence of an intracellular bacterium in *N. acidotum* (Wilcox and Wedemeyer, 1984). *N. amphidinoides* strain N. amph HLM just survived in the presence of bacteria and similar structures were also observed after DAPI staining within the cell. However, all other strains of *N. amphidinoides* as well as *N. aeruginosum/acidotum* survived under axenic condition. The cultivation of algae under axenic conditions is highly unnatural but absolutely necessary to study their interaction with bacteria. The combination of studies on natural populations with studies under contolled laboratory conditions seems to give the best results. It is quite interesting why exactly cryptophytes seem to be one of the most prefered sources of kleptoplasts in dinoflagellates but also in ciliates. They are also widely ingested to supplement the nutrition of mixotrophic and heterotrophic dinoflagellates (Hansen, 1992, Stoecker et al., 1997, Calado et al., 1998, Lee et al., 2014). A possible explanation might be that this group of mostly photosynthetic unicells occurs in nearly all kinds of aqueous habitats, both freshwater and marine and often occurs in high abundancies (Klaveness, 1988, Clay et al., 1999, Novarino, 2003).

4.4 The granular kleptoplast – a primitive prestage of the U-shaped kleptoplast?

The presence of kleptoplasts differing in both, stability and morphology makes the dinoflagellate *N. aeruginosum/acidotum* interesting for evolutionary questions concerning the necessary steps to establish stable plastids from taken up prey. The granular kleptoplasts found after feeding experiments with *Chroomonas* strains of subclade 3 completely differed from the U-haped kleptoplast usually found in nature. Many studies concerning the kleptoplasts in *N. aeruginosum/acidotum* were published in the past. Most of them just showed the presence of one large U-shaped kleptoplast. However a few publications already indicated that *N. aeruginosum/acidotum* is able to make additional types of kleptoplasts. Farmer and Roberts (1990) showed the presence of two enlarged cryptophyte nuclei in both, DAPI staining as well as TEM sectons of *N. acidotum*. As these observations were done natural populations, they could only speculate about this result and hypothesized that the kleptokaryon devided inside the dinoflagellate. In the study presented here, this situation would be categorized as the intermediate kleptoplast type. In another study published by Fields and Rhodes (1991), *N. acidotum* was fed with three strains of *Chroomonas*. All strains were ingested and figure 6 of their publication shows a cell with taken up prey that clearly does not contain the typical U-shaped kleptoplast but instead multiple small kleptoplasts that highly resemble the granular type. However the authors did not go into detail with this observation. One of the most striking observations comes from a publication on *G. eucyaneum* that is not widely known due to the fact that it was written in Chinese (Shi et al., 1983). The authors wrote that the number of cryptophyte nuclei could vary from 0-10 although 0-4 nuclei were more frequently (information adopted from Xia et al., 2013). Such numbers of kleptokarya were also observed in for the granular type. Studies on *G. aeruginosum* from a saline lake ecosystem in Australia showed a change in the number of 'chromatophores' as well as the cell size during the year. Cells observed in spring 1969 had a size of approximately 35µm and a relatively small number of chromatophores distributed in the hypocone whereas the cells observed in autumn 1970 were relatively small (approx. 18 µm) but were densely packed with chromatophores (Walker, 1973). However, the salinity of the studied lake varied beteen 23-56 g/l and the pH was highly alkaline with 9.8, conditions that do not fit to the natural freshwater habitat where *N. aeruginosum* is usually found. Personal observatons on *N. aeruginosum/acidotum* under increased salinities (0-10g/l) showed that this dinoflagellate already dies at 2g/l NaCl (data not shown). *N. aeruginosum* was never described again to occur in saline waters and therefore it remains unclear whether

Walker really saw *N. aeruginosum* or another dinoflagellate that just simply had a similar morphology. Another strong hind for the ability to form differently stable kleptoplasts by *N. aeruginosum* comes from a review by Nowack and Melkonian (2010) in which the following sentence was published: 'Whereas many *Chroomonas* strains can serve as prey for *G. aeruginosum*, only few are apparently able to re-establish kleptoplastidy (Melkonian, M. 2006 (unpublished observations))'. Despite all these observations, no publication focused on these observations yet. The granular kleptoplasts derived from subclade 3 seem to represent a more primitive type compared to the highly modified and more stable U-shaped kleptoplasts. Granular kleptoplasts resembled freshly taken up prey but nevertheless were stable for about 14 days. Moreover the kleptokarya were stable for up to 10 days. Whereas granular kleptoplasts were just distributed to the daughter cells, U-shaped kleptoplasts were able to divide. Therefore granular kleptoplasts can be defined as 'more primitive' compared to the U-shaped kleptoplast. The comparison with kleptoplasts of *N. amphidinoides* and *N. amphidinoides* small epicone that can also be regarded as granular, shows that different degrees in prey modification exist between different organisms. In contrast to *N. aeruginosum/acidotum*, the kleptokarya in *N. amphidinoides* seem to be digested soon after prey uptake. Nevertheless the stability of kleptoplasts in *N. amphidinoides* was similar to the granular kleptoplasts in *N. aeruginosum/acidotum*.

The ciliate species *Mesodinium rubrum* shows a lot of similarities to the species *N. aeruginosum/acidotum*. It also ingests different cryptophyte species but just a few can be retained for an extended period of time. Feeding and starvation eyperiments with two different cryptophyte prey strains showed that one was stable for at least 37 days whereas the other was digested within 14 days. Moreover differences in division rates could be observed. Ciliates fed with *Teleaulax amphioxeia* that induced the more stable kleptoplasts showed a five fold higher division rate compared to cells fed with *Pyrenomonas* sp. (Myung et al., 2011).

4.5 kleptoplast stability among different eukaryotic groups

The ability to retain kleptoplasts for an extended period of time makes kleptoplastic organisms interesting for the study of the evolutionary mechanisms that led to the formation of stable plastids. Kleptoplast stability can range from 10-14 days as observed for *N. amphidinoides* or for *N. aeruginosum/acidotum* (granular state) up to one year in the marine sea slug *Elysia chlorotica* (Mondy and Pierce, 2003). Shedding light on the strategies of such

organisms has always been one of the central goals. However, the high diversity of kleptoplastic organisms as well as their diverse strategies exacerbates the search for common patterns. One of the most extensively studied organisms is the marine sea slug *Elysia chlorotica*. This animal incorporates plastids of its algal prey species *Vaucheria litorea* during its juvenile stages and is able to live its whole life completely autotrophic (West, 1979, West et al., 1984, Rumpho et al., 2010). *Vaucheri*a is a xanthophyte alga with plastids of red algal origin that are surrounded by four membranes. Just two enveloping membranes were observed after incorporation of the plastids by *Elysia*. The absence of associated algal nuclei in *E. chlorotica* raised the question about the mechanism for the longevity of the kleptoplasts (Rumpho et al., 2001). Several hypotheses have been proposed in the past such as a very large plastid genome that encodes for all necessary proteins, unusual plastid stability or long-lived algal RNA molecules (Pelletreau et al., 2011). All of these hypotheses were disproven and the results clearly support the presence of horizontal gene transfer from the algal nuclei to the host nuclear genome (Pierce et al., 2012). Indeed several alga-derived genes were observed in the transcriptome of *Elysia chlorotica*. One of such genes is *psbO* that is involved in oxygenic photosynthesis (Rumpho et al., 2008). Schwartz et al. (2010) demonstrated the presence of transcripts coding for light harvesting complexes 3 and 4 in the *Elysia* transcriptome and in another study the *ftsH* gene encoding for a D1 quality control protease essential for photosystem II repair has been detected (Vries et al., 2013). However, some researchers did not trust these transcriptomic data and interpreted the found genes as ‘algal contaminations’ within the transcriptomes. Moreover they hypothesized that kleptoplasts in *Elysia* seem to be food reserves that are slowly digested and do not perform photosynthesis (Christa et al., 2013). One of the most convincing evidences that algal genes were indeed transferred to the *Elysia* genome was a positive FISH-labeling of a *Vaucheria*-derived gene on a chromosome of *Elysia chlorotica* isolated from eggs that never came in contact with algae (Schwartz et al., 2014). On the other hand no algal genes have been found in the nuclear sea slug genome (Bhattacharya et al, 2013). Despite all these results, researchers are still far away from understanding this system. One of the biggest problems in *Elysia* but also other systems is that comparisons between differently stable kleptoplasts can just be done by comparison of different species as just one type of kleptoplast is formed by one organism. The study of the dinoflagellate *N. aeruginosum/acidotum* has the big advantage that the same organism is able to form kleptoplasts of different morphology and stability thus making comparisons possible. The comparison of the ‘unstable’ granular kleptoplasts with the ‘stable’ U-shaped kleptoplasts indicated that the kleptokaryon plays a central role in kleptoplast longevity. This is supported

by the observation that cells containing the kleptokaryon maintained their kleptoplast longest in the U-shaped situation. As soon as the kleptokaryon was digested in both, the granular and U-shaped situation, also the kleptoplast was digested. Moreover the polyploidization of the kleptokaryon likely contributed to kleptoplast stability. This hypothesis is supported by the comparison with other members within the genus *Nusuttodinium. N. poecilochroum* was described to digested the cryptophyte nuclei already 3 hours after ingestion and also harbored less stable kleptoplasts (Onuma and Horiguchi, 2013). A dinoflagellate described from the Ross Sea Antarctica was able to maintain its haptophyte-derived kleptoplasts for up to 8 months in the presence of the kleptokaryon (Gast et al., 2007). However, also within the genus *Nusuttodinium* it remains unclear how *N. amphidinoides* is able to maintain its kleptoplasts for up to 14 days in the absence of kleptokarya. Comparative analyses of the transcriptomes of both species could help to shed light on such questions in future. Another group of unicellular protists, the Foraminifera harbor diatom-derived kleptoplasts for several months in the absence of kleptokarya and therefore show a similar situation like *Elysia chlorotica*. Attempts to understand kleptoplast stability by transcriptome sequencing failed due to probable contamination of the used cell material as well as missing cultures (Pillet and Pawlowski, 2012). The ciliate *Mesodinium rubrum* is able to harbor cryptophyte plastids as well as nuclei for more than three months and shows similarities to *N. aeruginosum/acidotum* (Kim et al., 2016). Feeding experiments revealed that *M. rubrum* in addition is able to harbor kleptoplasts of different stability depending on the prey offered. However no data about the morphology of the kleptoplasts were published (Myung et al., 2011). In contrast to *N. aeruginosum/acidotum*, cryptophyte nuclei still divided inside the ciliate (Kim et al., 2016). Transcriptional activity between 4 and 13 weeks was detected for the kleptokarya. Genes involved in light and dark reactons of photosynthesis, chlorophyll assimilation and DNA methylation were upregulated that could explain the longevity of the kleptoplasts (Johnson et al., 2007, Kim et al., 2016). The transcriptome sequenced from a natural population of *M. rubrum* feeding on the cyptophyte species *Teleaulax ampioxeia* revealed that genes encoding for photosynthesis and cell division were highly expressed in the cryptophyte transcriptome. Moreover genes encoding for intramembrane ammonium transporters were upregulated in both, ciliate and cryptophyte transcriptome. The increased number of transporters could help to facilitate the exchange of metabolites between host and endosymbiont. Such an interaction was called '*Mesodinium*-farming-*Teleaulax*' as the host helps its endosymbiont (Qiu et al., 2016). Although the transcriptomes sequenced for *N. aeruginosum/acidotum* were not yet analyzed, an increase in genes encoding for photosynthesis is likely in the U-shaped condition

as the oxygen production of one U-shaped kleptoplast was measured to be 78 fold higher compared to the original plastid of one corresponding cryptophyte cell. As observed for the U-shaped situation in *N. aeruginosum/acidotum*, the kleptokaryon also enlarges within *M. rubrum* indicating that this process increases kleptoplast stability (Kim et al., 2016).

4.6 Prey scarcity – A driving force to acquire photoautotrophy

The formation of different types of kleptoplasts depending on the prey availability of *Chroomonas* strains belonging to subclade 4 in *N. aeruginosum/acidotum* indicates big importance of this factor that was previously not in the focus of kleptoplast research. In *N. aeruginosum/acidotum* high prey availability had an inhibitory effect on the formation of stable kleptoplasts. Instead of enlarging their kleptoplasts, dinoflagellate cells reacted with an accumulation of prey cells. Under permanent high prey availability these accumulated prey cells were simply digested similar to food vacuoles. However, if prey availability decreased, the collected prey cells could be distributed to the daughter cells in which they developed to more stable kleptoplasts. It is still unclear how long the taken up prey cells keep their potential to form U-shaped kleptoplasts within the dinoflagellate. A specific labeling of the prey cells could help to answer this question. The behavior of *N. aeruginosum/acidotum* highly resembles the so called 'luggage hypothesis'. This hypothesis describes the behavior of a predator under different concentrations of its 'microsymbiont' and suggests that the microsymbiont is simply digested at predator : microsymbiont ratios < 1 whereas at ratios >1 the microsymbiont is carried as a luggage promoting stable symbiosis (Wouters et al., 2009). Interestingly, just a predator:prey ratio of 1 led to the development of 100% U-shaped kleptoplasts within the density experiment that exactly fits to the predicted hypothesis. *N. aeruginosum/acidotum*, *N. amphidinoides* as well as *N. amphidinoides* small epicone were able to recognize increased prey densities and reacted with an increased uptake. Personal observations on the swimming behavior of the dinoflagellates suggest that the search for prey cells was not directed but occured randomly. Cells containing granular kleptoplasts derived from *Chroomonas* strains of sublade 3 also showed different treatments of their kleptoplasts under high or low prey availability. Under high prey availability no recognizable modifications of the kleptoplast shape could be seen whereas the kleptoplasts slightly increased in size and were slightly modified also in their shape when prey availability decreased (personal observations). The observation of an increased division rate of *N. aeruginosum/acidotum* with granular kleptoplasts compared to U-shaped kleptoplasts

indicates that energy output through digestion processes of prey is higher than through photosynthesis. A similar behavior has also been observed for the mixotrophic dinoflagellate *Fragilidium subglobosum*. This species was able to grow completely photoautotrophic but could also prey on other dinoflagellate species belonging to the genus *Ceratium* (Skovgaard, 1996, Hansen and Nielsen, 1997). The presence of prey resulted in direct uptake and a decrease in cellular chlorophyll a content to 47% and a decrease of the photosynthetic performance to 42% of the values measured for photoautotrophic cultures after 4-8 days. Growth rates in contrast were three fold higher under mixotrophic compared to photoautotrophic conditions (Hansen et al., 2000), a result that also correlates to the observations on *N. aeruginosum/acidotum*. Although the luggage hypothesis fits to the behavior of *N. aeruginosum/acidotum*, it gives no scientific explanation why stable kleptoplasts were formed under low prey availability whereas high prey availability resulted in non-stable kleptoplasts. One observation that has been made at the beginning of this study was that the uptake of just one prey cell belonging to subclade 4 always resulted in one U-shaped kleptoplast. Dinoflagellates that took up the prey cell did not divide until the typical U-shaped morphology was reached. The dinoflagellates enlarged in an immobilized state and only started moving when this development was completed. As dinoflagellates with enlarged kleptokarya did not take up new prey, the kleptokaryon might be inhibitory for this process. However, despite many attempts, it could not be found out at which timepoint during kleptoplast development the feeding behavior of the dinoflagellate stopped due to the fact that most dinoflagellates were already immobile one day after the ingestion of the cryptophyte cell. Cells of the genus *Chroomonas* usually divided once per day and cell division always occurred during the dark phase. The kleptokaryon might still continue to double its DNA content in the same rhythm. As it is not able to divide any more due to a lacking spindle apparatus, it increases in size. A doubling of DNA content might already be sufficient for an inhibitory effect. The analysis of single cell transcriptomes during this enlargement process could help to understand the genetic background behind this observation.

4.7 Polyploidy – A potential key to establish stable plastids

The enlargement of the kleptokaryon within the U-shaped situation as well as the results obtained from DNA-fluorescence measurements and semi-quantitative PCR indicate a polyploidization of the cryptophyte nuclear DNA. Polyploidy refers to the presence of more than two complete sets of chromosomes within the same nucleus (Vakchaure and Ganguly,

2016). Currently two different types of polyploidy are distinguished, autopolyploidy and alloploidy. Autopolyploidy is the doubling of chromosomes within the same species via endoreplication whereas allopolyploidy represents an increase of chromosome number through the addition of chromosomes from different species (Ramsey and Schemske, 1998). Another type of ploidisation event is aneuploidy. Aneuploidy represents a condition of having an imbalanced copy number of single chromosomes (Thompson et al., 2010). Polyploidy is widespread among eukaryotes and can be found in unicellular as well as multicellular organisms. Many important crop plants like wheat, cotton and strawberry are polyploid (Chen, 2010). Polyploids within plants often exhibit increased vigor compared to their diploid relatives (Sattler et al., 2016). It is supposed that all seed plants have experienced at least one round of Whole Genome Duplication (WGD) in their evolutionary history (Renny-Byfield and Wendel, 2014). Polyploidy can also occur within animals and is extensively studied with respect to human diseases like tumors (Holland and Cleveland, 2009, Gordon et al., 2012, Zasandil et al., 2013). Although polyploid situations occur, most cancers are ascribed to aneuploidy with irregular karyotypes (Storchova and Pellmann, 2004, Storchova and Kuffer, 2008). Characteristic for such tissues is an uncontrolled cell division (Szalad et al., 2009). The situation found in the kleptokaryon of *N. aeruginosum/acidotum* is more likely a WGD and therefore shows a higher similarity to the land plants than to cancer. Although the estimation of the DNA content as well as the semi-quantitative PCR strongly suggest a WGD, a final prove by counting of chromosomes would be necessary. The comparison of the granular situation with the U-shaped situation clearly showed the strong impact of the polyploid kleptokaryon on both, behavior of the dinoflagellate as well as kleptoplast stability. The inhibition of feeding behavior was just observed in the U-shaped situation that might be due to some inhibitory substances synthesized in the presence of the enlarged kleptokaryon. An inhibition of the uptake of new prey might also be a strategy to protect both, enlarged kleptoplast and kleptokaryon from digestion. The observation that the enlargement of the kleptokaryon went hand in hand with the enlargement of the kleptoplast indicates its necessity for the increased protein synthesis during this phase. As also described for the ciliate *M. rubrum,* the kleptokaryon was essential for kleptoplast stability in *N. aeruginosum/acidotum*. The prolongated life time of both, kleptokaryon as well as kleptoplast might facilitate endosymbiotic gene transfer to the host nucleus that is necessary for a stable integration of the kleptoplast. Interestingly the enlarged kleptokaryon lost its ability to divide. A similar situation has also been described for the nitrogen fixing *Rhizobium* bacterium that lives in symbiosis with legumes. The bacteria underwent a host directed differentiation process in

which they became polyploid. Associated with this feature were an enlargement in bacterial cell size, a branching of the cells and the loss of cell division ability (Maroti and Kondorosi, 2014). The inability of the kleptoplasts and kleptokarya to divide was also described for the katablepharid flagellate *Hatena arenicola* that inherited kleptoplasts and kleptokarya just to one daughter cell resulting in one colorless cell after each cell division. Although kleptoplasts were described to be enlarged compared to the free living prey *Nephroselmis*, no information was published about a potential polyploidy of the kleptokarya and the DAPI image shown cannot be interpreted as no reference image of a free living prey cell is shown (Okamoto and Inouye, 2005). In 2011 a marine dinoflagellate *Gymnodinium myriopyrenoides* (now *Nusuttoinium myriopyrenoides*) has been described that showed a remarkable similarity of its kleptoplast to *N. aeruginosum/acidotum*. It also contained one large blue-green U-shaped kleptoplast of cryptophyte origin. However TEM analysis did not clarify whether the associated kleptokaryon has also been enlarged. Due to the lack of a culture no further investigation of this species with respect to the study of polyploidy is possible (Yamaguchi et al., 2011). As observed for *N. aeruginosum/acidotum*, also the marine ciliate *M. rubrum* is able to enlarge its kleptokaryon. This process however just happens under starvation conditions as kleptokarya of freshly taken up prey did not enlarge (Kim et al., 2016). Onuma and Horiguchi (2015) observed in TEM sections of *N. aeruginosum* up to eight nucleomorphs within one enlarged U-shaped kleptoplast. Nucleomorphs were observed in kleptoplasts of cells with and without a kleptokaryon. This result suggests that in contrast to the kleptokaryon, the nucleomorph maintains its ability to divide. However it is still unclear what the function of so many nucleomorphs is but it is likely that they also contribute to kleptoplast stability.

4.8 A novel hypothesis about the formation of stable plastids

The comparison of kleptoplast biology of the dinoflagellates *N. aeruginosum/acidotum*, *N. amphidinoides* and *N. amphidinoides* small epicone allows the formulation of a novel hypothesis about the formation of stable plastids. The major idea behind this hypothesis came from Prof. Dr. Melkonian. The hypothesis presented here is splitted into 5 steps.

Step 1: Polyploidization of the endosymbiotic DNA

Kleptoplast stability in *N. aeruginosum/acidotum* was directly connected to the presence of a kleptokaryon. The comparison of granular kleptoplasts with U-shaped kleptoplasts indicates

that the stability increases with a multiplication of the genome copy number within the kleptokaryon. The increased copy number might help to produce enough transcripts encoding for plastid targeted proteins necessary to maintain kleptoplast stability. Further evidence that this process occurred multiple times came from a study on the copy numbers of the four genomes present in the only two known nucleomorph containing lineages, the chlorarachniophytes and the cryptophytes. This study revealed that the nucleomorph in the chlorarachniophyte *Bigelowiella natans* is diploid and that it is tetraploid in the cryptophyte *Guillardia theta* (Hirakawa and Ishida, 2014) (Fig. 36).

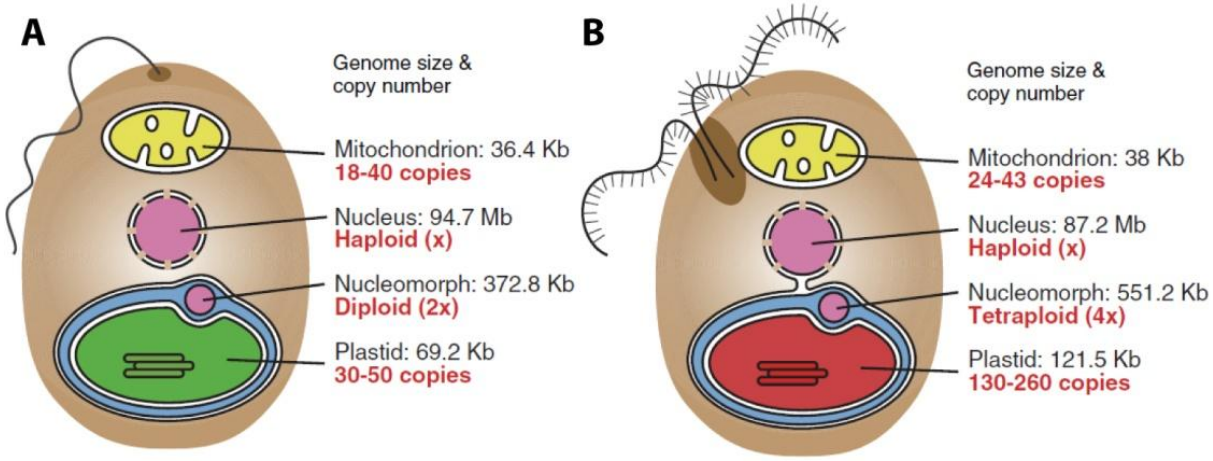

Figure 36. Copy numbers of the four genomes in the chlorarachniophyte *Bigelowiella natans* **A** and the cryptophyte *Guillardia theta* **B**. The figure is adopted from Hirakawa and Ishida, (2014).

Another support comes from the dinoflagellate *K. foliaceum* that is known to harbor a diatom endosymbiont. Although no studies have proven yet the polyploid nature of its diatom derived nucleus, the strong enlargement is highly similar to the situation observed for the enlarged kleptokaryon in *N. aeruginosum/acidotum*. However the bead chain-like shape as well as the permanent nature of the enlarged nucleus differs from the sphaeroid shape and transient nature in *N. aeruginosum/acidotum* (Fig. 37).

Step 2: Inhibition of feeding

Feeding experiments of dinoflagellates with and without an enlarged kleptokaryon clearly showed that feeding is inhibited by the presence of an enlarged kleptokaryon. A comparison with cells containing granular kleptoplasts and non-enlarged kleptokarya showed that just enlarged kleptokarya had an inhibitory effect. An inhibition of feeding is found in several eukaryotic lineages. The katablepharid flagellate *Hatena arenicola* is known to harbor kleptoplast derived from the prasinophyte green alga *Nephroselmis*. Similar to *N.*

aeruginosum/acidotum, also *Hatena* enlarges its kleptoplast. Cells with an enlarged kleptoplast lost their feeding apparatus indicating a completely photoautotrophic lifestyle although this still has to be proven on a stable culture that is not available yet (Okamoto and Inouye, 2006). The marine sea slug *Elysia chlorotica* is able lo live completely photoautotrophic after the uptake of plastids derived from the xanthophyte alga *Vaucheria litorea* during its juvenile stage (Rumpho et al., 2010). Feeding experiments on the diatom plastid harboring genus *Kryptoperidinium* with a variety of potential prey algae showed that prey uptake is also inhibited in this dinoflagellate (personal observations). A potential sense behind the inhibition of feeding might be the protection of the endosymbiont from digestive enzymes that might not distinguish between the prey vacuole and the kleptoplast. This hypothesis is supported by the observation that *N. aeruginosum/acidotum* was never observed to contain an enlarged kleptokaryon and a freshly taken up prey.

Step 3: Unequal distribution of the endosymbiotic nucleus

One of the most striking differences between the U-shaped and granular situation was the distribution of the endosymbiotic organelles to both daughter cells during cell division. Whereas both kleptoplasts as well as kleptokarya were distributed equally in the granular stage, an unequal distribution of the enlarged kleptokaryon was observed in the U-shaped situation. Once ingested by the dinoflagellates *N. aeruginosum/acidotum* and *N. amphidinoides*, the cryptophyte nuclei lost their ability to divide. Unequal distribution of the endosymbiotic organelles has also been described for *Hatena arenicola*. Just one cell inherited the endosymbiont during cell division whereas the other cell became colorless (Okamoto and Inouye, 2006). Studies on the two closely related dinoflagellate species *Kryptoperidinium foliaceum* and *Durinskia baltica* also support the hypothesis that the unequal distribution of the endosymbiotic organelles might be a key step that finally leads to the stable integration of plastids. Both dinoflagellates contain endosymbionts of diatom origin derived via tertiary endosymbiosis similar to the endosymbionts of *N. aeruginosum/acidotum*. Studies have shown that the endosymbiont is closely related to the pennate diatom genus *Nitzschia* (Chesnick et a., 1996, 1997, McEwan and Keeling, 2004). Characteristic is the presence of a second large endosymbiotic nucleus of highly irregular shape that lacks microtubules and as a consequence a spindle apparatus during any stage of division. Mitosis was absent. Interestingly the endosymbiotic nucleus was distributed unequally to both daughter cells and division occurred by simple cleavage (Dodge, 1971, Tippit and Pickett-Heaps, 1976) (Fig. 37). The spherical shape of the enlarged kleptokaryon im *N.*

aeruginosum/acidotum might not allow such a simple cleavage as observed for *Kryptoperidinium* and *Durinskia*.

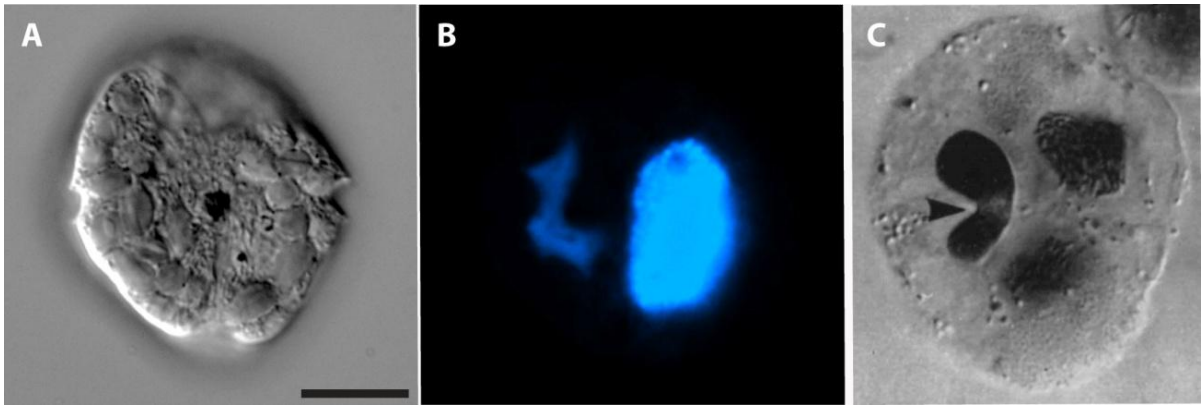

Figure 37. The binucleate dinoflagellates *Kryptoperidinium* and *Durinskia* contain an enlarged diatom derived nucleus. **A** DIC image of *Kryptoperidinium* sp. strain M4675. **B** DAPI fluorescence of the cell in **A** showing the presence of the dinokaryon and a second elongated diatom derived nucleus. **C** Cell of *Durinskia baltica* (formerly *Peridinium balticum*) during division stained with acetocarmine showing an unequal distribution of the endosymbiotic nucleus to both daughter cells. **C** is adopted from Tippit and Pickett-Heaps (1976) (bar in **A** also applies to **B** =10µm, the magnification of **C** approximately corresponds to those in **A** and **B**).

Studies on the life cycle of three strains of *K. foliaceum* showed that the binucleate condition is permanent in contrast to the transient nature in *N. aeruginosum/acidotum*. However, the size of the endosymbiotic nucleus varied between the cells. Larger cells contained a larger endosymbiotic nucleus than smaller cells (Figueroa et al., 2009). The endosymbiotic DNA content between different strains of *K. foliaceum* differed (Kempton et al., 2002). Personal observations showed that the maximum size of the kleptokarya reached in the U-shaped situation differed between the six established strains of *N. aeruginosum/acidotum* resembling the observations on different *K. foliaceum* strains. However this observation still needs to be confirmed statistically. Many studies exist on the species *K. foliaceum* and all of them were done on cells with an endosymbiotic nucleus. However in 2002, Kempton et al. described *K. foliaceum* from a South Carolina bloom in 2001/2002 to lack an endosymbiotic nucleus. Comparison of dinoflagellate nuclear SSUrDNA sequences showed that the cells from the bloom were identical with the binucleate culture strains UTEXLB1688 and CCMP 1326. This finding suggests that plastid establishment in this species is in process. It might be that gene transfer from the diatom to the host nucleus has already been accomplished for some lineages making the endosymbiotic nucleus no longer necessary and therefore allowing the dinoflagellate to discard it (Bhattacharya et al., 2007). Unfortunately, no culture was

established from the mononucleate strain to test this hypothesis via a transcrpitomic approach (Kempton et al., 2002).

A still unsolved question concerning dinoflagellates with tertiary endosymbionts deals with the loss of the original plastid. It is supposed that these dinoflagellates originally contained a peridinin plastid and lost it secondarily. No remnants of the original plastid have ever been described for members of the genus *Nusuttodinium*. *K. foliaceum* in contrast was described to contain an eyespot that is surrounded by three envelope membranes which resemble in appearance the membranes surrounding dinoflagellate plastids of the peridinin type (Dodge, 1968, Dodge and Crawford, 1969). Therefore the eyespot is thought to be a reduced remnant of the peridinin plastid although this has never been proven (Kempton et al., 2002). As observed for the presence of an endosymbiotic nucleus, also the presence of an eyespot seems to be variable among *K. foliaceum* strains. Kempton et al. (2002) investigated the strain UTEX LB 1688 and described it to lack an eyespot. Interestingly the same strain was originally described to contain an eyespot (Dodge and Crawford, 1969). These observations suggest that evolution in *K. foliaceum* is ongoing and that several characters can be changed or lost also under culture conditions. An interesting question concerning kleptoplastic dinoflagellates is how sexuality takes place in species like *N. aeruginosum/acidotum* and if the kleptokarya are also involved in this process. Until now no studies have been performed on this question yet, also because of lacking cultures. Observations of old cultures showed the presence of resting cysts but the fusion of cells has never been observed in culture. Therefore it is likely that the resting cysts were formed asexually. The genera *Kryptoperidinium* and *Durinskia* in contrast were described to contain a sexual lifecycle. In both genera small cells were produced that likely represented gametes (Chesnik and Cox, 1989, Figueroa et al., 2009). Observations made on the fusion process of such gametes revealed homothallism and a synchronization of karyogamy of the host- and endosymbiotic nuclei (Chesnick and Cox, 1987, Chesnick and Cox, 1989). However the S- phase of the endosymbiotic nucleus started 6-8 h later than the S-phase of the dinoflagellate nucleus. Mitosis of the endosymbiotic nucleus has not been observed (Figueroa et al., 2009). The enlarged kleptokarya within the U-shaped situation in *N. aeruginosum/acidotum* highly resemble the endosymbiotic nucleus of *Ktyptoperidinium* and *Durinskia* in also lacking mitosis. The next evolutionary step that *N. aeruginosum/acidotum* would have to undergo, is the permanent maintenance of the kleptoplast and kleptokaryon to become independent from the availability of prey in nature.

Step 4: Endosymbiotic gene transfer from the endosymbiotic nucleus and organelles to the host nucleus

Many studies have been performed on the question and importance of endosymbiotic gene transfer during the establishment of plastids. Eukaryotic plastid genomes usually just encode about 200 proteins of the predicted 1000-5000 that are required for chloroplast activity and maintenance indicating that EGT occured (Martin et al., 2002, Richly and Leister, 2004, Archibald, 2009). Investigations on the amoeba *Paulinella chromatophora*, that represents an example for a recent independent primary endosymbiosis, have shown that more than 30 genes derived from EGT and involved in photosynthesis and photosynthesis related processes were present in the nuclear genome (Nakayama and Ishida, 2009, Reyes-Prieto et al., 2010, Nowack et al., 2011). The two nucleomorph containing groups, cryptophytes and chlorarachniophytes represent good examples to study the impact of EGT during plastid establishment as their plastids were obtained from red- and green algae through secondary endosymbiosis, a process that led to an enormous diversity of photosynthetic eukaryotes. However, these two groups seem to have not yet completed the full reduction of the taken up prey to a plastid as both groups still contain the former red- and green algal nucleus. Studies on the nucleomorph of the chlorarachniophyte *Bigelowiella natans* show an extreme reduction of its genome just having three linear chromosomes that encode for 331 genes. This result strongly supports the importance of EGT. Comparisons with the genome of the host showed that 17 plastid protein genes still have to be transferred to the host nucleus to allow disappearance of the nucleomorph (Gilson et al., 2006). As no transcriptomic analyses have been performed on species of the genus *Nusuttodinium* yet, the impact of EGT for stable plastid integration remains unclear. Two groups, in which the impact of EGT on plastids derived from tertiary endosymbiosis has been investigated (also due to their simplicity in cultivation), are dinoflagellates with diatom and haptophyte derived plastids. Dinoflagellates with diatom derived plastids seem to have reached a more advanced evolutionary level compared to *N. aeruginosum/acidotum.* Especially the two genera *Kryptoperidinium* and *Durinskia* have been in the focus. Studies of their transcriptomes have shown that 9 genes of diatom origin have been transferred to the dinoflagellate nucleus (Burki et al., 2014). These genes were associated with plastid related pathways. To be able to distinguish transcripts of dinoflagellate origin from diatom derived transcripts, the GC content was taken into consideration that was approx 45-55% in diatom transcripts and 60-70% in dinoflagellate transcripts (Hehenberger et al., 2014). Moreover the N-terminal sequences were used to identify the transcript origin (Yokohama et al., 2011). In addition to the transcriptomes, also

the plastid genomes of *D. baltica* and *K. foliaceum* were sequenced. However both genomes were very similar in gene content to that of the pennate diatom *Phaeodactylum tricornutum* (Imanian et al., 2010). Also the genomes of the diatom mitochondria showed little change from free living diatoms (Imanian et al., 2007, Imanian et al., 2012). These results suggest that the first EGT during plastid establishment occurs from the prey nucleus to the host nucleus. As long as the prey nucleus remains intact, it might provide the associated plastids and mitochondria with transcripts encoding for all necessary proteins making EGT from these organelles to the host genome redundant. Although it is well known that the endosymbiotic nucleus within *Durinskia* and *Kryptoperidinium* is highly enlarged compared to the original situation of the closest relative *Nitzschi*a, no study has confirmed yet a polyploidization. Although the enlargement resembles the situation found in *N. aeruginosum/acidotum*, it remains unclear what caused the bead chain-like structure of the nucleus and what is the sense of such structure. As it has been shown by Tippit and Pickett-Heaps (1976), that the nucleus is inherited unequally to both daughter cells similar to the situation found in *N. aeruginosum/acidotum*, the hypothesis arises that this unequal distribution causes an evolutionary pressure that itself drives EGT to the host nucleus. The polyploid nature might help to still provide all necessary genes despite the unequal distribution during cell division. Personal observations on isolated small cells of *Kryptoperidinium* showed that they contained a smaller endosymbiotic nucleus than larger cells. However, after transfer to fresh medium, these cells enlarged and were not distinguishable from other large cells, indicating that they are able to re-synthesize the missing parts that were lost during cell division. Therefore the bead chain-like structure might be one of the reasons why the endosymbiotic association is permanent in contrast to *N. aeruginosum/acidotum* where all genetic information is lost in the daughter cell that does not inherit the kleptokaryon. An additional hypothesis concerning the positive effect of polyploidization on EGT in the case of *N. aeruginosum/acidotum* is that the increased gene copy number might help to increase the probability of gene transfer through EGT during the digestion process of the kleptokaryon. Dinoflagellates with haptophyte derived plastids seem to have reached the most advanced evolutionary level within tertiary endosymbiosis as the prey has been completely reduced to a plastid and the situation is permanent. Transcriptomic studies mainly focused on the two species *Karlodinium micrum* and *Karenia brevis*. In total 16 plastid associated genes within the *K. micrum* transcriptome were found to be of haptophyte origin (Danne et al., 2012). Analysis of the *Karenia* transcriptome even revealed 90 genes of haptophyte origin (Burki et al., 2014). Interestingly, also some genes of the former plastid have been identified (Patron et al., 2006) indicating that

each genome carries a genetic footprint. The *K. brevis* transcriptome even revealed genes belonging to other groups of eukaryotic organisms like ciliates, chlorarachniophytes and metazoans (Burki et al., 2014). A similar observation has been made in the case of the chlorarachniophyte *B. natans*. About 21 % of its genes have been acquired via HGT from various sources like streptophytes, red algae as well as bacteria. It is hypothesized that this observation is due to the trophic behavior of chlorarachniophytes that are known to ingest a variety of different algae (Archibald et al., 2003). Previous studies suggest that any kind of interaction such as trophic interaction or temporary association could lead to HGT (Keeling and Palmer, 2008). This hypothesis is supported by a recent study on the transcriptome and genome of *P. chromatophora*. At least 229 nuclear genes were acquired via HGT from various bacteria. Just 25% of these genes were obtained through EGT from the chromatophore genome. The rest of the genes are hypothesized to have been transferred via HGT. The genes obtained through HGT are supposed to compensate the loss of genes in the endosymbiont genome. *P. chromatophora* is supposed to be originally heterotrophic. As also assumed for *B. natans*, *P. chromatophora* acquired bacterial genes through the ingestion of various bacteria. After getting its cyanobacterial endosymbionts, it started to become mixotrophic and acquired genes through both, HGT and EGT. Finally, enough genes were present in the *P. chromatophora* genome leading to a stable integration of the chromatophores and a photoautotrophic lifestyle (Nowack et al., 2016). The dinoflagellate *N. amphidinoides* small epicone was observed to ingest a large spectrum of different algal groups. However, ingested plastids of the genus *Chroomonas* showed a higher stability compared to ingested plastids of other algal groups. It might be that this dinoflagellate already acquired a small set of genes that encode for *Chroomonas* plastid related proteins that help to elongate the life span of kleptoplasts of this group. The ingestion of a large spectrum of prey organisms might therefore be typical for organisms that are in a very early stage during plastid acquisition. An investigation of its transcriptome will help to confirm or reject this hypothesis.

Despite all these findings, HGT and EGT are just able to partially explain the stability of ingested plastids. Deep-sea foraminiferans are able to maintain their kleptoplasts for up to 12 months (Grzymski et al., 2002, Bernhard, 2003). However no transcriptionally active genes indicating gene transfer have been found in transcriptomes (Pillet and Pawlowski, 2013). A similar situation was found within different *Elysia* species where transcriptomic studies also revealed no evidence for transferred genes although the kleptoplasts were stable for several months (Green et al., 2005). A hypothesis that was formulated to explain kleptoplast stability

in such cases is the so called ‘Chloroplast Stability Hypothesis’ (Pillet, 2013). This hypothesis refers to the natural stability of the plastids and explains kleptoplast stability by the fact that plastids derived from different sources differ in their stability. Indeed experiments with extracted plastids from different algal sources and incubated under similar conditions differed in their stability (Green et al., 2005). However, as this hypothesis does not give a real scientific explanation, it is not accepted by many scientists. It is also speculated that the behavioral adaptation of the host contributes to kleptoplast longevity like specific shading behavior (Pierce et al., 2012). A study on *M. rubrum* showed that indeed the host is able to support the endosymbiont by the synthesis of membrane transporters that are incorporated into the endosymbiont membrane and facilitate metabolite exchange (Qui et al., 2016). Summarizing, there seems to be no common strategy among all eukaryotic organisms and the significance of HGT and EGT is highly variable. Interestingly, all groups where EGT is not able to explain kleptoplast stability lack species with permanent plastids that is in contrast to the groups in which EGT has been detected. All species that contain permanent plastids and have been investigated with respect to EGT were found to contain transferred genes. Therefore it seems that the acquisition of permanent plastids is coupled to this process.

Step 5: Loss of all endosymbiotic organelles except for the plastids

The reduction of the endosymbiont to a stable integrated plastid is the farthest developed stage during endosymbiosis. Whereas photoautotrophic lineages derived via primary endosymbiosis already reached this stage, examples from secondary and tertiary endosymbiosis exist that still seem to be in the process of plastid establishment. Examples for groups derived via secondary endosymbiosis are the nucleomorph containing cryptophytes and chlorarachniophytes. With respect to the observations made on *N. aeruginosum/acidotum* during this study and comparison to the other two groups of tertiary plastid containing dinoflagellates, a developmental gradient can be observed. The endosymbiotic association in *N. aeruginosum/acidotum* seems to be the most primitive compared to dinoflagellates with diatom endosymbionts and haptophyte plastids. This is due to the transient nature of the kleptoplast and kleptokaryon. The dinotom dinoflagellate genera *Durinskia* and *Kryptoperidinium* have already integrated the endosymbiotic nucleus and plastids. However, the endosymbiont is still separated by a membrane from the host cytoplasm and the endosymbiotic nucleus is still present. The farthest developed stage in tertiary endosymbiosis has been reached by the haptophyte plastid containing genera like *Karenia* and *Karlodinium* that reduced their endosymbiont to a stable plastid (Fig. 38).

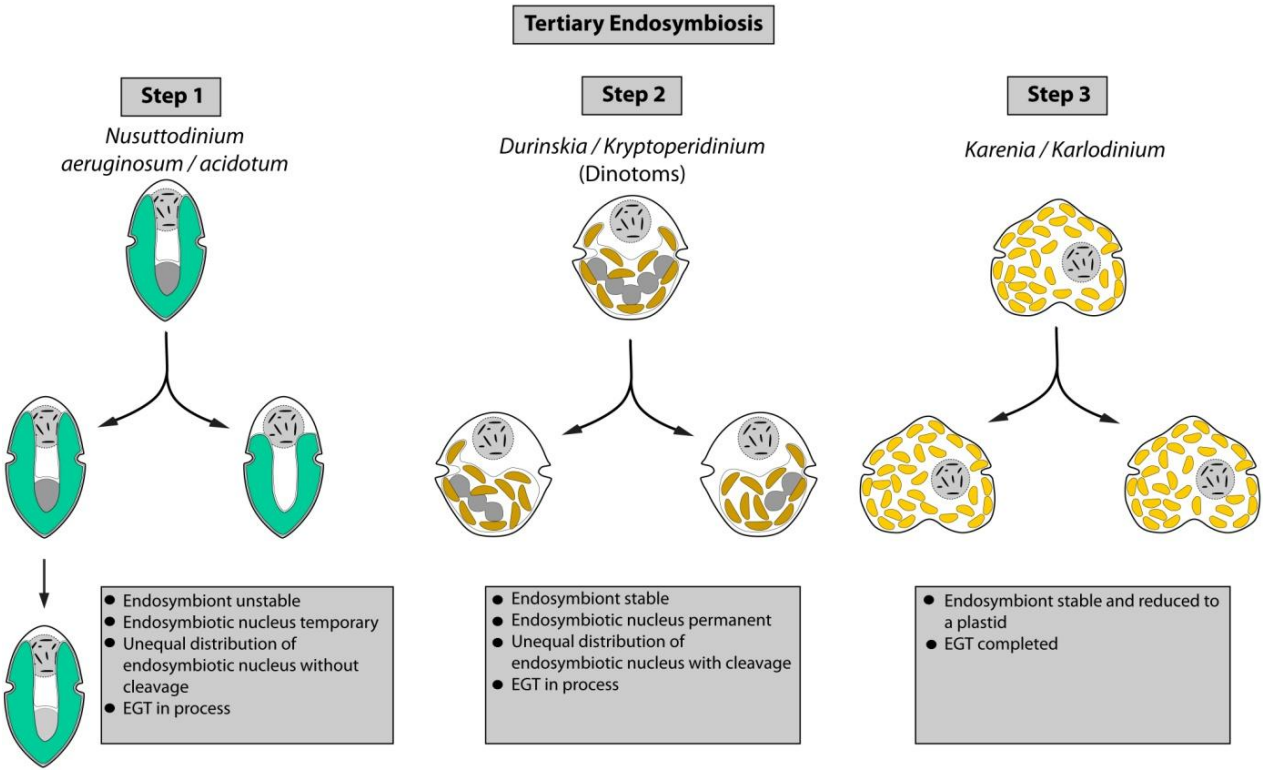

Figure 38. Hypothesis about the evolutionary steps during tertiary endosymbiosis (light color of the kleptokaryon indicates degradation, EGT = Endosymbiotic Gene Transfer).

5. Outlook

Observations on *N. aeruginosum/acidotum* and *N. amphidinoides* give rise for further study of these species. It remains unclear why just *Chroomonas* strains belonging to subclade 4 induce the U-shaped kleptoplast. Comparative analyses of the transcriptomes of *N. aeruginosum/acidotum* under different conditions could help to answer this question. Moreover the comparison with the transcriptome of the 'more primitive' species *N. amphidinoides* small epicone could give insight into the number of transferred cryptophyte genes that is expected to be higher in *N. aeruginosum/acidotum*. A dinoflagellate species that also forms a large U-shaped kleptoplast is *N. myriopyrenoides*. As the only study performed on this species has been done on natural samples it would be interesting if the species shows the same characteristics like *N. aeruginosum/acidotum*. Therefore it would have to be brought into culture first. The discovery of polyploidization in the kleptokaryon of *N. aeruginosum/acidotum* as well as the parallels to the polyploid nature of the nucleomorph raises the question if this is a general step during plastid acquisition. An investigation of the enlarged bead chain-like diatom derived nucleus in the dinotom dinoflagellates with respect to

polyploidization could support this hypothesis. Moreover it still remains unclear why the endosymbiotic nucleus of the dinotoms has a bead chain-like shape. A hypothesis is that each bead contains a full set of genes but also this still has to be tested. This study showed that prey availability has a strong impact on the type of kleptoplast formed in *N. aeruginosum/acidotum*. This has to be also investigated for other kleptoplastic species. Observations have shown that kleptoplasts in *N. amphidinoides* showed a similar stability as the granular kleptoplasts in *N. aeruginosum/acidotum*. However *N. amphidinoides* seems to directly digest the cryptophyte nuclei in contrast to *N. aeruginosum/acidotum*. It would be interesting to compare both strategies on the transcriptomic level. It is still unclear whether colorless dinoflagellates, which comprise about 50% of all described species, completely lost their original plastid or still contain a remnant. The analysis of the genome of a colorless dinoflagellate could help to eventually discover plastid targeted proteins. An antibody approach on such proteins could help to shed light on this question.

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7. Appendix

7.1 Video of the behavior of *N. aeruginosum/acidotum* CCAC 4851 with granular kleptoplasts derived from *Chroomoas* sp. CCAC3453 (subclade 3)

7.2 Video of the behavior of *N. aeruginosum/acidotum* CCAC 4851 with U-shaped kleptoplasts derived from *Chroomoas* sp. CCAC4832 (subclade 4)

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Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbstständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit- einschließlich Tabellen, Karten und Abbildungen, die anderen Werken im Wortlaut und Sinn entnommen sind, in jedem Einzelfall als Entlehnung erkenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität vorgelegen hat; dass sie - abgesehen von unten angegebenen Teilpublikationen – noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Prof. Dr. Michael Melkonian betreut worden.

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Studium

10. 2007 – 09. 2011	Biologie, Bachelor of Science an der Universität zu Köln: Thema der Bachelorarbeit: ‚Kleptoplastiden in *Gymnodinium aeruginosum*‘
10. 2011 – 03. 2014	Biologie, Master of Science in Biological Sciences an der Universität zu Köln. Thema der Masterarbeit: ‚*Prosoaulax* – A Dinoflagellate with Mysterious Plastids‘

Promotion

seit 09. 2014	Doktorarbeit im Botanischen Institut der Universität zu Köln (Lehrstuhl 1). Thema der Doktorarbeit: ‘Acquisition of phototrophy in kleptoplastidic dinoflagellates – *Nusuttodinium aeruginosum/acidotum* as a case study’

Berufspraktika

07. 2007 – 08. 2007	Praktikum in der Gärtnerei Lambertz
02. 2011 – 03. 2011	Praktikum im Max-Planck-Institut für Züchtungsforschung

Nebenberufliche Tätigkeiten

02. 2003 – 07. 2007	Aushilfskraft in der Gärtnerei Blumen Wunsch
04.2004 – 12. 2008	Aushilfskraft in der Gärtnerei Lambertz
04.2008 – 12. 2008	Zivildienst in einer Einrichtung für Menschen mit geistiger Behinderung

03. 2010 – 04. 2014	Studentische Hilfskraft in der Arbeitsgruppe Flügge der Universität Köln
10. 2010 – 03. 2011	Studentische Hilfskraft in der Arbeitsgruppe Hoef-Emden der Universität Köln
04. 2011 – 07. 2011	Tischassistent im Modul „Angewandte Ökologie“ der Universität Köln
07. 2011 – 04. 2014	Studentische Hilfskraft in der Arbeitsgruppe Melkonian der Universität Köln
seit 02. 2013	Gutachter für Kakteenschmuggel beim Zollamt Essen
04. 2014 – 07. 2014	Wissenschaftlicher Mitarbeiter im Modul „Angewandte Ökologie“ der Universität Köln

Auszeichnungen

2014	Beste pflanzenwissenschaftliche Masterarbeit an der Universität zu Köln (Deutsche Botanische Gesellschaft)
2016	Ernst Georg Pringsheim Preis (Deutsche Botanische Gesellschaft, Sektion Phykologie)

Köln, 09.01.2018 Sebastian Wittek