

Cellular aggregation in response to
ecological disturbances in the green algae
Chlamydomonas reinhardtii

-

Concomitant implications for the evolutionary
transition from unicellular to multicellular life

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Cellular aggregation in response to ecological disturbances in the green algae *Chlamydomonas reinhardtii*

Concomitant implications for the transition from unicellular to multicellular life

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Cellular aggregation in response to ecological disturbances in the green algae *C. reinhardtii* - Concomitant implications for the transition from unicellular to multicellular life

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Для Елизаветы. Моей родственной душе, с любовью.

Für Gertrud, Gerhard und Chiara. Für Alles. Seit jeher.

Til Markus. For vennskap og med takknemlighet.

Tusen takk Catharina Broch and Tom Andersen, without whom the realization of the project would not have been possible. Thank you for all of your invaluable time spent reading and revising the manuscripts and thinking about the project during its execution. Thank you again, Catharina for being an invaluable support and a grand help in all aspects – ranging from conversations, laboratory tasks, data analysis and writing during the year of 2017. Thank you, Kim Aalborg, Øyvind Torp, Louise Valestrand and Sunniva Reitan for conversations, encouragement and company.

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After listening to comments and criticism from supervisors and a lay-reader, I found it unavoidable to make explicit the following: The introduction to this thesis (and to a certain extent the discussion) paints a broad picture thematising an inherently complex and fascinating area of biological research. The reader may therefore be underwhelmed by the teeny implications the empirical results in this study yield and may state that the contribution to the concepts and the intelligibility of the topic is nil. I am in no position to object to that criticism since seeing this text from a phenomenological “other” perspective, it is fully reasonable. Nonetheless, I chose not to reduce the paragraphs to “the matter at hand” partly out of curiosity, a somewhat scholarly character and because I found the exposure to these grand thoughts and serious reflections of others motivating, stimulating and elucidating.

I hope the reader is not being “misled by hastily assuming that this particular empirical study was selected and designed and executed in such a way as empirically to test broader conceptions or assumptions”:

“Among abstracted empiricists, there is a recent tendency to preface empirical studies with a chapter or two in which they summarize “the literature of the problem”. This is of course a good sign and is, I think, in some part a response to criticism from the established disciplines. But in actual practice this work is all too often done after the data are collected and “written up”. The memorandum which he produces is then reshaped in an effort to surround the empirical study with “theory” and to “give it meaning” or – as is frequently said – to “get a better story out of it”. Even this, perhaps, is better than nothing. But it does often mislead the outsider who may hastily assume that this particular empirical study was selected and designed and executed in such a way as empirically to test broader conceptions or assumptions”.

- C. Wright Mills: *The Sociological Imagination* (1959)

0. Preface

Theoretical biology has important roots in the experimental tradition of early-twentieth-century Vienna. Paul Weiss and Ludwig von Bertalanffy were among the first to use the term theoretical biology in its modern sense. In their understanding, the subject was not limited to mathematical formalization or empirical investigation, as is often the case today, but extended to the conceptual foundations of biology. It is this commitment to a comprehensive and cross-disciplinary integration of theoretical concepts that fuels the investigations into some of the most intriguing questions in the biological sciences.

It is generally assumed that there was a protracted early period in geological time (~4250 – 1500 million years ago) when the only prokaryotic and eukaryotic organisms on planet earth were unicellular. They were the ancestors of all the multicellular organisms that exist today, including ourselves (Grosberg & Strathmann, 2007). The realization that multicellularity arose more than once is also generally appreciated by biologists. It was not a matter that was, or is, much discussed; it was simply a reasonable assumption. Becoming multicellular opens up the possibility of evolution almost wholly guided by natural selection – it is a novel lifeworld explored by organisms inhabiting a wholly different one of which has been written: “Life has a range of magnitude narrow indeed compared to that with which physical science deals; but it is wide enough to include three such discrepant conditions as those in which a man, an insect and a bacillus have their being and play their several roles. Man is ruled by gravitation, and rest on mother earth. A water-beetle finds the surface of a pool a matter life and death, a perilous entanglement or an indispensable support. In a third world, where the bacillus lives, gravitation is forgotten, and the viscosity of the liquid, the resistance defined by Stokes’s law, the molecular shocks of the Brownian movement, doubtless also the electric charges of the ionised medium, make up the physical environment and have their potent and immediate influence on the organism. The predominant factors are no longer those of our scale; we have come to the edge of a world of which we have no experience, and where all our preconceptions must be recast” (Thompson, 1942).

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1. Introduction

1.1. Individuality and the Major Transitions in Evolution

Evolution is a diachronically constructive process. Over large timescales it has driven the development from simple to more complex life forms, from prokaryotic to eukaryotic cells, from single cellular to multicellular organisms, from solitary insects to colonies, from animal groupings to human societies (Bonner, 1988; Carroll, 2001; Maynard Smith & Szathmáry, 1998; Tarnita et al, 2012). Evolution in the sense of the modern synthesis (MS), the current paradigm in biology, is based on the concept on natural selection which impinges on a set of objects¹ if there are heritable differences in fitness among them (Lewontin, 1970). Essentially, if variant entities in a population experience differential reproductive success, and if the variation is heritable, then the genetic, morphological and behavioural composition of a population will change over time (Okasha, 2005). Other concepts which are supposed to have played a substantial role in the development of more complex life forms and on which a part of the discussion e.g. the inheritance of cell colonies, relies, include neo-Lamarckian epigenetic inheritance, inclusive inheritance, niche construction theory, developmental (phenotypic) plasticity and inter- and intraspecific cooperative interactions (Sober, 1984; Pigliucci, 2007; Reid, 1985; Okasha, 2006, Nowak & Sigmund, 2004). In order to operationalize the “working” theory of natural selection biologists count individuals and their changing frequencies, as they are the loci of fitness (this is highly controversial and fuelled the ongoing “level of selection” debate) (Okasha, 2006). Canonical individuals have often been taken to be differentiated multicellular organisms. The hierarchy of life (e.g., genes, chromosomes, prokaryotic cells, eukaryotic cells, multicellular organisms, eusocial groups) implies that new forms of individuals with varying and often ambiguous

¹ For example, in systems biology, the theory of natural selection can equally well be used in a reductionist framework to make sense of complex transcription networks in the cell and the prevalence of certain network motifs whereby characteristics of a promoter region are selected for or against depending on the functionality provided to the cell (Alon, 2007). I chose the term “objects” due to the recent rise of research programs such as gene-culture evolution, evolutionary psychology, sociobiology or cultural evolution.

“nuanced” individualities (e.g. indicated by varying physiological unification and genetic uniformity) have evolved (Buss, 1987; Maynard Smith, 1988; Michod & Roze, 1997). **Table 1** depicts an overview of some of the cooperative group formations and major evolutionary transitions that have occurred in evolutionary history (O’Malley & Powell, 2016; Maynard Smith & Szathmáry, 1995). At various points in evolutionary history, the “higher-level” units in this hierarchy have emerged from interactions among the “lower-level” units. One can attempt to explain large-scale trajectories of life by targeting major turning points in evolution and connecting them all by a common theoretical thread. John Maynard Smith & Eörs Szathmáry conceptualized eight “major evolutionary transitions” in the history of evolution of life on earth which seek to identify large-scale patterns in life’s history as partly exemplified in **Table 1** (Maynard Smith & Szathmáry, 1984, 1995, 1999). In each transition, such as the transition from unicellular to multicellular life, a number of smaller units, originally capable of surviving and reproducing on their own, emerged as a larger unit, creating a new level in the hierarchical organization and most importantly, a new unit of selection – a transition in individuality that occurs when one level of individuality gives way to another (O’Malley & Powell, 2016).

Table 1. A selection of cooperative group formations of unitary “lower level” objects, followed by major evolutionary transitions and the emergence of “higher level” objects.

Original state	Cooperative group formation / New state	Major Evolutionary transition
Uncontained replicating molecules	Groups of compartmentalized molecules	Origin of life / prokaryotic cell
Prokaryotic cell (“limited complexity”)	Eukaryotic cell (“increased complexity”)	Origin of eukaryotes
Unicellular or facultatively multicellular organisms	Obligately multicellular organisms with developmental cell differentiation	Origin of Multicellularity
Individual multicellular organisms	Obligately social organisms with developmental and cultural differentiation of organisms	Origin of obligately social groups / Eusociality

The biological hierarchical structure is not a logically necessary feature of the biological world and since the earliest life forms were presumably not hierarchically complex, the various levels in the hierarchy must, like diversity on which natural selection acts, somehow have evolved in itself (Okasha, 2006).

Multicellular organisms did not come from nowhere, and a complete evolutionary theory must surely try to explain how they evolved, rather than just taking their existence for granted (Michod, 1999). Multicellularity has evolved at least 25 times across the tree of life including in bacteria, archaea, and eukaryotes (Bonner, 1998; Grosberg & Strathmann, 2007). Ecological factors are supposed to be major driving forces promoting the formations of groups from single celled organisms. Logically, the ecological conditions prevailing millions of years ago are essentially unknowable but approximations and educated guesses are nonetheless present in the scientific literature. One of the essential conditions for a “higher unit” to arise is the potential for cell-type specialization that enables protection from environmental toxins and opens up radiation-possibilities in new adaptive niches. The fungi *Saccharomyces cerevisiae* for example forms clusters as a protection strategy against toxins (Smukulla et al., 2008). Additionally, dispersal has played a major role in driving colony formation, as illustrated in the slime mould *Dictyostelium discoideum* (Watts & Ashworth, 1970).

In the framework of Godfrey-Smith (2009) and Clarke (2012), individuals are defined by their membership in Darwinian populations, those that are capable of adaptive evolution. This conceptual framework co-opts Richard Lewontin’s classical formulation of the minimum conditions of evolution in which he introduced the concept of a Darwinian population, that is, a population of agents with heritable variation in phenotypes that reproduce, but not with equal prospects of success, and in such a way that the descendants resemble their parents (Lewontin, 1970). A standard and general account for the evolution of multicellular organisms takes the following form: Collections of single cells, by virtue of heritable differences in reproductive output, exist as members of Darwinian populations. As members of such populations, cells participate in the process of natural selection, that is, they are units of selection. The conceptual switch from a gradual and teleological evolutionary process to a set of dynamic, mechanistic processes that can be altered in themselves is a fundamental

aspect when trying to make sense of the evolutionary transitions in individuality. During the major transition to multicellularity, individual cells became components of groups that eventually evolved the capacity for autonomous reproduction. This transition from a unicellular to a multicellular life-style acted in “a space of biological possibility that is evolving in itself” (Godfrey-Smith, 2009). Essentially, during the transition, natural selection was afforded opportunity to operate at the higher (group) level. In some cases, this led to the emergence of groups as Darwinian individuals—as units of selection—in their own right. Thus, the emerging Darwinian populations satisfy the minimum conditions for evolutionary change, and a Darwinian individual is simply a member of a Darwinian population. In this view, the major transitions in evolution modify core elements of the evolutionary processes and stand in stark contrast with Gould’s view that the morphospace has been explored by passive diffusion from a starting point of minimal complexity (Calcott & Sterelny, 2011; Gould, 1996). Inherent to this framework is the idea developed by Leo W. Buss in his book “The Evolution of Individuality” that individuality itself is a derived character, and that a unit of selection (e.g. a gene [Williams, 1966; Dawkins, 1989] or a group of organisms (Hamilton, 1963; Wilson, 1975; Griffin & Gardener, 2007)) could itself change over the course of evolution (Buss, 1987). In his own words, “... At each transition - at each stage in the history of life in which a new self-replicating unit arose - the rules regarding the operation of natural selection changed utterly. Novel selective scenarios dominate at times of transition between units of selection...” (Buss, 1987). At the beginning of such a transition there is a population of Darwinian agents interacting with others in fitness- affecting ways. That population is itself structured: Interactions are patterned so that local groups interact with one another in ways that contrast with their interactions with agents outside that local group. These are groups - collections - of interacting Darwinian agents. At the beginning of a transition, these collections are at best peripheral or marginal cases of Darwinian agents, and metapopulations of groups are at best peripheral examples of Darwinian populations. The group members, on the other hand, are core Darwinian agents. By the end of a transition, the *collections* have become *collectives*. They are now core Darwinian agents in an unambiguously Darwinian population. Their members are still present, but they have become parts of collectives, and the evolutionary fate of those descendants is now welded together. That new agent is more structurally complex than those

interacting at the beginning of the process which has selective drawbacks but also a multitude of advantages. The major transitions are therefore episodes in which the *vertical complexity* of life has increased through the transformation of a collection or group into a collective, an individual in its own right, without the actual goal (τέλος) of evolution being the ascension of complexity itself and without applying a progressivist view of evolutionary history (Godfrey-Smith, 2009; Maynard Smith & Szathmary, 1995; Michod, 1999; Okasha, 2006; O'Malley & Powell, 2016). There are multiple different kinds of non-equivalent individuals based on variation in genetic uniqueness, genetic homogeneity, and autonomy/physiological unity indicating a continuous variation in individuality (Santelices 1999). Individuality is often assumed to be constant across members of a given species but the context-dependency of individuality should be considered and the evolutionary consequences investigated and most importantly, the ecological context affecting individuality needs to be taken into account (Hanschen et al., 2017).

Egalitarian transitions in individuality begin with a partnership between non-closely related individuals, e.g. eukaryote evolution. *Fraternal* transitions are fused alliances between close relatives, e.g. multicelled organisms and eusocial animals (Queller, 2000). This involves a hierarchical shift in the level of selection (e.g. from multilevel selection 1 to multilevel selection 2 (Wilson et al. 2007)²) and with it the emergence of new kinds of biological individuals whose success very often curtailed the independent evolution of lower level entities (Buss, 1987; Godfrey-Smith, 2009; Okasha 2005). Intermediate – and indeterminate – life forms, endowed with a degree of heritability much weaker than what is typical among extant organisms, could nonetheless have been the “raw material” upon which natural selection acted at the collective level (De Monte & Rainey, 2014).

² The key difference between these two types of multilevel selection is that in MLS1, reproduction is at the level of the individuals themselves and group fitness is simply the total fitness of its constituent members, whereas in MLS2, reproduction at the higher level is decoupled from reproduction at the lower level, and group fitness is not an additive function of component fitness (Okasha 2006; Folse & Roughgarden, 2010). In MLS1, particles (i.e., lower level units) are the focal units (i.e., the units being tracked from generation to generation) while in MLS2 both particles and collectives (i.e., higher-level units) are focal units (Okasha, 2006; Shelton & Michod, 2009)

The transitions in the basic units of life share two common themes: (i) the emergence of cooperation among the lower level units in the functioning of the new “higher level” unit and (ii) regulation of conflict among the lower level units (Michod & Roze, 1999; Michod, 2003). The relative fitness at the “lower” level compared to the “higher” level presumably increased during an Evolutionary transition in individuality (ETI), whereby conflict mediators (a feature of the higher level (the group) that restricts the opportunity for fitness variation in fitness at the lower level (cells) and/or enhances the variation in fitness at the higher level (the cell-group or organism)) evolve in response to non-costly or synergistic forms of cooperation as shown in **figure 1**:

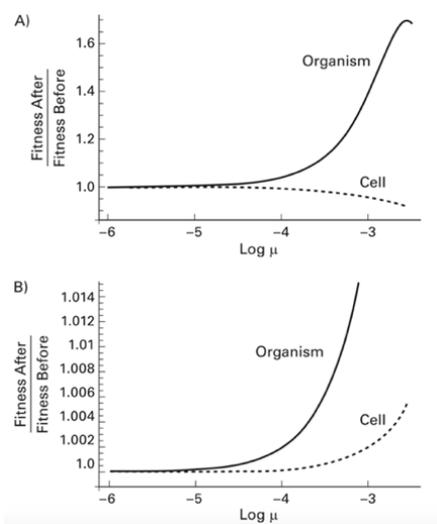


Figure 1. Effect of evolutionary transitions on the fitnesses of the lower and higher levels. The effect of an evolutionary transition on the heritability of fitness at the group and the cell level is shown as a function of the deleterious mutation rate, μ , for the case of (a) cell-cell altruism (selfish mutations) and (b) cell synergism (uniformly deleterious mutations). Calculation of average organism fitness and cell fitness and construction of the figure is explained in Section 6 of Michod & Roze (1999). Figure adopted from (Michod, 2003).

More complex and fit organisms are likely to have emerged during the transition from unicellular to multicellular life and propagated in higher frequency than less fit organisms, given the potential for positive feedback between natural selection and organismal forms, through innovations such as life cycles and mechanisms for suppressing particle-level conflicts (conflict mediation) (Buss, 1987; Griesemer, 2000).

Much experimental and theoretical research is aimed at elucidating the biological mechanisms, the genetic modifications (*e.g.* gene duplication events) and the ecological conditions that promoted a colonial lifestyle in the past which led to a major evolutionary transition in individuality. Model organisms have been the fungi *Saccharomyces cerevisiae*, the slime mould *Dictyostelium discoideum*, and the Volvocales, a lineage of green algae first described by Antony van Leeuwenhowek (1700). After briefly reviewing the evolutionary history of this lineage and their characteristics

as a model system, I consider potential ecological circumstances that were present during an evolutionary transition in individuality in this lineage. This thesis focuses on the major evolutionary transition from unicellular to potential multicellular, group-living entities within the Volvocales lineage, exclusively using the hypothesized unicellular ancestor *Chlamydomonas reinhardtii*, as a model organism.

1.2. The Volvocean Algae

A schism in the evolutionary past of a hypothetical ancestral green flagellate marks the origin of the green plant lineage: the kingdom Viridiplantae, which gave rise to two major lineages, one of which diversified in Earth's oceans and gave rise to a large diversity of marine and freshwater green algae (Chlorophyta) while the other gave rise to a diverse array of freshwater algae and the land plants (Streptophyta) (Finet et al., 2010; Lewis & McCourt, 2004; O'Kelly, 2007). The Viridiplantae is one of the major groups of oxygenic photosynthetic eukaryotes that have played a dominant role in global ecosystems for millions of years. Molecular clock analyses estimate the origin of this lineage at between 700 – 1500 mya (Douzery et al., 2004; Hedgres et al., 2004; Berney & Pawlowski, 2006; Roger & Hug, 2006; Herron et al., 2009)³. The Chlorophyta lineage includes a wide array of marine planktonic groups such as the paraphyletic class Prasinophyceae, marine benthic groups such as Ulvophyceae and the freshwater-dwelling core chlorophytes Chlorophyceae and Trebouxiophyceae (Lewis & McCourt, 2004; Lemieux et al., 2007; Finet et al., 2010). The Chlorophyceae are a large and morphologically diverse group, including non-motile and motile unicells, colonies, branched and unbranched filaments, and blade-like thalli. They are especially abundant in freshwater but also occur in terrestrial habitats. They are rare in marine waters but can dominate the phytoplankton of enclosed estuaries or enclosed lagoons, especially in late summer and fall. Green algae are important as components of the benthos and dominate intertidal soft-bottom seaweed assemblages (Levinton, 2014). Molecular and

³ Molecular clock analyses rely on the approximate constancy of the rate of amino acid substitutions in such proteins as hemoglobins, cytochrome c, and fibrinopeptides and should be interpreted with care. The discrepancy of 800 my can be explained by considering the inaccuracy due to the varying rate of molecular evolution among different proteins, the difficulty of distinguishing between functionally important and unimportant sites and the apparent consistency of the rate of amino acid substitutions per year rather than per generation, by which the method of the molecular clock has been deployed (Dayhof, 1972; Langley and Finch, 1974; Nei, 2013).

ultrastructural data identify five major clades within the Chlorophyceae: Chlamydomonadales, Sphaeropleales, Chaetophorales, Chaetopeltidales and Oedogales (Booton et al., 1998a; Buchheim et al., 2001; Wolf et al., 2002; Wolf et al., 2003; Turmel et al., 2008). The clade Chlamydomonadales contains some of the most well studied organisms. As with any model organism, they are a compromise between tractability and relevance. Some of what can be learned from them is likely to apply broadly to other multicellular groups; other features of their evolution will be uncommon or unique to the model system itself. The real strength of this model system (**Figure 2**) lies not in comparisons between the extremes of unicellular and fully differentiated multicellular species, but in the diversity of species of *intermediate size* and *intermediate complexity* as exemplified in **Fig. 2** and **Fig. 3** (Hanschen et al., 2017; Herron, 2016).

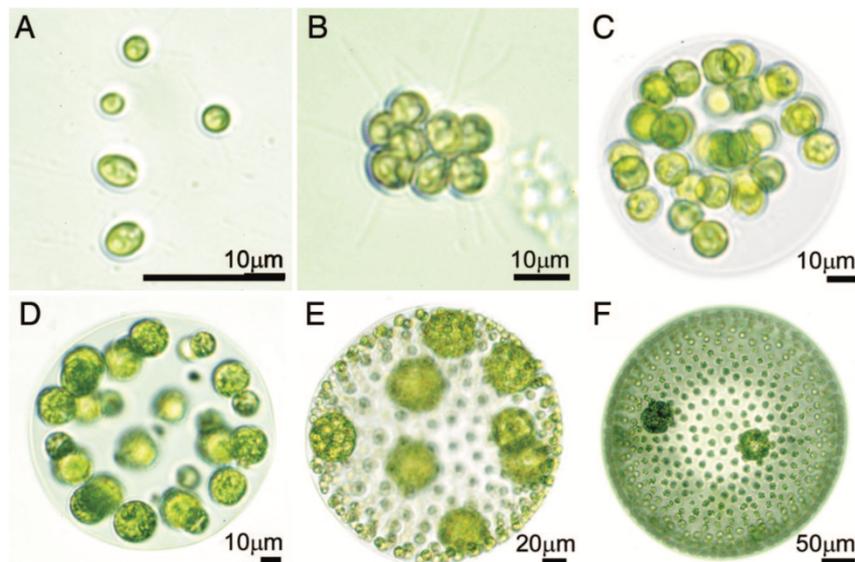


Fig. 2: Examples of volvocine species varying in cell number, colony volume, degree of specialization, and proportion of somatic cells. (A) *C. reinhardtii*, a unicell. (B) *Gonium pectorale*, a flat or curved sheet of 8-32 undifferentiated cells. (C) *Eudorina elegans*, a spherical colony of 16-64 undifferentiated cells. (D) *Pleodorina californica*, a spherical colony with 30-50% somatic cells. (E) *V. carteri*. (F) *Volvox aureus*. Where two cell types are present (D-F), the smaller cells are somatic cells and the larger cells are reproductive cells. Pictures were taken by C. Solari (University of Arizona). Figure adopted from (Michod, 2007).

Within the Volvocales, there exist about 800 species in 33 genera, of which the genus *Chlamydomonas* accounts for by far the greatest number (Kenneth, 2017). The volvocine green algae provide a unique window into the origin of multicellularity, division of labour, and the evolution of sexual reproduction (Michod, 2011). It has been speculated that the initial shift from unicellular to multicellular lifestyle in the Volvocales occurred

~ 223 million years ago (mya, Bayesian credibility interval ~24 mya), when the daughter cells of a *Chlamydomonas*-like ancestor became embedded in a common extracellular matrix, a speculated initial step in the evolution of e.g. the differentiated, multicellular *Volvox carteri* (Herron et al. 2008, 2016). Under which ecological conditions did *Chlamydomonas*-like ancestor single cells become bound to a welded-together evolutionary fate?

Unicellular *Chlamydomonas reinhardtii* is thought to resemble the unicellular ancestor of the multicellular species within the Volvocines, including the undifferentiated *Gonium pectorale* and the differentiated *Volvox carteri* (Hanschen et al. 2016; Coleman, 1999; Larson et al., 1992, Herron et al., 2008; Herron, 2016). The experimental advantages of *C. reinhardtii*, such as a short generation time, fairly easy and cost-effective cultivation, and a sequenced genome make it especially suitable for evolutionary and ecological studies. In general, the Volvocales, including *C. reinhardtii*, comprise biflagellated, photosynthetic, facultatively sexual, predominantly haploid (a diploid zygote is formed during sexual reproduction) eukaryotes.

One example of this seemingly simple reconfiguration of the organismic body plan (unicellular to multicellular) are exemplified by the members of the Tetrabeaceae (Basichlamys and Tetrabaena) (**Fig. 2, Fig. 3**), the smallest multicellular forms present in the Volvocalean lineage. They are made up of four *Chlamydomonas*-like cells held together by a common extracellular matrix, morphologically and developmentally similar to the hypothetical organism present after the initial shift from a unicellular to a multicellular lifestyle, when the daughter cells of a *Chlamydomonas*-like ancestor became bound to a common evolutionary fate. Since nature is what selects, the ecological conditions prevalent during the time of the evolutionary transition were presumably advantageous for multicellular organisms.

Many researchers interested in the major evolutionary transition from unicellular to multicellular life in this lineage adopted the “Volvocine lineage” hypothesis as a first-order approximation of Volvocine phylogeny (shown in **Fig. 3**). The evolution of the Volvocine algae has often been (and still is) viewed in a framework resting on this axiom which holds, that members of this group represent a progressive and linear increase in

size and complexity from unicellular *Chlamydomonas* to multicellular *Volvox* and that the phylogeny of the group accurately reflects the “progression” in their evolutionary history (Lang, 1963; Van de Berg & Starr, 1971; Pickett-Heaps, 1975). This hypothesis is seen by others as an over-simplified conceptualization of actual Volvocine phylogeny (Larson et al., 1992; Kirk, 1998; Nozaki, 2003).

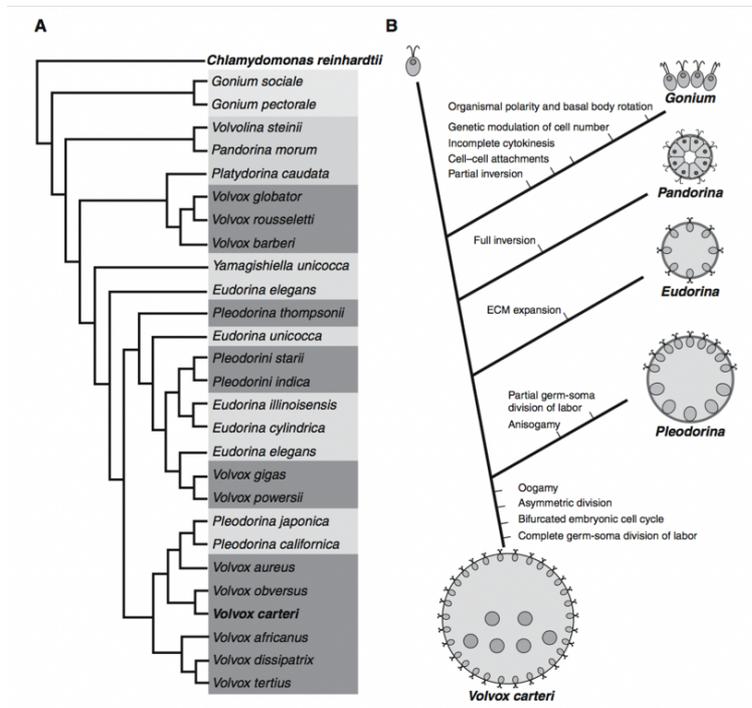


Fig. 3. Innovations in the Volvocine algae. (A) Cladogram based on data in Kirk (2005) and Umen and Olson (2012) showing algal lineages with shading highlighting levels of organization in each genus. (B) Simplified cladogram showing key innovations in the steps leading from *C. reinhardtii* to *V. carteri*. Adopted from (Umen, 2011).

It is likely that the history of morphological and developmental changes for the entire lineage is more complicated than those in the single lineage leading to *V. carteri* would indicate (Herron & Michod, 2008). The gradual lineage hypothesis can be considered a reasonable approximation of evolutionary relationships *only* if the majority of Volvocine diversity is ignored, stressing the need to give a more refined account of the evolutionary history of their phylogeny (Herron & Michod, 2008).

Herron & Michod (2007) conceived the evolution of complexity in the volvocean algae as a progressive series of small steps, each advantageous in itself, that lead to the more complex state, such as Charles Darwin himself conceived the evolution of the eye (Darwin, 1872; Herron & Michod, 2007). **Fig. 3** shows some of these steps, e.g. genetic modulation of cell number, extracellular matrix (ECM) expansion and complete germ-soma division of labour. The Volvocine algae do not have a multicellular ancestor and have evolved undifferentiated multicellularity only once (Herron & Michod 2008;

Leliaert et al. 2012). Their relatively recent radiation facilitates the identification of genetic and phenotypic changes associated with the evolution of multicellularity (Kirk 2005; Herron et al., 2009; Prochnik et al., 2010; Hanschen et al., 2016). The evolution of multicellular individuality from unicellular ancestors in the Volvocales likely involves minor changes in genetic homogeneity, genetic uniqueness, and spatial/temporal boundaries (Hanschen et al., 2016).

Examining the criteria of individuality in an ecological context suggests *four* kinds of multicellular individuals (whereby the first kind is introduced here for the first time): *uncommitted facultative* multicellular individuals (*C. reinhardtii* palmelloids), *uncommitted obligate* multicellular individuals (*Tetrabaena* and *Gonium*), *committed* multicellular individuals (*Pandorina* and *Eudorina*), and *committed, differentiated* multicellular individuals (*Pleodorina* and *Volvox*) (Fig. 2, this thesis). The transition from unicellular to multicellular life in the Volvocales is a paradigm example of an evolutionary transition in individuality (ETI), in which a new biological individual emerged from the interaction between previously independently existing and replicating subunits. However, while such entities are multicellular in the literal sense of consisting of multiple cells (such as *C. reinhardtii* aggregates), they lack a division of labour and functional organization, and therefore are by some not considered to constitute individual organisms. An undifferentiated colony may have potential adaptive, ecological advantages over single cells, such as the ability to resist ingestion or digestion from predators that are too small to capture an entire colony or temporal protection from the fluctuating environment (such as osmotic imbalance, strong currents, UV radiation). Yet, this is merely analogous to a school of fish, a flock of birds, or a herd of grazing buffalos, which aggregate in groups often but not solely for protection against predation via strength in numbers. One does not consider these groups to be individual organisms, primarily because the locus of fitness on which any form of natural selection can potentially act, remains the single, individual member rather than the whole group, and, secondarily, because these groups do not demonstrate functional organization demonstrating group-level adaptations. Only once the colony emerges as an individual *in its own right*, adaptations can begin to emerge and evolve at the colony level. From an *alignment-of-fitness* perspective, a collection of adhering cells

descendant from a single founder cell is an individual organism by virtue of the fact that the cells share the same genome, they are genetically homogenous.

Alternatively, according to the *export-of-fitness* perspective, an ETI has not occurred until actual cooperation and division of labour between cells exports fitness to the level of the emerging multicellular organism (Michod, 1999; Folse & Roughgarden, 2010). The export of fitness requires an alignment of fitness as a prerequisite of being able to be exported to the “next higher level”, that of the new individual. Germ-soma specialization such in *V. carteri* cannot evolve until there is cooperation in the first place. A linear sequence beginning with alignment of fitness by genetic relatedness, the export of fitness by e.g. a germ-soma specialization, and, finally, functional organization by adaption at the higher level combines both diverging approaches to individuality and embraces a multidimensional perspective on individuality.

One of the first premises, and the one that is being investigated from an ecological point of view in this thesis, for multicellularity to evolve is simply the formation of groups, cellular aggregation, of single cells. Cellular aggregation is a phenomenon observed in many unicellular and facultatively multicellular organisms and there are several environmental factors that can lead to this phenotype (Raper, 1984; Boraas et al., 1998; Kaiser, 2003; Niklasa & Newman, 2013; Sathe & Durand, 2016). *Chlamydomonas reinhardtii* has been used as a model organism for investigating ecological conditions and questions concerning cells coming together or staying together (Lurling & Beekman, 2006; Ratcliff et al., 2013; Moulton & Bell, 2013).

The environmental, ecological circumstances favouring the fitness of colony-formation are of major interest when trying to make sense of this major evolutionary transition. The next paragraph reviews environmental circumstances that lead *C. reinhardtii* to express an aggregative phenotype and summarizes the findings and hypothesis as to why, when and how this behaviour occurs. Most importantly, it shows the ecological benefits and drawbacks of a colonial life-style in aquatic environments.

1.3. Palmelloid Formation in *Chlamydomonas reinhardtii*

The facultative “multicellular” green algae *Chlamydomonas reinhardtii* is a ca. 10 µm in diameter, spherical or ellipsoidal photosynthetic organism with two equal-sized flagella for motility and mating located at the anterior of the cell (Kenneth, 20017; Merchant et al., 2012; Sahoo & Seckbach, 2015). This species can mostly be found in soil, fresh- and brackish water (Sahoo & Seckbach, 2015). *C. reinhardtii* has two modes of reproduction: Asexual and sexual. Asexual reproduction takes place mainly by zoospores but can also occur through aplanospores (a nonmotile asexual spore formed by rejuvenescence), hypnospores (a very thick-walled asexual resting spore) and a palmella stage (see below) (Sahoo & Seckbach, 2015). Zoospores are formed during the night, aplanospores under unfavourable conditions such as drought and the palmella stage is formed under certain “unsuitable” conditions. During the palmelloid stage, the parent cell divides to form 4-8 non-motile daughter cells that stay clustered together within a mucilaginous matrix formed by gelatinization and swelling of the parent cell wall (Sahoo & Seckbach, 2015). This stage is thought to be a temporary perennating stage (the ability of organisms to survive from one germinating season to another, especially under unfavourable conditions) and is usually of brief duration (Sahoo & Seckbach, 2015). Under favourable conditions the individual cells readily develop flagella and return to the motile condition. These cells then escape out from the mucilaginous matrix and mature into large vegetative cells.

In *C. reinhardtii*, cell division occurs through palintomy rather than by binary fission; this is, cells grow to many times their original size without dividing and then undergo several rounds of rapid cell division with little or no growth between successive division (Sleigh, 1989). The resulting offspring emerge from the mother cell wall and begin their lives as separate unicellular organisms. Most multicellular volvocine algae have retained this mode of cell division, producing large reproductive cells, each of which divides n times to produce 2^n daughter cells (n usually ranges from 2 to ~ 14) (Olson & Nedelcu, 2016). Together with *Volvox carteri*, *C. reinhardtii* it is one of the two organisms that represent the extremes of size and complexity within the clade and are used to understand the major evolutionary transition from unicellular to multicellular life (Herron, 2016; Okasha, 2006). In spite of the dramatic differences in size and complexity

between the two organisms, their genomes are similar in size and gene content despite 200 million years of independent evolution. The *V. carteri* nuclear genome is ~ 17 % larger than that of *C. reinhardtii* (138 Mb vs. 118 Mb), but this difference is largely due to non-coding sequences, as *V. carteri* has greater repeat content and, on average, longer introns (Prochnik et al., 2010). The total number of genes is similar, with ~ 14,500 genes in each species genome (Merchant et al., 2007; Prochnik et al., 2010, Leliaert et al., 2012).

The similarities between *V. carteri* and *C. reinhardtii* in genetic composition and taxonomic relatedness has stimulated a growing body of research on both model organisms whereby one focus lies on the “multicellular” clusters that *Chlamydomonas reinhardtii* forms under certain conditions. Such plastic behaviour exhibited by *C. reinhardtii* and other green algae (e.g. *Chlorella vulgaris*) was termed “palmelloid formation” as mentioned above. It is fundamentally a clustering together of a minimum of two to sixteen cells (numbers vary between publications investigating this trait and can be up to ~1000 cells) which remain embedded in a jelly-like material when subjected to certain stress- exerting environmental conditions supposedly inhibiting daughter cell hatching. Palmelloids have been described in a unicellular variety of Chlamydomonadineae under certain, not clearly established, culture conditions. They are generally presumed to result from either a failure of daughter cells to produce flagella or progressive gelatinization of the membranes during successive generations which prevents the liberation of dividing cells (Nakamura et al. 1977). Cells in these clusters show a significant reduction in growth rate and an increase in predator survivorship. One proposed advantage of aggregate formation is the effective shielding (itself) off from the influences of the environment via the production of its own internal environment (Bonner, 1998). At return of the favourable conditions, the daughter cells revert to the typical motile condition (Sharma, 1986).

Early publications investigating the behaviour of palmelloid formation in *C. reinhardtii* date back to as early as 1968 with a study conducted by Kozo Iwasa & Shohachi Murakami (1968, 1969) who found that *C. reinhardtii* forms colony-like cell aggregates when subjected to certain organic acids which inhibit photosynthetic growth (e.g., citrate, oxalate, succinate, fumarate, malate, glutamate, aspartate, glycolate and phtalate at neutral pH) (Iwasa & Murakami, 1968, 1969). Early observations of this phenomenon

were commented with: “In an aged culture of *C. reinhardtii*, there are bodies which consist of four to sixteen cells” and “Palmelloid formation in laboratory cultures is not uncommon and it is usually a nuisance” (Iwasa & Murakami, 1968, 1969; Khona et al., 2016). They postulated that the palmelloids result from a failure of daughter cells to produce flagella and that the progressive gelatinization of the membranes during successive generations prevents the liberation of the dividing daughter cells. Since then *C. reinhardtii* has been subjected to a variety of different environmental treatments as presented in **Table 2**.

Table 2. Studies investigating the formation of aggregates in *C. reinhardtii* (and *Scenedesmecea*) and the respective abiotic and biotic treatments explored therein.

Nature of Treatment	Treatment	Publication
Abiotic	Presence of divalent ions (Ca^{2+} , Mg^{2+})	U. Schlösser, 1966
	Medium devoid of Ca^{2+} or with EDTA	Iwasa & Murakami, 1968, 1969
	Chloroplatinic acid and Carbohydrates	Nakamura et al., 1975
	Herbicide paraquat	Franqueiro et al. 2000
	High salinity concentrations	Khona et al. 2016
	Copper(II)-sulfate	Cheloni et al. 2016
Biotic	Presence of the zooplanktonic predator <i>Brachionus calyciflorus</i>	Lurling & Beekman, 2006
	Rotifer grazing pressure	Becks et al. 2010
	<i>Peranema trichophorum</i> (Euglenoidea) physical presence and culture filtrate	Sathe & Durand, 2016
	Presence of the zooplanktonic predator <i>Daphnia magna</i>	Sarah Cossey (Kansas State University, unpublished)
	Presence of infochemicals from <i>B. calyciflorus</i> and <i>Daphnia magna</i> .	Verschoor et al. 2014

The literature investigating the phenomenon of palmelloid colony formation in *C. reinhardtii* is filled with a variety of (and sometimes opposing) conclusions as to what the “mechanism” behind the formation of colonies is. It is supposedly a *heritable trait* which was observed when previously grazed *C. reinhardtii* cultures were cultured without apparent grazing pressure and continued to form palmelloid colonies for nine generations whereas cultures of ungrazed *C. reinhardtii* lineages maintained a consistently high proportion of single cells (Becks et al., 2010).

The diverging results of Lurling and Beekman (2006) and Becks et al. (2010) suggest that under different circumstances, *C. reinhardtii* may evolve either an inducible or a heritable constitutive palmella morphology as a defence against rotifer grazing and, as exemplified above, the behaviour may occur even without grazing pressure, bringing uncertainty to the mechanism(s) behind palmelloid formation in *C. reinhardtii*. This is important since it is proposed that the first multicellular organisms were groups of undifferentiated cells and predation was the dominant ecological selective pressure driving this phenotype in several organisms including the Volvocines and other Chlorophytes (Herron, 2016). Ratcliff and colleagues (2013) selected *C. reinhardtii* for cluster formation (rapid settlement through liquid medium over multiple generations) and found that the artificial selected *C. reinhardtii* cells possessed a novel life cycle consisting of alternating phases: a dispersal phase in which clusters reproduce via motile unicellular propagules *which gave rise to new clusters* and a growth phase during which clusters produce few propagules and instead increase in cell number. Importantly, clusters developed clonally by adhering daughter cells after mitotic reproduction, not by aggregation of single cells that cohabit the same immediate environment (Ratcliff et al., 2013). This paragraph functions as a primer for the discussion in this thesis, in which I suggest that the constitutive palmella morphology by *C. reinhardtii* is both, plastic and heritable.

Chlamydomonas applanata, a species also belonging to the Volvocales, shows an aggregate response when cultures were exposed to a pH of 4.4 (Visviki & Santikul, 1999). The colonies consisted of several non-motile vegetative cells surrounded by a common extracellular matrix similar to *C. reinhardtii*. It is conceivable that *C. reinhardtii* would exhibit a similar response when subjected to various levels of pH (Visviki & Santikul,

1999). Nakamura et al. (1975) postulated that the palmelloids form due to an abnormality in cell wall formation rather than flagellar malfunction or loss (Iwasa et al., 1968; Khona et al., 2016). Iwasa & Murakami (1968, 1969) observed that the palmelloids can be dissociated by the calcium ion (Ca^{2+}) at a concentration of $6.8 \times 10^{-5} M$, but not by the magnesium ion (Mg^{2+}) and is suppressed by the phosphate ion (PO_4^{3-}) (Iwasa & Murakami, 1969 & 1968). The researchers suggest, that Ca^{2+} limitation induces the palmelloid stage in *C. reinhardtii*.

Lurling & Beekman (2006) postulated that at least one of the reasons why *C. reinhardtii* is capable of forming palmelloids is to cope with herbivory, among one of the largest selective pressures on phytoplankton, since the increase in size would give the colonies resistance to ingestion and the envelopment in an extracellular matrix would give resistance to digestion (Lurling & Beekman, 2006). Most importantly, they postulated that the trait constitutes grazer-induced *phenotypic plasticity* but do not define a concrete mechanism by which the behaviour may occur. In the unpredictable heterogenous environment in which *C. reinhardtii* can be found, grazing pressure fluctuates on the spatiotemporal scale and is supposed to be abundant. So are fluctuations in the chemical and physical composition of the micromilieu, the immediate environment surrounding the algal cell.

Cheloni et al. (2016) found that *C. reinhardtii* induced aggregate-colonies and inhibited growth when subjected to high levels of copper(II)-sulfate ($CuSO_4$), a toxic micropollutant that induces enzyme inactivation, DNA damages and lipid as well as protein degradation (Cheloni et al., 2016). Conceivable advantages of an increase in number and embedment in a ECM (extracellular matrix) over a strictly solitary lifestyle were/are the ability to stick to substratum, access to new ecological niches (most notably: size as a niche), survival in harsh environments such as preventing the cell aggregates from being carried away by strong currents; ensuring the safety of the germ line by producing a protected internal environment and the increase in speed due to the increase in size (Bonner, 1998).

It is noteworthy that due to the embedment into an extracellular matrix, motility in undifferentiated colonial cell aggregates such as the palmelloids formed by *C. reinhardtii*

under certain environmental stress conditions, is highly impaired due to the internalization of the flagella and increased swimming speed may *sensu stricto* not have been or be a selective or adaptive advantage of this type of colony formation. Rather, it is acknowledged that motility is inhibited during normal cell division. The inability to both divide and maintain flagellar activity is referred to as the *flagellation constraint* (Koufopanou, 1994). In most green flagellates, during cell division the flagellar basal bodies remain attached to the plasmamembrane and flagella, and behave like centrioles; however, in volvoclean algae, due to a coherent rigid cell wall the position of flagella is fixed and thus, the basal bodies cannot move laterally and take the position expected for centrioles during cell division while remaining attached to the flagella. Since Volvocales are negatively buoyant, the motility function so basic to survival is increasingly comprised during cell division and also during the *akinetes* resting state.

As for various criteria of individuality, the two main model organisms *Chlamydomonas*, as studied in this thesis, and *Volvox* can be characterised in the following way: *Chlamydomonas* palmelloids are facultatively sexual and therefore genetic uniqueness is similar to that in facultatively sexual *Volvox carteri* (Hanschen & Michod, 2017). Genetic homogeneity in *C. reinhardtii* colonies depends on the details of the aggregate response but can exhibit a unicellular bottleneck such as *V. carteri*. Cell clusters may form via aggregation between separate strains, suggesting *Chlamydomonas* clusters are not always genetically homogenous (Sathe & Durand 2015); however, in the other studies discussed above, the clusters are considered to be clonally formed and genetically homogenous. The case of palmelloid clusters illustrates the challenges involved in distinguishing the level of selection of levels of individuality leading to increasing physiological unity and the presence of true group adaptations (Clarke 2010, 2013). There are no clear examples of physiological unity or group-level adaptations in palmelloid clusters, although secretion of proteins constituting the extracellular matrix upon which cluster formation is based is a potential candidate. Alternatively, the secretion of extracellular matrix may be a cell-level adaptation which increases the inclusive fitness of secreting cells. Shelton & Michod (2014) analysed a model of simple clusters of genetically identical cells as a way to understand when group-level adaptations emerge. The conditions under which group adaptations emerge depend on how strongly

group membership affects life history variables such as mortality and growth. While the beneficial effect of group membership on lowering predation-rates is consistent with group adaptations being present in *Chlamydomonas* palmelloid clusters, it is not sufficient to clearly demonstrate their presence. The example of palmelloid *Chlamydomonas* serves as a null comparison for the undifferentiated colonial species in which group-level adaptation and integration are clearly present (Hanschen & Michod 2017). It is therefore worthwhile to further investigate this behaviour and additionally, studies of volvocine ecology are solely lacking. As a result, discussion of the evolutionary processes underlying the transition to multicellular life have largely excluded ecological and biogeographical context (Herron, 2016).

Understanding how and why groups of individuals become new kinds of individuals is a major challenge in explaining the history of life and the particulars of this transition depend not only on the nature of the unicellular ancestor, but on the specific selective pressures due to prevailing ecological conditions exerted by the internal and external environment that drove the transition towards more complex and integrated lifeforms. It is not clearly established if the aggregative trait exhibited by *C. reinhardtii* is in any way a precursor for the evolution of more complex, multicellular organisms but it fulfils the intuitively conceived, initial step towards the evolution of multicellularity. For the major multicellular lineages, the factors underlying their origins and evolution of mode of reproduction lay hidden deep in their evolutionary past, obscured by hundreds of millions of years of subsequent evolution. Accumulated genetic changes may be irrelevant to multicellularity and are only fragmentarily present in the fossil record due to extinction and evolutionary succession. Supposedly, one of the first steps to occur and a requirement for the evolution of multicellularity, during this transition was the transformation of the cell wall into a ECM, keeping the cells attached to each other, and the only traits that are common to, and probably ancestral to, all extant colonial species in the volvocean algae is this transformation of the cell wall into an ECM. (Herron & Michod, 2008).

1.4. Introduction to the Salinity Experiment

I chose to investigate the response to high levels of salinity in *C. reinhardtii* due to the universal nature of NaCl in aquatic ecosystems and, since it has been clearly shown that cells form palmelloids in the presence of NaCl (Khona et al. 2016), to assure that the four different strains used in this study were initially capable of forming palmelloids before investigating other, more elusive, biotic or abiotic factors.

Osmotic stress is linked to salt stress: the latter involves an excess of sodium ions whereas the former is primarily due to a deficit of H₂O without a direct role of sodium ions (Hsiao, 1986; Munns 2002, Sudhir et al., 2004). Excessive accumulation of ambient Na⁺ and Cl⁻ ions results in ionic imbalance and reduces the potential of the cell to take up any of the other vital minerals such as K⁺, Ca²⁺ and Mn²⁺ which has various effects on physiological processes in the cell such as increased respiration rate and ion toxicity, mineral redistribution, membrane instability resulting from calcium displacement by sodium and decreased efficiency of photosynthesis (Sudhir et al., 2004; Hasegawa et al., 2000). Furthermore, it affects the resistance to biotic and abiotic stresses (for reviews and examples, see: Boyer, 1976; Kirst, 1989; Munns, 2002; Sayed, 2003, Zuo et al., 2014, Marschner, 1986). Algae and other phytoplankton respond to hypotonic challenges and other environmental stressors via the accumulation of metabolites such as glycerol, polyalcohols, mannitol and glycosides which provide further osmotic balance and thereby counteract external disturbances (Husic et al., 1986; Amotz et al., 1983). The universal nature of metabolite-production in phytoplankton reflects the evolutionary necessity of these organisms to cope with osmotic disturbances exerted by their environment.

The response of algae to moderate changes of salinity is a well-regulated biphasic process. The first phase is characterized by rapid changes in turgor pressure (walled cells) or volume changes (wall-less cells) caused by massive water fluxes in and out of the organism following the osmotic gradient. The second phase represents osmotic adjustment: Cellular concentration of the osmotically active solutes (osmolytes) change until a new steady state is achieved. Both phases are part of a feedback loop that comprises the osmotic acclimation (Kirst, 1989; Cram, 1976; Gutknecht et al., 1978). *C.*

reinhardtii synthesises glycerol in response to osmotic stress and growth is strongly impaired (Husic et al., 1986; Gamboa et al., 1985; Reynoso et al., 1982). The inhibition of growth and photosynthesis of *C. reinhardtii* prevails because intracellular glycerol levels do not suffice to establish sufficiently fast intracellular equiosmolar conditions. *C. reinhardtii* accumulates proline upon salt stress which was also detected after short term salt stress in the brown alga *Ectocarpus siliculosus* (Dittami et al., 2011; Mastrobuoni, 2012). Upon exposure to high levels of salinity, *C. reinhardtii* loses motility due to resorption of flagella and cell division was found to be slowed down (Neelam et al., 2013). *C. reinhardtii* employs two contractile vacuole systems in the cell anterior, on opposite sides of the plane that include the flagellar basal bodies to uptake excessive water from the cytoplasm and expel it into the environment to achieve cellular homeostasis (Luykx et al., 1997; Xu et al., 2016). Interestingly, these contractile vacuoles in walled algae appear mainly to be confined to green flagellates belonging to the Volvocales. K^+ , a major osmolyte in contractile vacuoles, is predicted to create higher osmolarity for water influx in *C. reinhardtii* (Xu et al., 2016). The osmolytes in the contractile vacuole attract water that enters through the aquaporin water channels present on the contractile vacuole membrane (Komsic-Buchmann et al., 2014) and in *C. reinhardtii*, a voltage-gated K^+ channel is localized at the contractile vacuole membrane and crucial for the osmoregulatory system of the organism via regulation of K^+ entry into the organelle (Xu et al., 2016). The reduced uptake of K^+ due to ionic imbalance may result in malfunction of the osmoregulatory system (especially the dysfunction of the voltage-gated K^+ channel) and be one of the reasons why *C. reinhardtii* shows so many fitness-reducing characteristics (such as reduced growth, impaired motility, the need to allocate resources to the protein synthesis via the expression of genes coding for osmolytes) when exposed to high levels of salinity.

When subjected to hyperosmotic conditions, *C. reinhardtii* induces mRNA expression of the genes GAS28, GAS30, and GAS31 (Hoffmann & Beck, 2005). These gene products exhibit typical features of *C. reinhardtii* cell wall proteins: a ser(pro)_x-rich domain and encode for hyp-rich glycoproteins that presumably are cell wall constituents and are activated by zygote formation and by the application of osmotic stress. Loss of contact between the plasma membrane and the cell wall is postulated to trigger a signalling

pathway that results in the induction of the three GAS genes. This pathway also may be employed when cells are shifted to hyperosmotic conditions, since shrinking of the cytoplasmic membrane involves its partial detachment from the wall (Hoffmann & Beck, 2005). A response to stressful osmotic conditions is the formation of cell colonies and the additional production of an extracellular matrix that shields the organism from environmental perturbations as has been shown in Khona et al. (2016). In their study, *C. reinhardtii* has been subjected to high levels of salinity (50, 100, 150, 350 and 500 mM) and palmelloids were clearly present after 3 hours post exposure (Khona et al. 2016).

1.5. Introduction to the Infochemical Experiment

I chose to subject the four different *C. reinhardtii* strains to the culture filtrate of *B. calyciflorus* since it has so far been only investigated once (Sathe & Durand, 2016) and due to the increasing awareness of the importance and universal nature of interspecific chemical communication in aquatic ecosystems (Grasswitz & Jones, 2002; Van Donk et al. 2011). Furthermore, since cue concentration likely indicates the level of risk (Ferrari et al. 2010), prey should use cue concentration to adjust the intensity of their antipredator avoidance in a threat-sensitive manner to optimize fitness in the trade-off between costly predator avoidance and fitness-related activities. Accordingly, I subjected four *C. reinhardtii* strains to three different “concentrations” of predator (*B. calyciflorus*) infochemicals and expected a difference in (i) the strength of the adaptive response (high conc. > low conc.) and (ii) moment of colony formation (high conc. < low conc.).

The avoidance of predators may be almost inevitable. Predators are believed to be important agents of selection and to elicit a staggering variety of adaptations in organism (Vermeij, 1981). Predators can be successfully avoided, misdirected, or repelled for some time if prey act in an appropriate manner. Logically, appropriate behaviour will vary across species. For some, it will be increased vigilance and increased proclivity to flee when perceiving danger. In some cases, social behaviour will create effective group

defence mechanisms. In essence, the inducible defences can manifest as behavioural, morphological effects or shifts in life-history parameters. For many organisms, the proper response may be a morphological one, such as making shells or other hard outer coverings, growing spines or thorns, or producing or sequestering poisonous substances.

Phytoplankton species must survive, grow and reproduce in unpredictable environments while dealing with the risk of predation by zooplanktic grazers. Planktonic primary producers are not defenceless food particles that are easily harvested by their consumers. Rather, a number of phytoplankton taxa have evolved a variety of either *constitutive* or *inducible* defence mechanisms against zooplankton grazing. The ability to defend themselves against predation is considered to be a main driving force in their evolutionary histories and bears consequences on competition and trophic cascades within an ecosystem (Van Donk et al. 2011; Agrawal, 1998). Selection in favour of antipredatory traits can occur only when some members of a prey population survive to reproduce after being detected, pursued, or assaulted by a predator, that is, when predators are less than 100 % efficient at one or more stages of their interactions with prey.

In an eco-evolutionary feedback loop in which the behavioural response of the prey (aggregate formation of the algae in presence of predators) modifies the dynamics of predator-prey cycling, algae were shown to produce cell clumps too large for rotifers to consume effectively. Thus, “algal evolution” altered the food environments of the rotifers, with subsequent consequences for the environment of the algae when the *predominance of defended algal genotypes* caused the rotifer population to decline. This allowed the algal population to increase, but also led to expression of the cost for defence – a lower population growth when competing for limited resources (Becks et al. 2012).

Becks et al. (2010) brought forward the argument, that the formation of colonies in *C. reinhardtii* was a heritable inducible defensive trait that evolved due to the exertion of strong predation pressure by rotifer grazers (Becks et al. 2010). Grazing pressure fluctuates both on spatial and temporal scale and the evolution of plastic genotypes enabling algae to make sense of changes in their immediate environment is considered

to be a result thereof (Morales et al. 2002). It is reasonable to expect that chemoperception works in the both directions (from prey to predator and *vice versa*). Several studies have demonstrated that the formation of protective colonies in freshwater green algae such as *Actinastrum*, *Coelastrum*, *Desmodesmus*, and *Scenedesmus*, and the marine prymnesiophyte *Phaeocystis* might be evoked by zooplankton associated chemicals (Hessen & Van Donk 1993, Lurling 1998; Van Donk et al. 1999).

Chemical communication is a well-known phenomenon mediating interactions between organisms who depend strongly on these chemical stimuli to learn about their biotic and abiotic environments (Dicke & Sabelis, 1992). Chemosensory perception in locating and selecting prey is probably vital in the viscous world of micro-zooplanktic consumers and the capability may well be widespread among the planktic protists (Weisse, 2003). The ciliate *Euplotes* is known to react to predator-specific chemical factors produced by its amoeboid predators by producing giant, uningestible cells (Kusch, 1995). The chemical nature of the substances involved is not well known. Semiochemicals, chemicals that transmit information between conspecifics and other species, are apparently dominant in aquatic habitats (Ferrari et al. 2010). They are collectively referred to as kairomones, a class of infochemicals emitted by one species (predator) and being received by a second species (prey), evoking an adaptive, interspecific behavioural or physiological reaction in the receiver that is favourable for the receiver but not for the sender (Grasswitz & Jones, 2002).

Changes in morphology caused by predator presence is known as predator-induced polyphenism, and occurs across a variety of animals. Mice (*Mus musculus*), for example, are instinctively aware of their natural predators, including cats (*Felis domesticus*) and rats (*Rattus norvegicus*) via their receptiveness to major urinary proteins and upon reception initiate a flight- or hide-response (Rodrigues, 2010). The freshwater crustacean *Daphnia longicephala* adapts to changing predation risks (exposure to kairomones) by forming inducible defences (protective crests) which are only formed when they are advantageous, saving associated costs when the defence is superfluous [Weiss et al. 2015]. Furthermore, in response to fish kairomones, *Daphnia* reduce body size and

increase fecundity and reduce the amplitude of diurnal vertical migrations (Stibor 1992, Reede 1995, Latta et al. 2007).

In *C. reinhardtii* the adaptive phenotypically plastic response of colony formation falls under the category of inducible defences (Agrawal, 1988) and has been experimentally shown to occur rapidly within 25 h when cultured together with *B. calyciflorus* (Lurling & Beekman, 2006) and is not induced in the absence of predators, which is conceivable, considering the complexity of ruling out other experimental factors that may induce this phenomenon, the multitude of disadvantages and costs associated were the colony-formation-defence-mechanism a permanent morphological solution to predation, such as the selective pressure of resource acquisition favouring smaller sizes (Lehman, 1988), costly production of an ECM (Kohn et al. 2016) or enhanced sinking rate of colonies, increasing dispersal from the euphotic zone (Lurling & Van Donk, 2000). It has been shown, that *C. reinhardtii* may undergo vertical shifts of several meters due to kairomone-dependent phototaxis (Latta et al. 2009). Furthermore, results of Sathe & Durand (2016) show, that *C. reinhardtii* aggregates within 18-48 h in the presence of *Peranema* or its culture filtrate. Cue concentration likely indicates the level of risk (Ferrari et al. 2010). Hence, prey should use cue concentration to adjust the intensity of their antipredator avoidance in a threat-sensitive manner to optimize fitness in the trade-off between costly predator avoidance and fitness-related activities.

1.6. Introduction to the Calcium Experiments

I chose to investigate the response of the *C. reinhardtii* strains to Ca^{2+} depletion, substitution and chelation due to the fact that the studies aimed at investigating this phenomenon date back to 1966 – 1969 and have not been replicated but nonetheless been cited many times (e.g. in: Ellis, 1972; Lurling & Beekman, 2006; Khona et al. 2016).

The original incentive of observing the response of colony formation in *Chlamydomonas reinhardtii* to altered calcium concentrations was to investigate the nature of a postulated hatching enzyme. Unfortunately, there are no more than two publications following this line of thought, one from 1966, published as a dissertation by U. Schlösser in German, unavailable in English, and the other from 1969 by Kozo Iwasa & Shohachi

Murakami. Iwasa and Murakami found that media containing 12×10^{-5} M EDTA (a Ca^{2+} chelator) induced palmelloid formation and additionally observed palmelloid formation in calcium poor medium with a concentration of less than 3.5×10^{-6} M Ca^{2+} . Schlösser experimented with a *C. reinhardtii* mutant (*C. reinhardtii dystokos*) which was unable to release daughter cells from the common extracellular matrix. Schlösser postulated that there was a “Wirkfaktor”, an “operating factor”, to be responsible for the degradation of the cell wall that bound the daughter cells together. He found the factor responsible for the release for daughter cells constitutes a protein with enzyme-character that required either CaCl_2 or MgSO_4 in the medium to function. The factor dissolves the extracellular matrix even of the mutant cells (Fig. 4), is non-dialysable, heat-labile and pH-dependent and is only activated in the presence of divalent ions (Ca^{2+} , Mg^{2+}) and is deactivated by papain (a cysteine protease enzyme present in papaya and mountain papaya). Most notably, the factor does not initiate the dissolution of the cell walls of other green algae (Volvocales, Chlorococcales) species and the enzyme seems thus to be specific for *Chlamydomonas reinhardtii* (Schlösser, 1966).

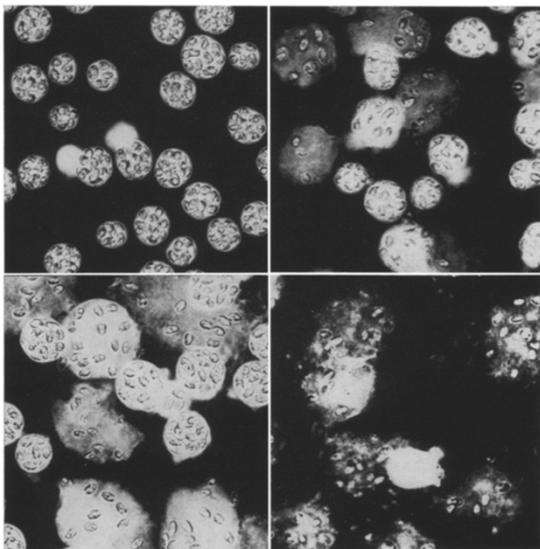


Fig. 4: Consecutive stages of the release of zoospores from sporangia of the *dystokis*-mutant *C. reinhardtii* clone / dissolution of the extracellular matrix, after addition of the “Wirkfaktor”, obtained by cultures of non-mutant *C. reinhardtii* cells. Adopted from (Schlösser, 1966).

1.7. Aims and Incentives

Observations of colony formation in *Chlamydomonas reinhardtii* due to inhibited desegregation of daughter cells in response to environmental variables has been investigated and documented in numerous studies (Khona et al. 2016; Cheloni et al. 2016; Sathe & Durand, 2016; Ratcliff et al. 2013; Lurling & Beekman, 2006; Iwasa & Murakami, 1968, 1969; Schösser, 1966). Some of these (and other) studies employ prefaces and introductions stating that the formation of palmelloids (*C. reinhardtii* colonies) constitutes an early stage during the major evolutionary transition from unicellular to multicellular life (Yamashita et al. 2016; Kianianmomeni, 2015; Miller, 2010; Olson & Nedelcu, 2016) which makes investigations into this phenomenon worthwhile. In this master thesis, I aim at quantitatively and qualitatively reproducing (and therefore consolidating or deconsolidating) experimental results regarding the formation of colonies in *C. reinhardtii*. I subject four different strains of this green algae to previously investigated ecological (environmental) treatment conditions that were previously shown to induce the aggregative phenotype. Accordingly, I subjected the algal cells to high levels of salinity, the absence/presence of calcium and to three different concentrations of *B. calyciflorus* infochemicals. The culture filtrate of *B. calyciflorus* was used in order to avoid potential selective feeding which may have been one reason for the observed palmelloid stage in the experiment conducted by Lurling & Beekman (2006). Different *C. reinhardtii* strains were used in this thesis to further investigate if there is strong genetic variability for the trait in question. Furthermore, a technique was used to produce a large amount of quantitative cell area measurements. This thesis therefore explores the feasibility of this method for investigations into colony formation in phytoplankton species as an induced defence in response to biotic or abiotic ecological conditions.

2. Material and Methods

2.1. Organisms

For the experiments, four different *C. reinhardtii* strains were obtained from the Chlamydomonas Resource Center. Prior to the succeeding experiments and since date of arrival, all four *C. reinhardtii* strains were cultured under controlled conditions as stock cultures in 250 ml tissue flasks (TC-Flasks T75, suspension) containing modified (M)sWC Medium for freshwater algae (**Table 4**, Guillard et al., 1972). The stock cultures were transferred every week into fresh sterile medium. 4 Osram 18 W Daylight lamps served as a continuous light source. No dark-light cycle was introduced. The temperature in the climate room where all experiments were conducted and the stock cultures were kept ranged from min. 21.47 °C to max. 24.16 °C (Mean: 23.80 °C). To simplify statistical analysis and comparison of the strains, the names of the four *C. reinhardtii* strains (**Table 3**) were given the following abbreviations (square brackets): CC-2932: [A], CC-2936 [B], CC-1010: [C], CC-4414: [D]. For simplicities sake, I will from here on refer to the four different *C. reinhardtii* strains either as Clone A, Clone B, Clone C, Clone D or simply A – D. *C. reinhardtii* cells are approximately 10 µm in length [Rochaix, 2001]. Cell area measurements in this thesis are given in square micrometer (µm²). A median (e.g. in **Fig. 12**) of e.g. 270 µm² corresponds to a cell diameter of ~ 20 µm² and a measurement of ~100 µm² corresponds to a cell diameter of ~ 11 µm respectively (see formulae below). It is important to keep in mind the excess fluorescence on the periphery of the cell(s) as being mentioned in **2.4.2**, but a value of 100 – 300 µm² may be interpreted as constituting a single *C. reinhardtii* cell.

$$\frac{100 \mu m^2}{\pi} = 31.83 \mu m^2; \sqrt{31.83 \mu m^2} = 5.64 \mu m; 5.64 \mu \times 2 = 11.28 \mu m$$

Table 3: *C. reinhardtii* algal strains used in all experiments*. Information regarding the original isolation (i.e., person, place and time) is given when available.

Strain	Information on Isolation
<i>Chlamydomonas reinhardtii</i> (Strain A) CC-4414 wild type mt ⁺ [DN2]	David Nelson, Lefebvre laboratory, University of Minnesota, Obtained by the Chlamydomonas resource center on: May 2011 Isolated from an environmental sample taken at 3,9624 km in Breckenridge, Colorado. The sample was collected Friday, August 10, 2007.
<i>Chlamydomonas reinhardtii</i> (Strain B) CC-2932 wild type mt ⁺ [North Carolina]	Elizabeth Harris, Chlamydomonas Genetics Center, Duke University, September 1993 Isolated from garden soil collected in Durham, North Carolina
<i>Chlamydomonas reinhardtii</i> (Strain C) CC-1010 wild type mt ⁺ [UTEX 90]	University of Texas, Algal Collection (UTEX), May 1980 Isolated in 1945 by G.M. Smith, near Amherst MA
<i>Chlamydomonas reinhardtii</i> (Strain D) CC-2936 wild type mt ⁺ [Quebec]	Graham Bell, McGill University, October 1993 Bell's isolate LEE-2 from Quebec

* All algae cultures were obtained from the Chlamydomonas Resource Center, Department of Plant and Microbial Biology at the University of Minnesota (www.chlamycollection.org).

Brachionus calyciflorus rotifer cysts were obtained from MicroBioTests Inc. (Nazareth, Belgium) and prior to hatching were stored at 4 °C in darkness. The cysts were obtained via rinsing two vials (each vial containing a minimum of 250 neonates according to distributor) with 1 ml (M)WC medium each and the cysts were subsequently transferred into a Petri dish containing 4 ml (M)WC medium. The cysts were kept in this condition at ca. 23 °C under constant fluorescent illumination of 3800 lux. After 24 h, most of the *B. calyciflorus* specimens hatched and were transferred into two 500 ml glass jars containing 350 ml (M)WC Medium. Hatchlings were fed the algae *Nannochloropsis sp.* concentrate (Nanno3600, Reed Mariculture Inc, Microalgae AS). The concentrate has an average of 0.27 mg total dry biomass per μl and a carbon content of 43.3 %, giving 0.12 mg C per μl of the concentrate (Catharina Broch, personal communication). Prior to experimentation, the *B. calyciflorus* cultures were subdivided into smaller batches of 250 μl each and after each second day, 100 ml of (M)WC medium and 200 μl of Nanno3600 were added. Nanno3600 medium was used as a substitute for (M)WC medium in all treatments (except control 1) in order to see if added *Nannochloropsis sp.* cells had an underlying effect on colony-formation and somehow altered the algal response. Additional to that, control cultures solely with (M)WC medium were set up in which the same amount of Nanno3600 concentrate as for the rotifer cultures was added every day. The control cultures served as medium for the controls. The jars were kept at ca. 23 °C, ca. 3800 lux light intensity and grown to a dense concentration prior to filtration.

2.2. Algal Media & Media Manipulation(s)

Distilled water (dH₂O) was used in all subsequent experiments and media preparations. To produce 5 litres of medium, a 5 litre Erlenmeyer flask was filled with 5 litres of dH₂O and placed on a stirring plate with a stirring bar placed at the bottom of the flask to assure homogenization. 0.575 g TES buffer was placed at the bottom of the Erlenmeyer flask prior to adding dH₂O. 5 ml of each of the prepared stock solutions (1 – 6) were added and the medium was mixed for 20 minutes. The medium was stored in a cold environment (4 °C). Vitamins and the 5 ml of the combined trace element mix (**Table 4**) were added when the medium had a temperature of ca. 4°C.

Table 4: Composition of algal growth medium: (M) WC (Modified WC Medium) for freshwater algae. This is the medium from which alterations for the subsequent experiments were deduced. The recipe is publicly available from the Culture Collection of Algae and Protozoa (Guillard R. R. L. & Lorenzen C. J., 1972).

Stock Chemicals	/ Litre
CaCl ₂ •2H ₂ O (1)	36.80 g
MgSO ₄ •7H ₂ O (2)	37.00 g
NaHCO ₃ (3)	12.60 g
K ₂ HPO ₄ •3H ₂ O (4)	11.40 g
NaNO ₃ (5)	85.00 g
Na ₂ O ₃ Si•5H ₂ O (6)	21.20 g
Combined trace elements	
Na ₂ •EDTA	4.36 g
FeCl ₃ •6H ₂ O	3.15 g
CuSO ₄ •5H ₂ O	0.01 g
ZnSO ₄ •7H ₂ O	0.022 g
CoCl ₂ •6H ₂ O	0.01 g
MnCl ₂ •4H ₂ O	0.18 g
Na ₂ MoO ₄ •2H ₂ O	0.006 g
H ₃ BO ₃	1.00 g
Vitamin Mix	
Thiamine HCL (C ₁₂ H ₁₈ C ₁₂ N ₄ OS)	0.1 g
Biotin (C ₁₀ H ₁₆ N ₂ O ₃ S)	0.0005 g
Cyanocobalamin (C ₆₃ H ₈₈ CoN ₁₄ O ₁₄ P)	0.0005 g
Buffer	
TES*	0.115 g

*TES = N-Tris (hydroxymethyl)-methyl-2-aminoethane-sulphonic acid (C₆H₁₅NO₆S); Sigma T-1375

2.2.1. Experimental setup

In all subsequent experiments, the four different algae strains were grown in 250 ml suspension tissue culture flasks (Tissue culture flask T-75; Sarstedt AG & Co) with a hydrophobic growth surface to assure suspension and to prevent algal wall growth. The cultures were bubbled with a continuous flow of sterile air to assure the prevention of CO₂-limitation and to enhance mixing in order to prevent algal settlement (**Fig. 5**). The culture flasks were continuously illuminated from below with two Osram Colour Proof Daylight lamps (L18W/950-daylight, Osram Licht AG). Irradiance [W/m²]: 15.99. Photon flux density [74.2 μmol quanta /m²/s]. Luminous flux [lux]: 4816.6. The flasks used contained 175 ml of dH₂O. The conduits connecting the aquarium air pumps with the culture flasks were made using low-volatile grade, platinum-cured silicon tubes (1.5 x 3 mm, 2 x 4 mm, 3 x 5 mm, VWR International). Cannulas (Kanyler, 2.0x80mm, plastansats, Jan F. Andersen AS) were used to connect the tubes from flasks to flasks.

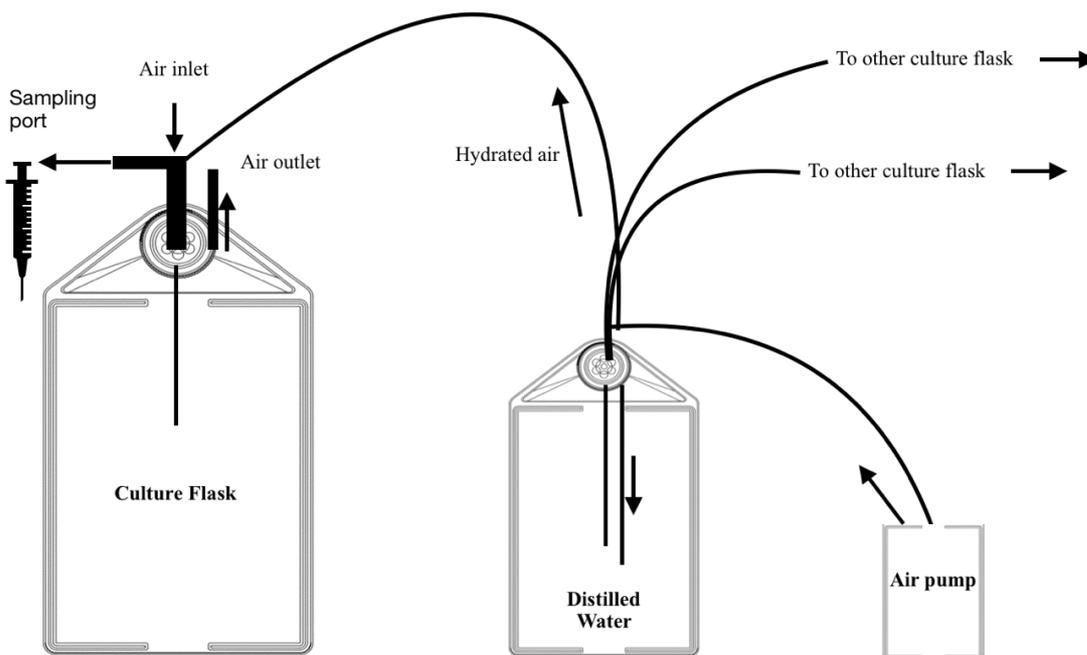


Fig. 5: Experimental setup scheme as being used in all subsequent experiments [personal communication: Catharina Broch].

Inoculation of the four different algal strains in their respective treatment media was conducted as follows: 3 ml of the original algae cultures were transferred into 200 ml centrifuge tubes (Falcon®, Corning Life Sciences, VWR International) and centrifuged for 5 min at 1100 rpm at 20 °C. The remaining medium was discarded and the algal pellets

were washed and re-suspended in 1 ml fresh (M)WC medium (or the respective treatment media) prior to transferring the cells into their respective treatment flasks. Prior to this procedure, cell densities of the original cultures were measured.

2.2.2. Salinity Experiment

To test whether the four different strains of unicellular *C. reinhardtii* exhibit the plastic aggregate response triggered by elevated levels of ambient salinity, independent triplicate cultures were grown in four different salinity concentration levels. Triplicate control cultures for each strain were grown in modified WC medium (Table 4). The triplicate cultures of each *C. reinhardtii* strain were reared in either 50 mM, 100 mM or 200 mM NaCl enriched (M) WC medium. To obtain different (M) WC media with varying salinity concentrations a 5 M NaCl stock solution was prepared by weighing 292.2 g⁴ sodium chloride (ACS, Reag. Ph. EVR, VWR Chemicals, Density: 2.16 $\frac{kg}{L}$) and dissolving it overnight in 1 L dH₂O via continuous stirring to assure homogenization. For the 50 mM NaCl medium, 2.5 ml of the 5 M stock solution were added to 250 ml of (M) WC medium. For the 100 mM NaCl medium, 5 ml of the 5 M stock solution were added to 250 ml of (M) WC medium. For the 200 mM NaCl medium, 10 ml of the 5 M stock solution were added to 250 ml of (M) WC medium. The following formula was used to calculate the volumes needed to obtain the varying treatment media prior to algal inoculation: $c1 \times v1 = c2 \times v2$.

E.g., 250 ml 200 mM (0.2 M) NaCl (M) WC medium from a 5 M NaCl stock solution:

$$5 M \times v1 = 0.2 M \times 250 ml$$

$$v1 = \frac{0.2 M \times 250 ml}{5 M}$$

$$v1 = 10 ml$$

Fresh water osmolality is typically below 7 mOsm, whereas the cytosolic osmolality of fresh water protozoa is estimated to be 45-117 mOsm and in *C. reinhardtii* it has been

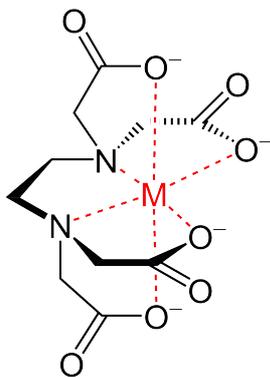
⁴ NaCl solubility in H₂O: 359 $\frac{g}{L}$. Molecular mass: 58.44 g (Na⁺ 22.990 g, Cl⁻ 35.453 g).

measured as 170 mOsm (Allen et al., 2002; Komsic-Buchmann et al., 2014). The osmolarities of 50 mM, 100 mM and 200 mM sodium chloride media as used in this experiment are therefore: 100 mOsm, 200 mOsm and 400 mOsm respectively⁵, assuming the osmolarity of the (M) WC medium to be the baseline.

2.2.3. Calcium Experiment(s)

To test the influence of the presence/absence of the Calcium ion (Ca^{2+}) on the plastic aggregation response of *C. reinhardtii* single cells, two media-manipulation experiments were conducted. Prior to deciding if a large-scale experiment with triplicate batch cultures was worth investigating, the response of *C. reinhardtii* to various media manipulations was explored using a single replicate setup. *C. reinhardtii* clones were reared in four different versions of the (M) WC medium: control medium (+ CaCl_2), calcium-deficient medium (- CaCl_2), calcium-deficient medium with MgCl_2 as a substitution (- CaCl_2 , + MgCl_2) and calcium-enriched medium with EDTA as a calcium ion chelator (Fig. 6).

Fig. 6: Metal(M)-EDTA complex.



Ethylenediaminetetraacetic acid (EDTA) is a hexadentate ligand and chelating agent and has the ability to sequester metal ions such as Ca^{2+} and Fe^{3+} by binding to the metal cation through two amines and four carboxylate groups. After being bound by EDTA into a metal complex, metal ions remain in solution but exhibit diminished reactivity [Caldwell P. C., 1970].

Picture from: <https://upload.wikimedia.org/wikipedia/commons/thumb/0/09/Metal-EDTA.svg/150px-Metal-EDTA.svg.png>

For medium lacking Ca^{2+} , CaCl_2 was left out of the chemicals added to dH_2O as shown in **Table 4**. 0.2503 M CaCl_2 was added to the medium containing no CaCl_2 but aimed at

⁵ Osmolarity is calculated by multiplying the molarity by the number of osmoles that each solute produces. In the case of NaCl, the complete dissociation in water to form Na^+ and Cl^- ions makes for an osmolarity of 2 Osmol/L in a 1 mol/L NaCl solution. (An osmole (Osmol) is 1 mol of particles that contribute to the osmotic pressure of a solution).

exhibiting balanced ionic strength and to investigate if calcium substitution has an effect on *C. reinhardtii* growth and palmelloid formation. Equal molarities of EDTA and CaCl₂ were combined for the chelation medium. The modified WC medium has a CaCl₂·2H₂O molarity of 0.2503 M per litre. Thus, 73.06 g EDTA were weighed and added to the TES buffer at the beginning of the media-making process to assure equal CaCl₂ and EDTA molarities (0.2503 M) in the final medium. The concentration and measurements for CaCl₂ are described in **Table 4**.

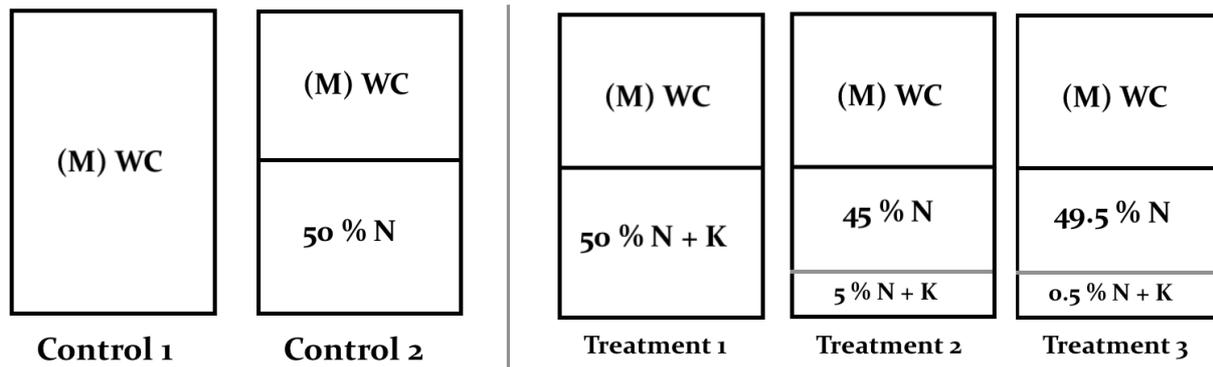
The second calcium-manipulation experiment was primarily based on the paper *Palmelloid Formation of Chlamydomonas II. Mechanism of Palmelloid Formation by Organic Acids* by Kozo Iwasa and Shohachi Murakami (Iwasa & Murakami, 1969). To verify their findings that *C. reinhardtii* forms aggregate colonies when grown in media containing certain amounts of EDTA or in media low in Ca²⁺, the four different *C. reinhardtii* clones used in this experiment were reared in media containing 12.5 μM EDTA and 68 μM CaCl₂ and in media containing solely 68 μM CaCl₂. Control cultures with un-altered (M) WC medium were set up. A 0.5 M EDTA stock solution was prepared by weighing 8.76 g EDTA in 60 ml dH₂O. The pH was steadily altered towards higher alkalinity by adding sodium hydroxide (NaOH) until the solution reached a pH of 8.0 in order for the EDTA to fully dissolve overnight. 250 μl (0.25 ml) of the 0.5 M EDTA stock solution were added to 1 L of (M) WC medium containing 68 μM CaCl₂. A 0.25 M CaCl₂ stock solution was prepared by weighing 6.9375 g of CaCl₂ in 250 ml dH₂O. To obtain (M) WC medium with 68 μM CaCl₂, 272 μl (0.272 ml) were added to the medium initially devoid of calcium.

The observation that *C. reinhardtii* readily forms cell aggregates due to inhibited desegregation of daughter cells in medium devoid of calcium or when supplemented with EDTA, has been cited many times, for example in Khona et al. (2016); Lurling & Beekman (2006); Ratcliff et al. (2013) and Cheloni et al. (2016), indicating that the aggregative response of *C. reinhardtii* as a response to these conditions is conceptualized as a well-established fact on which further investigations build on. The experimental methodologies and results presented here aimed at (i) investigating the influence of calcium chelation, calcium depletion and calcium substitution on the aggregative

response of *C. reinhardtii* and (ii) at the reproduction and verification of the experimental results originally conceived by Kozo Iwasa & Shohachi Murakami (1968, 1969). If a postulated hatching protein is calcium- or magnesium dependent (Schlösser, 1966), then *C. reinhardtii* cells in a medium devoid of calcium (either by non-addition, chelation or substitution) should theoretically form colonies due to the inability to release daughter cells from the ECM formed by the mother cell.

2.2.4. Kairomone Experiment

14-day-old *B. calyciflorus* cultures and control media solely containing *Nannochloropsis* sp. (as food source, Nanno3600) were collected and filtered to remove specimens, algal cells and any other debris present in the media which size exceeds 0.45 μm . Two filtration steps were conducted: *B. calyciflorus* medium and Nanno3600 medium were first filtered through a 1.2 μm mesh-size glass microfiber filter (4.7 cm, GF/C, Whatman, Balston LTD) suitable for a collection of suspended solids in potable water and natural and industrial wastes. As a second step, the filtered media were filtered again through a 0.45 μm CA Low Protein Binding filter (Corning Incorporated Life Sciences, Lowell, MA) under a laboratory bench. Both filtration processes were conducted using suction filtration at low vacuum. The filtrates were collected in fresh, sterile media bottles. The day of filtration was the same day as experimental inoculation of algal strains in their respective treatment media to minimize risk of contamination and kairomone degradation. Rotifer concentrations were measured one hour prior filtration. The rotifer concentrations were estimated to about 14 specimens per ml ($n = 3$, $sd = 2.16$).



Treatment Fig. 7: 50 %, 5 %, 0.5 % Kairomone Filtrate set-up: **Control 1:** 100 % (M) WC Medium; **Control 2:** 50 % (M) WC Medium + 50 % (M) WC Medium & Nanno3600 Medium; **Treatment 1:** 50 % (M) WC Medium + 50 % Kairomone Filtrate; **Treatment 2:** 50 % (M) WC Medium + 45 % (M) WC Medium + Nanno3600 Medium + 5 % Kairomone Filtrate; **3:** 50 % (M) WC Medium + 49.5 % (M) WC Medium + Nanno3600 Medium + 0.5 % Kairomone Filtrate. Triplicate cultures for each of the four *C. reinhardtii* strains and triplicate control cultures (1 and 2) were reared in conditions as described above.

Van Donk et al. (2011) stated that different strains of the same algal species showed varying responses to grazing pressure. To understand the ecology of phytoplankton defences it is advised to learn much more about such diversity and variation in the responses of different genotypes (strains), which is the reason why four different strains of *C. reinhardtii* are being used in this experiment (Van Donk et al. 2011). Grosberg & Strathmann (2007) argued that phagotrophic microorganisms consuming unicellular prey were probably the most important selective pressure exerted for the origin of multicellularity. Solari et al. (2015) showed that predation can be a strong selective pressure for the origin of the first cell clusters. When the predator *P. tricophorum* was present, colonial *G. pectoral* had a lower predation rate than unicellular *C. reinhardtii*, and there was an increase in the proportion of larger colonies in *G. pectoral* populations (Solari et al 2015). The experimental methodologies and results presented here investigated the response of four *C. reinhardtii* strains to culture filtrate of *B. calyciflorus* at three different infochemical concentrations.

2.3. Experimental parameters

2.3.1. Sampling procedure

In order to measure algal densities and cell areas of the *C. reinhardtii* strains in their respective culture flasks, 1 ml of each flask's content was sampled using disposable syringes (5ml, Norm-Ject® Luer Slip Bulk Syringe, Air-Tite) via the sampling port shown in **Fig. 5**. Samples were taken 1 h, 24 h and 48 h (or 1 h, 24 h, 48 h, 72 h, 96 h for the first calcium experiment) after inoculation of the algal strains in the respective manipulated media. The 1 ml samples were transferred into the wells of a 24-Well cell culture plate (Falcon™ Polystyrene Microplates, Thermo Fisher Scientific). Prior to measurements, the samples were further partitioned into duplicate technical replicates (**see 2.3.1.1.**) onto a 96-well culture plate (Nunclon™, Delta 96-Well MicroWell™ Plates, Sterile, Thermo Scientific, VWR). The 96-well plates were placed in a microwave and heated for 2 min at power 7500 (ca. 55 °C) to assure immobility and possibly cell death and afterwards centrifuged for 1 min at 1500 rpm at 20 °C to assure that the cells were located at the bottom of the wells such that the cells are stationary and lie at a similar focal level. To assure an equal volume in all wells containing algae samples, dH₂O was added to each well such that each well contained 200 µl in total volume. Samples were analysed using an imaging cytometer (iCys Research Imaging Cytometer, CompuCyte, Massachusetts, USA).

2.3.1.1. Technical Replicates

The 1 ml samples in the 24-Well cell culture plates were further portioned into 50 μ l and 100 μ l or 50 μ l and 20 μ l duplicate technical replicates to safeguard against saturation of the cell counts caused by cells overlapping (and thus be counted as 1 cell with twice the area) since this would give a false positive aggregation. **Fig. 8** shows cell counts per 50 and 100 μ l plotted against each other and gives a good indication that saturation was not a problem.

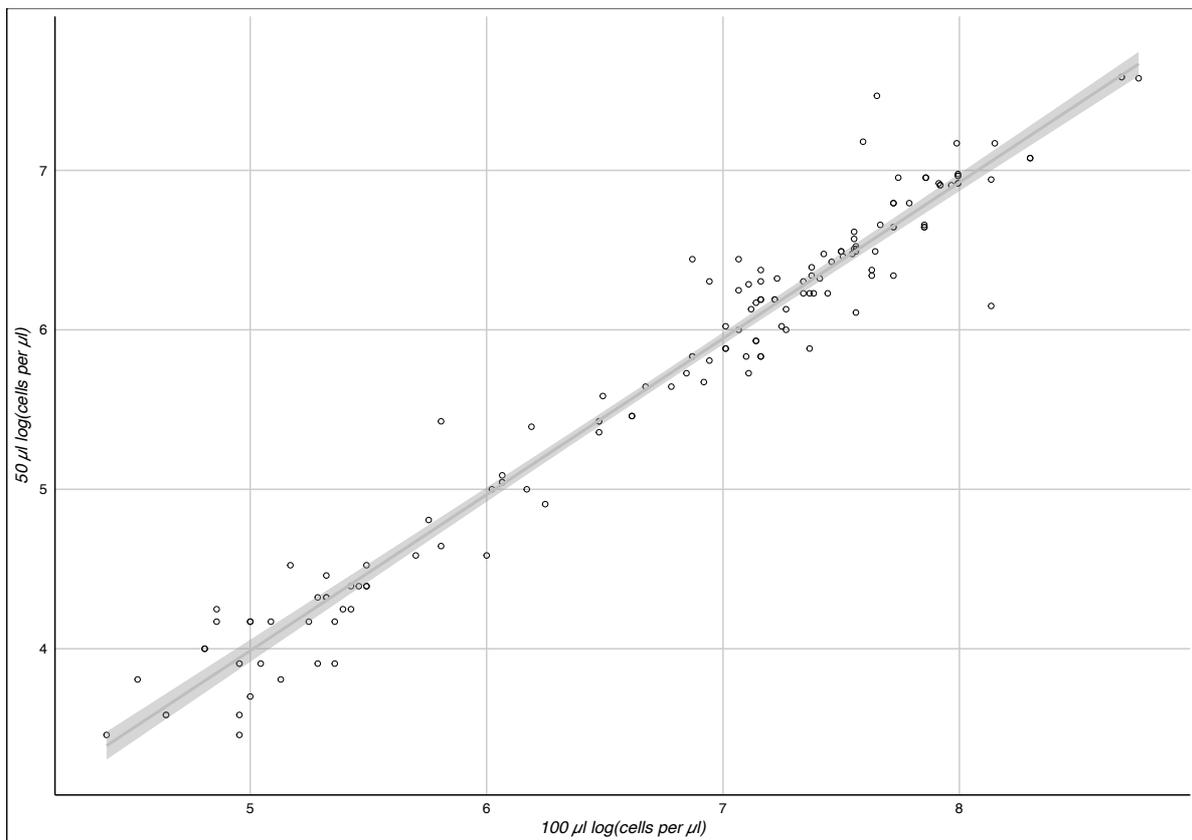


Fig. 8. To assess whether the use of different technical replicates has an influence on the quantity of cells and possibly on the cell area distributions in the analysed samples, cells per 50 μ l and per 100 μ l were plotted against each other. The graph indicates that there is a coherent relationship and that using different technical replicates in the method seems to be unproblematic.

2.3.2. Cell Area measurement

Empirical measurements regarding algal abundance and size of single cells and colony-sized cell aggregates were obtained using an imaging cytometer (iCys Research Imaging Cytometer, CompuCyte, Massachusetts, USA). The cytometer makes use of laser technology and photomultiplier detectors to measure various properties of cells based on their fluorescent characteristics. To excite chlorophyll a, a specific form of chlorophyll, abundant in the large cup-shaped chloroplasts of *C. reinhardtii* (Sahoo & Seckbach, 2015), a 488 nm argon ion laser was used. The automated scanning procedure of the cytometer allowed for the collection of data for multiple sets of cell samples. The scanning protocol for 96-well plates collects data on cell properties from each well by scanning an area of 0.77 mm². The same scanning protocol was used throughout all experiments. The data obtained by the scanning procedure contains the variable area, which essentially represents the area in square microns (μ^2) for each observed event. Briefly, the imaging cytometer produces estimates of the area of each cell or cell aggregate in the scanning field based on the fluorescence emitted by the event. One problematic is, that the fluorescence signal comes from the chloroplast(s) which, although cup-shaped and occupy a large space in *C. reinhardtii* cells, is not equivalent to the whole cell volume.

2.3.3. Microscopy

To visualize the emergence of algal colonies, pictures of the 1 ml samples obtained from the culture flasks were taken 1 h, 24 h and 48 h post inoculation. Pictures were taken with a Nikon Eclipse TS100 inverted routine microscope (Nikon Instruments) at 20x or 40x magnification depending on the algal densities in the well. Prior to microscopy analysis, the culture plate was spun for 1 min at 1500 rpm, 20 T °C to assure algal abundance at the bottom of the wells. **Figures 30 – 35** (Appendix).

2.3.4. Temperature, conductivity and pH

Temperature was continuously measured during experiments with an automated temperature logger submerged in 250 ml dH₂O and placed next to the culture flasks.

The temperature logger was placed in water to assure the accurate representation of the temperature conditions in the culture flasks (appendix). Electrical conductivity or specific conductance (measurement of a material's ability to conduct an electrical current, bearing the SI unit siemens per metre (S/m)) was measured using a B-771 LAQUAtwin Compact Conductivity meter (Horiba Scientific). The pH of the culture media was measured using a B-711 LAQUAtwin Compact pH meter (Horiba Scientific). The possibility that temperature or pH, fundamental parameters of fresh- and saltwater environments, had any influence on the experimental results were mitigated by measuring both parameters. Conductivity measurements (for the salinity experiment) and pH measurements can be found in the appendix (**Fig. 25 – Fig. 29**).

2.3.5. Specific Growth rate

Cell density calculations from the iCys raw data were used to calculate algal growth rates. The specific growth rate, S_{Gr}^* , is calculated as follows (Shuler & Kargi, 2002):

$$S_{Gr} = \frac{1}{x} \frac{dx}{dt}$$

where x is the concentration of algae (cells/ml) and t is the culture time. Hence, from concentration over time calculations, the specific growth rate can be determined by:

$$S_{Gr} = \frac{\ln\left(\frac{x}{x_0}\right)}{t}$$

S_{Gr} per day is calculated by: $S_{Gr} \times 24$

* The symbol S_{Gr} instead of μ is used to denote the specific growth rate since μ is frequently used in this thesis to denote microns.

2.3.6. Data Analysis

Mean values of the algal counts from the technical replicates per culture replica were used as an estimate for the algal densities of each continuous culture, whereas means, medians, quantiles from the same replicas were used. To better depict the distribution and presence of single celled and cell aggregates over time and per treatment, the median rather than the mean was used for further analysis since the calculated mean value of a set of recorded area values tended to skew the data (probably due to cell aggregates localized at the edges of the well due to the centrifugation process). When data are skewed, the median of the dataset may provide a more appropriate measure of central tendency (Hector, 2015). For each group of samples corresponding to the different treatments (salinity and presence/absence of infochemicals), a two-factor ANOVA as well as factor interaction effects was computed. The simplest data-driven model building approach is called forward selection. In this approach, one adds variables to the model one at a time. At each step, each variable that is not already in the model is tested for inclusion in the model. The most significant of these variables is added to the model, so long as it's P-value is below some pre-set level (in this case: > 0.05). This concept has drawbacks, including the fact that each addition of a new variable may render one or more of the already included variables non-significant. An alternate approach which avoids this is backward selection, the approach used here. Under this approach, one starts with fitting a model with all the variables of interest. Then the least significant variable is dropped, so long as it is not significant at the chosen critical level. One continues by successively re-fitting reduced models and applying the same rule until all remaining variables are statistically significant [<https://www.stat.ubc.ca/~rollin/teach/643wo4/lec/node41.html>].

All data analysis and visualization was done in the R Studio programming environment (R Core Team 2017, Version 1.0.136) and the Microsoft Excel environment (Microsoft® Excel for Mac, Version 15.13.3). Microscopy pictures were assembled in the LaTeX TeXShop editor environment for macOS (Version 3.82).

3. Results

3.1. Salinity Experiment

The addition of sodium chloride affected the growth of the freshwater algae *C. reinhardtii* (**Fig. 9**). There were notable differences regarding the growth of strain B and D between control cultures and cultures exposed to NaCl concentrations > 100 mM, whereby strains in the treatment cultures showed halted growth (**Fig. 9**). Contrastingly, no such differences for strains A and C were observed, suggesting that they are more halotolerant. This is reflected in their specific growth rates (**Table 5**). Palmelloid formation was clearly observed in all strains after 48 hours in the 50 and 100 mM salinity treatments, underlining that an aggregative response may be observed independent of which strain was used in the experiments (**Fig. 10, Table 6**). Palmelloids were not observed in the control (0 mM) and highest (200 mM) salinity conditions which was confirmed by microscopy images (**Fig. 27, Fig. 28, Fig. 29 - appendix**).

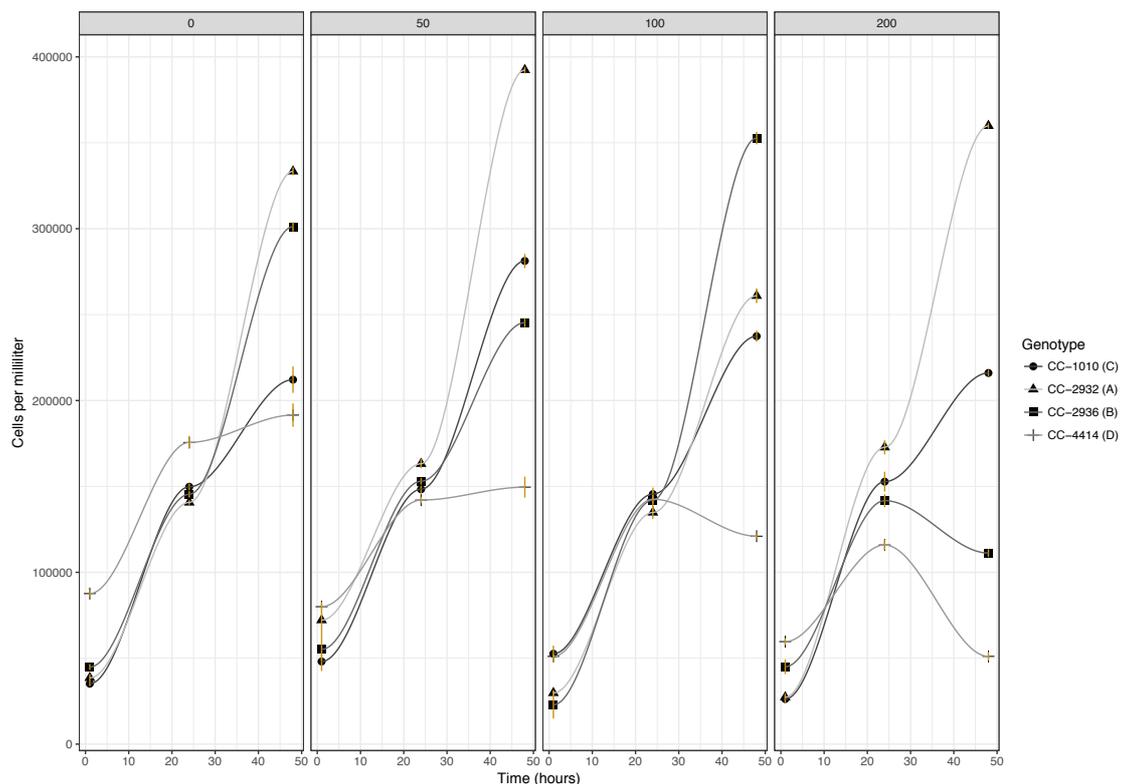


Fig. 9. The estimated cell densities (expressed as cells per ml) of four *C. reinhardtii* strains exposed to different sodium chloride concentrations (expressed as millimolar (mM) NaCl) after 1 hour, 24 hours and 48 hours of exposure. Data points represent the arithmetic mean of n=3 culture replicates of each strain and treatment. Vertical yellow lines represent the standard errors of the mean (max: ± 10137 cells per millilitre, min: ± 1111 cells per millilitre). Smoothing method: loess (since: < 1.000 observations).

Table 5: Specific growth rates (S_{Gr}/day) for *C. reinhardtii* strains reared in the respective osmotic culture conditions. Calculated between 1 hour and 24 hours. \pm gives the standard error of the mean calculated from three culture replicates. For formulae, see p. 41.

S_{Gr} per day / Strain	Treatment			
	0 mM	50 mM	100 mM	200 mM
S_{Gr} A	1.29 ± 0.05	1.11 ± 0.04	1.43 ± 0.05	1.31 ± 0.06
S_{Gr} B	1.14 ± 0.04	0.87 ± 0.03	1.16 ± 0.04	0.43 ± 0.01
S_{Gr} C	0.91 ± 0.03	0.87 ± 0.03	0.93 ± 0.04	1.04 ± 0.01
S_{Gr} D	0.408 ± 0.02	0.29 ± 0.03	0.36 ± 0.04	0.11 ± 0.02

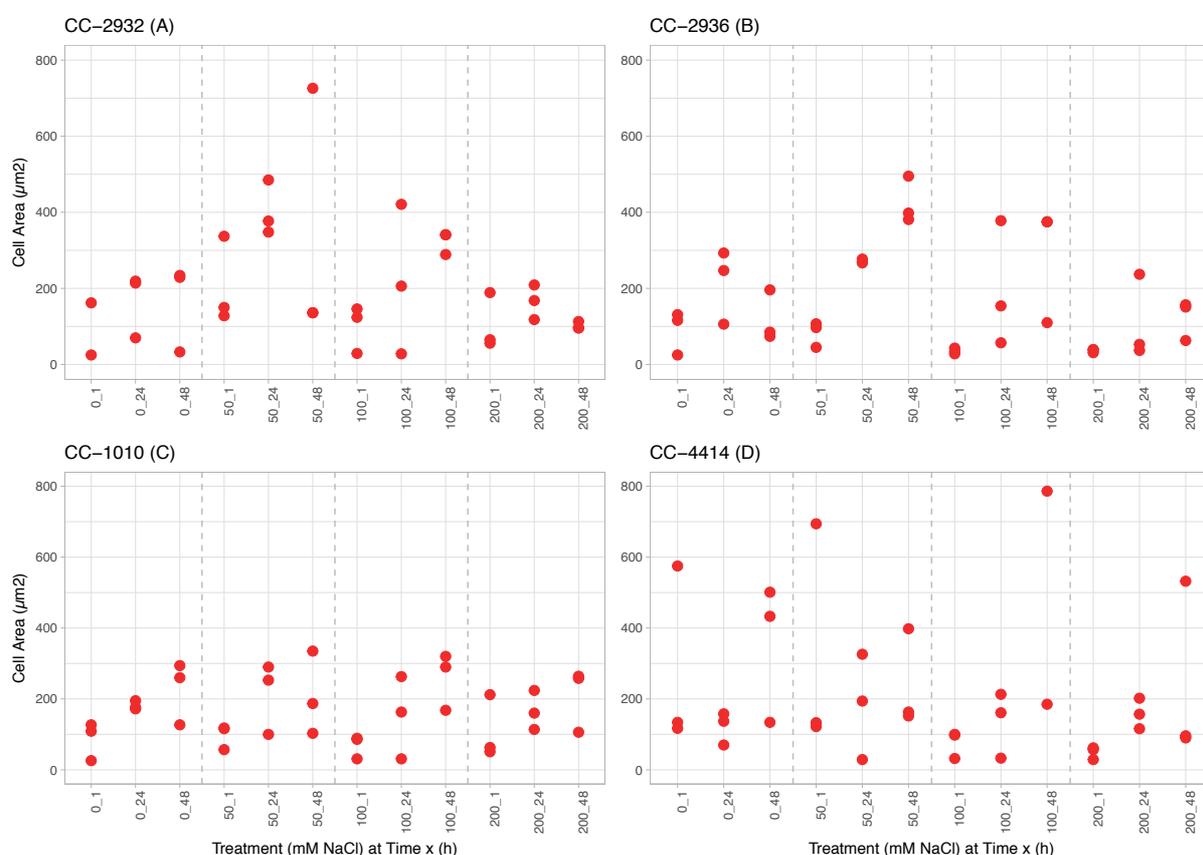


Fig. 10: Area medians (expressed as μm^2) of four *C. reinhardtii* strains grown in triplicate cultures at each salinity concentration at each time (h) of measurement post exposure (red points). For data distributions and boxplots showing the median values depicted here, see **Figure 31** in the appendix. E.g. CC-1010 (C) 50_48 conveys the information that this measurement was done for strain C in the 50 mM NaCl treatment after 48 hours of exposure.

Table 6: Two-way ANOVA Summary Table for Response Variable *Area Median (48 hours)*

Source	<i>df</i>	<i>F</i>	<i>p</i>	SS	pR^2
Treatment (A)	3	3.19	0.03186	284064	0.1725
Strain (B)	3	1.66	0.18036	147871	0.0898
A x B Interaction	9	1.18	0.33815	303440	0.1842
Residuals	32	-	-	911181	0.5533

Note. – *df* = degrees of freedom, *F* = *F*-values, *p* = significance probability of *F*-values, *SS* = sum of squares, pR^2 = partial R^2 (fraction of total variance explained). The data constitutes recorded area values (medians) for all strains after 48 hours of treatment.

The medians from the recorded distributions of detected *C. reinhardtii* cells (red points in: **Fig. 31** – appendix and **Fig. 10**) were used for statistical analysis. **Table 6** presents the results of a two-way ANOVA for medians recorded after 48 hours of treatment exposure. This measurement point was chosen for analysis since the most prominent palmelloid responses were observed 48 hours (all strains showed palmelloid formation). There was a statistically significant main effect for treatment, while no significant main effect for strain (genotype) was found, indicating that strains responded similarly to the treatments (**Table 6**). Furthermore, a two-way ANOVA for medians after 24 hours of treatment exposure was calculated but is not presented in tabloid form. In this analysis, as in **table 6**, a significant main effect for treatment ($p = 0.035$) but not for strain or for an interaction effect (p 's= non-significant) was found.

Strain A showed palmelloid formation in the 100 mM treatment after 1 hour and in the 50 mM treatment after 48 hours (**Fig. 27, 29** - appendix). This is partly reflected in **Fig. 10**, except that area values for the 100 mM treatment after one hour are approximately $175 \mu\text{m}^2$ which would not be considered to constitute palmelloids. Strain B also showed palmelloid formation after 48 hours in the 50 mM treatment, but not in the 100 mM treatment (**Fig. 27, Fig. 28, Fig. 29** - appendix) which is also reflected in **Fig. 10** in which high median values were recorded in this treatment after 48 hours. Similar to strains A and B, strain C showed pronounced palmelloid formation in the 50 mM treatment after 48 hours (**Fig. 29** – appendix) and also after 24 hours (**Fig. 28** – appendix) supported by highest recorded median values in the 50 mM treatment after 48 hours and slightly

lower median values after 24 hours (**Fig. 28, 29** - appendix, **Fig. 10**). Furthermore, strain C showed lesser pronounced palmelloids in the 100 mM treatment after 48 hours (**Fig. 29** - appendix). Similar to the other three stains, strain D showed palmelloid formation after 48 hours in the 50 mM treatment (**Fig. 25** - appendix) but not after 24 hours and in any other of the treatments (**Fig. 27, Fig. 28, Fig. 29** - appendix). For strain D, **Fig. 10** is inconclusive due the amount of high outlying median values (e.g. in 100 mM after 48 hours and 0 mM after 48 hours) such that **Fig. 27 – Fig. 29** were predominantly used for interpretation.

None of the strains showed palmelloid formation in the control and 200 mM treatments, indicating that a NaCl concentration equal or above 200 mM exceed the organism's capacity to respond to the osmotic conditions via the formation of colonies. Low median values in the 200 mM treatments also reflect the low specific growth rates (**Table 5, Fig. 10**).

3.2. Kairomone Experiment

The addition of *B. calyciflorus* culture filtrate did not seem to affect the growth of the freshwater algae *C. reinhardtii* (Fig. 11). It is difficult to be fully certain that the difference in filtrate concentration or another factor was the cause of increased growth of strain A in the 50 % filtrate treatment when compared other treatments (including control 1 and 2, Fig. 11) even though the strain was reared in identical culture conditions (Table 7). Surprisingly, no palmelloid formation could be observed in either of the strains independent of the duration of the experiments and independent of *B. calyciflorus* filtrate concentration in the culture medium (Fig. 12). This is clearly reflected in the microscopy pictures in which no palmelloid colonies were to be observed (Fig. 24, Fig. 25, Fig. 26 - appendix).

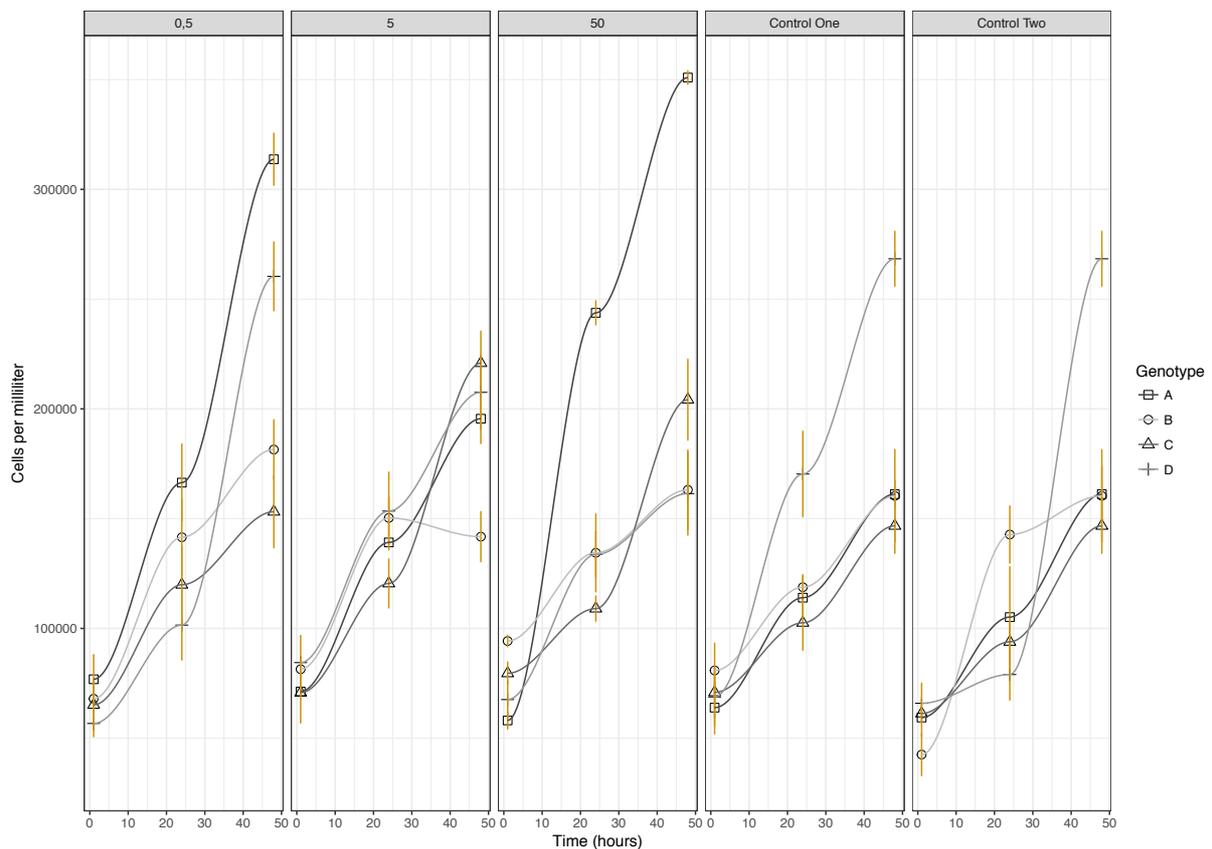


Fig. 11: The estimated cell densities (expressed as cells per ml) after 1 hour, 24 hours and 48 hours in the cultures of four *C. reinhardtii* strains reared either in Treatment 1 (50 % filtrate), Treatment 2 (5 % filtrate), Treatment 3 (0.5 % filtrate), Control 1 (WC) or Control 2 (50 % WC, 50 % N): See Material & Methods, p. 37, Fig. 6). Data points represent the arithmetic mean of n=3 culture replicates of each strain for each treatment. Vertical yellow lines represent the standard errors of the mean (max: ± 23008 cells per millilitre min: ± 1201 cells per millilitre). Smoothing method: loess (since: < 1.000 observations).

Table 7: Specific growth rates (S_{Gr}/day) for *C. reinhardtii* strains reared in the five different treatment cultures. Calculated between 1 hour and 24 hours and 24 hours and 48 hours. \pm gives the standard error of the mean calculated from three culture replicates. For exact treatment explanations, see p. 37, **Fig. 6** and for formulae, see p. 41.

S_{Gr} per day / Strain	Treatment				
	50 %	5 %	0.5 %	Control 1	Control 2
S_{Gr} A 24 h	1.43 ± 0.01	0.66 ± 0.02	0.76 ± 0.01	0.57 ± 0.02	0.56 ± 0.01
S_{Gr} B 24 h	0.35 ± 0.03	0.61 ± 0.01	0.73 ± 0.004	0.38 ± 0.02	1.21 ± 0.05
S_{Gr} C 24 h	0.31 ± 0.01	0.53 ± 0.01	0.61 ± 0.02	0.36 ± 0.01	0.42 ± 0.02
S_{Gr} D 24 h	0.68 ± 0.05	0.59 ± 0.01	0.58 ± 0.01	0.91 ± 0.01	0.18 ± 0.03
S_{Gr} A 48 h	0.63 ± 0.03	0.33 ± 0.02	0.63 ± 0.1	0.34 ± 0.04	0.42 ± 0.03
S_{Gr} B 48 h	0.19 ± 0.01	0.05 ± 0.01	0.24 ± 0.05	0.30 ± 0.02	0.11 ± 0.02
S_{Gr} C 48 h	0.24 ± 0.06	0.61 ± 0.05	0.23 ± 0.02	0.25 ± 0.02	0.44 ± 0.07
S_{Gr} D 48 h	0.19 ± 0.04	0.30 ± 0.01	0.94 ± 0.06	0.45 ± 0.03	1.22 ± 0.08

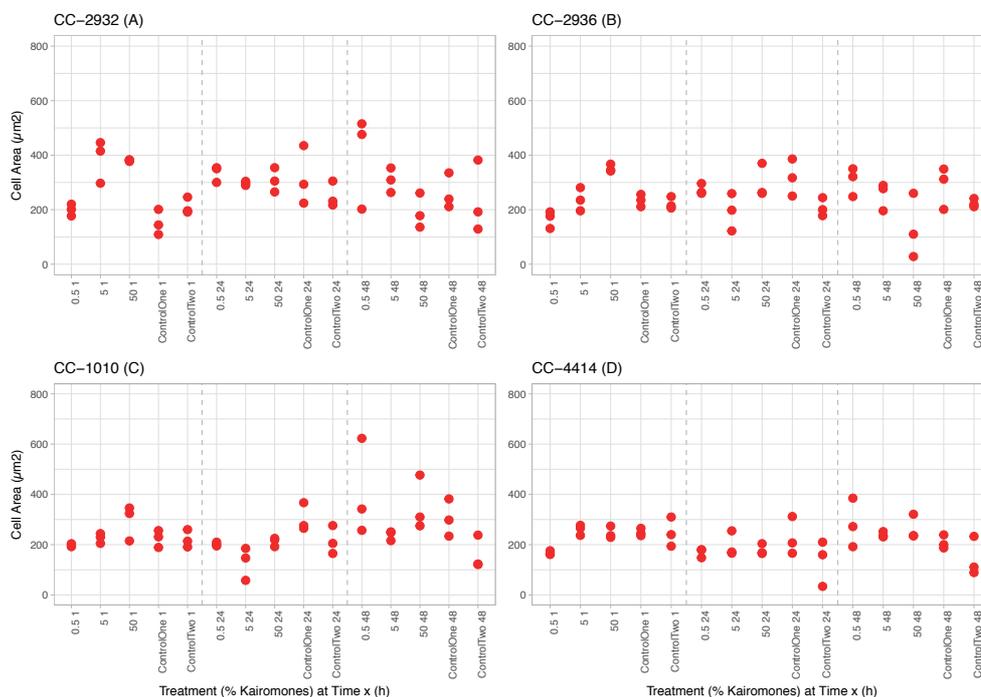


Fig. 12. Area medians (expressed as μm^2 , shown in red) of four *C. reinhardtii* strains grown in triplicate cultures at three different *B. calyciflorus* medium filtrate concentrations (%) and two control treatments (1: 100 % WC medium, 2: 50 % WC + 50 % N medium, see **Fig. 6**, p. 37), measured over time (h). For data distribution and boxplots showing the median values depicted here, see **Figure 32** in the appendix. E.g. CC-1010 (C) 0.5 24 conveys the information that this measurement was done for strain C in the 0.5 % filtrate treatment after 24 hours of exposure.

Table 8: Two-way ANOVA Summary Table for Response Variable *Area Median (48 hours)*

Source	<i>df</i>	<i>F</i>	<i>p</i>	SS	pR^2
Treatment (A)	4	4.99	0.00299	159238	0.24887
Strain (B)	3	1.78	0.166253	42626	0.06660
A x B Interaction	12	1.24	0.289351	118976	0.185944
Residuals	40	-	-	319014	0.49858

Note. – *df* = degrees of freedom, *F* = *F*-values, *p* = significance probability of *F*-values, SS = sum of squares, pR^2 = partial R^2 (fraction of total variance explained). Data constitutes recorded area values (medians) for all strains after 48 hours of treatment.

Table 9: Two-way ANOVA Summary Table for Response Variable *Area Median (24 hours)*

Source	<i>df</i>	<i>F</i>	<i>p</i>	SS	pR^2
Treatment (A)	4	5.49	0.001274	65531	0.18665
Strain (B)	3	13.8	0.000002	123852	0.35276
A x B Interaction	12	1.18	0.326247	42413	0.12080
Residuals	40	-	-	119294	0.33978

Note. – *df* = degrees of freedom, *F* = *F*-values, *p* = significance probability of *F*-values, SS = sum of squares, pR^2 = partial R^2 (fraction of total variance explained). Data constitutes recorded area values (medians) for all strains after 48 hours of treatment.

Likewise, to the salinity experiment, the medians from the recorded distributions of detected *C. reinhardtii* cells (red points in: **Fig. 32** – appendix and **Fig. 12**) were used for statistical analysis. **Table 8** presents the results of a two-way ANOVA for medians recorded after 48 hours of treatment exposure. This measurement point was chosen for analysis since it was also used in statistical analysis of the salinity experiment. There was a statistically significant main effect for treatment after 48 hours of treatment exposure (**Table 8**). No main effect for strain (genotype) was found (**Table 8**) and no statistically significant interaction effect was found and therefore excluded from the linear model analysis (**Table 8**). Figures depicting the raw data (distribution of data values, number of observations) can be found in the appendix (**Fig. 32**, **Fig. 33**, **Fig. 34** - appendix). Furthermore, an ANOVA was computed for data after 24 hours of treatment exposure (**Table 9**). There was a statistically main effect for treatment after 24 hours of treatment

exposure (**Table 9**). Surprisingly, there was a statistically significant main effect for strain (genotype, **Table 9**).

No palmelloids were observed in either of the treatments. This was confirmed by microscopy pictures (**Fig. 24, 25, 26**– appendix). It is logically sound to assume that the statistical significance for treatment (**Table 8, Table 9**) stems from either (i) different growth patterns of the *C. reinhardtii* strains indicated by analysed wells densely laden with *C. reinhardtii* cells and/or (ii) a strong distribution of low data values in the two control treatments for the kairomone experiment (**Fig. 33, 34** – appendix, further shown in the specific growth rates and growth trajectories in **Table 7** and **Fig. 11** as well as in section 3.4.). **Figure 12** shows no prominent differences in recorded area median values between strains, treatments and times of measurement. Recorded cell areas ranged from ca. 125 μm^2 to ca. 325 μm^2 with outliers in both directions (**Fig. 12**). Specific growth rates were notoriously low when compared to standard growth rates in the scientific literature (Janssen et al., 1999) except for strain A (**Table 7**).

3.3. Calcium Experiment I

This experiment lasted for 96 hours with only one set of replicates for each treatment-strain combination. Palmelloid formation was not observed in either strain, treatment or time of measurement (**Fig. 14**). Since no palmelloids were to be observed the experiment was not repeated with altered chemical composition or other changed experimental parameters. This experiment functioned as a primer for the experiment shown in 3.3.1. The goal was to initially explore possible effects of various media manipulations regarding Ca^{2+} on different strains and afterwards to explore algal responses to culture conditions as close as possible to the experiments that were aimed at being replicated. Calcium depletion ($-\text{CaCl}_2$), chelation ($+\text{EDTA}$) and substitution ($-\text{CaCl}_2, +\text{MgCl}_2$) had strong negative effects on algal growth (**Fig. 13, Table 10**). The EDTA treatment had similar effects on specific growth rates as did the Ca^{2+} depletion experiments (**Fig. 13, Table 10**).

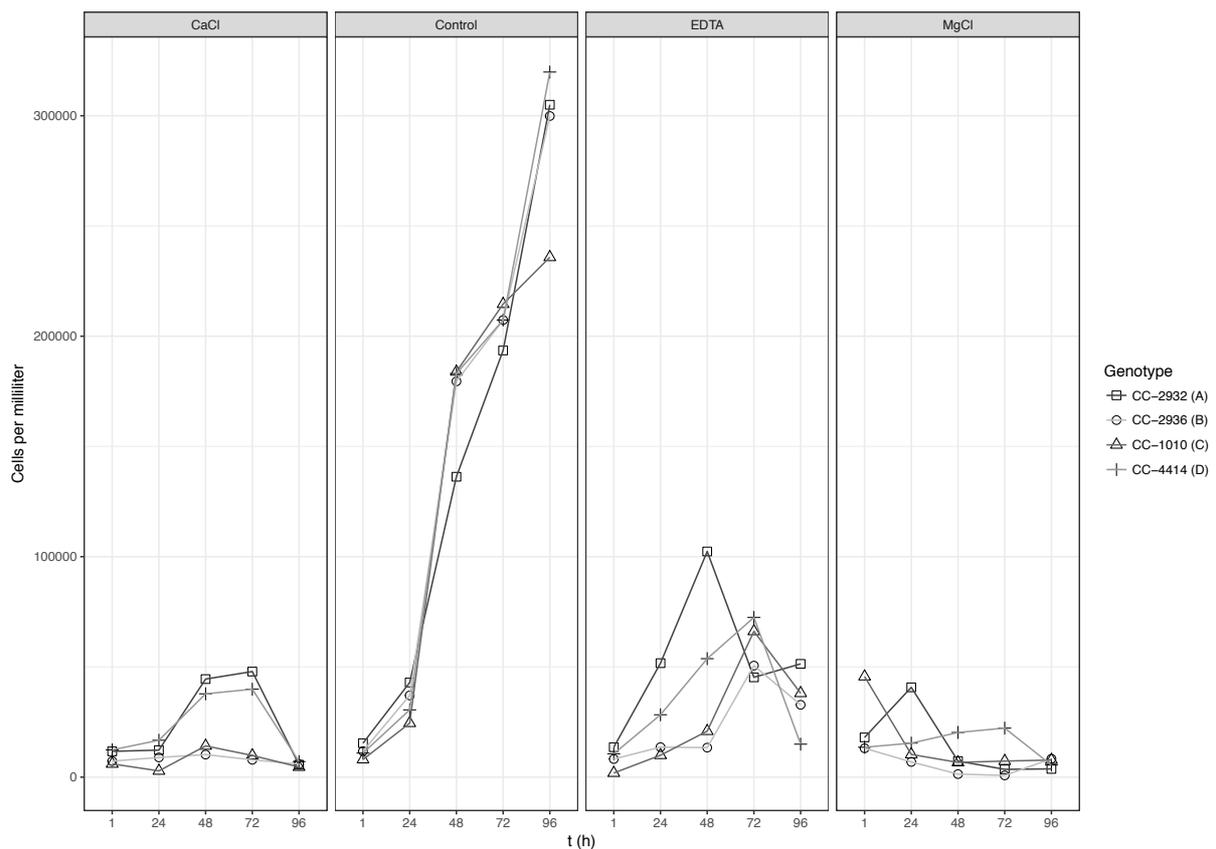


Fig. 13: The estimated cell densities (expressed as cells per ml) of cultures of four *C. reinhardtii* strains after 1 hour, 24 hours, 48 hours, 72 hours and 96 hours, cultured in medium containing either no CaCl_2 , WC medium (control), medium containing 0.2503 M EDTA and finally, medium containing 0.2503 M MgCl_2 and no CaCl_2 . Smoothing method: lm.

Table 10: Specific growth rates (S_{Gr}/day) for *C. reinhardtii* strains reared in the respective treatment culture conditions, calculated between 1 h and 24 h. For formulae, see p. 41.

S_{Gr} per day / Strain	Treatment			
	Control	MgCl ₂	CaCl ₂	EDTA
S_{Gr} A	1.069	0.789	0.049	1.394
S_{Gr} B	1.196	0.000	0.215	0.156
S_{Gr} C	1.166	0.000	0.115	0.686
S_{Gr} D	1.072	0.1336	0.314	1.025

As is shown in **Fig. 13**, strains reared in control cultures showed expected growth behaviour due to optimal culture conditions. Specific growth rates were as expected (**Table 11**, $S_{Gr} = 1.119$ (arithmetic mean for all strains)) for all strains in the control cultures. When cultured in full (M)WC medium with the addition of EDTA, growth was at first as expected over the course of 72 h but was markedly lower than in the control cultures, suggesting a chelation effect. Figures depicting the raw data (distribution of data values, number of observations, boxplots) can be found in the appendix (**Fig. 35**).

The cell areas of all four strains when reared in Ca²⁺ depletion or EDTA-rich conditions did neither increase nor decrease, but a notorious low number of observations (e.g. 17 for substitution at 72 h or 9 for depletion at 24 h, **Fig. 35**- appendix) reflects the impaired growth in these treatment conditions. There is an increase in recorded cell area values over time in the control cultures and the number of observations is high. The same seems to be given for the chelation cultures, even though the number of observations is less (**Fig. 35** – appendix). There is no increase in recorded cell areas over time in either the depletion or the substitution cultures and the number of observations is notoriously low (mean number of observations in the depletion treatment: 18, mean number of observations in the substitution treatment: 25, **Fig. 35** - appendix).

No statistical analysis (ANOVA or other) on this experiment has been performed due to the absence of true culture replicates for each treatment-strain combination. Nonetheless, since all four *C. reinhardtii* strains behaved similar (as in the previous two experiments), it is sound to assume that the absence of palmelloids in this experiment is good evidence that the trait may not be observed in the conditions described here.

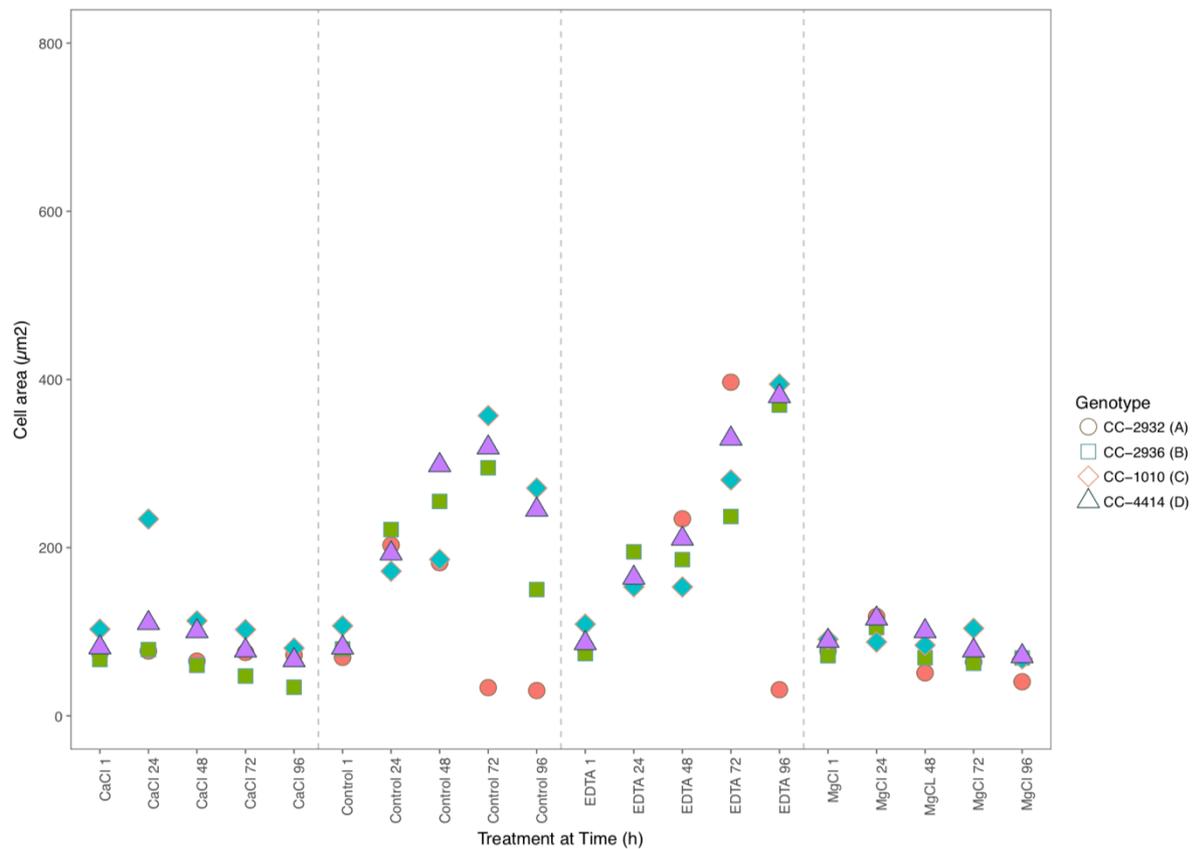


Fig. 14: Area medians (expressed in μm^2) of four *C. reinhardtii* strains reared in respective Ca^{2+} treatment media, measured over time x (hours). CaCl = (M)WC medium - CaCl_2 ; Control = (M)WC medium; EDTA = (M)WC medium + CaCl_2 + EDTA; MgCl = (M)WC medium - CaCl_2 + MgCl_2 . Labels showing the treatment and the time of measurement e.g.: EDTA 72 shows the median of a large dataset of measured cell areas after 72 h of culturing the strain in medium containing low concentrations of CaCl_2 and EDTA etc.). Colours and shapes of data-points represent *C. reinhardtii* strains (A = red circle, B = green square, C = blue diamond, D = violet triangle). Figures depicting the raw data (distribution of data values, number of observations, boxplots) can be found in the appendix (**Fig. 35**).

3.3.1. Calcium Experiment II

Following the results of the first calcium experiment, a new experiment was performed with modified experimental conditions. The treatments were now chosen to be as close as possible to the culture conditions chosen by Iwasa & Murakami (1968, 1969). As in the experiment described above, since no palmelloids were to be observed, this experiment was also set-up with only one culture replicate in contrast to the salinity and kairomone experiment in which triplicate cultures were set up. As in the first calcium experiment, the depletion and chelation of Ca^{2+} negatively affected the growth of all *C. reinhardtii* strains (Fig. 15, Table 11). None of the *C. reinhardtii* strains investigated in this experiment media formed palmelloids (Fig. 16). Figures depicting the raw data (distribution of data values, number of observations, boxplots) can be found in the appendix (Fig. 36 - appendix).

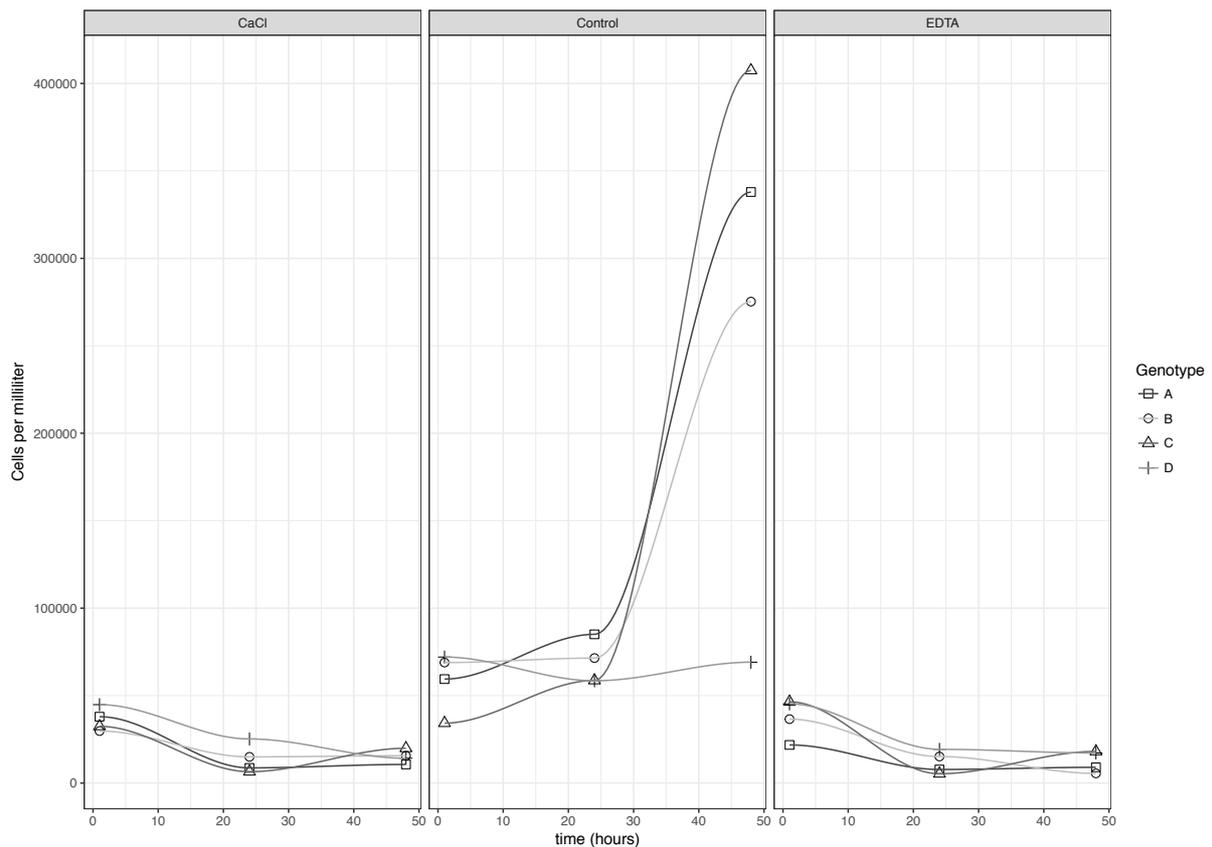


Fig. 15: Estimated cell densities (expressed as cells per ml) after 1 hour, 24 hours and 48 hours in the cultures of four *C. reinhardtii* strains cultured in medium containing either 6.8×10^{-5} CaCl_2 , full WC medium (Control) or medium containing 12.5×10^{-5} M EDTA and 6.8×10^{-5} CaCl_2 (EDTA). Data points represent the arithmetic mean of $n=2$ technical replicates of one culture replica each (sd = \pm max. 5567 cells per millilitre, min. 162 cells per millilitre). Smoothing method: loess (since: < 1.000 observations).

Table 11: Specific growth rates (S_{Gr}/day) for *C. reinhardtii* strains reared in the three different treatment cultures. Calculated between and 1 h and 24 h. For formulae, see p. 41.

S_{Gr} per day / Strain	Treatment		
	CaCl ₂	EDTA	Control
S_{Gr} A	0.934	0.000	2.277
S_{Gr} B	0.000	0.000	1.423
S_{Gr} C	0.000	0.000	1.236
S_{Gr} D	0.000	0.000	0.210

Although there are certain discrepancies in recorded cell area measurements (medians) between the treatment cultures of the four *C. reinhardtii* strains and the control cultures as shown in **Fig. 16**, the differences are not very different from previous experiments. All *C. reinhardtii* strains reared in the control and chelation treatments showed higher median values after 24 hours of culturing, whereby strain A showed the highest median values (**Fig. 16**). Since (i) the median distributions at 1 hour and 48 hours are overly similar in control and treatment cultures, (ii) there were no palmelloids observed after 24 hours of treatment, and (iii) the maximum median in **Fig. 16** ($270 \mu\text{m}^2$) corresponds to a cell diameter of ca. $20 \mu\text{m}$ which would constitute a dividing *C. reinhardtii* cell rather than a palmelloid colony, I infer that no palmelloids were present in either depletion, chelation or control treatments (**Fig. 16**, **Fig. 36** – appendix). Furthermore, the medians and the density distributions of recorded cell area measurements in both, the -CaCl₂ and the EDTA treatment are lower than the control cultures. This reduction in cell area and cell abundance is also reflected in the low specific growth rates (esp. EDTA: **Table 11**), the low number of observations and the distribution of medians (**Fig. 36** - appendix). The minimum median ($35 \mu\text{m}^2$) corresponds to a cell diameter of ca. $6.63 \mu\text{m}$ and likely constitutes a single *C. reinhardtii* cell.

As for the previous experiment, no statistical analysis on this experiment has been performed due to the absence of triplicate cultures for each treatment-strain combination. Nonetheless, since all four *C. reinhardtii* strains behaved similar to the previous calcium manipulation experiment, it is sound to assume that the absence of palmelloids in this experiment is good evidence that the trait may not be observed in the absence of the Ca²⁺ ion.

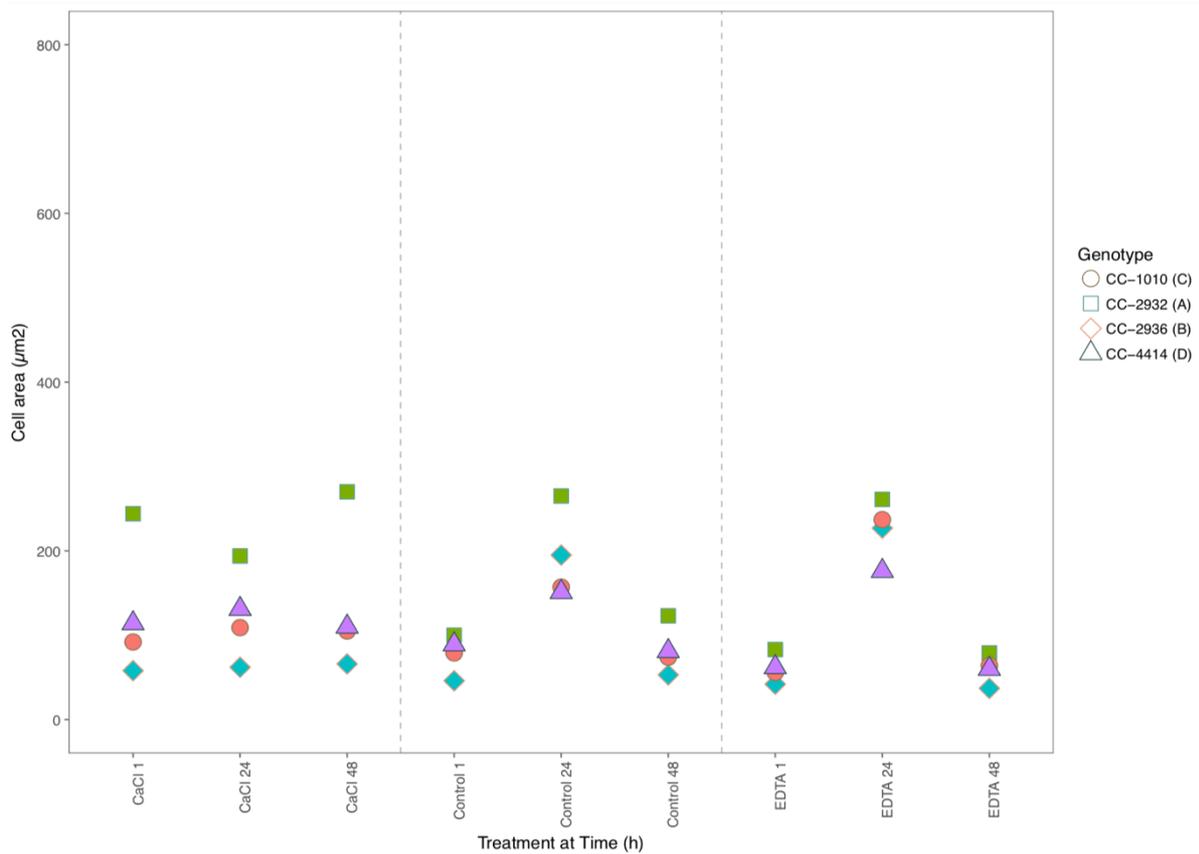


Fig. 16: Area medians (expressed in μm^2) of *C. reinhardtii* strains reared in the respective Ca^{2+} depletion ($68 \mu\text{M CaCl}_2$), control (full (M)WC medium) and chelation ($68 \mu\text{M CaCl}_2 + 12.5 \mu\text{M EDTA}$) treatments measured at time x (hours). Colours and shapes of data-points represent *C. reinhardtii* strains (A = green square, B = blue diamond, C = red circle, D = violet triangle). Figures depicting the raw data (distribution of data values, number of observations, boxplots) can be found in the appendix (**Fig. 36** - appendix).

3.4. Comparison of Control Cultures

To see if any change in *C. reinhardtii* cell areas resulted because of un-investigated independent variables, standard control cultures (100 % (M)WC medium) were set up in each experiment to try to eliminate experimental errors and experimenter bias. For all experiments, the *C. reinhardtii* strains were cultured under identical light, temperature and experimental set-up conditions. The expectation was, that growth and formation of palmelloids (the expected absence) were similar for the controls under these conditions. Since the experiments were conducted over the course of several months, independently from one another, I decided to compare the results (cell areas and growth trajectories) of the 100 % (M)WC medium control cultures between (Fig. 17, Fig. 19) and within (Fig. 18 – solely cell areas) experiments to detect possible unknown experimental and methodological factors that may have influenced the results.

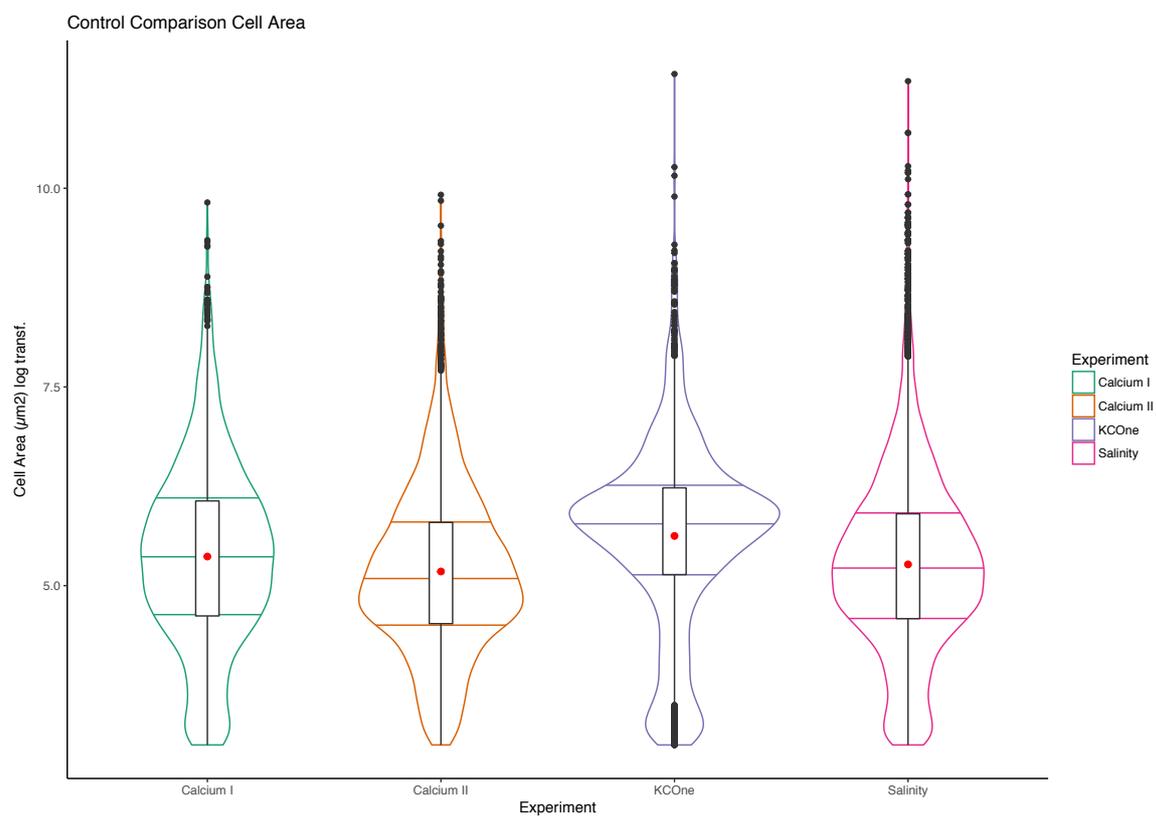


Fig. 17. Comparison between controls cultures of the first and second calcium experiments (100 % (M)WC medium), the first kairomone experiment control (100 % (M)WC medium) and the salinity experiment control (100 % (M)WC medium). “Pooled” data: all technical replicates, time points of measurements and strains. Cell area measurements (μm^2 , \log_2 -scale). Boxplots show median (red), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying violin plots and 0.25, 0.5 and 0.75 quantiles are shown as coloured lines. Green = Calcium I experiment, orange = Calcium II experiment, violet = Kairomone experiment, pink = Salinity experiment.

The control cultures are adequately consistent between the experiments with no grave deviations from one another (**Fig. 17**). This suggests, that there were no methodological factors that changed during the experimental period. An exception is the data distribution of the culture(s) of the kairomone experimental control, which show a somewhat bigger abundance of larger cells than the others and also a slightly higher median value (**Fig. 17**). Due to the unreliability of the method (e.g. too dense pictures used for cell area analysis, crowding on the periphery of wells due to centrifugation), used to determine cell area sizes, it is probable that this reflects measurement errors. It can be deduced, that over time, the conditions in which the control cultures were reared in, were overall identical and may be used as accurate representations of a negative control in which the phenomenon of palmelloid formation was not observed.

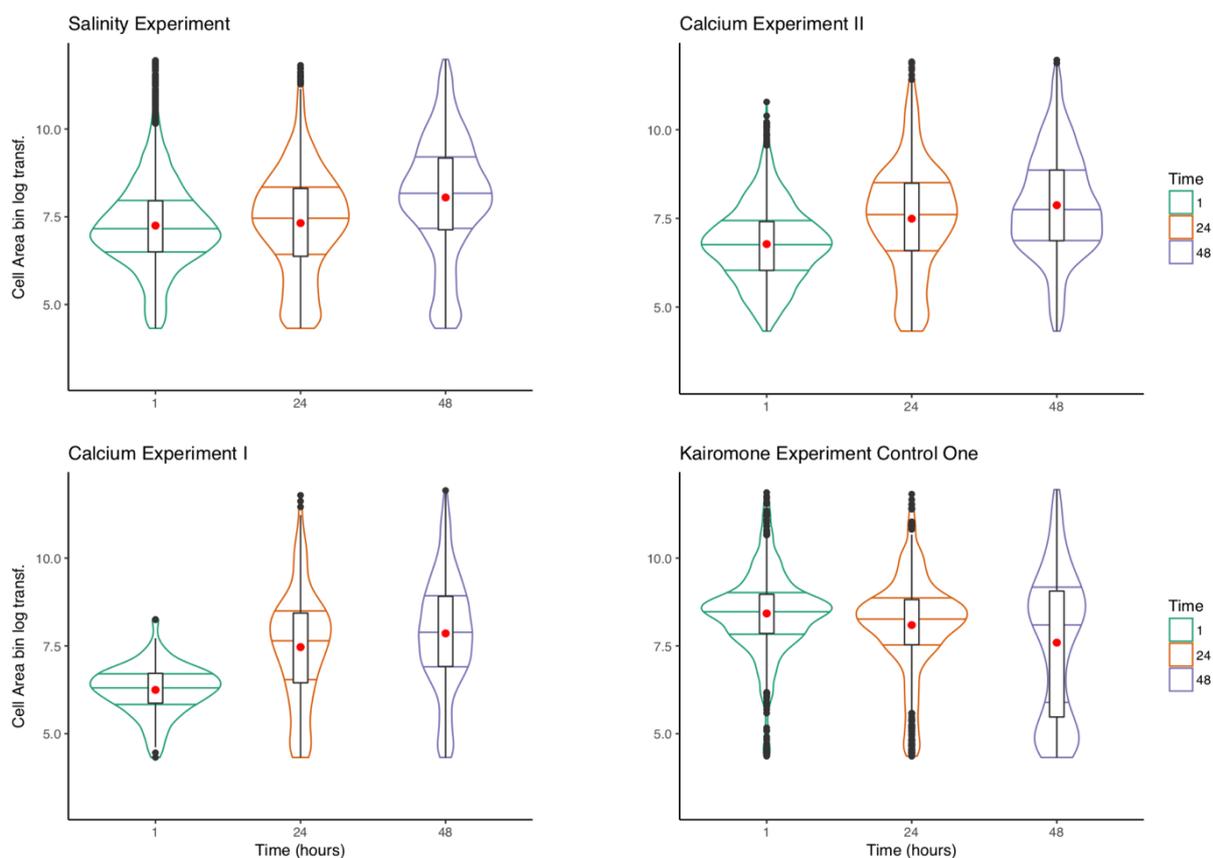


Fig. 18. Comparison within (and between) experimental treatment controls at the three measured time points (1, 24 and 48 hours). As in **Fig. 17**: salinity experimental, calcium experimental I & II, kairomone experimental controls (all: 100 % (M)WC medium). Cell area measurements (μm^2 , \log_2 -scale). Boxplots show median (red), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying violin plots and 0.25, 0.5 and 0.75 quantiles are shown as coloured lines. Green = 1 hour, red = 24 hours, violet = 48 hours.

When the control cultures are compared within (different measurement times) and between (experiments), it is evident, that there are some variations between the

distributions, but there are no clear signs of any trends that might be due to consistent methodological changes (**Fig. 18**). The strongest deviations are the cell area distributions in the kairomone control cultures after 48 hours of the experiments (**Fig. 18**). It seems that there is a difference in sampling time, reflecting the ascension of cell area over time in the control cultures. There are time points that deviate unexpectedly from the rest, indicating higher cell are measurements with later measurement (**Fig. 18**). This could reflect the higher abundance of cells (due to growth) and the higher probabilities of having more cells in the wells used in the cell area measurements at later times. As expected, strains grew similar in the control treatments between experiments (**Fig. 19**, **Table 13**). Cells in the second calcium experiment increased more strongly after 48 hours of culturing (**Fig. 19**). This is also indicated by the exalting median values over time shown in **Fig. 18** and by their higher specific growth rates (except strain D, **Table 12**).

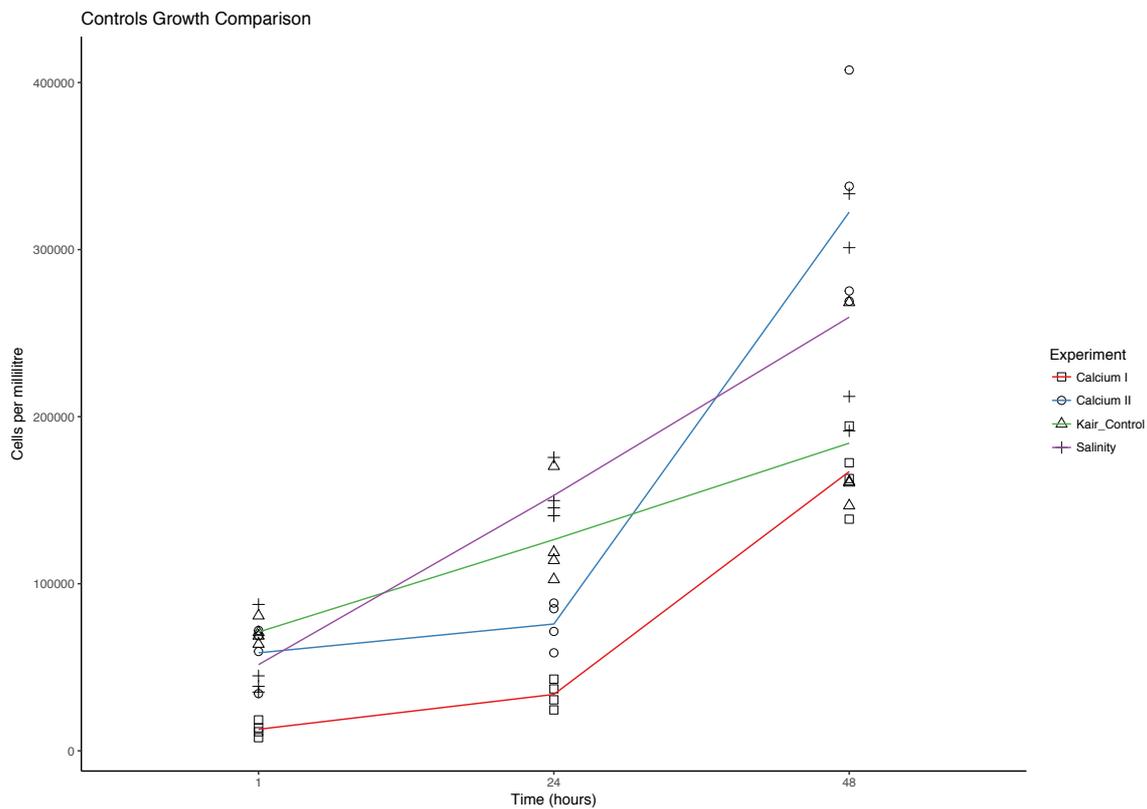


Fig. 19. The estimated cell densities (expressed as cells per ml) after 1 hour, 24 hours and 48 hours of four *C. reinhardtii* strains in the different experimental control cultures expressed as shaped points (square = Calcium I experiment, circle = Calcium II experiment, triangle = Kairomone experiment, plus sign = Salinity experiment). As in **Fig. 17** and **Fig. 18**: salinity experimental, calcium experimental I & II, kairomone experimental controls (all: 100 % (M)WC medium). Smoothing method: loess (since: < 1.000 observations). Red line: Calcium I experiment; blue line: Calcium II experiment; green line: Kairomone experiment; violet line: Salinity experiment.

Table 12. Specific growth rates (S_{Gr}/day) for all four *C. reinhardtii* strains reared in control treatment cultures (100 % (M) WC medium) in the four experiments. Calculated between and 1 hour and 24 hours*.

S_{Gr} per day / Experiment	<i>C. reinhardtii</i> strain			
	Strain A	Strain B	Strain C	Strain D
S_{Gr} Calcium I	1.06	1.19	1.16	1.07
S_{Gr} Calcium II	2.277	1.423	1.23	0.21
S_{Gr} Kairomone	0.57	0.38	0.36	0.91
S_{Gr} Salinity	1.29	1.14	0.91	0.41

*Standard errors of the mean for the kairomone and salinity experiments (triplicate cultures) can be found in **table 5** and **table 8**. Specific growth rates for the Calcium II experiment were calculated between 24 hours and 48 hours.

4. Discussion

In quintessence, it was possible to observe *C. reinhardtii* palmelloid colonies in one (salinity) out of three (salinity, calcium, kairomone) experiments. Growth was strongly impaired in the absence and chelation of Ca^{2+} , in salinity concentrations > 100 mM but seemingly not affected by *B. calyciflorus* filtrate at various concentrations.

Salinity Experiment

The results from the salinity experiment consolidate previous findings by Khona et al. (2016) and show that the plastic trait is present in at least all *C. reinhardtii* strains that have been investigated here, whereby some of the strains (e.g. strain A) seem to be more halotolerant than the others (Khona et al. 2016). In the experiment presented here, the aggregative plastic response of palmelloid formation depended on the concentrations of NaCl in the environment (> 200 mM seems to exceed the adaptive capacity of *C. reinhardtii*) and that, independent of which strain investigated, *C. reinhardtii* forms palmelloids in the presence of high salinity concentrations (50 mM and 100 mM). Investigating the plastic physiological responses of this aquatic primary producer in response to salinization in this experiment shows, that different strains of *C. reinhardtii* may potentially not only assure the immediate survival of the present organism, but is hypothetically also able to protect future progeny by the construction of a niche via the formation of cell colonies and the production of an extracellular matrix that shields the organisms from environmental perturbations. In this experiment and others it has been shown that varying salinity concentrations are an ecological factor that facilitates the formation of palmelloid colonies in *C. reinhardtii* (Khona et al., 2016; Krohnholm et al., 2017). Therefore, since this important ecological factor is (and has been) present in many habitats (e.g. *C. reinhardtii* can be found in soil (Sahoo & Seckbach, 2015)), the (active or inactive) ability to form palmelloids potentially directed (and allowed for) the evolution (after Lewontin, 1970) of physiological adaptations (such as partial inversion of the embryo, establishment of organismal polarity, genetic modulation of cell number,

partial germ/soma division of labour (Fig. 3, Herron & Michod, 2007)) that were of crucial importance for the transition from unicellular to multicellular life in the Volvocean algae lineage. Nonetheless, it appears doubtful to me since this can (potentially) never be known through the approximate recreation of potential past ecological conditions investigated in the present. Nonetheless, it is important to know how freshwater organisms may react to changes in salinity levels in their environment and this study shows the multitude of physiological adaptations “simple” organisms such as green algae may have towards altered ecological conditions.

It is therefore interesting to confirm, that the freshwater green algae *C. reinhardtii* (among others) is able to cope with salinization conditions < 400 mOsm via the formation of protective colonies. Furthermore, *C. reinhardtii* was demonstrated to be a promising source of sustainable liquid transportation fuels (Scranton et al., 2015) and, since the chemical nature of sodium and chloride does apparently not alter the chemical structures of biofuel chemicals (Shirazi et al., 2013), may be used as a substrate to flocculate the algal cells such that they sink to the bottom of the container and may be harvested in an industrious and cost-efficient manner such to increase productivity (Shirazi et al., 2013; Scranton et al., 2015).

Kairomone Experiment

There is a large body of studies that have shown, that phytoplankton species respond to the physical presence of predatory species and/or infochemicals released by them (e.g.: Latta et al. (2009); Lurling & Beekman, (2006); Van Donk et al. (2011); Lass & Spaak, (2003)). By assembling as a “multicelled” organism, prey may escape the upper limits of a predator’s capacity to ingest foreign objects (Lurling & Beekman, 2006). This has been investigated in a study by Boraas et al. (1998) in which *Chlorella vulgaris* was selected for multicellularity via predation by the phagotrophic predator *Ochromonas vallescia* (Boraas et al., 1998). Furthermore, *C. reinhardtii* undergoes vertical shifts due to infochemical-dependent phototaxis (Latta et al., 2009) and aggregates within 18-48 hours in the presence of *Peranema* or its culture filtrate (Durand, 2016) or in the presence of *B. calyciflorus* within 25 hours (Lurling & Beekman, 2006).

It comes as a surprise that none of the *C. reinhardtii* strains used in this experiment underwent an aggregative response when subjected to different concentrations of *B. calyciflorus* culture filtrate even though the strains have clearly been shown to exhibit the ability to respond morphologically via the formation of colonies. Van Donk et al. (2011) defined three main factors triggering induced defences in both marine and freshwater phytoplankton (Van Donk et al., 2011). Many studies did not disentangle the exact mechanism(s) governing defence induction in any particular species (Van Donk et al., 2011). Van Donk et al. (2011) identified three distinct chemical cues:

- (i) Chemical cues associated with mechanical damage
- (ii) Chemical cues associated with herbivore presence
- (iii) Chemical cues associated with grazing/feeding

To test if palmelloids were induced by herbivore-specific cues⁶, the design of the experiment at hand excluded the possibility that chemicals released upon mechanical damage alone, in other words products released from lysed cells of the focal algal species (*C. reinhardtii*), triggered the induced defence of palmelloid formation since *B. calyciflorus* was not being cultured together with *C. reinhardtii*. Additionally, it excluded the possibility that specific chemical compounds that are feeding-related (i.e. when cells and/or their contents of *C. reinhardtii* come into contact with the feeding apparatus and digestive system of *B. calyciflorus*) by feeding *Nannochloropsis sp.* to the hatched *B. calyciflorus* specimens. Nonetheless, it cannot be excluded that chemical compounds that induce palmelloids in *C. reinhardtii* may not be also released by *B. calyciflorus* when the grazer comes into contact with another algal species (in this case *Nannochloropsis sp.*). Furthermore, the possibility of selective feeding on single cells and the concomitant increase in cell colonies was also ruled out since predators were not physically present and thus did not feed on single celled *C. reinhardtii*.

One methodological explanation for the absence of an induced anti-predatory response is the possibility that there were no assumed *B. calyciflorus* infochemicals present in the

⁶ For example, if herbivores use sex pheromones or aggregation pheromones, these strictly intra-specific signals could actually be used or “spied upon” by algae and cyanobacteria. These signals would be highly reliable cues for herbivore presence and identity, as pheromones are strongly species-specific (Van Donk et al., 2011).

filtrated media, that they were biochemically inactive or in too low concentration to elicit a response in *C. reinhardtii*. This may have had several reasons. First, the *B. calyciflorus* specimens might not have emitted the infochemicals in question or if they did, it may not have been the chemicals to which *C. reinhardtii* responds with an anti-predatory phenotypically behaviour even though other studies (e.g. Fischer et al., 2014) show that that behaviour occurs. Furthermore, *C. reinhardtii* cells have been shown to form palmelloids only after ca. 40 days of culturing them together with natural predators or after a much shorter time (hours) (e.g. Broch, 2015). This elucidates a rather obvious complication in these studies. If one does not exactly know, and prior to experimentation determines, to which chemical structures prey may react, or if the chemicals are not present in the used medium in the first place, then how is one to know to which chemical(s) prey react, if they react. Imagine there were no infochemicals present, but *C. reinhardtii* elicits an aggregative response (to something other, such as light regime, chemical composition of the medium, selective feeding or prolonged culturing (Broch, 2015)) then one would wrongly infer a causal *explanans* (infochemicals are responsible for the formation of palmelloids) from correlative data for the *explanandum* (why does *C. reinhardtii* form palmelloids?) (Hempel & Oppenheim, 1958, p. 152). For future experiments, it would be beneficial to carefully design the studies such that it is, by chemical analysis, exactly known to the researcher what chemicals he or she infers to be the reasons for an adaptive plastic response in an experiment aimed at elucidating complex ecological community dynamics.

In this experiment, it has been anticipated that *C. reinhardtii* would induce colonial phenotypes in relative response to filtrate concentration (showing the strongest and arguably most rapid response in the 50 % treatment). In a series of large-scale experiments, Verschoor et al. (2014) showed that 38 different strains of *Scenedesmacea* (Chlorococcales, Chlorophyta) formed colonies in response to grazing-released infochemicals from the herbivorous zooplankters *Brachionus calyciflorus* and *Daphnia magna* (Verschoor et al., 2014). In these studies, colony size was found to increase with *B. calyciflorus* infochemical concentrations and could be described by sigmoid function (Verschoor et al., 2004). In the light of the abundant evidence, that colonial formation of phytoplankton is indeed an adaptive response to grazing pressure, it is reasonable to

assume that there have been multiple (or one) methodological errors in the experiment conducted here. For example, the potential degradation of infochemicals in the filtrate medium (even though the filtrate was used in the experiments the exact same day) or that the infochemical concentrations were too low. The data presented here do not deconsolidate the multitude of previous findings as cited above. The results document an experimental system in which the hypothesized presence of infochemicals released by a phagotrophic protist did not result in the rapid proliferation of a predator-resistant multicellular morphology within populations of *C. reinhardtii*. Furthermore, the data presented in this thesis do not support Stanley's (1973) hypothesis for the origins of multicellular organisms: the emergence of phagotrophic unicells in the Precambrian could have been rapidly selected for colonial prey, eventually resulting in the Metaphyta and Metazoa (Booras et al., 1998).

Calcium Experiments

The results for the calcium manipulation experiments clearly showed the dependency of *C. reinhardtii* growth and proliferation on the availability of calcium in their environment. Palmelloid formation was not observed in either experiment and the findings of Iwasa & Murakami (1968, 1969) could not be consolidated. In the introduction to this experiment I provided references to research that did observe palmelloid formation when subjected to the conditions as investigated here. One explanation for the inability to form palmelloids is that, since the formation of colonies requires growth and division of cells, it is conceivable that the low concentrations of Ca^{2+} in the experiments presented here were too low and thus hindered these processes and therefore also hindered the formation palmelloids. This was also the conclusion of the first calcium experiment conducted here which is why the treatments were modified.

The calcium concentrations in this experiment matched the ones by Iwasa & Murakami (1968, 1969) such that the formation of palmelloids, as in their studies, was expected. There may have been differences in the medium used to culture *C. reinhardtii* by Iwasa & Murakami (1968, 1969), such that an unknown nutritional (or other) factor in the WC medium used here detained *C. reinhardtii* cells from forming colonial aggregates.

Comparing the “normal medium” by Iwasa & Murakami (1968, 1969) and the medium used in the current study (**Table 3**), notable differences were the presence of Na₂EDTA, silicon (Si) and sodium (Na⁺) in the latter medium. Other than that, the chemical composition was similar, and all elements present in the WC medium were also present in the medium used by Iwasa & Murakami (1968, 1969). While silicon is an essential element for growth in diatoms, there is ample evidence, that it stimulates the growth in the non-silicon-dependent green algae (and other), *Chlorella vulgaris*, in which the increase in growth rate upon the addition of silicon could be directly correlated with an increase in the biologically available phosphorous fraction (Exley et al., 1993). Since the presence of silicon mainly has been shown to affect the availability of phosphorus, it is unlikely that the presence of silicon in the medium used in this study is responsible for the non-formation of palmelloids in *C. reinhardtii*. However, the presence of Na₂EDTA in the WC medium might have been an important factor, and (since it has two molecules of water) the dissolution in H₂O compared to the anhydrous form is more effective, there likely has been a higher concentration of EDTA in the experimental medium than in the medium used by Iwasa & Murakami (1968, 1969) which may have been the reason for the unobserved formation of palmelloids in this experiment. Unfortunately, these aspects have not been recognized prior to experimentation and highlight the importance of rigorous detailing of nutritional medium used in aquatic experimental systems. Interestingly, the addition of EDTA gave similar algal growth and area distribution responses as low Ca²⁺ levels. It is therefore of crucial importance to provide aquatic experimentalists with sufficient knowledge about the underlying chemical factors (and interactions thereof) that may crucially alter experimental conditions and influence the study organism’s behaviour, genotype (potentially) and phenotype from laboratory to laboratory and from experiment to experiment.

Considering the possibility of a higher-than assumed EDTA concentration in the experimental medium used in this study, uncovers a possible *a priori* error in the design of the experiment that may be important to be considered in future experiments investigating the formation of palmelloids in *C. reinhardtii*. Due to the experimental shortcomings as laid out above, the empirical findings presented in this thesis do not provide evidence for or against the observation that *C. reinhardtii* forms colonies in the

absence (or chelation) of the calcium ion. It shows the complexity of laboratory experiments without a “universal standard” of media and the implications for the complex process of replicability of scientific studies of this kind and the need for a deeper understanding about the multiple possible implications.

C. reinhardtii palmelloids – Disentanglement

At the absolute minimum, multicellularity requires adhesive interactions between cells to maintain a coherent, physically connected entity that binds multiple individuals to a common evolutionary fate (Umen, 2014). It has been shown that colony formation in *C. reinhardtii* is, at the most fundamental level, a temporary perennating stage, inhibiting the release of daughter cells from an extracellular matrix formed by a mother cell in response to certain environmental conditions. The formation of colonies has so far been understood as either a fixed genetic trait that arises in response to selection pressure(s) (Becks et al. 2010; Ratcliff et al. 2013; Fischer et al., 2014) and as a phenotypically plastic character induced by a variety of environmental conditions such as altered calcium concentration, high salinity levels and the presence of toxins (Iwasa & Murakami 1968; 1969; Lurling & Beekman 2006; Khona et al., 2016; Krohnholm et al., 2017; Cheloni et al., 2016).

Herron and Michod (2008) suggest that the initial cooperative trait to spread among a common ancestor of multicellular volvocine algae was the transformation of the outer cell wall into an extracellular matrix (ECM). Multicellular volvocine algae all have ECM, which consists of glycoproteins and is homologous with the outer layer of the *C. reinhardtii* cell wall (Herron & Michod, 2008). In extant multicellular volvocine alga, the ECM functions to keep cells in a colony together and also acts as a kind of commons for the storage of temporarily abundant nutrients. Herron & Michod (2008) interpret the initial evolution of the ECM as cooperative because individual cells could contribute to a common resource which was beneficial to all cells in the group (Herron & Michod, 2008). Following their argumentation, palmelloid formation may have “set the stage” for subsequent evolutionary processes (**Fig. 3**) resulting in differentiated multicellular

Volvocales (**Fig. 2**). In the light of this hypothesis, I think it is worthwhile to dialectically disentangle the different explanations of palmelloid formation in *C. reinhardtii*.

In the experiments conducted by Ratcliff et al. (2013) *C. reinhardtii* colonies emerged in response to settling selection (centrifugal forces) (Ratcliff et al., 2013). Colonies developed clonally by daughter cells staying together after mitotic reproduction (Ratcliff et al., 2013). Becks et al. (2010) presented data that they argued showed palmelloid formation in response to rotifer grazing and concluded that palmelloid formation in *C. reinhardtii* constitutes a heritable trait. The premise of their study was that the variation they observed is genetically based and not due to plasticity or fluctuations in the population's age structure. They confirmed that palmelloid colony formation in their system was a heritable trait by conducting an independent experiment in which they followed the percentage of single cells vs. colonies over the course of nine cycles of cell division: the cells at the end were seven-greats granddaughters of the starting population, what they argued was more than what is typically considered a sufficient number of generations to detect induced phenotypic effects. Previously grazed *Chlamydomonas* cells cultured without rotifers continued to form palmelloid colonies for the full nine generations whereas cultures of the un-grazed *Chlamydomonas* lineage maintained a consistently high proportion of single cells from which Becks et al. (2010) deduced that the trait is heritable, not induced. The implication that colony formation is based on differences in individual cell's genomes is that the formation of colonies evolved independently, and at a rapid pace, in all of the algal cultures that were inoculated with unicellular algae. This implies that the trait arose independently due to the rapid evolution of genetic differences in *C. reinhardtii* cells. These results are distinct from that reported by Lurling & Beekman (2006), who found that a strain of *C. reinhardtii* growing as single cells began exhibiting clumping within 25 hours of being cultured with rotifers (Lurling & Beekman, 2006). The results by Lurling & Beekman (2006) may have been due to selective feeding, that is, rotifers were only able to ingest and digest single cells and not colonies, such that the frequencies of palmelloids in their studies increased due to the progressive decrease of predated single cells. This is reflected in **Fig. 2** in their study, which shows a large number of 4-celled and 8-celled *C. reinhardtii* palmelloids in the absence (control) of *B. calyciflorus* (Lurling & Beekman,

2006). Furthermore, empirical evidence amounts that suggests that non-genetic (epigenetic) inheritance may play an important role in the formation of palmelloids (Krohnholm et al. 2017).

The contrasting results of Lurling & Beekman (2006) and Becks et al. (2010) is, as the last authors noted, intriguing, and suggest that under different circumstances *C. reinhardtii* may exhibit either an inducible or a heritable constitutive palmella morphology as a response to rotifer grazing or the presence of predator infochemicals in the culture medium. Furthermore, it shows the complexity of the colony formation trait, especially when considering the multitude of other environmental factors inducing the response in *C. reinhardtii*. Fischer et al. (2014) compared gene expression profiles of different *C. reinhardtii* strains exhibiting different propensities for colony formation in the presence of *B. calyciflorus*. Their results show that gene expression differed strongly between strains in absence of *B. calyciflorus* and that the presence of *B. calyciflorus* induced changes, both in morphology and gene expression, which varied among the strains (Fischer et al. 2014). Collectively, these results indicate that the propensity to form palmelloids is a variable character among strains of *C. reinhardtii*, i.e. there is an aspect of heritability in the induced response (Broch, 2015).

Colony formation in *C. reinhardtii* as investigated by Lurling & Beekmann (2006), Becks et al. (2010) and others may be of the same nature. The trait can be induced under certain conditions (phenotypic plasticity in the broad definition) *and* may constitute a heritable, genetic and/or non-genetic trait that can be passed on mitotically and/or meiotically from mother to daughter cells. I think it is therefore worthwhile to define the phenotypically plastic character more closely and furthermore explain the phenomenon in the light of contemporary evolutionary theory.

Phenotypic Plasticity

The ways in which an organism reacts to different environments is as much part of its characteristics as its appearance and qualities in a single environment. The common (broad) definition of phenotypic plasticity is: the ability of a single genotype to produce

more than one alternative form of morphology, physiological state, and/or behaviour in response to novel or heterogeneous environmental conditions (West-Eberhard, 1989). This definition entails the danger of too broad a definition since all biological processes are to some extent influenced by the environment. Thus, most responses of biological entities to their environment fall under the umbrella term of phenotypic plasticity. Plasticity, as seen in the conceptual framework of the recently advocated (and controversial) extended evolutionary synthesis is a cause, not simply a consequence, of phenotypic evolution (Pigliucci, 2007; Laland & Wray, 2014). Phenotypic plasticity plays a central role as a generator of novel, yet potentially functional and coordinated, phenotypic variation (Pigliucci, 2007; Agrawal, 2001). It facilitates colonization of novel environments and potential niches, affects population connectivity and gene flow, contributes to temporal and spatial variation in selection and may increase the chance of adaptive peak shifts, radiation and speciation events (Pigliucci, 2008; Laland & Wray, 2014). If plasticity is broadly (or, as is often the case, not at all) defined by the researcher, then it is necessary to distinguish multiple possible manifestations, keeping in mind that one should take great care to clarify how one interprets and uses the plasticity concept to avoid misunderstanding. The following concepts are distinguished: (a) *morphological* and (b) *physiological* as well as (a₂) *active* and (b₂) *passive* plasticity if conceptualized in a broader distinction and (c) *labile effects* (d) *cross-generational effects* (e) *acclimation* (f) *developmental switches* and (g) *developmental pathologies* (West-Eberhard, 1989; Huey and Berrigan, 1997). The concepts are being explained in the next paragraph. Making a conceptual distinction of phenotypic plasticity may help to classify the palmelloid formation response of *C. reinhardtii* and to form an understanding of the nature of the trait on which further investigations can build on.

Many changes in the phenotype of an organism are physiological (and genetic) in origin, so fundamentally all plasticity is physiological. Where physiological changes have predominantly morphological teleological (end) effects however (such as the expression of protective crests in *Daphnia magna* as a response to predator presence), one can talk about morphological plasticity. Active plasticity is used predominantly anticipatory, and often highly integrated, phenotypic change in response to some environmental cue or signal, and reflect modifications of developmental pathways and regulatory genes

(West-Eberhard, 1989). Passive plasticity may stem from direct environmental processes, and is generally not considered anticipatory, but a mere consequence of the environment, such as stunted growth owing to low resource levels (Forsman, 2015). When the environment acts directly on the expression of the trait (passive), phenotypic changes are often proportional to environmental differences, in contrast, the magnitude of the induced phenotypic response(s) in active plasticity is not necessarily correlated with the strength of the environmental signal (Schreiner, 2006). Labile effects are rapid phenotypic modifications as function of the organism's immediate environment. Cross-generational effects are maternally or paternally transmitted phenotypic modifications. Developmental switches result in an irreversible fixation of a phenotype due to environmental conditions during a critical phase of development (e.g. temperature dependent sex determination, royalactin induced queen differentiation in honeybees (Kamakura, 2011)). Plasticity is itself an evolvable property of the genetic-developmental system of living organisms (i.e., it can be selected for and become adaptive), whereas at other times it may buffer the action of selection, leading to the build-up of genetic redundancy (Lenski et al., 2006). Genetic variation for plasticity exists in virtually every species in which it has been looked for, and the understanding of the phenomenon is such that one now appreciates the fact that it can both be the result of natural selection as well as influence the effectiveness of selection (Forsman, 2015). The inherent plasticity of most developmental systems may in some circumstances lead the way toward evolutionary change, preceding genetic change in a population (Price et al., 2003). One explanation for the way by which plastic traits may become genetically based lies in the process known as genetic assimilation (Waddington, 1961): The population is initially occupying one environment (e.g. low osmotic conditions), although there is an unexpressed capacity for plasticity, should the environment change. If the environment does change (e.g. high osmotic conditions), the pre-existing reaction norm allows the population to persist, producing a novel phenotype with no initial genetic change (palmelloid formation). Finally, if natural selection (or artificial) keeps operating in the new environment, the novel phenotype may become genetically fixed (assimilated), and the original reaction norm may lose plasticity, for example because of drift or costs associated with maintaining plasticity when it is not favoured by natural selection (because the old environment is no longer experienced) (Pigliucci et al., 2006). The

latter changes would then in some sense fix and perhaps fine tune what was initially a (pre)adaptive phenotypic response to a change in the environment. Traditionally it has been argued that environmentally induced variation slows down the rate of adaptive evolution by shielding the genotype from the effects of selection (Levis & Pfennig, 2016). The alternative argument (plasticity first hypothesis) posits, that phenotypic plasticity is initially established in a population which may produce phenotypes that are favoured under novel conditions, and thus facilitate survival during environmental change (Levis & Pfennig, 2016). If genetic or epigenetic variation exists, the genotype(s) with the advantageous trait value but less (costly) plasticity may be favoured. Plasticity decreases and genetic assimilation may occur. From this point of view plasticity may facilitate and speed up the process of adaptive evolution (Pigliucci et al., 2006).

The emergence of *C. reinhardtii* palmelloids as conceived and described e.g. by Lurling & Beekman (2006) and other studies showing the induced defence response of *C. reinhardtii* (Becks et al., 2010; Sathe & Durand, 2016; Verschoor et al., 2015) clearly falls under the broad definition of phenotypic plasticity. For whatever it is worth, the physiological response observed in this study is hereby defined as *morphological, active, labile* and *cross-generational* when defined more attentively. The palmelloids by Schlösser (1966) do not fall under any phenotypic plasticity definition since it was a mutant strain that he investigated (Schlösser, 1966). Interestingly, it is not clear under which definitions of phenotypic plasticity the palmelloid response of *C. reinhardtii* in response to high salinity concentrations (Khona et al. 2016; Krohnholm et al., 2017; this thesis) or to e.g. toxins or heavy metals (Cheloni et al., 2016; Franqueiro et al., 2000; Nakamura et al., 1975) falls. Since, in contrast to active (in that the response involves multiple regulatory genes and processes acting at different hierarchies to produce a complex, coordinated change (Whitman & Agrawal, 2009)), other environmentally induced phenotypic alterations appear to be simple susceptibilities to physical or chemical environmental stresses (passive plasticity) (Whitman & Agrawal, 2009). Toxins, poor nutrition, and extreme temperatures, pH, O₂ levels, and osmolarities can directly alter chemical, enzymatic, cellular, and developmental processes, producing passive (not regulated by the organism) changes to the phenotype (Whitman & Agrawal, 2009). Since most forms of plasticity likely contain active and passive components,

distinguishing them can be difficult (Whitman & Agrawal, 2009). Palmelloid formation is in my eyes nonetheless, when defined as passive plasticity, labile (it has been shown in this thesis that *C. reinhardtii* may form palmelloids in ca 1 hour to environmental and cross-generational changes (as shown in e.g. Krohnholm et al., 2017 or Ratcliff et al., 2013)).

The aspect of cross-generationally is of crucial importance in the light of recent results stemming from experimental evolution experiments and is taken up in the next paragraph. It is reasonable to say that the formation of palmelloids in *C. reinhardtii* constitutes phenotypic plasticity. With this definition, nothing is lost but little is won. It is e.g. conceivable, that the phenotypically plastic character of palmelloid formation was (and potentially is) under selective pressures and has been a precursor to multicellular evolution in this lineage that became fixed via genetic assimilation (see p. 71 - 72). It is intuitive that plasticity should often increase under selection, if the most plastic individuals are the most capable of colonizing a novel environment or persist in a fluctuating environment (Pigliucci et al., 2006).

Intergenerational Epigenetic Inheritance

Whereas Maynard Smith and Szathmáry (1995) see all evolution, from the emergence of the first DNA-based cells to the acquisition of language by hominids, in terms of changes in the genetic system, Jablonka & Lamb (1994, 1995, 2005) have argued that new types of information and modifications of existing non-genetic information systems were fundamental to the major transitions in evolution (Smith & Szatmáry, 1995; Jablonka & Lamb, 1994, 1995, 2005). The transition to multicellularity has occurred several times in the evolution of life on earth (Bonner, 1998), but the most interesting of the transitions are those that led to present-day plants, animals and fungi (Bonner, 1998). In these transitions, a division of labour between genetically identical component cells has resulted in many interdependent, phenotypically different cell-types. Because the determined and differentiated states of these cell types are inherited in cell lineages, the importance of non-DNA information transmission (epigenetic inheritance or “cell memory”) in the evolution of complex multicellular organisms has been widely

recognized (e.g. see Jablonka, 2004, Jablonka & Lamb, 1995; Maynard Smith & Szathmáry, 1995; Wolpert, 1990).

The view that biological information is transmitted from one generation to next by the DNA sequence alone has been increasingly questioned over the recent years (there are many examples but see e.g. Liu, 2007). Information not directly encoded in the DNA sequence can also be transmitted between generations. For example, non-genetic information can be transmitted when DNA or its associated proteins (e.g. histones) are modified, as is the case for DNA methylation and acetylation and histone modifications such as ubiquitylation, sumoylation and phosphorylation (Verhoeven & Van Gurp, 2012, Krohnholm et al. 2017). This process is often referred to as Neo-Lamarckian inheritance, which occurs whenever *a stimulus-dependent character in one generation, becomes stimulus-independent in the following generations* (Jablonka & Lamb, 1999). There is good evidence that unicells transmit non-DNA information about their structure, their environment and state of activity to daughter cells (Jablonka & Lamb, 1999), so it can be argued that during the evolution of multicellular organisms, transmission mechanisms that existed in ancient unicells were recruited and selectively modified in ways that improved their efficiency and fidelity (Jablonka & Lamb, 2005). Without efficient transmission of epigenetic information, the cells of new multicellular organisms would have switched to inappropriate states that would have compromised the success of the individual as a whole (Jablonka & Lamb, 2005). The non-vertical (inheritance may be affected horizontal- that is, not from parent to offspring alone) components of genetic and non-genetic inheritance may help to explain the enigma of major evolutionary transitions - that is, the emergence of new levels of organismal complexity as shown in **Table 1**. Initially, autonomous cells are thought to have cooperated through molecular communication (Jablonka & Lamb, 2005). When such communication eventually led to the reconstruction of the same trait value in a descendant multicellular organism, inheritance was thus generated (either genetic or non-genetic) within the lineages of the multicellular organisms (Jablonka & Lamb, 2005). Additionally, pre-transition epigenetic alterations (which might have constituted a form of cell plasticity or immunity against parasite strands of DNA) are thought to have been recruited for cell differentiation, which allowed the division of labour among cell lineages (as seen in V.

carteri), thereby potentially stabilizing the new organism. According to such scenarios, mitotic epigenetic inheritance is thought to have played a major part in the emergence of mechanisms that prevent individual cells from behaving independently.

The results of many studies investigating the role of DNA (histone) methylation⁷ in plasticity remain largely correlative, that is, differences in methylation are associated with environmental gradients or treatments: Weiner and Toth (2012) reviewed evidence for the role of methylation in caste determination in social insects. Holeski (2007) found that *Mimulus guttatus* plants that were induced to produce more trichomes in response to herbivory also produced offspring with elevated trichome levels even if offspring were not damaged. Scoville et al. (2011) also studied *M. guttatus* trichome production, investigating the role of epigenetic marking. They found that MYB MIXTA-like8 expression was negatively correlated with trichome production, and was significantly downregulated in both damaged parental leaves and in undamaged progeny, suggesting that downregulations were transmitted epigenetically to offspring (Scoville et al., 2011). These studies offer a glimpse into mechanisms of environmentally dependent epigenetic modification, but the evolutionary implications are rudimentary at best and substantial further work is necessary (Grativol et al. 2014). Similarly, it is conceivable that *C. reinhardtii* cells that were induced to produce palmelloids in response to environmental conditions (e.g. high salinity, toxins) or herbivory (or chemical cues associated therein) may also produce offspring with elevated palmelloid occurrence even if offspring were not exposed to the environmental conditions or to herbivory.

Essentially, many epigenetic marks are environmentally induced. Plasticity itself (such as palmelloid formation in *C. reinhardtii*) can be mediated by epigenetic modification, and transgenerational (maternal) effects may be due to maternal plasticity (e.g.,

⁷ A process by which methyl groups are added to the DNA molecule. Methylation may change the activity of a DNA segment without changing the sequence. When located in a promoter region, DNA methylation typically acts to repress gene transcription. This process is essential for normal development and is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, repression of transposable elements, aging and carcinogenesis (Bird, 2017). DNA methylation, catalysed by the DNA methyltransferases (DNMTs), is regarded as a key player in epigenetic silencing of DNA transcription (Jin et al. 2011).

provisioning) or to epigenetic inheritance. Krohnholm et al. (2017) carried out experiments in four different high salinity environments culturing *C. reinhardtii* to investigate if methylation is correlated with adaptation to a high-stress environment. They manipulated the production and transmission of epigenetic variation either genetically or chemically and reared four *C. reinhardtii* strains in high salinity environments (see Krohnholm et al., (2017) for details). Krohnholm and colleagues (2017) expected the high salt environment to exert stress on the cells which should respond (adapt in their sense) to the environment by an increase in growth rate. This assumption does not take the formation of colonies and the associated reduction in growth rate into account which has been previously observed when *C. reinhardtii* was reared in high salinity environments (Khona et al. (2016) and confirmed in this study). Therefore, the assumption that *C. reinhardtii* should respond to high salinity conditions with higher growth rates seems untenable. In the study by Krohnholm et al. (2017) the genomes and methylomes were sequenced after the exposure to high levels of salinity. The authors noted, that if it is the case that epigenetic changes are used in an adaptive response, it is expected that manipulating the amount of epigenetic variation available for the populations will reduce adaptation in the different environments. When comparing the methylation patterns of evolved populations, the strains cultured in high salinity environment clustered together (colony formation) based on methylation differences. This observation clearly shows, that there is an epigenetic, heritable factor to the palmelloid formation in *C. reinhardtii*. If variant *C. reinhardtii* with variant genomes and methylomes experience differential reproductive success (e.g. cells with the ability to withstand osmotic perturbation are more fit) and if the variant methylomes are heritable, then the composition of a population would shift towards cells with the ability to form a *milieu interior* (Bernard, 1978) via the manifold process of natural selection as well as additional processes such as mutation and genetic drift among others. The hypothesis posed above, that the phenotypically plastic trait of palmelloid formation is heritable *but not genetic* is thereby supported with empirical evidence. Populations subjected to control or only slight epigenetic manipulation were able to “adapt” to high NaCl regardless of strain, while the more severe epigenetic manipulation impeded adaptation to high salt. Overall, Krohnholm et al. (2017) saw, that decreasing epigenetic variation decreased or impeded adaptation to the high salt environment. The results from

the high NaCl environment most closely matched the prediction that reducing the amount of epigenetic variation available with the chemical treatments decreased adaption. Nonetheless, as has been written above, it is unsure if the *C. reinhardtii* palmelloid formation constitutes active or passive plasticity when the cells are subjected to e.g. hyperosmotic conditions and if the formation of palmelloids therefore is an “active” “adaption” on the side of the organism. Taken together, the data support that epigenetic changes can be important in adapting to osmotic environmental changes and can be intergenerational inherited (Krohnholm et al. 2017; Jablonka & Lamb, 2005). If hypothesized unicellular *C. reinhardtii* ancestor populations formed palmelloids in e.g. hyperosmotic conditions, these ecological conditions prevailed for long enough a time for evolutionary change to occur and the cells forming palmelloids having had the advantage of forming a *milieu interieur* (Bernard, 1978) and therefore survived (due to homeostatic advantages over non-protected cells in the population(s)) and were selected by nature to beget more offspring (after Lewinton, 1970), passing the phenotypically plastic active or passive trait epigenetically on to their offspring which formed palmelloids also after the stimulus vanished (Jablonka & Lamb, 2005), then one potential outcome of this scenario may have been the by chance (e.g. via mutation⁸) evolution of permanent colonial individuals (ETI) and therefore one of the precursory life-forms to differentiated Volvocean algae may have occurred. Furthermore, since cellular specialization (e.g. the division of cellular labour between germ and soma in *Volvox carteri*), whether via genetic co-option or other means, after the initial encapsulation of related clones in an extracellular matrix, could take place *because* the cells were able to freely exchange information in the internal milieu of the collective without losing it to noise created by the external environment, they were therefore also able to exert cooperate and developmental pressure on one another (Reid, 1985; Woods & Wilson, 2013; Michod, 1999). This “how possibly” explanation (see conclusion, p. 80) serves as a heuristic starting point for future investigations (see areas of future research, p. 82).

⁸ E.g. it has been shown by Vallon & Wollman (1995) that a mutation in a gene involved in the posttranslational modification of glycoproteins, the primary component of *C. reinhardtii* cell walls, can cause *C. reinhardtii* palmelloid colonies to form (Vallon & Wollman, 1995).

In essence, colony formation in *C. reinhardtii* (and other algae such as *Chlorella vulgaris*) occurs via (i) artificial selection (e.g. settling selection) (ii) predation pressure (predator presence, infochemicals, selective feeding) (iii) and a variety of environmental stimuli (e.g. hyperosmotic conditions, toxins, acids etc.) (**Table 2**). The sheer multitude of factors inducing a colonial morphotype in these algae shows the complexity of the response and no factor alone should be held responsible for having been the sole selective pressure that was part of the evolutionary dynamics that lead to more complex multicellular algal body plans. Furthermore, there is a recurring logical fallacy when trying to make sense of adaptive colony-formation in *C. reinhardtii* in the light of the evolutionary transition to multicellularity. It is neither factual nor necessary, that “staying together” (e.g. the formation of colonies in e.g. the presence of predation pressure) has been a trait or a physiological response of a hypothetical *Chlamydomonas*-like ancestor prior to the emergence of multicellular life in this lineage. We tend to imagine the ancestors of multicellular groups as being similar to their extant unicellular relatives, but that is not a given. In fact, these unicellular species have been evolving just as long since their divergence from their multicellular relatives as the multicellular lineages have. There is no evidence whatsoever that the physiological response to environmental stimuli was or even had to be in place before e.g. differentiated *Volvox carteri* evolved. Colony formation in these organisms (algae) might have evolved at a point in geological time where multicellular and even differentiated multicellular organisms in these lineages were *already present* and the hypothesized *Chlamydomonas*-like ancestor was not ancestor, but bystander to this transition and “adopted” the plastic physiological response of colony-formation at a later point in evolutionary history. Furthermore, the selection pressure “predation, grazing pressure” does only constitute a selective pressure *prior* and during colony formation. *Post* colony formation, the selective pressure is either transformed (e.g. lowered in strength) or abrogated (since predators avoid colonies due to their inability to ingest them) (recall that the transition from a unicellular to a multicellular life-style acted in “a space of biological possibility that is evolving in itself” (Godfrey-Smith, 2009)). Since the evolution of multicellularity involved mainly changes that occurred *post* colony formation (**Fig. 3**), the selective pressure (grazing pressure, predation) might not be relevant for these organismal changes. I therefore think that predation pressure may have been a selective force that

fostered the evolution of colonies but that it might not have been important for/during the evolutionary transition in individuality from unicellular to multicellular life.

5. Conclusion

In this project, I aimed at investigating if palmelloid formation occurs in different strains of *C. reinhardtii* as a response to predator infochemicals, high salinity levels and various calcium media alterations. Furthermore, I conceptualized theoretical starting points for further investigation into the phenomenon of colony formation of this green algae. I was able to replicate the findings, that different *C. reinhardtii* strains form palmelloids in high salinity environments. It was not feasible to replicate earlier findings that altered Ca^{2+} and Mg^{2+} concentrations in the medium or the presence of predator-derived infochemicals induces palmelloid formation in the four distinct *C. reinhardtii* strains. For environmental factors that might differ in environments, one would expect that there is a variance in the traits associated with the response to them. The fact that all strains used in the experiments were capable of colony formation (showed the same response), suggests that this trait is a fundamental response among *C. reinhardtii* strains. I conclude that the response might constitute either active phenotypic plasticity (in the case of biotic stressors) or passive phenotypic plasticity (in the case of abiotic stressors). I further conclude, that the phenomenon of colony formation in *C. reinhardtii* constitutes a far more complex phenomenon than has been previously assumed.

Biologists in many fields of research give how-possibly explanations of the phenomena they study. Although such explanations lack empirical support, and might be regarded by some as “unscientific”, they play an important heuristic role in biology by helping biologists develop theories and concepts and suggesting new areas of research. How-possibly explanations serve as a useful framework for conducting research in the absence of adequate empirical data, and they can even become how-actually explanations if they gain enough empirical support (Resnik, 1991). The explanation for the evolution of colony formation in *C. reinhardtii* as a process of intergenerational epigenetic inheritance of an active or passive phenotypically plastic character presents such a “how-possibly” explanation and may open up areas of future research, integrating ecological experimentation with molecular biological methodology such as methylome sequencing in order to further shed a dim light on the major evolutionary transition from unicellular to multicellular life in the Volvocean green algae lineage. Particularly important is the shift away from the conception that the trait of colony formation is solely based on

genetic inheritance. I aimed at replicating frequently-cited studies since science moves forward by corroboration – when researchers verify other’s results instead of taking them for granted. In a recent survey of 1576 researchers, 52 % stated that there is a significant “crisis”. More than 70 % of researchers have tried and failed to reproduce another scientist’s experiments, and more than half have failed to reproduce their own (Nature, Vol. 533, 26 May 2016).

6. Areas of future research

The discrepancy between the biological relevance and the lack of data about infochemicals in the environment (or in laboratory studies) reveals the necessity of further research. According to many studies, infochemicals are so decisive for the interactions in an ecosystem that they should not be neglected in ecotoxicology and ecological research in general (Vet & Dicke, 1992). Furthermore, it is important to note that results of simple 2-species experiments cannot be extrapolated to more complex natural communities (Kratina et al., 2007). Regarding future research, it would be interesting to know whether infochemical detection in *Chlamydomonas* uses a common pathway or whether the mechanisms are predator-infochemical specific. Furthermore, it would be worthwhile to start an interdisciplinary approach towards (i) identifying the chemical structure and nature of infochemicals and (ii) using that knowledge to design experiments with uttermost certainty of infochemical presence as well as chemical concentration. Knowledge of this kind would allow for more complex multiple-species chemical-communication-community-dynamic studies that may be important for addressing e.g. the complex outcomes of shifts in trophic structures, species composition and the influence of physical water properties (such as pH, T°C) on inter- and intraspecific chemical communication. Regarding the study of influence of Ca²⁺ on palmelloid formation in *C. reinhardtii*, it might be interesting to set up a NaCl stress experiment as has been done in this thesis and additionally supply various Ca²⁺ concentrations *post* palmelloid formation to observe if the cation plays an important role in the stress response after the response has already been established.

During the end of the experiments, there has been an opportunity to participate in a workshop offered by the University of Oslo. Multiple samples of *C. reinhardtii* strain A reared in 50 mM NaCl conditions via a IN Cell Analyser 2500HS, a wide-field high-content imaging system were analysed. The process was done in a much faster fashion, with higher resolution and automatized identification of colonies via the provided software. An example of a microscopy picture showing *C. reinhardtii* palmelloid colonies can be found in the Appendix.

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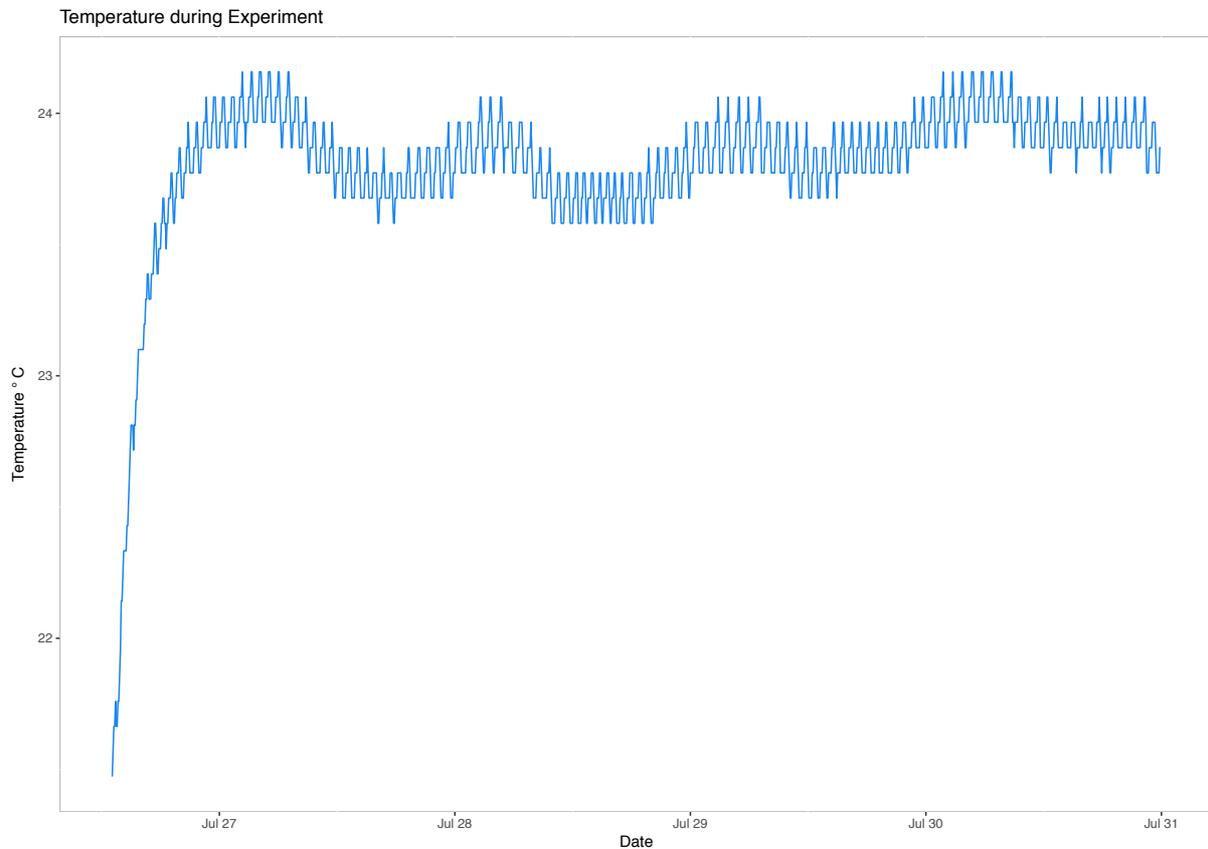
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Appendix



Temperature (°C) measured as stated in 2.2.4 during the first calcium manipulation experiment. Excerpt of the temperature logger data to show the relative consistency of temperature in the climate room during the experiments.

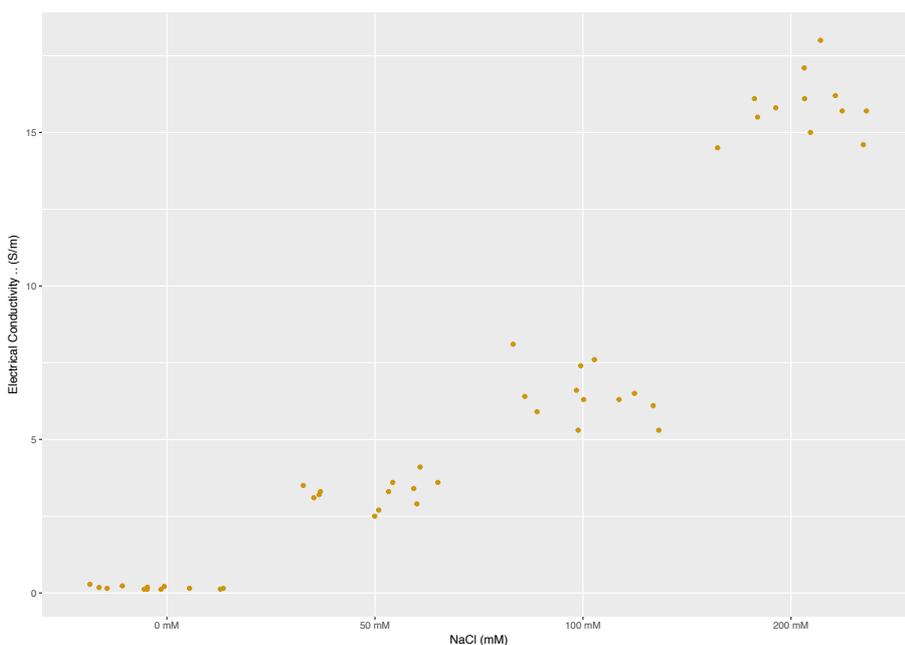


Fig. 20. Specific conductivity σ (S/m) measured as stated in 2.2.4 during the salinity experiment. Ochre points represent mean conductivity values of $n = 3$ culture replicates for each *C. reinhardtii* strain for each salinity treatment (0 – 200 mM NaCl).

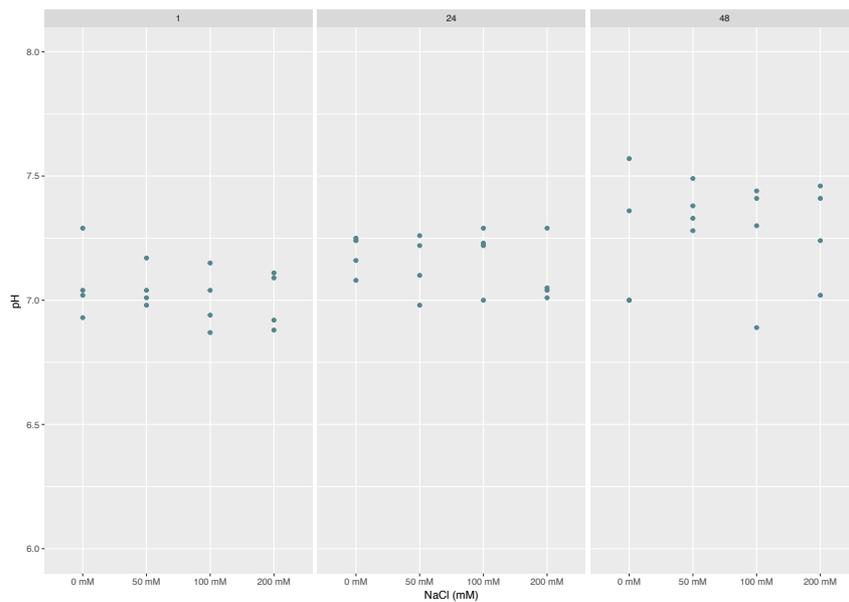


Fig. 21. pH measured as stated in 2.2.4. during the salinity experiment. Dark blue points represent mean pH values of $n = 3$ culture replicates for each *C. reinhardtii* strain for each salinity treatment (0 – 200 mM NaCl) faceted by different times of measurement (post inoculation).

In the salinity experiment, the (neutral) pH values of the media tended to be slightly higher (+ ~ 0.3) after 48 hours of culturing the strains in the various treatment flasks when compared to 1 hour or 24 hours of treatment. This could not be observed in the infochemical and calcium II experiments such that it is inconclusive as to what may have caused the (negligible) increase in pH over time. Unfortunately, due to an instrument malfunction, the pH values for the first calcium experiment cannot be presented here.

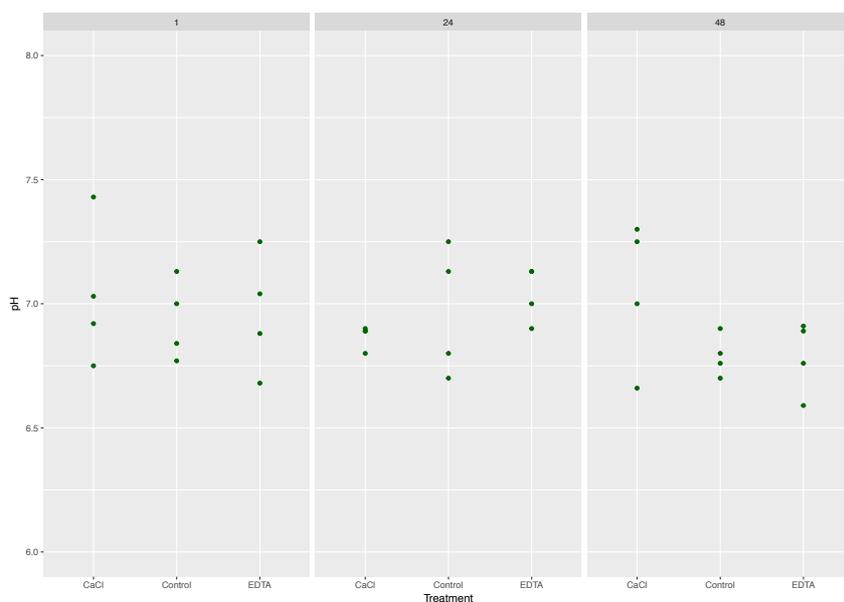


Fig. 22. pH measured as stated in 2.2.4 during the infochemical experiment. Brown points represent mean pH values of $n = 3$ culture replicates for each *C. reinhardtii* strain for each infochemical concentration treatment (0.5 % - 50 %) faceted by different times of measurement (post inoculation).

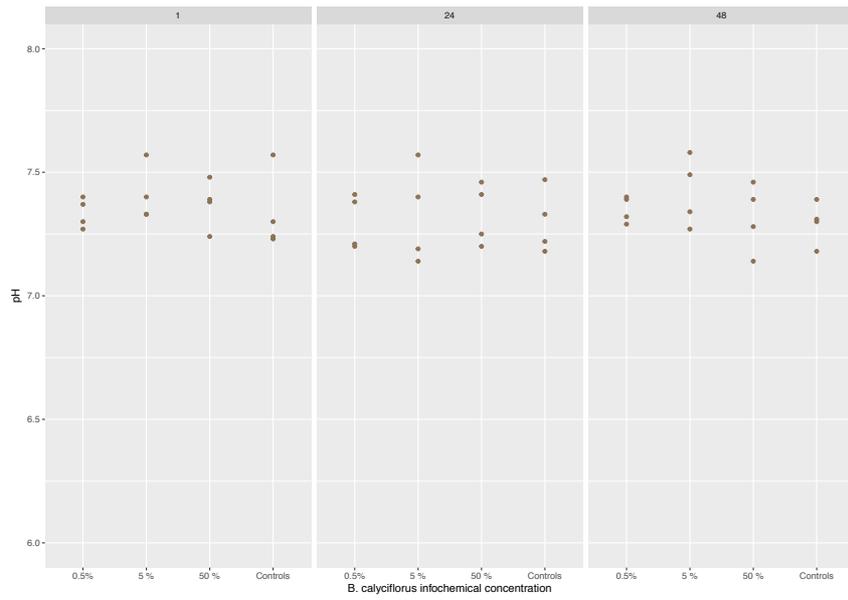


Fig. 23. pH measured as stated in 2.2.4 during the second calcium experiment (Iwasa). Dark green points represent pH values of $n = 1$ culture replicates for each *C. reinhardtii* strain for each treatment (Control, -CaCl₂, +EDTA) faceted by different times of measurement (post inoculation).

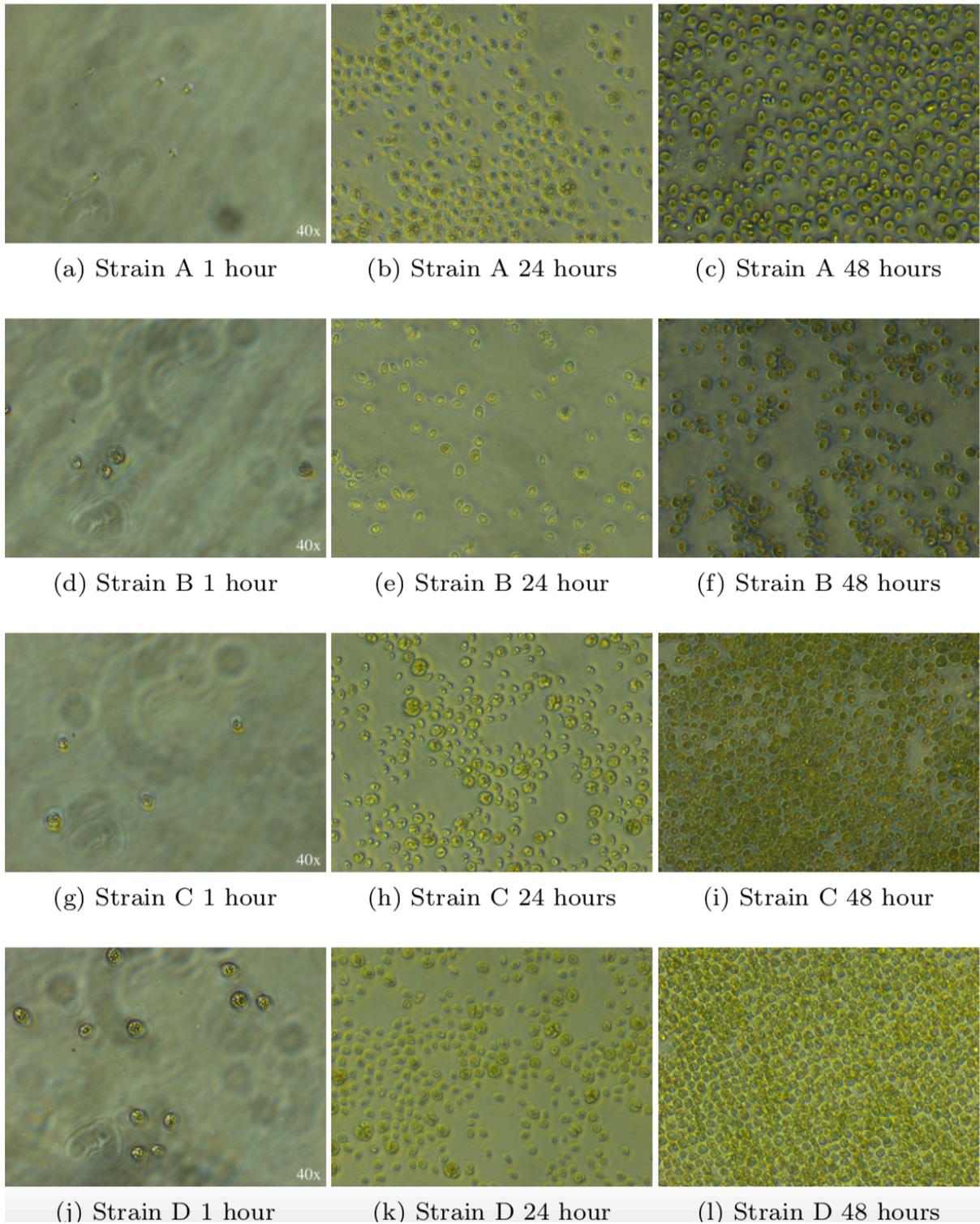


Figure 24. Microscopy pictures of *C. reinhardtii* strains A – D reared for 1 hour, 24 hours and 48 hours in the 50 % infochemical treatment (see Material & Methods section for further elaboration). (a) – (c): Strain A, (d) – (f) Strain B, (g) – (i) Strain C, (j) – (l) Strain D – 1 hour – 24 hours – 48 hours.

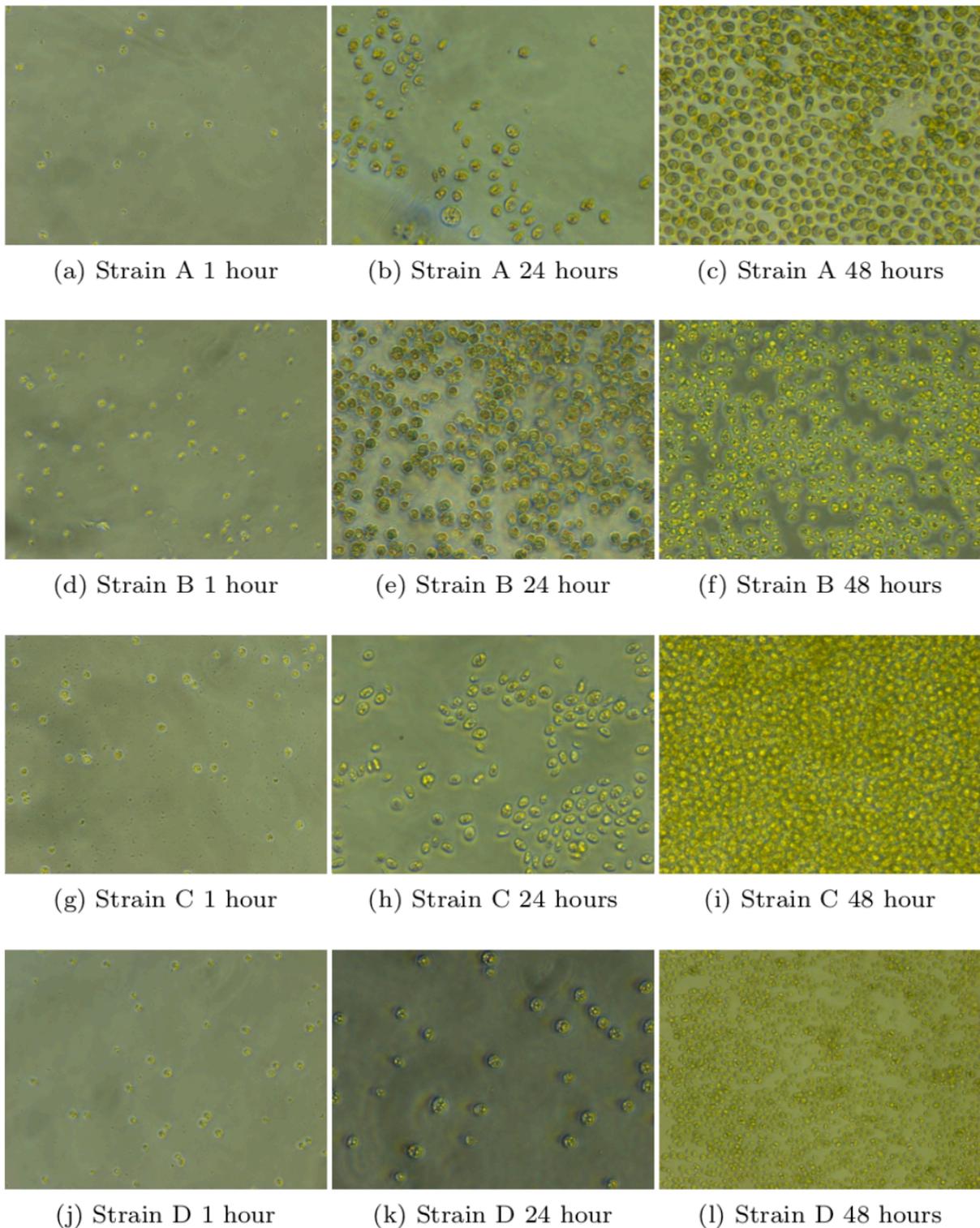


Figure 25. Microscopy pictures of *C. reinhardtii* strains A – D reared for 1 hour, 24 hours and 48 hours in the 5 % infochemical treatment (see Material & Methods section for further elaboration). (a) – (c): Strain A, (d) – (f) Strain B, (g) – (i) Strain C, (j) – (l) Strain D – 1 hour – 24 hours – 48 hours.

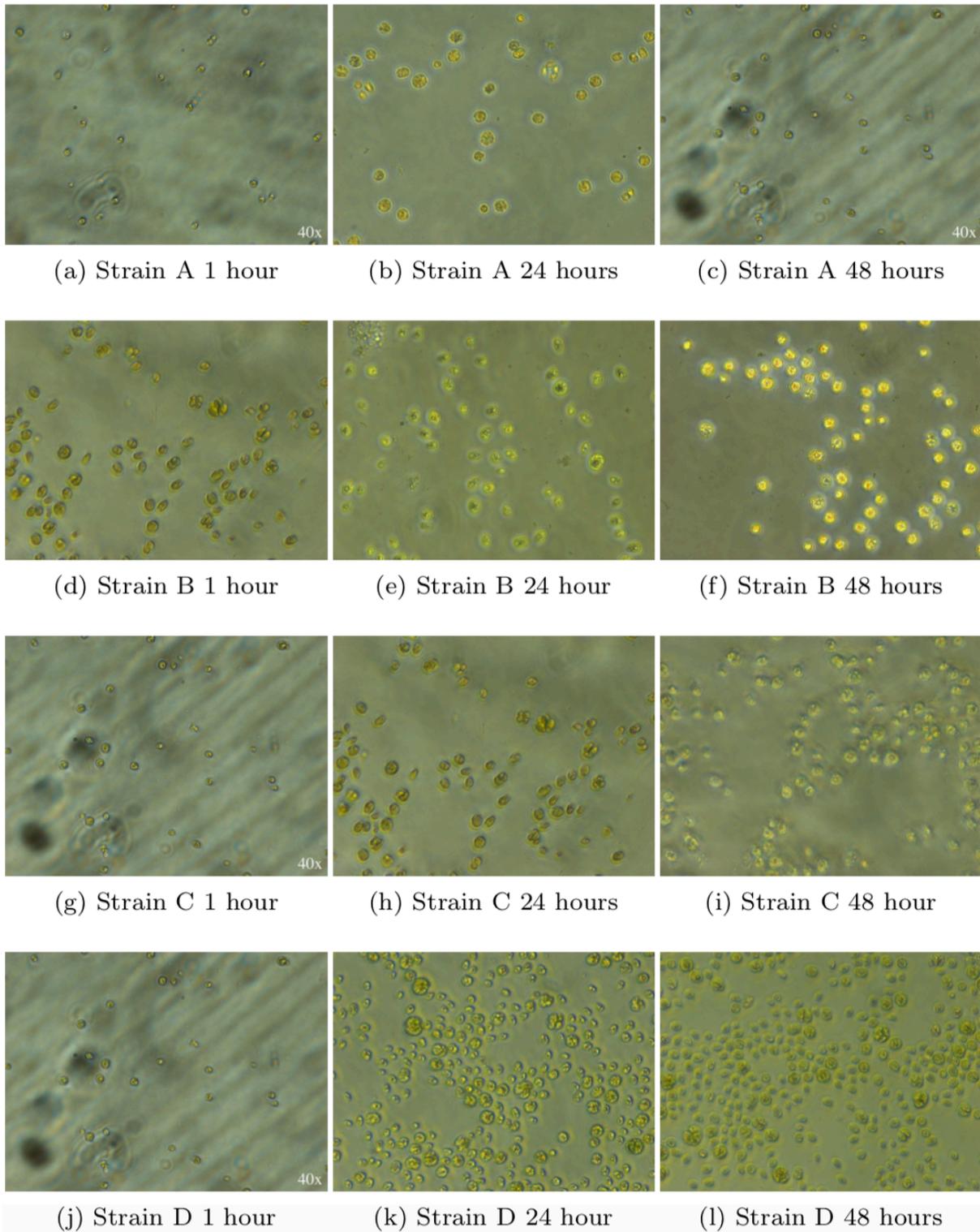
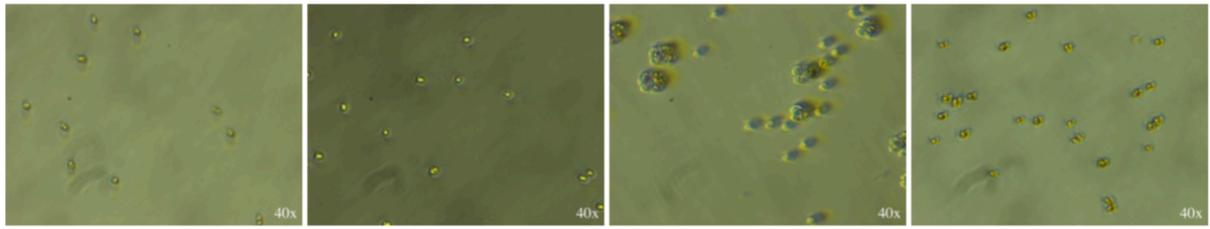
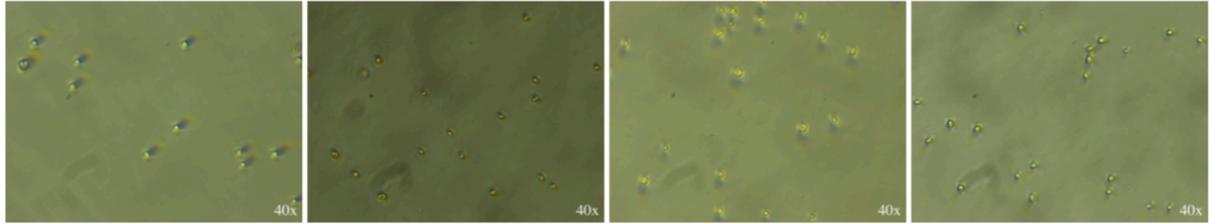


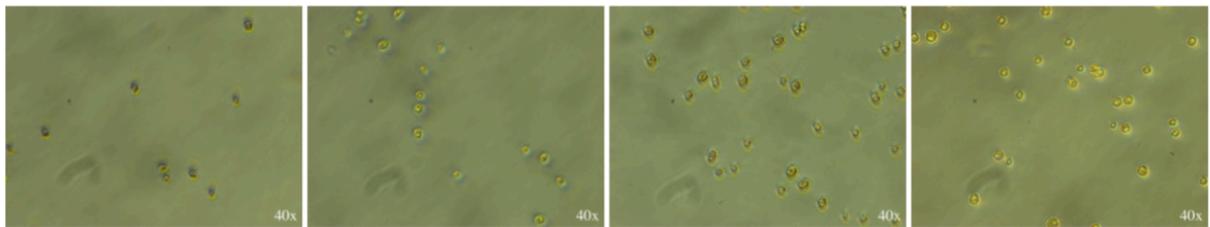
Figure 26. Microscopy pictures of *C. reinhardtii* strains A – D reared for 1 hour, 24 hours and 48 hours in the 0.5 % infochemical treatment (see Material & Methods section for further elaboration). (a) – (c): Strain A, (d) – (f) Strain B, (g) – (i) Strain C, (j) – (l) Strain D – 1 hour – 24 hours – 48 hours.



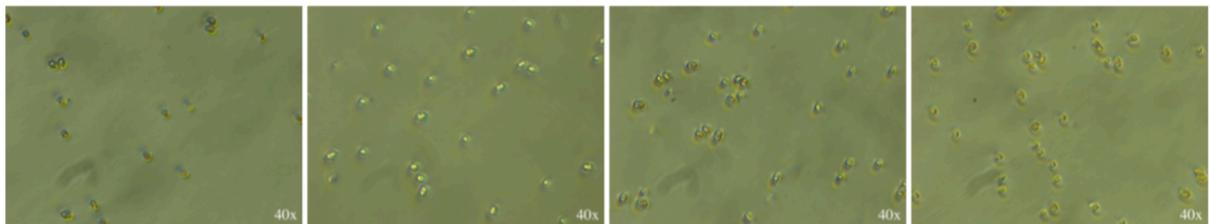
(a) Strain A 1 hour 0 mM (b) Strain A 1 hour 50 mM (c) Strain A 1 hour 100 mM (d) Strain A 1 hour 200 mM



(e) Strain B 1 hour 0 mM (f) Strain B 1 hour 50 mM (g) Strain B 1 hour 100 mM (h) Strain B 1 hour 200 mM

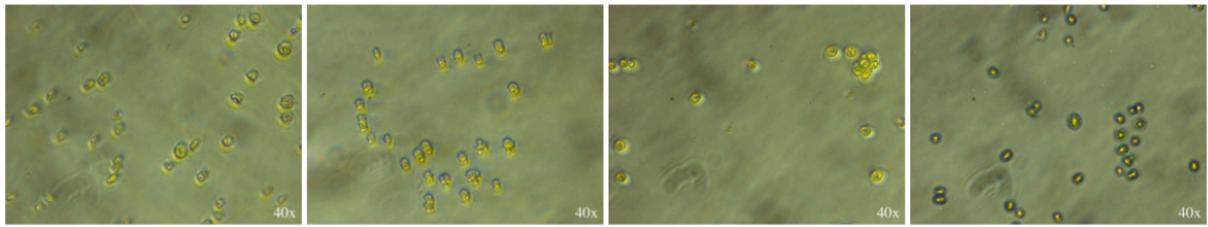


(i) Strain C 1 hour 0 mM (j) Strain C 1 hour 50 mM (k) Strain C 1 hour 100 mM (l) Strain C 1 hour 200 mM

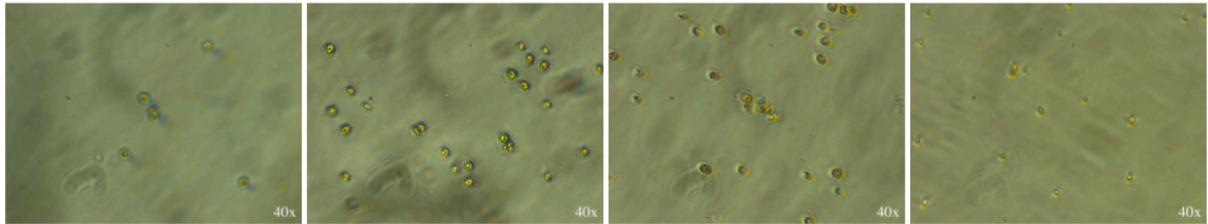


(m) Strain D 1 hour 0 mM (n) Strain D 1 hour 50 mM (o) Strain D 1 hour 100 mM (p) Strain D 1 hour 200 mM

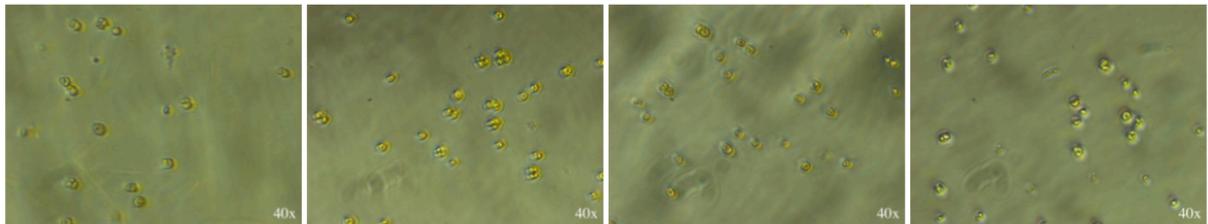
Figure 27. Microscopy pictures of *C. reinhardtii* strains A – D reared for 1 hour in the respective salinity treatments (see Material & Methods section for further elaboration). (a) – (d): Strain A, (e) – (h) Strain B, (i) – (l) Strain C, (m) – (p) Strain D – 0 mM, 50 mM, 100 mM, 200 mM.



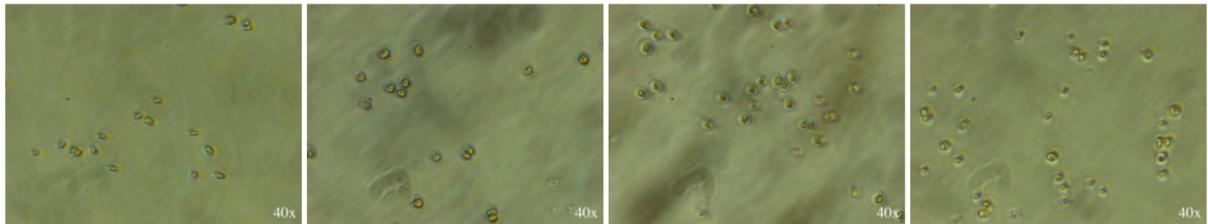
(a) Strain A 24 hours 0 mM (b) Strain A 24 hours 50 mM (c) Strain A 24 hours 100 mM (d) Strain A 24 hours 200 mM



(e) Strain B 24 hours 0 mM (f) Strain B 24 hours 50 mM (g) Strain B 24 hours 100 mM (h) Strain B 24 hours 200 mM



(i) Strain C 24 hours 0 mM (j) Strain C 24 hours 50 mM (k) Strain C 24 hours 100 mM (l) Strain C 24 hours 200 mM



(m) Strain D 24 hours 0 mM (n) Strain D 24 hours 50 mM (o) Strain D 24 hours 100 mM (p) Strain D 24 hours 200 mM

Figure 28. Microscopy pictures of *C. reinhardtii* strains A – D reared for 24 hours in the respective salinity treatments (see Material & Methods section for further elaboration). (a) – (d): Strain A, (e) – (h) Strain B, (i) – (l) Strain C, (m) – (p) Strain D – 0 mM, 50 mM, 100 mM, 200 mM.

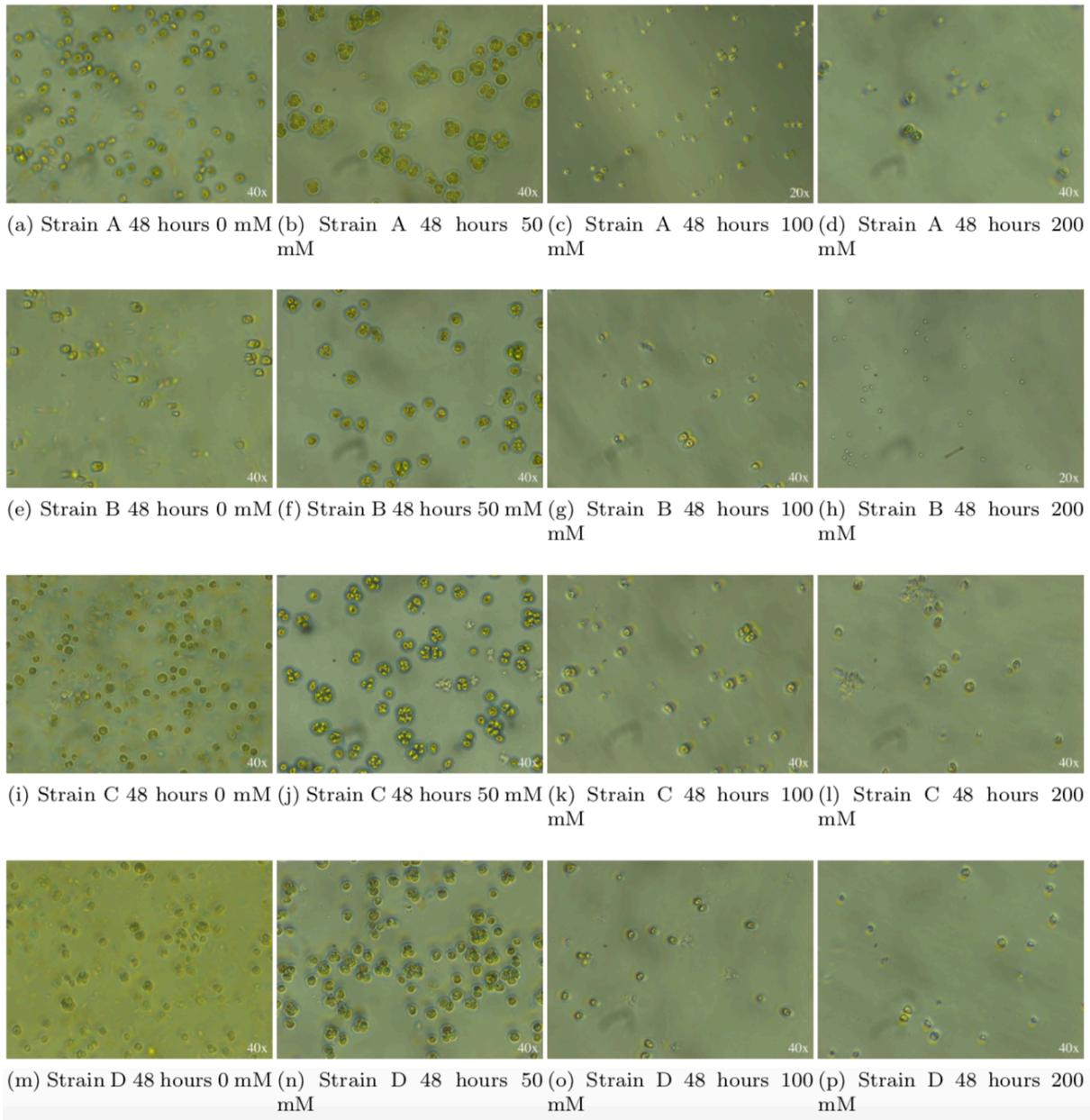


Figure 29. Microscopy pictures of *C. reinhardtii* strains A – D reared for 48 hours in the respective salinity treatments (see Material & Methods section for further elaboration). (a) – (d): Strain A, (e) – (h) Strain B, (i) – (l) Strain C, (m) – (p) Strain D – 0 mM, 50 mM, 100 mM, 200 mM.

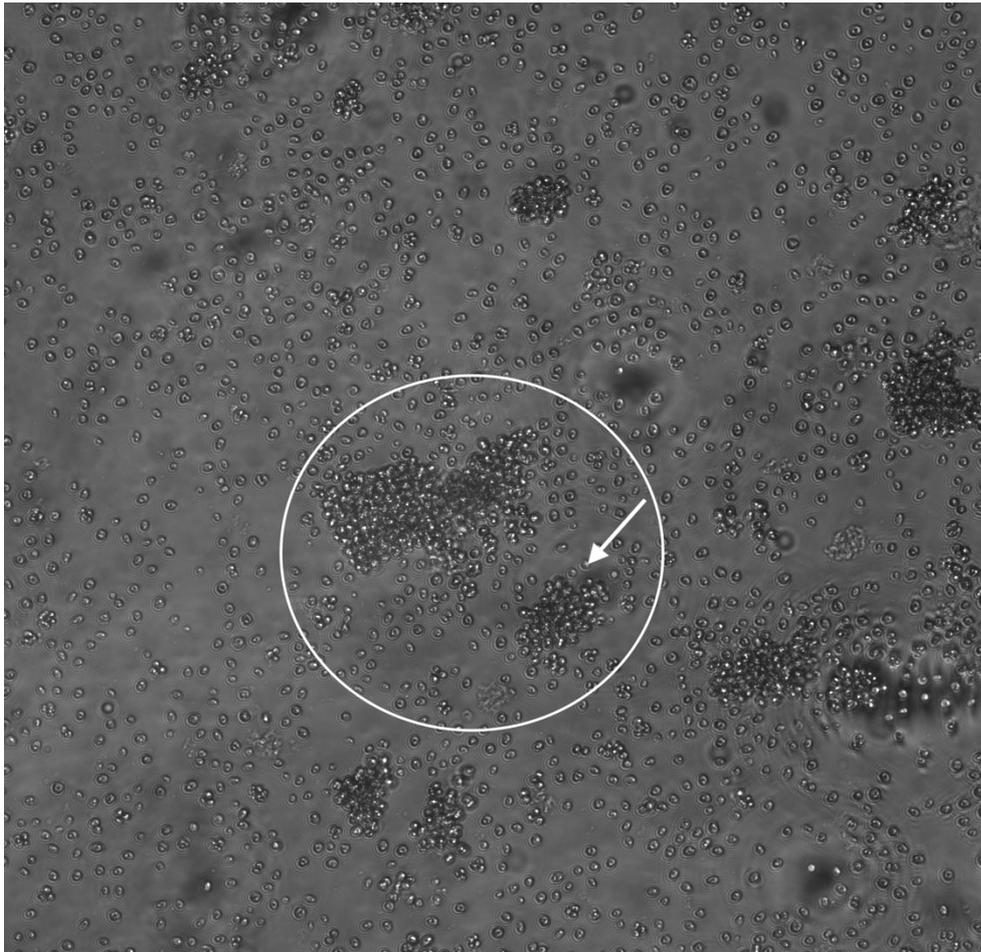


Figure 30. A microscopy picture of *C. reinhardtii* strain A reared for 24 hours in a 50 mM NaCl treatment, showing clearly the emergence of palmelloid colonies. The image was done during a workshop using an IN Cell Analyzer 2500 HS high content analysis (HCA) imaging system (GE Healthcare Life Sciences).

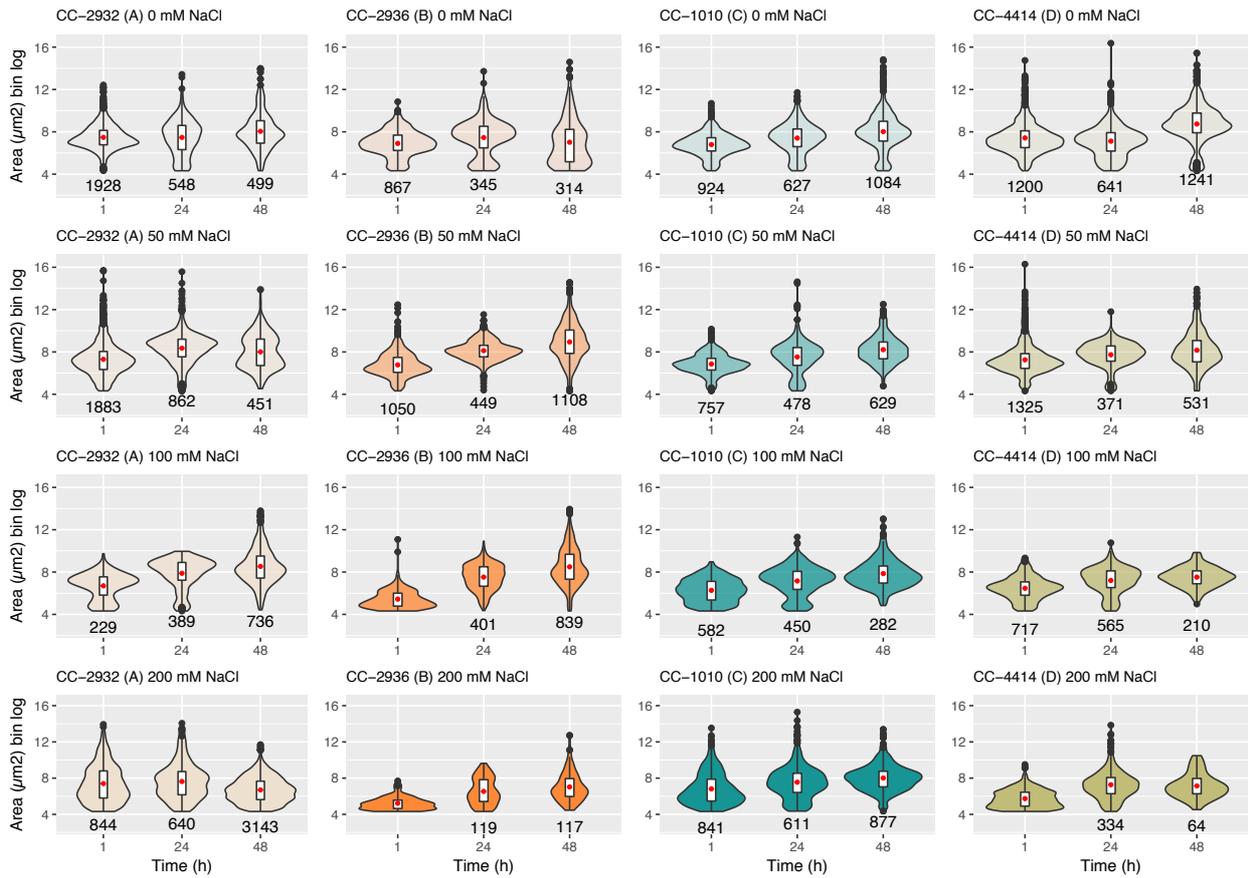


Fig. 31: Cell area measurements ($\log_2 \mu\text{m}^2$) of *C. reinhardtii* strains in respective treatment cultures (mM NaCl), indicated by plot transparency (lowest transparency: 0 mM – highest transparency: 200 mM) measured over time (h). Boxplots show median (red circle), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying violin plots coloured by strain (beige = A, orange = B, cyan = C, olive = D). Number of observations (n) is given underneath individual plots. The data for this plot represents aggregated data from all three replicate cultures.

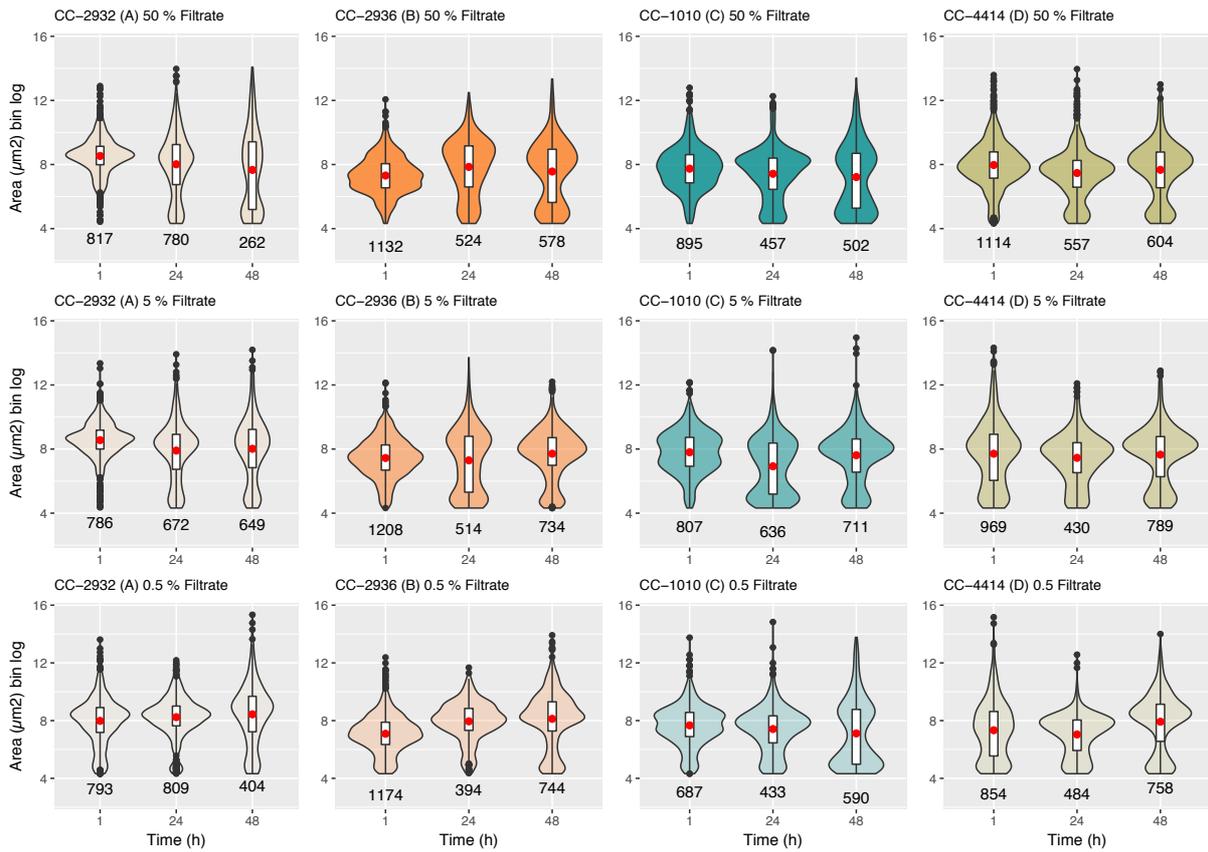


Fig. 32: Cell area measurements (μm^2 , \log_2 -scale) of each *C. reinhardtii* strain reared in either Treatment 1, Treatment 2 or Treatment 3 (see 5.3.3, indicated by plot transparency (lowest transparency: 50 % filtrate – highest transparency: 0.5 % filtrate), measured over time (h). Boxplots show median (red), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying, coloured violin plots (beige = A, orange = B, cyan = C, olive = D). Number of observations (n) is given underneath individual plots. Plots for the control cultures 1 and 2 are found below (**Fig. 33**, **Fig. 34**).

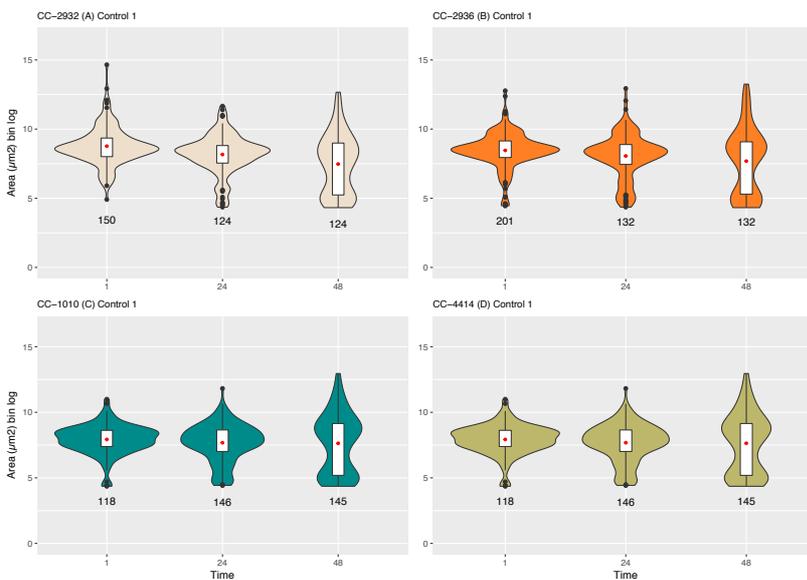


Fig. 33. Cell area measurements (μm^2 , \log_2 -scale) of each *C. reinhardtii* strain reared in control treatment 1, measured over time (h). Boxplots show median (red), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying, coloured violin plots (beige = A, orange = B, cyan = C, olive = D).

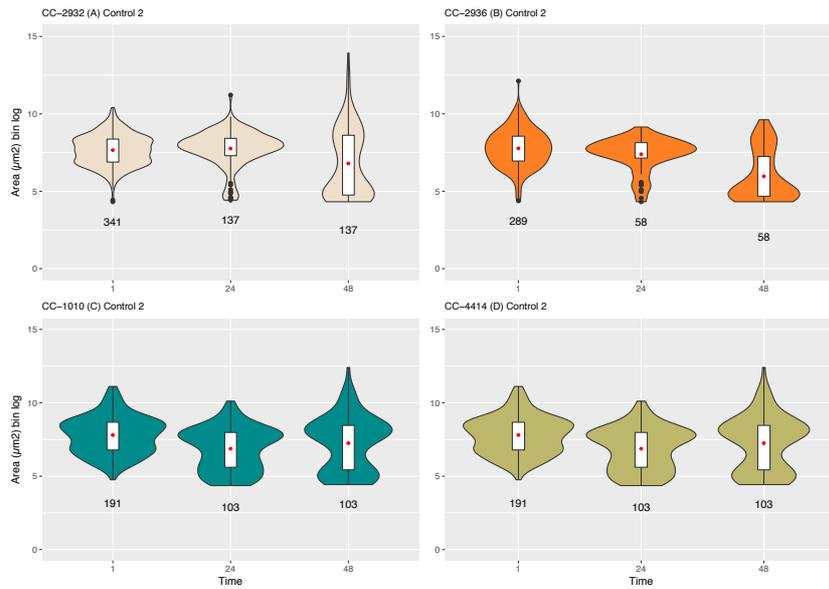


Fig. 34. Cell area measurements (μm^2 , \log_2 -scale) of each *C. reinhardtii* strain reared in control treatment 2, measured over time (h). Boxplots show median (red), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying, coloured violin plots (beige = A, orange = B, cyan = C, olive = D).

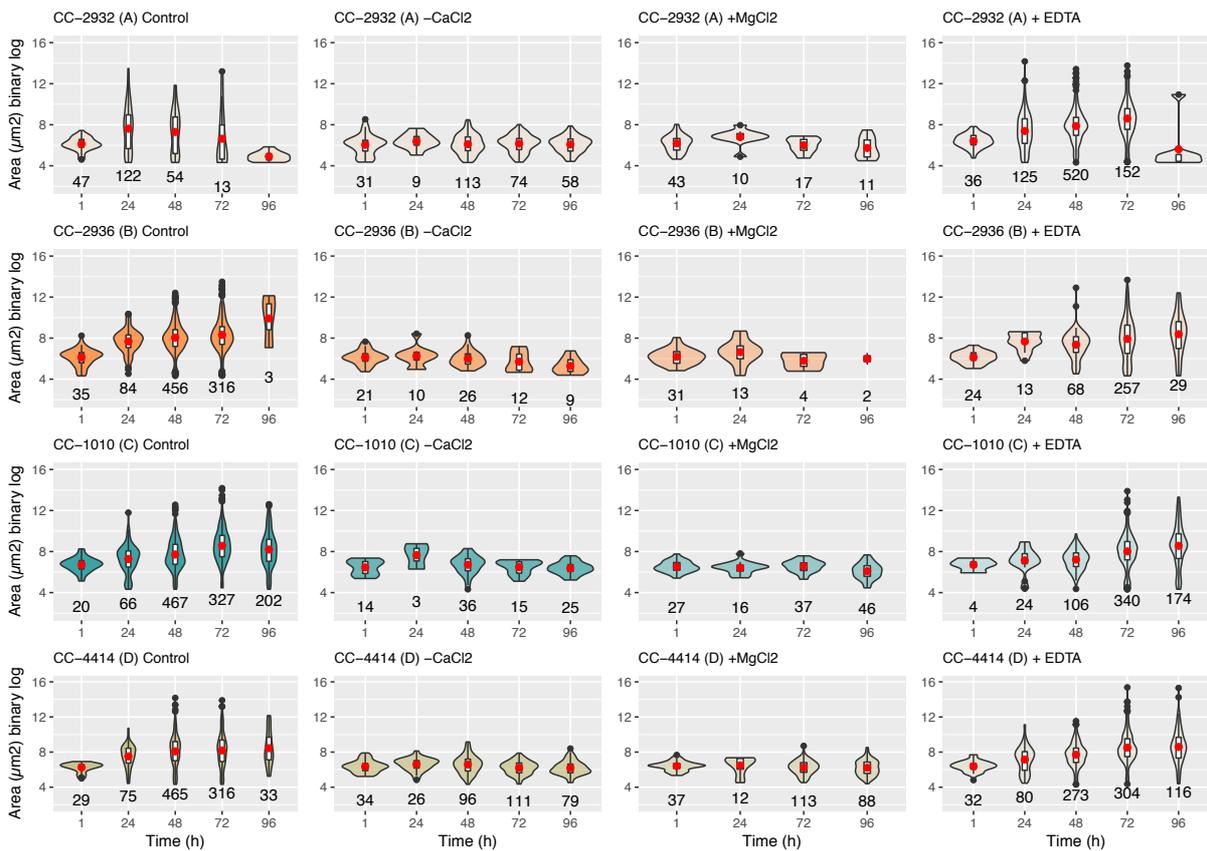


Fig. 35: Cell area measurements (\log_2 , μm^2) of *C. reinhardtii* stains in respective treatment cultures (indicated by plot transparency (low to high): Control, $-\text{CaCl}_2$, $+\text{MgCl}_2$, $+\text{EDTA}$), measured over time (h). Boxplots show median (red), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying, coloured violin plots (beige = A, orange = B, cyan = C, olive = D). Number of observations (n) is given underneath individual plots.

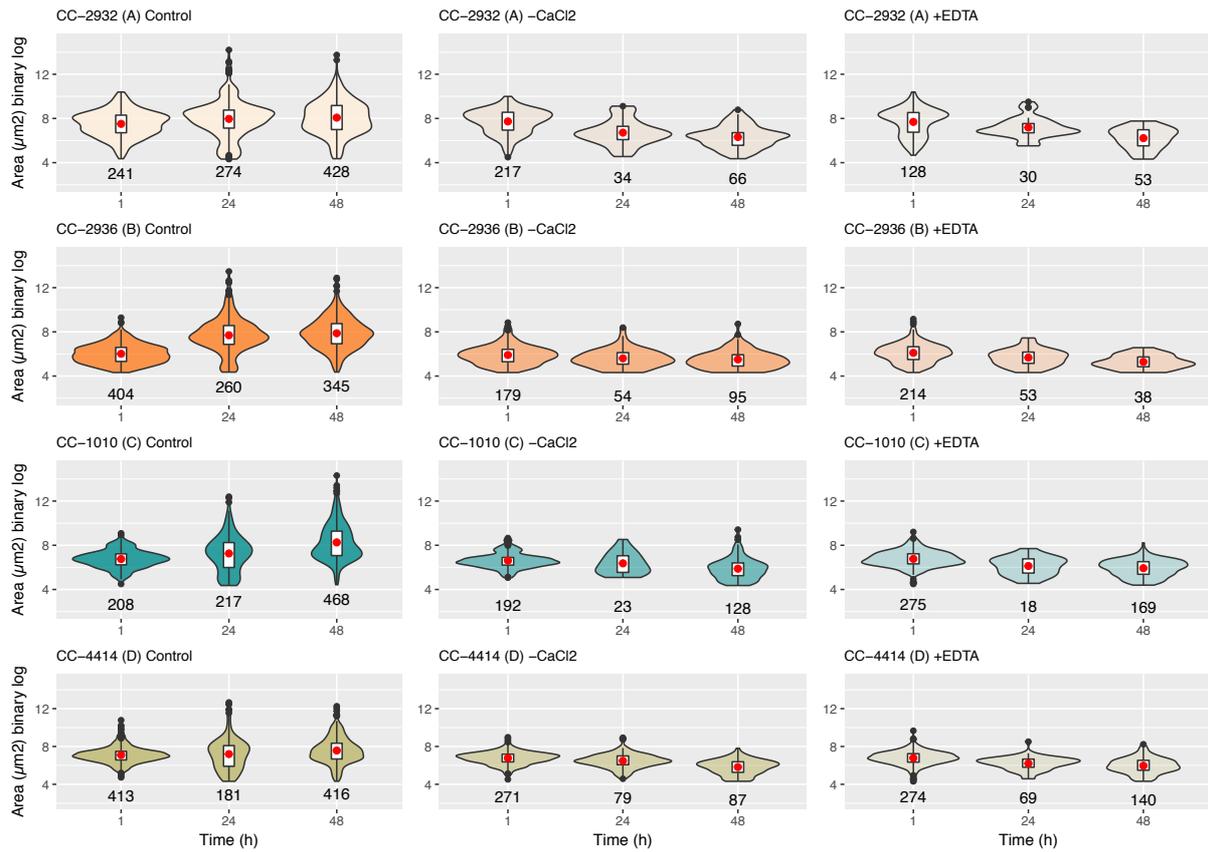


Fig. 36: Cell area measurements (μm^2 , \log_2 -scale) of each *C. reinhardtii* strain reared in either WC medium (control), 6.8×10^{-5} CaCl_2 treatment, and 12.5×10^{-5} M EDTA + 6.8×10^{-5} CaCl_2 treatment (indicated by plot transparency (lowest transparency: Control, middle: CaCl_2 , highest transparency: EDTA), measured over time (h). Boxplots show median (red), upper and lower quartiles and whiskers. Density information (distribution of data values) is given by underlying, coloured violin plots (beige = A, orange = B, cyan = C, olive = D). Number of observations (n) is given underneath individual plots.