UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA E RECURSOS NATURAIS

Morte celular programada em microalgas verdes cocoides e seus possíveis efeitos ecológicos

Marcelo Malisano Barreto Filho

Orientadora: Profa. Dra. Inessa Lacativa Bagatini

São Carlos – SP
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Dissertação de mestrado apresentada ao Programa de Pós-Graduação em Ecologia e Recursos Naturais da Universidade Federal de São Carlos, como parte dos requisitos para obtenção do título de MESTRE EM ECOLOGIA E RECURSOS NATURAIS, área de concentração em Ecologia e Recursos Naturais.

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Certifico que a defesa realizou-se com a participação à distância do(s) membro(s) John Alexander Berges e, depois das arguições e deliberações realizadas, o(s) participante(s) à distância está(ao) de acordo com o conteúdo do parecer da banca examinadora redigido neste relatório de defesa.

Profa. Dra. Inessa Lacativa Bagatini

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Marcelo *in memorian* e Mara,
por todo o carinho, compreensão
e amor.

Aos meus irmãos,
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que amo muito,
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"Não sou nada,
Nunca serei nada,
Não posso ser nada,
À parte isso, tenho em mim todos os sonhos do mundo"
(Fernando Pessoa)

"Nada na biologia faz sentido exceto à luz da evolução"
(Theodosius Dobzhansky)

Resumo

A morte celular programada (MCP) pode desempenhar importantes efeitos intra e interespecíficos nas populações fitoplanctônicas, e consequentemente na ecologia dos ecossistemas aquáticos. Entretanto, antes de quaisquer estudos ecológicos ou interpretações evolutivas, é essencial a correta documentação das alterações fenotípicas associadas à MCP, pois os morfotipos e marcadores podem ser diferentes em um mesmo organismo em resposta a diferentes estímulos. Além disso, as interpretações podem ser conflitantes devido à aplicação e interpretação incorretas de métodos e marcadores de MCP. Desta forma, no Capítulo 1, apresentamos uma revisão sistemática dos diferentes métodos e marcadores de MCP, e fizemos sugestões sobre como a MCP deve ser medida no fitoplâncton eucariótico. No capítulo 2, acrescentamos informações inéditas sobre a MCP em *Ankistrodesmus densus*, pertencente ao importante grupo de microalgas verdes da família Selenastraceae (Chlorophyta, Chlorophyceae, Sphaeropleales). Baseado na revisão realizada, a microscopia eletrônica de transmissão (TEM) parece ser essencial, como primeira abordagem, para se detectar adequadamente a MCP. Imagens ultra-estruturais devem ser comparadas e complementadas com métodos bioquímicos e genéticos. Em ordem de preferência, foram sugeridos a degradação do DNA (DNA *laddering*) e a externalização da fosfatidilserina (PS). Ensaios bioquímicos como a atividade de enzimas caspases e o aumento de espécies reativas de oxigênio devem ser usados com cuidado porque estes não são suficientemente específicos para MCP. Assim, nós utilizamos mudanças morfológicas ultraestruturais, externalização da PS e DNA *laddering* para documentação da MCP em *A. densus* em resposta à privação de luz, nitrogênio e ao choque de calor e frio. Nossos achados sugerem que a privação total de luz e nitrogênio podem levar a uma fração pequena, mas significativa, da comunidade à MCP. Por outro lado, há evidências de que

diferentes fenótipos de morte podem emergir em *A. densus* dependendo dos indutores ambientais, o que é essencial para a interpretação dos resultados de estudos ecológicos. Além disso, nossos resultados indicam que a morte celular do tipo vacuolar/autofágica já existia em microalgas verdes (Chlorophyta) unicelulares e evolutivamente pode ter sido importante para a homeostase tecidual em plantas.

Abstract

Programmed cell death may have important intra and inter-specifics effects in phytoplanktonic populations, and consequently in the ecology of freshwater ecosystems. However, before any ecological studies or evolutionary interpretations, it is essential to document the phenotypic changes associated with PCD since the morphotypes and markers may be different in the same organism in response to different environmental stimuli, implying in different ecology evolutionary interpretations. Moreover, these interpretations can also be conflicting due to incorrect application and interpretation of the measures and markers of PCD. Thus, in Chapter 1, we presented a systematic review of the different methods and measures of PCD, and offered suggestions as how PCD should be measured in eukaryotic phytoplankton. Additionally, in chapter 2 we provided a first-time report of PCD in *Ankistrodesmus densus,* a member of the important microalgal group of the family Selenastraceae (Chlorophyta, Chlorophyceae, Sphaeropleales). Based on our revision, transmission electron microscopy (TEM) appears essential, as the first approach, for properly detecting PCD. Ultrastructural images should be compared and complemented with biochemical and genetic assays. In order of preference, we suggested DNA laddering and PS externalization. Biochemical markers such as caspase activity and the increase in reactive oxygen species (ROS) should be used with caution since these are not specific enough for PCD. Therefore, we have used ultrastructural morphological changes, PS externalization and DNA laddering to document PCD in *A. densus* in response to darkness, nitrogen starvation, and heat and cold shock. Our findings suggest that light and nitrogen deprivation may lead to a small but significant fraction of community to undergo PCD. On the other hand, we also present evidence that different death morphotypes can emerge in *A. densus* depending on different environmental inducers, which is essential for the interpretation of the results of

ecological studies. Moreover, our results indicate that the vacuolar/autophagic like cell death morphotype existed in single-celled green microalgae (Chlorophyta) before land plants evolved and might have been an important development for tissue homeostasis in higher multicellular plants.

Lista de siglas e abreviaturas

Ac-DEVDCHO - Caspase-3-inhibitor

APAF-1 - Apoptosis Activating Factor-1

APX - Ascorbate PeroXidase

AsA-GSH - Ascorbate-glutathione

ATG - AuTophaGy Related

Boc-D-FMK – Broad spectrum caspase inhibitor

CAT - Catalase

CCMA - Coleção de Cultura de Microalgas verdes de Água doce

Cyt C – Cytochrome C

Cyt F - Cytochrome F

dad-1 - Defense Against Death factor-1

DD- Derived Diatoms

DEVDase - Caspase ability to cleave the sequence DEVD

DHAR - Dehydroascorbate reductase

E-64 -Protease inhibtor

EhMCs - *Emiliania huxleyi* metacaspases

EhVs - *Emiliania huxleyi* viruses

FITC - Fluorescein isothiocyanate

FITC-VAD-FMK - Fluorescently labeled caspase inhibitor

H2DVFDA - The cell-permeant ROS staining 2',7'-dichlorodihydrofluorescein diacetate

MCI, MCII, MCIII- Metacaspases 1, 2 and 3

MCP - Morte Celular Programada

MDHR - Monodehydroascorbate reductase

NCCD - Nomenclature Committee on Cell Death

NO – Nitric oxide

PCD - Programmed cell death

PE - Phosphatidylethanolamine

PG- Phosphatidylglycerol

PI - Propidium iodide

PS- Phosphatidylserine

PSII - Photosystem II

ROS - Reactive oxygen species

RT-PCR - Reverse transcriptase Polymerase Chain Reaction

RuBisCO - Ribulose-1,5-Biphosphate carboxylase/oxygenase

SOD - Superoxide dismutase (SOD)

TpMCs - *Thalassiosira pseudonana* metacaspases

TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labelling

UV - Ultraviolet

WEHD, DEVD, VEID, IETD or LEHD – Sequence substrates for caspases 1, 3, 6 ,8, and 9 respectively

z-VAD-FMK – Caspase-8-caspase inihibtor

Lista de figuras

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Capítulo 2

Figs. 3-22. Representative transmission electron micrographs revealing the ultrastrutural changes in *A. densus* exposed to different stress stimuli. Nu, nucleus; No, nucleolus; Mtc, mitochondria; Cw, cell wall; Cyt, cytoplasm; Clh, chloroplast; Thy, thylakoid; Vc, Vacuole; Chm, Chromatin; Pm, Plasm membrane; Pyk, Pyknosis; Gc, golgi complex. The morphological alterations described in the text are indicated by the numbered arrows. (Figs 3-6) Normal vegetative cells growing on WC complete medium under regular light and temperature conditions. Note the cytoplasm without vacuolization; (Figs 7-10) Cells after 4 days in complete darkness. Note the extensive cytoplasmic vacuolization (arrow 1), mitochondrial elongation (arrow 2), and pyknosis without chromatin marginalization (arrow 3); (Figs 11-14). Cells after 4 days under nitrogen starvation. Cells experienced vacuolization (arrows 1) followed by chloroplast alterations (arrows 3). Note arrows 2 on Fig. 11 which might be highly disorganized thylakoids; also mitochondria did not elongate (number 4). In addition, note the nucleolar condensation and the movement of the chromatin to the nuclear membrane (arrows 5) with ultimate marginalization (arrows 6); (Figs 15-18). Cells after exposure to 44°C for 1 hour and returned to standard conditions for 12 hours. Note the shrunken, damaged cytoplasm (arrows 1), possible membrane disruption (arrows 2), altered thylakoids (arrow 3) and balloon shaped mitochondria (arrow 4); (Figs 19-22). Cells after exposition to cold shock (0 °C) for 3 hours and returned to regular growth conditions for 12 hours. Overall, cells were not quite affected. Note, however, some chromatin condensation (arrow 1), slightly dilated

Lista de tabelas

Capítulo 1

APRESENTAÇÃO DA DISSERTAÇÃO

A dissertação foi elaborada para conter os itens: (1) Introdução geral; (2) Objetivos e Hipóteses; (3) Capítulos (com resultados e discussão) e (4) Discussão geral. A introdução geral, no início, bem como a discussão geral, no final, estão escritas em português, enquanto os capítulos estão apresentados em inglês. Os objetivos e as hipóteses estão escritos em ambas as linguagens.

Cada capítulo será apresentado no formato de artigo científico: com resumo, introdução, material e métodos, resultados, discussão e referências bibliográficas. Este formato foi escolhido para facilitar a publicação dos resultados obtidos.

O primeiro capítulo encontra-se formatado para a publicação na revista *Methods in Ecology and Evolution*. Neste capítulo, apresentamos uma revisão sistemática dos diferentes métodos e medidas do PCD, oferecendo sugestões sobre como a MCP deve ser medida no fitoplâncton.

No segundo capítulo da dissertação documentamos as alterações fenotípicas associadas à MCP na microalga Selenastraceae *A. densus* em resposta a diferentes e relevantes estímulos ambientais, que é fundamental na interpretação de futuros estudos evolutivos em ecologia. Este artigo foi formatado nos moldes da *European Journal of Phycology*, periódico escolhido para submissão.

DISSERTATION PRESENTATION

The dissertation was prepared to contain the items: (1) General introduction; (2) Objectives and Hypotheses; (3) Chapters (with results and discussion) and (4) General discussion. The general introduction, in the beginning, as well as the general discussion in the end are written in Portuguese, while the chapters are presented in English. The objectives and hypotheses are presented in both languages.

Each chapter is presented in the format of a scientific paper: with abstract, introduction, material and methods, results, discussion and references. This format was chosen to make easier the publication of the obtained results.

The first chapter was formatted for publication in the journal Methods in Ecology and Evolution. In this chapter, we present a systematic review of the different methods and measures of the PCD, and make suggestions on how PCD should be measured in phytoplankton.

In the second chapter of the dissertation we have documented the phenotypic changes associated with PCD in the Selenastracean microalgae *A. densus* in response to different relevant environmental stimuli, which is essential for the interpretation of ecology evolutionary studies. This article was formatted according to the lines of the European Journal of Phycology, the chosen periodic for submission.

Sumário

Introdução geral

A seleção natural, importante processo na evolução dos organismos, pode operar em níveis distintos - genes, células, grupos de células, organismos multicelulares, populações e comunidades (OKASHA, 2006). Fenômenos como a morte celular programada (MCP), por exemplo, ocorrem em nível celular, mas podem beneficiar o organismo multicelular. A morte programada em células selecionadas do organismo multicelular pode aumentar a aptidão geral, evitando que células individuais se repliquem indefinidamente às custas da organização multicelular superior (MICHOD, 2003, 2007; MICHOD; ROZE, 2001). Em razão disso, a MCP é considerada uma marca da multicelularidade com implicações importantes para o desenvolvimento, diferenciação celular e manutenção do correto funcionamento dos tecidos nos animais (KERR, 1971; SAUNDERS, 1966). Desta forma, no mundo multicelular, a MCP faz sentido uma vez que as células cooperam para aumentar o valor adaptativo do organismo como um todo.

No entanto, nos microrganismos unicelulares a morte da célula não traz benefícios diretos óbvios para o valor adaptativo dos indivíduos (DURAND *et al.*, 2014). Embora a origem da MCP nos organismos unicelulares possa ser pleiotrópica (NEDELCU *et al.*, 2011), evidências apontam para a possibilidade de uma natureza adaptativa da MCP (DURAND; RAMSEY, 2019). A maioria das explicações para a MCP no mundo unicelular se baseia na teoria de seleção de parentesco e / ou de grupo (AMEISEN, 2002; NEDELCU *et al.*, 2011; PEPPER *et al.*, 2013). Nesse cenário, a MCP é um mecanismo pelo qual o valor adaptativo dos organismos que morrem é transmitido para os parentes que permanecem vivos no ambiente (DURAND *et al.*, 2014; DURAND; RASHIDI; MICHOD, 2011).

Assim, a MCP pode desempenhar papéis ecológicos importantes (BIDLE, 2016; CHOI; BERGES, 2013; FRANKLIN; BRUSSAARD; BERGES, 2006). O fenômeno é ativado no fitoplâncton por uma ampla variedade de fatores abióticos, tais como estresse salino (AFFENZELLER *et al.*, 2009), escuridão (SEGOVIA *et al.*, 2003) e choque de calor (ZUPPINI; ANDREOLI; BALDAN, 2007), e bióticos, como infecção viral (BIDLE *et al.*, 2007) e alelopatia (VARDI, 2008). Mediado pela MCP, o material orgânico dissolvido liberado pode ter importantes implicações intraespecíficas (DURAND *et al.*, 2014; ORELLANA *et al.*, 2013). Em *Chlamydomonas reinhardtii*, por exemplo, a ativação da MCP pelo choque de calor libera substâncias que possibilitam que as células sobreviventes fiquem maiores e produzam mais indivíduos, aumentando o valor adaptativo. Além disso, a MCP também tem efeitos interespecíficos pois afeta substancialmente o fluxo de nutrientes orgânicos e inorgânicos nesses ambientes (BIDLE, 2016). Em adição a MCP também pode regular a propagação e dispersão durante condições desfavoráveis de crescimento em cianobactérias ao intermediar a formação de necrídeos para liberação de hormôgonias (BERMAN-FRANK *et al.*, 2004). Portanto, estudos sobre o papel ecológico da MCP são fundamentais para a compreensão da dinâmica de populações e comunidades frente ao estresse ambiental nos ecossistemas aquáticos.

No entanto, antes da realização de estudos ecológicos e/ou evolutivos sobre o papel da MCP, é essencial a correta documentação das alterações fenotípicas associadas ao fenômeno, pois os morfotipos e marcadores podem ser diferentes em uma mesma espécie em resposta a diferentes estímulos, implicando em diferentes interpretações. Além disso, é fundamental conhecer e definir os diferentes marcadores e os métodos mais apropriados para a documentação da MCP, uma vez que a aplicação e interpretação incorreta de métodos tradicionalmente utilizados, sem levar em consideração suas

limitações, podem levar à interpretação incorreta dos resultados. Desta forma, é fundamental se ter conhecimento sobre o que está sendo medido, dos processos biológicos nos quais os métodos são baseados, além de possíveis limitações técnicas, antes da atribuição de significado aos marcadores encontrados.

Atualmente, existem ensaios morfológicos, bioquímicos e celulares usados para identificar formas programadas de morte celular. Os marcadores clássicos procurados remetem à apoptose, que é um modo de morte comum nos animais (KERR; WYLLIE; CURRIE, 1972; SAUNDERS, 1966) e que inclui alterações morfológicas, como encolhimento celular, condensação de cromatina, exposição da fosfatidilserina (PS) (um fosfolipídeo) na membrana celular, e degradação do DNA, além de marcadores bioquímicos, como a ativação da caspases e aumento de espécies reativas de oxigênio (BIDLE & FALKOWSKI, 2004). No entanto, existem muitos outros modos de MCP além da apoptose (GALLUZZI *et al.*, 2012). A morte celular do tipo autofágica, por exemplo, é um importante modo de morte para plantas (MININA; SMERTENKO; BOZHKOV, 2014; VAN DOORN *et al.*, 2011) e algas verdes de parede celular (AFFENZELLER *et al.*, 2009), onde apoptose em sua visão clássica não é esperada devido à rigidez das paredes celulares que impedem a fragmentação da célula em "corpos apoptóticos" (Danon et al., 2000; van Doorn et al., 2011b).

A documentação da MCP em organismos fitoplanctônicos unicelulares é complexa. Nesses organismos, a complexidade dos caminhos moleculares de morte celular em conjunto com uma ampla gama de fatores ambientais resulta numa imensa variedade morfológica e bioquímica de marcadores de MCP mesmo dentro do mesmo táxon (JIMENEZ *et al.*, 2009). Além disso, táxons diferentes podem exibir marcadores distintos quando o mesmo estímulo é usado (AFFENZELLER *et al.*, 2009; VAVILALA *et al.*, 2015). Portanto, não existe um conjunto de métodos e marcadores pré-determinado

para se medir MCP no fitoplâncton, embora existam estudos que reportam os diferentes marcadores para diferentes estímulos e táxons.

Muitos destes estudos foram realizados com microalgas verdes da divisão Chlorophyta, que compreende espécies onipresentes de macro e microalgas (KRIENITZ; BOCK, 2012). Nesta importante divisão, muitas espécies de microalgas foram testadas por uma ampla gama de gatilhos ambientais, e muitos marcadores foram descritos (AFFENZELLER *et al.*, 2009; JIMENEZ *et al.*, 2009; SEGOVIA *et al.*, 2003; VAVILALA *et al.*, 2015; ZUPPINI; ANDREOLI; BALDAN, 2007). Assim, as clorófitas podem fornecer importantes respostas para o entendimento do papel ecológico e fisiológico da MCP no fitoplâncton (BIDLE, 2015; FRANKLIN; BRUSSAARD; BERGES, 2006).

Por outro lado, dentro de Chlorophyta, a documentação de MCP em algumas famílias ainda é escassa. Este é o caso, por exemplo, da família Selenastraceae (Sphaeropleales, Chlorophyceae) que compreende um grupo de microalgas cocoides altamente diversificado e muito comum em corpos de água doce (GARCIA DA SILVA *et al.*, 2017; KRIENITZ *et al.*, 2001). Apesar de sua importância nos sistemas aquáticos, pouco se sabe sobre os programas de morte celular que desempenham um papel em sua ecofisiologia (KOZIK *et al.*, 2019). O único relato de MCP em microalgas desta família foi realizado *in situ* em ambiente eutrófico, durante transição entre primavera e outono, por meio de um único marcardor, a externalização de fosfatidilserina (KOZIK *et al.*, 2019). No entanto, dada a complexidade de variáveis em um ecossistema aquático, não foi possível encontrar correlação da MCP com as variáveis ambientais medidas.

Desta forma, exposta a complexidade e a dispersão de dados sobre métodos e marcadores de PCD em fitoplâncton, nosso primeiro objetivo neste estudo foi realizar uma revisão sistemática dos diferentes métodos e marcadores de MCP já reportadas para

o fitoplâncton, com ênfase em algas verdes. A revisão é apresentada em forma de artigo (Capítulo 1) e traz sugestões sobre os métodos e marcadores mais adequados para medir a MCP no fitoplâncton, baseado na significância e interpretação de cada um deles. Dessa forma, esperamos auxiliar pesquisadores na escolha de métodos em futuros estudos de documentação de MCP no fitoplâncton. O outro objetivo desta dissertação, foi contribuir com informações sobre a MCP no importante grupo de microalgas da Família Selenastraceae. A caracterização dos fenótipos de morte celular induzidos por diferentes estímulos ambientais representa um estudo de documentação inédito de MCP em *Ankistrodesmus densus* (Clorophyta, Chlorophyceae, Sphaeropleales) e é apresentada também na forma de artigo (Capítulo 2). Ao examinar as respostas fenotípicas à morte celular induzidas por mudanças ambientais drásticas em luminosidade, temperatura e flutuações em macronutrientes, nós buscamos oferecer informações importantes que ajudarão pesquisadores a elucidar os papéis evolutivos e ecológicos da MCP em futuros estudos nesta importante família de microalgas verdes.

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Objetivos

1) Apresentar uma revisão sistemática dos diferentes métodos e medidas de MCP e oferecer sugestões sobre como ela deve ser medida no fitoplâncton.

2) Documentar as alterações fenotípicas associadas à MCP no importante grupo de microalgas da família Selenastraceae, a fim de fornecer dados úteis para futuros estudos evolutivos e ecológicos.

Objectives

1) To present a systematic review of the different methods and measures of PCD, and offer suggestions on how PCD should be measured in phytoplankton.

2) To document the phenotypic changes associated with PCD in the important microalgal group of the Family Selenastraceae, in order to provide useful data for future evolutionary ecological studies.

Hipóteses

Um processo de MCP pode ser ativado na microalga *A. densus* em resposta a diferentes estímulos ambientais.

Os marcadores de MCP podem variar em um mesmo taxón dependendo dos estímulos ambientais a qual o organismo é submetido.

Hypothesis

A PCD process can be activated in the microalgae *A. densus* in response to different environmental stimuli.

The MCP markers can vary in the same taxon depending on the environmental stimuli on which the organism is submitted.

Measures of programmed cell death in eukaryotic phytoplankton

Running title: Measuring PCD in eukaryotic phytoplankton.

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Abstract

1. To address questions concerning the ecological and evolutionary role of Programmed Cell Death (PCD) in unicellular organisms, clarity on how to measure it is essential. The misapplication of some traditional, widely used methods without considering their limitations leads to erroneous interpretations. Here, we present a systematic review of the different methods and markers of PCD in phytoplankton, with an emphasis on the green algae, and suggest suitable approaches.

2. We provide an historical overview on how PCD has traditionally been measured in microalgae. We critically analyze the markers and measures used in different eukaryotic phytoplankton. We have assigned significance to the measures and complemented it by providing assessments of their frequency of use and their detection efficiency.

3. Transmission electron microscopy (TEM) appears essential, as the first approach, for detecting whether a cell is dying in an organized way or whether it is incidental, haphazard death. The ultrastructural alterations indicate whether death includes changes in gene and protein regulation and is thus dying by a 'programmed' mechanism. DNA laddering, a marker that is specific to PCD, was detected in 63% of cases. Phosphatidylserine (PS) externalization was consistently demonstrated, except in dinoflagellates, but there are constraints in terms of its specificity. Caspase-like activity was used in 50% of published works reporting PCD and detected in 87% of cases where PCD was observed. This is noteworthy since phytoplankton lack caspases. Reactive oxygen species (ROS) and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining, were used in 40% of reports with an 80% and 70% positivity, respectively. The latter assays, however, are not specific to PCD.

4. We suggest possible ways for measuring PCD reliably. Overall, the adoption of a polyphasic approach that uses TEM, in conjunction with genetic and biochemical assays

seems most appropriate. TEM is labour intensive but is helpful for corroborating the findings of other methods. For the biochemical methods, we suggest DNA laddering, PS externalization (annexin V), followed by the other markers. In addition, the upregulation of PCD-related genes (e.g. metacaspase, ROS enzymes) and chloroplast biochemical alterations (e.g. degradation of Rubisco and PSII) can be important markers of cells undergoing PCD. Caspase-like and metacaspase activity and inhibition assays, and TUNEL staining are less specific and should be used with caution.

Key words: PCD, microalgae, phytoplankton

Introduction

Phytoplankton are central to global primary productivity (Worden, Nolan, & Palenik, 2004) supporting secondary (i.e. zooplankton) and tertiary consumers (e.g. fish, crustaceans, marine mammals) through complex food webs (e.g. Fig.1 in Bidle, 2016). They are also key components in the recycling of nutrients (Falkowski, 1994) by absorbing carbon dioxide (CO_2), acting as a carbon sink to the deep sea and recycling biological minerals and compounds in the oceans (Bidle, 2016; Hülse, Arndt, Wilson, Munhoven, & Ridgwell, 2017). Factors that control phytoplankton losses, therefore, have enormous ecological implications across aquatic systems. Phytoplankton loss rates are controlled by grazing, sinking, hydraulic washout, and death (Reynolds, 1963). However, the evolutionary ecology of death in aquatic systems is poorly understood (Franklin, Brussaard, & Berges, 2006). The mechanisms of phytoplankton death can be attributed to viruses (Brussaard, 2004), parasites (Kühn, 1998), incidental physicochemical damage, senescence, and programmed cell death (PCD), which is emerging as a much more important component than previously thought. It has emerged that PCD is of great significance for understanding death in the dynamics of aquatic communities (Franklin, Brussaard, & Berges, 2006). For example, PCD impacts the fitness of others in the population and community (Vardi et al., 1999; Moharikar, D'Souza, Kulkarni, & Rao, 2006; Durand, Rashidi, & Michod, 2011; Durand, Choudhury, Rashidi, & Michod, 2014; Yordanova, Woltering, Kapchina-Toteva, & Iakimova, 2013). PCD-derived dissolved organic materials (DOMs) from dying cells can be re-used by conspecifics and/or by co-occurring bacteria for reproduction (Franklin, Brussaard, & Berges, 2006; Orellana, Pang, Durand, Whitehead, & Baliga, 2013; Berges & Choi, 2014; Bidle, 2016). Thus, not surprisingly, "the ways in which

cells die" (Segovia et al., 2003) impact the evolution of microbial systems (Durand et al., 2016) and evolutionary transitions (Blackstone & Green, 1999; Michod & Nedelcu, 2003; Blackstone, 2013, 2016; Iranzo, Lobkovsky, Wolf, & Koonin, 2014; Durand, Filho, & Michod, 2019).

There are different definitions of PCD, but mechanistically it can be defined as an "active, genetically controlled, cellular self-destruction driven by a series of complex biochemical events and specialized cellular machinery" (Berman-Frank, Bidle, Haramaty, & Falkowski, 2004). The important component is that death is realized by a genetic programme as opposed to incidental mechanisms (Durand & Ramsey, 2019). The programme can be activated in freshwater and marine phytoplankton species by a wide range of abiotic (e.g. Segovia, Berges, Litti, & Falkowski, 2003; Zuppini, Andreoli, & Baldan, 2007; Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009; Vavilala, Sinha, & D'Souza, 2014; Sathe, Orellana, Baliga, & Durand, 2019), and biotic factors (Evans, Malin, Mills, & Wilson, 2006; Bidle, Haramaty, Barcelos e Ramos, & Falkowski, 2007; Vardi, 2008; Kozik, Young, Sandgren, & Berges, 2019).

The field of PCD in unicells is fraught with difficulties. One of the main reasons, perhaps even the most important reason, for this can be attributed to the methods used to detect PCD. They have been imported, sometimes without due consideration (Nedelcu, Driscoll, Durand, Herron, & Rashidi, 2011), from the multicellular literature, where they were developed to detect metazoan cell death based on simple cause-effect relationships (Franklin, Brussaard, & Berges, 2006; Kozik, Young, Sandgren, & Berges, 2019). But, the ecological scenarios in the unicellular world are much more complex. The ecological contexts are very different from multicellular organisms. In some instances, the trait appears cooperative (Fabrizio et al., 2004; Herker et al., 2004; Büttner et al., 2006; Durand, Rashidi, & Michod, 2011; Durand, Choudhury, Rashidi, &

Michod, 2014; Orellana, Pang, Durand, Whitehead, & Baliga, 2013; Yordanova, Woltering, Kapchina-Toteva, & Iakimova, 2013), and co-ordinated at the group level (Vardi et al., 1999; Choi, Brosnahan, Sehein, Anderson, & Erdner, 2017). In others it appears non-adaptive (Segovia, Berges, Litti, & Falkowski, 2003), and in others still it impacts community dynamics (Bidle, 2016). Furthermore, the biotic and abiotic interactions in highly variable environments make the interpretations of the causes of death more difficult (Franklin, Brussaard, & Berges, 2006; Kozik, Young, Sandgren, & Berges, 2019). For example, in *D. salina*, the morphotypes and markers of PCD change depending on different stimuli (Jimenez et al., 2009) with different ecological outcomes. For instance, in *Dunaliella salina* darkness-induced PCD in a hypersaline environment was interpreted as a means of sustaining the population and in terms of nutrient recycling (Orellana, Pang, Durand, Whitehead, & Baliga, 2013). But in the congeneric *Dunaliella tertiolecta* PCD can be interpreted as a non-adaptative phenomenon (Segovia, Berges, Litti, & Falkowski, 2003). Two different species were studied, which of course may explain the different findings, but the implications for understanding the evolutionary histories are grave. In the first instance, PCD is an adaptation. In the second it is maladaptive. In these particular examples, the methods were sound, but for comparative purposes the same methods should be used. Furthermore, the death morphotypes in the two instances were different and only by using standardized methodologies and contexts can results be compared. Thus, the first step towards disentangling all the philosophical and evolutionary questions (Durand & Ramsey, 2019) as well as the downstream ecological interpretations (Franklin, Brussaard, & Berges, 2006; Berges & Choi, 2014) depends on knowing that PCD is being detected accurately.

To begin to address the confusion concerning the measures and markers of PCD there are two important starting points. First, it must be clear what is meant by PCD. The evolutionary definitions are dealt with elsewhere (Durand & Ramsey, 2019). Here, it is only important to provide a broad mechanistic definition of PCD, which we refer to as "any form of death that is non-incidental and involves a genetic programme, but is not specific to a particular morphotype" (Sathe, Orellana, Baliga, & Durand, 2019). Second, for empiricists, it must be clear how PCD should be detected. In this review, we will deal with the latter.

Clearly, the methods for documenting PCD in unicellular phytoplankton need to be sound. This, however, is often where the problem lies because there is no single pathognomonic sign of PCD, except of course at a late stage when the cell has completely self-destructed. The assays used to detect some markers in phytoplankton are usually very sensitive but not always specific to PCD (Berges & Choi, 2014) and there is sometimes only superficial knowledge concerning what the finding actually means. There are also technical difficulties, which in conjunction with the complexity of the cell death activation pathways, the inconsistencies in methods and the various ecological contexts, are a source of contrasting interpretations (Reece, Pollitt, Colegrave, & Gardner, 2011; Berges & Choi, 2014). Without suitable comparative approaches that take into account the limitations and strengths of the methods, it is difficult to draw meaningful conclusions. At least within taxonomic groups, it seems reasonable to adopt a strategy that can be used consistently for comparative and interpretative purposes.

We present a systematic review that compares different methods and markers of PCD in microalgae with a specific emphasis on the green algae. Before proceeding with this, it is necessary to provide an overview on the mechanisms of PCD because the

methods are based on these underlying mechanisms. A general overview of the main developments in methodologies will follow. We will critically review the methodologies as well as the frequencies of use, efficiencies of detection, and make suggestions for harmonizing the different approaches.

Unicellular model organisms in cell death research

Unicellular algae are used extensively in PCD research (Bidle, 2016). They are also enormously diverse. The *green algae,* for example, comprise more than 10000 species (Guiry and Guiry, 2019), most of them unicellular. Although recent systematics has split this group in two phyla: Chlorophyta and Streptophyta (Leliaert et al., 2012), here we will use the term chlorophytes to include all green algae.

There are a range of morphological, biochemical and cellular assays used to identify programmed forms of cells death in phytoplankton. PCD is typically mediated by metacaspase enzymes (Uren, 2000; Aravind & Koonin, 2002; Franklin, Brussaard, & Berges, 2006; Minina et al., 2020), although there are also metacaspase-independent mechanisms (Broker, Kruyt, & Giaccone, 2005), leading to a series of biochemical and morphological phenotypes. Morphological changes include cell shrinkage, chromatin condensation and externalization of phosphatidylserine, while biochemical markers involve activation or inhibition of proteases, and the degradation of DNA into oligo fragments (Bidle & Falkowski, 2004). Although a categorization of morphotypes and the mechanisms implicated in death have been proposed in metazoa (Galluzzi et al., 2009, 2012), and yeast (Carmona-Gutierrez, Fröhlich, Kroemer, & Madeo, 2010), a similar framework for unicellular microalgae is still missing, despite our understanding that in microalgae there are "different ways to die". (Jimenez et al., 2009). In fact, there is considerable controversy over the kinds of cell death. The categories of cell death in

animals and yeast have sometimes been employed in phytoplankton, but that has largely been unhelpful and led to confusing interpretations (animal and yeast cells lack plant cell walls, chloroplasts and many other intracellular organelles). The PCD morphotypes observed in plant cells are more appropriate for green algae (van Doorn et al., 2011; Minina, Smertenko, & Bozhkov, 2014).

Death phenotypes vary depending on the nature of the stimuli (heat, nutrient depletion, oxidative stress, UV light, anti-metabolites and physical trauma), and may change depending on the ecological context (Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009; Jimenez et al., 2009; Vavilala, Sinha, & D'Souza, 2014). Additionally, the PCD molecular pathways can overlap with related stress responses like encystment (Vardi et al., 1999), aging (Johnson, Janech, & Van Dolah, 2014), sexual reproduction (Nedelcu, Marcu, & Michod, 2004), biofilm formation (Vardi, 2008), acclimation and cell-survival mechanisms (Choi et al. 2017, Minina et al. 2017). The differing responses between and within taxa to different environmental stimuli, the overlapping stress responses, and the variations in assay sensitivities and specificities have led to misapplications and misinterpretations. A detailed examination of the current measures will be the first important step to resolving these issues.

Literature review and analysis

To compare the detection methods of PCD in eukaryotic phytoplankton, we conducted an exhaustive search by way of the web search engine, *Google scholar*. This on its own in sometimes considered an adequate searching tool for systematic reviews (Beckmann & von Wehrden, 2012), but we included the databases web of science (https://clarivate.com/webofsciencegroup/solutions/web-of-science/), pubmed (https://www.ncbi.nlm.nih.gov/pubmed/), research gate (https://www.researchgate.net)

and science direct (https://www.sciencedirect.com). The keywords “Programmed Cell Death (PCD)”, “Cell death”, “Apoptosis” “Phytoplankton”, “Unicells”, “Microalgae”, “Chlorophyta” “Dinoflagellates”, “Diatoms”, “Haptophytes” were used in conjunction with specific methodological words such as “Transmission electron microscopy (TEM)”, “Caspases”, “Reactive oxygen species (ROS) ”, Annexin V, “DNA laddering”, Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), “Metacaspase” and “Phosphatidylserine (PS)”. Manuscripts related to prokaryotic phytoplankton (e.g. cyanobacteria) were not used. As a general framework for measuring PCD in unicellular phytoplankton, we have included the findings in other organisms (e.g. plants, yeast) to characterize and describe the traditional measures and markers.

Twenty eight original manuscripts (table 1) that reported PCD-related phenotypes were selected. For calculation purposes, works that replicated findings in the same model organism, exposed to the same stimulus and over the same set of conditions were used only once to avoid bias (e.g. caspase-like activity was measured each time in dark-exposed *Dunaliella tertiocleta* in Segovia et al., 2003, 2005 and 2009; and in heat-stressed *Chlorella saccharophila* in Zupinni et al., 2007, 2009). For each study we have identified author, organism, stimulus, and taxonomy. Taxonomy was assigned according to algaebase (https://www.algaebase.org) (Guiry and Guiry, 2020).

The frequency of use and the efficiency of detection were assessed for each method (table 2). The frequency of use is the number of times the method was used (as a percentage) to detect PCD in the 28 studies. The efficiency of detection is the percentage of positive findings for a particular method when PCD was concluded. The methods used were based on known PCD mechanisms including genes, cell biochemistry and physiology, membrane changes, and morphology.

Significance of each method was assigned according to two important criteria: sensitivity and specificity. Based on this, it is possible to classify each method as a hard sign, soft sign, and a potential gold standard (Durand, 2020). Hard signs are very specific markers but are not sensitive, in other words they may report false negatives. Soft signs on the other hand include markers which are highly sensitive, but not unique to PCD. They may report false positives. Gold standards are both, specific and sensitive markers of PCD (Durand, 2020).

This approach was used to evaluate which methods are most reliable in detecting PCD in eukaryotic phytoplankton. The reference to compare the method to, was the authors' own conclusion that PCD had occurred. It is acknowledged that there is an inherent bias and the logic is circular because PCD was declared in the publications based on the methods used and we use the method to assess its efficiency in detecting PCD. However, because there were multiple methods used and not all the methods were simultaneously positive, the bias is to some degree eliminated. Furthermore, there is no established 'gold standard' in phytoplankton and we have to rely on the authors' data analysis. This is reasonable since the papers were all published by reputable journals. It is worth noting as well, that unlike in prokaryotes where there is sometimes a single PCD gene, which is the molecular gold standard, there is no such single gene for PCD in eukaryotes (at least not in all the taxa examined here). The only possible metric therefore for determining whether PCD occurred was to rely on the authors' own conclusions.

Mechanisms of PCD

A broad outline of the PCD mechanisms across phytoplankton taxa highlights the stages at which methodological assays for detection are implemented. (Fig. 1). PCD can be induced in phytoplankton in response to a variety of environmental stimuli (see table 1) including heat shock (Nedelcu, 2006; Zuppini, Andreoli, & Baldan, 2007; Durand, Rashidi, & Michod, 2011), nitrogen starvation (Berges & Falkowski, 1998; Sathe et al., 2019), Ultraviolet (UV) radiation (Moharikar, D'Souza, Kulkarni, & Rao, 2006), darkness (Berges & Falkowski, 1998; Segovia, Berges, Litti, & Falkowski, 2003; Orellana, Pang, Durand, Whitehead, & Baliga, 2013), acetic acid (Zuo, Zhu, Bai, & Wang, 2012), salt stress (Ferroni et al., 2007; Zuppini, Andreoli, & Baldan, 2007; Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009), iron availability (Bidle & Bender, 2008), CO_2 availability (Vardi et al., 1999), oxidative stress (Vardi et al., 1999; Darehshouri, Affenzeller, & Lütz-Meindl, 2008; Vavilala, Gawde, Sinha, & D'Souza, 2015), viral infection (Bidle et al., 2007; Bidle & Vardi, 2011), anti-metabolites (Yordanova, Woltering, Kapchina-Toteva, & Iakimova, 2013), and diatom derived (DD) aldehydes (Vardi, 2008).

The above triggers are communicated in cells by way of secondary messengers such as, for example, intracellular Ca^{2+} transients (Vardi, 2008; Bidle, 2016) Fig. 1 [1]). These signals can lead to the release of mitochondrial (cytochrome C) and chloroplast (cytochrome f) components into the cytosol (Fig.1 [2]) (Zuppini et al., 2007; Murik, Elboher, & Kaplan, 2014a). Cyt F and Cyt C elicit an intracellular burst of reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) and superoxide (O_{2-}) (Vardi et al., 1999, 2007; Murik, Elboher, & Kaplan, 2014; Bidle, 2016) (Fig.1 [3]). The increase in ROS upregulates antioxidant enzymes of the AsA-GSH cycle such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX)

monodehydroascorbate reductase (MDHR) and dehydroascorbate reductase (DHAR) in an attempt to deal with the redox change (Fig.1 [4]) (Murik & Kaplan, 2009; Zuo, Zhu, Bai, & Wang, 2012; Murik, Elboher, & Kaplan, 2014; Vavilala, Sinha, & D'Souza, 2014). In severe stress situations, these enzymes cannot deal sufficiently with intracellular oxidation, leading to an increase in levels of the metabolite dehydroascorbate (DHA), a product of the enzyme APX (Murik et al., 2014a). The higher dehydroascorbate (DHA) levels in relation to ascorbate (AsA) leads to cell death via the activation of metacaspases, which are common executioners of PCD (Fig.1 [5]) (Murik, Elboher, & Kaplan, 2014). DHA can also directly lead to PCD without ROS mediation, which occurs via oxidative independent pathways (Fig.1 [6]) (Murik, Elboher, & Kaplan, 2014). Simultaneously, infochemicals (e.g. diatom-derived aldehydes DD) can also trigger the intracellular release of Ca^{2+} leading to a burst of another free radical, Nitric oxide (NO), via activation of a nitric-oxide-associated protein (NOA) (Vardi et al., 2006; Vardi, 2008), and the chloroplast enzymes nitric oxide synthase (NOS) and nitrate reductase (NR) (Sakihama, Nakamura, & Yamasaki, 2002; Foresi et al., 2010; Bidle, 2016). The increase in intracellular NO can also induce the expression of metacaspases (Fig.1 [7]) (Mannick et al., 2001; Vardi et al., 2008). Metacaspases can activate downstream responses which lead to the loss of membrane asymmetry represented by the externalization of PS (Fig.1 [8]), an earlier marker of PCD as well as late responses like DNA degradation and loss of membrane integrity (Fig.1 [9]).

How has PCD traditionally been detected in eukaryotic phytoplankton?

The conventions for detecting PCD in eukaryotic phytoplankton have mostly been developed in green algae (chlorophytes), but are also employed in diatoms,

dinoflagellates and haptophytes (here represented by the coccolithophore *E. huxleyi)* (Table 1).

Chlorophytes

Some of the earliest studies of PCD in chlorophytes were reported in the genus *Dunaliella*. In *Dunaliella tertiolecta* (Chlamydomonadales)*,* laboratory cultures undergo cell death after 6 days in complete darkness (Berges & Falkowski, 1998). It was only later, however, that biochemical hallmarks of PCD were detected in *Dunaliella tertiolecta* (Segovia, Berges, Litti, & Falkowski, 2003). Specifically, transmission electron microscopy (TEM) revealed that under prolonged darkness, this microalga exhibited apoptotic-like features including chromatin marginalization and nuclear degradation. These morphological changes were followed by biochemical events like DNA degradation (detected by TUNEL staining) and caspase-like activities, which were detected by highly-specific cleavage of diverse substrates (cleavage occurs after an aspartate (D) residue in substrates WEHD, DEVD, VEID, IETD or LEHD). The addition of caspase inhibitors suppressed the caspase-like activities and prevented cell death. This was an important development because for the first time, death-related features were documented and linked to caspase activity.

PCD in the model organism *Chlamydomonas reinhardtii* (Chlamydomonadales) revealed apoptotic-like features after prolonged exposition to ultraviolet light (Moharikar, D'Souza, Kulkarni, & Rao, 2006) and heat shock (Nedelcu, 2006). TEM images detected markers such as cell shrinkage, cytoplasmic vacuolization and nuclear fragmentation (Moharikar, D'Souza, Kulkarni, & Rao, 2006). As in *Dunaliella*, these features occurred in parallel with an increase in caspase-3-like activity. In addition, hallmarks of PCD like translocation of phosphatidylserine (PS) using an annexin-V

labelling fluorometric assay, increased ROS using the cell-permeant 2',7' dichlorodihydrofluorescein diacetate (H2DCFDA), and DNA laddering, using nucleic acid gel electrophoresis were observed.

Heat shock was also found to induce an apoptotic-like death in *Chlorella sacharophila* (Chlorellales) which was characterized by morphological and biochemical changes similar to those observed above in *Dunaliella* and *Chlamydomonas* (Leu & Hsu, 2005; Zuppini, Andreoli, & Baldan, 2007; Zuppini, Gerotto, Moscatiello, Bergantino, & Baldan, 2009). TEM micrographs revealed classic ultrastructural features like chromatin condensation and marginalization, which were followed by high caspase-like DEVDase activity, ROS production and DNA degradation, all of which were abolished with the addition of the caspase-3-inhibitor Ac-DEVDCHO. Chloroplast involvement was identified as a role-player in phytoplankton PCD (Zuppini, Gerotto, Moscatiello, Bergantino, & Baldan, 2009). The morphological features included alterations in thylakoid patterns and photosynthetic membrane damage, among other structural modifications. The biochemical changes were assessed by way of the western blot analysis and detected a rapid disappearance of the large subunit of the enzyme Ribulose-1,5-Biphosphate carboxylase/oxygenase (Rubisco) and the photosystem II (PSII) of the light-harvesting complex, followed by a rapid reduction in pigment content (Zuppini, Andreoli, & Baldan, 2007).

As indicated above, the detection of markers and morphotypes of PCD depends on the environmental stress. For example, in *Dunaliella viridis* (Chlamynomonadales), exposure to different inducers led to five different morphotypes of PCD and many different markers (Jimenez et al., 2009). All cases tested positive for increased caspase-3-like (DEVDase) activity and tested positive for human antibodies against Caspase-3-like proteins. TEM revealed different degrees of chromatin condensation, organellar

disintegration, and cytoplasmic vacuolization. This was an important finding because it revealed the possible regulatory functions of caspase-like enzymes in addition to their executioner role under diverse environmental stimuli. A year later, corroborating evidence was demonstrated in the congeneric *Dunaliella tertiolecta* exposed to heat shock, where the addition of the broad spectrum caspase inhibitor, Boc-D-FMK, not only abrupted caspase activity and prevented death, but also prevented earlier apoptotic-like events such as PS externalization, increased membrane permeability and ROS generation (Segovia & Berges, 2009). The PCD studies in chlorophytes, however, have also reported forms of death that may be independent of caspase-like activity. For instance, the microalgae *Micrasterias denticulata* (Streptophyta) was negative for caspase-3-like (DEVDase) activity in response to salt stress. The organism exhibited a mix of apoptosis (ROS and DNA laddering) and autophagy markers (degradation of organelles in conjunction with highly cytoplasmic vacuolization).

Haptophytes

Among the haptophytes, the coccolithophores, so-called because of the calcified scales known as coccoliths (Hagino & Young, 2015), are an important model group for studying haptophyte death. *Emiliania huxleyi* is of particular importance because it produces massive ocean blooms (Tyrrell & Merico, 2004). *E. huxleyi* is vulnerable to infection by large double-stranded DNA viruses called coccolithoviruses or EhVs (Wilson, 2015), that activate PCD in this microalga as part of its lytic infection strategy (Bidle, Haramaty, Barcelos e Ramos, & Falkowski, 2007; Vardi et al., 2009; Bidle & Vardi, 2011). Earlier work revealed an elevated production of ROS in E. *huxleyi* following viral infection (Evans, Malin, Mills, & Wilson, 2006), but more specific markers of PCD were only detected a year later (Bidle et al., 2007). Detection and

measurement of caspase-8-like-activity were assessed in EhV-infected *E. huxleyi* cells using the highly specific fluorogenic substrate IETD-AFC and confirmed by in-vivo staining with the fluorescently labeled caspase inhibitor FITC-VAD-FMK, which binds irreversibly to activated caspases. *E. huxleyi* recombinant metacaspase (EhMC) polyclonal antibodies were generated and used to identify EhV-infected cells with western blot analysis. The addition of the caspase inhibitor (z-VAD-FMK) abolished caspase-like activity, protein expression and resulted in an acute reduction in viral replication.

Diatoms

The sinking of dead diatoms is the primary way in which carbon and silica are transported to the deep ocean and studying their modes of dying is important for this one reason alone (Benoiston et al., 2017). PCD has been extensively reported in the diatoms *Thalassiossira pseudonana* and *Phaerodactylum tricornutum* under iron limitation (Bidle & Bender, 2008; Luo et al., 2014) and exposure to reactive aldehydes (Vardi, 2008), respectively. One of the first reports of the death mechanism in diatoms was in the marine centric diatom, *Thalassiosira weissflogii (*Thalassiosirales*,)* after nitrogen limitation. The cells experienced a gradual loss of proteins (especially the enzyme Rubisco), the activity of proteases doubled, and a specific protease was induced. These changes, however, were not be specifically linked to PCD (Berges & Falkowski, 1998). PCD was further characterized in the congeneric *Thalassiosira pseudonana* under iron-limited conditions (Bidle & Bender, 2008). Quantitative reverse transcription (RT-PCR) detected the increased expression of six *Thalassiosira pseudonana* metacaspases (TpMCs), and cells also tested positive for purified polyclonal antibodies previously raised against EhMCs (Bidle, Haramaty, Barcelos e

Ramos, & Falkowski, 2007). While not all the metacaspases were associated with PCD, two specific TpMCs could be correlated with positive annexin assays and high caspase-like activity.

Important new developments came later in the pennate diatom *Phaeodactylum tricornutum.* Exposure to reactive aldehydes, led to Ca^{2+} release from internal stores leading to a rapid generation of nitric oxide (NO) and an upregulation in metacaspase activity (Vardi, 2008). This was an important finding because it revealed similar effects of ROS and NO in PCD . An NO-surveillance system was proposed where subthreshold levels of NO immunized cells against environmental stress while high NO concentrations was involved in the catastrophic death observed in the rapid demise of some blooms (Vardi, 2008).

Dinoflagellates

Dinoflagellates are key components of marine food webs and in the balancing of the energy flux in aquatic ecosystems (Akbar, Ahmad, Usup, & Bunawan, 2018). Most of the current understanding of PCD in dinoflagellates came from the marine dinoflagellate *Peridinium gatunense (Peridiniales)* (Vardi et al., 1999, 2007). Evidence from lake Kinneret, Israel, suggested that in *P. gatunense,* limiting concentrations of CO_2 triggers death in the population (Berman-Frank, Zohary, Erez, & Dubinsky, 1994). Further investigations revealed that CO_2 limitation leads to ROS production, DNA fragmentation, and a PCD phenotype (Vardi et al., 1999). The detection of ROS and antioxidant activity was performed by way of specific staining, and hydrogen peroxide (H_2O_2) was the primary molecular species implicated. Epifluorescence microscopy images revealed cell shrinkage, while TUNEL staining indicated DNA nicking. PCD was corroborated with the addition of a specific cysteine caspase inhibitor, E-64, which

prevented cell death. Later research in *P. gatunense* also revealed that exposure to a specific thiol protease, which is excreted by *P. gatunense* senescing cells, can turn young, healthy cells sensitized to oxidative stress, causing PCD; while purifying the medium from this protease prevents cell death (Vardi et al., 2007). More recently, yet caspase 3/7 activity, DNA degradation, and previously undescribed morphotypes have been detected in aging *Karenia brevi,* a toxic bloom forming dinoflagellate (Johnson, Janech, & Van Dolah, 2014). The authors confirmed, via the development and successful cleavage of a novel caspase 3/7 substrate, the existence of many dependent and independent caspase-like proteins that may play a role in bloom termination. The role of PCD in bloom termination, detected by caspase-like and metacaspase activity, was also demonstrated in the toxic diatom *Alexandrium tamarense* (Jauzein & Erdner, 2013).

How accurately do the different markers and measures predict PCD?

Researchers appear to assign varying significance to the different markers used (Segovia, Berges, Litti, & Falkowski, 2003; Vardi, 2008; Nedelcu, Driscoll, Durand, Herron, & Rashidi, 2011; Berges & Choi, 2014; Bidle, 2016). However, the measures have not been used consistently, which may lead to contrasting interpretations. Here, a detailed examination of the current measures to resolving these issues is presented. Significances were assigned according to Durand (2020), and complemented with an analysis of the frequencies of use and the detection efficiencies based on the 28 PCD studies selected.

It is worthwhile emphasizing again that not all markers are associated with each case. The cross-talk and pleiotropy of the cell death pathways in conjunction with the highly variable environments in which phytoplankton thrive can lead to different

responses even in the same taxon (Jimenez et al., 2009; Vavilala, Gawde, Sinha, & D'Souza, 2015). At the same time, different markers can be observed in different taxa even when the same stimulus is used.

Ultrastructural alterations

The standard method for observing ultrastructural morphological alterations is transmission electron microscopy (TEM). Sample preparation involves fixation with glutaraldehyde and osmium tetroxide staining (Nozaki et al., 1993), followed by dehydration and embedding the sample in resin. Sections of samples are then stained with heavy metals like uranyl acetate and lead citrate and observed under TEM.

TEM was the second most used method to study PCD in phytoplankotn (46%) – along with an increase in ROS (caspase activity was the most commonly employed method). Ultrastructural changes associated with PCD had a high detection efficiency (0.9). TEM is considered a sensitive method. If PCD is occurring, the direct observation of death-related ultrastructural alterations indicated changes in gene and protein regulation, which are crucial for evaluating whether the cell is dying in an organized way. Thus, it avoids false negatives. TEM is also specific because it generally excludes other non-PCD explanations for the ultrastructural changes observed, avoiding false positives. TEM is both specific and sensitive. As a result of this, it can be regarded as the essential, gold standard method for detecting PCD.

The following are some of the PCD-related ultrastructural changes reported in phytoplankton in response to different stimuli: *cell shrinkage* (Leu & Hsu, 2005; Moharikar, D'Souza, Kulkarni, & Rao, 2006; Zuppini, Andreoli, & Baldan, 2007), *organellar degradation* (Franklin & Berges, 2004; Bidle, Haramaty, Barcelos e Ramos, & Falkowski, 2007; Bidle & Bender, 2008; Affenzeller, Darehshouri, Andosch, Lutz, &

Lütz-Meindl, 2009), *cytoplasmic vacuolization* (Franklin & Berges, 2004; Moharikar, D'Souza, Kulkarni, & Rao, 2006; Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009; Sathe, Orellana, Baliga, & Durand, 2019), *plasma membrane detachment from the cell wall* (Zuppini, Andreoli, & Baldan, 2007; Durand, Sym, & Michod, 2016), *chloroplast and mitochondrial alterations* (Leu & Hsu, 2005; Zuppini, Andreoli, & Baldan, 2007; Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009), *chromatin condensation* (Segovia, Berges, Litti, & Falkowski, 2003; Zuppini, Andreoli, & Baldan, 2007; Jimenez et al., 2009), and *chromatin or nucleolar degradation* (Moharikar, D'Souza, Kulkarni, & Rao, 2006; Sathe, Orellana, Baliga, & Durand, 2019).

Many of the death-related features in chlorophytes resembled those found in plants (van Doorn et al., 2011; Minina, Smertenko, & Bozhkov, 2014). This is unsurprising since chlorophytes likely have a common ancestor with plants (Leliaert et al., 2012). Both groups have hard cell walls, which prevent the fragmentation of cells into "apoptotic bodies". As a result of this, alternative ways of dying may have evolved (van Doorn et al., 2011). Among this, cytoplasmic vacuolization is a common death-related feature found in chlorophytes and plants (Moharikar, D'Souza, Kulkarni, & Rao, 2006; Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009; Jimenez et al., 2009; Sathe, Orellana, Baliga, & Durand, 2019).

Caspase-like/metacaspase activity

Caspases

Caspases (cysteinyl aspartate-specific proteases) are a large family of highly specific and evolutionary conserved proteases (Chang & Yang, 2000) , some of which play a key role in metazoan cell death (Kumar, 2007). Its specificity is due to the

requirement for cleaving substrates after an acidic aspartate at the P1 position, and the recognition of at least four amino acids to the left of the cleavage site (Pop & Salvesen, 2009). This substrate specificity has been used as a sensitive diagnostic method of PCD in unicellular phytoplankton. It is known, however, that caspases are also active during cellular processes other than PCD (Pop & Salvesen, 2009; Bell & Megeney, 2017), and there are also caspase-independent forms of PCD (Broker, Kruyt, & Giaccone, 2005; Tait & Green, 2008). Additionally, phytoplankton do not encode for orthologous caspases, but distant homologous enzymes known as metacaspases, which present distinct cleavage requirements (Enoksson & Salvesen, 2010; Tsiatsiani et al., 2011). Metacaspases hydrolyze proteins after lysine or arginine (basic residues) at the P1 position, not after an acidic aspartate (Enoksson & Salvesen, 2010; Tsiatsiani et al., 2011), and will be discussed in the following subsection. For these reasons, caspase assays, including the variants caspase inhibition and immunoreactive assays, are not specific enough to prove PCD, and are classified as soft signs (Durand, 2020).

Caspase-like activity was the most used assay to detect PCD in phytoplankton (50%) and had a detection efficiency of 0.8 . Caspase activity is usually detected with highly specific caspase substrates (specific sequences containing “D” aspartate residues) in conjunction with a fluorophore, which are cleaved by different caspase types (Segovia, Berges, Litti, & Falkowski, 2003). For example, the caspase-3-like DEVDase assay measures the cleaving activity of the sequence DEVD ([DEVD is a cleavage site found in poly-(ADP-ribose)-polymerase], where the last D means aspartate). Additionally, it is also possible to measure the caspase -1, 6 ,8, and 9-like activities, using respectively the substrates WEHD, VEID, IETD or LEHD. The ability of cleaving these substrates in phytoplankton, even though they lack true caspases, suggest that metacaspases might also cleave after aspartate residues, but, probably, not

as well as they do after arginine. Alternatively, caspase-like proteins might also reflect caspase-like downstream responses to the point of metacaspase regulation (Tsiatsiani et al., 2011). This view is supported by the findings of (Madeo et al., 2002) in yeast, which reported decreased caspase VEIDase and IEDTase activities, after disruption of the Yeast Caspase-1 (YCA1) gene (a yeast protease homologous to mammalian caspase) with addition of the caspase inhibitor Z-VAD-FMK.

Caspase-like activity, detected by immunoreactive assays (i.e. western blot), test for the presence of proteases raised against human caspase antibodies, and were observed at 25% of the reports, (e.g. Segovia, Berges, Litti, & Falkowski, 2003; Moharikar, D'Souza, Kulkarni, & Rao, 2006; Vavilala, Sinha, & D'Souza, 2014). Yet, another frequently used strategy to detect PCD was to use substances with known caspase inhibiting activity (21%) (e.g. Segovia, Berges, Litti, & Falkowski, 2003; Bidle, Haramaty, Barcelos e Ramos, & Falkowski, 2007; Zuppini, Andreoli, & Baldan, 2007; Bidle & Bender, 2008; Segovia & Berges, 2009). For example, the broad spectrum caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) has been showed to prevent death in diatoms (Bidle & Bender, 2008), chlorophytes (Segovia, Berges, Litti, & Falkowski, 2003) and coccolithophores (Bidle et al., 2007; Vardi et al., 2012). An additional elegant method reported is to replace the carbobenzoxy group (Z) N-terminal blocking group in Z-VAD-FMK for the fluorogenic substrate FITC to create a fluorescent substrate that can also be used to the detect *in situ* caspase activity by epifluorescence microscopy (Bidle, Haramaty, Barcelos e Ramos, & Falkowski, 2007; Bidle & Bender, 2008; Vardi et al., 2012).

Metacaspases

Classification and nomenclature for metacaspases have been proposed for

metacaspases (Minina et al. 2020). Three metacaspases (MCs) types were reported in phytoplankton, MCI, MCII and MCIII. MCI and MCII distinguish from one to another based on the presence or absence of a prodomain structure, while the recently discovered MCIII have distinct rearrangements of domain structures between N- and C-terminus (Choi & Berges, 2013b). While most phytoplankton seem to have MCI, *Clamydomonas reinhardtii* and *Volvox carteri* were the only taxa presenting both , MCI and MCII. However, diatoms, dinoflagellates, and haptophytes, and cryptophytes resisted classification into MCI and MCII, and proeminietly presented MCIII (Choi & Berges, 2013).

The upregulation of metacaspases was highly detected (detection efficiency=1) by RT-PCR (14%) and immunoreactive assays (10%) when PCD was investigated. Despite the fact that metacaspases are the effectors of PCD in phytoplankton, these enzymes can also be associated with other cellular responses such cell cycle regulation (Lee, Puente, Kærn, & Megeney, 2008) and clearance of proteins (Lee, Brunette, Puente, & Megeney, 2010). As a results of this, metacaspase activity is not only associated with PCD and should also be considered as a soft sign of PCD (Durand, 2020). It is true that some studies have reported that high metacaspase transcription activity can be positively correlated with caspase-like activity (e.g. in the green algae *C. reinhardtii* (Murik & Kaplan, 2009; Murik, Elboher, & Kaplan, 2014), the diatom *T. pseudonana* (Bidle & Bender, 2008) and the coccolith *E. huxlei* (Bidle, Haramaty, Barcelos e Ramos, & Falkowski, 2007; Vardi et al., 2012). However, because metacaspases and caspases have very distinct cleavage requirements (see above), caspase specific substrates probably do not accurately measure metacaspase activity, and otherwise conclusions can be regarded as premature (Tsiatsiani et al., 2011). For example, in the archaebacterium *Haloferax volcanii*, highly caspase-like activity was

detected in response to salt stress, even though no genetic homologous could be detected (Bidle, Haramaty, Baggett, Nannen, & Bidle, 2010). Thus, it has been urged that metacaspase activity must be specifically measured with metacaspase substrates (Tsiatsiani et al., 2011). A list of developed fluorogenic tri-or tetrapeptide substrates with Arg or Lys at the P1 position have been done before and can be found in (Tsiatsiani et al., 2011). The same reasoning is true for caspase inhibitors, which should be replaced by arginine protease inhibitors.

DNA degradation

DNA degradation can be detected by two different ways when the PCD phenotype was investigated. First, it can be detected by DNA gel electrophoresis, which represented 32% of the reports . DNA laddering had a detection efficiency of 0.6 , which is relatively less than other biochemical markers of PCD (see table 2). It is worthwhile to mention, however, that even though DNA laddering does not emerged as so often as caspase activity, when it does, it is regarded as a hard sign of PCD (Durand, 2020). The breaking of DNA in oligonucleossomal fragments is evidence of endonuclease activity, which is very specific of PCD (although the measurements of these enzymes have not yet been reported) (Leu & Hsu, 2005). DNA laddering may not be easily detected for some reasons: factors related the age or growth of the culture (Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009), blurring of the laddering by smeared necrotic (Dingman & Lawrence, 2012), and the small proportion of cells undergoing PCD, which would require higher biomass amounts for visible detection (unpublished observations).

The TUNEL assay (terminal deoxynucleotidyl transferase dUTP nick end labelling) which detects single and double stranded DNA nicking, was used as an

alternative to gel electrophoresis on 40% of the reports (Kumari, Rastogi, Singh, Singh, & Sinha, 2008). TUNEL staining had a slightly higher detection efficiency compared to gel electrophoresis (0.73). It is true, however, that the TUNEL assay can detect DNA damage, which may or may not lead to PCD (Engelbrecht, Durand, & Coetzer, 2012), and for this reason it is considered as a soft sign of PCD (Durand, 2020), although TUNEL staining is also positive when DNA laddering is detected (Leu & Hsu, 2005; Moharikar, D'Souza, Kulkarni, & Rao, 2006).

DNA degradation was also reported with the "alkaline comet assay" (7%), which detects single-cell DNA damage and presented a detection efficiency of 1. Similar to the TUNEL assay, however, the alkaline comet assay is also considered not specific enough to PCD (soft sign).

The above assays were combined with endonuclease inhibitors (e.g. Zn^{2+}) in 7% of the reports. Zn^{2+} prevents the endonucleolytic activity that leads to DNA degradation, and can be regarded as analogous to caspase inhibitors in that it can prevent cell death. (Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009; Zuo, Zhu, Bai, & Wang, 2012).

Loss of membrane asymmetry

The loss of membrane asymmetry is an early marker of PCD, that was used to detect PCD in unicellular phytoplankton in 25% of the reports (Moharikar, D'Souza, Kulkarni, & Rao, 2006; Durand, Choudhury, Rashidi, & Michod, 2014). This is measured with the annexin-V labelling fluorometric assay (Kroemer et al., 2009), and is considered a hard sign of PCD (Durand, 2020). In healthy cells, phosphatidylserine (PS) is confined to the cytoplasmic surface, but during early PCD it is externalized to the outer leaflet of the cell membrane. The cellular protein Annexin-V conjugated to a

fluorogenic substrate binds to PS, and can be detected by fluorescent microscopy and/or by flow cytometry.

Frequently, the annexin-v assay combined with nucleic acid stainings like propidium iodide (PI) and sytox blue to detect non-PCD death. These stains can intercalate into DNA once plasma membrane integrity has been damaged, which indicates late cell death or necrosis (Kroemer et al., 2009; Durand, Choudhury, Rashidi, & Michod, 2014; Sathe, Orellana, Baliga, & Durand, 2019).

Although chlorophytes have constantly been found positive to Annexin-V (detection efficiency=1) (e.g. Moharikar, D'Souza, Kulkarni, & Rao, 2006; Segovia & Berges, 2009; Durand, Choudhury, Rashidi, & Michod, 2014; Voigt & Woestemeyer, 2015), PS is not present in the PCD model-system *Chlamydomonas reinhardtii* (Giroud, Gerber, & Eichenberger, 1988). However, the jury is still out about its occurrence in other chlorophytes (Guschina and Harwood 2006). Thus, it is not clear if Annexin-v is specific for PS as first thought, and it is possible that Annexin-v can bind to other phospholipids such as phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (Weingärtner et al., 2012).

Upregulation of PCD associated genes

The upregulation of PCD associated genes was one of the most used measures when the PCD phenotype was investigated (33%). The increase in transcription activity can be semi-quantitatively detected by reverse transcription polymerase chain reaction (RT-PCR) (e.g. Nedelcu, 2006; Zuppini, Gerotto, Moscatiello, Bergantino, & Baldan, 2009) or quantitatively detected with real-time reverse transcription polymerase chain reaction (qRT) PCR (e.g. Murik & Kaplan, 2009; Murik, Elboher, & Kaplan, 2014). PCD-related genes can also be indirectly detected by western blot analysis with reactive

antibodies (Moharikar, D'souza, & Rao, 2007). On the other hand, RNA-seq is a new research method that is still quite unexplored in the field, but that has an enormous potential to lead to a better understanding of the mechanisms of PCD in phytoplankton. For example, a comparative transcriptomic analysis in the diatom *Thalassiosira pseudonana* revealed a total of 1313 possible PCD-related genes involved in acclimation to Fe and oxidative stress (Thamatrakoln, Korenovska, Niheu, & Bidle, 2012).

PCD associated genes had a high detection efficiency (1). It is true, however, that the association of PCD-related genes is always subjected to further experimentation as many genes can also be associated with other stress responses in the cells (e.g.Thamatrakoln, Korenovska, Niheu, & Bidle, 2012). Thus, the upregulation of PCD-related genes per se are not specific enough to prove PCD, and is considered as a soft sign of PCD (Durand, 2020).

Experimental designs can combine genetic and immunoreactive assays to explore the role of genes that encode major components of the PCD machinery. For example, in UV stressed *C. reinhardtii,* the upregulation of the apoptosis activating factor-1 (APAF-1) gene, detected by RT-PCR, was associated with the downregulation of the defender against apoptotic death (dad-1), detected by western blot (Moharikar, D'souza, & Rao, 2007).

Increase in reactive oxygen species (ROS)

The increase in ROS was assessed by three different ways: directly, by specific oxidative indicators (46% of the reports), and indirectly by antioxidant enzyme assays (14% of the reports), and antioxidant transcriptional levels (discussed above). The cell-permeants indicators 2',7'dichlorodihydrofluorescein diacetate (H2DCFDA) or variants

like CM-H2DCFDA (6-chloromethyl-2,7-dichlorodihydrofluorescein diacetate, acetyl ester) are nonfluorescent indicators which are converted to highly fluorescent forms after the cleavage of the acetate group by intracellular esterases under oxidative stress (Murik, Elboher, & Kaplan, 2014). Positive responses can be observed directly via epifluorescence microscopy or flow cytometry in phytoplankton. The fluorescent stain Xylenol orange can also be used as an alternative to H2DCFDA for specifically measuring the activity of the enzyme peroxidase (Jiang, Woollard, & Wolff, 1990). This technique has been used specifically to detect H_2O_2 content in *C. reinhardtii* under acetic acid, hydrogen peroxide and quinones (Zuo, Zhu, Bai, & Wang, 2012; Vavilala, Sinha, & D'Souza, 2014).

The increase in ROS represent the second most commonly used method to assess PCD (along with ultrastructural changes, detected via TEM) (e.g. Moharikar, D'Souza, Kulkarni, & Rao, 2006; Zuppini, Andreoli, & Baldan, 2007; Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009), and had a highly detection efficiency (0.8) when PCD was investigated. Admittedly, however, the increase in ROS is not only associated PCD, but also with most forms of stress in the cells (Nedelcu et al., 2004). Furthermore, ROS can play a role in cellular processes such as allelopathy, growth promotion and iron acquisition (Diaz & Plummer, 2018). As a result of these, the increase in ROS is not specific to PCD, and should be considered as a soft sign (Durand, 2020).

Some reports also measured the increase in ROS with biochemical assays which measure the activity of antioxidant enzymes (the enzymes which deal with the oxidative stress, e.g. SOD, CAT, APX) (Zuo, Zhu, Bai, & Wang, 2012; Murik, Elboher, & Kaplan, 2014; Vavilala, Gawde, Sinha, & D'Souza, 2015). Measuring the specific activity of each enzymatic type relies on different methodologies, which can be found in

(Zuo, Zhu, Bai, & Wang, 2012; Murik, Elboher, & Kaplan, 2014; Vavilala, Sinha, & D'Souza, 2014).

Nitric oxide (NO) generation

Evidence shows that NO plays a key role in metacaspase induction in marine diatoms (Vardi et al., 2006; Vardi, 2008), and could play similar roles in other phytoplankton taxa (although remains to be tested). Measuring the NO generation was found at 7% of reports and presented a highly detection efficiency (detection efficiency=1). However, it is fact that the increase in NO, similar to ROS, is not only associated with PCD but with other cellular processes like regulating growth, protection and regulation of photosystems (Li, Liu, Liu, Zhang, & Wang, 2013; Weisslocker-Schaetzel et al., 2017; Astier, Jeandroz, & Wendehenne, 2018). Therefore, the increase in NO can be regarded as a soft sign of PCD (Durand, 2020). The intracellular levels of NO can be directly measured with the specific NO indicator, NO-sensitive dye 4-amino-5-methylamino-29,7'-difluorofluorescein diacetate (DAF-FM) (Vardi et al., 2006). In addition, the increase in the transcript levels of NO-associated genes indicates indirectly NO generation (Guo et al, 2003). For example, in *Phaeodactylum tricornutum* overexpressing a nitric-oxide-associated protein (PtNOA) leads to metacaspase activation and PCD (Vardi, 2008; Vardi et al., 2008).

Mitochondrial and chloroplast biochemical alterations

Mitochondrial alterations were used as a marker of PCD at 10% of the reports, and had a detection efficiency of 0.75. During PCD, the mitochondrial membrane potential ($\Delta\Psi m$) is ruptured (Heiskanen, Bhat, Wang, Ma, & Nieminen, 1999; Andronis & Roubelakis-Angelakis, 2010). This change in $\Delta\Psi m$ can be assessed by commercial kits, using cationic fluorescent dyes that accumulate in healthy but not in impaired

mitochondria. This is detected qualitatively and quantitatively with epifluorescence microscopy and flow cytometry, respectively (Abcam, USA). Mitochondrial disruption has been demonstrated in *C. reinhardtii* under oxidative stress and exposure to quinones (Vavilala, Sinha, & D'Souza, 2014; Vavilala, Gawde, Sinha, & D'Souza, 2015). However, the change in $\Delta\Psi m$ is not considered as specific to PCD, and mitochondrial changes should be regarded as soft signs of PCD.

Unlike cytochrome c, which has not yet been implicated in phytoplankton death (Affenzeller, Darehshouri, Andosch, Lutz, & Lütz-Meindl, 2009), evidence of the upregulation and translocation of Cty f into the cytosol while cells are undergoing PCD was demonstrated in heat-stressed *Chlorella saccharophila* (Zuppini, Gerotto, Moscatiello, Bergantino, & Baldan, 2009). This is strong evidence of the role of chloroplast in PCD. Chloroplast biochemical-associated changes, which follows Cyt f release, corroborate the chloroplast's involvement in PCD, and were showed in 17% of the reports, presenting also a high detection efficiency (1). These alterations include a gradual loss of proteins (i.e. degradation of Rubisco and the light-harvesting complex of PSII), detected by western blot analysis (Zuppini, Andreoli, & Baldan, 2007), and also by SDS-page total protein analysis (Leu & Hsu, 2005). A reduction in photosynthetic pigments (i.e. chlorophylls and carotenoids) was also reported, detected by a spectrophotometric determination of pigments following stress (Leu & Hsu, 2005; Zuppini, Andreoli, & Baldan, 2007; Vavilala, Sinha, & D'Souza, 2014). Chloroplast biochemical alterations are currently classified as soft signs of PCD since they can be associated with other stress processes, but our data indicates they might be important markers for chlorophytes.

Suggestions for measuring PCD in eukaryotic phytoplankton

In this study we have presented a systematic examination of the mechanisms, methods and markers which have been used to measure PCD in eukaryotic phytoplankton. Here, based on the method's significances, frequencies of use and efficiencies of detection of the different markers and measures (table 2), we aim to make suggestions for comparative and interpretative purposes for measuring PCD reliably.

Overall, our data showed that ultrastructural alterations, caspase-like activity, and the increase in ROS were the most commonly used, and also the only diagnostic markers shared among chlorophytes, diatoms, dinoflagellates and haptophytes (table 2). Interestingly, diatoms were not tested or reported for DNA degradation, neither by gel electrophoresis nor by TUNEL staining. Unlike diatoms, dinoflagellates had DNA degradation in two cases, in *P. gatunense,* under CO_2 limitation (tested positive for TUNEL staining), and in symbiont zooxanthella under heat stress (showed DNA laddering) (Dunn, Bythell, Le Tissier, Burnett, & Thomason, 2002; Dunn, Thomason, Le Tissier, & Bythell, 2004). PS exposure have not been reported or tested in dinoflagellates, but was identified twice in the diatom *T. pseudonana* (Bidle & Bender, 2008; Luo et al., 2014).

The frequency of use and efficiency of detection data seems to corroborate ultrastructural alterations, detected by TEM, as a key method for measuring PCD in unicellular phytoplankton. However, our findings also indicate that caspase activity, the increase in ROS, and DNA nicking (detected by TUNEL staining), described in the last section as non-specific markers of PCD, are commonly used and detected as well. On the one hand, the high efficiencies of detection suggest a link between the latter and phytoplankton cell death, but on the other hand, the data suggest they might not be

specific to PCD, but also occur during stress and other cellular processes. This duality reflect earlier observations which realize that cause-effect relationships in phytoplankton cell death are not simple as metazoa (Franklin, Brussaard, & Berges, 2006). Phytoplankton thrive in a highly fluctuating environment (Descamps-Julien & Gonzalez, 2005), and constantly interact with parasites (Kühn, 1998) viruses (Brussaard, 2004), and con-specifics (Nedelcu, Driscoll, Durand, Herron, & Rashidi, 2011). As such, many of molecular pathways previously associated with PCD in animals (specially the non-specific ones) could also be involved in the physiological changes related to phytoplankton's acclimation in aquatic environments (Minina et al. 2017). For example, in dinoflagellates caspase's inhibition is linked to encystation (Vardi et al. 1999, Choi et al. 2017), and in marine diatoms NO generation and metacaspase activation plays key roles in allelopathy and biofilm development (Vardi, 2008). Yet, in heat-stressed *C. reinhardtii*, ROS is involved with the induction of sexual reproduction (Nedelcu et al., 2004). Thus, the adoption of measuring strategies, based on methods' significance, is essential for comparative purposes and to avoid premature conclusions and incorrect interpretations. Here, we make suggestions for accurately measuring PCD in eukaryotic phytoplankton.

If the aim is to document PCD for the first time, the adoption of a polyphasic approach that uses TEM, in conjunction with other genetic and biochemical assays seems to be the most appropriate. It is worthwhile emphasizing again that within this approach, we regard TEM as a key method for detecting whether a cell is dying in a PCD or non-PCD manner. DNA laddering and PS exposure, in this order of preference, which were considered as hard signs of PCD should be used in conjunction with TEM. The concomitant demonstration of ultrastructural alterations, DNA laddering and PS externalization are strong indications that cells are undergoing PCD. According to our

data ,however, DNA laddering is not detected in all cases (detection efficiency=0.64), which supports previous observations (Madeo et al. 1999, Vardi et al. 1999, Zuppini et al. 2007). Additionally, the annexin v assay also presents issues concerning its action mechanism in microalgae. It is uncertain where annexin v operates (i.e. on the inner or outer side of the cellular membrane) and to which phospholipid, specifically, it binds to. Thus, more accurate measurements can include soft markers such as biochemical (e.g. upregulation of PCD-related genes) and genetic (e.g. upregulation of PCD-related genes) methods, that will provide a better understanding about the molecular pathways which follow the ultrastructural morphological changes under a specific ecological set.

It is true that TEM also presents some important disadvantages. First, quantification under TEM is challenging. This, however, can be overcome by analyzing different fields of view under the smallest magnification, following by a calculation of the frequency of PCD positive cells off the total abundance (Jimenez et al., 2009). Additionally, the ultrastructural alterations in unicells can be many and complex, and can be different depending on the ecological set (Jimenez et al., 2009). As a result of this, there is no general pre-determined set of ultrastructural alterations. For helping and comparative purposes, one can refer to the list of markers described earlier in the *ultrastructural alterations* subsection. Finally, there are concerns regarding preparation and fixation artefacts, and micrographs may look different in different species. We advocate this can be attenuated by comparing ultrastructural images against similar phytoplankton species exposed to similar ecological sets.

TEM may be impractical for some experimental designs because it is laboring and time consuming. In this case, the second strategy is to use at least one hard sign of PCD (i.e. Annexin v or DNA laddering) in conjunction with other soft signs (i.e. biochemical and genetic markers). For example, in *C. reinhardtii* exposed to menadione

and oxidative stress, PCD was demonstrated with DNA laddering in conjunction with the increase in ROS, the upregulation of PCD associated-genes (e.g. AsA–GSH cycle genes and metacaspases), and DNA nicking (detected by TUNEL staining) (Vavilala, Sinha, & D'Souza, 2014; Vavilala, Gawde, Sinha, & D'Souza, 2015). Soft markers, however, may also present different significances. Our data showed that the upregulation of PCD-associated genes is the 5th most commonly used marker to assess PCD (32%), and had a high detection efficiency (0.9). Some of these genes had consistently increased transcription rates when cells were undergoing PCD. For example, the gene families of the ascorbate-glutathione pathway (AsA–GSH cycle genes), which combat the oxidative stress characteristic of PCD, have been independently demonstrated in *C. reinhardtii* (Zuo, Zhu, Bai, & Wang, 2012; Murik, Elboher, & Kaplan, 2014; Vavilala, Sinha, & D'Souza, 2014) under different stress and may be an important marker to detect PCD in chlorophytes. Similarly, chloroplast biochemical alterations which follow the oxidative stress were independently detected in the congeneric species *Chlorella pyrenoidosa* and *Chlorella saccharophila*, and may also be appropriate markers of PCD in Chlorophyta (17% of reports). In contrast, caspases should be used with caution since there is superficial knowledge regarding its enzymatic specificity. Since phytoplankton lacks true caspases, it was recommended before that these assays should be replaced by metacaspase-specific substrates (i.e. that have Lys and Arg at the P1 position) (Tsiatsiani et al., 2011). The same is true to caspase inhibitors, which should be replaced by arginal inhibitors (Tsiatsiani et al., 2011). Similar issues lies behind the DNA degradation, detected by TUNEL staining assay, which detects DNA nicking which may or not be related to PCD (Engelbrecht, Durand, & Coetzer, 2012).

Our third strategy is related to experimental designs on which the aim of the research is another from simple documentation (e.g. testing ecology evolutionary hypothesis). If PCD has already been consistently demonstrated using TEM in conjunction with other genetic and biochemical markers (e.g. *Chlamydomonas reinhardtii* (Moharikar et al., 2006; Durand et al., 2011; Murik et al., 2014a)*, Dunaliella tertiolecta* (Segovia, Berges, Litti, & Falkowski, 2003; Segovia & Berges, 2009)*, Chorella saccharophila* (Zuppini, Andreoli, & Baldan, 2007; Zuppini, Gerotto, Moscatiello, Bergantino, & Baldan, 2009)*,* it acceptable to use soft signs which are known to be positive under an specific set of ecological conditions (e.g. Orellana, Pang, Durand, Whitehead, & Baliga, 2013; Durand, Choudhury, Rashidi, & Michod, 2014).

Strategies can be flexible depending on the research aim and/or time and technical limitations, but in overall, the adoption of an polyphasic approach, which applies ultrastructural alterations in conjunction with genetic and biochemical measures, seems to be the most accurate strategy for measuring PCD in chlorophytes and other eukaryotic phytoplankton. We considered transmission electron microscopy (TEM) as a key method for detecting whether cells are dying in an organized, programmed way or in an incidental, uncoordinated manner. Complementary measurements should be chosen based on the method' specificity. Highly sensitive but not specific assays such as caspase-like activity, the increase in ROS and TUNEL staining should be used with caution since they can provide false positives.

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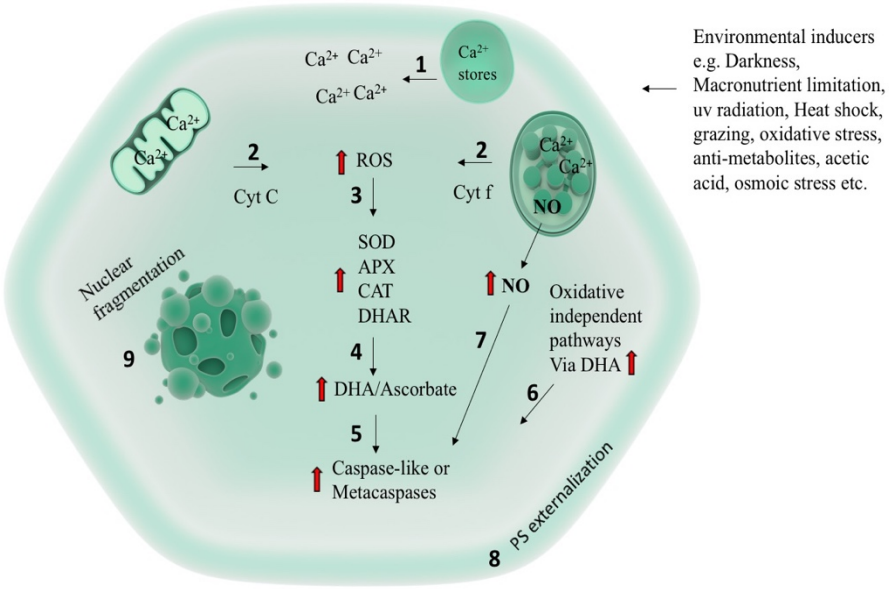

Fig. 1. A diagrammatic view of the mechanisms of PCD in phytoplankton. Only the essential steps and components are indicated. See text for details. Thin black arrows indicate the steps. Thick red arrows indicate upregulation. Based on images in *(Vardi, 2008; Bidle, 2016)*

Table 1. PCD documentation in eukaryotic phytoplankton. Taxonomy according to Guiry and Guiry (2020).

AUTHOR	STIMULUS	ORGANISM	PHYLUM	ORDER
(Nedelcu, 2006)	Heat	*C. reinhardtii*	Chlorophyta.	Chlamynomodales
(Moharikar et al., 2006)	Heat	*C. reinhardtii*	Chlorophyta.	Chlamynomodales
(Durand et al., 2011, 2014)	Heat	*C. reinhardtii*	Chlorophyta	Chlamynomodales
(Murik & Kaplan, 2009; Murik et al., 2014a)	Oxidative stress	*C. reinhardtii*	Chlorophyta.	Chlamynomodales
(Yordanova et al., 2013)	Mastoporan	*C. reinhardtii*	Chlorophyta	Chlamynomodales
(Zuo et al., 2012)	Acetic acid	*C. reinhardtii*	Chlorophyta.	Chlamynomodales
(Vavilala et al., 2014)	Menadione	*C. reinhardtii*	Chlorophyta	Chlamynomodales
(Vavilala et al., 2015)	Salt stress	*C. reinhardtii*	Chlorophyta	Chlamynomodales
(Vavilala et al., 2015)	Oxidative stress	*C. reinhardtii*	Chlorophyta.	Chlamynomodales
(Sathe & Durand, 2016)	Nitrogen limitation	*C. reinhardtii*	Chlorophyta	Chlamynomodales
(Orellana et al., 2013)	Darkness	*D. Salina*	Chlorophyta.	Chlamynomodales
(Segovia et al., 2003; Segovia & Berges, 2005, 2009)	Darkness	*D. tertiolecta*	Chlorophyta	Chlamynomodales
(Jimenez et al., 2009)	Many	*D. viridis*	Chlorophyta	Chlorelalles
(Leu & Hsu, 2005a)	Heat	*C. Pyrenoidosa*	Chlorophyta.	Chlorelalles
(Zuppini et al., 2007, 2009)	Heat	*C. Saccharophila*	Chlorophyta	Chlorelalles
(T.-C. Lee & Hsu, 2013a)	Heat	*S. Vacuolates*	Chlorophyta	Sphaeropleales
(Zuppini, Gerotto, & Baldan, 2010)	Salt stress	*C. Saccharophila*	Chlorophyta	Chlorelalles
(Darehshouri et al., 2008)	Oxidative stress	*M. denticulate*	Charophyta.	Desmidiales
(Affenzeller et al., 2009)	Salt stress	*M. denticulate*	Charophyta	Desmidiales
(Bidle et al., 2007)	Viral infection	*E. huxleyi*	Haptophyta	Isochrysidales
(Bidle & Bender, 2008)	Iron limitation	*T. pseudonana*	Bacillariophyta	Thalassiosirales
(Vardi et al., 2006; Vardi, 2008)	DD aldehydes	*P. tricornutum*	Bacillariophyta	Navicolales
(Luo et al., 2014)	Fe limitation	*T. pseudonana*	Bacillariophyta	Thalassiosirales
(Dunn et al., 2002, 2004)	Heat	*Symbiodinium spp*	Miozoa	Suessilales
(Franklin & Berges, 2004)	Senescence/Darkness	*A. carterae*	Miozoa	Gymnodiniales
(Vardi et al., 1999a, p. 199)	CO_2	*P. gatunense*	Miozoa	Peridiniales
(Johnson et al., 2014)	Aging	Karenia brevis	Miozoa	Gymnodiniales
(Jauzein and Erdner 2013)	Temperature	*A. tamarense*	Miozoa	Gonyaulacales

Table 2. The measures of PCD in chlorophytes, streptophyta, diatoms, dinoflagellates and haptophytes, including the methods for detection, main findings, frequency of detection, detection efficiency and significance. Not all studies might be represented in the table. The frequency of use is the number of times the method was used (as a percentage) to detect PCD in the 28 studies. The efficiency of detection is the percentage of positive findings for a particular method when PCD was concluded. Significances were assigned according to Durand (2020).

MARKERS OF PCD	METHOD (S)	MAIN FINDINGS	FREQUENCY OF USE	EFFICIENCY OF DETECTION	SIGNIFICANCE
Ultrastructural alterations	***Transmission electron microscopy*** ***Chlorophytes****:* (Jimenez et al., 2009; Moharikar et al., 2006; Sathe and Durand, 2016; Segovia, 2003; Yordanova et al., 2013; Zuppini et al., 2007); **Streptophyta:**(Affenzeller et al., 2009; Darehshouri et al., 2008) ***Diatoms:*** (Bidle and Bender, 2008); ***Dinoflagellates****:* (Dunn et al., 2004; Franklin and Berges, 2004). **Haptophytes:** (Bidle et al., 2007)	Cell shrinkage, membrane contraction and blebbing, cytoplasmic vacuolization, chloroplast and mitochondrial alterations, nuclear chromatin condensation and/or marginalization.	46%	0.92	Gold standard / hard sign
DNA degradation	***TUNEL staining*** ***Chlorophytes:*** (Jimenez et al., 2009; Moharikar et al., 2006; Segovia, 2003; Vavilala et al., 2014; Zuo et al., 2012; Zuppini et al., 2007); **Streptophyta:**(Affenzeller et al., 2009)**;** ***Dinoflagellates:*** (Johnson et al., 2014; Vardi et al., 1999b).	double or single strand DNA nicking;	39%	0.73	Soft sign
	DNA gel electrophoresis ***Chlorophytes:*** (Durand et al., 2014; Moharikar et al., 2006; Nedelcu, 2006; Yordanova et al., 2013; Zuo et al., 2012); **Streptophyta:**(Affenzeller et al., 2009; Darehshouri et al., 2008) **Dinoflagellate:** (Dunn et al., 2002)	DNA laddering;	32%	0.61	Hard sign
	Alkaline comet assay ***Chlorophytes***: (Yordanova et al., 2013; Sathe and Durand, 2016)	Single-celled DNA degradation	7%	1	Soft sign
	Nuclease activity assay and Zn^{2+} inhibition of endonucleases ***Chlorophytes:*** (Leu and Hsu, 2005a; Zuo et al., 2012).	Nuclease activity.	7%	1	Soft sign
Loss of membrane asymmetry	***Annexin-V labelling fluorometric or flow cytometric assay*** ***Chlorophytes****:* (Durand et al., 2014; Leu and Hsu, 2005a; Moharikar et al., 2006; Orellana et al., 2013; Segovia and Berges, 2009);	Externalization of PS in conjunction with retention of plasma membrane integrity	25%	1	Hard / Soft sign?

	Diatoms: (Bidle and Bender, 2008; Luo et al., 2014). **Haptophytes:**(Bidle et al., 2007)				
Caspase-like-activity	***High specific caspase biochemical assays (conjugated WEHD, DEVD, VEID,IETD or LEHDases fluorometric assays)*** ***Chlorophytes:*** (Orellana et al., 2013; Sathe and Durand, 2016; Segovia, 2003; Vavilala et al., 2014; Yordanova et al., 2013; Zuppini et al., 2007); **Streptophyta:**(Affenzeller et al., 2009; Darehshouri et al., 2008) ***Diatoms:*** (Bidle and Bender, 2008; Luo et al., 2014); **Dinoflagellates:**(Johnson et al., 2014) ***Haptophytes****:* (Bidle et al., 2007).	Caspase 1, 3, 6, 8, 9 activity;	50%	0.87	Soft sign
	Caspase inhibitors (like Ac-VAD-FMK, Boc-D-FMK and AC-YEVD-CMK) ***Chlorophytes:*** ; ***Diatoms:*** (Bidle and Bender, 2008); ***Dinoflagellates:*** (Vardi et al., 1999b); ***Haptophytes:*** (Bidle et al., 2007).	Prevention of cell death;	21%	1	
	Western blot analysis **Chlorophytes:** (Moharikar et al., 2006; Segovia, 2003; Vavilala et al., 2014). **Dinoflagellates:**(Johnson et al., 2014)	Caspase proteins detection.	25%	1	
Metacaspase gene or protein activity	***(qRT)- PCR*** ***Chlorophytes:*** (Murik et al., 2014a; Murik and Kaplan, 2009); ***Diatoms:*** (Bidle and Bender, 2008);	Metacaspase gene transcription;	14%	1	Soft sign
	Western blot analysis ***Haptophytes****:* (Bidle et al., 2007)		10%	1	
	High specific metacaspase activity assay **(fluorogenic substrates with Arg or Lys residues at the P1 position)** *Remains to be tested in eukaryotic phytoplankton** : (Tsiatsiani et al., 2011)	Metacaspase activity;	-	-	
	Metacaspase inhibtors like (Arginal protease inhibitors) *Remains to be tested in eukaryotic phytoplankton** : (Tsiatsiani et al., 2011)	Prevention of cell death.	-	-	
Upregulation of PCD associated genes (e.g.	***RT-PCR and (qRT)- PCR***	Presence and increase in factors like cytochrome f	32%	0.9	Soft sign

ChspetA, dad-1, APAF-1, AsA–GSH cycle genes, p-53 like genes, PtNOA)	***Chlorophytes**:* (Moharikar et al., 2007; Nedelcu, 2006; Vavilala et al., 2014; Zuppini et al., 2009); *(Murik et al., 2014a; Murik and Kaplan, 2009)* ***Diatoms:*** (Vardi et al., 2006)	(ChspetA) apoptotic regulators (dad-1 and APAF-1 factors), p-53 like protein and AsA–GSH cycle genes; Quantitative measurenment of antioxidant enzymes (AsA–GSH cycle genes); Quantitative measurenment of NO-associated gene.			
ROS	**H2DCFDA or CM- H2DCFDA; Xylenol orange staining** ***Chlorophytes:*** (Affenzeller et al., 2009; Durand et al., 2011; Murik et al., 2014a; Nedelcu, 2006; Zuppini et al., 2007); ***Dinoflagellates:*** (Vardi et al., 1999b). ***Chlorophytes:*** (Vavilala et al., 2014; Zuo et al., 2012). **Antioxidant enzymatic assays** ***Chlorophytes*:** (Murik et al., 2014a; Vavilala et al., 2014); **DAF-FM staining**	Burst in ROS/Burst in Oxygen Peroxide (H_2O_2); Increased activity of antioxidant enzymes	46% 14%	0.8 0.8	Soft sign
NO species	***Diatoms:*** (Vardi et al., 2006)	Burst in Nitric oxide.	3%	1	Soft sign
Mitochondrial-associated biochemical alterations	**Measurement of mitochondrial transmembrane potential; Succinate dehydrogenase activity** ***Chlorophytes:*** (Vavilala et al., 2014); (Lee and Hsu, 2013b)	Disruption of the mitochondrial transmembrane potential; Mitochondrial activity.	11%	0.75	Soft sign
Chloroplast-associated biochemical alterations	**Western blot or SDS-page total protein analysis; Spectrophotometric determination of chlorophyll and carotenoids** ***Chlorophytes:*** (Leu and Hsu, 2005a; Zuppini et al., 2007); (Lee and Hsu, 2013a; Leu and Hsu, 2005a; Vavilala et al., 2014; Zuppini et al., 2007).	Disappearance of large unit of RUBISCO and reduction of light-harvesting complex of PSII; Reduction in chlorophyll and/or carotenoid contents.	10%	1	Soft sign

Capítulo 2: Programmed cell death in the coccoid green microalga *Ankistrodesmus densus* Korshikov (Sphaeropleales, Selenastraceae)

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ABSTRACT

Reports of programmed cell death (PCD) across the taxonomic spectrum of photosynthetic unicellular organisms raise questions concerning its ecological and evolutionary roles. However, before any ecological studies or evolutionary interpretations, it is essential to document phenotypic changes associated with PCD at single-cell level, since the death-related responses may vary between taxa and within a single taxon depending on the environmental stimuli. Here, we report responses to rapidly changing light, temperature and fluctuations in macronutrients in the common Selenastracean green microalga *Ankistrodesmus densus* (Clorophyta, Chlorophyceae, Sphaeropleales). Specifically, the stimuli of darkness, nitrogen starvation, heat and cold shock were tested since these occur naturally and the PCD phenotypes examined by way of ultrastructural changes, phosphatidylserine (PS) externalization and DNA degradation. Flow cytometric Annexin V FITC analyses revealed that darkness and nitrogen starved cultures had significantly higher proportion of cells with PS externalization than control cultures ($p<0.05$), while heat and cold treatments did not affect the PS externalization. Transmission electron microscopy (TEM) revealed that light deprived cells presented with, among other ultrastructural changes, high cytoplasmic vacuolization suggesting vacuolar cell death, a subtype of the autophagic morphotype of PCD. Nitrogen starved cells had less vacuolization but presented typical ultrastructural markers of PCD such as chromatin marginalization and pyknosis. In contrast, the heat and cold shock treatments resulted in necrotic-like features. These findings suggest that under darkness and nitrogen starvation a small but significant fraction of the community undergoes PCD. *A. densus* may experience different death-related phenotypes depending on different environmental inducers, which is essential knowledge for interpreting the results from ecological studies. Furthermore, our results

indicate that the vacuolar/autophagic like cell death morphotype existed in single-celled phytoplanktonic organisms before land plants evolved, and might have been an important development for tissue homeostasis in higher multicellular plants.

Key words: *Ankistrodesmus*; autophagic/vacuolar cell death; PCD; phytoplankton, ultrastructural, TEM, unicells.

Introduction

Reports of programmed cell death (PCD) in unicellular phytoplankton (Bidle, 2016; Pepper et al., 2013) are increasingly raising questions concerning its ecological and evolutionary roles (Berges & Choi, 2014; Bidle, 2015; Franklin et al., 2006; Nedelcu et al., 2011). PCD plays important intra and inter-specific ecophysiological roles in phytoplankton populations and communities (Bidle, 2016; Durand et al., 2016; Franklin et al., 2006). In chlorophytes, for example, PCD may impact others in the population (Durand et al., 2011, 2014; Orellana et al., 2013; Yordanova et al., 2013). A variety of environmental triggers can also lead to PCD in marine and freshwater communities affecting the biogeochemical flow of nutrients between and within trophic levels e.g. (Bidle, 2015, 2016; Orellana et al., 2013; Vardi, 2008). Additionally, PCD can also regulate propagation and dispersal during unfavourable growth conditions and plays a role in evolutionary transitions in individuality (Berman-Frank et al., 2004).

Prior to interpreting the data on death in any ecological or evolutionary study, it is essential to document the environmental triggers and cell death morphotypes associated with PCD in a particular organism. Distinct markers and phenotypes reflect the organism's plasticity in adapting to environmental stresses (Bursch et al., 2006; Eisenberg-Lerner et al., 2009). Documenting the features of death in unicellular phytoplankton , however, can be challenging (Berges & Choi, 2014) and the distinction between death-related phenotypes has been a problem in the field (Berges & Choi, 2014; Nedelcu et al., 2011). The reason for this is that researchers have used the general experimental framework imported from the multicellular world (Nedelcu et al., 2011; Proto et al., 2013). These include morphological changes such as cell shrinkage, chromatin condensation and phosphatidylserine (PS) inversion on the membrane outer surface, as well as biochemical markers like activation of caspases and DNA

fragmentation (Bidle & Falkowski, 2004). In phytoplankton, however, a variety of morphologically and biochemically distinct morphotypes emerge upon different stimuli operating in the same taxon (Ameisen, 2002; Bidle, 2015, 2016; Franklin et al., 2006; Nedelcu, 2006). Moreover, different taxa can display similar or different and distinct markers when the same stimulus is used (Affenzeller et al., 2009; Vavilala et al., 2014) Comparing experiments, therefore, require one to know the morphotype associated with a specific scenario. In addition, typical mammalian assays are very sensitive, but not specific for detecting some markers in unicellular organisms. The Annexin-V assay, for example, that detects PS exposure, can also be positive for other phospholipids (Weingärtner et al., 2012), while caspase assays are usually positive in phytoplankton, even though they do not have orthologous mammalian caspases, but instead harbour distant homologous genes known as metacaspases (Tsiatsiani et al., 2011).The consequence is that, when documenting PCD, not all markers are observed for one taxon or between taxa in each cell death scenario and the results of experiments can easily become conflicted.

In metazoa, a variety of different morphotypes of PCD have been proposed by the Nomenclature Committee on Cell Death (NCCD) (Galluzzi et al., 2012). Although recommendations and terminology has been published for yeast (Carmona-Gutierrez et al., 2018), a similar nomenclature is missing for phytoplankton although here are many "different ways to die" (Jimenez et al., 2009). Autophagic-like cell death, for example, is associated with cytoplasmic vacuolization and is regulated by AuTophaGy Related (ATG) proteins (Duszenko et al., 2011; Minina et al., 2014; Tsujimoto & Shimizu, 2005). This morphological subtype of PCD is an important death-related mechanism in higher plants and its presence in cell-walled phytoplankton is not clear. Vacuolar death and autophagy in general is also not distinct, in that there is cross-talk between these

mechanisms and apoptosis pathways (Bursch, 2001; Bursch et al., 2006; Eisenberg-Lerner et al., 2009). The indicators of both mechanisms may overlap one another in phytoplankton depending on the nature and intensity of the stimuli. (Affenzeller et al., 2009; Jimenez et al., 2009)

Chlorophytes are used extensively as model organisms to study PCD in phytoplankton (Berges & Choi, 2014; Bidle, 2015, 2016; Durand et al., 2016). Chlorophyta represent the biggest division of the "green algae" comprising ubiquitous species of macro and microalgae (Krienitz & Bock, 2012). Several chlorophytes have been investigated under a wide range of environmental triggers, and many different cell death phenotypes have been described (Affenzeller et al., 2009; Jimenez et al., 2009, 2009; Moharikar et al., 2006; Zuppini et al., 2007) and are valuable group in ecological and physiological studies of phytoplankton PCD (Bidle, 2015; Franklin et al., 2006).

Within the Chlorophyta, the asexual coccoid microalgae from the family Selenastraceae (Sphaeropleales, Chlorophyceae) are particularly helpful as a model organism for PCD studies. They comprise a highly diverse clade and are very common in inland waters (Garcia da Silva et al., 2017; Krienitz et al., 2001). Despite being present in most freshwater ecosystems, little is known about how cell death programs affect their ecophysiology (Franklin et al., 2006). Recently, evidence of PCD-like death was reported in Selenastraceae in a temperate eutrophic pond in the USA (Kozik et al., 2019) during the transition between spring and autumn. However, correlation between changing conditions of irradiance, temperature, and macronutrient fluctuations (e.g. N:C) and cell death was not identified (Kozik et al., 2019). This in itself was slightly unexpected. The ecological stimuli that induce cell death (presumably PCD does occur in this lineage) and the associated death-related morphotypes in this important microalgal group is missing.

In this study, we have documented different modes of death in the common selenastracean green microalga *Ankistrodesmus densus* (Clorophyta, Chlorophyceae, Sphaeropleales) under rapidly changing conditions of light, temperature and fluctuations in macronutrients, all of which are ecologically crucial stimuli. Considering that the PCD-related features can involve subtle markers, we have used more stringent conditions of total darkness, nitrogen starvation, heat and cold shock to identify possible death-related morphotypes. The following markers of death were documented: ultrastructural-related changes, phosphatidylserine (PS) externalization and DNA degradation.

Material and Methods

Organism

The strain of *Ankistrodesmus densus* (CCMA-UFSCar 3) was maintained in axenic conditions at the Culture Collection of Freshwater Microalgae at the Universidade Federal de São Carlos, Brazil (CCMA-UFSCar, WDCM 835).

Culture conditions

Cell cultures of *A. densus* CCMA-UFSCar 3 with an initial concentration of 1 $\times 10^4$ cells mL^{-1} were maintained in WC medium, pH 7.0 (Guillard & Lorenzen, 1972), temperature at 23ºC (± 2°C), and irradiance of 200 µmol photons m^{-2} s^{-1} under 12-12 hours (h) light-dark cycle. Prior to the experiments, the cultures were tested for axenic conditions in WC medium at pH 7.0 added with peptone and glucose (250 mg L^{-1} each) , and there was no bacterial contamination. Growth curves were measured 3 days a week *by in vivo* chlorophyll *a* using spectrophotometry (HACH DR 5000), using the

subtraction between the wavelengths of 680 and 750 nm according to Griffths et al. (2011).

Programmed cell death induction

Cells were subjected to four environmental acute stress treatments: darkness, nitrogen starvation, heat and cold shock. Control cultures were maintained under the standard growth conditions as previously described. All experimental cultures were performed in triplicate test tubes of 10 mL each, prepared as follows.

Mid-exponential cultures of *A. densus* were counted using a hemocytometer, washed and resuspended to 150 mL of fresh WC medium at a density of 1×10^6 cells mL^{-1}. One hundred twenty ml of this suspension were equally redistributed to 12 test tubes (i.e. 10 ml for each tube), which were subsequently exposed in triplicates to darkness, heat and cold shock. Three tubes were used as control. The remaining suspension was washed three times with nitrogen-free WC medium at pH 7.0, cells were resuspended in 30 mL of the same medium, and aliquots of 10 ml were placed in 3 test tubes for nitrogen depletion treatment.

The control cultures, as well as the cells exposed to nitrogen starvation (nitrogen-free WC medium), were incubated for 4 days under standard conditions, i. e. 23°C (± 2°C) and 12-12h light-dark cycle at 200 μmol photons m^{-2} s^{-1}. The darkness treatment was kept with no light for the same period and under the standard temperature. For the heat induction treatment, cells were heated to 44°C for one hour and returned to standard conditions for 12-14 hours; whereas for the cold shock treatment, cells were kept at approximately 0°C for 3 hours returning to standard conditions for 12-14 hours. The temperature choice for heat cultures 44°C was defined based on earlier shock experiments in green algae (Durand et al., 2011, 2014; Nedelcu,

2006). For the cold treatment, the temperature was randomly defined 0°C motivated by earlier unpublished laboratory observations indicating that cold could induce DNA fragmentation in *Chlamydomonas reinhardtii*. PCD analysis were performed in induced cells immediately after the incubation period described above for each treatment.

Programmed cell death analyses

The most informative assay for programmed cell death relies on integrating data from direct observation (e.g. TEM) with biochemical assays. Based on our previous work (Durand, 2020), we have used three signs of PCD: phosphatidylserine (PS) externalization in conjunction with retention of membrane integrity, observation of PCD ultrastructural morphological changes and DNA degradation. These measures of PCD are very specific markers but are not always sensitive. This means that PCD is not excluded if they are negative, for example. On the other hand, biochemical assays such as for metacaspase activity, increase of reactive oxygen species (ROS) and TUNEL staining were not used because they are regarded as soft signs of PCD (Durand, 2020). Soft signs include markers which are sensitive to detect PCD, but not unique to PCD.

Loss of membrane asymmetry and retention of membrane integrity

For quantification of PCD, PS externalization was detected by flow cytometry using the Apoptosis detection kit, BD Pharmingen, following the manufacturers' suggested protocol. Briefly, approximately 1×10^6 cells were harvested, washed with PBS and resuspended in Annexin V binding buffer. From this suspension, approximately 1×10^5 cells were harvested by centrifugation and stained with annexin-V and propidium iodide (PI) for 15 min in the dark. Annexin V and PI positivity were

assessed using a FACSCalibur cell analyzer (Becton-Dickinson, San Jose, CA, USA) using standard FL1 (530/30 nm emission) and FL2 filter sets (610/20). The data was acquired using BD FACDiva software. Prior to the acquiring, control (unstained) cells were gated on the SSC and FSC channels, and an analysis of excitation and emission spectra was performed to minimize chlorophyll autofluorescence spillover in the detection channels.

Flow cytometry data analysis

Cytograms were analyzed using FlowJo software (10.5.3) with a minimum of 1 $\times 10^4$ cells acquired for each sample (n=3, for each treatment). The quadrants were set based on unstained cells. Total cell abundance in each quadrant was determined by the proportion of cells bound to FITC and PI under the green (FL1 filter set) and orange fluorescence (FL2 filter set). It is worth to mention that in to PS externalization assays in mammalian cells, compensation for fluorescence spillover between channels is not usually performed for phytoplankton because it is unclear what might constitute good positive controls(Gasol & Morán, 2015).

Statistical analyses were performed with GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). We compared the percentage of annexin V and PI binding cells of control and treated cultures using one-way analysis of variance (ANOVA) and Dunnet's multiple-comparison posttest. Prior to the analysis, Annexin-V and PI data sets were log-transformed (Dingman & Lawrence, 2012a), and the assumptions of homogeneity of variance and normal distribution were assessed using Brown-Forsythe and Shapiro–Wilk's tests.

Transmission electron microscopy (TEM)

Biological materials were collected at the end of the incubation period and fixed for 4 hours at room temperature (20-25 ºC) with 2% glutaraldehyde in 0.025 sodium cacodylate buffer (pH 7.5) as follows: 3 mL of the cell suspension from each biological replicate were pooled, obtaining of 9 mL for treatment. These cell suspensions were added to equal volumes of 4% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.5) (Nozaki et al., 1993). After fixation, samples were washed 3 times in 0.025M sodium cacodylate buffer for 5 minutes each. After each washing, cells were re-peletized in an IKA mini G centrifuge at 3000 × *g*. Cell pellets were then postfixed with 2% osmium tetroxide in 0.025 M sodium cacodylate buffer for 2 h at room temperature followed by two washes with 0.025 mM sodium cacodylate buffer. The fixed materials were then dehydrated for 10 min each in 5, 10, 25, 40, 70, 80, 95 and 100 % ethanol followed by a final dehydration in 100 % ethanol for 20 min. After dehydration, cells were transferred to increasing ethanol:LR white resin ratios of 3:1; 2:1; 1:1 for 20 min each, then twice in 100 % LR white resin for 45 min each and finally left overnight at 4°C. The following day, cells were embedded in fresh LR White resin in gelatin capsules and allowed to polymerize at 65 ºC. The hardened resin blocks were sectioned at 90 nm thickness with a ultramicrotome (Reichert-Jung, Vienna, Austria) and the sections were collected on copper grids (5- 10 grids per sample; each grid had several thin sections). The grids were stained with 5% aqueous uranyl acetate and lead citrate (Reynolds, 1963), dried and examined with an FEI Tecnai G2 Transmission Electron Microscope at 120kV.

DNA laddering

To detect DNA fragmentation, control and treated cultures were collected as follows: 3 ml of each replicate of experimental cultures were pooled, totalizing 9 ml for each treatment and for the control culture. The cell suspensions were centrifuged, and the genomic DNA was extracted using a DNeasy Powersoil kit (Qiagen) and electrophoresed in 1% agarose gel (45 min, 80V). Unlike the flow cytometric detection DNA fragmentation provides a qualitative result of PCD since not all cells in the population are engaged in the process.

Results

Loss of membrane asymmetry and retention membrane integrity

Flow cytometric analysis of PS externalization revealed that the darkness and nitrogen starvation treatments were significantly different than the control cultures ($p<0.05$, n=3, Fig. 1, a). However, temperature (heat and cold cultures) did not affect significantly the PS externalization ($p=0.44$ and $p=0.99$, n=3, respectively, Fig. 1, a). On the other hand, the heat shock stressed cultures were significantly different from control cultures in terms of loss of membrane integrity and PI binding to DNA ($p=0.0015$, n=3 Fig. 1, b). In light-limited and nitrogen-starved cultures, respectively, 8.4 ± 3.5 and 7.42 ± 2.6% of the cells showed PS externalization, while in control, heat and cold stressed cultures, these proportions were, respectively, 2.9 ± 0.3, 2.1 ± 0.4, and 3.1 ± 0.5% of cells showed the PCD phenotype (Q3 in Fig. 2). In contrast, heat shock cultures had 15.6 ± 4% of cells presenting membrane permeabilization compared to 5.42 ± 1.7%, 5.27 ±1 and 6.49 ± 1.3% of PI binding in control, darkness and nitrogen treatments, respectively (Q1 in Fig. 2).

We found no significant difference between control and treatments in the proportion of cells positive for both FITC and PI (Q2 in Fig. 2). Although in the

nitrogen depletion treatment 8.29 ± 1.7% of cells were found positive for both stains, this result was not significantly different (p=0.28) from the proportion found in control (4.45 ± 0.9%). Nevertheless, quadrant 2 provides less information about the mode of death, since cells positive for both FITC Annexin V and PI could have died by an apoptotic pathway or as a result of a necrotic process.

The higher proportion cells bound to FITC-Annexin V in darkness and nitrogen depleted treatments can also be seen in the histograms (Fig.2, f) by a small shift to the right of those treatments after superposition to control cultures. In contrast, heat and cold treatments showed complete overlapping of the control histogram (Fig. 2, g).

It is worthwhile to mention that although chlorophytes have been repeatedly showed to bind Annexin-V (Durand et al., 2014; Moharikar et al., 2006; María Segovia & Berges, 2009), PS is not present in green algae like *Clamydomonas* (Giroud et al., 1988). The reason for this is that Annexin-V possibly binds to other phospholipids such as phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) (Weingärtner et al., 2012), that has the same significance for PCD.

Ultrastructural changes by transmission electron microscopy (TEM)

Ultrastructural micrographs of *A. densus* cells growing actively in control cultures (WC complete medium and standard conditions) (Figs 3-6) showed one large nucleus with a central nucleolus surrounded by uncondensed chromatin (Fig. 3); one parietal chloroplast with stacked thylakoids (Figs 4-6); and a dense cytoplasm with some small mitochondria (Figs 4, 5).

Cultures exposed to the darkness treatment (Figs 7-10) underwent high cytoplasmic vacuolization, with each vacuole containing electron dense materials (Figs 7-9, arrows 1). Mitochondria, chloroplast, plasma and nuclear membranes remained

intact, although mitochondrial elongation (Fig. 9, arrows 2) was eventually observed. A single mass of chromatin condensation (pyknosis) in the center of the nucleus but without marginalization could be seen (Fig. 10, arrow 3).

Nitrogen-starved cells (Figs 11-14) also showed cytoplasmic vacuolization although much less than light-deprived cells (Fig. 11, arrows 1). We also observed a structure that resembled a plastid with blebbing (Fig. 11, arrow 2). Although unclear, the internal structures resemble double membranes, and might possibly be highly disorganized thylakoids (Fig.11, arrow 2). In contrast to the darkness treatment, cells expressed impairment of the photosynthetic membranes which is represented on the micrographs by electron-lucent spaces between the thylakoids (Fig. 12, arrows 3). Mitochondria did not elongate (Fig. 12, arrow 4). Nucleolar shrinkage/condensation and movement of the chromatin to the nuclear membrane was observed (Fig. 13, arrows 5), eventually resulting in chromatin marginalization (Fig. 14, arrows 6).

Heat-shock stressed *A. densus* cells (Figs 15-18) presented a shrunken, damaged protoplasm with membrane detachment from the cell wall (Fig. 15-16, arrows 1). Complementing to the PI data from flow cytometry (Fig. 2), TEM micrographs also revealed possible spots of membrane rupture (Fig. 16, arrow 2). Although thylakoids also showed membrane impairment similar to nitrogen starved cells (Fig. 17, arrow 3), only in the heat treatment could alterations be seen as, balloon-shaped and swollen mitochondria (Fig. 18, arrow 4).

Cold-stressed cells were similar to control cultures with respect to membrane structure, and similarly did not show the ultrastructural changes observed in other treatments. Cytoplasmic vacuolization was not observed, and the nucleus and other organelles presented intact (Figs 19-22), although some chromatin condensation and protoplasm shrinkage could be noted (Fig.19, arrows 1 and 3 respectively). Yet, only

discrete alterations were noted on mitochondria, which presented as slightly dilated (Figs 19-21, arrows 2), and also some impairment between thylakoids in the chloroplast (Fig. 22, arrow 4).

DNA fragmentation

Neither the control, nor the stressed cells displayed DNA laddering as measured by gel electrophoresis (data not shown).

Discussion

The aim of this study was to investigate the death-related responses in the common selenastracean alga *A. densus,* which can provide essential information when interpreting results from evolutionary ecology studies in the family Selenastraceae. We have exposed this alga to a range of ecologically important stimuli including darkness, nitrogen starvation, heat and cold shock. We documented the following markers of PCD: ultrastructural-related changes, loss of membrane asymmetry and DNA degradation. Our results suggested that rapidly changing light and nitrogen levels induce PCD-related phenotypes in *A. densus.* Furthermore, our findings showed that the same taxon may exhibit different markers depending on the nature of the stimulus. However, despite the differing responses vacuolar cell death, that is an autophagic morphotype of PCD, seemed to be an important mechanism of PCD in this cell-walled green microalgae, similar to that demonstrated in higher plants (Bassham, 2007; Minina et al., 2014; van Doorn et al., 2011).

Reports of PCD in the important and ubiquitous group of Selenastraeae are scarce. One of the few PCD studies in these organisms to date comes from (Kozik et al., 2019) and suggested a link between cell death and a mixture of abiotic and biotic factors. Although the authors correlation between thresholds of irradiance, temperature,

and nitrogen fluctuations and the PCD phenotype could not be found, here we have showed that more severe changes, that remain ecologically relevant, such as light and nitrogen concentration can induce death-related responses in *A.densus*. Rapidly changing environmental conditions may be rare in natural systems - although they do occur (e.g. nitrate depletion) - and other factors such as pathogens and allelopathy can play greater roles in cell death (Bidle, 2016; Kozik et al., 2019). This has also been identified in a wide range of unialgal taxa (Affenzeller et al., 2009; Jimenez et al., 2009; Moharikar et al., 2006; Segovia, 2003).

After sudden exposure for 4 days in complete darkness, *A. densus* cells had a statistically significant result for the Annexin V staining (Fig.1, a). These findings, however, seem to indicate that only a relatively small fraction of the community was undergoing PCD (approximately 8%). Interestingly, the TEM micrographs of light-deprived cells revealed a massive cytoplasmic vacuolization which presented "electron dense material" inside. Vacuolization during cell death was described before in phytoplankton, including chlorophytes (Affenzeller et al., 2009; Jimenez et al., 2009), cyanobacteria (Berman-Frank et al., 2004; Ning et al., 2002), social amoebas (Cornillon et al., 1994; Otto et al., 2003), vascular plants (Danon et al., 2000; van Doorn et al., 2011b), and even in mammals (Henics & Wheatley, 1999). This morphology have been usually associated with vacuolar/autophagic cell death, a special type of PCD (Affenzeller et al., 2009; Bursch et al., 2006). Consistent with this morphotype, the finding of a single pyknotic mass in the center of the nucleus seems to indicate autophagic PCD (Bursch, 2001). The appearance of elongated mitochondria is also suggestive of autophagy. During autophagy, mitochondria can fuse and elongate to sustain cell viability, since longer mitochondria are protected from degradation and produces more ATP which is necessary under stress conditions (Gomes & Scorrano,

2011).

Although autophagy might also be involved in cell survival (Das et al., 2012; Galluzzi et al., 2012; Mizushima & Komatsu, 2011), the small but significantly proportion of *A. densus* cells positive for annexin V along with retention of membrane integrity is interesting. PS is a known apoptosis marker but can sometimes also be associated with vacuolar autophagy. The presence of vacuolization and PS externalization then corroborates the literature which indicates that apoptotic and autophagic-like cell death processes should not be seen as "mutually exclusive phenomena" (Bursch, 2001; Bursch et al., 2006; Lockshin & Zakeri, 2004). The interplay between autophagy and apoptosis depends on the stimuli and the cell's environment (Bursch et al., 2006; Eisenberg-Lerner et al., 2009), and their overlapping markers have been showed in chlorophytes before (Affenzeller et al., 2009; Jimenez et al., 2009; Yordanova et al., 2013). It is worth mentioning once more that, despite the finding that PS is absent in chlorophytes (Giroud et al., 1988), the annexin appears to be picking up PE and PG externalization, which has the same meaning for PCD.

These findings are important for disentangling the evolutionary ecology of PCD en route to multicellularity in plants. In higher plants, autophagy is a mechanism activated during "*vacuolar cell death*" which is one of two major types of cell death in plants, along with necrosis (Minina et al., 2014; van Doorn et al., 2011). Vacuolar cell death is thought to have evolved in plants due to their rigids cell walls, which prevents the fragmentation of the cells, and also the absence of macrophages (van Doorn et al., 2011b). During vacuolar cell death, the cytoplasm is gradually reduced by lytic vacuoles with subsequent degradation of cellular contents (Minina et al., 2014, 2014; van Doorn et al., 2011). This is fundamentally different from necrosis where general swelling and early rupture of the plasma membrane release cellular contents (Kroemer et al., 2009) . The green lineage

comprises Chlorophyta, that includes *A. densus,* and the Streptophyta, that includes algae ("Charophyta") and plants (Embryophyta) (Leliaert et al., 2012; Lewis & McCourt, 2004). Even though the green ancestor of plants is within streptophyte algae (Leliaert et al., 2012), it is possible that this vacuolar mode of death might have evolved earlier in the green lineage. Accordingly, autophagic PCD have also been described in the streptophyte microalgae *Micrasterias denticulate* in response to salt stress (Affenzeller et al., 2009).

Cells under nitrogen starvation demonstrated ultrastructural apoptotic-like hallmarkers, which were observed in phytoplankton elsewhere, such as the movement of condensed chromatin from the nucleolus to the nuclear membrane (Figs 13, arrow 5) (M. Segovia, 2003), pyknosis and the the margination of chromatin (Fig 14, arrow 6) (Zuppini et al., 2007). And these cells also exhibited a small but statistically significant positive result for PS exposure (7,4%) in conjunction to retention of plasma membrane integrity (Fig. 1, a and b). These findings specifically implicate in an apoptotic-like morphotype in *A. densus*. However, nitrogen-starved cells also presented cytoplasmic vacuolization, that is a marker of autophagic PCD, although less than in the darkness treatment (Fig. 11, arrow 1). This may indicate that, in fact, cytoplasmic vacuolization is a morphological feature encoded in *A. densus* cell death program, supporting the hypothetical interplay between apoptosis and autophagy in this microalgae.

Interestingly, nitrogen-deprived *A. densus* cells also presented alterations in plastid ultrastructure (Fig. 12, arrows 3; see also Fig. 11, arrow 2). Chloroplast alterations suggest that the observed vacuoles might be involved in the degradation of chloroplastic proteins. In plants, senescence associated vacuoles (SAVs) are under a genetic death program, and are responsible for nutrient recycling via degradation of plastid proteins to provide nitrogen to other parts of the plant (Costa et al., 2013; Ishida et al., 2014; Jones, 2001). Chloroplast morphological alterations along with protein

degradation were also observed during autocatalytic death in heat stressed *Chlorella* cells (Leu & Hsu, 2005; Zuppini et al., 2007). Additionally, in cyanobacteria, isolated pseudovacuoles from dying *Anabaena* cells were reported to be positive for protease activity (Ning et al., 2002). Similar to the systemic response that is observed in multicellular plants during senescence, chloroplast-degrading vacuoles in the clonal *A. densus*, under nitrogen depletion conditions may play a role in kin survival. This hypothesis, however, remains to be tested under further experimentation.

The fact that we were unable to observe DNA laddering in DNA isolated from cells in the darkness and the nitrogen starvation treatments does not rule out a programmed mode of death in the cells exposed to these stimuli. The literature have reported other organisms which undergo PCD without visible DNA laddering e.g. (Madeo et al., 1999; Vardi et al., 1999; Zuppini et al., 2007).

Exposure of cells to sudden heat shock revealed a shrunken cytoplasm, detachment of the plasma membrane from the cell wall (Fig. 15-16, arrows 1), and ultrastructural chloroplast alterations (Fig 17, arrow 3). Although these features may indicate PCD (Zuppini et al., 2007), the statistically significant proportion of cells with damaged membranes (Fig. 1, b) indicates necrosis (also known as incidental death). In addition, cell shrinkage is not specific to PCD and may occur in incidental death (van Doorn et al., 2011b). For example, plant protoplasm's may shrink in response to abiotic stress but plasma membrane integrity usually is not retained (Heath, 2000). This is supported in heat-shocked cells by damaged balloon shaped and swollen mitochondria, which indicate necrosis (Affenzeller et al., 2009). The different markers found in *A. densus* in comparison to other microalgae under acute heat shock e.g. (Dingman & Lawrence, 2012b; Durand et al., 2011; Lee & Hsu, 2013; Zuppini et al., 2007) support earlier observations that indicate that different taxa may respond differently to the same

stimulus (e.g. heat shock response in *Scenedesmus* (Lee & Hsu, 2013) and *Chlorella* (Zuppini et al., 2007).

In contrast to heat, cold shock had little if any effect upon the cells, at least at the intensity and exposure time tested here. Under exposure to cold shock, our cells presented non-significant results for PS externalization (Fig.1, a), and damaged membranes (PI-positive) compared to control cultures (Fig. 1, b). On the other hand, the discovery of some enlarged mitochondria (Figs 19-21, arrows 2) may indicate that the cold shock might have led to incidental death in at least a few cells (Kroemer et al., 2009). This may be supported by the slightly higher (although not significant) mean of cells positive to PI compared to control cells. Moreover, some chromatin condensation spots and cytoplasm shrinkage were noted, but these markers are not specific to PCD and may also be presented under necrosis (Hou et al., 2016; Morel & Dangl, 1997; van Doorn et al., 2011b).

Natural systems are fundamentally different from laboratory unialgal cultures. Although in nature many other factors are likely to play a role in Selenastracean losses (Kozik et al., 2019), this study supports evidence of a link between rapidly changing environmental conditions and PCD-related responses. Most importantly, our results revealed specifically markers and morphotypes to be considered in PCD studies in Selenastraceae. And that there might be an interplay between autophagic and apoptotic-like markers in unicells depending on the stimuli. Therefore, these markers and their possible ecological implications should be taken into account in further evolutionary ecological studies of this family of chlorophytes.

Moreover, we have presented evidence of an autophagic/vacuolar-like cell mode of death in chlorophytes, that is also a PCD mechanism in their Streptophyta descendants, including land plants. This is an important finding because it indicates that

this form of PDC existed in single-celled phytoplanktonic organisms before land plants evolved, and might have been an important development for tissue homeostasis in higher multicellular plants.

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Author contributions

MMBF, ILB & PMD: conceived the original ideas, designed the study; MMBF: Culture experiments, data analysis, drafting and editing manuscript; ILB: Main supervisor, data

analysis, drafting and editing manuscript; PMD: Co-supervisor, data analysis, drafting and editing manuscript; KVN: culture experiments, flow cytometry; NEA: culture experiments; AJ: Transmission electron microscopy; HS: flow cytometric analysis.

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Figure legends

Fig. 1. PCD and cell Viability Assessment on *Ankistrodesmus densus*. (A) Proportion of cells positive (log transformed) for Annexin V FITC in the Control, Darkness, Nitrogen starvation, Heat and Cold treatments. (B) Proportion of cells with permeabilized membranes (log transformed) for the same stress stimuli. Asterisk (*) marked values = statistically significant ($p < 0,005$, n=3).

Fig. 2. PCD flow cytometric detection in *A. densus*. (a) to (e) are dot-plots of samples (10.000 cells each), in triplicates, of the control, darkness, nitrogen starvation, heat and cold treatments, respectively. The axes x represents the FITC fluorescence and the axes y describes the PI fluorescence. Q1= represents necrotic cells (PI positive and FITC negative cells); Q2=represents late PCD cells (FITC and PI positive cells); Q3= represents early PCD cells (FITC positive and PI negative cells) and Q4= represents healthy cells (FITC and PI negative cells). (f) comprised the overlapped histograms of the control, darkness and nitrogen starvation treatments while (g) represents the overlapping of the control, heat and cold shock treatments histograms graphs. (h) displays the average percentage value and the standard deviation found on each of the four quadrants (Q1, Q2, Q3 and Q4) for each of the four environmental stimuli. Asterisk (*) marked values = statistically significant ($p < 0,005$, n = 3).

Figs. 3-22. Representative transmission electron micrographs revealing the ultrastrutural changes in *A. densus* exposed to different stress stimuli. Nu, nucleus; No, nucleolus; Mtc, mitochondria; Cw, cell wall; Cyt, cytoplasm; Clh, chloroplast; Thy, thylakoid; Vc, Vacuole; Chm, Chromatin; Pm, Plasm membrane; Pyk, Pyknosis; Gc, golgi complex. The morphological alterations described in the text are indicated by the numbered arrows. (Figs 3-6) Normal vegetative cells growing on WC complete medium under regular light and temperature conditions. Note the cytoplasm without vacuolization; (Figs 7-10) Cells after 4 days in complete darkness. Note the extensive cytoplasmic vacuolization (arrow 1), mitochondrial elongation (arrow 2), and pyknosis without chromatin marginalization (arrow 3); (Figs 11-14). Cells after 4 days under nitrogen starvation. Cells experienced vacuolization (arrows 1) followed by chloroplast alterations (arrows 3). Note arrows 2 on Fig. 11 which might be highly disorganized thylakoids; also mitochondria did not elongate (number 4). In addition, note the nucleolar condensation and the movement of the chromatin to the nuclear membrane (arrows 5) with ultimate marginalization (arrows 6); (Figs 15-18). Cells after exposure to 44°C for 1 hour and returned to standard conditions for 12 hours. Note the shrunken, damaged cytoplasm (arrows 1), possible membrane disruption (arrows 2), altered thylakoids (arrow 3) and balloon shaped mitochondria (arrow 4); (Figs 19-22). Cells after exposition to cold shock (0 °C) for 3 hours and returned to regular growth conditions for 12 hours. Overall, cells were not quite affected. Note, however, some chromatin condensation (arrow 1), slightly dilated mitochondria (arrows 2) and

protoplasm shrinkage (arrow 3). Thylakoids showed also slight disorganization (arrow 4). Scale bars: Figs 3-13 = 0.2 µm; Fig. 14 = 0.1 µm; Figs 15, 18, 20, 21 = 2 µm; Figs 16, 17, 19, 22= 5 µm.

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Fig. 1.

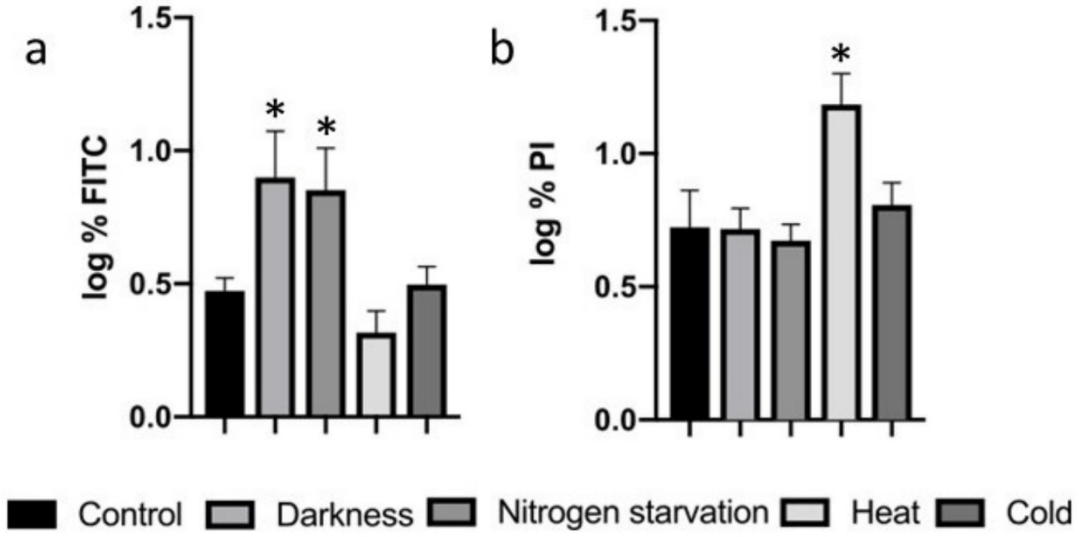
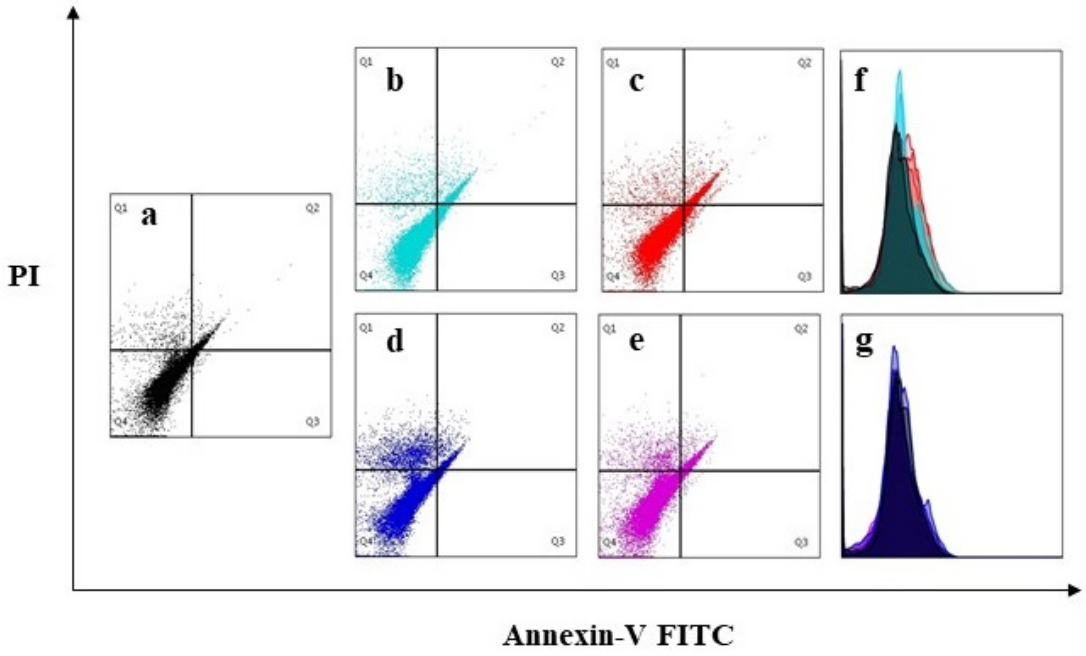
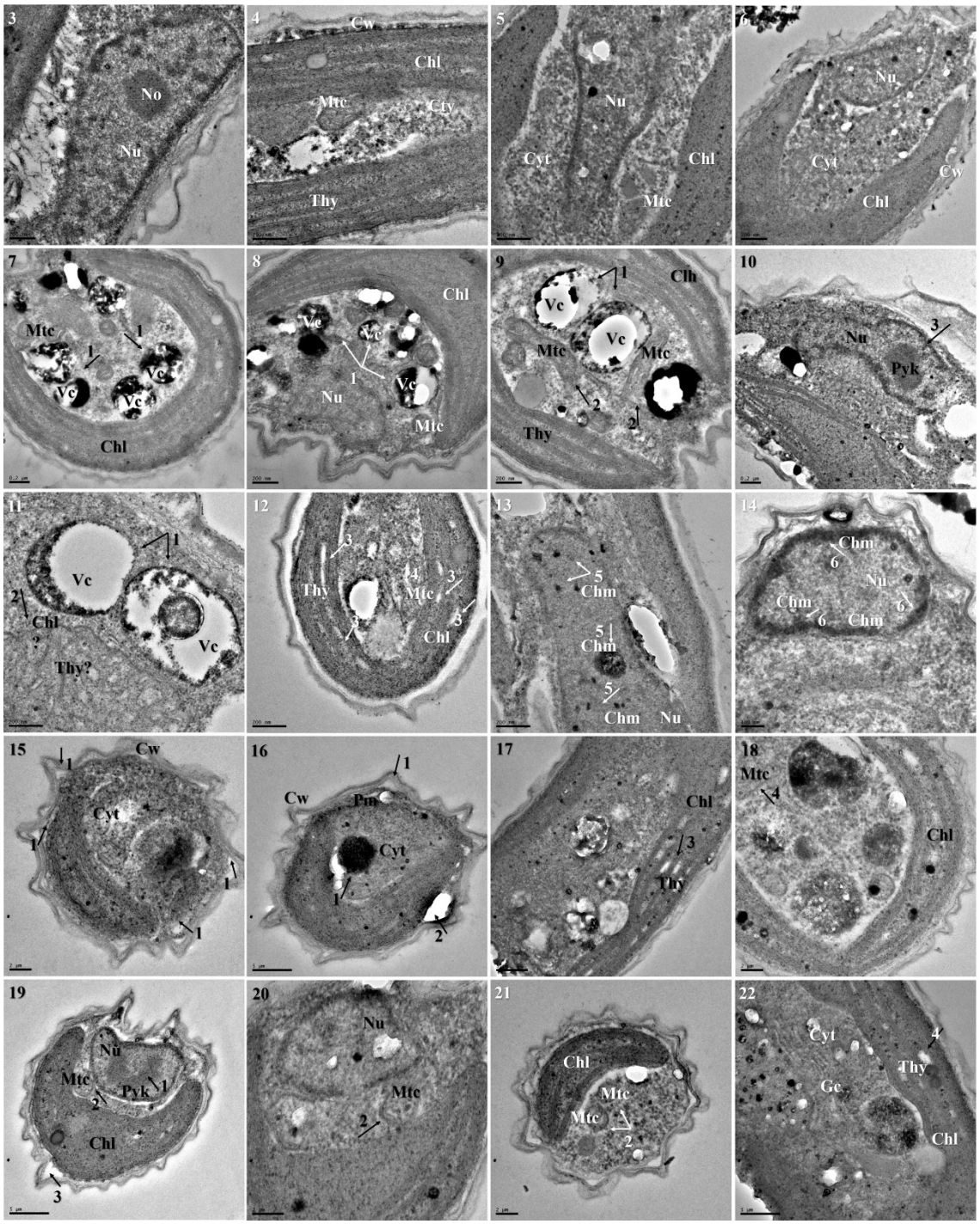


Fig. 2.



h	Control ■	Darkness ■	Nitrogen starvation ■	Heat ■	Cold ■
Q1	5.4 ± 1.7	5.2 ± 1.0	4.7 ± 0.7	15.6 ± 4.0*	6.4 ± 1.3
Q2	4.4 ± 0.9	5.4 ± 3.3	8.2 ± 1.7	4.2 ± 1.4	6.1 ± 2.3
Q3	2.9 ± 0.3	8.4 ± 3.5*	7.4 ± 2.6*	2.1 ± 0.4	3.1 ± 0.5
Q4	87.1 ± 2.2	80.9 ± 7.8	79.5 ± 4.4	77.9 ± 3.8	84.2 ± 1.9

Figs. 3 – 22.


3
No
Nu
4
Cw
Chl
Mtc
Cty
Thy
5
Nu
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Vc
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8
Chl
Vc
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Clh
Vc
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Pyk
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Vc
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Thy?
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Thy
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Discussão

A documentação de fenótipos relacionados à MCP e dos indutores ambientais associados ao processo devem ser um pré-requisito à realização de estudos sobre o papel ecológico da MCP na evolução do fitoplâncton eucariótico. Diante do cenário complexo de se realizar tal medição, porém, é necessária a definição dos métodos e marcadores que serão utilizados, como etapa anterior ao estudo de documentação. No capítulo 1, nós realizamos uma revisão dos diferentes ensaios e marcadores de MCP no fitoplâncton e fizemos sugestões de como o processo deve ser mensurado, baseado numa análise de significância e frequência dos principais marcadores encontrados, especialmente em Chlorophyta, divisão a qual pertence a microalga *A. densus,* foco do nosso estudo no capítulo 2. Três marcadores sugeridos no capítulo 1 foram utilizados para a realização do estudo de documentação de MCP em *A. densus.* Os resultados e conclusões apresentados servirão como base para futuros estudos de ecologia evolutiva em Selenastraceae.

Os marcadores emergem e podem ser usados para medir MCP com base nos mecanismos subjacentes (Fig. 1, Capítulo 1). Deve-se ter em mente, no entanto, que nem todos os marcadores serão positivos para um táxon em cada cenário de ocorrência de MCP. Além disso, diferentes marcadores podem ser observados entre os táxons, mesmo quando o mesmo estímulo é usado. Estes marcadores podem ser agrupados no fitoplâncton em: alterações ultraestruturais, degradação do DNA, perda da assimetria da membrana plasmática, atividade de enzimas semelhantes as caspases, atividades de genes ou proteínas metacaspases, produção de espécies reativas de oxigênio e nitrogênio, regulação positiva de genes associados à MCP, e alterações bioquímicas mitocondriais e cloroplásticas (Capítulo 1, Tabela 1). Enquanto nem todos os mecanismos foram demonstrados para todos os taxa, é possível que os caminhos moleculares possam ser compartilhados dentro do grupo taxonômico de organismos fitoplanctônicos unicelulares, e assim podem prover um modelo-conceito pelo qual hipóteses possam testadas (BIDLE,

2016). No entanto, de acordo com nossos dados, somente a atividade de caspases e ROS foram compartilhados por todos os grupos. Por outro lado, DNA laddering não foi encontrado em diatomáceas, enquanto que a perda de assimetria da membrana não foi descrita em dinoflagelados. Embora conclusões definitivas não possam ser atribuídas a esses dados, eles indicam que ROS e caspases são comuns nesses organismos sob estresse, e mesmo que amplamente utilizados, talvez estes marcadores não sejam tão específicos para MCP nesses organismos.

Não existe, de fato, um conjunto de métodos e marcadores especifico para se medir a MCP no fitoplâncton, mas a adoção de uma estratégia de medição, baseada na significância dos métodos e marcadores (Tabela 1, Capítulo 1), pode ser útil na escolha de marcadores mais adequados para se medir a MCP no fitoplâncton. A interpretação e significância dos métodos e marcadores de MCP devem se basear em dois importantes critérios: sensibilidade e especificidade (Durand, 2020). Com base nestes, é possível classificar funcionalmente os marcadores de MCP em sinais rígidos (hard) e suaves (soft), e padrão ouro (gold standards). Sinais "rígidos" de MCP são marcadores muito específicos, mas que nem sempre são sensíveis (e.g. DNA laddering). Isso significa que o diagnóstico de MCP não é excluído se o resultado for negativo, por exemplo. Os sinais "suaves", por outro lado, incluem um conjunto de marcadores que são sensíveis à detecção, mas que não são exclusivos da MCP (e.g. ROS, caspases). Em adição, nós também realizamos uma análise da frequência de detecção e eficiência de detecção com base em uma seleção de 27 estudos de documentação de MCP em microalgas. A frequência de deteção mede quantas vezes o método foi usado para documentação da MCP, enquanto que a eficiência de deteção considera também resultados negativos, em que o método foi usado mas não foi detectado. Aqui, para a documentação de MCP em *A. densus*, dois sinais rígidos, e um padrão ouro foram utilizados: a externalização da

fosfatidilserina (PS) em conjunto com a retenção da integridade da membrana, observação de alterações morfológicas ultra-estruturais e a degradação do DNA.

Geralmente, ensaios bioquímicos como aqueles que medem a atividade de caspases, metacaspases e de espécies reativas de oxigênio (ROS) são agrupados na categoria "soft signs". A razão para isso é estes ensaios podem apresentar resultados positivos falsos, na medida em que podem estar relacionados a outros fatores como por exemplo, a comunicação celular e formação de biofilmes (VARDI, 2008), encistamento (VARDI *et al.*, 2007) e reprodução sexual (NEDELCU; MARCU; MICHOD, 2004). O exemplo mais evidente disso são ensaios que detectam a atividade de caspases. Nossos resultados indicam que estes ensaios são os mais utilizados para se detectar a MCP (48%) (junto com modificações ultraestruturais), e também apresentam uma alta frequência de detecção (por volta de 90%). No entanto, microalgas não apresentam caspases verdadeiras e sim metacaspases. Apesar das duas classes representarem proteases, metacaspases são homólogos distantes de caspases e não apresentam descendência comum (genes ortologos). Dessa forma, ensaios que detectam caspases não são completamente específicos para MCP no fitoplancton, uma vez que não se sabe ao certo o que está sendo medido, e não podem ser utilizados por si só para conclusões definitivas. Assim, nós sugerimos que os marcadores selecionados, dependerão do objetivo do estudo. Se o objetivo é documentar a PCD pela primeira vez, recomendamos o uso de mudanças ultra-estruturais em combinação com outros sinais rígidos como a fragmentação do DNA e externalização da fosfatidilserina. Por outro lado, se a MCP já tenha sido demonstrada por microscopia eletrónica acompanhada de outros sinais rígidos, e o objetivo da pesquisa é outro além da simples documentação (e.g. teste de hipóteses referentes ao papel ecológico da MCP), é aceitável o uso sinais suaves que são conhecidos por serem positivos sob condições específicas

Após a definição dos marcadores, as respostas relacionadas à morte frente a três estresses ecologicamente importantes - mudanças de luminosidade, temperatura e flutuações nos macronutrientes - foram documentadas. Diante do fato de que os fenótipos de MCP podem ser muito sútis, nós utilizamos condições extremas de escuridão total, privação de nitrogênio, choque de calor e frio numa tentativa de destacar os morfotipos relacionados à morte. As culturas axênicas de *A. densus* foram então expostas as condições descritas acima, de acordo com uma metodologia específica detalhada no capítulo 2 seguida pela documentação imediata dos marcadores de MCP acima definidos.

Os marcadores foram documentados pelos seguintes métodos: 1) detecção da externalização de PS e verificação do estado de integridade das membranas, que foram detectados usando citometria de fluxo em conjunto com um kit de detecção de Apoptose (Sigma). No início da MCP, a integridade da membrana é mantida, mas PS, que é geralmente encontrada na superfície citoplasmática, é translocado ao lado externo da membrana. O corante Anexina V-FITC (ou AV-FITC) é capaz de se ligar a PS quando esta é externalizada. Por outro lado, a integridade da membrana foi verificada utilizando o iodeto de potássio (ou PI), que é capaz de se ligar ao DNA quando ocorrer a ruptura da membrana, que ocorre durante as fases finais da MCP ou durante a morte incidental (ou necrótica). Células necrosadas por outros meios que não MCP são AV-FITC - e PI+, enquanto células induzidas a MCP são positivas para ambos ou somente para AV-FITC; 2) documentação de alterações morfológicas ultra-estruturais relacionadas a MCP, realizada células fixadas imediatamente após o período indutivo, e analisado posteriormente sob microscopia eletrônica de transmissão; 3) análise do estado do DNA das culturas, realizada por extração do DNA genômico seguido por uma eletroforese de gel.

Nossos resultados sugerem que os tratamentos de escuridão total e privação de nitrogênio podem induzir fenótipos relacionados à MCP em *A. densus*. A existência da alta vacuolização citoplasmática em conjunto com outras mudanças ultra-estruturais específicas (Capítulo 2, Fig. 3) indicam a morte celular autofágica, que é um tipo especial de MCP. Embora a autofagia possa estar relacionada à sobrevivência sob condições de limitação de nutrientes (DAS; SHRAVAGE; BAEHRECKE, 2012; MIZUSHIMA; KOMATSU, 2011), os resultados significativos para a externalização de PS, que é conhecido como um marcador apoptótico (Capítulo 2, Figs. 1 e 2), entre outras alterações ultra-estruturais apoptóticas clássicas (especificamente no tratamento de depleção de nitrogénio) são achados interessantes. Esses resultados indicam que pode haver uma interação entre marcadores autofágicos e apoptóticos, dependendo dos estímulos e do ambiente (BURSCH, 2001; BURSCH *et al.*, 2006; LOCKSHIN; ZAKERI, 2004).

Em plantas superiores, a autofagia é um mecanismo ativado durante a "morte celular do tipo vacuolar", considerado um dos dois principais tipos de morte celular em plantas junto com a necrose (MININA; SMERTENKO; BOZHKOV, 2014; VAN DOORN *et al.*, 2011). Esse modo de MCP pode ter surgido nas plantas como uma alternativa a apoptose, devido às rígidas paredes celulares, que impedem a fragmentação das células e também devido ausência de macrófagos (VAN DOORN *et al.*, 2011). Durante o processo, a degradação do conteúdo celular por vacúolos citoplasmáticos líticos (VAN DOORN *et al.*, 2011), contrasta fundamentalmente do inchaço geral e ruptura precoce da membrana plasmática que ocorre durante a necrose (KROEMER *et al.*, 2009). As algas verdes compreendem duas linhagens relacionadas: Chlorophyta, que inclui *A. densus*, e Streptophyta, que inclui algas (“Charophyta”) e plantas (Embryophyta) (LELIAERT *et al.*, 2012; LEWIS; MCCOURT, 2004). Embora o ancestral das plantas esteja dentro de Streptophyta (LELIAERT *et al.*, 2012), nós sugerimos o modo vacuolar

de morte possa ter evoluído mais cedo na linhagem verde. Evidência que corrobora esta hipótese, foi encontrada em *Micrasterias denticulata,* uma microalga Streptophyta que pode morrer por MCP autofágica sob estresse salino (AFFENZELLER *et al.*, 2009). Dessa forma, autofagia parece ser um importante mecanismo de MCP nas microalgas verdes de parede celular, semelhante ao processo que ocorre em plantas (MININA; SMERTENKO; BOZHKOV, 2014; VAN DOORN *et al.*, 2011).

Além disso, a vacuolização citoplasmática relatada nestes tratamentos pode desempenhar um papel semelhante ao que é observado durante MCP autofágica em Streptophyta, incluindo plantas. Em plantas, por exemplo, vacúolos associados à senescência (SAVs) são responsáveis pela reciclagem de nutrientes via degradação de proteínas do cloroplasto para fornecer nitrogênio a outras partes da planta (COSTA *et al.*, 2013; ISHIDA *et al.*, 2014; JONES, 2001). De forma similar, os vacúolos associados a degradadação de proteínas plastídicas em *A. densus*, sob condições de depleção de nitrogênio, podem desempenhar um papel na sobrevivência de parentes. Esta hipótese, no entanto, precisaria ser testada em experimentos futuros.

Neste estudo, evidências de diferentes modos de morte celular foram apresentadas em *A. densus* em resposta a diferentes fatores ambientais extremos. No entanto, somente os estímulos de escuridão e provação de nitrogênio induziram respostas que puderam ser relacionadas à MCP. Tais respostas revelaram marcadores autofágicos e apoptóticos, sendo que pode haver também uma interação entre estes, dependendo dos estímulos. Em conclusão, os marcadores e fenótipos de MCP discutidos neste trabalho e suas possíveis implicações ecológicas fornecem informações importantes para estudos ecológicos e/ou evolutivos adicionais em Selenastraceae. Além disso, nós também apresentamos evidencia de um modo de morte celular autofágico/vacuolar em clorófitos, que também é um mecanismo de PCD em Streptophyta, incluindo plantas terrestres. Este também é

um achado importante, pois indica que essa forma de MCP existia em organismos microalgas unicelulares antes da evolução das plantas terrestres e pode ter tido um papel importante no desenvolvimento da homeostase tecidual de plantas multicelulares.

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