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# Genotypic and phenotypic variation in the astaxanthin producing green algal genus *Haematococcus*

Promotor: Prof. Dr. Wim Vyverman
Co-promotor: Dr. Pieter Vanormelingen

Academic year 2016-2017

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CÉLINE C. ALLEWAERT

**Dutch translation of the title**

Genotypische en fenotypische variatie in het astaxanthine producerende groenwier-genus *Haematococcus*

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**Ghent University - Faculty of Sciences**

**Department of Biology**

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**Promotor: Prof. Dr. Wim Vyverman**
**Co-promotor: Dr. Pieter Vanormelingen**

***Thesis submitted in fulfillment of the requirements for the degree of Doctor in Science - Biology***

**Academic year 2016-2017**

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## Notation index

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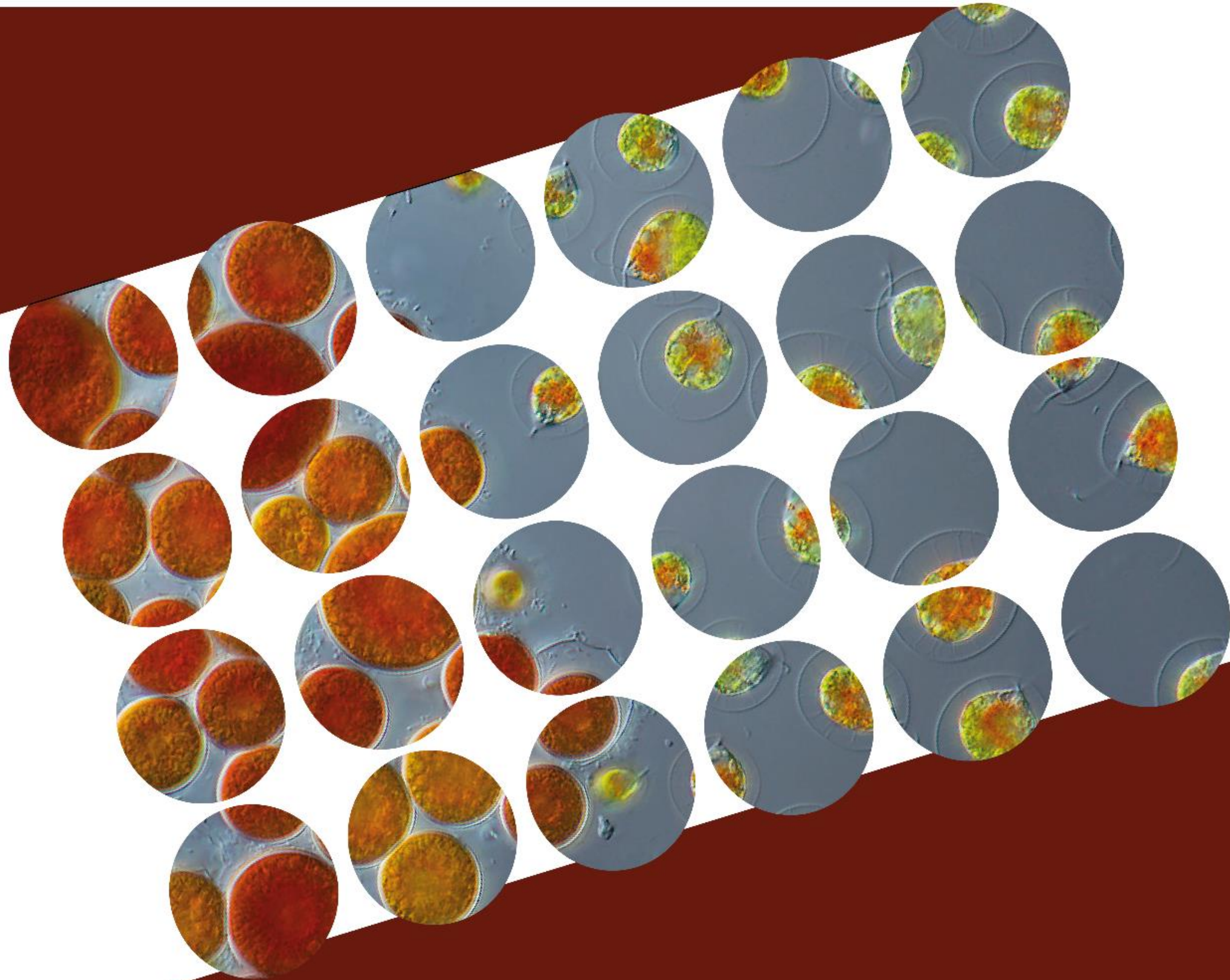
| | |
|---|---|
| **18S** | Small subunit of the ribosomal operon |
| **26S** | Large subunit of the ribosomal operon |
| **5.8S rDNA** | Ribosomal DNA located between the SSU and LSU rDNA |
| **AIC** | Akaike Information Criterion |
| **ANOVA** | Analysis of variance |
| **BI** | Bayesian Inference |
| **BBM** | Bold Basal Medium |
| **BBM-3N** | Bold Basal Medium enriched with 3 fold the nitrate concentration |
| **BOLD** | Barcode of Life Data System |
| **bp** | Base pair(s) |
| **CBC** | Compensatory Base Change |
| **CCAP** | Culture Collection of Algae and Protozoa |
| **DGGE** | Denaturing Gradient Gel Electrophoresis |
| **DMSO** | Dimethylsulfoxide |
| **DNA** | Deoxyribonucleic Acid |
| **DW** | Dry Weight |
| **EFSA** | European Food Safety Authority |
| **FDA** | Food and Drug Administration |
| **G x E** | Genotype - Environment interaction |
| **GMYC** | Generalized Mixed Yule Coalescent method |
| **GWAS** | Genome-Wide Association Study |
| **HPLC** | High Performance Liquid Chromatography |
| **ISSR** | Inter Simple Sequence Repeat |
| **ITS** | Internal Transcribed Spacer |
| **ITS-1** | Internal Transcribed Spacer region 1 located between SSU and 5.8S rDNA |
| **ITS-2** | Internal Transcribed Spacer region 2 located between SSU and 5.8S rDNA |
| **LSU rDNA** | Large Subunit of the ribosomal operon |
| **ML** | Maximum Likelihood |
| **NIES** | Culture Collection at the National Institute for Environmental Studies |
| **OFAT** | One-Factor-at-A-Time approach |
| ***P*** | Statistical p-value obtained |

| | |
|---|---|
| **PAM** | Pulse Amplitude Modulation |
| **PCA** | Principal Component Analysis |
| **PDA** | Photodiode array detector |
| ***pds*** | Gene Phytoene Desaturase |
| **PCR** | Polymerase Chain Reaction |
| **PS** | *Paraphysoderma sedebokerense* |
| **PSII** | Photosystem II (or water-plastoquinone oxidoreductase) |
| **QTL** | Quantitative Trait Loci |
| **RAPD** | Random Amplified Polymorphic DNA |
| ***rbc*L** | RuBisCO large subunit |
| **rDNA** | Ribosomal DNA |
| **ROS** | Reactive Oxygen Species |
| $R_f$ | Response factor |
| **SAG** | Sammlung von Algenkulturen der Universität Göttingen (Culture Collection of Algae at Göttingen University) |
| **SCCAP** | Scandinavian Culture Collection of Algae & Protozoa |
| **SD** | Standard Deviation |
| **SNV** | Single Nucleotide Variant |
| **SSU rDNA** | Small Subunit of the ribosomal operon |
| **tufA** | Translation Unstable Factor |
| **UTEX** | Culture collection of algae at the University of Texas at Austin |
| **WGA** | Wheat Germ Agglutinin, Lectin from *Triticum vulgaris* (wheat) |



# SAMENVATTING

Deze studie beoogt om tot een beter begrip te komen van de genotypische en fenotypische variatie in de astaxanthine producerende groenalg *Haematococcus pluvialis*. We wilden meer bepaald 1) onderzoeken of *H. pluvialis* één soort is of uit meerdere soorten bestaat door het analyseren van variatiepatronen in moleculaire merkers, morfologische kenmerken en ecofysiologische voorkeuren, 2) de genetisch gebaseerde component van fenotypische variatie op inter- en intraspecifiek niveau kwantificeren bij *Haematococcus* met nadruk op kenmerken gerelateerd aan de astaxanthine productiviteit, 3) ons een beeld vormen over de manier waarop responsen van verschillende stammen behorend tot verschillende genotypes kunnen uiteenlopen bij milieuveranderingen, meer bepaald bij variërende lichtintensiteit, 4) de gastheer-specificiteit van *Paraphysoderma sedebokerense*, een courant pathogeen van *H. pluvialis* beter begrijpen.

Het eerste deel van dit proefschrift beoogde het genereren van een fylogenetisch kader voor verder vergelijkend onderzoek.

In **Hoofdstuk 3** werden genetische, morfologische en ecofysiologische patronen bestudeerd bij Europese *Haematococcus* stammen geïsoleerd uit 15 locaties verspreid over Europa om de mogelijke soortsniveau variatie te evalueren. Ter vergelijking werd de Europese verzameling aangevuld met geïsoleerde stammen uit Zuid-Amerika en stammen uit cultuurcollecties. De genetische differentiatie van de stammen werd onderzocht op basis van de volledige ITS[1] rDNA regio (ITS1-5.8S-ITS2) en voor een selectie stammen ook het *rbc*L[2] chloroplast gen. De aanwezigheid van CBCs[3] in ITS1 en ITS2 werd ook geëvalueerd. Fylogenetische analyse resulteerde in zes duidelijke ITS clades - onderbouwd door zowel GMYC[4] als statistische parsimonie netwerk analyse[5] – waarvan er drie alle Europese stammen bevatten en het grootste deel van de cultuurcollectiestammen. De drie bijkomende clades aanwezig in *Haematococcus,* worden voorlopig *Haematococcus sp.* genoemd totdat hun taxonomie verder wordt uitgezocht. De *rbc*L groep vertoonde veel minder variatie dan de ITS, maar onderscheidde wel twee van de drie ITS groepen waar onze Europese stammen toe behoorden. We hebben drie CBCs gevonden bij elk van de drie Europese clades in de ITS1 secondaire structuur, terwijl er één CBC werd geïdentificeerd bij clades in de ITS2 secondaire structuur. Samen leidde deze resultaten tot het identificeren van drie soorten *Haematococcus:* een epitype werd voorgesteld voor *H. pluvialis* en we beschreven twee nieuwe soorten: *H. rubicundus* en *H. rubens*.

[1] **ITS** Internal transcribed spacer, ITS regio's uit het ribosomale cistron.
[2] ***rbc*L** ribulose 1-2 bi-phosphate carboxylase Large subunit gene, chloroplast DNA.
[3] **CBC** Compensatory Base Change, dubbelzijdige verandering van een nucleotide paar in een helix die deel uitmaakt van de secondaire structuur van het ITS.
[4] **GMYC** Generalized Mixed Yule Coalescent method, statistische soortsafbakenings methode gebaseerd op het verkiezen van best passende vertakingsmodellen voor binnen en tussen soorten variatie om fylogenetische stambomen te reconstrueren.
[5] **Statistical parsimony network analysis**, afbakening van soorten gebaseerd op het aansluiten van de meest nauw verwante knoopunten in een fylogenetische boom tot de maximale"parsimony" is bereikt.

Ook werd DGGE[6] aangewend om deze drie soorten te onderscheiden zonder te moeten sequeneren. De aanwezigheid van bijkomende variatie in *Haematococcus* laat ons vermoeden dat verdere cryptische soortdiversiteit bestaat. We hebben verder de congruentie met morfologie en temperatuurvoorkeur bepaald voor een selectie stammen die deel uitmaakten van de drie beschreven soorten. Ondanks een grote intraspecifieke variatie en aanzienlijke overlap tussen kenmerken, vonden we significante verschillen in morfologie en groeisnelheid tussen de soorten, hetgeen onze soortafbakening ondersteunde. *H. pluvialis* en *H. rubens* hadden gemiddeld meer langwerpige cellen, duidelijkere cytoplasmatische strengen en peervormige protoplasten. De optimale groeitemperaturen waren gelijkaardig voor *H. pluvialis*, *H. rubicundus* en *H. rubens*, nl. variërend tussen 17 en 23 °C, maar *H. pluvialis* vertoonde gemiddeld een lagere maximale groeisnelheid dan *H. rubicundus* en *H. rubens*.

In **Hoofdstuk 4** werd de genetisch gebaseerde component van **fenotypische variatie** gekwantificeerd in een “common garden” experiment, waarbij de zes volgende fenotypische kenmerken van genetisch verschillende stammen werden vergeleken onder strikt identieke omgevingsomstandigheden: groeisnelheid, stationaire fase densiteit, percentage palmelloide cellen, post stress droog gewicht, astaxanthine inhoud en aplanospore biovolume. De verschillen in fenotype die overblijven wanneer alle omgevingsvariatie uitgeschakeld wordt, zijn dan te wijten aan genetische verschillen. Via deze methode hebben we de genetisch gebaseerde intra- en interspecifieke (co-) variatie onderzocht bij zes kenmerken gerelateerd aan astaxanthine productiviteit. Hiervoor werd een totaal van 30 stammen behorend tot *H. pluvialis* en *H. rubicundus* onderzocht. Naast 24 eigen stammen werden ook zes *H. pluvialis* stammen uit cultuurcollecties gebruikt. Dit gebeurde onder (pre-stress) groeivoorwaarden en onder voorwaarden waarbij astaxanthine productie werd opgewekt (post-stress). We vonden een significant reservoir van intraspecifieke variatie in alle zes kenmerken, bepaald door hoge heritabiliteit[7], wat de genetisch achtergrond van deze kenmerken bevestigd. Ondanks een significante overlap van soorten voor de onderzochte kenmerken (zowel pre- als post-stress) vonden we soortverschillen in vijf van de zes kenmerken. Tussen de slechtst en de best presterende stammen vonden we zelfs tot een vijftienvoudige factor aan variatie in astaxanthine productiviteit. Het was opvallend dat de stammen van de cultuurcollecties een lagere astaxanthine productiviteit hadden vergeleken met het recent geïsoleerde materiaal behorend tot *H. pluvialis,* hetgeen waarschijnlijk een verlies van fotoprotectieve capaciteit weerspiegelde gedurende hun langdurige bewaring in cultuur.

[6] **DGGE** Denaturing Gradient Gel Electrophoresis elektroforetische scheidingstechniek om dubbelstrengige DNA fragmenten te scheiden van elkaar.

[7] De **heritabiliteit** of erfelijkheidsgraad van een kenmerk in een bepaalde populatie ($H^2$) geeft weer welk gedeelte van de totale fenotypische variatie verklaard wordt door genetische verschillen.

De variatie in totale astaxanthine productiviteit in het algemeen hing voornamelijk af van de post-stress kenmerken, eerder dan de pre-stress kenmerken; focus voor verbetering tijdens astaxanthine productie.

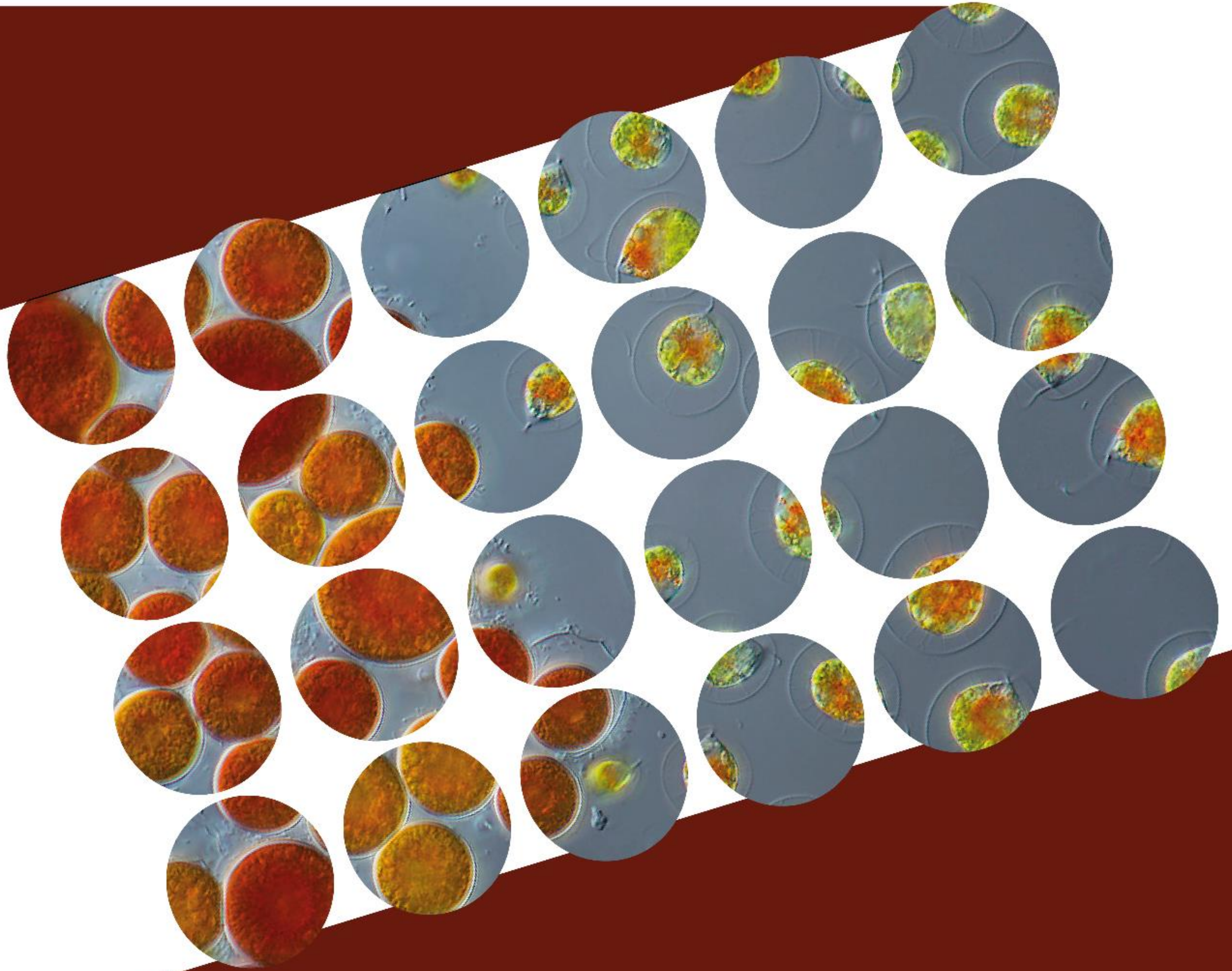
In **Hoofdstuk 5**, bestudeerden we de **ecofysiologische reacties** van verschillende stammen (behorend tot verschillende genotypes) op lichtintensiteit gedurende de groeifase. De reacties van zes verschillende *Haematococcus* stammen behorend tot twee soorten (*H. pluvialis* en *H. rubicundus*), werden geëvalueerd bij blootstelling aan een lichtgradiënt bestaande uit negen verschillende intensiteiten (2,7,15,30,52,79,114,154 en 222 µmol fotonen $m^{-2}$ $s^{-1}$). Er werden systematische vergelijkingen gerealiseerd van batch gekweekte stationaire culturen van de zes stammen voor volgende kenmerken: celtype, celgrootte, mortaliteit, groeisnelheid, celdensiteit, fotosynthetische activiteit evenals kwantitatieve en kwalitatieve pigmentsamenstelling. Significante stam effecten werden gevonden voor alle kenmerken uitgezonderd groeisnelheid en celdensiteit. Verder had lichtintensiteit een significante invloed op alle kenmerken uitgezonderd biovolume. Bovendien vonden we significante lichtintensiteit x stameffecten, of G x E[8] (genotype x milieu) invloeden op cel mortaliteit, ratio $F_v/F_m$[9], celdensiteit alsook op pigmentsamenstelling.Dit houdt in dat het niet mogelijk was één uniek optimale lichtintensiteit aan te duiden die resulteerde in verhoogde groei en biomassa-accumulatie die geldig bleek voor alle onderzochte *Haematococcus* stammen.

In het laatste deel van dit proefschrift wilden we fenotypische variatie gebruiken om resistente stammen te zoeken bij *Haematococcus.* **Hoofdstuk 6**, concentreerde op het verband tussen de infectiviteit van het veelvoorkomend pathogeen (in grootschalige kwekerijen van *H. pluvialis*) *P. sedebokerense* en het gastheer genus *Haematococcus.* Kwantitatieve fenotypering werd aangewend om resistente *Haematococcus* stammen te identificeren. De gastheerspecificiteit van *P. sedebokerense* (stam PS1) werd namelijk *in vitro* onderzocht op 44 *Haematococcus* stammen in een gecontroleerde infectiviteitproef, waarbij de groei en de fotosynthetische activiteit werden gemeten in de aan- en afwezigheid van PS1. Bovendien hebben we op het einde van de test de aanwezigheid van PS1 in de gecontamineerde culturen gekwantificeerd met een hiervoor nieuw ontwikkelde methode, gebruik makend van een fluorescentie metingen na het binden van een *P. sedebokerense* specifieke kleurstof (WGA[10]-fluoresceïne). De resulterende metingen werden omgezet in drie infectivitieit-proxies, hetgeen vergelijkin gen onder stammen mogelijk maakte. Significante stam verschillen werden gevonden tussen alle 44 *Haematococcus* geteste stammen voor de drie onderzochte infectiviteit proxies.

[8] **G x E interactie** of genotype –milieu interactie duidt aan wanneer verschillende genotypes verschillen in hun respons ten opzichte van milieu veranderingen.
[9] $F_v/F_m$ is een maat voor het nuttig fotosynthethisch vermogen van microalgen. Hoe hoger $F_v/F_m$, hoe beter het fotosynthetisch vermogen (er is dan relatief weinig fluorescentie verlies).
[10] **WGA** "Wheat Germ Agglutinin" is een lectine dat bescherming biedt aan tarwe (*Triticum vulgaris*) tegen insecten, gisten en bacteriën.

We vonden geen correlatie tussen deze patronen en de fylogenetische achtergrond, noch met de oorsprong van de stam. De helft van de stammen die potentieel een lage gevoeligheid vertoonden voor PS1 hadden dominant geflagelleerde cellen, in tegenstelling tot de overige waar palmelloïde en aplanosporen domineerden. Op een analoge wijze konden we aantonen dat de kwetsbaarheid voor PS1 van een erg gevoelige *H. pluvialis* stam verminderde in een selectie-experiment op lange termijn door de overheersing van geflagelleerde fenotypes over verschillende generaties van infectie. Onze bevindingen toonden aan dat stamresistentie genetisch bepaald kan zijn, door onder andere verschillen in morfologie.

Samengevat dragen de resultaten van dit onderzoek bij tot het begrip van de genetische, morfologische en fysiologische diversiteit van *Haematococcus*, zowel op intraspecifiek als interspecifiek niveau. Deze studie draagt bij tot drie van de belangrijkste zuilen voor de ontwikkeling van microalgen (genetische diversiteit, aanpassing aan het milieu, fenotypische karakterisering) en viseert een bredere toegepaste context, waarbij de uiteindelijke bedoeling is hoogwaardige stammen te verkrijgen met een breed aanpassingsvermogen en een hoge weerstand tegen pathogenen.



# SUMMARY

The present study aimed to obtain a better understanding of the genotypic and phenotypic variation in the astaxanthin producing green algae *Haematococcus pluvialis*. More specifically, we wanted to find out 1) whether *H. pluvialis* is a single species or comprises multiple species by analyzing variation patterns in molecular markers, morphological characteristics and ecophysiological preferences, 2) to quantify the genetically based component of phenotypic variation at the inter- and intraspecific level in *Haematococcus* with a focus on traits related to astaxanthin productivity, 3) to obtain a first view on how responses of different strains belonging to different genotypes may diverge with environmental changes, specifically with varying irradiance 4) to better understand the host specificity of *P. sedebokerense,* a common pathogen of *H. pluvialis*.

The first part of this thesis was designed to generate a phylogenetic framework for further comparative studies.

In **Chapter 3,** genetic, morphological and ecophysiological patterns were studied in European *Haematococcus* strains isolated from 15 locations across Europe to assess potential species level variation. For comparison, the European strains collected were complemented with South American isolates and public culture collection strains. Genetic differentiation between strains was investigated using the complete ITS[1] rDNA region (ITS1-5.8S-ITS2) and for a subset of strains the *rbc*L[2] chloroplast gene. Also the presence of CBCs[3] in ITS1 and ITS2 secondary structures was evaluated. Six lineages could be resolved in the ITS rDNA phylogeny - supported by both GMYC[4] and statistical parsimony network analysis[5] - of which three contained all the newly collected European strains and most of the culture collection strains. The *rbc*L alignment showed less variation than the ITS, yet resolving two out of the three ITS lineages to which our European strains belonged. Up to three CBC's were found between each of the three European lineages in the ITS1 secondary structure, while one CBC was found between lineages in the ITS2 secondary structure. Combined, these results allowed the identification of three species of *Haematococcus:* an epitype for *H. pluvialis* was proposed and two new species were described: *H. rubicundus* and *H. rubens*. DGGE[6] was performed as a tool to distinguish the three species from each other, without the need to sequence. The presence of three additional lineages within *Haematococcus* suggest that further cryptic species diversity exists and remains to be explored.

[1] **ITS** nuclear Internal Transcribed Spacer region.
[2] ***rbc*L** ribulose 1-2 bi-phosphate carboxylase Large subunit gene of the chloroplast DNA.
[3] **CBC** Compensatory Base Change, a double-sided base change of a nucleotide pair in a helix, retaining the secondary structure.
[4] **GMYC** Generalized Mixed Yule Coalescent a model of a phylogenetic tree that separately considers branching within species (neutral coalescent model) and branching between species(Yule model)
[5] **Statistical parsimony network analysis**, method for delimiting species which separates groups of sequences into different networks if genotypes are connected by long branches that are affected by homoplasy.
[6] **DGGE** Denaturing Gradient Gel Electrophoresis a form of electrophoresis which uses a chemical gradient to denature samples moving across an acrylamide gel.

For a subset of strains belonging to the three described species, the further congruence with morphology and temperature preferences was determined.

Although showing a high degree of intraspecific variation and considerable overlap, significant interlineage differences in morphology and growth rate were found, supporting our species boundaries. *H. pluvialis* and *H. rubens* had on average more elongated cells, more noticeable cytoplasmic strands and pear shaped protoplasts. Optimal temperatures for growth were similar for *H. pluvialis*, *H. rubicundus* and *H. rubens*, varying between 17 and 23 °C, yet *H. pluvialis* had on average a lower maximal growth rate than *H. rubicundus* and *H. rubens*.

The second part of this thesis aimed at exploring the phenotypic diversity in *Haematococcus* in relation to its capacity to produce biomass and astaxanthin. This was done through the study of differences in physiological responses and the intraspecific variation between strains.

In **Chapter 4**, the genetically based component of **phenotypic variation** was quantified in a common garden experiment, where comparisons of phenotypic traits of genetically distinct strains were made under strict identical environmental conditions. Specifically, using this approach under growth conditions (pre-stress) and under astaxanthin inducing conditions (post-stress), we examined genetically based intra- and interspecific (co-) variation in six traits related to astaxanthin productivity using a total of 30 strains belonging to *H. pluvialis* and *H. rubicundus.* Besides including 24 newly isolated strains, six *H. pluvialis* strains from culture collections were examined. A significant reservoir of intraspecific variation was found for all six traits both pre- and post-stress, characterized by high broad sense heritability estimates[7], reinforcing the genetic determinism of this variation. Next, overlap between species was found in the six traits. Yet despite the overlap, species differences were found in five out of six traits. As much as fifteen fold variation in astaxanthin productivity was found between the poorest and the best performing strain. Strikingly, strains from culture collection had a lower astaxanthin productivity compared to natural isolates of *H. pluvialis*, possibly reflecting loss of photo protective capacity, during their long term maintenance. The variation in total astaxanthin productivity largely co-varied with post stress traits, rather than pre-stress traits, focus for future astaxanthin productivity improvement

In **Chapter 5**, we focused on the **ecophysiological responses** of different strains to irradiance during the growth phase. The responses of six different *Haematococcus* strains, belonging to two species (*H. pluvialis* and *H. rubicundus*) were assessed when exposed to a light gradient comprised of nine different irradiances (2, 7, 15, 30, 52, 79, 114, 154 and 222 µmol photons m$^{-}$

[7] **Broad sense heritability estimate** reflects all the genetic contributions to a population/species phenotypic variance.

$^{2}$ $s^{-1}$). Systematic comparisons of batch grown stationary cultures of the six strains were made in terms of cell type, cell size, mortality, growth rate, cell density, photosynthetic activity as well as quantitative and qualitative pigment composition. Significant strain differences were found for all traits with few exceptions, namely growth rate and cell density. Significant irradiance effects were found for all traits except cell biovolume. Moreover, irradiance x strain, or G x E interaction[8] effects were found for cell mortality, $F_v/F_m$[9] ratio, cell density as well as for pigment composition implying that is was not possible to denote a single optimum irradiance resulting in increased growth and biomass accumulation valid for all *Haematococcus.*

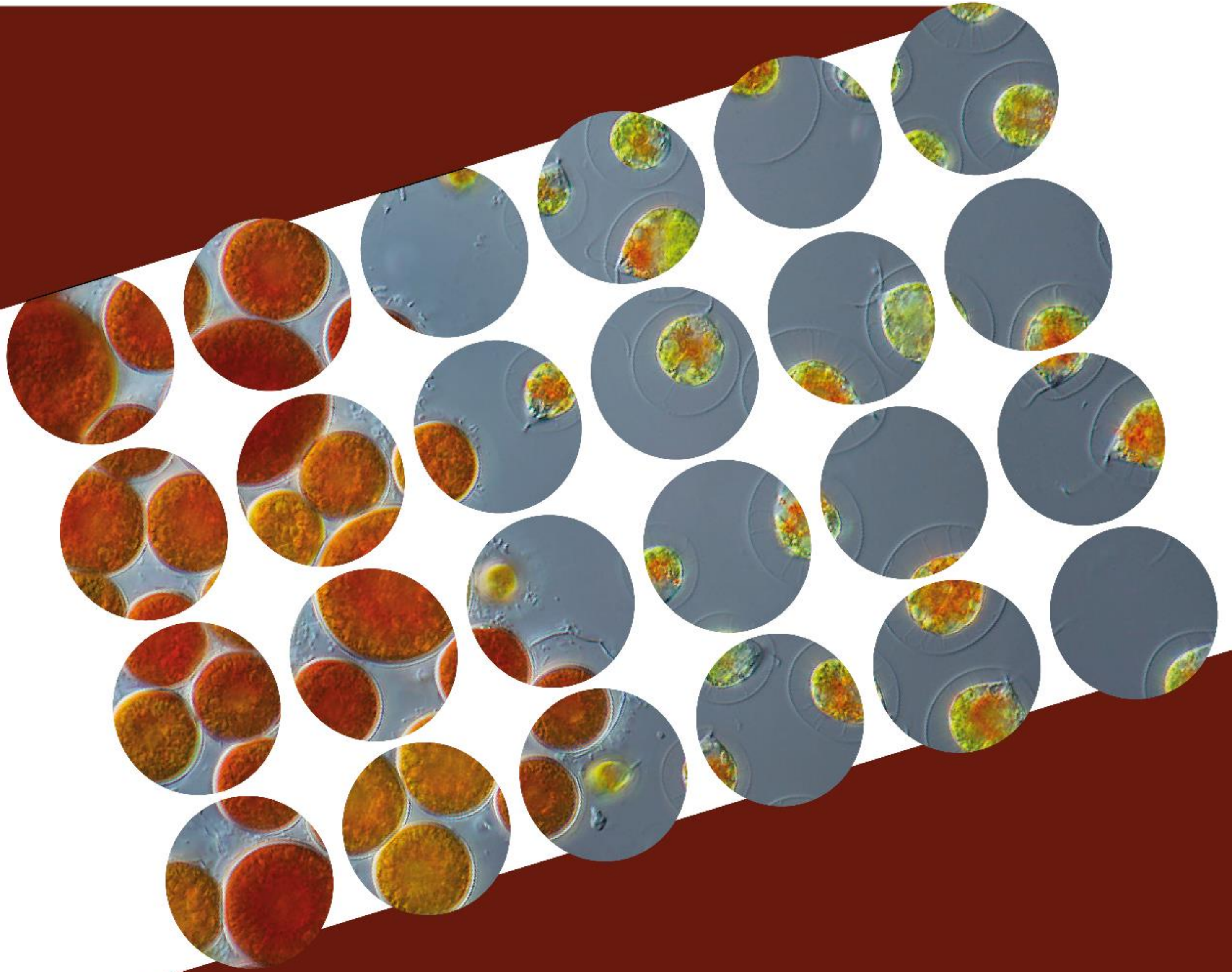
The final part of this thesis aimed at utilizing phenotypic variation in the search for resistant strains amongst *Haematococcus.*

**Chapter 6**, focused on the relation between the highly threatening pathogen *P. sedebokerense* and *Haematococcus,* through quantitative phenotyping to identify resistant *Haematococcus* strains. Specifically, the host specificity of *P. sedebokerense* (strain PS1) was examined on 44 *Haematococcus* strains in a laboratory controlled infectivity assay, where growth and photosynthetic activity was measured in presence and absence of PS1. Moreover, at the end of the trial, the presence of PS1 in infected cultures was quantified through a novel method developed for this purpose, by measuring the fluorescence intensity after staining with fluorescein labeled WGA[10], specific to *P. sedebokerense*. Altogether the measurements resulted in three infectivity proxies allowing comparative studies across strains. All 44 *Haematococcus* strains differed significantly in susceptibility to infection for all three infectivity proxies. Differences were was not related to phylogenetic background nor strain sampling origin. Half of the strains exhibiting potential low susceptibility to PS1, possessed cells in flagellated state, unlike the remaining which were palmelloid and aplanospore dominated. Correspondingly, we showed that vulnerability to PS1 of a highly susceptible *H. pluvialis* strain was decreased in a long term selection experiment through the dominance of flagellated phenotypes over several generations of infection. Our results demonstrated that strain resistance may be genetically determined and that the morphological flagellated state among others, may provide protection.

In conclusion, the results of this thesis contribute to the understanding of genetic, morphological and physiological diversity in *Haematococcus* strains both at the intraspecific as at the interspecific level. This work contributes to three of the main pillars for microalgae development (genetic diversity, environmental adaptation, phenotypic characterization)

[8] **G x E interaction** Gene/genotype–environment interaction is when two different genotypes respond to environmental variation in different ways.
[9] $F_v/F_m$ tests whether or not stress affects photosystem II in a dark adapted state.
[10] **WGA** Wheat germ agglutinin lectin that protects wheat (*Triticum vulgaris*) from insects, yeast and bacteria.

embodied within the applied pipeline, where the ultimate goal is to obtain high yielding strains with wide adaptability and high resilience to pathogens.



# CHAPTER 1:
# Preface

## 1.1. Problem statement

Microalgae in biotechnology are presently the focus of much interest and investment worldwide (Cadoret *et al.*, 2012). Microalgae offer complementary products (e.g. phycobilins) to land plants with the advantage of being easily manipulated, producible on non-arable land and the possibility to be harvested batch-wise or continuously almost all year round (Piganeau, 2012). Although only a fraction has been described to date, the biological diversity of microalgae provides an exceptional range of adaptability and represents a vast potential as sources of food, feed, biomaterials, original molecules and applications in the broad field of biotechnology (Georgianna & Mayfield, 2012; Ratha & Prasanna, 2012; Murray *et al.*, 2013).

Currently no more than 20 microalgae species from marine, estuarine and freshwater ecosystems are employed in biotechnology, representing just a minuscule fraction of the currently described species (Chu, 2012). While bioprospection or the search of new species and associated compounds is ongoing, parallel efforts are made to keep the production costs of current commercial species as low as possible. Although targeted selection and domestication as widely implemented by early farmers throughout history for improved agricultural crops are expected to develop in a near future (Georgianna & Mayfield, 2012), the improvement of species for increased yield and productivity is presently done either through manipulations of their genetic and cellular metabolism or through (so far prioritized) technological developments of production processes (Shurin *et al.,* 2014, Shurin *et al*., 2016). Typically, efforts to increase yields and productivity are based on research using single strains (Sayegh & Montagnes, 2011). Although rigorous studies on single strains are essential, a huge body of literature has shown that strains of phylogenetically distint algal species may considerably vary in a range of morphological, ecological and physiological traits (Lundholm *et al.*, 2006; Wilson *et al.*, 2006; Zhang *et al.*, 2014; Harvey *et al.*, 2015; John *et al.*, 2015; Malcom *et al.*, 2015). The broad congruence of this variation with environmental conditions on local, regional and global scales has fostered the concept that ecophysiological characteristics of microalgae are well adapted to their local growing conditions (Rynearson & Armbrust, 2004). Thus each habitat may contain locally adapted genotypes with different properties. The prospect of quantifying this phenotypic variation among genotypes of a species from different sources is hardly explored in microalgae biotechnology and generally, little is known on how species/strains belonging to one taxonomic group/species from different source locations vary in yield and productivity.

In this doctoral thesis, we compared strains from different origins belonging to the genus *Haematococcus* amongst which, the blood algae, *Haematococcus pluvialis* is a unicellular green alga and is the richest known source of the natural "super anti-oxidant" astaxanthin (Boussiba, 2000). *H. pluvialis* therefore represents an important natural source of this pigment which is frequently used in cosmetics, nutraceuticals and as animal feed (Lorenz & Cysewski, 2000). Additionally, the species is gaining importance as a model for carotenoid synthesis and accumulation studies (Shah *et al.*, 2016). Increasing the astaxanthin yield in *H. pluvialis* remains an active research target due to the many bottlenecks and challenges faced during cultivation. Although technological developments (ranging from cultivation, harvest and post-harvest bioprocessing) as well as metabolic control of astaxanthin biosynthesis and genetic engineering have been achieved over the years (Shah *et al.*, 2016), there are currently some gaps in fundamental knowledge on this species, particularly for the following points:

Firstly, relatively little is known on strain diversity at the phylogenetic level. Over the years, many strains with interesting properties have been described. These properties have not yet been coupled to molecular diversity. No complete study has been undertaken to resolve relationships among currently available strains (Klochkova *et al.*, 2013). Phylogenetic variation was already described in two studies among isolates of *H. pluvialis* (Noroozi *et al.*, 2011; Buchheim *et al.*, 2013). However, whether this variation is associated with undescribed species diversity remains unknown. Documenting this diversity is important in a biotechnology context since it opens opportunities to select strains with interesting properties, or strains that may be better adapted to local climate conditions.

Secondly, the lack of phylogenetic studies on *H. pluvialis* along with the generalization of findings obtained from experiments using single *Haematococcus* strains, result in difficulties in the interpretation of physiological responses to various environmental factors, nutrient requirements and productivity as these can be due to differences between strains. A random selection of 80 papers with keyword *"Haematococcus pluvialis"*, published between 2010 and 2016, showed that a majority (63 %) of the strains used were obtained from culture collections (SAG, SCCAP, UTEX, CCAP, FACB or NIES), while for roughly 37 % publications, the strain was either taken from a local culture collection or from the wild (=Others)(Fig. 1), with no actual control on strain identity and lack of molecular data to do so. Although this type of disparity is not uncommon in scientific research, it leads to a great deal of confusion when attempting to compare results for different strains/isolates between laboratories. Consequently,

we do not know which portion of the variation between strains is due to genetic variation. Quantifying these differences is essential since it may offer a potentially large reservoir of functional variation which may aid in further understanding the link between genes and functions. Moreover, many algal strains from culture collections have been propagated by continuous culture under specific selection conditions over long periods of time, rather than being cryopreserved. Continuous culture may result in adaptation to the selection conditions and the accumulation of mutations that can compromise the robustness or phenotypes of the original wild-type organisms and their performance. Whether newly established strains differ from long cultured strains has not yet been investigated for *H. pluvialis*.

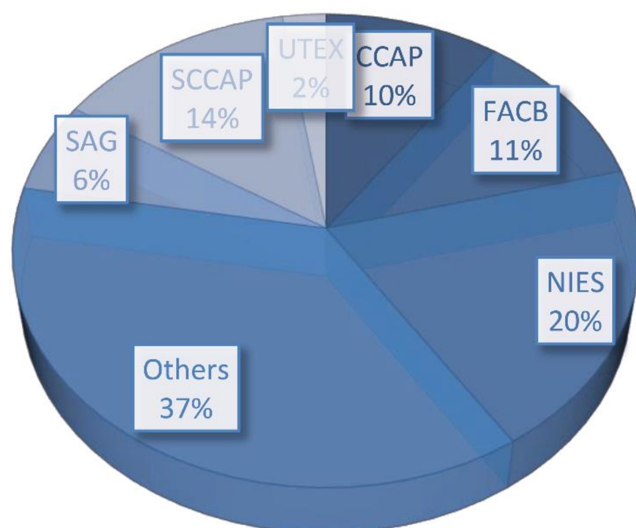



***Figure 1.*** *Frequency of different culture collections from which strains were obtained in a random selection of 80 publications on Haematococcus between 2010 and 2016.*

Thirdly, the physiology and biochemistry of *H. pluvialis* present some challenges, which make it an unique organism to study (Shah *et al.*, 2016). *H. pluvialis* is highly plastic throughout its life cycle (Hazen, 1899, Elliott, 1934, Kobayashi *et al.*, 1997b, Wayama *et al.*, 2013). While interesting life cycle changes associated with astaxanthin accumulation (Chapter 2), i.e. the transition to aplanospores, have been explored extensively, others are still under documented. In this respect, the transition from flagellated state to the palmelloid stage, accompanied principally by the loss of flagella and the formation of a tough cell wall, is highly interesting. Both flagellated and palmelloid cells possess properties that are important in the astaxanthin production process. Yet this transition is often overlooked in culture experiments impeding the interpretation of results. Although known to be strain specific (Han *et al.*, 2012), the triggers for the onset and reversion to palmelloid cells are not yet well described and necessitate careful monitoring.

Finally, contamination is a significant factor affecting production in any cultivation system. Since stringent sterilization is expensive, labor intensive and often hazardous to the environment, strains will need to have sufficient defenses against- or tolerance of pathogens to achieve high yields. Over last years, *H. pluvialis* large scale production has been threatened by the destructive blastocladialean fungus, *Paraphysoderma sedebokerense*, responsible for complete collapses of *Haematococcus* populations over very short time spans. Not much is known on the interaction between both organisms, particularly on the host specificity. A better understanding of this relation, in a context of finding resistant strains, is important for the development of future mitigation strategies.

## 1.2.Thesis outline

Despite the recent advances in research and development of *H. pluvialis*, some of the following **key questions** remain unresolved. Answering these questions is a priority, given the likely convergence of several areas of research on *H. pluvialis* now that the whole genome sequence is on its way (QIBEBT, 2012).

(1) Is *H. pluvialis* a single species or does it comprise multiple (cryptic) species based on the analysis of variation patterns in selected molecular markers and morphological characteristics?

(2) How large is the intra- and interspecific genetically based phenotypic variation in traits related to astaxanthin productivity as well as traits affecting mass scale production such as biomass accumulation, growth rate etc..?

(3) Do physiological responses vary between strains and how does a variable environment, specifically irradiance affect morphology, physiology and pigment composition? Are there G x E interactions for the traits assessed?

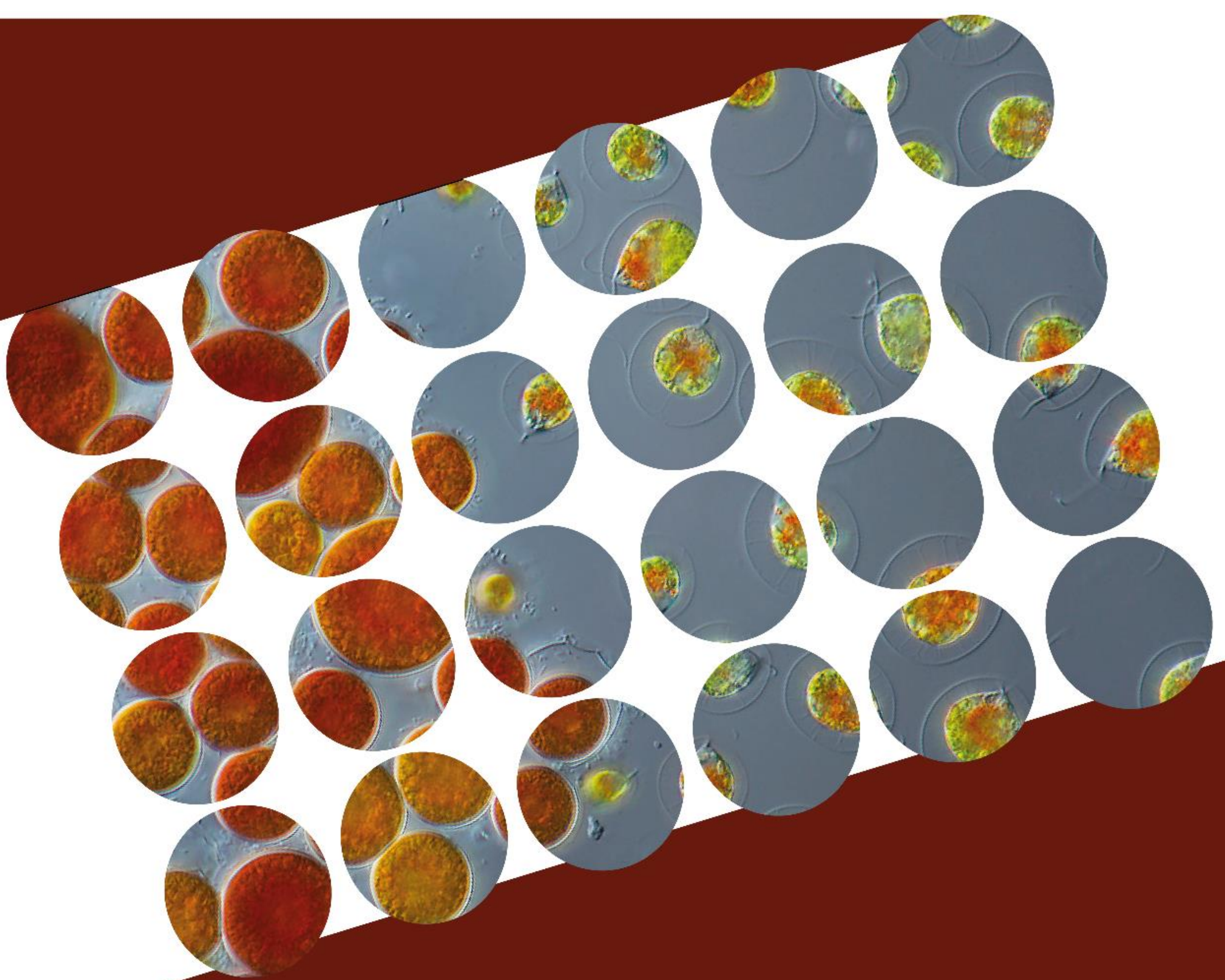
(4) Is there genetically based variation in the vulnerability to the pathogen *P. sedebokerense* (Blastocladiomycota)?

Overall, the main objective of this doctoral thesis is to obtain a better insight into the species diversity and phenotypic variation in *Haematococcus,* in particular with respect to potential astaxanthin productivity. The thesis is organized as follows:

- An overview of the genus *Haematococcus,* its taxonomy, ecology, occurrence, distribution, life cycle, reproduction, cultivation as well as a synthesis of the challenges faced throughout cultivation is presented in the introductory Chapter 2.

- **Species boundaries** in *H. pluvialis* are investigated by studying genetic, morphological and ecophysiological variation in European populations of *H. pluvialis* in combination with an assessment of the nomenclature of the genus (Chapter 3). A total of 150 isolates from 15 different locations across Europe as well as existing isolates from official culture collections were generated. Genetic variation is assessed on a selection of strains by using the ITS1 – 5.8S – ITS2 rDNA region as well as the *rbc*L gene and correlated with morphological differences. Differences in temperature dependency of growth rate are investigated for the different strains. DGGE is developed as a tool to aid in the identification of strains.
- The variation in traits related to **astaxanthin productivity** in *Haematococcus* is studied using a common garden approach (Chapter 4). Specifically, genetically based intra- and interspecific (co-) variation in six traits related to astaxanthin productivity is determined for 30 natural isolates including culture collections strains belonging to two *Haematococcus* species.
- The **ecophysiological responses** of six *Haematococcus* strains to irradiance during the growth phase are assessed in Chapter 5. Effects on morphological changes, growth rate, cell density, photosynthetic efficiency and pigment composition were measured for six strains when they enter stationary phase, exposed to a irradiance gradient compromising nine different irradiance levels.
- The final research chapter (Chapter 6) focuses on **interactions** between *Haematococcus* strains and the blastocladialean fungus *P. sedebokerense* responsible for mass mortality of *Haematococcus* populations in algal cultivation systems. Specifically, the host specificity of *P. sedebokerense* (strain PS1) on 44 *Haematococcus* strains in a laboratory controlled infectivity assay is examined. Strains are scored for their susceptibility to PS1 through the use of different infection proxies with the two fold objective of better understanding the host-parasite interaction as well as identifying resistant strains.

- Finally, in the general discussion the **implications** and **applications** of this research are discussed, identifying key areas that require further investigation (Chapter 7).



# CHAPTER 2:
# General introduction

## 2.1. Microalgae: from biology to biotechnology

Though the term *algae* has no formal taxonomic standing, phycologists routinely use *algae* to indicate a polypheletic, non-cohesive and artificial assemblage of $O_2$-evolving, photosynthetic organisms (Barsanti & Gualtieri, 2014). Given that this definition encompasses plants as well, it is important to denote the differences between both groups. Algae lack the various structures that characterize land plants, such as organ structures (roots, stems leaves) and vascular tissues (Raven *et al*., 1992), they further do not form embryos and their reproductive structures consist of cells that are all potentially fertile and lack sterile cells protecting them (Barsanti & Gualtieri, 2014). Algae *sensu lato* enclose both eukaryotic or prokaryotic organisms (Cyanobacteria), depending on the presence or not of the typical eukaryotic flagella and organelles (chloroplast, mitochondria and nuclei) (Guiry, 2012). Most algae possess the photosynthetic pigment chlorophyll *a* (Chl *a*) and are photosynthetic, yet some are mixotrophic, while others may have become heterotrophic (Richmond, 2008).

Algae further comprise organisms with a wide range of morphologies belonging to unicellular genera (e.g. *Chlorella*) or multicelullar genera (e.g. *Macrocystis*) and are further categorized in **macroalgae** and a highly diversified group of microorganisms known as **microalgae.** The latter are unicellular microscopic algae which are widespread across aquatic (including marine, brackish and freshwater) and non aquatic habitats. To ease reading, in this thesis the term **microalgae** is used for both eukaryotic and prokaryotic microscopic algae, regardless their nutritional strategy.

Estimates of microalgae biodiversity range between 200,000 and several million species, compared to 250,000 in higher plants (Norton *et al.*, 1996). Unlike land plants, microalgae achieve high growth rates (through exponential growth phases) and photosynthetic efficiencies, requiring only water, $CO_2$, phosphate, nitrate and some specific trace elements.

Microalgae come with unique oil and protein chemistries which were already discovered by the ancient Aztecs who collected algae of the genus *Spirulina* from alkaline lakes for food consumption (Costa & de Morais, 2011). The actual exploitation of microalgae started in Mexico, Africa and Asia, approximately 100 years ago, in the fight against famine, when "backyard" cultivation of natural populations of blue-green algae *Nostoc, Aphanizomenon* and *Spirulina* were common practices (Pulz & Gross, 2004; Spolaore *et al.*, 2006). Large-scale commercialization of microalgae is a more recent development which initiated 60 years ago along with the need of live feed for aquaculture species (fish, molluscs, and shrimps)

(Borowitzka, 2013). To date, this fraction of the production together with other applications in feed (pets and farmed animals), is still massive in quantity, representing 30 % of the worldwide algal manufacturing market (Hamed, 2016). In the early 1960's a *Chlorella* industry was founded in Japan and Taiwan for the use of health food and nutritional supplements (Borowitzka, 1999; Spolaore *et al.*, 2006). In the 1970's, the first *Spirulina* plant was established in Mexico (Borowitzka, 1999), though this was rather a "managed harvest" than a controlled production facility. *Spirulina* plants started erupting from then on, mainly in the USA (California and Hawaii), Thailand and in China. The next successful large scale production was in the early-mid 1980's, of *Dunaliella salina* as source of β-carotene (Spolaore *et al.*, 2006). Pilots were established in the USA, Israel and Australia. From then on, the cultivation of algae as sources of common and fine high value chemicals was set. Commercial production of *Haematococcus pluvialis* for astaxanthin became a reality in the late 1990s in Hawaii, by Cyanotech. The first attempt to produce microalgae heterotrophically, was in the 1990s in the USA with the production of *Crypthecodinium cohnii* as a source of eicosapentaenoic acid. To date, 5000-7500 tons of non-transgenic microalgae biomass are harvested each year from commercial algal biotechnology (Pulz & Gross, 2004). Applications range from human nutrition, animal feed, aquaculture, to the production of chemicals and pharmaceuticals, generating an average annual income of $ 1.25 billion (Spolaore *et al.*, 2006). Currently, Asia and Australia are showing the highest growth and are the main producers of microalgae-based commercial products, still the actual production volumes are not competitive with traditional agriculture (Vigani *et al.*, 2015).

### 2.1.1. Products from microalgae – present state

There is a wide discrepancy between the vast diversity of microalgal species and the rather limited number of taxa currently cultivated at large scale. This inconsistency is due to the many practical hurdles limiting the expansion and establishment of new species in microalgal biotechnology. In fact, the commercialization of new bioproducts requires selective biorefining of the biomass and technology readiness on cultivation and commercialization development which are not always straightforward (Ruiz *et al.*, 2016). For these reasons, present microalgae production is restricted to niche markets with almost no competition and inflated product prices (Draaisma *et al.*, 2013).

At present, two main product categories can be obtained for which exhaustive lists are published (Vigani *et al.*, 2015; Hamed, 2016). The first include dried algae, with a high protein,

carbohydrate and vitamin content, which are usually included as bulk commodities (for feed/food markets) or sold as dietary supplements. The second product category comprises specialty products isolated and extracted from microalgae that can be added to feed and food to improve their nutritional value. These generally include pigments (astaxanthin, phycocyanin, β-carotene), antioxidant, proteins (phycobiliprotein), polysaccharides, triglycerides, vitamins and fatty acids (EPA, DHA). Altogether, the majority of these products are derived from a few extremophilic species. These have very specific culture requirements and are therefore easier to control during cultivation, such as *Spirulina* which favors high alkalinity, *Dunaliella* high salinity, *Chlorella* high nutrient concentrations etc.. Other cultivated species include *Nannochloropsis, Porphyridium, Haematococcus, Tetraselmis, Phaeodactylum, Pavlova, Skeletonema, Thalassiosira* and *Chaetoceros* (Hamed, 2016).

Besides above mentioned applications, microalgae have been successfully implemented as food source and feed additive in the commercial rearing of many fresh water and marine aquaculture species (crustaceans, fish, mollusks etc..). The most frequently used genera in aquaculture include *Chlorella*, *Tetraselmis*, *Scenedesmus sensu lato*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira,* generally chosen for their rapid growth and stability in culture. Moreover, these species lack toxic compounds, have a good nutrient composition and may contain bioactive compounds, which are transferred to the aquatic food chain (Sirakov *et al.*, 2015).

Microalgae have further re-gained attention as a promising and exciting source of biofuels since the proposition that commercially viable biofuel production could be possible if the other constituent of the biomass were simultaneously exploited as co-product(s) together with the triglycerides, necessary for biofuel production (Borowitzka, 2013). Nevertheless, there is currently still a high level of controversy around this topic, since many life cycle analyses have shown a negative energy balance (more fossil fuel energy used than energy contained in the algal biofuel produced) during the production process (Lardon *et al.*, 2009; Sander & Murthy, 2010).

### 2.1.2. Role of diversity in microalgal biotechnology development

Next to technological developments, present progress in the microalgae biotechnology sector comprises firstly the exploitation of novel algal strains with new properties and secondly improving the inherent properties of the currently implemented wild type strains to reduce the production costs.

On one side, bioprospecting, or the process of discovery and commercialization of novel strains, is of importance and will continue to lead to new strains (Georgianna & Mayfield, 2012). Algae indeed possess a potential genetic pool that is orders of magnitude larger than that of animals or land plants (Tirichine & Bowler, 2011). Bioprospecting may be performed in search for specific strains of a particular microalgae species, or for a particular property (e.g. high concentrations of a secondary metabolite). New discoveries are made regularly, and new compounds and applications for industrial purposes are to be expected in the near future (Gangl *et al.*, 2015; Murray *et al.*, 2013).

On the other side, currently used strains, producing well established microalgal products are further developed and improved through classical breeding approaches based on crossing, selection and modern genetic engineering techniques (Hlavová *et al.*, 2015).

Regardless of how both prospects will evolve, the implementation of a species into an applied biotechnological pipeline involves initial species designations to be completed. In this respect, determining a species framework becomes important.

### 2.1.3. Species delimitation in microalgae

Species are fundamental natural units in biology and their proper circumscription is essential for both biodiversity assessments and a correct understanding of their ecology, biogeography, evolutionary history and speciation mechanisms. Moreover, species are important as they represent groups of strains with the capacity of changing genes (classical breeding) and allow to perform research on genetic correlations/covariation. Nevertheless, the concept of “species” is perhaps the most debated subject in evolutionary biology as demonstrated by the existence of more than twenty definitions or **species concepts,** founded on different methods and criteria due to the difficulty in assigning an organism to a biologically meaningful category (Mayden, 1997; Hey, 2001). The most common concepts are here discussed.

Ernst Mayr was the first to introduce the **biological species concept** (Mayr, 1942), defining species as “a group of interbreeding natural populations that are reproductively isolated from other such groups”. Probably the greatest pitfall of this definition relates to its applicability. The definition is constrained to sexually reproducing microalga groups (Coleman, 1977; Amato *et al.*, 2005; Mann, 2010), able to reproduce under laboratory conditions (which does not apply to many Chlorophyta members) (Leliaert *et al.*, 2014; Fučiková *et al.*, 2015) and cannot be employed for allopatric populations.

For a long time, species classifications were based on discontinuities in morphology between organisms (**morphological species concept**). In green microalgae, morphological species delineation often presents a challenge for both ecologists and taxonomists due to the scarcity of visual characters useful for this purpose and their simple morphology, as in e.g. *Chlorella* (Leliaert *et al.*, 2014; Krienitz *et al.*, 2015). In addition, morphological features may be largely influenced by phenotypic plasticity, convergent morphological evolution, morphological stasis and polymorphism (Luo *et al*., 2006; López-Bautista *et al*., 2007; Darienko *et al*., 2015). The view that this concept led to a high degree of misinterpretation on species limits, was confirmed with the advent of molecular studies, upon the review of morphologically based classifications showing e.g. highly cryptic species to belong to very distant lineages (Coesel & Krienitz, 2008; Krienitz & Bock, 2012). From then on, the use of DNA sequence data for species delimitation became the most convenient solution to the problems affecting other types of data (Carstens *et al*., 2013). The **phylogenetic species concept** (Mayden, 1997), defines species as "the smallest biological entities that are diagnosable and /or monophyletic". Molecular data may also harbor substantial downsides, such as incomplete lineage sorting, trans-species polymorphism, hybridization and introgression (Fujita *et al*., 2012; Camargo & Sites, 2013, Leliaert *et al*., 2014). Also cases where nuclear markers failed to resolve clear species boundaries whereas non-recombinant DNA (e.g. plastid or mitochondrial markers) pleaded for genetic isolation have been illustrated and show that the choice of (a) marker(s) is critical (Lane *et al*., 2007, Roy *et al*., 2009). In the last decades, there have been numerous attempts to define species algorithmically and many new methods for species delimitation based on DNA have been proposed which although having considerable potential also suffer from several extensively discussed limitations (Puillandre *et al*., 2012; Carstens *et al*., 2013; Leavit *et al*., 2015).

The above described species concepts and the many others that have been proposed, may not be completely satisfactory and even incompatible since leading to different conclusions concerning species boundaries and numbers, resulting in the over or underestimation of true species diversity within a genus (De Queiroz, 2007). De Queiroz (2007) proposed a new "concept", **the unified species concept** which retains the critical idea that species are "separately evolving metapopulation lineages". All the characteristics that may or may not be acquired over the course of existence of a species are considered as "contingent properties" or "secondary species criteria" (e.g. phenetic uniqueness, diagnosability, monophyly, reproductive isolation, etc.) and can be used to support its specific status. In this sense, the more of the different contingent properties a 'species' has, the more lines of evidence we have to

support its status. Since morphological and breeding data are often problematic, the best current procedure on which most taxonomists consent, is to base species delimitations on multi-locus molecular data with good resolution, obtained from multispecies coalescent methods. The "separately evolving metapopulations lineages" obtained may then be strengthened by additional lines of evidence (or contingent properties) including morphological, ultrastructural, biochemical, geographic, ecological and/or breeding data (Leliaert *et al.*, 2014). Each of these properties and the congruence between them can be used as a line of evidence for assessing species limits.

## 2.2. Carotenoids

Carotenoids are lipophilic, isoprenoid molecules which are synthesized *de novo* by photosynthetic plants, algae, fungi, and bacteria (Christaki *et al.*, 2013) and are categorized in xanthophylls and carotenes, depending on the presence of oxygen in their molecular structure. Typically, carotenoids are orange, yellow or red and for that reason often deliberately added to food as colorant, to make it attractive for consumption. Over the last years, concerns regarding the adverse effects of synthetic food on human health have led to an increasing shift from chemically to naturally produced colorants (Mulders *et al.*, 2014). Compared to vegetables and fruits, microalgae offer the advantage of producing a wide spectrum of pigments (viz., lutein, zeaxanthin, astaxanthin, canthaxanthin, and β-carotene), some of which are unique to microalgae (e.g. phycoerythrin, phycocyanin, and allophycocyanin specific to Cyanophyta, Rhodophyta) (Mulders *et al.*, 2014). Other advantages include the high productivity, limited seasonal variation and their easier extraction (Christaki *et al.*, 2013).

### 2.2.1. The red antioxidant astaxanthin

Astaxanthin (3,3'-dihydroxy-ß-carotene-4,4'-dione; $C_{40}H_{52}O_4$) belongs to the family of the xanthophylls, the oxygenated derivatives of carotenoids and possesses a molecular weight of 596.86 $g.mol^{-1}$ (Foppen, 1971; Straub, 2013). It owes its red color to the extended chain of conjugated, alternating double bounds at the center of the molecule (Miki, 1991). The molecule is composed of a long double-bonded polyene chain with a six-membered ring polar end group, rendering both lipophilic and hydrophilic properties (Kidd, 2011)(Fig. 1A). In addition to providing the red color, the chain of conjugated double bonds provides the antioxidant function of astaxanthin as it results in a region of decentralized electrons that can be donated to reduce a reactive oxidizing molecule (ROS) (Ambati *et al.*, 2014). Altogether, astaxanthin has a unique structure allowing it to span biological membranes and is by far the most potent antioxidant

known among all commercially available carotenoids (Palozza & Krinsky, 1992) such as vitamins C and E which do not posses this ability (Kurashige *et al.*, 1989; Ambati *et al.*, 2014).

Since the astaxanthin molecule has two identical chiral centers at the positions of 3 and 3', it can exist in four different configurations which yield three different stereoisomers: (3R,3′R), (3R,3′S) and (3S,3′S) depending on the spatial orientation of the hydroxyl (OH) groups in chiral carbon (Fig. 1B)(Shah *et al.*, 2016). When the hydroxyl group is attached so that is projects above the plane of the molecule, it is said to be in the R configuration, and inversely, it is said to be in the S configuration. The chirality of astaxanthin varies in function of the organism from which it is obtained and can therefore be used to trace the astaxanthin source (Lorenz & Cysewski, 2000). Synthetic astaxanthin is composed of the (3R,3′S), (3R,3R) and (3S,3′S) isomers in a ratio of 2:1:1, respectively, yielding only 25 % of the naturally occurring (3S,3′S) present in *H. pluvialis*, the currently only accepted form in the nutraceutical market (Shah *et al.*, 2016) (cf. 2.2.1.2.).

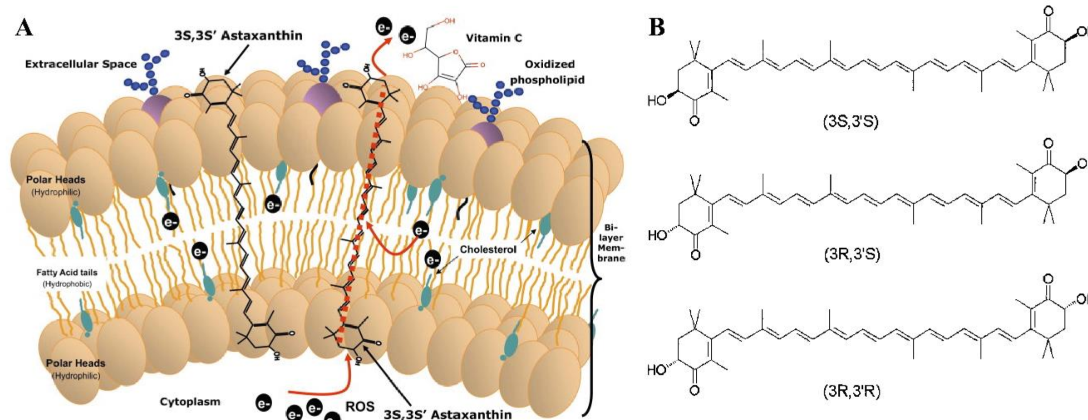



***Figure 1. A.*** *Transverse cell membrane Orientation of 3S,3S' astaxanthin, the major molecular form from H. pluvialis. ROS = reactive oxygen species. (Pashkow et al., 2008)* ***B.*** *Astaxanthin configurational isomers (Higuera-Ciapara et al., 2006)*

### 2.2.1.1.Sources of astaxanthin: synthetic and natural

Astaxanthin can be derived from various natural sources. Although natural astaxanthin is present in seafood (salmon, trout, red seabream, shrimp, lobster and fish eggs etc..) and in several birds (flamingoes, quails etc…) only a limited number of organisms are able to synthesize astaxanthin *de novo*. From all natural producers of astaxanthin, *H. pluvialis* accumulates by far the highest concentrations (Table 1).

Astaxanthin can also be produced through chemical synthesis. Synthetic astaxanthin is made of petrochemicals, raising issues of food safety, pollution and sustainability (Li *et al.*, 2011).

Moreover, the isomerism and racemic nature of synthetic astaxanthin make it less stable and efficient for coloration compared to its natural counterpart (Boussiba, 2000; Dufossé *et al.*, 2005).

The production cost of synthetic astaxanthin is further considerably high (€ 880/kg)(Panis & Carreon, 2016). Estimates on the production costs of natural astaxanthin are highly variable and range between $ 718/kg (Li *et al.*, 2011) in China, € 1536/kg in Greece and € 6403/kg in the Netherlands (Panis & Carreon, 2016). Although, clear market opportunities are opened for natural astaxanthin and studies have predicted the potential market value to be over $ 1.5 billion by 2020 (Nguyen, 2013), currently the European natural astaxanthin is not a competitive alternative for synthetic astaxanthin, at least not for aquaculture feeding purposes (Panis & Carreon, 2016).

***Table 1****. Organisms capable of de novo synthesis of astaxanthin*

| Organism | Astaxanthin content (% dry weight)(=dw) | Reference |
|---|---|---|
| **MICROALGAE** | | |
| *Chlamydomonas nivalis* | 0.04 | Bidigare *et al.* (1993) |
| *Chlorella zofingiensis* | 0.02-0.15 | Ip and Chen (2005) |
| *Chlorococcum* | <0.2 | Zhang *et al.* (1997) |
| *Coelastrella striolata var.multistriata* | 1.5 mg/g dw | Abe *et al.* (2007) |
| *Haematococcus pluvialis* | 2.3 – 7.7 | Kang *et al.* (2005) |
| *Monorpahidiumm sp.* | 0.25 | Fujii *et al.* (2006) |
| *Nannochloropsis gaditana* | <0.3 | Lubian *et al.* (2000) |
| *Neochloris wimmeri* | 0.6 | Orosa *et al.* (2000) |
| *Scenedesmus komarekii* | Not reported | Hanagata & Dubinsky (1999) |
| *Scenedesmus obliquus* | Not reported | Qin *et al.* (2008) |
| *Scenedesmus vacuolatus* | 0.01 | Orosa *et al.* (2000) |
| **MACROALGAE** | | |
| *Enteromorpha intestinalis* | 0.02 | Banerjee *et al.* (2009) |
| *Ulva lactuca* | 0.01 | Banerjee *et al.* (2009) |
| *Catenella repens* | 0.02 | Banerjee *et al.* (2009) |
| **YEAST** | | |
| *Xanthophyllomyces dendrorhous* | 0.4 | Jacobson *et al.* (2000) |
| *Candida utilis* | 0.04 | Miura *et al.* (1998) |
| **BACTERIA** | | |
| *Mycobacterium lacticola* | 0.003 | Simpson & Chichester (1981) |
| *Agrobacterium aurantiacum* | 0.01 | Yokoyama *et al.* (1994) |
| *Paracoccus carotinifaciencs* | 2.2 | EFSA (2006) |
| *Brevibacterium sp.* | 0.003 | Nelis & De Leenheer (1989) |
| *Sphingomonas sp.* | 1.2 μg/g cdw (cells dw) | Ma *et al.* (2016) |
| **PLANTS** | | |
| *Adonis amurensis* | Not reported | Seybold & Goodwin (1959) |
| **AMOEBAE** | | |
| *Thraustochytrium sp.* | 0.2 | Yamaoka *et al.* (2004) |

#### 2.2.1.2. Astaxanthin applications: from aquaculture to nutraceutical

Currently, commercial astaxanthin is available from petrochemicals, or solvent based extraction from natural sources, such as the yeast *Xanthophyllomyces dendrorhous* (formerly known as *Phaffia rhodozyma*) (Miller *et al.*, 1976). According to Industry Experts (2015), astaxanthin would also be available from genetically mutated *X. dendrorhous, Paracoccus carotinifaciens* and *Lactobacillus sp.* bacteria and from collected shrimp processing waste. All these sources principally find application as pigment in animal nutrition and have not been considered for direct human consumption. Currently, synthetic astaxanthin is the chief ingredient in the aquaculture industry and is used to impart the attractive red color to seafood. Besides providing coloration, astaxanthin has proven to contribute to an overall better fitness in aquaculture species, resulting in an increased resistance against pathogens and diseases (Petit *et al.*, 1997; Merchie *et al.*, 1998; Nakano *et al.*, 1999; Pan *et al.*, 2001).

Presently, astaxanthin derived from *H. pluvialis* is the only available source for human consumption since its approval by the FDA (Food and Drug Administration) (FDA, 1999), however there is no EFSA (European Food Safety Authority) approval for its therapeutic application so far (Shah *et al*., 2016). Dietary supplements containing *Haematococcus* derived astaxanthin were proven to possess potential activities and health benefits with no adverse side-effects from *in vitro* and even *in vivo* controlled trials (Shah *et al.*, 2016). Some of the main patented activities include: anti-oxidant activity, protection for UV rays, anti-skin cancer, anti-inflammatory, anti-gastric activity, hepato-protective, anti-diabetes, cardiovascular prevention, improving immune response and neuroprotection (Guerin *et al.*, 2003; Ambati *et al.*, 2014). Astaxanthin products from *H. pluvialis* are presently marketed as pure or blended and dosage forms as tablets, capsules, syrups, oils, soft gels, creams, biomass and granulated powders.

## 2.3. *Haematococcus pluvialis*

### 2.3.1. Classification and taxonomy of *H. pluvialis*

The fresh water unicellular alga *Haematococcus pluvialis* Flotow belongs to the class Chlorophyceae, order Volvocales and family Haematococcaceae (Bold & Wynne, 1985) and was first noticed by Girod-Chantrans (1802). The actual description of *Haematococcus* was conducted by Flotow (1844) who described the genus in great detail in a well-illustrated report. Hazen (1899) later described its biology and life cycle. The inclusion of five distinct species within the genus led to continuous debate among algal systematists (Smith, 1950; Pocock, 1960). Very recently, the analysis of 18S and 26S ribosomal DNA sequences (Buchheim *et al.*,

2013), confirmed Droop's suggestions to move all species except *H. pluvialis* to another genus, *Balticola* (Droop, 1956a). Until the research conducted for this thesis, *Haematococcus* was a monospecific genus composed of *H. pluvialis.* Through time, many synonyms have been employed for this species. Droop (1956b) revealed that many diagnoses (Agardh, 1828-1835; Sommerfelt, 1824; Greville, 1826) were based entirely on the non-motile stages, without indication that the species had a flagellated state, so are probably not *H. pluvialis*. Further ambiguous descriptions arose from sampled habitats other than the temporal fresh water bodies *H. pluvialis* typically inhabits (cf. section 2.3.2.).

Until recently, the circumscription of *H. pluvialis* was based solely on morpho-physiological attributes (Guiry & Guiry, 2014). The levels of genetic variation within this species have been poorly documented. Currently, two molecular studies have explored the variation amongst *H. pluvialis* isolates. Firstly, DNA fingerprint data showed variation among 10 different strains of *H. pluvialis.* The authors did not find any consistent geographical signal (e.g. based on the diversity in ISSR and RAPD molecular markers, strains obtained from Europe (Switzerland) grouped in a major branch of USA obtained strains) (Noroozi *et al.*, 2011). Secondly, highly variable ITS2 gene sequences were found among 42 isolates from a global range of provenances (Buchheim *et al.*, 2013). A good understanding of species limits within this genus is therefore lacking. This knowledge is essential however to comprehend patterns of diversity, biogeography and evolutionary history. Moreover, delimiting species in this commercially important species is a prerequisite for further studies at the species level and below, which may allow to perform comparative physiological studies in the search for candidate production strains for mass culture. The challenge of delineating 'species' or ecologically distinct populations will depend on a combination of genetic, morphological and physiological analyses (cf. section 2.1.2.).

### 2.3.2. Ecology, occurrence and distribution

Considerable attention has been paid to *H. pluvialis* with respect to its capacity to synthesize astaxanthin, yet detailed knowledge on its presence and abundance in natural systems, its ecology and biogeography is currently missing. *H. pluvialis* has been predominantly found colonizing temporary continental and coastal rock pools, water holes and other small natural or artificial water habitats (e.g. rocky depressions, bird baths, concrete basins and gutters) as well as water bodies held by terrestrial plants, better known as phytotelmata (Droop, 1953; Proctor, 1957; Gebuhr *et al.*, 2006)(Fig. 2). Reports of *H. pluvialis* from larger permanent fresh water

bodies are uncertain (Genitsaris *et al.*, 2016). Overall, *H. pluvialis* has evolved as an obligate inhabitant of temporary freshwater bodies defined as "standing fresh water bodies experiencing intermittently dry phases" (Williams, 1996) which vary more in their physico-chemical attributes than neighboring permanent fresh waters (Jocque *et al.*, 2010). Species inhabiting such water bodies are controlled by unstable conditions and extreme differences in temperature, salinity, irradiance (UV) and nutrients, accompanied by fluctuations in water level. Their tolerance limits are continuously challenged and therefore, temporal fresh water bodies are highly interesting systems from an ecological and evolutionary perspective (Jocque *et al.*, 2010). Unfortunately, until today, they have been the subject of rather limited ecological research.

*H. pluvialis* is well-adapted to survive in these unstable habitats by efficiently responding to environmental changes (Droop, 1953; Proctor, 1957; Sarada *et al.*, 2002). Firstly, cells exert rapid transition from macrozooids to aplanospores and *vice versa* under favorable and less favorable conditions. Aplanospores can withstand, extreme light conditions, and have a wide thermal tolerance (Kobayashi, 2000). Secondly, according to Proctor (1957), *H. pluvialis* would suffer competitive exclusion in the presence of other competitors, specifically Chlorophytes, preventing it to thrive in larger lacustrine environments. These observations are however not entirely supported by most recent literature. In their trials, Genitsaris *et al.,* (2011) and Oncel *et al.,* (2011) demonstrated consistent growth inhibition patterns in presence of *Chlamydomonas*, however, when *Scenedesmus* and *Chlorella* were co-cultured with *H. pluvialis*, its growth was not repressed. *H. pluvialis* was further found to be highly sensitive to grazing as judged by correlations between decreases in growth rate with increasing grazing pressure (Genistaris *et al*., 2016). Finally, since *H. pluvialis* is placed amongst one of the most high light adapted algae, tolerating high irradiances, due to the presence of astaxanthin (cf. section 2.4.) it is is thought to be specially adapted to shallow waters, where shading from other species is avoided (Falkowski & Owens, 1980).

*H. pluvialis* has been reported from all continents, except Antarctica (Guiry & Guiry, 2014) yet little is known on its actual dispersion mode. Since its discovery in air samples, *H. pluvialis* is believed to have developed an effective air-dispersion strategy, that allows it to successfully colonize and establish new populations in water bodies (Genitsaris *et al.*, 2016).

***Figure 2**. Some examples of the typical habitats of Haematococcus. **A.** Water puddle on green trash bin, Merelbeke, Belgium (Céline Allewaert) **B**. Dehydrated puddle on white chair, Třeboň, Czech Republic (Picture credits: Otakar Strunecký), **C.** Grey rain water barrel, Wageningen, The Netherlands (Picture credits: Suzanne Naus-Wiezer) and **D.** Small water puddle in rock depression, Province of Pescara, Italy (Picture credits: Griet Casteleyn)*

### 2.3.3. Life cycle and reproduction

Hazen (1899) was the first to present details of the life history of *H. pluviali*s with hand drawn images of the different stages of *Haematococcus*, which included following four cardinal categories: "*quiescens*" the encysted condition, "*agilis*", the motile condition, "*versatilis*" the vegetative phase and "*porphyrocephalis*" the gametes. Although the terminology of these stages has been debated, the existence of at least four different morpho-types remains widely recognized today (Collins *et al.*, 2011)(Fig. 3).

*H. pluvialis* proliferates mainly through asexual vegetative reproduction by direct division in the motile stages and by cell budding in the non-motile stages (Liu *et al.*, 1999). The sexual life cycle of *H. pluvialis* remains ambiguous (Zhang *et al.*, 2016). Peebles (1909) initially reported gametic fusions along with Schulze (1927), yet their findings were not supported by Elliott (1934), convinced that the division stages of microzooids could have been mistaken for fusions. Fusion was long considered homothallic (Hartmann, 1943), yet Droop (1956b) obtained

successful fusion of clones from different origin, suggesting heterothallic reproduction. Recent observations are scarce and only increase the doubts on sexual reproduction in *H. pluvialis*. Lee and Ding (1994) found a doubling of DNA content, suggesting gamete fusion. Triki *et al.*, (1997) managed to induce gametogenesis on starved cultures, yet conjugation was not observed. Clearly, the need for cytological and cytogenetic studies confirming or contradicting these findings is imminent.

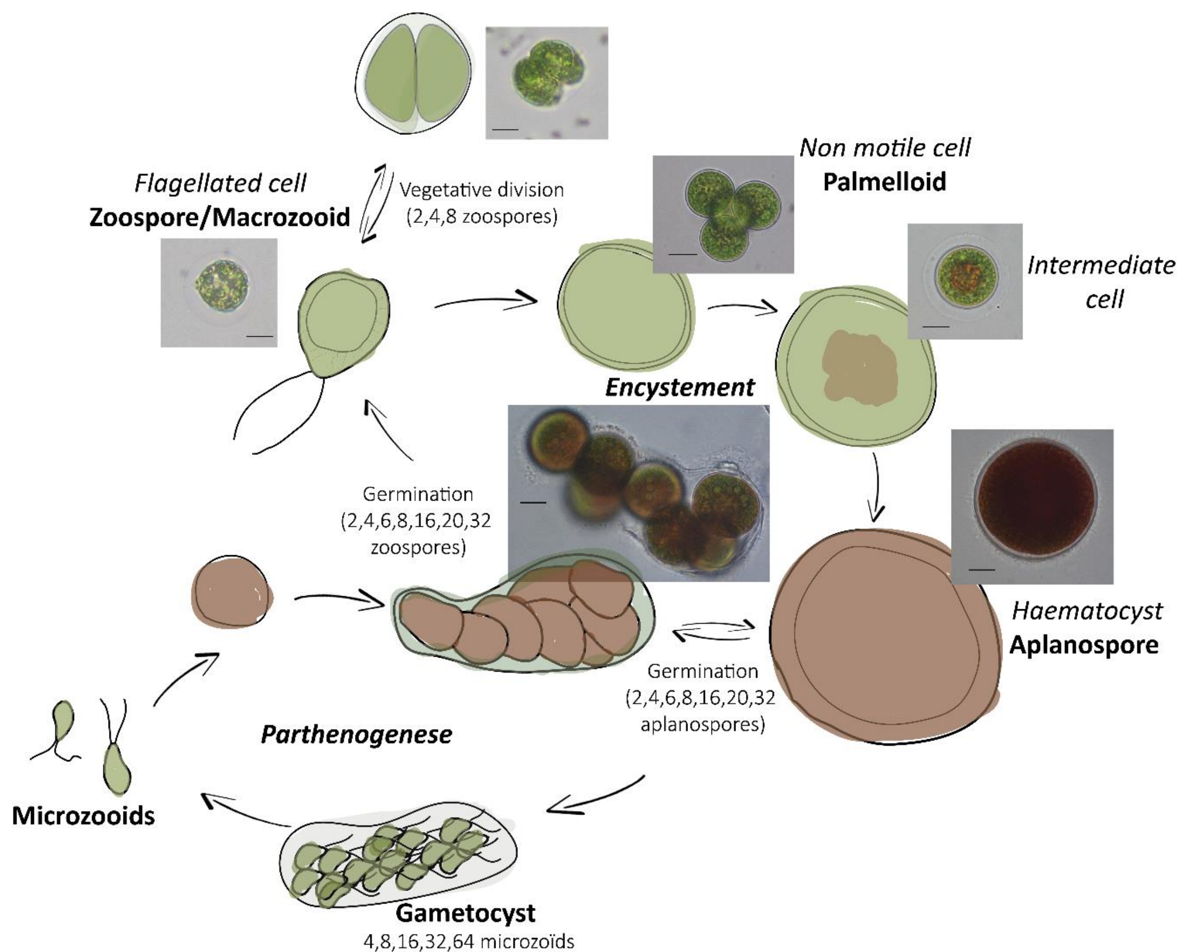



***Figure 3.*** *Illustration of life cycle of H. pluvialis (asexual and scenario for sexual reproduction), based on (Hazen, 1899; Triki et al., 1997; Han et al., 2013; Zhang et al., 2016) and on personal observations. Scale bars = 10 µm*

The different life stages of *H. pluvialis* include:

**ASEXUAL STAGES:**

***Green vegetative phase***

- **Macrozooids** or **zoospores,** cells are spherical, pear shaped or ellipsoidal (20-50 µm) with two flagella of equal length emerging from the anterior papilla perforating the cell wall at wide angles. Cup-shaped chloroplast, characterized by the possession of multiple scattered

pyrenoids. Cell wall appears as halo, separated from the plasma membrane, hence connected by cytoplasmic strands. Vegetative cells which reproduce asexually by simple division through mitosis (2,4,8 daughter cells) along the longitudinal plane (Wayama *et al.*, 2013). The daughter cells are formed with the parent cells, which remain motile until their flagella is shed and the walls dissolve to release the daughter cells.

- **Palmelloids** are macrozooids which have lost their flagella and expand in size due to conditions becoming unfavorable (yet the actual triggers remain unknown). The primary cell wall is an amorphous multilayered structure in the inner regions of the extracellular matrix. Palmelloid cells may gradually start accumulating astaxanthin beginning from the cell center, in which case they are called intermediate cells.

***Red astaxanthin accumulating phase***

- **Haematocysts** or **aplanospores,** formed under continued unfavorable conditions are large in size and characterized by a thick and rigid trilaminar sheath, and a secondary cell wall of acetolysis-resistant material. Mature aplanospores are bright red since they start accumulating large amounts of secondary carotenoids, particularly astaxanthin in lipid droplets deposited in the cytoplasm. When returning to favorable conditions, haematocysts germinate, releasing flagellated cells to initiate a new vegetative growth cycle. Although these may still contain some astaxanthin from their parent spore, it is rapidly catabolized (Elliott, 1934; Lee & Ding, 1994; Fábregas *et al.*, 2003, personal observations).

**SEXUAL STAGE:**

- **Microzooids** are small (<10 µm) ovoid cells released during gametogenesis from aplanospores (max. 64 microzooids) possessing a closely appressed cell wall. Microzooids exhibit high speed motility after release.

### 2.4. Carotenogenesis in *H. pluvialis*

The biochemistry and enzymology of astaxanthin biosynthesis as well as its genetic control are relatively well studied in *H. pluvialis* and extensively reviewed in Han *et al*., (2013). Briefly, astaxanthin is biosynthesized through the isoprenoid pathway which is also responsible for the vast array of lipid soluble molecules such as sterols, steroids, prostaglandins, hormones, vitamins D, K and E. The pathway initiates at acetyl-Co-A and proceeds through phytoene, lycopene, β-carotene, and canthaxanthin before the last oxidative steps to astaxanthin formation

(Cunningham & Gantt, 1998). It is important to know that all biosynthesis steps up to the formation of β-carotene, the most direct precursor of astaxanthin, take place in the chloroplast. β-carotene is then exported from the chloroplast into the cytoplasm (Grünewald & Hagen, 2001). The actual astaxanthin synthesis occurs in cytoplasmic lipid vesicles as was shown by its close relationship both in time and space with the accumulation of triglycerides (TAG), essential for deposition of astaxanthin inside lipid bodies (Solovchenko, 2015). The majority of astaxanthin is deposited as fatty acid mono or diesters or mono- or diesters of palmitic (16:0), oleic (18:1), or linoleic (18:2) acids. Approximately 70 % monoesters, 25 % diesters and only 5% of the free ketocarotenoid is present in the mature aplanospores of *H. pluvialis* (Zhekisheva *et al.,* 2002; Solovchencko *et al*., 2015).

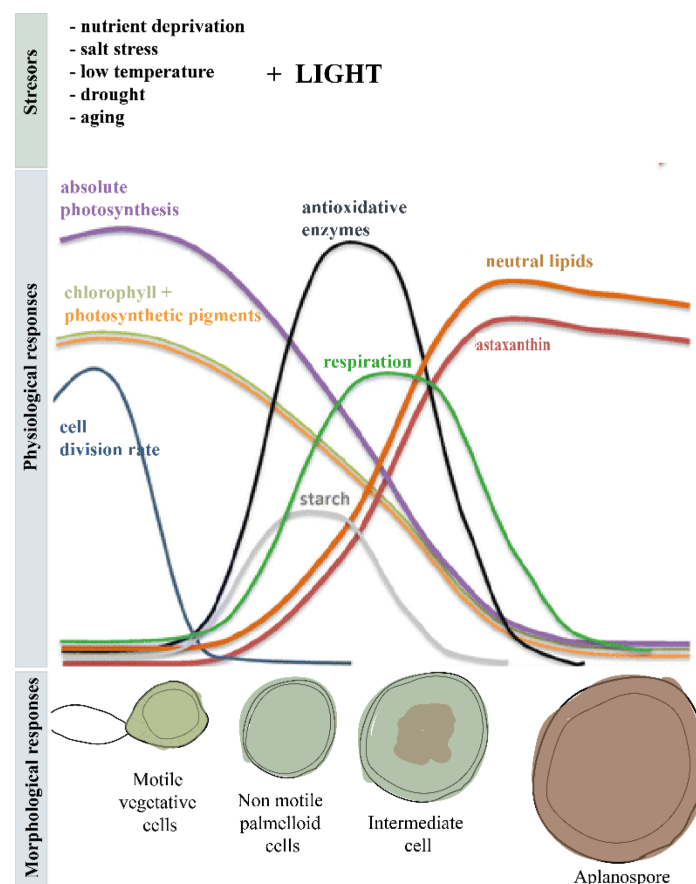



***Figure 4.*** *Schematic representation of stressors and their respective physiological and morphological responses during the process of carotenogenesis in H. pluvialis cells, adapted from (Boussiba, 2000; Solovchenko, 2015).*

As shown in Fig. 4, the process of carotenogenesis involves a complex interplay of physiological changes at the cellular level, resulting in morphological changes. At the initial stages of the astaxanthin accumulation, the cell retains a significant amount of photosynthetic pigments and photosynthetic activity driving accumulation of starch. At this stage, the up-regulated antioxidative enzymes protect the cell. Later, the starch synthesis is followed by its

degradation, the reduction of photosynthetic activity, increased respiration rate and the induction of fatty acid and astaxanthin biosynthesis resulting in the appearance of red lipid droplets in the cell. Finally, the decline in photosynthetic pigments proceeds and the antioxidative enzyme activity reverts to the basal level (Boussiba *et al*., 2000; Zhekisheva *et al.,* 2002, Solovchenko, 2015).

### 2.4.1. Multi-level stress protection

Astaxanthin functions as a secondary carotenoid (Mulders *et al.*, 2014), meaning that it is not functionally or structurally coupled to the photosynthetic apparatus in *H. pluvialis* (Solovchenko, 2013). It is currently accepted that astaxanthin accumulation is a carotenogenic response which allows increased cell tolerance to adverse environmental conditions (Boussiba 2000; Wang *et al*., 2003). Although the exact role of astaxanthin at intracellular level remains largely questioned, current available literature allows to distinguish at least four plausible functions (Solovchencko, 2015):

1) astaxanthin absorbs excessive light and shields the chloroplast and other cell structures from photooxidative damage (Hagen *et al.,* 1994, Han *et al*., 2012)
2) lipid droplets containing astaxanthin are suggested to form an antioxidant barrier surrounding the nucleus and the chloroplast, protecting them as well as the lipids containing unsaturated fatty acids from peroxidation (Boussiba 2000, Hagen *et al*., 1993)
3) since astaxanthin biosynthesis consumes $O_2$ it is believed that astaxanthin accumulation may protect from excessive $O_2$ which forms a potential danger for the cell under stress (Li *et al*., 2008).
4) the biosynthesis of astaxanthin and fatty acids may provide sinks for the photosynthates that cannot be directly used for cell growth and division during stress (Han *et al*., 2012).

## 2.5. Commercial cultivation of *H. pluvialis* for astaxanthin production

Currently, 16 leading companies are commercially producing *H. pluvialis* for astaxanthin and astaxanthin related products (Shah *et al.*, 2016). Production can either occur indoors, in open raceways or in closed photobioreactors (typically tubular, bubble column and airlift bioreactors) and carried out in batch, fed batch or continuous mode (Shah *et al*., 2016)(Fig. 5). The complexity of the *H. pluvialis* life cycle has determined the design of current production systems. Traditionally, *H. pluvialis* production is divided in two steps, with a biomass accumulation phase where vegetative cells are produced, separated from an astaxanthin accumulation phase where cysts are exposed to one or multiple stresses to accumulate

astaxanthin. Since underlying biochemical processes vary, the production requires optimization of both phases. Vegetative cells for biomass accumulation are both shear and light sensitive, therefore illumination, cell density and mixing strategies should be carefully chosen to avoid photo bleaching and cell death (Vega-Estrada *et al.*, 2005; Issarapayup *et al.*, 2009; Li *et al.*, 2011; Wang *et al.*, 2013). Moreover, due to so far unidentified triggers, vegetative cells have a limited amount of divisions before losing their flagella and converting to palmelloid cells (Lee & Ding, 1994). The latter are less vulnerable to light, but then divide at very low rates. In order to maintain cell division and guarantee high biomass accumulation, cultures can be maintained either, heterotrophically in the dark (Kobayashi *et al.*, 1992) or under a low N/P ratio (Tocquin *et al.*, 2012). After a sufficient volume of vegetative cells is produced, cultures are generally transferred to another system under conditions where cell growth and motility is halted, aplanospores can start forming and carotenoid synthesis is initiated (Wayama *et al.*, 2013). Conditions promoting this metamorphosis are exhaustively discussed in the literature and well-reviewed by Shah *et al.,* (2016). Astaxanthin production is generally induced by nutrient limitation (nitrate or phosphate deprivation), high temperature and light intensity or by the addition of sodium chloride to the culture medium (Lorenz & Cysewski, 2000). The above mentioned triggers for astaxanthin production are those proposed from literature, although one may suspect similar procedures are employed for the commercial scale production, at present, very little is known on the actual operation modes and strains chosen by companies. The insufficient collaboration between universities and commercial enterprises is a major problem for future improvement of *H. pluvialis* production (Shah *et al.*, 2016).

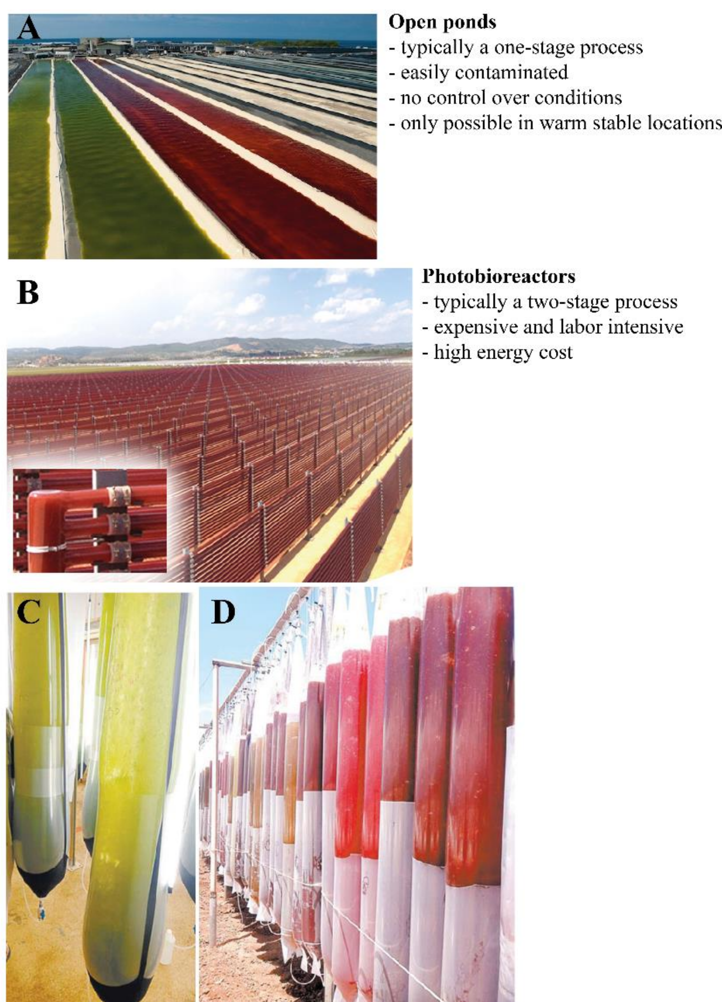



***Figure 5. A.*** *Open raceways ponds of Cyanotech Corporation, Hawaii.* ***B.*** *80-Acre astaxanthin production facility showing sealed, glass photobioreactors in Ben-Gurion University of the Negev, Israel.* ***C.*** *120 L bag of Haematococcus in the green stage, kept indoors* ***D.*** *Red stage; bags are placed outside in direct sunlight for up to 20 day in Maui Tropical Algae Farm, USA.*

### 2.6. Challenges affecting *H. pluvialis* cultivation

Today increasing the astaxanthin yield from *H. pluvialis* remains an active research target. The production of *H. pluvialis* still faces some major challenges, which are magnified as processes scale up and therefore require advanced technology and/or methods to be controlled. If not this may lead to economically unsustainable production. Although these challenges affect all production stages from cultivation to harvesting and post harvesting (e.g. cell disruption), only those shortcomings affecting the cultivation of *H. pluvialis* are here discussed.

To begin with, *H. pluvialis* has intrinsically slow growth rates and low biomass yields during the vegetative phase, compared to other coccoid green algae (Ip *et al.*, 2004). Cells are further highly sensitive to environmental stress, leading to either rapid transitions in morphology or in

the worst case, to cell death. A next rather slow process is the intrinsic induction of astaxanthin (Sharon-Gojman *et al.*, 2015). Finally, since *H. pluvialis* grows in a neutral culture medium (Lorenz & Cysewski, 2000; Zhang *et al.*, 2009), which is attractive to a broad range of organisms, a major bottleneck in the production of astaxanthin is its vulnerability to microbial contaminants and the lack of effective solutions to prevent and treat them. The latter point is discussed in detail in the following section.

### 2.6.1. Aquatic parasites and possible control measures

Similar to natural systems, where grazers, pathogenic bacteria and parasites play an important role in the composition of communities and the food web, cultivation of algae in artificial habitats, (e.g. algal ponds, photobioreactors) implies the presence of microbial contaminants. These can play an important role in modulating algal populations. With the intensification of microalgae mass cultivation, aquatic parasites form a significant biological threat and economic challenge to the commercial cultivation of algae. There is a general lack of knowledge on how parasites act in such artificial habitats. Clearly, the development of real-time and sensitive high throughput methods of contaminant identification and detection is of paramount importance for the further economic development of this industry (Carney *et al.*, 2016). Once detected, contaminants need to be either reduced or eliminated, necessitating rapid control measures. Although several mitigation measures have been proposed, such as the manipulation of nutrient concentration, pH, temperature, light, turbidity, or even more drastic solutions such as the addition of toxins, there is still a long road ahead in contaminant detection and remediation. Moreover, each group may have specific requirements (Gachon *et al.*, 2009).

Pathogens that hamper microalgae cultivation belong to different genera. Mass cultures of *H. pluvialis* are predominantly contaminated with fungal parasites and zooplanktonic predators (amoebae, ciliates and rotifers), cyanobacteria and several other eukaryotic microalgae (Han *et al.*, 2013). One of the potentially less controllable pathogen, is the recently described parasitic blasotoclad fungus *Paraphysoderma sedebokerense* (Boussiba, Zarka & James) (James *et al.*, 2006, 2012). The species has gained considerable attention since it was found responsible for the complete collapse of *H. pluvialis* cultures in no more than three days in laboratory conditions (Hoffman *et al.*, 2008). Although no numbers on the economic consequences of *P. sedebokerense* infection are available at present, its occurrence, together with the speed and persistence of infection in *H. pluvialis* cultures is alarming (Hoffman *et al.*, 2008; Gutman *et al.*, 2009; Gutman *et al.*, 2011; Carney & Lane, 2014).

### 2.6.1.1. *Paraphysoderma sedebokerense*

*P. sedebokerense* was first isolated from closed bioreactors in Israel (Hoffman *et al.*, 2008) after which it also appeared in cultures in Arizona, US (Carney *et al.*, 2016) and in Portugal (Strittmatter *et al.*, 2016). Currently, four strains of *P. sedebokerense* are identified and characterized (Table 2). *P. sedebokerense* was first thought to belong to the Chytridiomycota, yet, based on morphological characteristics in line with molecular evidence, *P. sedebokerense* was allocated to a new phylum with a single class and order: Blastocladiomycetes, Blastocladiales. Traits which are unique to the Blastocladiales include the possession of 1) zoospores with a characteristic nuclear cap of membrane-bounded ribosomes 2) a thick walled dark resting sporangium which cracks upon germination, with a endosporangium protruding from the opening 3) haploid and diploid phases with sporophytic and gametophytic stages throughout the life cycle 4) meiosis with spores instead of zygotes (James *et al.,* 2006, Porter *et al*., 2011). Four families of the Blastocladiales, were recently recognized, based on available molecular data of the nuclear ribosomal genes. As a separate clade, *Paraphysoderma* is embedded within the Physodermataceae, the most basal branch of the Blastocladiales along with the sister clades *Physoderma* and *Urophlyctis.* Curiously, the Physodermataceae are obligate plant parasites, while *P. sedebokerense* is so far the only parasite of unicellular green algae (*Scenedesmus dimorphus* and *H. pluvialis*).

***Table 2*** *Paraphysoderma sedebokerense isolates currently described*

| PS strain | Host | Geographic origin | Genbank accession (SSU) | Reference |
|---|---|---|---|---|
| TJ-2007a | *H. pluvialis* | Israel | EF565163 | Hoffman *et al.*, (2008) |
| JEL821 | *H. pluvialis* | Arizona, US | - | Carney (2015) |
| PS1 | *H. pluvialis* | Portugal | KT270356 | Strittmatter *et al.,* (2015) |
| FD61 | *S. dimorphus* | New Mexico, US | KJ563218 | McBride *et al.,* (2014) |

### 2.6.1.2. *Paraphysoderma sedebokerense* life cycle - state of the art

Considerable progress has already been made in better understanding the complex life cycle of *P. sedebokerense* yet many details still remain to be resolved (Hoffman *et al.*, 2008; Strittmatter *et al.*, 2016).

In *H. pluvialis* cultures, infection by *P. sedebokerense* can be unambiguously perceived by the initial attachment of amoeboid spores on to *Haematococcus* aplanospore cell walls (Fig. 6A). Upon attachment, the parasite develops into a cyst further forming a germ tube structure at the attaching point, which penetrates the cell wall and grows into the cytoplasm. The cyst begins

to grow rapidly at the expense of the host cell nutrients and develops into a sporangium within 1-2 days. As the infection proceeds, new amoeboid spores are formed within the sporangium (Fig. 6B) and then released, leaving an empty sporangium attached to the host cell (Fig. 6C-D). Macroscopically, *P. sedebokerense* presence in *H. pluvialis* cultures is noticeable through the initial appearance of clumps consisting of living cells, cell debris and particulate organic matter. Culture suspensions rapidly turn brown after which they gradually start bleaching, finally forming white clumps floating in suspension. So far, the parasite has been observed to infect both green palmelloid cells and red aplanospores, while infection of flagellated cells of *Haematococcus* has not yet been observed (Gutman *et al.*, 2011).

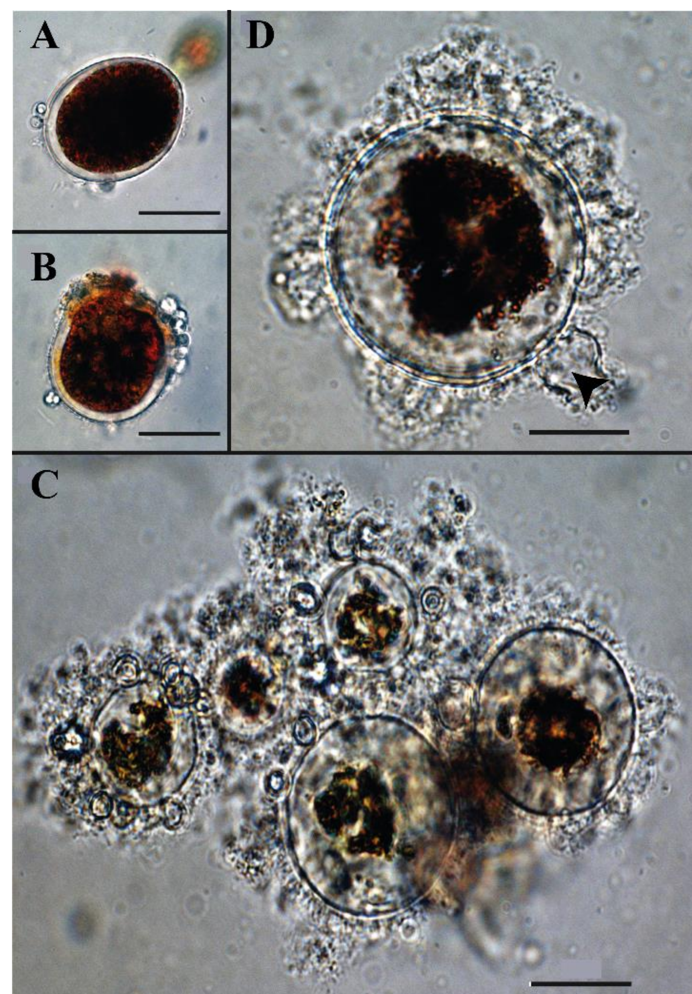



***Figure 6.*** *Microscopic evolution of infection of Haematococcus by the parasitic fungus P. sedebokerense strain PS1.* ***A-B.*** *First unambiguous signs of P. sedebokerense: the emergence of cysts growing on the surface of a Haematococcus aplanospore.* ***C.*** *Cysts develop into sporangia upon feeding on the protoplast of Haematococcus cells, which gradually shrinks.* ***D.*** *Flagellated and/or non-flagellated amoebae get released over time, leaving empty sporangia behind (indicated by an arrow). Scale bar =20 µm.*

## 2.7. Overcoming challenges – improving phenotypes

Clearly, *H. pluvialis* has not been genetically programmed for optimized mass production of astaxanthin under large-scale operational conditions, at least not in a way that it may completely replace synthetic astaxanthin for the far larger aquaculture feed market. Although optimization of photobioreactor design and engineering, may offer a contribution to relieve some of the species limitations, it is not a complete solution to the intrinsic problems as well as the contamination issues of this algae. Many traits require careful improvement and new highly efficient stable phenotypes need to be created either through modern or traditional upgrading techniques.

### 2.71. Genetic improvement

Genetic manipulation of microalgae have been the focus of growing interest. Their short generation times together with the availability of successful UV or chemical mutagenesis methods, make microalgae easy targets for manipulation (Larkum *et al.*, 2012). New strains can be developed by specifically targeting genes through reverse or forward genetic strategies. In this respect, advancements in basic and applied research on *H. pluvialis* are hampered by the lack of publically available genome sequence information. In fact, the full genome of *H. pluvialis* has not yet been published, although projects intend to do so (QIBEBT, 2012).

Although so far over microalgae 30 species, including *H. pluvialis,* have been genetically engineered, resulting in successful transient transgene expression, in most cases, obtaining a stable, hereditary genetic modification is still a work in progress (Shah *et al.*, 2016). In *H. pluvialis*, obtaining stable, reproducible nuclear transformation and successful engineering is hindered by the lack of suitable shuttle vectors and adequate transformation frequencies (Sharon-Gojman *et al.*, 2015). *H. pluvialis* has however been successfully transformed by microparticle bombardment with the pPlat-*pds* vector developed by Steinbrenner and Sandmann (2006), which carries a mutated copy of the *pds* conferring resistance to the herbicide norflurazon. Recently, stable chloroplast (Gutierrez *et al.*, 2012) and nuclear genome transformation (Sharon-Gojman *et al.*, 2015) was reported. Due to the transformation and engineering constrains, genetic improvement in *H. pluvialis* has long been limited to classical mutagenesis (Shah *et al.*, 2016). Several mutants with up to three fold improvement in astaxanthin content were created upon treatement with *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) and ethyl methanesulfonate respectively, with high frequency mutation and low lethality (Hu *et al.*, 2008; Gómez *et al.*, 2013).

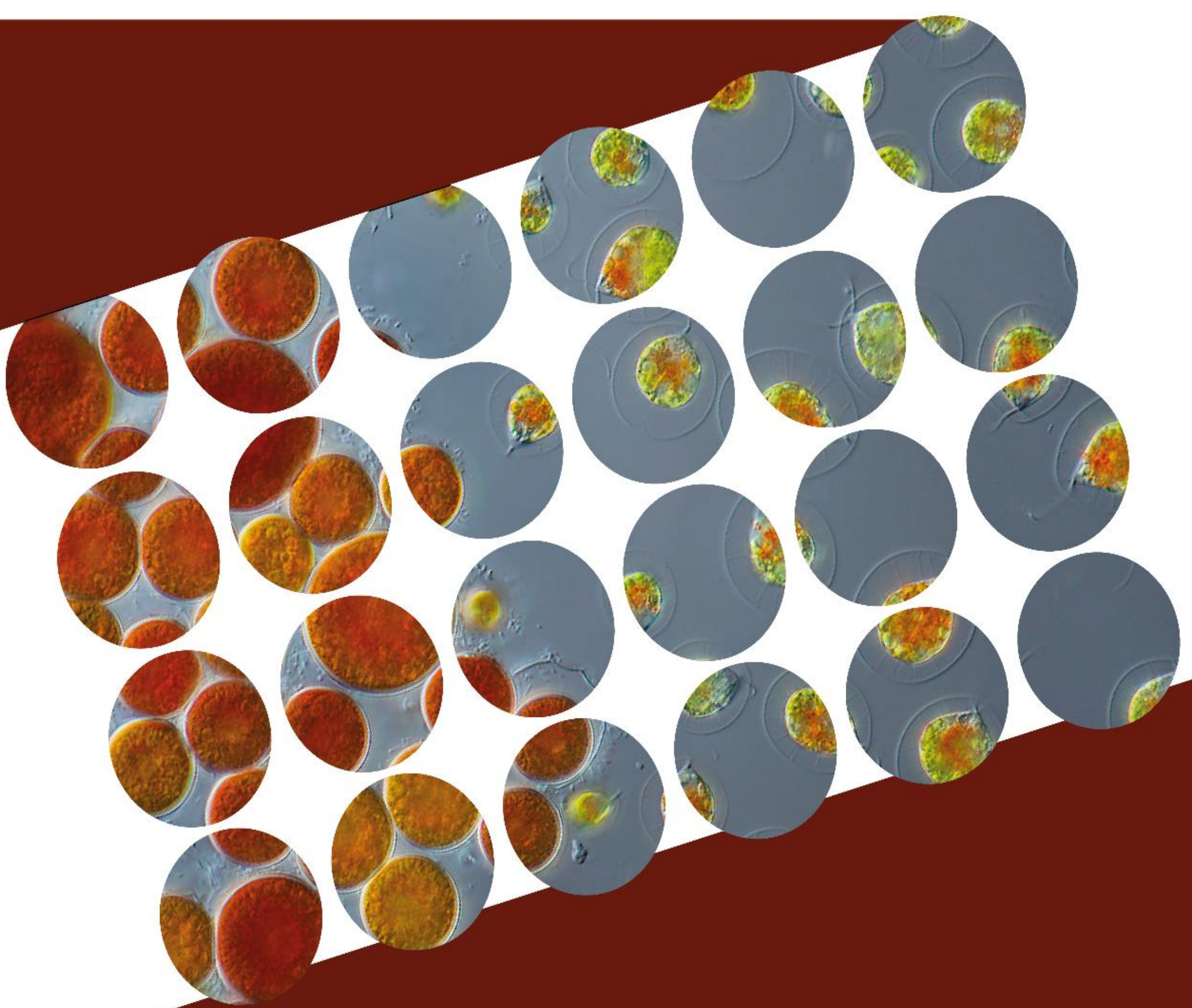
These new advancements are very challenging especially for the development of this organism as a model for carotenoid synthesis and for industrial astaxanthin production. Unfortunately, the genetic modification of an organism may present many drawbacks which still need to be overcome, before their actual acceptance and further implementation. The first problem lies in the difficulty of enclosing the transformed strains in confined areas. Therefore, regulations on genetically modified algae should be internationally enforced, since the spread of modified strains could have global consequences to ecosystems and livelihoods (Flynn *et al.*, 2013). Secondly there is the consumers acceptance, mutants are more readily accepted than transgenics by consumers, since the procedure is more natural and can occur spontaneously (Gómez *et al.*, 2013). Other limitations to genetic modification approaches include the potential for trade-offs. For example, a mutant microalgal strain that demonstrates good parasite resistance may not perform well in other aspects. The breadth of resistance could be quite narrow with resistance to one parasite species extending only to closely related species. Finally, because of selective pressure, there is the potential for the parasite to rapidly evolve to overcome the host algae resistance.

### 2.7.2. Breeding

When compared to plant crop developments, algal strain development is lacking one main component: a breeding program (Georgianna & Mayfield, 2012). In fact, specific phenotypes may also be obtained by searching for interesting properties within interfertile wild-type isolates. Microalgae possess many advantages for breeding strategies to be applied: 1) a short life cycle allowing quick selection from a large genotypic pool, 2) a small size allowing small scale breeding, 3) sexual and asexual reproduction increasing the rate of obtaining genetic diversity, 4) phenotypes which are amendable for high throughput screening and finally, 5) modification is applied through simple yet specific selection pressures (Chepurnov *et al.*, 2011; Georgianna & Mayfield, 2012; Larkum *et al.*, 2012). To date, *H. pluvialis* is not amenable for sexual breeding since its sexual life cycle cannot yet be controlled in laboratory conditions.

Both modern and classical techniques for strain optimization are currently subject of many limitations and rely on extensive manipulations necessary for the introduction of complex processes in microalgal traits. There is still much to be learned from the natural variation in phenotypic traits that already exists between species and strains given their long history of evolution. The physiological or genetic mechanisms underlying natural intraspecific and interspecific variation are largely untapped resources that firstly provide valuable information

on strain/species capacity and performance under varying environmental conditions and secondly may serve as invaluable genetic resource that can be used to improve yields. In fact, knowledge on this natural diversity will encourage the use of new strain backgrounds (with desirable traits) onto which additional genetic modification or breeding can be targeted in an attempt to improve yields. Since phenotyping of natural strains is very time consuming and labor intensive (Houle *et al.*, 2010), it compares poorly with the increasingly complete genotype data available. Phenotyping however, remains compulsory even upon applying genetic engineering techniques (Araus & Cairns, 2014). In the laboratory, clonal organisms such as *H. pluvialis* make it possible to study natural phenotypic variation in traits, since the species is capable of vegetative growth which, in contrast to species relying solely on sexual reproduction, allows the maintenance or multiplication of specific genotypes over prolonged periods of time, without alteration of trait combinations.



# CHAPTER 3: Species diversity in European *Haematococcus* (Chlorophyceae, Volvocales)

**Modified from Allewaert CC., Vanormelingen P., Pröschold T., Gómez PI., González MA., Bilcke G., D'hondt S., & Vyverman W. (2014) Species diversity in European *Haematococcus pluvialis* (Chlorophyceae, Volvocales). *Phycologia* 54(6): 583–598.**

## Abstract

*Haematococcus pluvialis* has received much interest because it can accumulate large quantities of the pigment astaxanthin. While different isolates of *H. pluvialis* seem to differ considerably in their physiology, their phylogenetic diversity has not yet been thoroughly studied. We studied the species diversity in a set of temperate European strains of *H. pluvialis* based on internal transcribed spacer (ITS) rDNA and *rbc*L molecular phylogenies and determined their congruence with morphology and temperature preferences. The ITS rDNA phylogeny resolved three lineages with the European strains. Three additional lineages were not represented by our European strains. Both statistical parsimony network analysis and the general mixed Yule coalescent recovered these six lineages as different species. An ITS1 structure analysis revealed up to three compensatory base changes (CBCs) between each of the three European lineages, while one CBC was found between lineages in the ITS2 secondary structure. Yet the CBCs were not always present in all strains of each lineage. The *rbc*L was much less variable and separated only two of the three ITS lineages. We propose an epitype for *H. pluvialis* and describe the two lineages containing European strains as new species, *Haematococcus rubicundus* and *Haematococcus rubens*. Although there is broad morphological overlap, strains of *H. pluvialis* and *H. rubens* have, on average, more elongated cells, more noticeable cytoplasmic strands and a more (extreme) pear-shaped protoplast apex than *H. rubicundus*. Optimal temperatures for growth were similar for the three species and varied between 17 °C and 23 °C, which is lower than reported earlier from other strains. This study provides a robust phylogenetic framework for further ecophysiological study of *Haematococcus*.

## 3.1. Introduction

The 'blood rain alga' *Haematococcus pluvialis* Flotow 1844 (Chlorophyceae) is widely studied for its capacity to produce mono- and di-esters of the red keto-carotenoid astaxanthin (3,39-dihydroxy-b,b-carotene-4,49-dione). Astaxanthin is a highly demanded pigment for its colouring and antioxidant properties with applications in nutraceuticals, cosmetics, food and feed industries (Vílchez *et al*., 2011; Murray *et al.,* 2013). *Haematococcus pluvialis* is not the only natural source of this pigment but is by far the most potent producer, accumulating up to 2.5-3.5 % (Li *et al.,* 2011) or even 4 % (Han *et al.,* 2013) astaxanthin of its dry weight. Several companies commercially produce astaxanthin from *H. pluvialis* in open ponds, closed bioreactors or a combination of both (Markou & Nerantzis 2013; Ambati *et al.,* 2014), commonly through a biphasic approach. In a first phase, high densities of actively growing flagellated cells are obtained in nutrient-rich media. In a second stage, the algae are transferred to a stressful environment to induce the formation of aplanospores and associated synthesis of astaxanthin (Han *et al.,* 2013).

In recent years, several new strains of *H. pluvialis* with interesting properties were isolated. Klochkova *et al.,* (2013) discovered an Arctic strain differing from other *H. pluvialis* by its capacity to grow and produce astaxanthin at low temperatures (4–10 °C). Chekanov *et al.,* (2014) reported a novel strain with elevated salt tolerance (up to 25 %). Han *et al.,* (2012) observed that some culture collection strains predominantly grew as green motile cells under laboratory conditions, while others transformed rapidly into non flagellated palmelloid cells. The latter were able to accumulate more astaxanthin (1.4 instead of 0.4 mg $g^{-1}$ dry weight). Finally, many studies reported significant physiological variability among strains, with up to threefold differences in biomass production, growth rate and/or astaxanthin content when grown under identical conditions (González *et al.,* 2009; Zhang *et al.,* 2009; Noroozi *et al.,* 2012; Gao *et al.,* 2015). Likewise, substantial genetic variation was found among 10 strains of *H. pluvialis* using intersimple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) molecular markers (Noroozi *et al.* 2011). Taken together, both genetic and physiological data suggest substantial diversity in *H. pluvialis*, which may (or may not) be associated with yet undescribed species diversity. Documenting this diversity is important in the context of biotechnology, as it provides opportunities for selection of fast growing, productive species and strains that are adapted to local growing conditions and for a reduction of economic and environmental costs of production plants. Assessing species boundaries and the selection of a suitable marker to aid the identification of *Haematococcus* species is essential

in this respect. As with other organisms, species delimitation in algae is increasingly based on molecular phylogenies (Leliaert *et al.,* 2014). Moreover, several automated phylogenetic species delimitation methods, such as the general mixed Yule coalescent (GMYC) (Pons *et al.,* 2006; Monaghan *et al.,* 2009) and statistical parsimony network analysis (Hart & Sunday 2007), have been developed based on different properties of DNA sequence variation indicative of the species level. Phylogenetic relationships among species have commonly been inferred from patterns of DNA sequence variation observed using a single marker. Yet single gene genealogies can in some cases be misleading, and the use of additional molecular markers is proposed as a way to improve species delimitation (Dupuis *et al.,* 2012; Leliaert *et al.,* 2014). Species boundaries are also more certain when combined with reproductive compatibility, morphological, geographic or ecological features (Bock *et al.,* 2011; Pröschold *et al.,* 2011; Dupuis *et al.,* 2012; Puillandre *et al.,* 2012; Hamilton *et al.,* 2014; Pardo *et al.*, 2014). In microscopic green algae, internal transcribed spacer 2 (ITS2) rDNA sequence regions are most commonly used for phylogenetic analysis, preferably in combination with the occurrence in their secondary structure of so-called compensatory base changes (CBCs), which appear correlated with reproductive compatibility (Coleman, 2003, 2007; Müller *et al.,* 2007; Wolf *et al,.* 2013). Generally, these findings are combined with morphological data (Pröschold & Leliaert 2007; Pröschold *et al.,* 2011). In some cases, the chloroplast *rbc*L gene is used as an additional marker (Rindi *et al.,* 2007; Fučiková *et al.,* 2011).

Despite the economic value of *H. pluvialis*, its phylogenetic diversity has not been thoroughly addressed. Recently, small subunit and large subunit rDNA phylogenies showed the polyphylethic nature of *Haematococcus*, resulting in a reduction of the genus from six species to one, *H. pluvialis* (Buchheim *et al.,* 2013; Pegg *et al.,* 2015). Buchheim *et al.,* (2013) confirmed the erection of the genus *Balticola* by Droop (1956a) for the other *Haematococcus* species. Droop (1956a) based his revision on morphological differences between *H. pluvialis* and the formerly described *Haematococcus droebakensis* (Wollenweber 1907, 1908) including its varieties and *Haematococcus buetschlii* Blochmann (1886). *Haematococcus pluvialis* mainly differed in its cell structure, by the possession of thin, cytoplasmic strands branched only at the extreme distal ends free of pigments and the possession of more than two pyrenoids (Droop, 1956a). Further distinguishing characters described were related to the mode of cell division, gametogenesis and encystment. Droop (1956a) established the genus *Balticola* and transferred *H. droebakensis* and *H. buetschlii* to this genus. In addition, he tried to clarify the complicated nomenclature of *Haematococcus* by arguing in favor of *H. pluvialis* against

*Haematococcus lacustris* (Girod-Chantrans) Rostafinski (Droop, 1956b). However, the erection of *Balticola* and the preference of *H. pluvialis* were rejected by Pocock (1960) and Almgren (1966).

The ITS2 rDNA phylogeny of 42 *H. pluvialis* strains presented by Buchheim *et al.,* (2013) showed the existence of five well-supported lineages in *H. pluvialis*, called A, B, C, D and E. Despite this, the authors suggested the conspecificity of all *H. pluvialis* strains based on the absence of compensatory base changes in the ITS2 secondary structure. In this study, we investigated species boundaries in European *Haematococcus* using strains isolated from 15 locations across Europe. For comparison, this European strain collection was complemented with South American isolates and public culture collection strains. Phylogenies were constructed based on sequences of the complete ITS rDNA (including ITS1-5.8S-ITS2) and, for a subset of strains, *rbc*L. GMYC and statistical parsimony network analysis were applied for species delimitation, and their congruence with morphology and temperature preferences was determined. In addition, the presence of CBCs in ITS1 and ITS2 was evaluated. Two new species are formally described, and denaturing gradient gel electrophoresis (DGGE) of the ITS1 was developed as a method for rapid species designation of European *Haematococcus* strains.

## 3.2. Material and methods

A total of 150 single cell isolations of *H. pluvialis* were made from 15 locations across Europe (Belgium, the Netherlands, Czech Republic, Hungary, Italy and Switzerland) (Table 1; Table S1, Supplementary Data). All isolated strains fit into the definition of *H. pluvialis* (Droop, 1956b). They were easily recognized based on the presence of protoplasmic strands and several pyrenoids in flagellated cells and their ability to transform into astaxanthin-rich aplanospores when stressed. Upon sampling, the cells were mostly in the aplanospore stage, often forming granular dehydrated crusts. Monoclonal cultures were obtained through isolation of single aplanospores by micromanipulation using a binocular microscope. All strains were grown in modified Bold's basal medium (3N-BBM+V; Bischoff & Bold 1963), with the initial pH adjusted to 7, at 23 °C and a 16:8 light:dark (L:D) regime provided by cool white fluorescent lamps at 20 µmol photons $m^{-2}$ $s^{-1}$. Two types of stock cultures were made of each strain for long-term preservation. The first are vegetative cultures with minimal cell division, kept at 6 °C with an 8:16 L:D cycle and ~5 µmol photons $m^{-2}$ $s^{-1}$. The second consisted of cultures of aplanospores obtained by applying light stress (irradiance up to 100 µmol photons $m^{-2}$ $s^{-1}$) to 3 week old cultures of which the cells were mostly in the palmelloid stage. A representative

number of strains, including the type strains BE08_06 and BE05_11 (see below), are kept cryopreserved at the Belgian Co-ordinated Collections of Micro- Organisms/Diatom Collection Ghent (BCCM/DCG) culture collection (http://www.bccm.belspo.be/catalogues/dcg-catalogue-search).

***Table1.*** *List of all Haematococcus sampling locations with information on sampling location, substrate type, geographical coordinates, sampling data and sample supplier.*

| Sample name | Sampling location | Type of substrate | Geographic coordinates | Sampling date | Collected by |
|---|---|---|---|---|---|
| BE01 | Ghent, Belgium | Dry crust on outdoor white table | 51°01'55.5"N, 03°43'48.4"E | 2 Sep. 2012 | Allewaert C. |
| BE02 | Munkzwalm, Belgium | Water puddle in aluminum wheelbarrow | 50°52'40.1"N, 03°43'59.0"E | 11 Sep. 2012 | Delbare D. |
| BE03 | Ghent, Belgium | Water puddle in concrete depression | 51°03'12.4"N, 03°42'26.9"E | 6 Aug. 2012 | Allewaert C. |
| BE04 | Aaigem, Belgium | Water puddle in white porcelain sink | 50°52'44.0"N, 03°55'58.9"E | 8 Feb. 2013 | Vyverman W. |
| BE05 | Ghent, Belgium | Water puddle on white bucket | 51°01'27.5"N, 03°42'38.8"E | 6 May. 2012 | Allewaert C. |
| BE07 | Ghent, Belgium | Water puddle on concrete floor | 51°01'04.3"N, 03°41'38.3"E | 25 Oct. 2012 | Allewaert C. |
| BE08 | Merelbeke, Belgium | Water puddle on green trash bin | 51°00'28.1"N, 03°45'38.5"E | 10 Feb. 2013 | Allewaert C. |
| BE09 | Mol, Belgium | Water puddle on blue boat on pond | 51°13'47.2"N, 05°10'40.2"E | 17 Jul. 2013 | Allewaert C. |
| BE10 | Ghent, Belgium | Rain water barrel | 51°04'29.6"N, 03°43'56.6"E | 17 Oct. 2013 | Vanormelingen P. |
| CZ01 | Třeboň, Czech Republic | Water puddle on white chair | 49°0'18.3"N, 14°46'23.8"E | 12 Feb. 2013 | Strunecky O. |
| HU01 | Ebes Hajdu-Bihar, Debrecen, Hungary | Rain water barrel | 47°28'15.3"N, 21°29'25.6"E | 1 May. 2012 | Basci I. |
| NL01 | Wageningen, The Netherlands | Grey rain water barrel | 51°59'15.5"N, 05°40'14.3"E | 6 Mar. 2013 | Wiezer S. |
| IT01 | Province of Pescara, Italy | Small water puddle in rock depression | 42°09'23.4"N, 14°00'11.4"E | 1 Apr. 2013 | Casteleyn G. |
| NL02 | Wageningen, The Netherlands | Water puddle on white bioreactor | 51°59'46.4"N, 05°39'29.4"E | 6 Jun. 2013 | Allewaert C. |
| NL03 | Vlissingen, The Netherlands | Water puddle on top of a container | 51°26'59.5"N, 03°35'19.2"E | 25 Jun. 2013 | Allewaert C. |

ITS rDNA sequences were obtained for two strains per sampled location from Europe (Table 1) and for strains from the Chilean culture collection (www.ficolab.cl), originating from South America (Culture Collection of Microalgae (CCM) of the Universidad de Concepción, Chile (UDEC) (020, 021, 022, 023, 024, 031, 032, 033, 034, 035, 036, 037, 038, 039, 041), Austria (CCM-UDEC 029), the United States (CCM-UDEC 019) and Japan (CCM-UDEC 0028). For comparison, these strains were complemented with 14 often cited strains from several culture collections; the Culture Collection of Algae and Protozoa, UK (CCAP) (34/7, 34/1D, 34/12, 34/13 and 34/14), Sammlung von Algenkulturen at the University of Göttingen (SAG) (34-1a, 34-1h, 34-1f, 192.80, 49.94 and 44.96), the Scandinavian Culture Center for Algae and Protozoa (SCCAP) (K-0084, K-0404) and the Culture Collection for the National Institute for

Environmental Studies, Japan (NIES) (144). Additionally, *rbc*L sequences were obtained for a subset of 13 strains representative of the three ITS rDNA lineages to which our strains belong (see results) to support species delimitation. For DNA analysis, subsamples (1 mL) of the cultures in exponential phase were concentrated by centrifugation and frozen at 80 °C. DNA extraction followed Zwart *et al.,* (1998), excluding the last purification step.

The ITS rDNA region was amplified by polymerase chain reaction (PCR) using the universal primers ITS4 (White *et al.,* 1990) and 1800F (Friedl, 1996). PCR reaction mixtures contained 1 µL of the template DNA, 0.5 µM of primers, 200 µM of each deoxynucleoside triphosphate, 0.4 µg $\mu L^{-1}$ of bovine serum albumin, 2.5 µL of 10× PCR buffer (Tris-HCl, $(NH_4)_2SO_4$, KCl, 15 mM MgCl2, pH 8.7 at 20 °C; 'Buffer I', Applied Biosystems, Foster City, California USA) and 1.25 U of Taq polymerase (AmpliTaq, Perkin-Elmer, Wellesley, Massachusetts USA). The mixtures were adjusted to a final volume of 25 µL with high performance liquid chromatography water (Sigma, St. Louis, Missouri USA). The PCR conditions were as follows: 30 cycles (1 min at 94 °C, 1 min at 48 °C and 2 min at 72 °C) with an initial denaturing step at 7 min at 94 °C and a final step of 10 min at 72 °C. The DNA extraction and PCR amplification of the additional strains from Chilean culture collection (strains CCM-UDEC) were conducted following the protocol described in Demchenko *et al.,* (2012).

Primers used for *rbc*L amplification and sequencing were chosen and combined from Nozaki *et al.,* (1995, 1997). The PCR reaction mixture was the same as for ITS. Because of the presence of introns in several of the first obtained sequences, the complete *rbc*L was obtained using three separate PCR and sequencing reactions. The first PCR product was obtained using primers P1–20 (Nozaki *et al.,* 1995) and P686–665 (Nozaki *et al.,* 1997). The second product was obtained using primers P650–671 and P1421–1402 (Nozaki *et al.,* 1995). Both PCR reactions were performed under following conditions: 30 cycles of 94 °C for 1 min to denature, annealing at 46 °C for 2 min and an extension step at 68 °C for 3 min, with an initial denaturing step of 3 min 94 °C and a final step at 72 °C for 15 min. For the third step PCR primers P320–341 and P1181–1160 (Nozaki *et al.,* 1995) were used. The reaction conditions were 35 cycles of denaturation at 94 °C for 1 min, followed by annealing at 50 °C for 1 min and an extension step at 72 °C for 1 min, with an initial denaturation step of 5 min at 94 °C and a final step at 72 °C for 10 min. The three PCR products were sequenced with their respective primers. All sequencing (ITS and *rbc*L) was performed by Beckman Coulter Genomics. Sequences were

assembled and partial *rbc*L sequences combined in BioNumerics 5.10 (Applied Maths, Sint-Martens-Latem, Belgium).

ITS and *rbc*L sequences were aligned automatically with subsequent manual correction using MEGA version 5.1 (Tamura *et al.,* 2011). Previously obtained yet unpublished ITS rDNA sequences from 14 *H. pluvialis* (from the Chilean culture collection) were also included for comparison (Table S1, Supplementary Data), as well as all ITS2 sequences available on Genbank on 15/8/2014. *Rbc*L sequences were complemented with sequences from Genbank 1000 bp long. Prior to phylogenetic analysis, the *rbc*L intron was left out, resulting in 1341 positions in the *rbc*L alignment. The ITS rDNA alignment consisted of 624 positions that were used for phylogeny reconstruction. Alignment of the ITS1 and ITS2 was guided by thermodynamically stable secondary structures that were computed by the Mfold server (Zuker, 2003); the ITS2 was constrained according to the sequence-structure alignments proposed by Buchheim *et al.,* (2013). CBC matrices were derived by 4SALE (Seibel *et al.,* 2006, 2008) and secondary structure figures made using VARNA (Darty *et al.,* 2009).

Phylogeny reconstruction was done using maximum likelihood (ML) and Bayesian Inference (BI). Separate analyses were done for the ITS rDNA (including only complete ITS sequences) and the ITS2 rDNA, given that most sequences on Genbank comprise only the ITS2. The most appropriate nucleotide substitution model was selected using jModeltest (Posada 2008) using default settings, under the Bayesian information criterion (Posada & Buckley 2004). For ML, it concerned TPM2 +G for both ITS and ITS2 and TrN+G for the *rbc*L. For BI, SYM+G (ITS), K2P+I (ITS2) and GTR+G (*rbc*L) were used. ML phylogeny reconstruction was done in MEGA 5.1 (Tamura *et al.,* 2011). The number of different rate categories was set at six for ITS and four for ITS2, and positions with gaps or missing data were not included in the analysis when they were less than 50 % complete. The robustness of the resulting clades was tested using a bootstrap analysis with 1000 replications. BI was performed in MrBayes 3.2 (Ronquist & Huelsenbeck 2003; Ronquist *et al.,* 2011). No initial values were assigned to the model parameters. Two runs of four Markov Chains (one cold and three heated) were run for 10 million generations and sampled every 500 generations. This yielded a posterior probability (PP) distribution of 20,001 trees. After exclusion of 10,000 'burn–in' trees, PPs were calculated by constructing a 50 % majority-rule con*sensu*s tree. For ITS, no outgroup was specified because of alignment difficulties in the most variable parts, which are the most informative for closely related lineages. *Chlorogonium fusiforme* Matvienko was used as an outgroup in an

*rbc*L tree, as it was found to be closely affiliated with *H. pluvialis* (Buchheim *et al.,* 2013). Uncorrected pairwise *P* distances and the number of differences were calculated in MEGA 5.1. All sequences were submitted to Genbank [accession numbers are given in (Table S1, Supplementary Data) and the DNA barcode database (www.boldsystems.org).

For statistical parsimony network analysis of the ITS rDNA region, the 95 % connection limit, indicative of species limits (Hart & Sunday 2007), was calculated using TCS v1.21 (Clement *et al.,* 2000). The sequences of strains NL01_04, BE04_09 and BE10_09 were not included in the analysis because the ends were missing. Positions containing ambiguous or missing base(s) were omitted before analysis. For GMYC analysis based on the ITS rDNA region, a BI phylogeny was constructed as specified above but under a fixed molecular clock model. When several strains had exactly the same sequence, only one sequence was included for the BI analysis. GMYC was performed on the most likely BI tree under a single threshold model (Monaghan *et al.,* 2009) using the SPLITS package in RStudio version 0.98.1102 (R Core Team, 2013).

To identify strains without sequencing, a DGGE of the ITS1 rDNA (400 bp region) was developed. Amplification of fragments for DGGE analysis was done using the 1800F forward primer (Friedl 1996), with the addition of a GC clamp (van Gremberghe *et al.,* 2011) and the DITS2 reverse primer (Zechman *et al.,* 1994). PCR reaction conditions for the ITS rDNA were as described above. Band separation was achieved using an acrylamide gel with a 35–55 % denaturant gradient (100 % denaturant corresponding to 7 M urea and 40 % (v/v) formamide). Other conditions for electrophoresis were as described in van Gremberghe *et al.,* (2011).

Growth rate as a function of temperature was determined for 13 strains. Strains for which the ITS rDNA was not sequenced were identified using DGGE (Table S1, Supplementary Data). Five strains from different geographic locations for the two most-represented lineages were selected. For the third lineage the three available strains, all from one location, were used. Additionally, four culture collection strains, NIES 144, SCCAP K-0084, CCAP 34/1D and SAG 192.80, were included. Two months before the experiment, the strains were taken from stock culture (6 °C) and cultivated in exponential growth phase. Four days before the start of the experiment, they were all reinoculated at the same density, corresponding to an $F_0$ of 0.003 (as measured using a PAM fluorometer; see below) to 24 well plates, to make sure they were all in the same physiological condition at the start of the experiment. The plates were placed on a temperature gradient table (Labio Ltd., Prague, Czech Republic) with three randomly

positioned replicates per strain per temperature. Cultures were exposed to nine different temperatures: 11.6 ± 0.6 °C, 14.8 ± 0.5 °C, 18.0 ± 0.3 °C, 21.2 ± 0.2 °C, 24.3 ± 0.3 °C, 27.3 ± 0.3 °C, 30.3 ± 0.5 °C, 33.4 ± 0.6 °C and 36.2 ± 0.7 °C (as measured using a calibrated TTX 100-thermometer, Ebro, Germany). The light regime was continuous with an intensity of 25.4 ± 0.7 μmol photons $m^{-2}$ $s^{-1}$. Culture growth was monitored daily for 12 days by pulse amplitude modulated (PAM) fluorescence (MAXI Imaging PAM fluorometer, Walz, Germany) using settings 3, 1 and 2 for intensity, gain and damping, respectively. Otherwise default settings were used. The minimum fluorescence yield $F_0$ was used as proxy for biomass (Honeywill *et al.,* 2002; Consalvey *et al.,* 2005). Prior to measurement, cultures were dark acclimated for 15 min at room temperature. After measurement, the different well plates exposed to the same temperature were replaced in random order on the temperature gradient table. The experiments were carried out in triplicate. Because of the limited number of wells that could be exposed to each temperature on the table, the experiment was performed twice, with the replicates for each strain randomly divided over both experiments.

Growth rate was calculated as the slope of the linear regression of the log2-transformed $F_0$ fluorescence vs time for each culture during the exponential phase. The relation between growth rate and temperature was modelled as in Yan & Hunt (1999) using the function:

$$\mu = \mu max \cdot \left(\frac{Tmax - Topt}{Tmax - T}\right) \cdot \left(\frac{T}{Topt}\right)^{\frac{Topt}{Tmax - Topt}},$$

where $T_{max}$ and $T_{opt}$ are the maximum and optimum temperature for growth, and $\mu_{max}$ is the maximum rate of growth at $T_{opt}$. The equation was fitted to the growth at different temperatures for each replicate in R version 3.0.2 (R Core Team, 2013) using the nonlinear least squares function. Growth rates for temperatures above 33 °C were omitted before making the fits, since cultures were either dying or encysted in these conditions, and negative growth rates could not be quantified. Estimated optimal and maximal temperatures ($T_{opt}$ and $T_{max}$) as well as maximal growth rates ($\mu_{max}$) were compared between strains and species by one-way ANOVA using Statistica version 6.0 for Windows (StatSoft, Tulsa, Oklahoma USA).

A total of 35 strains (17, 13 and 5, respectively, of each of the three lineages) were examined morphologically in exponential growth phase. Up to three strains per source location were randomly chosen. Strains for which the ITS rDNA was not sequenced were identified using DGGE. Our own strains were complemented with eight culture collection strains, CCAP

(34/1D, 34/7, 34/12, 34/13, 34/14), SAG 192.80, NIES 144 and SCCAP K-0084. Living cells were observed and photographed using a Zeiss Axioplan 2 Universal microscope equipped with an AxioCam MRm monochrome digital camera (Zeiss Gruppe, Jena, Germany). The following morphological characters were determined based on the description proposed by Droop (1956b): the number of pyrenoids, the shape of the protoplast apex (round, round/pear, pear, extreme pear) the presence and characteristics of cytoplasmic strands (delicate or thick, branched or unbranched, all over the cell or concentrated). In addition, the length and width of the cell and protoplast were measured (n = 7–25 for each strain) and the difference between length and width determined. Morphology of the encysted stages was not studied, as cyst size and morphology was highly variable within each strain using light microscopy. All measurements were made using the Axiovision Release 4.4 (Zeiss Gruppe) camera software. Principal component analysis (PCA) was carried out to visualize patterns of morphological variation using CANOCO version 4.5 for windows (Biometrics–Plant Research International, Wageningen, the Netherlands). Owing to the absence of cytoplasmic strands in several strains, the characteristics of the cytoplasmic strands were not used for the analysis. Morphological variables were standardized before performing the PCA. The significance of species identity (included as dummy variables, significance of all canonical axes together, 9999 permutations, default settings) for the morphological variation among strains was tested using a redundancy analysis (RDA).

## 3.3. Results

### 3.3.1. Phylogenetic analyses

Six lineages were resolved in the ITS rDNA phylogeny (Fig. 1). All the European strains studied here and most of the culture collection strains belonged to one of three well- supported lineages. These corresponded to three of the five ITS2 rDNA lineages outlined by Buchheim *et al.,* (2013) (named A, C and E; see Fig. 1 and the ITS2 rDNA phylogeny in Fig. S1, Supplementary Data). Lineages A and C also contained all strains from the Chilean culture collection, except strain CCM-UDEC 028 from Japan. Three additional lineages were only represented by a single culture collection strain; one of them corresponding to Buchheim's lineage D, the second contained a strain (SAG 34-1f) included in lineage C by Buchheim *et al.,* (2013) but which had to be excluded from lineage C based on our findings. Finally, the third lineage contained the strain from Japan (CCM-UDEC 028). Although we included several strains from the United States (CCAP 34/12, CCAP 34/13, CCAP 34/13) in our phylogeny, no

representatives of the lineage B *sensu* Buchheim (from which all strains originated from the US) were present (Fig. S1, Supplementary Data). Given that our strains belonged to the lineages A, C and E, we further focused on these lineages. Uncorrected *P* distances were 11.2–13.3 % (68–77 bp) between A and E, 10.0–12.2 % (58–71 bp) between A and C and 12.2– 13.1 % (70–75 bp) between E and C. *P* distances within these three lineages were 0.0–2.4 % (0–14 bp). Lineage C differed by 10.4–11.3 % (58–64 bp) from strain SAG 44.96 (lineage D), while strains CCM-UDEC 028 and SAG 34-1f differed by 4.1–5.6 % (24–34 bp) from lineage C.

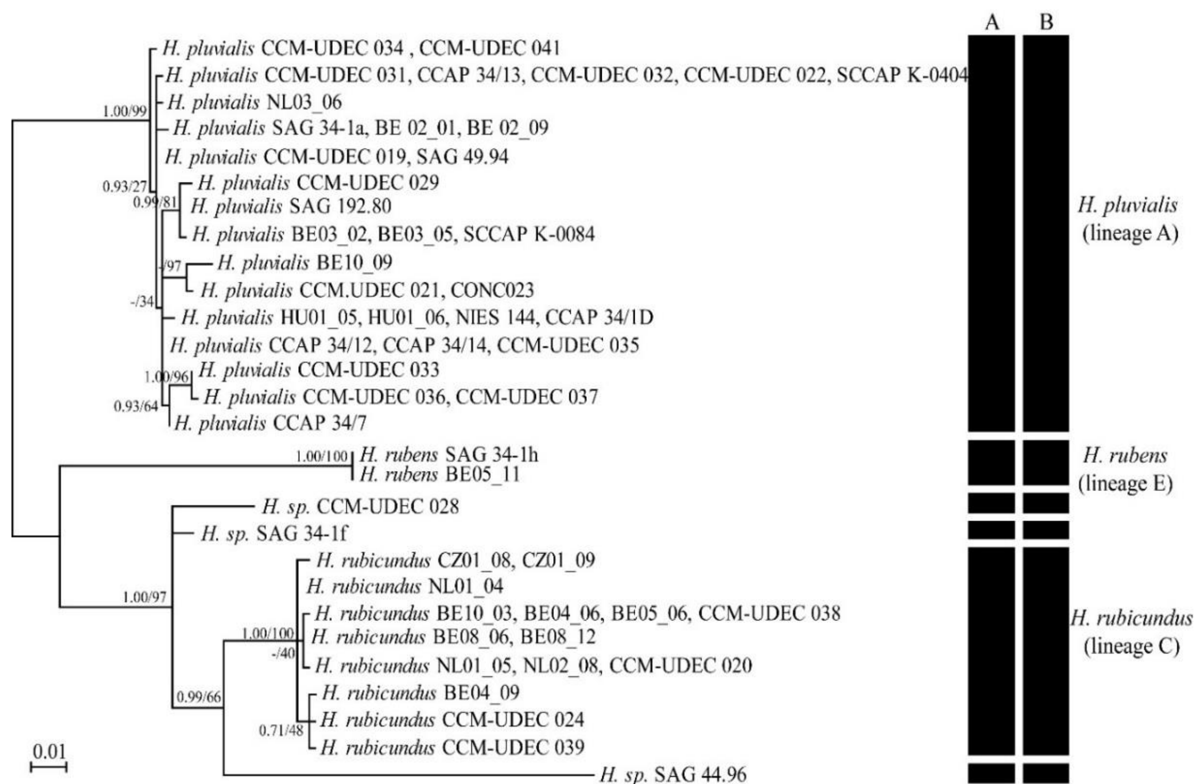



***Figure 1.*** *Haematococcus maximum likelihood phylogeny based on ITS rDNA (ITS1-2.8S-ITS2) sequences. Bayesian posterior probabilities (left) and ML bootstrap values (right) are indicated at the nodes. Species names are given according to the taxonomic changes made in the present paper. The vertical black bars indicate the different species as outlined using (A) statistical parsimony network analysis and (B) GMYC. All strains were sequenced in this study.*

The statistical parsimony network analysis recovered six independent haplotype networks corresponding to the six lineages outlined above (indicated on Fig. 1). The same six independent haplotype networks were recovered whether gaps were treated as a fifth base or as missing data. The GMYC model was highly significant (*P* = 0.00095). Two clusters (confidence interval 2–

3) and 6 ML entities (confidence interval 6–7) were outlined, again corresponding to the six lineages mentioned above (indicated on Fig. 1; see also Fig. S3, Supplementary Data).

The ITS1 and ITS2 secondary structure models of strain BE08_06 (lineage C) are shown in Fig. 2. The secondary structures were very similar for all strains, including four helices for both ITS1 and ITS2. The original ITS2 structure folded with Mfold differed from that in Buchheim *et al.,* (2013). The basal part of Helix III was thermodynamically very stable; as a result, Helix IV presented by Buchheim *et al.,* (2013) was incorporated as side loop in Helix III. We therefore constrained the structure not to bind centrally, forcing it to have a common core structure comprising four helices (Mai & Coleman 1997; Caisová *et al.,* 2013). This did not result in any changes in the number and position of CBCs. Generally, only a few CBCs were found. Three CBCs were found in the ITS1 secondary structure. A CBC was found between lineage E and lineages A and C in Helix I. A second CBC was found in Helix I between strains of lineage C and lineage A, not in E (as indicated in Fig. 2). A third CBC was found between lineage C and strains BE10_09, CCM-UDEC 021 and CCM-UDEC 023 in Helix III. Within the ITS2 secondary structure, one CBC in Helix III was found between strains BE04_09, CCM-UDEC 024 and CCM-UDEC 039 (lineage C) and all strains of lineage A and E. Finally, the lineage with strain SAG 44.96 (clade D in Buchheim *et al.,* 2013) differed from the others by two to three CBCs in ITS1 and one in ITS2. No CBCs were found in the ITS1 and ITS2 between strains CCM-UDEC 028 and SAG 34-1f (belonging to separate lineages) and strains from lineage A, C or E.

The *rbc*L alignment showed much less variation than the ITS, with only 0–45 variable positions (0–0.4 %). Nevertheless, three lineages could be distinguished in the *rbc*L phylogeny (Fig. 3). All isolated European strains and culture collection strains belonged to two lineages with very high support. The third *rbc*L lineage was represented by a Genbank sequence from a strain (KORDI03) for which the ITS rDNA sequence is not available. *P* distances between the two lineages containing our strains were 0.2–0.4 % (29–45 bp). The first lineage corresponded to two sister lineages A and E in the ITS rDNA phylogeny. All four ITS lineage A strains had identical *rbc*L sequences. The *rbc*L of the single included ITS lineage E strain, which differed 1 bp from the ITS lineage A strains. Apart from our own strains, this lineage also contained several Genbank sequences from *H. pluvialis*, which differed from our strains by 0–0.19 % (0–22 bp). However, given that these differences were concentrated at the 30 end of the *rbc*L alignment, most might concern sequencing errors rather than existing variation. The second

*rbc*L lineage corresponded to ITS lineage C, and all eight *rbc*L sequences of this clade were identical.

The three ITS rDNA lineages within our European *H. pluvialis* isolates were supported by both automated phylogenetic species delimitation methods, and we concluded that these represented three species. Moreover, two of those lineages were supported by the *rbc*L phylogeny, despite its limited resolution. The lineage A containing most of our strains contains the eptiype strain SAG 192.80 (see proposal below); the two other lineages are described below as new species, *Haematococcus rubicundus* and *Haematococcus rubens*, corresponding to lineages C and E, respectively. Hereafter, we refer to the three lineages A, C and E by species name.

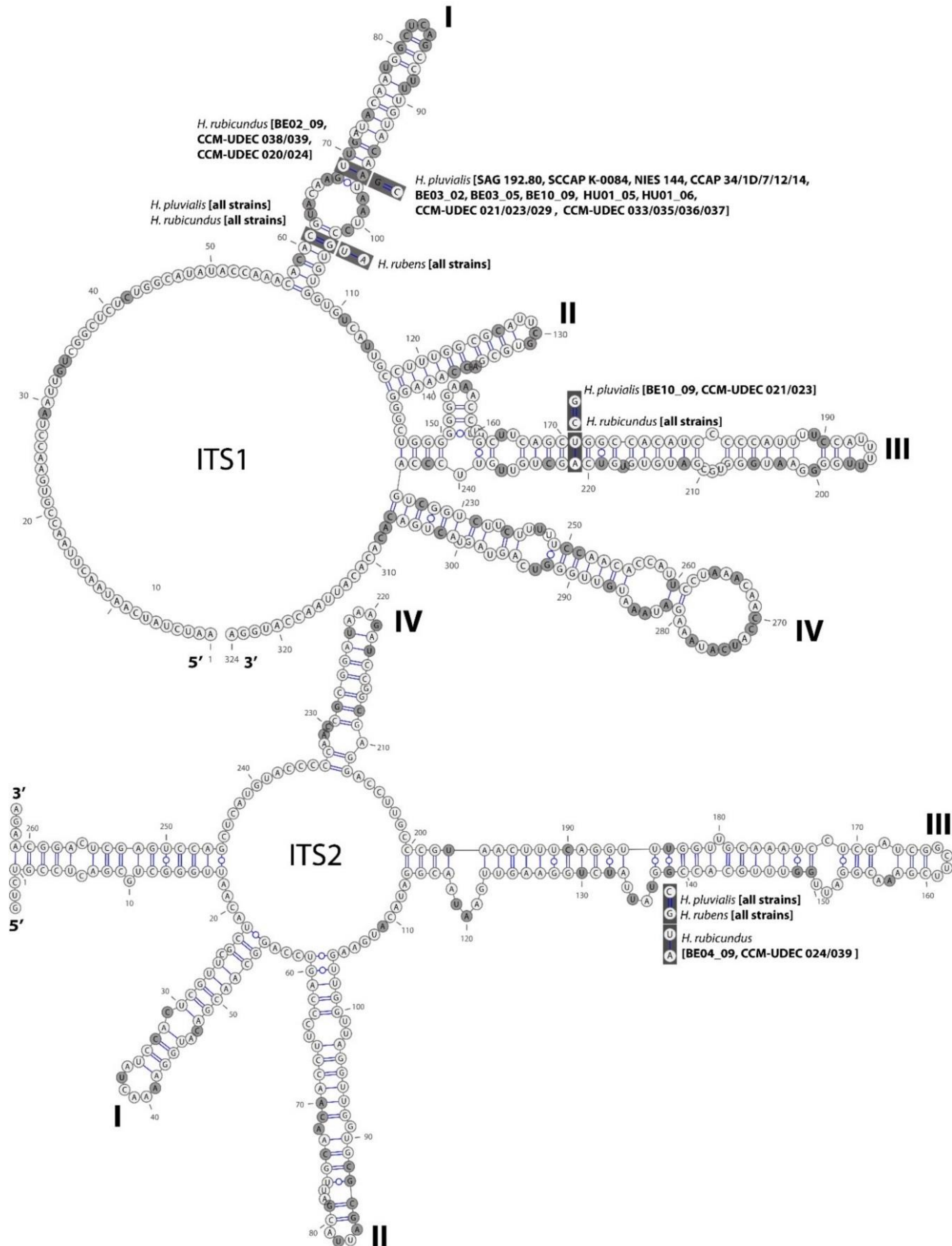



***Figure 2.*** *Diagrams of the secondary structures of ITS1 and ITS2 of Haematococcus rubicundus strain BE08_06. Variable bases within ITS1 and ITS2 are indicated in grey; compensatory base changes between and within different lineages are marked in black boxes.*

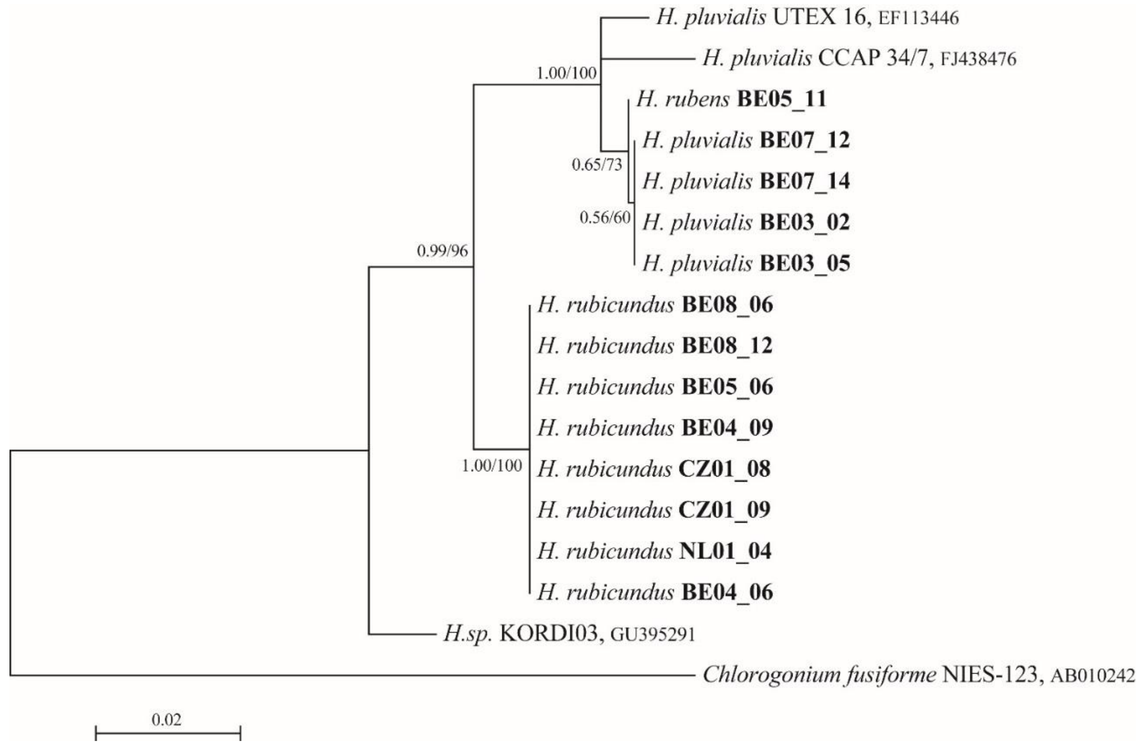



***Figure 3.*** *Haematococcus maximum likelihood phylogeny based on rbc*L *sequences. (Left) Bayesian posterior probabilities and (right) ML bootstrap values are indicated at the nodes. Chlorogonium fusiforme was chosen as outgroup. Strains in bold were sequenced.*

### 3.3.2. DGGE

DGGE separated the ITS1 rDNA variants of *H. pluvialis*, *H. rubicundus* and *H. rubens* (Fig. 4). As a result, the method could be used for rapid identification of additional European temperate strains for temperature preference and morphology assessments. This also showed the co-occurrence of *H. pluvialis* and *H. rubicundus* in samples originating from Italy (IT01) as well as *H. rubicundus* and *H. rubens* in Belgian samples (BE04, BE05)(Table 1).

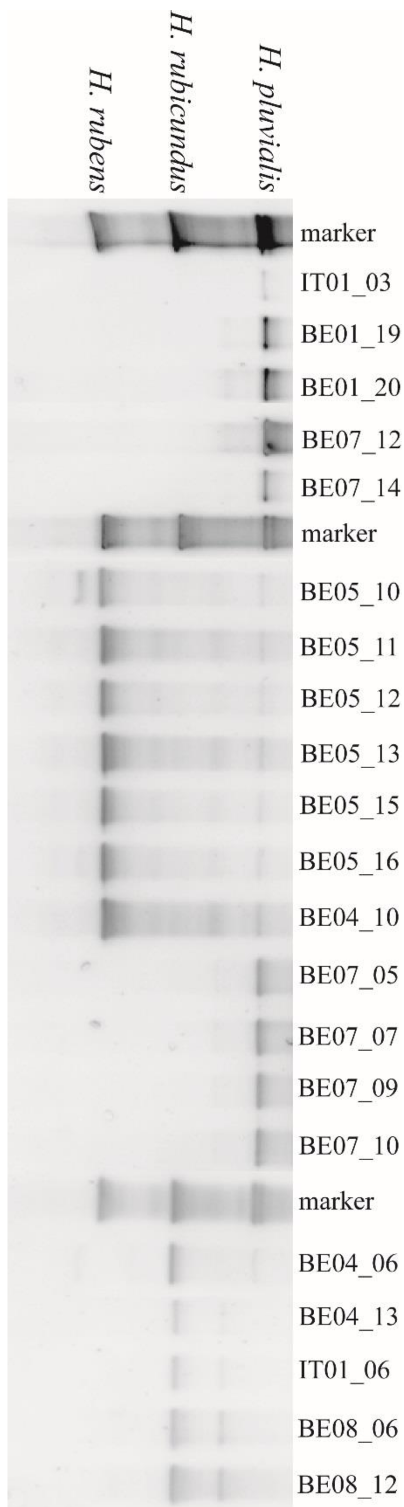



***Figure 4.*** *Denaturation gradient gel electrophoresis of the ITS1 rDNA sequences of Haematococcus strains belonging to three lineages. DGGE gel from natural strains of Haematococcus. The marker contains a mixture of DNA from three strains belonging to different lineages, mixed before PCR. Species names are given according to the taxonomic changes.*

### 3.3.3. Temperature preferences

Average growth rates with respect to temperature for the three species are shown in Fig. S2, Supplementary Data. Generally, growth was close to maximal between 15 °C and 27 °C and dropped steeply at more extreme temperatures. The maximal growth rate ($\mu_{max}$) of all strains examined varied between 0.41 ± 0.01 divisions day$^{-1}$ and 0.74 ± 0.14 divisions day$^{-1}$ (Fig. 5). Strains of *H. pluvialis* had on average a lower $\mu_{max}$ than *H. rubicundus* and *H. rubens* ($P$ = 0.048). There were also differences between strains within *H. pluvialis* ($P < 0.001$), *H. rubicundus* ($P = 0.002$) and *H. rubens* ($P < 0.002$). Modelled optimal temperatures ($T_{opt}$) for the strains varied between 17.4 ± 0.8 °C and 23.1 ± 0.7 °C. While there were highly significant strain differences in $T_{opt}$ within both *H. pluvialis* and *H. rubicundus* ($P < 0.001$ and $P = 0.006$, respectively), there was no significant difference between species ($P = 0.157$). Finally, the maximal temperature ($T_{max}$) of the strains varied from 30.4 ± 1.3 °C to 36.3 ± 0.1 °C. There was no significant difference in $T_{max}$ between species ($P = 0.959$) but strains differed significantly from one another within *H. pluvialis* ($P < 0.001$) and *H. rubicundus* ($P < 0.001$). The three strains of *H. rubens* showed no significant strain differences in $T_{max}$.

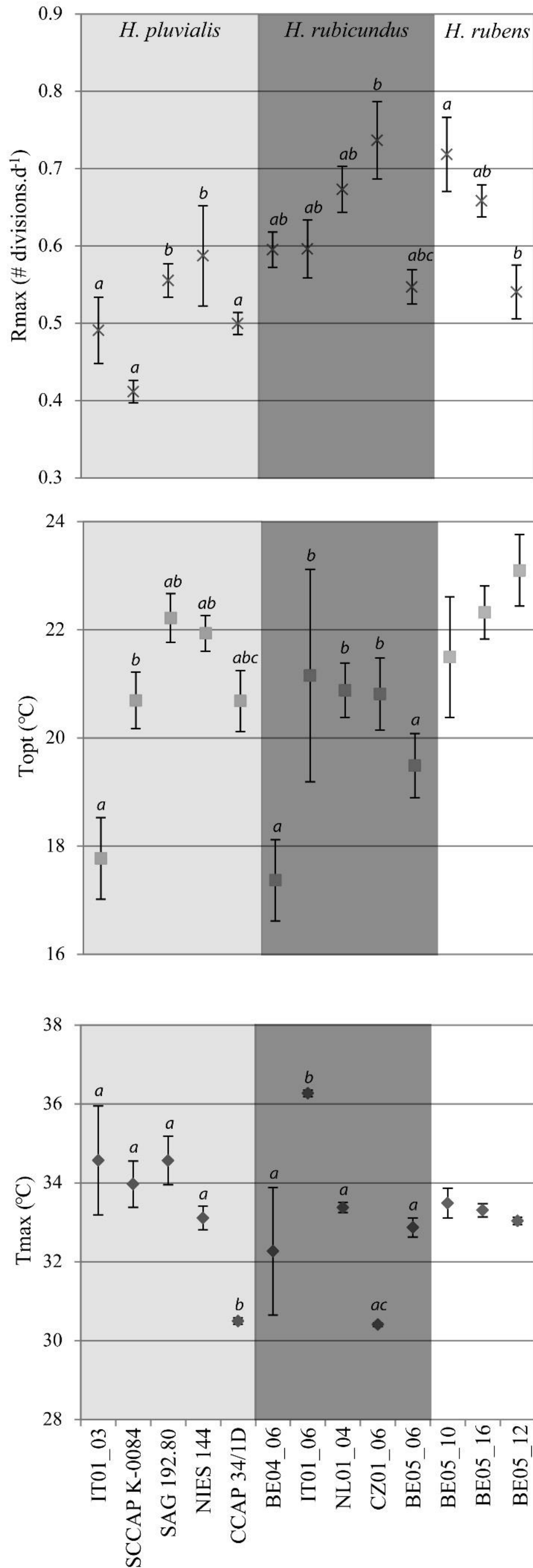


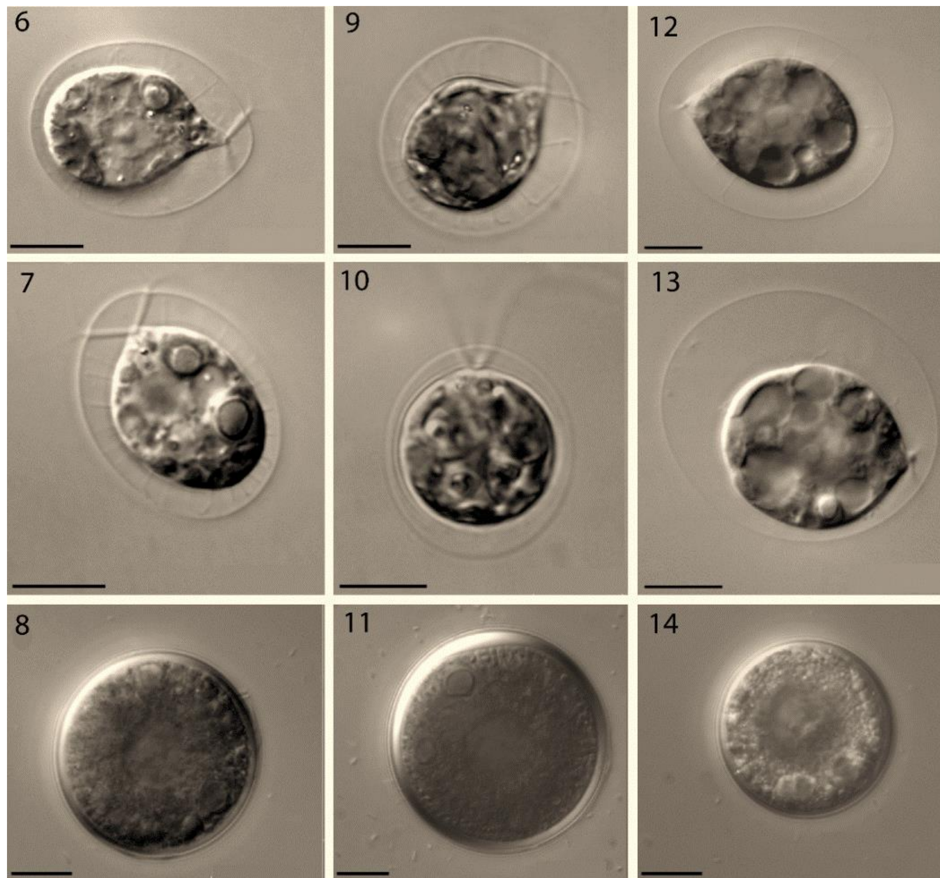

***Figure 5.*** *Graphs showing the optimal growth temperature (Rmax), average optimal temperature for growth ($T_{opt}$) and average maximal temperature for growth ($T_{max}$) of 13 strains of H. pluvialis, H. rubicundus and H. rubens determined by fitting the Yan & Hunt (1999) equation to the growth at different temperatures for each replicate. The error bars correspond to the standard deviation for each strain. Different letters show significant differences between the groups at each time point ($P < 0.05$).*

### 3.3.4. Morphology

Light microscopy pictures of vegetative cells and aplanospores of the three species are shown in Figs. 6–14. Morphological measurements and observations are given in the DNA barcode database BOLD (project entitled *Haematococcus*) and summarized in the biplot of a principal component analysis (Fig. 15). First, a large variation within and among the strains in morphology was not related to species-level differences for following morphological characteristics: protoplast and cell size (length and width) and the number of pyrenoids but also in the protoplast apex shape and when present, the characters of the cytoplasmic strands. Second, despite the large overlap, species identity did influence strain morphology, as indicated by an RDA ($P$ = 0.021) for following characteristics. Strains combining a large difference in protoplast length and width (>2) and at least some protoplasts with an (extreme) pear shape and cytoplasmic strands (14 out of 22 strains) invariably belonged to *H. pluvialis* or *H. rubens*. Strains of *H. rubicundus* had cells that were on average less elongated, had a rounded protoplast apex and delicate cytoplasmic strands that were difficult to discern. *H. pluvialis* and *H. rubens* did not differ morphologically from each other (Fig. 15). Also, thickness, branching type and location of the cytoplasmic strands were highly variable between the strains and did not show clear differences between *H. pluvialis* and *H. rubens* (but note the small number of strains investigated in the latter).

### 3.3.5. Nomenclatural and taxonomic changes

The genus *Haematococcus* was originally described by Agardh (1828–1835) with three species: *Haematococcus noltii* (= *Euglena sanguinea* Ehrenberg), *Haematococcus sanguinea* (= *Gloeocapsa sanguinea* (Agardh) Kützing) and *Haematococcus grevillei* (= *Haematococcus pluvialis* Flotow). The last species clearly represented *Haematococcus*; however, the original description by Agardh was incomplete and contained some uncertainties. Therefore, Silva (1980) proposed to conserve the name *Haematococcus* described by Flotow (1844) against *Haematococcus* Agardh (1828–1835), which was accepted by the International Code for Botanical Nomenclature (Voss & Greuter 1981). The complicated nomenclatural history of *Haematococcus* in the context of earlier described genera such as *Sphaerella* and *Protococcus* was clarified by Wille (1903). The nomenclatural status was recently resolved by Doweld (2013), and the genus *Haematococcus sensu* Flotow, as emended by Wille, has been accepted by the scientific community.




***Figures 6–14.*** *Light microscopy pictures of the vegetative cells and aplanospores of Haematococcus strains.* ***Figs. 6–8.*** *Vegetative cells of (6) H. pluvialis strain BE02_09 and (7) strain SCCAP K-0084 and (8) cyst of strain BE02_09. The vegetative cell has an elongated shape, discernable threadlike cytoplasmic strands and an extremely pear-shaped apex.* ***Figs. 9–11.*** *Vegetative cells of (9) H. rubicundus strain IT01_06 and (10) strain NL01_04 and (11) cyst of strain IT01_06. Cell and protoplast are less elongate, thick branched cytoplasmic strands, if present (9) are distributed over the entire protoplast.* ***Figs. 12–14****. Vegetative cells of (12) H. rubens strain BE05_11 and (13) strain BE05_12 and (14) cyst of strain BE05_11. Vegetative cells have a more elongated shape, protoplasmic strands are difficult to discern. Scale bar = 10 µm.*

**_Haematococccus_ Flotow emend Wille, _nom. cons_.**

ORIGINAL DESCRIPTION: Flotow (1844). Über *Haematococccus pluvialis*. Novorum Actorum Academia Caesareae Leopoldinae-Carolinae Naturae Curiosorum 20: 537–557, pl. XXV, fig. 70.

TYPE SPECIES: *Haematococccus pluvialis* Flotow emend Wille, *typ. cons*.

**_Haematococccus pluvialis_ Flotow emend Wille, _typ. cons._**

ORIGINAL DESCRIPTION: Flotow (1844). Über *Haematococccus pluvialis*. Novorum Actorum Academia Caesareae Leopoldinae-Carolinae Naturae Curiosorum 20: 537–557, pl. XXV, fig. 70.

EMENDED: Wille (1903). Nytt Magazin for Naturvidenskapene 41: 104.

LECTOTYPE (DESIGNATED HERE): Flotow (1844), pl. XXV, fig. 70.

EPITYPE (DESIGNATED HERE IN SUPPORT OF THE LECTOTYPE): The strain SAG 192.80 (proposed here as an authentic strain of *H. pluvialis*) is cryopreserved in the Sammlung von Algenkulturen (SAG) University of Göttingen, Germany.

COMMENTS: As mentioned in the introduction, the nomenclatural situation of *H. pluvialis* and *H. lacustris* (Girod- Chantrans) Rostafinski was a controversial topic discussed in several publications. From the original publications it is very difficult to decide whether *H. lacustris* (=*Volvox lacustris* Girod-Chantrans) is identical to *H. pluvialis*. We recommend following the arguments presented by Wille (1903) and Droop (1956b).

### *Haematococccus rubicundus* **Allewaert & Vanormelingen** *sp. nov*.

Figs. 9-11

DESCRIPTION: Vegetative cells generally spherical to ovoid, average 26.5 µm long (range 23.7–34.5 µm), 23.5 µm wide (range 20.3–31.3 µm), with a length: width difference of 3.0 µm (range 1.1–5.2 µm). Papillae absent, cell membrane hyaline, wide. Average protoplast length 21.3 µm (range 18.2–31.4 µm) and average width 19.5 µm (range 17.0–25.3 µm), with a mostly rounded off apex but sometimes (extremely) pear shaped. Cytoplasmic strands generally absent, or at least invisible; when present, mostly branched and spread over the entire protoplasm. In some cases cytoplasmic strands limited to the cell side opposite the flagella. Two isokont anterior flagella present, these as long as the cell, surrounded by short divergent tubes at the base. Chloroplast cup-shaped, occupying 1/3 of the protoplast and containing 1–11 scattered spherical pyrenoids. Nucleus centrally located. Cells in old cultures turn red/orange, lose their flagella, gradually increase in size, forming a resistant round spore enclosed by a thick cell wall. Found in temporary water pools or puddles. Differs in ITS and *rbc*L sequence from *H. pluvialis* and *H. rubens*. Morphologically very similar to and overlapping with *H. pluvialis* and *H. rubens*. On average, vegetative cells and protoplasts had a smaller length: width ratio. The

protoplast apex was more often round, and cytoplasmic strands were more often missing or so fine that they were not visible compared with the other two *Haematococccus* species.

HOLOTYPE: Strain BE08_06 collected by Allewaert C., cryopreserved at the BCCM/DCG culture collection (accession number DCG 0575).

TYPE LOCALITY: From a water puddle on a trash bin, Merelbeke, Belgium.

ETYMOLOGY: *Rubicundus* (Latin for red) for its capacity to produce the red keto-carotenoid astaxanthin.

GENBANK ACCESSION NUMBER: ITS (KR914742) and *rbc*L (KR914700).

ADDITIONAL MATERIAL: Strains BE04_06, BE04_09, BE04_13, BE05_06, BE08_12, BE10_03, CZ01_06, CZ01_08, CZ01_09, NL01_04, NL01_05, IT01_06, NL02_08 cryopreserved at BCCM/ DCG culture collection. Strains CCM-UDEC 038, CCM-UDEC 039, CCM-UDEC 024, CCM-UDEC 020 and strains SAG 34-1m, SAG 34-1c and SAG 34-1f preserved at SAG. Finally, strain HP065 collected by Buchheim *et al.,* (2013).

DISTRIBUTION: Specimens were found in Europe (Belgium, the Netherlands, Czech Republic, Spitsbergen, Switzerland, Italy), the United States (Tulsa) and South America (Bolivia, Chile).

***Haematococcus rubens* Allewaert & Vanormelingen *sp. nov***

Figs. 12-14

DESCRIPTION: Cells dominantly ovoid, average 31.4 µm long (range 24.2–34.8 µm) and 26.9 µm wide (range 20.2–31.2 µm) with a length: width ratio of 4.5 (range 3.6–5.5 µm). Papilla absent; cell membrane hyaline, wide. Protoplast on average 23.0 µm long (range 17.7–25.3 µm) and 19.4 µm wide (range 15.8–21.6 µm). Apex predominantly pear to extremely pear shaped. Cytoplasmic strands mostly but not always present. When present, thick, generally branched and distributed over the entire protoplasm. Two isokont anterior flagella present, these as long as the cell and surrounded by short divergent tubes at the base. Chloroplast cup-shaped, occupying 1/3 of the protoplast and with several scattered spherical pyrenoids (2–6). Nucleus centrally located. Old cultures turn red/ orange, lose their flagella, gradually increase in size, forming a resistant round spore enclosed by a thick cell wall. Found in temporary water pools

or puddles. Differs from *H. pluvialis* and *H. rubicundus* in ITS rDNA and from *H. rubicundus* in *rbc*L. No morphological differences with *H. pluvialis* for the morphological parameters considered.

HOLOTYPE: Strain BE05_11, collected by Allewaert C., cryopreserved at the BCCM/DCG culture collection (accession number DCG 0570).

TYPE LOCALITY: Collected from a water puddle on a white bucket, Ghent, Belgium.

ETYMOLOGY: *Rubens* (Latin for red) for its capacity to produce the red keto-carotenoid astaxanthin.

GENBANK ACCESSION NUMBER: ITS (KR914735) and *rbc*L (KR914695).

ADDITIONAL MATERIAL: Other strains from the same location, including BE05_12, BE05_16 assigned to the clade by DGGE as well as strain BE04_10 from Aaigem, Belgium, cryopreserved at the BCCM/DCG culture collection. Strain SAG 34-1h collected from Tvärminne, Finland, preserved at SAG.

DISTRIBUTION: So far only collected in Europe, strains were isolated from Belgium (BE05_11, BE05_12, BE05_16 and BE04_10) and Finland (SAG 34-1h).

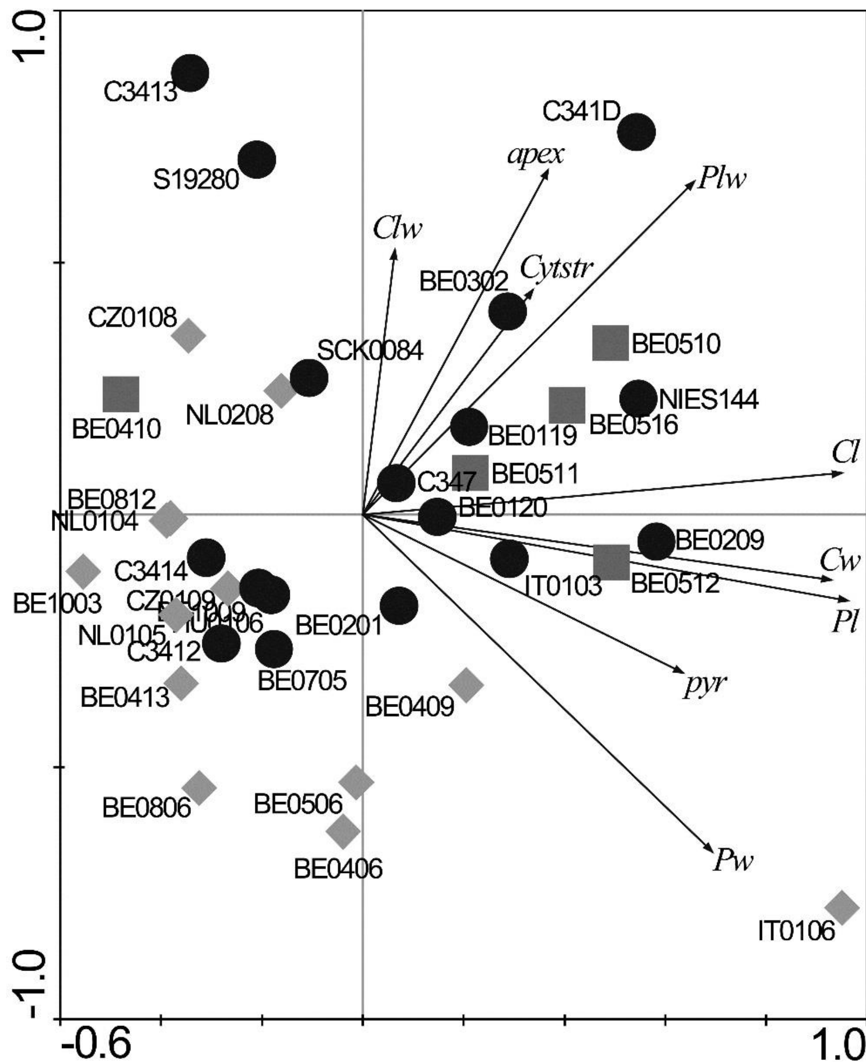



***Figure 15.*** *Biplot of the first two axes of a PCA of morphological measurements on strains of Haematococccus pluvialis (black circles), H. rubicundus (light grey diamonds) and H. rubens (squares). The first axis explains 47 % and the second 22 % of total variation. The morphological variables included are Cl (cell length), Cw (cell width), Clw (difference between cell length and width), Pl (Protoplast length), Pw (Protoplast width), Plw (difference between protoplast length and width), pyr (number of pyrenoids), apex (protoplast apex shape) and Cytstr (presence of cytoplasmic strands).*

### 3.4. Discussion

We took a molecular phylogenetic approach to investigate the species diversity of European *H. pluvialis*, and this was complemented with sequence data from South American and culture collection strains. Buchheim *et al.,* (2013) reported the presence of five *H. pluvialis* lineages (A–E) in an ITS2 rDNA phylogeny but given the lack of CBCs in the secondary structures of

the ITS2, the authors supported the unity of the species. We increased taxon sampling and used the complete ITS rDNA region instead of only ITS2 for phylogeny reconstruction. This is because more phylogenetic information is present in the complete region than in part of it, while the complete ITS rDNA region is still short enough to be sequenced using two sequencing primers (meaning no additional costs). Both the statistical parsimony network and GMYC analysis confirmed that the repetition of different lineages that were distinguished in the ITS rDNA phylogeny corresponded to different species. This allowed us to conclude that three species were present in our European strains, one of which corresponds to *H. pluvialis*. In the ITS rDNA phylogeny, two of these species consist of a mixture of strains from Europe and South America. While some sequence variation might be attributed to geographic origin (there are no ITS types shared between both continents) this is not larger than the sequence variation found within continents and is much smaller than the interspecific differences. This suggests that there is little to no (phylo)geographic influence on species delimitation in the genus.

The recent transfer of most *Haematococccus* species to the new genus *Balticola* (Buchheim *et al.,* 2013) has left the genus with one representative: *H. pluvialis*. Many strains previously designated as *H. lacustris* are identical with those of *H. pluvialis* (see comments and citations above; Fig. 1). Another described species, *Haematococccus thermalis* (Lemmerman, 1905), more likely belonged to *Chloromonas* than to *Haematococccus* based on its original description (lack of flagella, astaxanthin and pyrenoids). We therefore formally describe the two additional European *Haematococccus* lineages found here as the new species, *H. rubicundus* and *H. rubens*. Epitypification of known *H. pluvialis* was undertaken given that the lectotype was a drawing made by Flotow, and our data indicate that there is more than one species going under the name *H. pluvialis*. Strain SAG 192.80 was selected as epitype, as it is kept cryopreserved (and thus in an inactive metabolic state) and its source location (Bruchberg, Harz Mountains, Germany, Central Europe) is closest to the type locality described by Flotow (1844): ‘in the shallow cavity of a granite slab on the footpaths between Hirschberg and Grunau’ (Grunau, near Hirshberg in Silesia, Germany). Three other *Haematococccus* ITS rDNA lineages, represented by a single strain, are probably also new species. Moreover, clade B *sensu* Buchheim was not represented in our ITS rDNA phylogeny (Fig. 1). Although we consider it premature to describe these as new species here because of a lack of strains, it does indicate that additional species diversity is present in *Haematococccus*. These are best referred to as *Haematococccus sp*. until their taxonomy can be resolved.

The *rbc*L gene proved much less variable than the ITS rDNA region, as judged by the two orders of magnitude lower *P* distances and number of differing positions between strains. Although there seemed to be a small difference in *rbc*L sequence between *H. pluvialis* and *H. rubens*, there was no reciprocal monophyly between the two species in the *rbc*L phylogeny. *Rbc*L has proved useful for species delimitation (Fawley *et al.,* 2011), as also illustrated in this study with the separation of *H. rubicundus* and *H. pluvialis*/*H. rubens* but its relatively low discriminatory power is well known in green algae (Kress *et al.,* 2009; Kazi *et al.,* 2013). Authors confronted with organelle genes with low discrimination power are therefore advised not to make final conclusions about species limits unless validated by nuclear gene data (Payo *et al.,* 2013; Leliaert *et al.,* 2014). We therefore rely on the ITS rDNA phylogeny to separate *H. rubens* from *H. pluvialis*. The availability of an additional molecular marker with similar resolution to ITS rDNA would be highly informative but there is at present no such marker that lends itself to routine identification. A promising candidate is the tufA gene, a plastid marker successfully used in several other green algae (Hall *et al.,* 2010; Saunders & Kucera 2010; Fučiková *et al.,* 2011; Moniz *et al.,* 2014).

CBCs in the secondary structures of ITS1 and ITS2 are considered to indicate reproductive isolation (Coleman *et al.,* 1998; Coleman, 2000). The presence of such CBCs among the ITS1 secondary structures supports the separation of the three *Haematococccus* species recognized here. In ITS2, we only found one CBC between three strains of *H. rubicundus* and strains of *H. pluvialis* and *H. rubens*. With observations similar to ours, Buchheim *et al.,* (2013) found only one CBC between strain SAG 34-1m (*H. rubicundus*) and the other *H. pluvialis* and *H. rubens* strains. CBCs do not form a causal basis for speciation (Müller *et al.,* 2007; Leliaert *et al.,* 2014), so their interpretation requires caution. Coleman (2003) pointed out that CBCs should serve as a rough measure of phylogenetic relationship and reported an error rate of 7 % within the Volvocaceae. Mating data for *Haematococccus* are still missing. Gamete formation was described in response to stress factors such as desiccation, freezing, nitrogen deficiency and nutrient starvation (Hazen 1899; Triki *et al.,* 1997) but gamete induction seems difficult to reproduce routinely, and the mating system is unknown. As a result, mating trials have to our knowledge never been successfully executed.

The species diversity in European *H. pluvialis* examined here represents a new addition to the many cases of morphologically scarcely different to indistinguishable chlorophyte species for which the traditional diagnostic characters using light microscopy have high intraspecific

variation and interspecific overlap (Kooistra, 2002; Vanormelingen *et al.,* 2007; Hofmann *et al.,* 2010; Subirana *et al.,* 2013). Although the aplanospores of the different isolates were not subjected to morphological study, preliminary observations have shown that strains SAG 34-1a and SAG 192.80 differed in morphology and encystment from strains SAG 34-1f, SAG 34-1h and SAG 44.96 based on culture conditions (personal observations).

Cell wall ultrastructures (visible using electron microscopy) have proved to be well correlated with species limits derived from molecular phylogenies (Vanormelingen *et al.,* 2007; Fawley *et al.,* 2011; McManus & Lewis 2011) but not all green algal genera possess ultrastructural details allowing their differentiation. According to Hagen *et al.,* (2002), flagellated cells of *H. pluvialis* possess a typical volvocalean multilayered extracellular matrix with a central tripartite crystalline layer, while the aplanospores contain traces of sporopollenin-like material. Whether ultrastructural details can provide diagnostic characters requires further investigation. The large variation in the morphological characters within and between *H. pluvialis* strains reported here was noted earlier by several taxonomists. Droop (1956b) had already observed 'small morphological and physiological differences' between different *Haematococccus* strains, though he did not provide further details. Pocock (1960) later mentioned 'there is considerable variation in behaviour in different strains of the species even when treated similarly'. Likewise, Pringsheim (1966) observed slight physiological deviations in *H. pluvialis* strains from different localities but claimed that they were 'so similar morphologically that they should not be described as varieties'. The large morphological overlap between *Haematococccus* species hinders the use of morphology as sole criterion for species delimitation or identification, since it is based on the detection of morphological discontinuities. Therefore, we developed a DGGE method that allows rapid and cost-efficient identification of *H. pluvialis*, *H. rubicundus* and *H. rubens*.

Temperature is a basic factor influencing volvocalean growth, directly affecting cell processes mainly by control of the photosynthetic rate (Zachleder & Vandenende 1992; Vítová *et al.,* 2011). *Haematococccus* inhabits small to very small temporal habitats and is exposed to strong seasonal and diurnal temperature fluctuations (Borowitzka *et al.,* 1991; Tripathi *et al.,* 2002). The optimal temperature for growth of our strains was between 17 °C and 23 °C, which is considerably lower than the reported 25 °C (Fan *et al.,* 1994) and 28 °C (Wan *et al.,* 2014) for *H. pluvialis*. Tjahjono *et al.,* (1994) exposed strain NIES 144 to different temperatures and found 20 °C to be the optimal temperature for production of vegetative cells. This is similar to

the values found here for the same strain (optimum temperature of 21.9 °C). All of our strains started encysting or died above 33 °C, as in previous reports (Fan *et al.,* 1994; Wan *et al.,* 2014). There was no relation between temperature optimum and species identity, indicating that the three species have similar temperature preferences. In contrast, there was a considerable intraspecific variation in temperature preference. At the interspecific level, we found that at the selected culture conditions *H. pluvialis* had a lower maximal growth rate than *H. rubicundus* and *H. rubens*. It is widely accepted that conditions favouring high growth rates do not necessarily favour astaxanthin accumulation and *vice versa* (Evens *et al.,* 2008). We mainly concentrated on the vegetative growth phase of *Haematocolcccus*. It would be of interest to examine effects of temperature on aplanospore formation and associated astaxanthin accumulation as well as the degree of variation between strains for this trait, particularly in a context of astaxanthin production in more temperate regions (given the origin of most strains).

### 3.5. Conclusions

In conclusion, an ITS rDNA phylogeny of a large set of European *Haematococcus* strains allowed the identification of three species of *Haematococcus* as described above. The presence of three additional lineages represented by a single strain suggests higher cryptic species diversity within *Haematococcus*. *Rbc*L was much less variable and provided support for only two of the three species outlined using the ITS rDNA region. A molecular marker that is more variable than *rbc*L would be highly valuable to provide additional support for species discovery based on the ITS rDNA. Despite considerable overlap, significant differences in morphology and growth rate support our species boundaries. A high degree of intraspecific variation was found in optimal and maximal temperature and maximal growth rate. The phylogenetic characterization of *Haematococcus* strains provides a solid phylogenetic framework for further comparative physiological studies and for the selection of candidate production strains for mass culture adapted to local climatic conditions.

### 3.6. Acknowledgements

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indebted to the Belgian Coordinated Collection of Micro-organisms (http://bccm.belspo.be) for cryopreserving strains from this study.

## 3.7.Supplementary Data

***Table S1***. *Isolates of Haematococcus species used in this study. The sampling location substrate type, as well as date of collection is given. Genbank accession numbers are given for the respective sequences. Further strains subjected to DGGE and to the temperature preferences experiment are indicated. Isolates that were observed for morphological analyses are also indicated.ND = Not determined*

| Species | Strain number | Substrate type | Location | Geographical Coordinates | Collected by | Date | BOLD | Genbank (ITS complete) | Genbank (*rbc* L) |
|---|---|---|---|---|---|---|---|---|---|
| *H. pluvialis* * | SAG 34-1a | Freshwater | Aneboda, Sweden | 57°06'59.3"N, 14°32'53.9"E | Pringsheim E.G. | 1966 | HAEMA001-15 | KR914704 | ND. |
| *H. pluvialis* | SAG 34-1b | N/A | Former Czechoslovakia | N/A | Mainx F. | 1931 | HAEMA002-15 | ND | ND |
| *H. pluvialis* | SAG 49.94 | Freshwater, birdbath | Ferrum, Virginia, USA | 36°55'22.5"N, 80°0'48.1"W | Ott F.D. | 1959 | HAEMA025-15 | KR914707 | ND |
| *H. pluvialis* | SAG 34-1d = CCA | Puddle in Bot. Gard. | University of Basel, Zwitserland | 47°33'33.5"N, 7°34'56.9"E | Vischer W. | 1923 | HAEMA027-15 | KR914705 | ND |
| *H. pluvialis* | SAG 34-1e | Roof of Botany School | Cambridge, United Kingdom | 52°12'09.3"N, 0°07'17.4"E | George E.A. | 1950 | HAEMA005-15 | ND | ND |
| *H. pluvialis* | SAG 34-1n | Little "blood pond" | Near Samnun, Graubünden, | 46°57'01.6"N, 10°22'09.1"E | Zehnder A | 1953 | HAEMA003-15 | ND | ND |
| *H. pluvialis* | SCCAP K-0404 | Freshwater, birdbath | Ramløse Sand, North Zealand, Denmark | N/A | Larsen N.H. | Sep. 1989 | HAEMA019-15 | KR914713 | ND |
| *H. pluvialis* | SCCAP K-0084 | Small rock pool | Island of Trutbådan, Sweden | 58°42'0.0"N, 17°16'0.0"E | Christensen T. | 1950 | HAEMA021-15 | KR914711 | ND |
| *H. pluvialis* | SAG 192.80 | Bog pool | Bruchberg/Harz Mts, Germany | 51°46'60.0"N, 10°31'0.0"E | Koch W. | 1959 | HAEMA022-15 | KR914710 | ND |
| *H. pluvialis* | NIES 144 | Freshwater, lake water | Sapporo Hokkaido, Japan | 43°3'43.5"N, 141°21'15.7"E | Ichimura T. | 16 Jul. 1964 | HAEMA023-15 | KR914709 | AB084336 |
| *H. pluvialis* | CCAP 34/7 | Freshwater | Ostpicken Island, Tvärminne, Finland | 59°50'35.4"N, 23°14'38.5"E | Droop M.R. | 1953 | HAEMA020-15 | KR914712 | FJ438476 |
| *H. pluvialis* | CCAP 34/12 | Freshwater | Ottowa, Kansas, USA | 38°36'56.8"N, 95°16'7.1"W | Ott F.D. | 1977 | HAEMA024-15 | KR914708 | ND |
| *H. pluvialis* | CCAP 34/13 | Freshwater, birdbath | Ferrum, Virginia, USA | 36°55'22.5"N, 80°0'48.1"W | Ott F.D. | 1959 | HAEMA025-15 | KR914707 | ND |
| *H. pluvialis* | CCAP 34/14 | Freshwater, cement urn | Cattonsville, Maryland, USA | 39°16'19.4"N, 76°43'54.9"W | Ott F.D. | 1989 | HAEMA026-15 | KR914706 | ND |
| *H. pluvialis* | CCM-UDEC 029 | Water fountain | Vienna, Austria | 48°12'20"N, 16°22'23"E | González M.A. | 2005 | HAEMA006-15 | KR914726 | ND |
| *H. pluvialis* | CCM-UDEC 021 | Marble vessel | Cemetery, Concepción, Chile | 36°50'00"S, 73°03'00"W | González M.A., Cifuentes A.S. | 2001 | HAEMA007-15 | KR914725 | ND |
| *H. pluvialis* | CCM-UDEC 022 | Marble vessel | Cemetery, Concepción, Chile | 36°50'00"S, 73°03'00"W | González M.A., Cifuentes A.S. | 2001 | HAEMA008-15 | KR914724 | ND |
| *H. pluvialis* | CCM-UDEC 023 | Granitic small fountain | University of Concepción, Chile | 36°50'00"S, 73°03'00"W | González M.A. | 2001 | HAEMA009-15 | KR914723 | ND |
| *H. pluvialis* | CCM-UDEC 034 | Granitic flowerpot | Cemetery, Osorno, Chile | 40°34'00"S, 73°09'00"W | Gómez P.I., González M.A. | 2006 | HAEMA010-15 | KR914722 | ND |
| *H. pluvialis* | CCM-UDEC 035 | Granitic flowerpot | Cementery, Osorno, Chile | 40°34'00"S, 73°09'00"W | Gómez P.I., González M.A. | 2006 | HAEMA011-15 | KR914721 | ND |
| *H. pluvialis* | CCM-UDEC 019 | N/A | Steptoe, Nevada, USA | 39°24'25"N, 114°45'50"W | Provided by Lewin R. | 1989 | HAEMA012-15 | KR914720 | ND |
| *H. pluvialis* | CCM-UDEC 032 | Plastic bottle | Cemetery, Valdivia, Chile | 39°48'30"S, 73°14'30"W | Gómez P.I., González M.A. | 2006 | HAEMA013-15 | KR914719 | ND |

***Table S1*. Continued**

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *H. pluvialis* | CCM-UDEC 031 | N/A | Brazil | N/A | Provided by Lavin P. | N/A | HAEMA014-15 | KR914718 | ND |
| *H. pluvialis* | CCM-UDEC 041 | Cement pool | Cemetery, Castro, Chile | 42°47'21"S, 73°77'31"W | Gómez P.I., González M.A | 2006 | HAEMA015-15 | KR914717 | ND |
| *H. pluvialis* | CCM-UDEC 036 | Plastic bottle | Cemetery Osorno, Chile | 40°34'00"S, 73°09'00"W | Gómez P.I., González M.A. | 2006 | HAEMA016-15 | KR914716 | ND |
| *H. pluvialis* | CCM-UDEC 037 | Plastic bottle | Cemetery Osorno, Chile | 40°34'00"S, 73°09'00"W | Gómez P.I., González M.A. | 2006 | HAEMA017-15 | KR914715 | ND |
| *H. pluvialis* | CCM-UDEC 033 | Granitic flowerpot | Cemetery, Valdivia, Chile | 39°48'30"S, 73°14'30"W | Gómez P.I.; González M.A. | 2006 | HAEMA018-15 | KR914714 | ND |
| *H. pluvialis* | BE01_19 | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | HAEMA028-15 | ND | ND |
| *H. pluvialis* | BE01_20 | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | HAEMA029-15 | ND | ND |
| *H. pluvialis* | BE02_01 | Water puddle in aluminum | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | HAEMA030-15 | KR914734 | ND |
| *H. pluvialis* | BE02_09 | Water puddle in aluminum | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | HAEMA031-15 | KR914733 | ND |
| *H. pluvialis* | BE03_02 | Water puddle in concrete | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | HAEMA032-15 | KR914732 | KR914694 |
| *H. pluvialis* | BE03_05 | Water puddle in concrete | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | HAEMA033-15 | KR914731 | KR914693 |
| *H. pluvialis* | BE07_05 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA034-15 | ND | ND |
| *H. pluvialis* | BE07_07 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA068-15 | ND | ND |
| *H. pluvialis* | BE07_09 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA069-15 | ND | ND |
| *H. pluvialis* | BE07_10 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA070-15 | ND | ND |
| *H. pluvialis* | BE07_12 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA035-15 | ND | KR914692 |
| *H. pluvialis* | BE07_14 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA036-15 | ND | KR914691 |
| *H. pluvialis* | BE10_09 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct. 2013 | HAEMA037-15 | KR914730 | ND |
| *H. pluvialis* | HU01_05 | Rain water barrel | Ebes Hajdu.Bihar, Debrecen, Hungary | 47°28'15.3"N, 21°29'25.6"E | Basci I. | May 2012 | HAEMA038-15 | KR914729 | ND |
| *H. pluvialis* | HU01_06 | Rain water barrel | Ebes Hajdu.Bihar, Debrecen, Hungary | 47°28'15.3"N, 21°29'25.6"E | Basci I. | May 2012 | HAEMA039-15 | KR914728 | ND |
| *H. pluvialis* | IT01_03 | Small water puddle in rock | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | HAEMA040-15 | ND | ND |
| *H. pluvialis* | NL03_06 | Water puddle on top of a | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | HAEMA066-15 | KR914727 | ND |
| *H. rubicundus* | BE04_06 | Water puddle in white porcelain | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | HAEMA041-15 | KR914739 | KR914698 |
| *H. rubicundus* | BE04_09 | Water puddle in white porcelain | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | HAEMA042-15 | KR914738 | KR914697 |
| *H. rubicundus* | BE04_13 | Water puddle in white porcelain | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | HAEMA043-15 | ND | ND |
| *H. rubicundus* | BE05_06 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA044-15 | KR914737 | KR914696 |

***Table S1***. Continued

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| *H. rubicundus* * | BE08_06 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA045-15 | KR914742 | KR914700 |
| *H. rubicundus* | BE08_12 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA046-15 | KR914741 | KR914699 |
| *H. rubicundus* | BE10_03 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct. 2013 | HAEMA053-15 | KR914746 | ND |
| *H. rubicundus* | CZ01_06 | Water puddle on white chair | Třeboň, Czech Republic | 49°0'18.3"N, 14°46'23.8"E | Strunecky O. | 12 Feb. 2013 | HAEMA071-15 | ND | ND |
| *H. rubicundus* | CZ01_08 | Water puddle on white chair | Třeboň, Czech Republic | 49°0'18.3"N, 14°46'23.8"E | Strunecky O. | 12 Feb. 2013 | HAEMA047-15 | KR914751 | KR914703 |
| *H. rubicundus* | CZ01_09 | Water puddle on white chair | Třeboň, Czech Republic | 49°0'18.3"N, 14°46'23.8"E | Strunecky O. | 12 Feb. 2013 | HAEMA048-15 | KR914750 | KR914702 |
| *H. rubicundus* | NL01_04 | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar. 2013 | HAEMA049-15 | KR914749 | KR914701 |
| *H. rubicundus* | NL01_05 | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar. 2013 | HAEMA050-15 | KR914748 | ND |
| *H. rubicundus* | NL02_08 | Water puddle on white bioreactor | Wageningen, The Netherlands | 51°59'46.4"N, 05°39'29.4"E | Allewaert C. | 6 Jun. 2013 | HAEMA052-15 | KR914747 | ND |
| *H. rubicundus* | IT01_06 | Small water puddle in rock | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | HAEMA051-15 | ND | ND |
| *H. rubicundus* | CCM-UDEC 024 | N/A | Titicaca Lake, Bolivia | 15°45'00"S, 69°25'00"W | N/A | 2003 | HAEMA054-15 | KR914745 | ND |
| *H. rubicundus* | CCM-UDEC 020 | Granitic pool | Puerto Varas, Chile | 41°19'00"S, 72°58'60"W | Aguilera A. | 2001 | HAEMA055-15 | KR914744 | ND |
| *H. rubicundus* | CCM-UDEC 038 | Granitic pool | Cemetery, Castro, Chile | 42°47'21"S, 73°77'31"W | Gómez P.I. & González M.A. | 2006 | HAEMA067-15 | KR914740 | ND |
| *H. rubicundus* | CCM-UDEC 039 | Plastic bottle | Cemetery, Castro, Chile | 42°47'21"S, 73°77'31"W | Gómez P.I. & González M.A. | 2006 | HAEMA056-15 | KR914743 | ND |
| *H. rubens* | SAG 34-1h | Supralittoral rock pool (20000m) | Tvärminne, Finland | 59°49'51.6"N, 22°57'40.9"E | Droop M.R. | 1951 | HAEMA057-15 | KR914736 | ND |
| *H. rubens* | BE05_10 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA059-15 | ND | ND |
| *H. rubens* ** | BE05_11 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA058-15 | KR914735 | KR914695 |
| *H. rubens* | BE05_12 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA061-15 | ND | ND |
| *H. rubens* | BE05_13 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA072-15 | ND | ND |
| *H. rubens* | BE05_15 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA073-15 | ND | ND |
| *H. rubens* | BE05_16 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA060-15 | ND | ND |
| *H. rubens* | BE04_10 | Water puddle in white porcelain | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Febr. 2013 | HAEMA062-15 | ND | ND |
| *H. sp.* | CCM-UDEC 028 | Soil | Ueno Park, Tokyo, Japan | 35°42′44″N, 139°46′16″E | Donated by Coleman A.W. | 2004 | HAEMA063-15 | KR914752 | ND |
| *H. sp.* | SAG 34-1f | Dry crust on a stone | Spitsbergen, Norway | 53°10'32.9"N, 6°49'26.0"E | Pringsheim E.G. | 1932 | HAEMA064-15 | KR914753 | ND |
| *H. sp.* | SAG 44.96 | Soil from marshland | Cape flats "De Klip", Cape town, | 34°1'16.6"S, 18°35'23.3"E | Schlösser U.G. (leg. Pocock | 1969 | HAEMA065-15 | KR914754 | ND |

***Figure S1.*** *Haematococcus maximum likelihood phylogeny based on ITS2 rDNA. Bayesian posterior probabilities (left) and ML bootstrap values (right) respectively are indicated at the nodes. Strains in bold were sequenced for this study.*

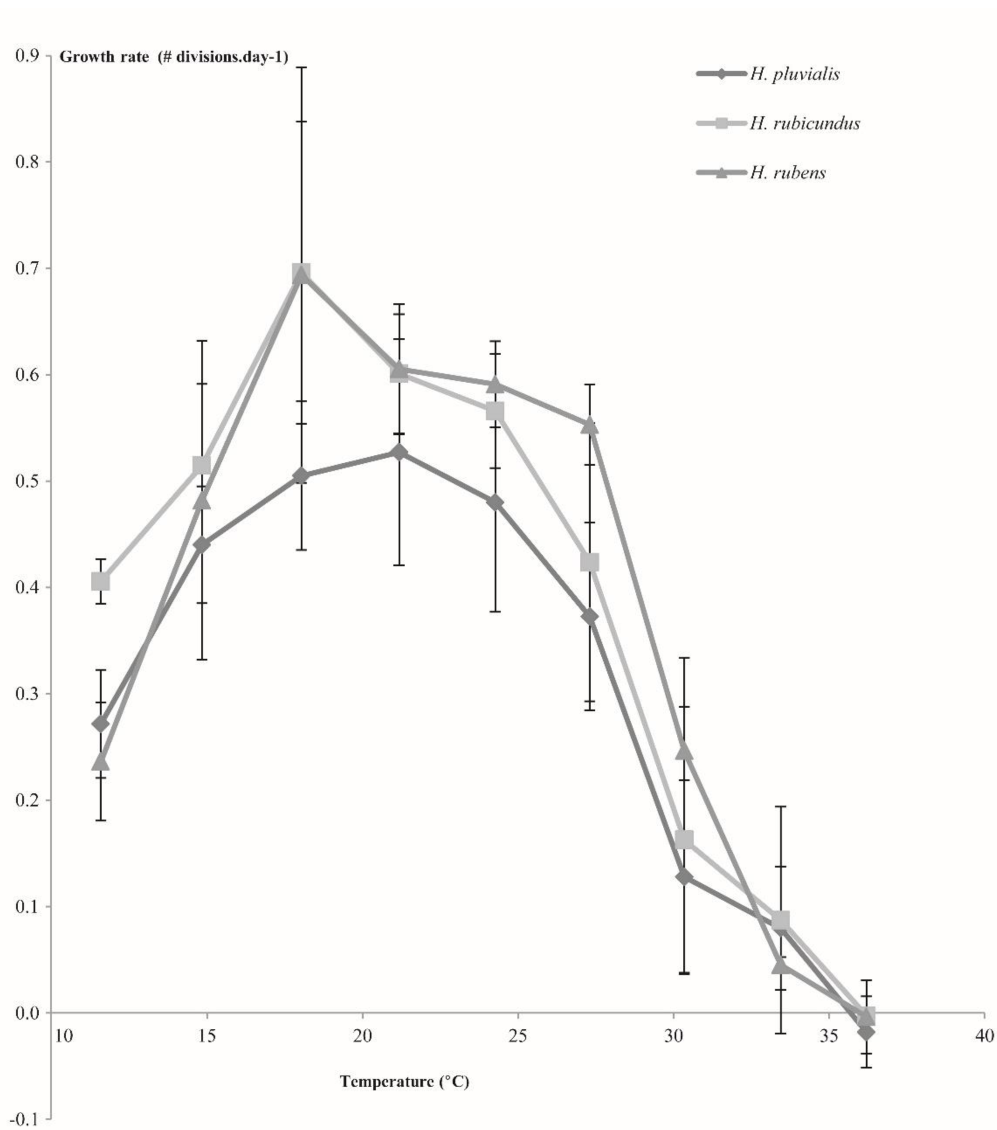



***Figure S2.*** *Temperature-dependent growth rates of H. pluvialis, H. rubicundus and H. rubens. The graph give the growth rate for each of the nine temperatures used, averages per ITS rDNA lineage, error bars represent the standard deviation.*

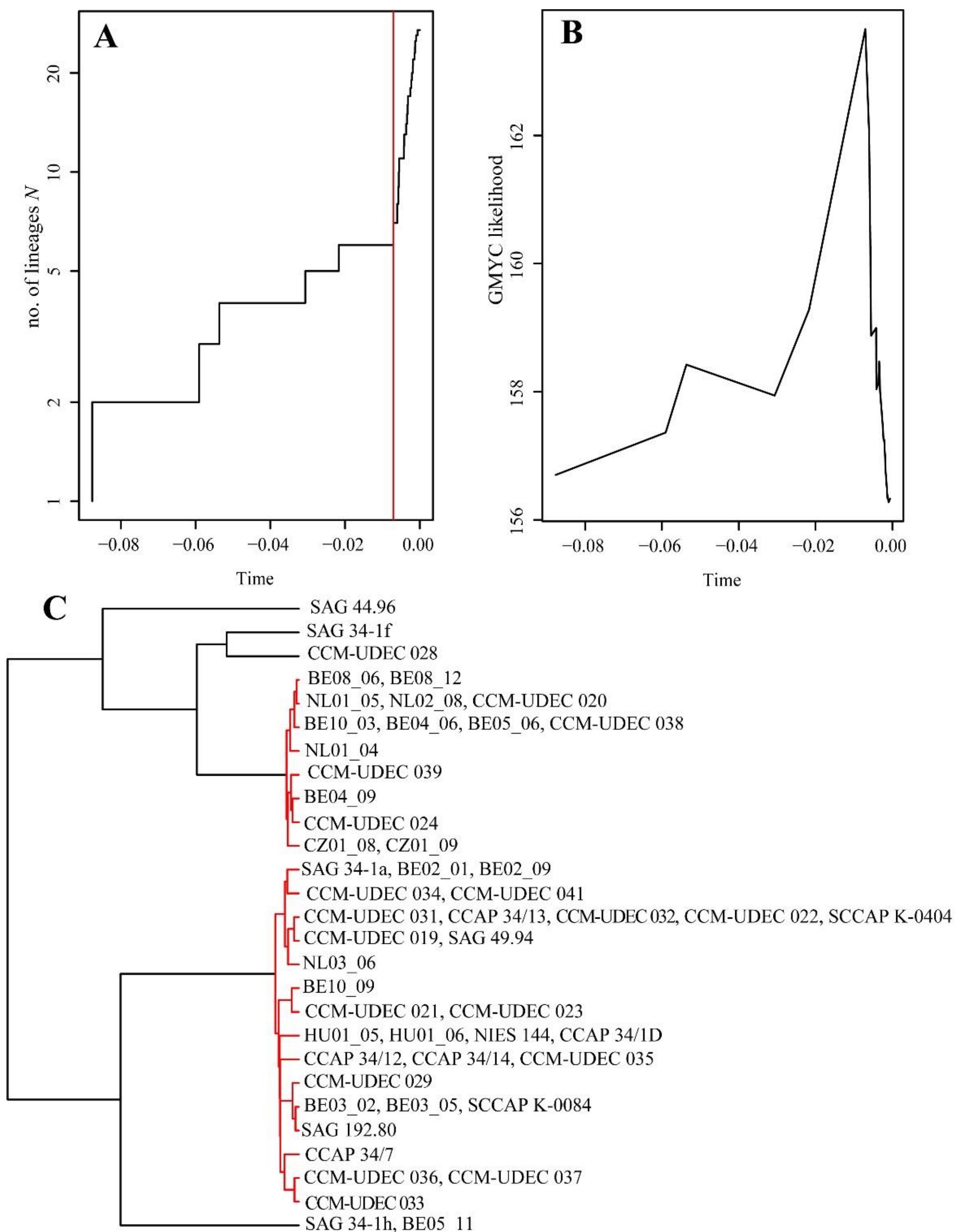
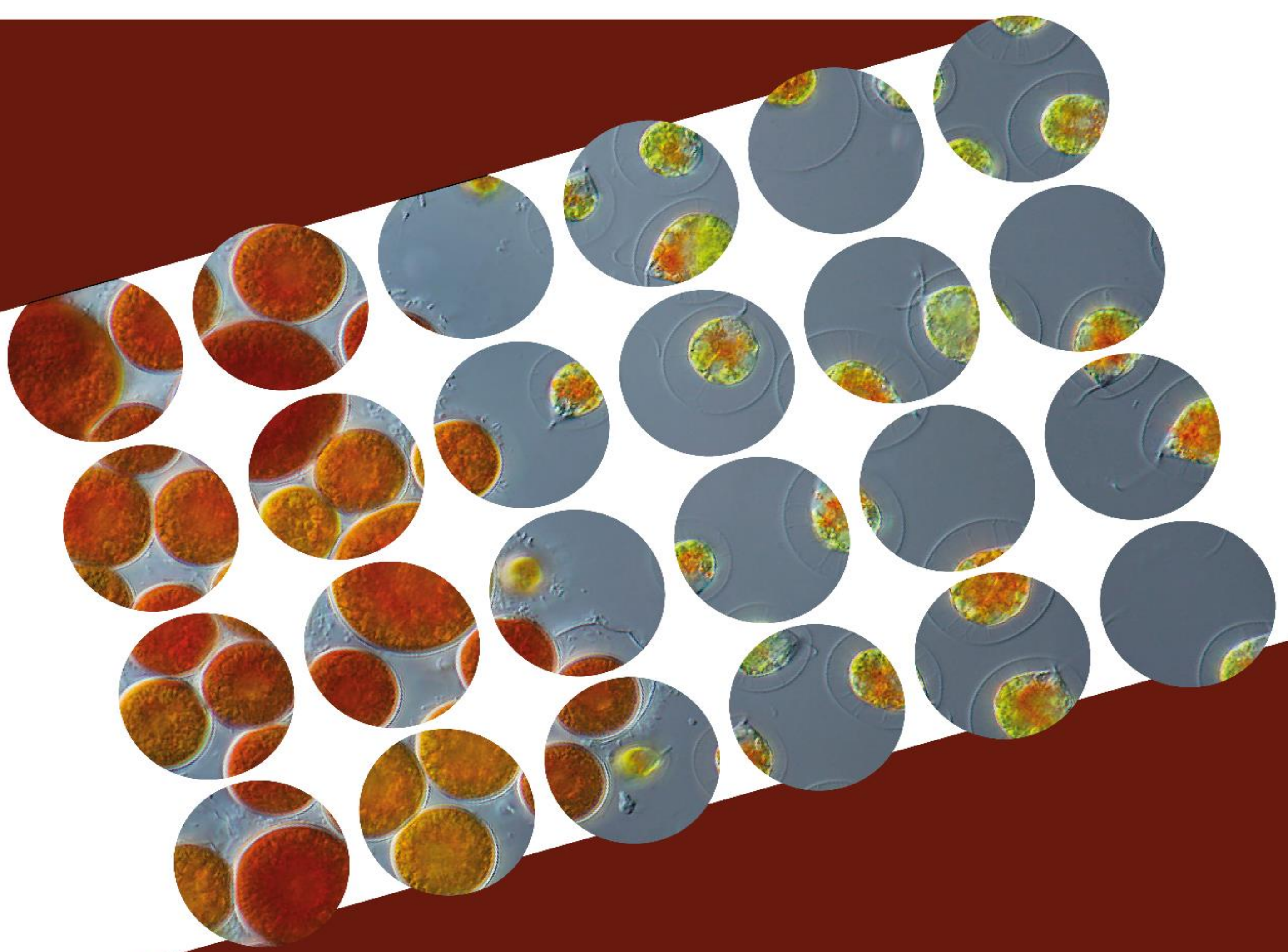



***Figure S3.*** *Results of a GMYC analysis of a Haematococcus ITS rDNA phylogeny.* ***A.*** *Plot of the number of branches through time. The vertical lines shows the most likely point in time of the increase in branching rate typical for the species level.* ***B.*** *Plot of the likelihood of an increase in branching rate through time. The time point where the likelihood peaks is taken as the transition point from inter-to intraspecific variation.* ***C.*** *Species clusters within Haematococcus. Strains belonging to the same species cluster are indicated in red.*



# CHAPTER 4: Intraspecific trait variation affecting astaxanthin productivity in two *Haematococcus (Chlorophyceae)* species

**Modified from Allewaert CC., Vanormelingen P., Daveloose I., Verstraete T. & Vyverman W. (2017) Intraspecific trait variation affecting astaxanthin productivity in two *Haematococcus* (Chlorophyceae) species. *Algal Research* 21: 191-202.**

**Abstract**

Microalgae are increasingly used as commercial sources of high-value compounds. However, the nature and genetic basis of variation in commercially relevant traits remain understudied. This study focuses on the green alga *Haematococcus pluvialis*, well-known for accumulating the carotenoid astaxanthin. We examined intra- and interspecific variation and correlations between six traits related to astaxanthin productivity among 30 natural isolates and cultivated strains of two *Haematococcus* species, *H. pluvialis* and *H. rubicundus*. Significant intraspecific genotypic variation was found for all traits assessed in both species (broad sense heritability estimates $H^2$= 0.48-0.89), resulting in a fifteen-fold variation in astaxanthin productivity between the poorest and the best-performing strain. The two species differed in five of the six traits. Cultivated strains had a lower astaxanthin productivity compared to natural isolates of *H. pluvialis*, possibly reflecting loss of photoprotective capacity during long-term cultivation. In general, trait correlations were weak yet stronger in *H. rubicundus* than in *H. pluvialis*. Most of the variation in overall astaxanthin productivity could be explained by the differences in post-stress traits. Our results reveal extensive trait variation among isolates of a commercially interesting microalga. We recommend natural strain panels as a valuable tool for cost-efficient trait mapping and to select targets for genetic engineering, marker-assisted strain selection or breeding aiming at the optimization of astaxanthin productivity.

## 4.1. Introduction

Modern evolutionary research, particularly population genomic studies, aims to explore the genetic basis of phenotypic variation resulting in a better understanding of the mechanisms governing trait variation (Lee *et al.*, 2014). In an era of omics, technological advances have led to increasing amounts of genomic data, from whole genome sequencing- to extensive transcriptomic, methylomic and metabolomics data. Complementing these -omics data with phenotypic data is an essential, yet time consuming and labor intensive step of many biological experiments (Houle*,* 2010; Chepurnov *et al.*, 2011; Bougaran *et al.*, 2012; Araus & Cairns, 2014). As natural sources of value added commodities such as chlorophylls, carotenoids, antioxidants, enzymes, polymers, toxins, sterols and fatty acids (Ghosh *et al.*, 2016), microalgae represent a promising and sustainable feedstock supplying food and nonfood markets (Herrero & Ibanez, 2015). From a commercial perspective, however, current yields and processes are often insufficient to sustainably produce bioproducts. In the face of growing competition and significantly larger market opportunities, one major goal in microalgae biotechnology is the screening, selection and development of strains with increased productivity and resilience (Mutanda *et al.*, 2011; Nascimento *et al.*, 2013). Although still rare, some studies on model microalgae with economical potential have screened natural populations with the aim to identify better performing strains either for enhanced pigment productivity (Mendoza *et al.*, 2008; Sathasivam *et al.,* 2014) or fatty acid production (Bougaran *et al.*, 2012; Doan & Obbard, 2012).

The green microalga *Haematococcus pluvialis* Flotow (1844) is exploited as the richest biological source of the high value red carotenoid, astaxanthin. The predicted market value for astaxanthin is expected to be over $ 1.5 billion by 2020 (Nguyen, 2013). Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) accumulates in the cytoplasmic oil globules of the aplanospores under various stress conditions (Zhekisheva *et al.*, 2002) where it acts as a protective pigment (Solovchenko, 2015) and reaches up to 1-5 % of cell dry weight (Wang *et al.*, 2013a; Wan *et al.*, 2014). Synthetic astaxanthin is widely used in the aquaculture sector as salmon colorant (Ahmed *et al.*, 2015), while natural astaxanthin from *H. pluvialis* is principally commercialized as a biological nutraceutical with attractive properties (Pérez-López *et al.*, 2014; Solovchenko & Chekanov, 2014). To successfully compete with synthetically prepared astaxanthin, *Haematococcus* production processes faces some major challenges (Shah *et al.*, 2016), among which the most important biological issues are the slow cell growth rate, changes in cell morphology under various conditions and vulnerability to contamination. So far,

research has prioritized the production conditions, including the engineering of culture systems and the manipulation of culture conditions (nutrient composition, light intensity, photoperiod, temperature etc..) using single strains from culture collections (González *et al.*, 2009). However, the use of genetically different strains, as well as the differences in experimental designs, hamper accurate comparisons. Consequently, it is not known to which extent the variation in astaxanthin productivity between different studies is due to strain differences. In fact, little is known about the phenotypic variation spectrum in *H. pluvialis*. A few assessments among strains of *Haematococcus* have been made (González *et al.*, 2009; Zhang *et al.*, 2009; Noroozi *et al.*, 2011; Gao *et al.*, 2015; Gómez *et al.*, 2016), which revealed a great deal of physiological diversity between *H. pluvialis* isolates. Yet a better understanding of the nature of this variation is essential for the selection of promising isolates and for the setup of any future selection program.

One prerequisite for assessing the genetic basis of phenotypic variation patterns (at intra- and interspecific level), for future breeding programs, is knowledge on species boundaries. Until recently, all *Haematococcus* isolates were assigned to a single species, *Haematococcus pluvialis*, although internal transcribed spacer (ITS) rDNA sequences showed the existence of different lineages (Buchheim *et al.*, 2013). In a recent multi-locus study of the phylogenetic diversity of European isolates of *Haematococcus,* six species-level lineages were found, and two of these formally described as *H. rubicundus* and *H. rubens* (Allewaert *et al.*, 2015). Although the newly described species are also astaxanthin producers, it is not known to what extent their astaxanthin productivity capacities differ from each other and from *H. pluvialis*.

To quantify the genetically based component of phenotypic variation, common garden experiments are used in which typically comparisons of phenotypic traits of genetically distinct strains, families or populations are made under strict identical environmental conditions (Ljungfeldt *et al.*, 2014). Common garden approaches are becoming increasingly popular over recent years, particularly since the gap between available phenotypic data versus genotypic data is increasing. Currently, these experiments are employed in several disciplines, in applied contexts e.g., in invasion research (Moloney *et al.*, 2009), climate change research (Aspinwall *et al.*, 2013) and in biotechnology for the selection of promising candidate strains for biodiesel production (Vello *et al.*, 2014). In this study, we used a common garden approach using a total of 30 strains belonging to two *Haematococcus* species, *H. pluvialis* and *H. rubicundus* represented by 17 and 13 strains respectively. Six *H. pluvialis* strains from culture collections were included. The genetically based intra- and interspecific (co-)variation in traits considered

to be related to astaxanthin productivity was studied within and between both species, and traits which determine the overall astaxanthin productivity were identified.

## 4.2. Material and Methods

### 4.2.1. Cultures and experimental set-up

Except for the culture collection strains included (see below), all unialgal strains used for this study are new natural isolates, obtained via single cell isolation (Table 1), all of which belong to different genotypes based on intraspecific differences in ITS sequences (Fig. 1) with the exception of the strain pairs BE03_02 and BE03_05, BE02_09 and BE02_05, NL02_08 and NL02_10, CZ01_06 and CZ01_08 as well as NL03_06 and NL03_07. Finally BE08_04, BE08_06 and BE08_12 also had identical ITS sequences. Strains were identified to species based on their ITS rDNA sequence (Allewaert *et al*., 2015). Seventeen *H. pluvialis* and thirteen *H. rubicundus* strains were selected from a larger set of strains for the common garden experiment. Generally, two isolates per sampled location were selected (Table 1). For *H. pluvialis*, six additional strains were included from culture collections (Culture Collection of Algae and Protozoa, UK (CCAP) (34/7, 34/13, 34/14), Sammlung von Algenkulturen at the University of Göttingen (SAG) (192.80, 49.94=CCAP 34/13), the Scandinavian Culture Center for Algae and Protozoan (SCCAP) (K-0084) and the Culture Collection for the National Institute for Environmental Studies, Japan (NIES) (144). All new isolates are cryopreserved and maintained at the Diatom Collection Ghent of the Belgian Coordinated Collections of Microorganisms BCCM/DCG) (http://www.bccm.belspo.be/catalogues/dcg-catalogue-search).

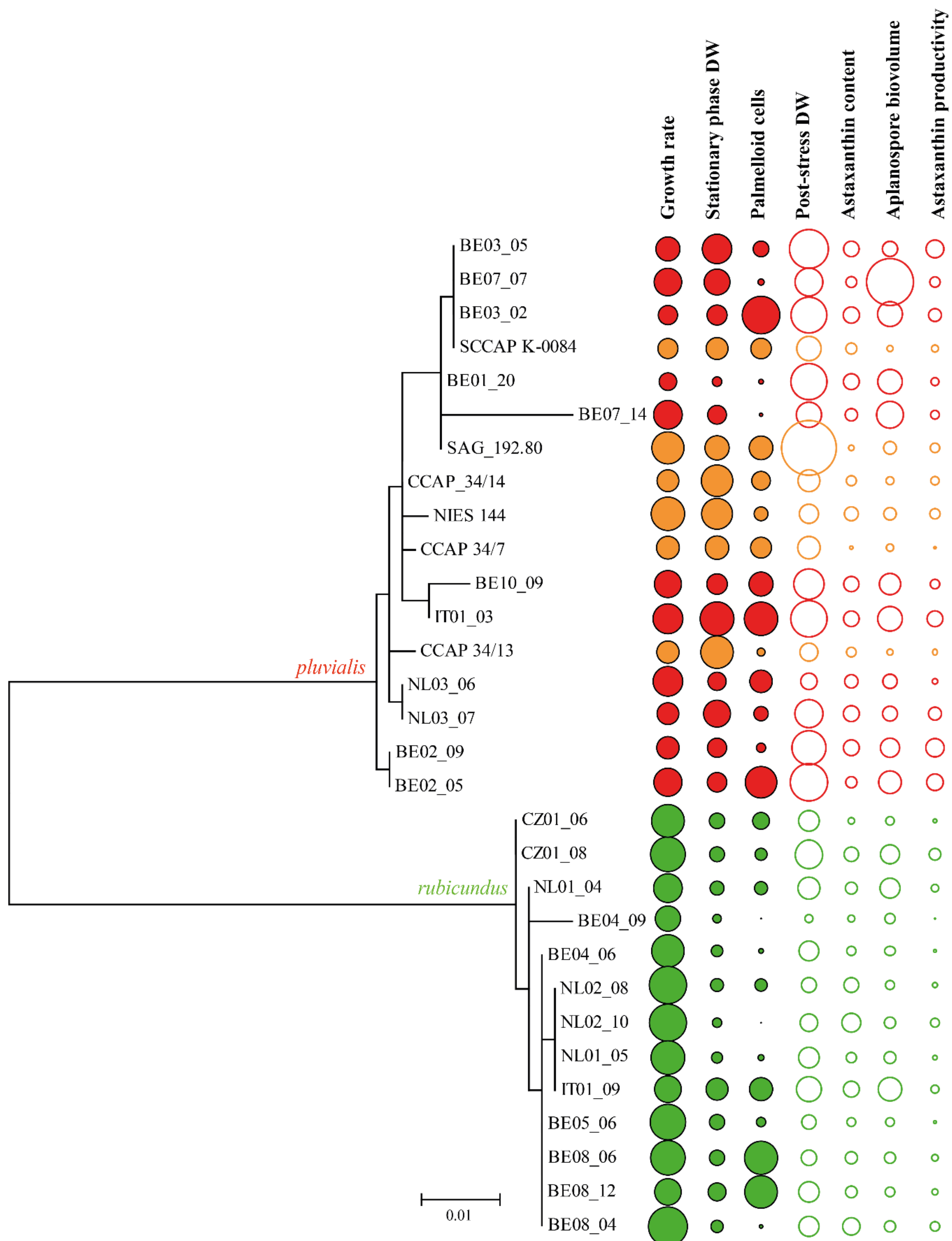



***Figure 1.*** *Haematococcus Maximum Likelihood (ML) phylogeny based on ITS rDNA (ITS1-2.8S-ITS2) sequences of the thirty strains used in this study (methodology as in Allewaert et al., 2015). Trait means in each column are in proportion to the size of circles for each species, where larger circles indicate higher values. Filled circles correspond to pre stress traits and unfilled circles to post stress traits, red circles correspond to H. pluvialis natural strains, orange circles to H. pluvialis cultured strains and green circles correspond to H. rubicundus. DW= dry weight.*

Four replicates per strain were grown up at 23°C from stock cultures (preserved at 6°C) in Bold Basal Medium modified with 3-fold Nitrogen (BBM-3N (Bischoff & Bold, 1963)) using a 16:8 h light:dark regime and a light intensity of 20 ± 2 µmol photons $m^{-2}$ $s^{-1}$. Strains were re-inoculated twice with an interval of three days at the same initial $F_0$ value of 0.023 (settings 6-1-2, as measured using a PAM fluorometer; see below) before starting the experiment, to ensure that they were in exponential and flagellated stage. Cells were harvested by centrifugation (3000 rpm at 20 °C during 5 min) in 15 mL falcon tubes, and re-suspended in 12 multi-well plates (Greiner Bio-One) with a working volume of 4 mL using BBM-3N at an initial $F_0$ value of 0.023 (settings 6-1-2, see below). Each of the four replicates per strain was split in two separate wells at the start of the experiment, for each replicate two technical replicates needed to measure all traits (see below). A bi-phasic approach was used for the cultivation of *Haematococcus* strains for astaxanthin synthesis, involving different conditions for biomass and for astaxanthin accumulation (Fig. 2).

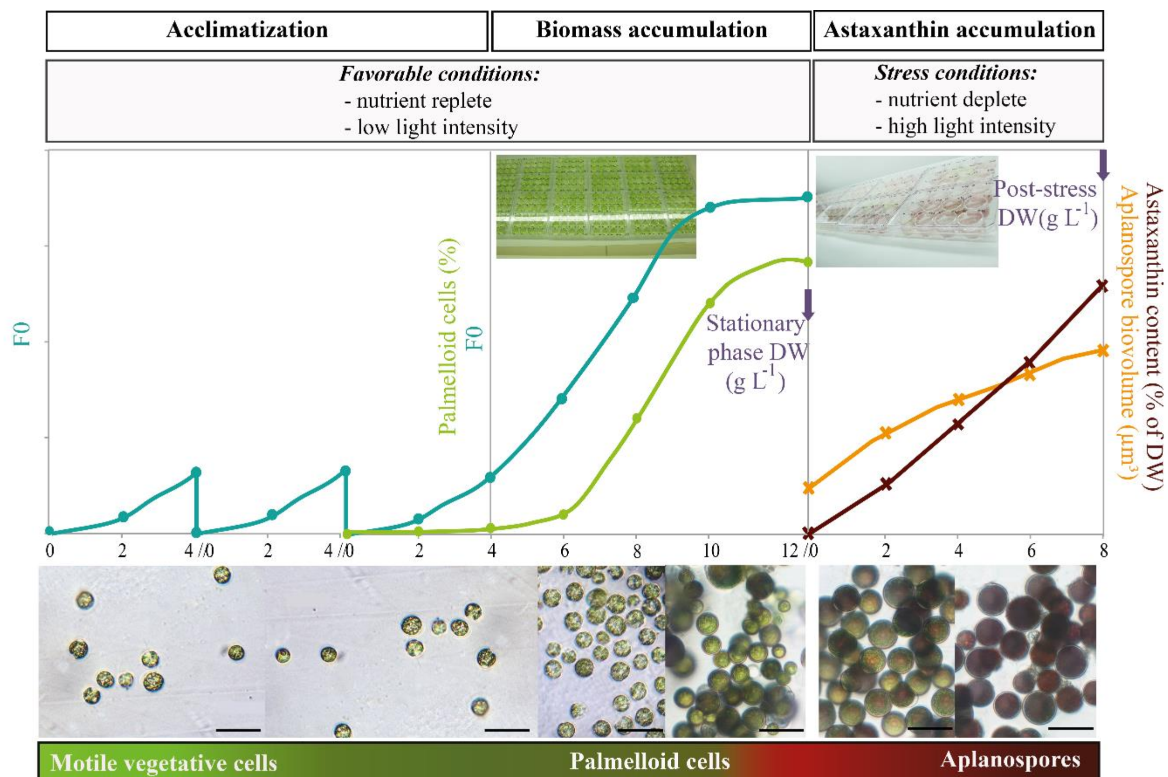



***Figure 2.*** *Schematic representation of the bi-phasic (cultivation and stress) protocol used in this common garden experiment for Haematococcus strains. Strains were first acclimated in exponential phase by two sequential inoculations to ensure their flagellated state, under conditions favorable for vegetative cell division. Next cultures were grown under nutrient replete conditions and low light intensity in batch until reaching stationary phase (time point of harvest was different for each strain). Three traits were measured, including the growth rate, the stationary phase dry weight (DW) and the relative percentage of palmelloid cells. After reaching stationary phase, cultures were harvested, washed twice and inoculated at same densities, under phosphate deficient conditions and high light intensity. At the end of this phase (day 8), three traits were subsequently measured, including aplanospore biovolume, astaxanthin content and dry weight. Photographs on the bottom of the figure were taken during the experiment, scale bar corresponds to 50 µm.* $F_0$*= minimal fluorescence, DW= dry weight.*

***Table 1.*** *Collection data of the thirty Haematococcus strains included in this study (Allewaert et al., 2015). Specifically, substrate type, geographic origin, person who collected the sample, collection date, BOLD (The Barcode of Life Data Systems) & Genbank accession numbers (ITS & rbcL) are shown.ND: Not determined*

| Species | Strain number | Substrate type | Location | Geographical Coordinates | Collected by | Date | BOLD | Genbank (ITS) | Genbank (*rbc* L) | Accession number DCG/BCCM culture collection |
|---|---|---|---|---|---|---|---|---|---|---|
| *H. pluvialis* | SCCAP K-0084 | Small rock pool | Island of Trutbådan, Sweden | 58°42'0.0"N, 17°16'0.0"E | Christensen T. | 1950 | HAEMA021-15 | KR914711 | ND | - |
| *H. pluvialis* | SAG 192.80 | Bog pool | Bruchberg/Harz Mts, Germany | 51°46'60.0"N, 10°31'0.0"E | Koch W. | 1959 | HAEMA022-15 | KR914710 | ND | - |
| *H. pluvialis* | NIES 144* | Freshwater, lake water | Sapporo Hokkaido, Japan | 43°3'43.5"N, 141°21'15.7"E | Ichimura T. | 16 Jul. 1964 | HAEMA023-15 | KR914709 | AB084336, AB084337 | - |
| *H. pluvialis* | CCAP 34/7* | Freshwater | Ostpicken Island, Tvärminne, Finland | 59°50'35.4"N, 23°14'38.5"E | Droop M.R. | 1953 | HAEMA020-15 | KR914712 | FJ438476 | - |
| *H. pluvialis* | CCAP 34/13 = SAG 49.94 | Freshwater, birdbath | Ferrum, Virginia, USA | 36°55'22.5"N, 80°0'48.1"W | Ott F.D. | 1959 | HAEMA025-15 | KR914707 | ND | - |
| *H. pluvialis* | CCAP 34/14* | Freshwater, cement urn | Cattonsville, Maryland, USA | 39°16'19.4"N, 76°43'54.9"W | Ott F.D. | 1989 | HAEMA026-15 | KR914706 | ND | - |
| *H. pluvialis* | BE01_20 | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | HAEMA029-15 | ND | ND | - |
| *H. pluvialis* | BE02_05 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | HAEMA073-15 | ND | ND | DCG 0588 |
| *H. pluvialis* | BE02_09 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | HAEMA031-15 | KR914733 | ND | DCG 0563 |
| *H. pluvialis* | BE03_02 | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | HAEMA032-15 | KR914732 | KR914694 | DCG 0564 |
| *H. pluvialis* | BE03_05 | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | HAEMA033-15 | KR914731 | KR914693 | DCG 0565 |
| *H. pluvialis* | BE07_07 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA068-15 | ND | ND | DCG 0573 |
| *H. pluvialis* | BE07_14 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA036-15 | ND | KR914691 | - |
| *H. pluvialis* | BE10_09 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct. 2013 | HAEMA037-15 | KR914730 | ND | DCG 0589 |
| *H. pluvialis* | NL03_06 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N , 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | HAEMA066-15 | KR914727 | ND | DCG 0577 |
| *H. pluvialis* | NL03_07 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N , 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | HAEMA075-15 | ND | ND | DCG 0578 |
| *H. pluvialis* | IT01_03 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | HAEMA051-15 | ND | ND | DCG 0581 |
| *H. rubicundus* | BE04_06 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | HAEMA041-15 | KR914739 | KR914698 | DCG 0566 |
| *H. rubicundus* | BE04_09 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | HAEMA042-15 | KR914738 | KR914697 | DCG 0567 |
| *H. rubicundus* | BE05_06* | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA044-15 | KR914737 | KR914696 | DCG 0569 |
| *H. rubicundus* | BE08_04 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA076-15 | ND | ND | DCG 0574 |
| *H. rubicundus* | BE08_06 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA045-15 | KR914742 | KR914700 | DCG 0575 |
| *H. rubicundus* | BE08_12 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA046-15 | KR914741 | KR914699 | DCG 0576 |
| *H. rubicundus* | CZ01_06 | Water puddle on white chair | Třeboň, Czech Republic | 49°0'18.3"N, 14°46'23.8"E | Strunecky O. | 12 Feb. 2013 | HAEMA071-15 | ND | ND | DCG 0580 |
| *H. rubicundus* | CZ01_08 | Water puddle on white chair | Třeboň, Czech Republic | 49°0'18.3"N, 14°46'23.8"E | Strunecky O. | 12 Feb. 2013 | HAEMA047-15 | KR914751 | KR914703 | - |
| *H. rubicundus* | NL01_04* | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar 2013 | HAEMA049-15 | KR914749 | KR914701 | DCG 0582 |
| *H. rubicundus* | NL01_05 | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar 2013 | HAEMA050-15 | KR914748 | ND | DCG 0583 |
| *H. rubicundus* | NL02_08 | Water puddle on white bioreactor | Wageningen, The Netherlands | 51°59'46.4"N, 05°39'29.4"E | Allewaert C. | 6 Jun. 2013 | HAEMA052-15 | KR914747 | ND | DCG 0584 |
| *H. rubicundus* | NL02_10 | Water puddle on white bioreactor | Wageningen, The Netherlands | 51°59'46.4"N, 05°39'29.4"E | Allewaert C. | 6 Jun. 2013 | HAEMA077-15 | ND | ND | DCG 0585 |
| *H. rubicundus* | IT01_09 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | | ND | ND | DCG 0676 |

For biomass accumulation the above-described growing conditions were used to measure the following pre-stress traits:

***Growth rate -*** Growth rates of the strains under assessment were determined based on daily measurements of Chl *a* fluorescence by Pulse-Amplitude-Modulated (PAM) fluorescence using a Waltz MAXI-Imaging (PAM) with default settings, intensity of 1, gain of 1 and damping of 2. Minimal fluorescence $F_0$ (Schreiber, 1986) was used as proxy for Chl *a* content of the algal cultures (Honeywill *et al.*, 2002). Before measurement, the cultures were dark acclimated for 15 min. After each measurement, the well plates were randomly displayed under the experimental conditions. Growth rate was calculated as the slope of the linear regression of the log2-transformed $F_0$ fluorescence vs time for each culture during the exponential phase (using minimal 6 measurements).

***Stationary phase DW -*** Algal biomass concentration was measured gravimetrically on the second day of the stationary phase (based on $F_0$ fluorescence) for each strain separately. One of the two technical replicates was harvested by filtering 3 mL of homogenized culture through a pre-weighed and muffled (550 °C for 2 h) glass fiber filter (GF/F, diam. 25 mm, pore size 0.7 µm; Whatman, Kent, UK) and washing twice with de-ionized water (5 mL) to remove remaining salts. Filters were further freeze-dried at -80°C overnight before determining the dry weight (DW).

***Palmelloid cells -*** Palmelloid cells were distinguished based on (Han *et al.*, 2012) as follows: non-motile coccoid green cells of which the spherical protoplast is enveloped within a closely adherent palmella membrane, the primary cell wall. In a preliminary experiment, the evolution of the percentage of palmelloid cells was followed during batch culture growth (as followed using $F_0$) with increases in $F_0$ for six strains (Fig. S1, Supplementary Data). All strains produced palmelloid cells yet at a different rate: for four of the six strains a maximal percentage palmelloid cells was reached when cultures where in stationary phase for two consecutive days ($F_0$-based), while for the two remaining strains at this same time point, the percentage palmelloid cells kept increasing gradually. Based on these preliminary data, the proportion of palmelloid cells in the cultures was quantified on the second consecutive day in stationary phase (time at which palmelloid cell formation reached a maximum) as follows. Subsamples of 50 µL of each homogenized culture were taken and fixed with formaldehyde borax (final concentration 1 %). After settlement, pictures of each replicate were taken using a digital camera (Powershot G3, Canon) and the relative abundance of flagellated cells, bleached cells

(without pigment) and palmelloid cells ($n \geq 200$, per replicate) determined using Image J software (version 1.48; National Institutes of Health, USA). The final palmelloid cells (expressed as % of the total live cells) was used as a proxy for the rate at which cells transformed from flagellated stage to palmelloid.

Experimental stress conditions for astaxanthin induction (the second phase of the experiment) were evaluated in a preliminary experiment using six strains (Figs. S2 & S3, Supplementary Data). Cultures cultivated under conditions described above were transferred to four different stress-inducing conditions by omitting several nutrients from the basal BBM-3N medium as following: a) without nitrate, b) without phosphate, c) without nitrate and phosphate and d) without nitrate, phosphate and trace elements, (leaving only $CaCl_2.2H_2O$, $MgSO_4.7H_2O$ and NaCl). Of the four different treatments, phosphate deprivation led to the highest mean astaxanthin content (% of DW) in four of the six strains tested. In the two remaining strains, the absence of nitrate led to highest astaxanthin content, but not significantly higher than the astaxanthin content under P limitation. (Fig. S2, Supplementary Data). Therefore, P limitation was used to induce stress and astaxanthin production.
After two consecutive days in stationary phase (see above for palmelloid cell induction), the other technical replicate was harvested by centrifugation (3000 rpm at 20 °C during 5 min) and washed twice with a BBM-3N devoid of phosphate. Cultures were subsequently diluted to a concentration corresponding to a $F_0$ of 0.1 (settings 1-1-2) in 12 multi-well plates (Greiner Bio-One) with a working volume of 4 mL phosphate-free BBM-3N medium. Each plate was randomly placed under a continuous light regime and a light intensity of 103 ± 4 µmol photons $m^{-2}$ $s^{-1}$ at 23 °C. All cultures were harvested after eight days of stress, as determined in the preliminary experiment to be sufficient for pronounced strain differences and maximal increases of the post stress traits to be quantified:

***Post stress DW* -** Algal biomass concentration was measured gravimetrically (see stationary phase DW) to determine the accumulated DW.

***Astaxanthin content* -** Samples (0.5 mL) were taken from each well and immediately frozen at -80°C, lyophilized and stored in dark at -80°C before extraction. Astaxanthin was extracted using a modification of Sarada *et al.*, 2006. The freeze-dried pellets were treated with 2 mL HCl (1.5 M) to ensure cell wall lysis, vortexed for 10 s and placed in a warm water bath (70°C) for 5 min. After cooling down, samples were centrifuged for 10 min at 4000 rpm. The acid layer

was removed and washed twice with MillQ water. Extraction was performed by adding 100 % acetone to the washed pellet, followed by sonication at 40 Hz during 1 min in an ice bath. Serial extractions were performed until the extract was colorless and showed absorbance values equal to those of acetone (100 %). The extracts from these series were combined and their absorbance read at 475 nm for astaxanthin and 750 nm for turbidity correction with a spectrophotometer (Shimazu UV-1601, Japan). Astaxanthin was calculated based on the calibration curve of authentic astaxanthin (Sigma Chemical, St. Louis, MO, USA). Standard stock solutions of astaxanthin were prepared in 100 % acetone and serially diluted for the preparation of a standard solution for calibration. All processes were conducted in the dark. The calibration curves were then obtained for known standard solutions and the curves for unknown samples were compared with the calibration curves. To obtain the astaxanthin content (% of DW), the astaxanthin concentration (g $l^{-1}$) was divided by the accumulated post stress DW (g $l^{-1}$). Since all strains were brought to identical $F_0$ at the start of the stress phase, the dilution factor (DF) was taken into account for astaxanthin productivity calculation. Mean astaxanthin productivity (g $l^{-1}$ $d^{-1}$) was calculated as follows:

$$Astaxanthin\ productivity\ = \frac{DW \times astaxanthin\ concentration}{cultivation\ days} \times DF$$

***Aplanospore biovolume* -** Aplanospore biovolume was determined using the same sampling and fixing procedure are palmelloid cell quantification. Aplanospore biovolume (µm³) was estimated from pictures by measurements of mean cell length and cell width ($n \geq 50$, per replicate) using the best fitting ellipse particle sizing function in Image J software (version 1.48; National Institutes of Health, USA). Aplanospore biovolume was calculated assuming aplanospores were spherical.

### 4.2.2. Statistics

Two way nested ANOVA's were applied to test for effects of strain (random factor nested within species) and species for the six traits investigated (including growth rate (number of divisions $d^{-1}$), stationary phase DW (g $l^{-1}$), post stress DW (g $l^{-1}$), astaxanthin (% of DW), palmelloid cells (%), aplanospore biovolume (µm³) and astaxanthin productivity (g $l^{-1}$ $d^{-1}$). Four replicates were available for all traits, except growth rate, which was measured in eight separate wells per strains. As exception, for growth rate, eight replicates were available so this data was treated separately. To obtain a fully balanced design, with thirteen strains per species, three randomly chosen strains of *H. pluvialis* were omitted from the analysis being BE03_02,

BE02_09 and NIES 144. Due to the loss of one of the well plates during the experiment, all replicates belonging to this well plate were removed from the dataset and subsequent random replicates were removed to obtain a balanced design with three replicates of each strain (instead of four). Given the random nature of experimental error, normal distribution was assumed. Before performing the nested ANOVA, homogeneity of variances was checked using the Levene's test, with a critical significance level of $P = 0.01$ for each variable. All variables had a significance level greater than 0.05, indicating homogeneity of variances. The ANOVA was performed in R version 3.0.2 (R Core Team, 2013) using the built in function aov applying a significance level of 0.05. Pairwise comparisons between strains were performed using one way ANOVA's followed by post hoc Tukey tests, with strain as fixed factor and the individual traits as dependent variables on the total dataset (4 replicates, excluding however the replicates associated with the lost well plate), including all strains. The latter were performed with *Statistica* version 7.0 for Windows (StatSoft) by applying a significance level of 0.05.

To test for correlation between traits for the different genotypes in *H. pluvialis* and *H. rubicundus*, Pearson correlation coefficients (r) were calculated and the associated *P*-values determined to test the hypothesis of no correlation against the alternative of non-zero correlation. Correlations were considered statistically significant if $P \leq 0.05$. Correlation analyses were made in R version 3.0.2 using the lattice package (R Core Team, 2013). To quantify the portion of growth, palmelloid cells, DW (stationary and post stress), astaxanthin content, aplanospore biovolume and astaxanthin productivity variation attributable to genetic variation, broad-sense heritabilities ($H^2$) were calculated for *H. pluvialis* and *H. rubicundus*. For clonal organisms, the broad-sense heritability of a trait represents the portion of total phenotypic variance comprised by the total genetic variance: $H^2 = \sigma^2_G / \sigma^2_T$ where $\sigma^2_G$ is the genetic variance (between strain variance) and $\sigma^2_T$ is the total trait variance (Lynch & Walsh, 1998). Using the results from the one-way ANOVA for each trait with strain as random factor, $H^2$ was calculated as the ratio of among-lineage variance ($\sigma^2_G$) to total phenotypic variance ($\sigma^2_T$), where $\sigma^2_T$ is the sum of all variance components.

Principal component analysis (PCA) of the six traits together with astaxanthin productivity was carried out to visualize patterns of phenotypic variation among and between species using the devtools package in R version 3.0.2. Due to different units of measurements all data were standardized before analysis (Z score transformation).

## 4.3. Results

For the strains tested, *H. pluvialis* and *H. rubicundus* differed significantly (Fig. 3, nested ANOVA (Table 2)) in growth rate, stationary phase DW, palmelloid cell percentage, post stress DW and aplanospore biovolume, while no species differences were found in astaxanthin content. The strain-effect (nested in species) was significant both for all pre-stress and post-stress traits, meaning there was significant intraspecific variation for all traits.

Pre-stress mean exponential growth rates varied between 0.44 ± 0.02 divisions $d^{-1}$ and 0.98 ± 0.06 divisions $d^{-1}$. Strains of *H. pluvialis* had on average a lower growth rate than *H. rubicundus* (Fig. 3A). Within *H. pluvialis* and *H. rubicundus*, the mean growth rate differed significantly between strains ($P$ = 1.10 $e^{-16}$ and $P$ = 2.22 $e^{-16}$, respectively). The mean stationary phase DW varied between 0.22 ± 0.06 g $l^{-1}$ and 0.85 ± 0.27 g $l^{-1}$ and was significantly higher for *H. pluvialis* than for *H. rubicundus* (Fig. 3B). In both species, the mean stationary phase DW differed significantly between strains within *H. pluvialis* ($P$ = 1.13$e^{-04}$) and *H. rubicundus* ($P$ = 3.33$e^{-04}$). The mean percentage of palmelloid cells after two days in stationary phase, varied between 1.4 ± 1.7 and 94.7 ± 4.5 % and significantly differed between *H. pluvialis* and *H. rubicundus* (Fig. 3C). Here again, mean palmelloid cell percentage differed significantly between strains within both *H. pluvialis* ($P$ = 2.95$e^{-08}$) and *H. rubicundus* ($P$ = 1.02$e^{-06}$).

Post stress mean DW varied between 0.20 ± 0.07 g $l^{-1}$ and 1.37 ± 0.18 g $l^{-1}$ (Fig. 3D) and strains of *H. pluvialis* accumulated on average more biomass than strains of *H. rubicundus*. There were also differences in mean post stress DW between strains within both *H. pluvialis* ($P < 3.57e^{-11}$) and *H. rubicundus* ($P$ = 7.99$e^{-04}$). The final astaxanthin content (expressed as % of DW) varied between 0.82 ± 0.26 % of DW and 4.72 ± 0.29 % of DW (Fig. 3E) yet did not differ significantly between species ($P$ = 0.081), while significant differences between strains within *H. pluvialis* ($P$ = 1.15$e^{-10}$) and within *H. rubicundus* ($P$ = 3.52$e^{-12}$) were found. Finally, the aplanospore biovolume varied between 3.04$e^{+04}$ ± 6.27$e^{+03}$ µm³ and 2.35$e^{+05}$ ± 6.54$e^{+04}$ µm³ and *H. pluvialis* strains had on average a significantly higher mean aplanospore biovolume than *H. rubicundus* (Fig. 3F). Within *H. pluvialis* ($P$ = 1.42$e^{-08}$) and *H. rubicundus* ($P$ = 1.60$e^{-06}$), the mean aplanospore biovolume differed significantly between strains.

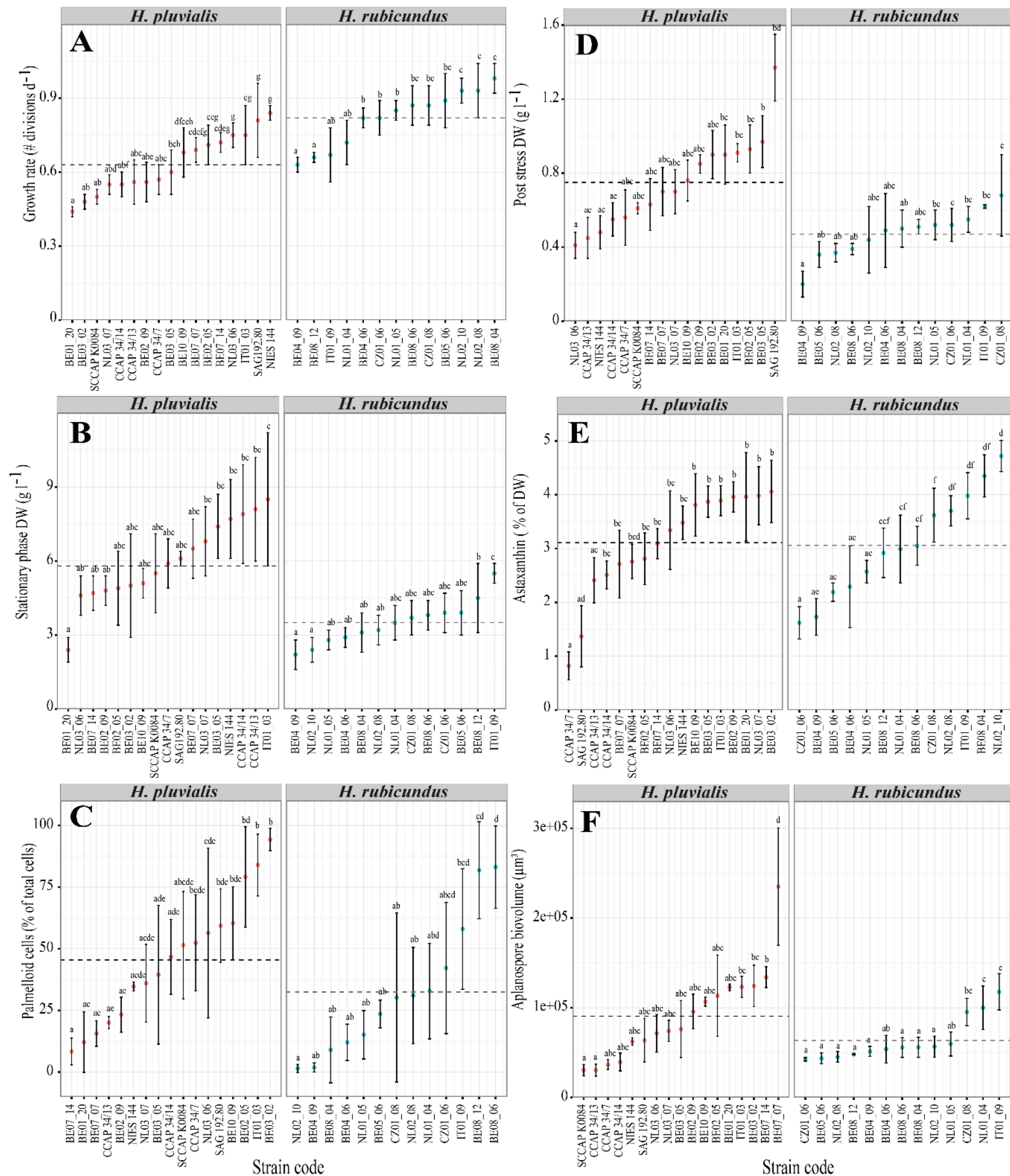



***Figure 3**. **A.** Means (± SD) of the specific growth rate (number of divisions d$^{-1}$), **B.** stationary phase dry weight (DW) (g l$^{-1}$), **C.** relative percentage of palmelloid cells, **D.** post stress DW (g l$^{-1}$) , **E.** astaxanthin content (% of DW) and **F.** aplanospore biovolume (µm³) of the thirty different strains of Haematococcus pluvialis and Haematococcus rubicundus. Within each species, strains are ordered according to increasing mean for each trait. The horizontal black and grey dashed bars represent the mean for each trait for H. pluvialis and H. rubicundus respectively. Significant differences between strains as evaluated by post hoc Tukey comparisons are indicated with letters where strains with the same letter are not significantly different at P ≤ 0.05. DW= dry weight.*

Nested ANOVA's showed that there was a significant main effect of species as well as species and strain on average astaxanthin productivity (Table 2). The mean astaxanthin productivity of all *Haematococcus* strains varied between 0.23 ± 0.07 and 4.59 ± 1.29 mg $l^{-1}$ $d^{-1}$ (Fig. 4). *H. pluvialis* had significantly higher astaxanthin productivity than *H. rubicundus*. There were highly significant differences in astaxanthin productivity between strains within *H. pluvialis* ($P = 9.83e^{-06}$) and within *H. rubicundus* ($P = 3.25e^{-04}$).

***Table 2.*** *Summary statistics for the nested two-way ANOVA to determine strain specific effects within Haematococcus pluvialis and Haematococcus rubicundus (species) for the traits investigated; *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns, not significant.*

| | Growth rate (# of divisions.$d^{-1}$) | | | Stationary phase DW ($g\ l^{-1}$) | | | Palmelloid/total live cells (%) | | | Post stress DW ($g\ l^{-1}$) | | | Astaxanthin (% of DW) | | | Aplanospore biovolume (µm³) | | | Productivity ($mg\ l^{-1}\ d^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | df | F | *P* | df | F | *P* | df | F | *P* | df | F | *P* | df | F | *P* | df | F | *P* | df | F | *P* |
| *Species* | 1 | 227.18 | *** | 1 | 42.59 | *** | 1 | 11.58 | ** | 1 | 58.23 | *** | 1 | 0.33 | ns | 1 | 42.59 | *** | 1 | 27.42 | *** |
| *Species : Strain* | 24 | 11.09 | *** | 24 | 12.18 | *** | 24 | 6.35 | *** | 24 | 4.13 | *** | 24 | 10.70 | *** | 24 | 12.18 | *** | 24 | 4.73 | *** |

Patterns of association were analyzed among traits for *H. pluvialis* and *H. rubicundus* separately. Overall, we found five significant positive trait correlations in *H. rubicundus* against two significant trait correlations in *H. pluvialis* ($P \leq 0.05$)(Fig. 5). The calculated astaxanthin productivity showed a strong positive association with the three measured post stress traits, including post stress DW, astaxanthin content and aplanospore biovolume in *H. rubicundus*. Similarly, in *H. pluvialis* this was the case, for the exception of aplanospore biovolume which did not significantly correlate with astaxanthin productivity. However, upon removal of the outlier strain BE07_07, -which formed extremely large aplanospores (up to $2.35e^{05}$ µm³) yet contained low astaxanthin yields-, both traits were correlated. Additionally, in *H. rubicundus*, a significant positive correlation between aplanospore biovolume and post stress DW was found and between stationary phase DW and palmelloid cells.

All estimated broad-sense heritability $H^2$ estimates were superior to 0.48 for *H. pluvialis* and 0.43 for *H. rubicundus* (Table S1, Supplementary Data), yet average values over the different traits were similar for both species ($H^2$=0.68). Astaxanthin content showed the highest $H^2$ for *H. rubicundus* (0.83) while aplanospore biovolume was highest for *H. pluvialis* (0.79).

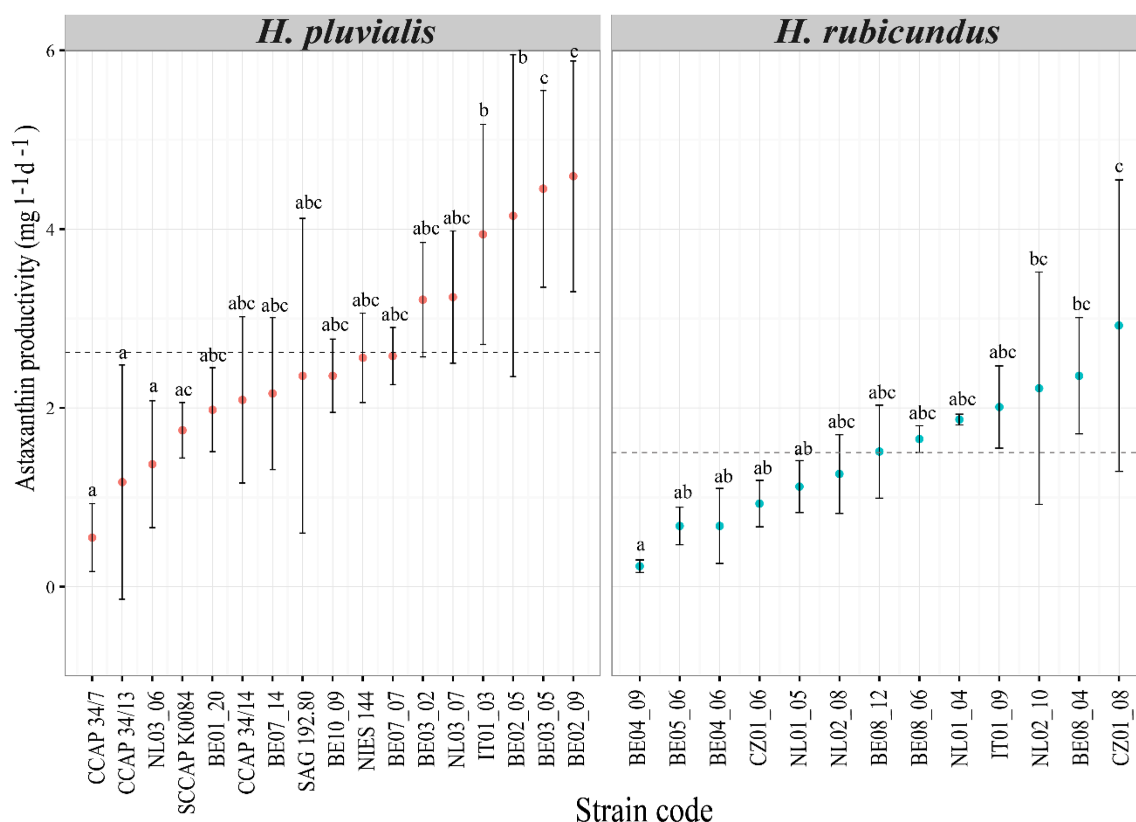



***Figure 4.*** *Means (± SD) of astaxanthin productivity (mg $l^{-1}$ $d^{-1}$) of the thirty different strains of Haematococcus pluvialis and Haematococcus rubicundus. Within each species, the strains are ordered according to increasing mean for each trait. The horizontal black and grey dashed bars represent the mean for each trait for H. pluvialis and H. rubicundus respectively Significant differences between strains as evaluated by post hoc Tukey comparisons are indicated with letters where strains with the same letter are not significantly different at P ≤ 0.05.*

The principal component analysis (PCA) extracted two major components that cumulatively accounted for 60.8 % of the variance in all traits. Principal component 1 (PC1, X-axis, Fig. 6, Table S2, Supplementary Data) accounted for 40.0 % of total variation among strains and positively correlated with astaxanthin productivity, post stress DW, stationary phase DW and aplanospore biovolume. Principal component 2 (PC2, Y-axis, Fig. 6, Table S2, Supplementary Data) accounted for 20.8 % of the variation among strains and was negatively correlated with astaxanthin content and growth rate, and positively with stationary phase DW and palmelloid cells. All other traits were weakly related with the first two PCs. PCA sample scores showed clustering of strains according to species along the first axis and according to wild type vs cultured strains along the second axis. *H. pluvialis* strains from culture collections (including CCAP 34/7, CCAP 34/13, CCAP 34/14, SCCAP K0084, SAG 192.80 and eventually NIES 144) formed a cluster, isolated from *H. rubicundus* and *H. pluvialis* wild type clusters, determined by a low astaxanthin content.

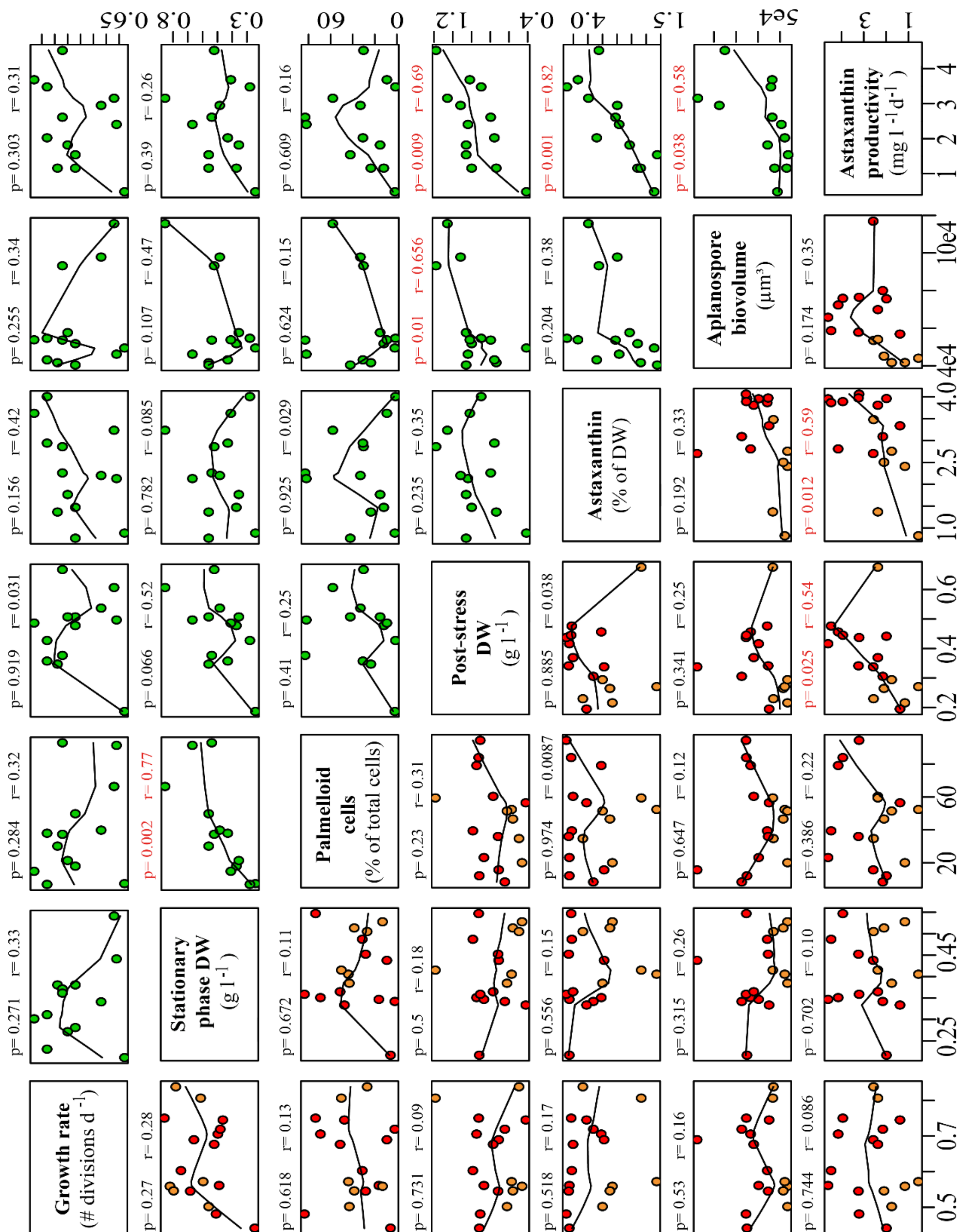



***Figure 5.*** *Correlations among the six traits measured (and astaxanthin productivity) for Haematococcus pluvialis (lower panel) and Haematococcus rubicundus (upper panel). The trait name and unit is shown in the diagonal. Each point represents the mean per strain for each trait: red circles correspond to H. pluvialis natural strains, orange circles to H. pluvialis cultured strains and green circles correspond to H. rubicundus. The Pearson correlation for each pair of variables (r) and their statistical significance (P-value) are given above each correlation graph. DW= dry weight.*

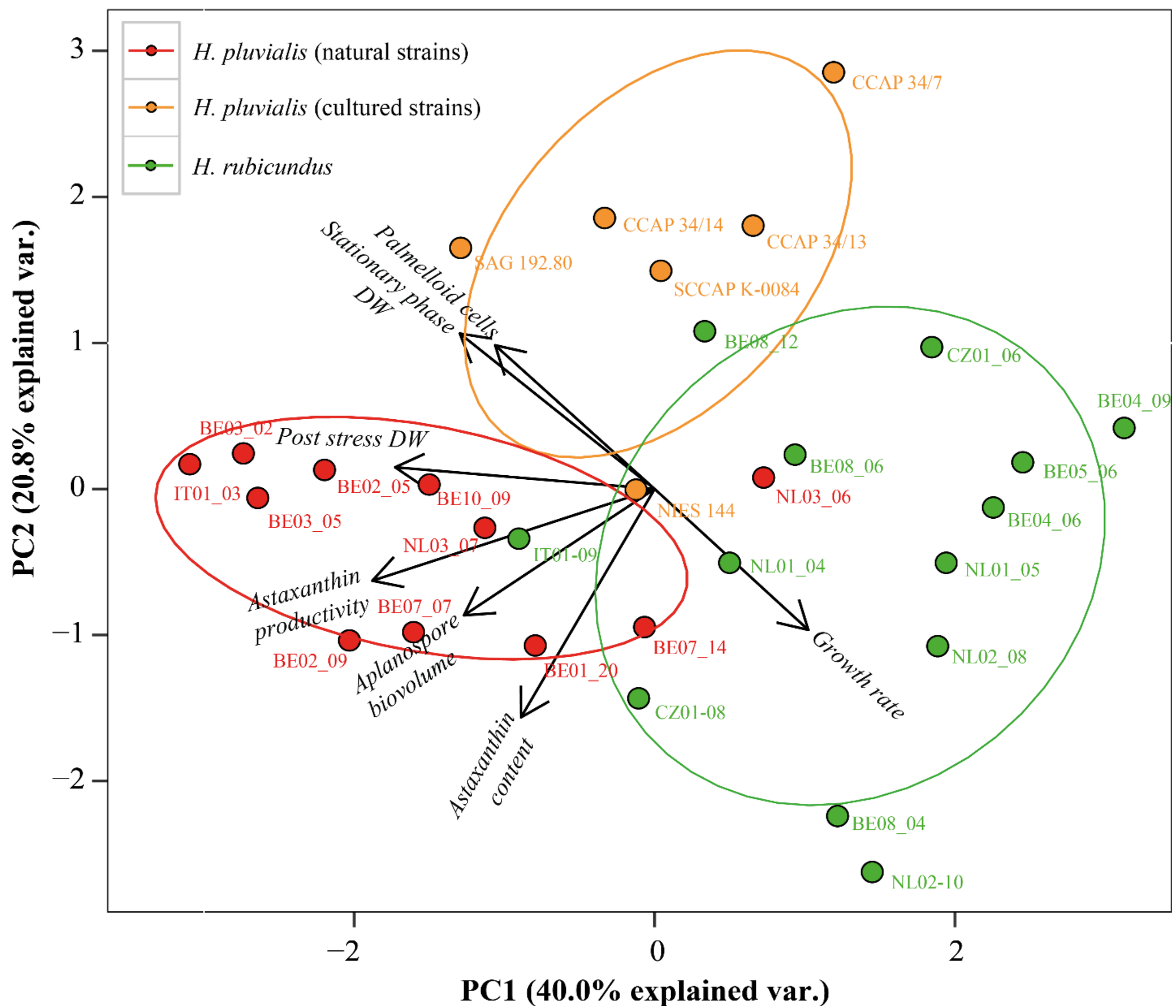



***Figure 6.*** *Biplot on the first two axes of a PCA of the traits under assessment on natural (red circles) and cultured (orange circles) strains of Haematococcus pluvialis, Haematococcus rubicundus (green circles). DW= dry weight.*

## 4.4. Discussion

In the present study, we explored the genetically based variation in a series of phenotypic characters among thirty strains of two *Haematococcus* species. Differences between *H. pluvialis* and *H. rubicundus* were also assessed. We found a large reservoir of intraspecific variation for each of the six traits measured in both pre-stress and post stress conditions. At the interspecific level, a strong overlap in trait values was found between both species, yet significant species differences were found.

Regarding pre stress traits, the maximal growth of the flagellated stage differed twofold between the slowest and the fastest growing strain. Strain BE08_04 of *H. rubicundus* grew fastest with a growth rate (0.98 divisions $d^{-1}$), which is higher than earlier reported for *Haematococcus* strains (0.633 $d^{-1}$, 0.72 $d^{-1}$and 0.29 $d^{-1}$; (Gong & Chen, 1997; Cifuentes *et al.*,

2003; Göksan & Ak 2006), respectively). Higher growth rate could be the result of the medium used in our study which was enriched with nitrate, positively influencing growth and steady state density (Fábregas *et al.*, 2000). Likewise, early stationary phase DW was found to be highly variable between strains with a fourfold difference between the strains with lowest DW and highest DW, respectively. Strains were further found to differ in their capacity to form palmelloid cells during early stationary phase. Recent studies have already advanced behavioral differences in strains regarding palmelloid formation (Han *et al.*, 2012; Wang*et al.*, 2014). This temporary phase of development is usually considered as a response to unfavorable environmental or culture conditions (Han *et al.*, 2013). In batch culture as used in this study, palmelloid cells percentage (and subsequently flagellated cell percentage) was related to culture age, yet the rate at which this transition occurs, strongly differs between strains. Today, very little is known on the conditions that contribute to the onset or the reversion of the palmelloid stage, yet, from an astaxanthin productivity perspective, palmelloid cells are preferred over flagellated cells as they are believed to be more resistant to the stress imposed to cultures for astaxanthin induction (Wang *et al.*, 2003). Regarding the post stress traits, firstly the lowest mean DW differed almost seven fold with the highest. Secondly, the maximal astaxanthin content (4.7 % DW), was up to six times higher than the minimal astaxanthin content (0.82 % DW) obtained from this study and was similar to maxima from previous indoor cultures of *H. pluvialis* (4 % and 2.7 % of DW reported by Aflalo *et al.*, (2007) and Harker *et al.* (1996a). The final aplanospore biovolume strongly varied between strains. Converted to cell diameter, the largest was found for strain BE07_07 (*H. pluvialis*) 37 µm and shortest of 18 µm for strain SCCAP K-0084 (*H. pluvialis*). Aplanospore diameters in literature vary between 25 µm, 28 µm and 30 µm. (Hagen *et al.*, 1994; Grünewald *et al.*, 1997; Wang *et al.*, 2003; Torzillo *et al.*, 2005; Wang *et al.*, 2013a), reported cell diameter increases of 23 % and 10 % respectively while according to Torzillo *et al.,* (2005), cell diameter remained constant during stress.

Since astaxanthin productivity is the product of astaxanthin content and biomass productivity, this measure gives an indication of the pigment produced on a basis of both volume and time and is a critical variable for evaluating and comparing strains for astaxanthin production. We found up to fifteen-fold differences between the most productive and the less productive strains and *H. pluvialis* had a higher overall astaxanthin productivity than *H. rubicundus*. Maximal astaxanthin productivities were in agreement with previous findings (4.8 and 4.3 mg l $d^{-1}$, López *et al.,* (2006) and Zhang *et al.,* (2009) although several higher values have been reported (5.6 and 6.3 mg $l^{-1}$ $d^{-1}$ by Del Río *et al.,* (2008) and Kang *et al.,* (2005), respectively).

Noteworthy is the fact that recently isolated strains had higher mean astaxanthin productivities than strains from culture collections, implying that cultivated strains and wild type strains responded differently to the culture and stress conditions imposed. All six culture collections strains from this study have a long history (oldest strain since the 1950's) of cultivation and sub-cultivation on agar and on liquid medium. They have for long been exposed to constant growth conditions, detaching them from their 'natural state' or original phenotype. Lakeman *et al,.* (2009) reviewed this matter in detail and have listed some examples of in-culture evolution. There is an increasing body of evidence that even slight changes in culture conditions may drive directional evolution towards physiological changes through adaption of genotypes (Goho & Bell, 2000; Martins *et al.*, 2004; von Dassow *et al.*, 2005; Borowitzka & Moheimani, 2013). It seems likely that the contrast between cultured strains and our recent isolates (reduced astaxanthin productivity) is the result of selection in culture conditions or neutral mutation accumulation resulting in mutant strains with characteristics deviating from the original phenotypes. Altogether, our results emphasize the significance of isolation and screening of new strains, as well the importance of maintaining strains in a metabolically inactive state, i.e., through cryopreservation.

For five of the six traits examined, despite significant overlap in trait values, significant differences between species were found: *H. rubicundus* strains on average grew faster, while accumulating less DW at the stationary phase than *H. pluvialis*. *H. pluvialis* strains had an overall tendency to rapidly transform to palmelloid stages. *H. pluvialis* was already reported to have a lower specific growth rate than *H. rubicundus* (Allewaert *et al.*, 2015). *H. rubicundus* further accumulated less biomass after stress and concomitantly formed smaller aplanospores than *H. pluvialis*. Although we found that trait variations were linked to species, we did not find any correlation with the source environment (data not shown). PCA analysis of the traits assessed in function of strain origin (geographical) did not reveal any correlations. It is important to note, however, that our study was confined to two closely-related *Haematococcus* species sampled from European temperate habitats.

Overall, our results suggest that the variability of astaxanthin productivity in both *Haematococcus* species is driven by a combination of DW accumulation, astaxanthin accumulation and aplanospore biovolume increases, all traits determined during the stress phase. It is well known that correlations between traits can be affected by taxonomic biases, implying that single species may be more appropriate to distinguish trait correlations (Wright *et al.*, 2007; Fajardo & Piper, 2011). Our study emphasizes that patterns of trait associations

should be investigated at the species level. Genus level correlations in our case would largely be determined by within *H. rubicundus* correlations and to a lesser extent *H. pluvialis.* The absence of correlations in *H. pluvialis* (at least in the biomass accumulation phase) could be due to the presence of culture strains, which had contrasting phenotypic patterns, as also revealed by PCA. Very little information on *Haematococcus* trait correlations is present in literature. Firstly, from our data set, there is no evidence showing that strains producing more palmelloid cells also have a higher astaxanthin productivity. This contradicts the findings of Han *et al.,* (2012), where two *H. pluvialis* strains-one dominantly palmelloid and one dominantly flagellated-where compared for astaxanthin productivity. Secondly, in accordance with Kang *et al.,* (2005), we would expect aplanospore size and astaxanthin content to be positively correlated, yet our results imply that the strains with large aplanospores did not necessarily result in higher astaxanthin content, regardless of the species. The correlation of aplanospore biovolume with post stress DW suggests that a common genetic architecture governs both traits. Since all strains were brought to identical $F_0$ before applying astaxanthin inductive conditions, increases in post stress DW could be attributable to either aplanospore enlargement (as is the case in *H. rubicundus*) or cell division, as in Wang *et al.,* (2013b). Other morphological changes, such as the production of palmelloid cells were more pronounced in *H. rubicundus* since they also affected changes in DW.

Although these strain differences could be exploited by careful selection for potential gains in productivity, direct implementation of the most performant strains from this study– strains with highest astaxanthin productivity- for industrial purposes would not be meaningful at this stage. Since the screening was performed under a standard set of laboratory conditions, it remains to be investigated whether our findings are amenable to large scale production conditions. In any case, this study not only demonstrates the feasibility of phenotyping for several traits, it also provides strain engineers/breeders with a well-documented strain panel to further improve astaxanthin productivity in *Haematococcus* through the different motifs discussed here.

Firstly, the broad variation in phenotypic traits among natural isolates can provide insights into the underlying biological processes and mechanisms in future studies of gene function, the search for quantitative trait loci (QTL) and targeted selection experiments. Comparative studies of gene function for quantitative genetic traits are emerging. Recently, Gao *et al.,* (2015) reported that carotenogenesis regulation at a posttranslational level was highly strain-specific in *H. pluvialis*. Secondly, only positive associations were found between traits and since astaxanthin productivity was correlated predominantly with post stress traits in both species,

this part of the astaxanthin production process should be targeted during bi-phasic cultivation. Thirdly, to help predict responses to selection for desired traits, the broad sense heritability of each trait was quantified. The overall high $H^2$ estimates found (particularly for astaxanthin content) imply that progeny will respond rapidly and with high gains, while for traits with low heritability, responses will be slower and less marked (Falconer *et al.*, 1996).

Finally, breeding programmes, as widely implemented in agriculture for crop selection, clearly have potential in microalgae development since they can be executed much faster using interfertile wild-type isolates given their short sexual cycles and rapid growth (Georgianna & Mayfield, 2012). Unfortunately, so far, we have not been able to observe mating processes in any of our *Haematococcus* strains collected, although *H. pluvialis* is known to reproduce sexually under special circumstances (Triki *et al.*, 1997; Sun *et al.*, 2008).

## 4.5. Conclusions

In broader terms, our study builds on classical ecological genetics and provides a more in depth physiologically based understanding of genetic divergence within and between species. In light of massive comparative genomic studies at intra and interspecific level, as in several plant species (Cao *et al.*, 2011; Xu *et al.*, 2012; Li *et al.*, 2013; Wang, B *et al.*, 2014) and recently in microalgae (Flowers *et al.*, 2015), studies as ours may be important for targeting loci that control or influence phenotypes. In the end, this will contribute to the development of engineered strains with increased growth, resilience and carotenoid productivity.

## 4.6. Acknowledgements

The authors are indebted to the Belgian Coordinated Collection of Micro-organisms (http://bccm.belspo.be) for cryopreserving and providing all strains from this study (accession numbers in Table 1). PV is a postdoctoral research fellow with the Research Foundation – Flanders (FWO). This work was supported by a Ph.D. grant (contract number: 121449) from the Agency for Innovation by Science and Technology (IWT). The authors wish to thank the editor and anonymous reviewer for the helpful suggestions.

## 4.7. Supplementary Data

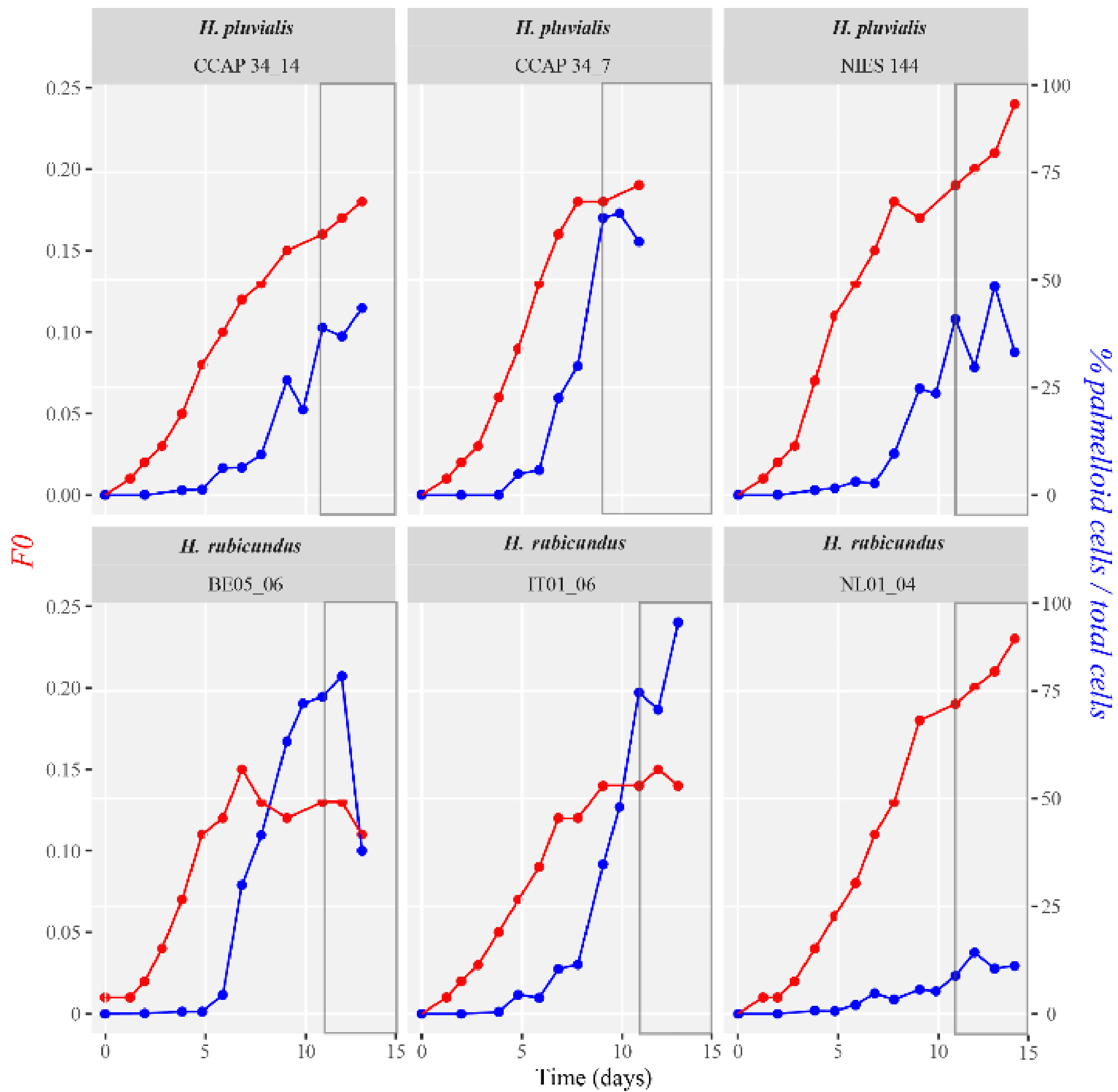



***Figure S1.*** *$F_0$ obtained by PAM (red line) values and relative percentage palmelloid cells (blue line) through time during the biomass accumulation phase for six strains of H. pluvialis and H. rubicundus obtained for the preliminary experiment.*

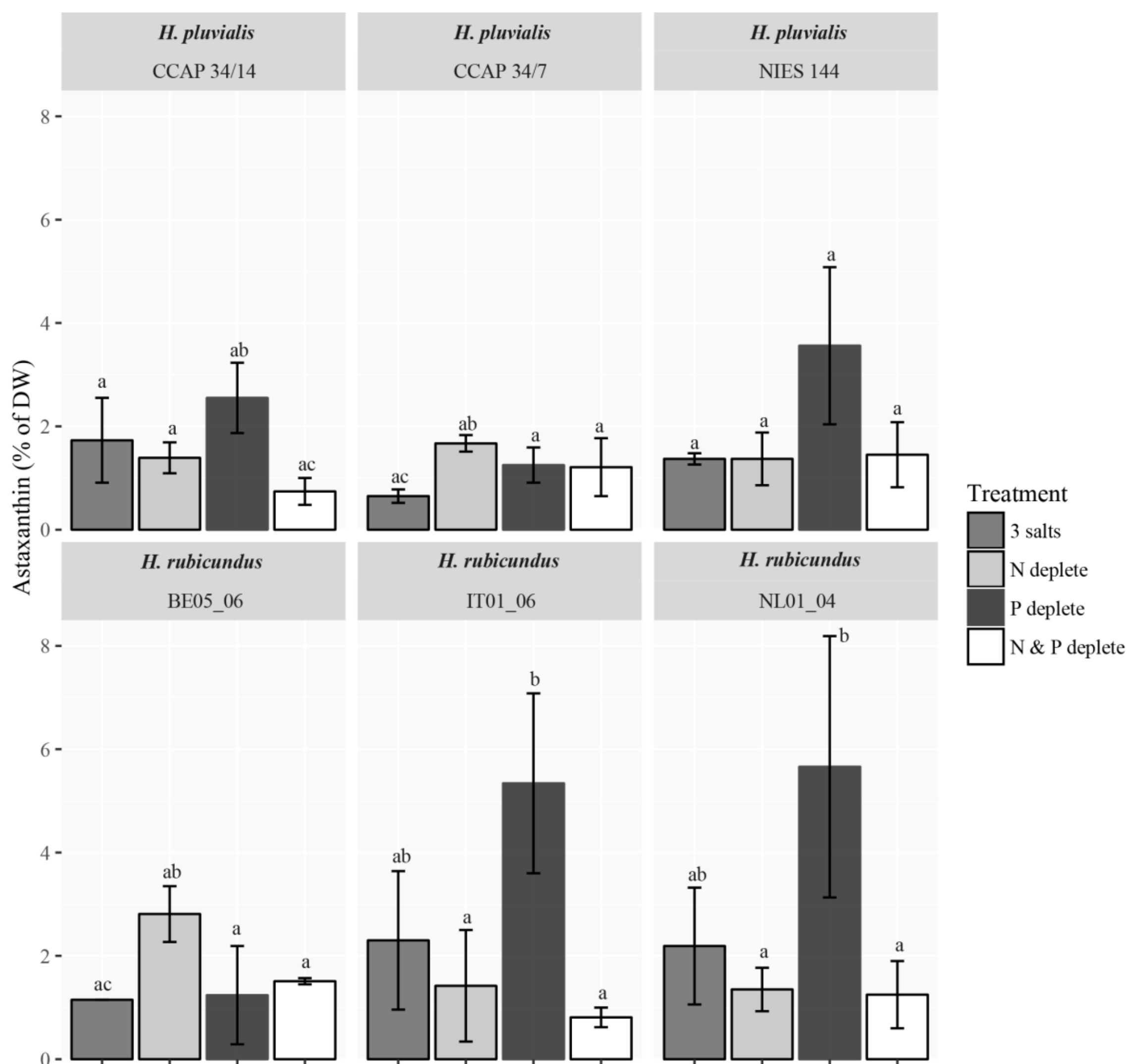



***Figure S2.*** *Percentage astaxanthin (% of DW) on day 12 obtained for the six strains of H. pluvialis and H. rubicundus under four different stress conditions (including –N nitrate limited. -P phosphate limited. -P&N: phosphate and nitrate limited. 3 salts: culture medium containing only three salts. Different letters indicate significant differences (P < 0.05 for all significant differences).*

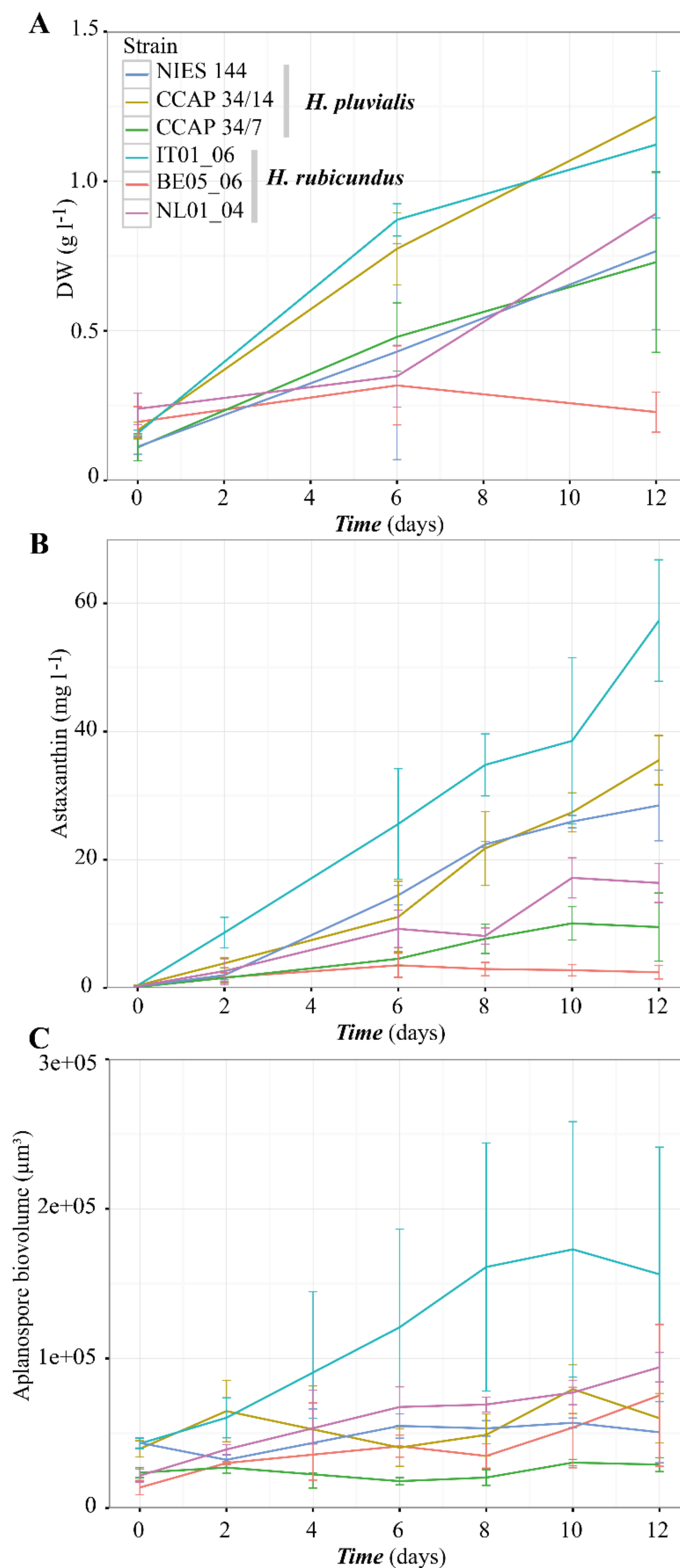


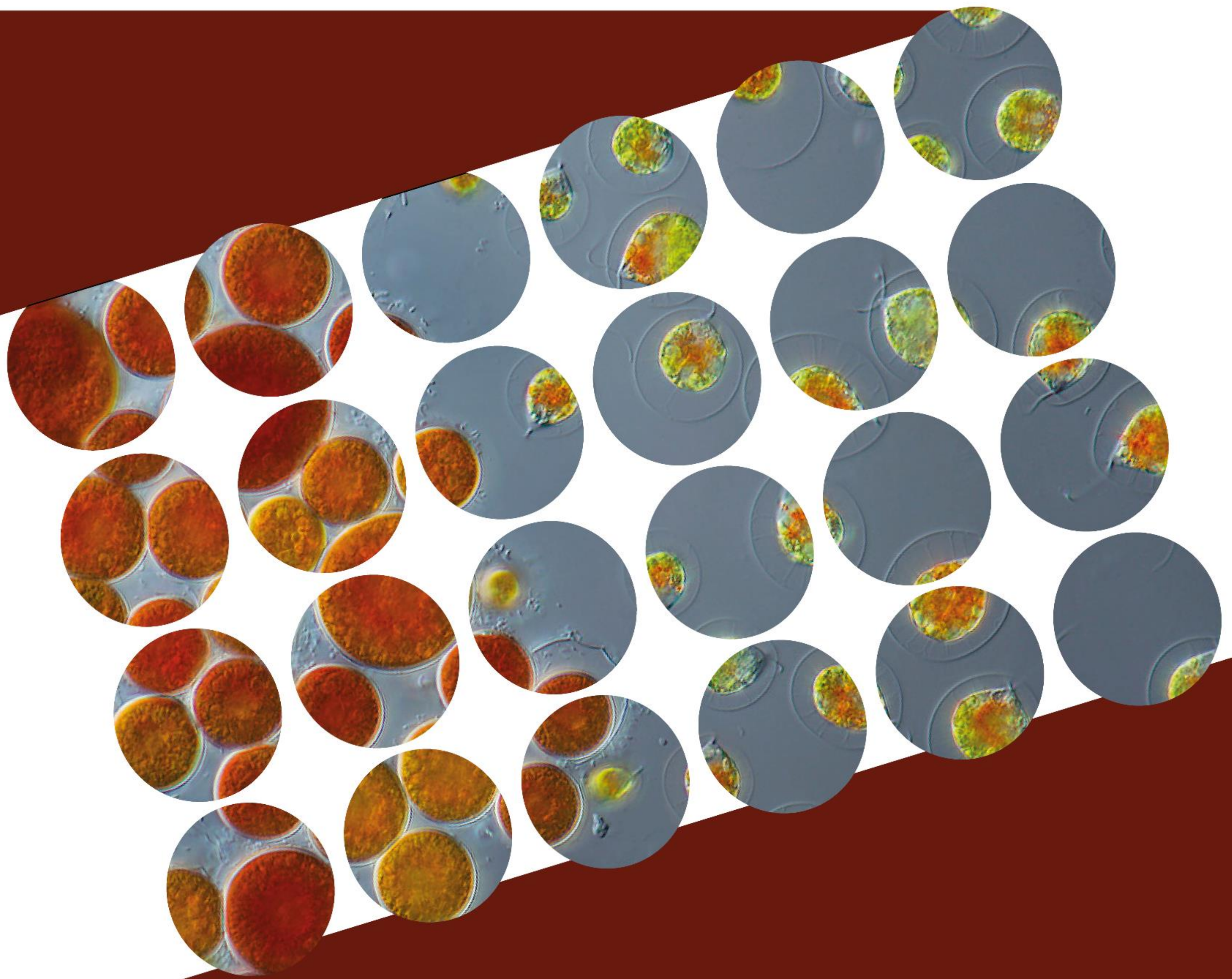

***Figure S3. A.*** *Mean post stress DW (g $l^{-1}$),* ***B.*** *astaxanthin (mg $l^{-1}$) and* ***C.*** *aplanospore biovolume (µm³) (± SD) increases of the six strains of H. pluvialis and H. rubicundus throughout the astaxanthin accumulation phase under phosphate limitation obtained for the preliminary experiment.*

***Table S1.*** *Broad sense heritability estimates ($H^2$) and the level of significance based on one way ANOVA's within H. pluvialis and H. rubicundus for each of the six traits and astaxanthin productivity.*

| | ***H. pluvialis*** | ***H. rubicundus*** |
|---|---|---|
| Growth rate | 0.67<br>P<0.001 | 0.69<br>P<0.001 |
| Stationary phase DW | 0.48<br>P<0.001 | 0.63<br>P<0.001 |
| Palmelloid cells | 0.68<br>P<0.001 | 0.66<br>P<0.001 |
| Post stress DW | 0.55<br>P<0.001 | 0.50<br>P<0.001 |
| Astaxanthin | 0.72<br>P<0.001 | 0.83<br>P<0.001 |
| Aplanospore biovolume | 0.79<br>P<0.001 | 0.77<br>P<0.001 |
| Astaxanthin productivity | 0.59<br>P<0.001 | 0.43<br>P<0.001 |
| Average | 0.64 ± 0.11 | 0.64 ± 0.14 |

***Table S2.*** *Principal component analysis (PCA) for the first two principal components based on six traits and astaxanthin productivity of thirty Haematococcus strains. PC loadings and the percentage of variance explained by the first two PCs are reported. For each PC, the first four variables with highest loadings are in bold.*

| | PC1 | PC2 |
|---|---|---|
| Growth rate | 0.29 | **-0.37** |
| Stationary phase DW | **-0.36** | **0.41** |
| Palmelloid cells | -0.30 | **0.38** |
| Post stress DW | **-0.48** | 0.06 |
| Astaxanthin | -0.25 | **-0.61** |
| Aplanospore biovolume | **-0.35** | -0.33 |
| Astaxanthin productivity | **-0.52** | -0.24 |
| Total variance % | 40.0% | 20.8% |
| Cumulative variance % | 40.0% | 60.7% |



# CHAPTER 5:
## Genotype by irradiance interactions in six *Haematococcus* strains *(Chlorophyceae, Volvocales)* during vegetative growth

Unpublished Manuscript. Allewaert CC., Vanormelingen P., Daveloose I. & Vyverman W. (2016).

## Abstract

*Haematococcus pluvialis* Flotow has received considerable attention given its capacity to accumulate high concentrations of astaxanthin for use in animal feed and as nutraceutical for humans. Since the conditions for cultivation greatly vary from those for astaxanthin production, *H. pluvialis* is produced in two steps: a green (biomass accumulation) and a red (astaxanthin accumulation) phase. Many studies have investigated the optimal irradiance for astaxanthin accumulation, yet currently, considerable disagreements exist regarding the green phase, with optima for growth and yield varying between studies from 30 and 200 µmol photons $m^{-2}$ $s^{-1}$. Whether these discrepancies arise from strain variability or from poorly established experimental designs is currently not known.

In this study, the responses of six different *Haematococcus* strains (different genoytypes), belonging to two species (*H. pluvialis* and *H. rubicundus*) were assessed when exposed to a light gradient comprised of nine different irradiances (2,7,15,30,52,79,114,154 and 222 µmol photons $m^{-2}$ $s^{-1}$). We made systematic comparisons of batch grown steady state unialgal cultures in terms of cell type, cell size, mortality (bleaching), growth rate, cell density, maximum quantum yield of PSII ($F_v/F_m$) and quantitative and qualitative pigment composition.

Significant strain and irradiance effects were found for almost all traits (except growth rate and relative percentage flagellated cells, strain effect) (cell density and biovolume, irradiance effect), implying that is was not possible to denote a single optimum irradiance resulting in increased growth and biomass accumulation valid for all *Haematococcus* strains from this study. Moreover, irradiance x strain, or G x E effects were found for cell mortality, $F_v/F_m$ ratio, cell density as well as for pigment composition. Since strains differed in their genetic ranking order in the respective reaction norms, responses to these factors cannot be predicted from one environmental to the next. We conclude that investigated strains of *H. pluvialis* and *H. rubicundus* likely employ different strategies to respond to varying irradiance.

## 5.1. Introduction

Studies on the cultivation of the green microalgae *Haematococcus pluvialis* at laboratory scale are numerous and growing as this organism has emerged as an outstanding producer of astaxanthin under stress conditions and as reference species for the exploration of astaxanthin biosynthesis pathways (Chen *et al.*, 2015; Shah *et al.*, 2016). Astaxanthin (3-3-dihydroxy-β,β′-carotene-4,4′-dione) is a secondary carotenoid which has a long tradition as dye in the aquaculture industry (Fujii *et al.*, 2006; Parisenti *et al.*, 2011; Basri *et al.*, 2015). Besides acting as a pigment, astaxanthin has a super anti-oxidant potential, believed to be greater than β-carotene and even α-tocopherol (Ambati *et al.*, 2014), which makes it an appealing product in the nutraceutical industry.

Compared to other green microalgae, studies on the morphogenesis and ultrastructure of *H. pluvialis* proceed slowely. Understanding the complex morphology and life cycle of this specis is becoming increasingly important due to its interest as a biotechnological source of astaxanthin (Klochkova *et al.*, 2013). Since different conditions are required for production of green cells and the accumulation of astaxanthin in aplanospores, two stage production processes are most commonly used, where the biomass accumulation is separated from the astaxanthin accumulation phase (Olaizola, 2000; Fábregas *et al.*, 2001). Numerous papers investigate how nutritional and environmental factors influence astaxanthin synthesis, but very few concentrate on the biomass accumulation stage. During this stage, the algae may undergo substantial morphological and physiological changes, which are often overlooked in experiments, leading to highly heterogeneous results amongst studies (Ohnuki *et al.*, 2013). Moreover, due to poor experimental design and lack of culture synchronization prior experiments, cultures may contain mixtures of flagellate, palmelloid and aplanospore cells (Kakizono *et al.*, 1992; Harker *et al.*, 1996b; Fábregas *et al.*, 2003). One particular and essential transition which is often overlooked, is the transformation of flagellated cells into palmelloid cells (Kobayashi *et al.*, 1997b). Characteristics of both cell types were thoroughly summarized by Shah *et al.* (2016). Some of the major transformations between flagellated cells and palmelloids include 1) the loss of motility 2) the possession of a thick amorphous multilayered structure against a gelatinous extracellular matrix as primary cells wall and 3) the gradual increases in cell size. From a production perspective, both flagellated and palmelloid cells own characteristics which may be advantageous for different causes.

If biomass accumulation is the priority, producing high amounts of flagellated cells - or zoospores - in a continuous way is important since only flagellated cells are capable of rapid

vegetative reproduction while palmelloid cells barely divide (Hata *et al.*, 2001). Therefore, conditions should be chosen as such that their transformation to palmelloids is not induced. Zoospores are known to be highly sensitive to light and mechanical breakage (Aflalo *et al.*, 2007). Studies claim they are best grown in medium containing saturating levels of nitrate (Tocquin *et al.*, 2012) at temperatures between 20 and 27/28 °C (Evens *et al.*, 2008, Klochkova *et al*., 2013) and under low light intensity. However "low light intensity" may considerably vary form one laboratory to another (Evens *et al.*, 2008). According to Hata *et al.* (2001) light was not an obligate requirement during the biomass accumulation stage, though heterotrophic conditions were at cost of low growth rates and high contamination risks. Chekanov *et al.* (2014) and Zhang *et al.* (2014) continuously supplied moderate light intensities of 30 and 40 µmol photons $^{-2}$ $s^{-1}$ respectively, to obtain large amounts of green cells. In turn, Saha *et al.* (2013) found that 80 µmol photons $m^{-2}$ $s^{-1}$ was suitable for biomass accumulation, but observed the transition of flagellated cells to intermediate cellular morphologies with immobile green cells and large immature cysts under these conditions. Several authors have claimed that growth rate became saturated at much higher irradiances, specifically 90 and 100 µmol photons $m^{-2}$ $s^{-1}$ (Fan *et al.*, 1994; Harker *et al.*, 1995, respectively). Finally, Domínguez-Bocanegra *et al.* (2004) achieved a maximum growth under continuous illumination by 177 µmol photons $m^{-2}$ $s^{-1}$ which was close to the 200 µmol photons $m^{-2}$ $s^{-1}$ reported as ideal for biomass productivity by Torzillo *et al.,* (2005). Conclusively, it is currently difficult to predict on optimal irradiance for *H. pluvialis* since the determined irradiances differ highly from one study to another. Moreover, from the available literature, irradiances for optimal growth rate appear higher than those for biomass accumulation. Given that light exploitation varies for each variable, it has been highly recommended to consider different traits when comparing studies on irradiance, particularly, culture density, growth rate, transition from flagellates to palmelloids (Wang *et al.*, 2003; Göksan & Ak 2006). Moreover, culture vessel attributes should be considered as additional source of heterogeneity since they may highly influence the available light (e.g. mixing rate, light path lengths, geometry). Finally, other sources of heterogeneity include, illumination cycles and light quality as proven by e.g. the increased phototactic behavior of flagellated cells upon exposure to blue light (Kobayashi *et al.*, 1992). Next to all these factors, optimal irradiances were also found to be highly strain specific, as suggested in the synopsis of strain optima proposed by Evens *et al.* (2008).

If producing astaxanthin is anticipated, palmelloid cell formation should be induced. Palmelloids possess a thick cell wall with closely adherent membrane offering protection from

the high irradiances necessary to accelerate astaxanthin accumulation (Han *et al.*, 2012). Also since they lack flagella, palmelloids may easily settle to the bottom of culture vessels, facilitating the process of harvesting for subsequent re-inoculation into stressful conditions. Currently not much is known on the causal factors leading to the transformation of actively swarming and dividing zoospores to cell division arrest in palmelloids. Some physico-chemical causes have been proposed, including culture age/maturity thus suggesting the transformation would be the result of resource limitation (Wang *et al.*, 2003; Wayama *et al.*, 2013; Allewaert *et al.*, 2017). Boussiba & Vonshak, (1991) triggered palmelloid cell formation through the application of cell division inhibitors. So far, the causes of this morphogenesis have not yet been intentionally investigated. Several authors have however noticed that some strains were predominantly in palmelloid stage while others remained flagellated (Han *et al.*, 2012; Klochkova *et al.*, 2013). Yet, from more recent observations, *Haematococcus* strains were found to differ in the rate at which palmelloid cell formation was intitiated (Allewaert *et al.*, 2017). Given its important impact on the astaxanthin production process, the transition from flagellated cells to palmelloids demands to be further investigated, particularly with respect to irradiance.

The current knowledge on *Haematococcus* responses to irradiance are limited to individual responses of single strains (exclusively belonging to *H. pluvialis*) to irradiance or the comparison of available data from specific literature (Evens *et al.*, 2008). The study of reponses of mutiple strains belonging to different genotypes to a light gradient may be particularly useful since it may provide information on G x E interactions. G x E interactions occur when the mean trait value of a genotype varies across environments and the ranking order of genotypes, with respect to their mean trait value, varies among environments (or graphically, if genotype-specific reaction norm slopes are nonparallel or even cross (De Jong, 1990). Given that due to G x E interactions, the genetic rank of strains may vary, so that the best performing genotype in one environment might not be the best in another environment, G x E are important in selection and breeding programs (Falconer *et al.*, 1996).

In this study, we targeted the green vegetative phase of the *Haematococcus* production process, focusing on the responses of synchronized, non-self-shading unialgal strains when exposed to a gradient of nine different irradiances. The *Haematococcus* strains included three *H. pluvialis* strains obtained from international culture collections and three *H. rubicundus* strains recently isolated from different locations throughout Europe, all belonging to different genotypes (Allewaert *et al.*, 2015). Besides performing strain comparisons for the two species across

irradiances, irradiance and strain x irradiance interaction effects were investigated for the following parameters: cell biovolume, density, mortality, growth rate, photosynthetic efficiency of the photosystem II and pigment composition (quantitative and qualitative). Particular attention was paid to the morphological transition from flagellated to palmelloid upon varying irradiance.

## 5.2. Material and methods

### 5.2.1. Strains and cultivation conditions

All six strains from this study belong to different genotypes based on the ITS rDNA phylogeny of *Haematococcus* (Allewaert *et al.*, 2015). Three strains of *H. pluvialis* were included from culture collections (NIES 144, CCAP 34/7, CCAP 34/14) and three strains of *H. rubicundus* were recent isolates from continental Europe (IT01_06, BE 05_06, NL01_04) (Table 1).

Each strain was grown at 23 °C from stock cultures (preserved at 6 °C) in 12 well plates (Greiner Bio-One) with a working volume of 4 mL, plates were daily randomized. The culture medium used was Bold Basal medium with three times the nitrate concentration (BBM - 3N (Bischoff & Bold, 1963)). Cultures were illuminated with cool white fluorescent light at an intensity of 19.05 ± 2.09 µmol photons $m^{-2}$ $s^{-1}$ and a 16:8 light:dark regime in a climate chamber at continuous temperature of 23 °C. Strains were acclimatized under these conditions. The acclimatization was further performed as in Allewaert *et al.* (2017) before starting the experiment to ensure all cells were in identical physiological state and subsequently brought to an initial $F_0$ value of 0.023 corresponding to ± 700 cells $mL^{-1}$ (settings 6, 1, 2 for intensity, gain and damping respectively, see below).

***Table 1*** *Haematococcus strains used in this study: strain code, geographical origin and coordinates, collection date and person, method of preservation and Genbank ITS accession.*

| Species | Strain | Geographical origin | Geographical Coordinates | Collected by | Date of isolation | Preservation | ITS accession |
|---|---|---|---|---|---|---|---|
| ***H. pluvialis*** | NIES 144 | Sapporo Hokkaido, Japan | 43°3'43,5"N, 141°21'15,7"E | Ichimura, Terunobu | 16/07/1964 | Cryopreservation | KR914709 |
| | CCAP 34/7 | Ostpicken Island, Tvärminne, Finland | 59°50'35,4"N, 23°14'38,5"E | Droop | 1953 | Sub + Cryopreservation | KR914712 |
| | CCAP 34/14 | Cattonsville, Maryland, USA | 39°16'19,4"N, 76°43'54,9"W | Ott | 1989 | Sub | KR914706 |
| ***H. rubicundus*** | BE05_06 | Ghent, Belgium | 51°01'27,5"N, 03°42'38,8"E | Allewaert C. C. | 06/05/2012 | Cryopreservation | KR914737 |
| | IT01_06 | Province of Pescara, Italy | 42°09'23,4"N, 14°00'11,4"E | Casteleyn G. | 1/04/2013 | Cryopreservation | ND |
| | NL01_04 | Wageningen, The Netherlands | 51°59'15,5"N, 05°40'14,3"E | Wiezer S. | 06/03/2013 | Cryopreservation | KR914749 |

ND Not determined

Homogeneous light conditions were achieved by attaching three cool white fluorescent tubes (Phillips, cool white 840, 36W, 3350lm) above a shelf, causing a constant light intensity over the length and width of the shelf. Strains were randomly distributed over multi-well plates (Greiner Bio-one 12 wells plates), fully covered (including the edges) with neutral density light filters and combinations of filters (Lee Technical filters) with different percentage of light transmission (100 %, 69.3 %, 51.2 %, 35.48 %, 23.5 %, 13.7 %, 6.6 %, 3.3 %, 1.55 %). This resulted in nine different light intensity conditions (2.0 ± 0.1, 7.5 ± 0.4, 14.7 ± 0.7, 30.5 ± 1.5, 52.3 ± 2.6; 79.0 ± 3.9; 114.0 ± 5.6; 154.3 ± 7.5 and 222.6 ± 10.9 µmol photons $m^{-2}$ $s^{-1}$). Well plates were incubated in a climate chamber at constant temperature (18 °C) and illuminated with 16:8 light:dark regime. Irradiances were measured using a quantum light meter (MQ-100, Apogee Instruments, USA). Four replicates per strain were included per light condition. To achieve constant temperature conditions, two ventilators were placed on the shelf. Temperature in the wells was measured by using a calibrated precision thermometer (Ebro Ttx 100 thermometer, Germany) and was on average 21.3 ± 1.5 °C. Plates were daily randomized over the experimental surface.

### 5.2.2. Microscopy

Subsamples (100 µL) of each strain at each light intensity were taken when cultures were stationary as judged by constant or decreasing values of $F_0$ (see below) over two days (Allewaert *et al*., 2017) and fixed with formaldehyde borax (final concentration 1 %). Pictures of settled cultures were taken with a camera (Canon PowerShot G11) connected to an inverted microscope (Zeiss Axiovert 200 M). Cells, were counted using the Image J imaging software (version 1.48; National Institutes of Health, USA) ($n \geq 250$ of 3/4 replicates). At the same time, cells were categorized as flagellated, palmelloid and/or bleached (without pigment) and cell biovolume of palmelloid and flagellated cells was determined ($n \geq 50$, 3/4 replicates, for the following intensities: 30; 114; 222 µmol photons $m^{-2}$ $s^{-1}$) as in Allewaert *et al.* (2017).

### 5.2.3. Growth rate and photosynthetic parameter $F_v/F_m$

Growth rates of the six strains were determined based on daily monitoring of the minimum fluorescence yield $F_0$ at a fixed time point (Schreiber, 1986) - a proxy for Chl *a* content of algal cultures (Honeywill *et al.*, 2002; Consalvey *et al.*, 2005) - by pulse amplitude modulated (PAM) fluorescence (MAXI Imaging PAM fluorometer, Walz, Germany) with settings as follow: 3, 1 and 2 for intensity, gain and damping respectively. Before each measurement, cultures were dark acclimatized for 15 min. The quantum yield of PSII was measured by $F_v/F_m$ calculated as

($F_m$ - $F_0$)/$F_0$ and represents the maximum potential quantum efficiency of PSII if all capable reaction centers are open. $F_v/F_m$ for each strain and irradiance was normalized to their respective initial $F_v/F_m$. Specific growth rate (μ; $d^{-1}$) was calculated as the slope of the linear regression from log-transformed $F_0$ against time, between day 1 and 5 of exponential growth. A light response curve was fitted to the specific growth rate (μ; $d^{-1}$) and irradiance ($I$; μmol photons $m^{-2}$ $s^{-1}$) for each replicate to obtain parameters characterizing growth dependence on light. Several growth irradiance models were tested and the best fitting model with smallest Akaike Information Criterion (AIC) was used. The relationship between μ and I, from our acclimated, non-self-shading cultures at various light intensities, was found to be best approximated by a Monod-type function, $\mu(I) = \frac{\mu max * I}{I + \frac{\mu max}{\alpha}}$ where μ ($d^{-1}$) is growth rate as a function of irradiance ($I$, μmol photons $m^{-2}$ $s^{-1}$). From this model, following parameters were obtained: maximum growth rate ($\mu_{max}$; $d^{-1}$) and the initial slope of the growth-irradiance curve (α, μmol photons $m^{-2}$ $s^{-1}$)(Litchman, 2000; Schwaderer *et al.*, 2011). Model fits were performed in R, using nonlinear least squares (nls) function from the stats package (R Core Team, 2013).

### 5.2.4. Pigment composition

Pigment composition of the six strains was determined for the following three light intensities: 30; 114 and 222 µmol photons $m^{-2}$ $s^{-1}$ (3/4 replicates). For this purpose HPLC analysis was performed on pigment extracts generated according to Allewaert *et al.*, (2017). HPLC analysis was performed on the Agilent 1100 HPLC system, equipped with a pump, auto sampler and photodiode array (PDA) detector. Pigment extracts (80 µL aliquots injected) were analyzed with a Zorbax Eclipse XBD-C18 (Agilent) analytical column (5 µm, 250 × 4.6 mm, PN:990967-902) and a Zorbax Eclipse Plus C-18 (Agilent) pre-column (5 µm, 12.5 × 4.6 mm, PN:820950-936) at 40 °C. The mobile phase consisted of solvent A (acetone 100 %) and solvent B (an isocratic mixture of methanol and water (90:10 (v/v))). For the simultaneous separation of pigments, the following gradient procedure was used: a linear gradient from 80 to 20 % B for 25 min., 20 % B for 10 min, and a linear gradient from 20 to 80 % B for 5 min. The flow rate was maintained at 1 mL $min^{-1}$. The detection wavelength of the PDA detector was set between 350 and 700 nm, and the chromatographic peaks were measured at a wavelength of 472 nm to facilitate astaxanthin detection. Chromatographic peaks were identified by comparing retention times and spectra against known standards. The standards of Chl *a,* Chl *b* and *all-trans*-astaxanthin were obtained from Sigma-Aldrich Chemical Co., canthaxanthin, lutein, echinenone, α-carotene, β-carotene from DHI. Astaxanthin esters were identified based on shape (free astaxanthin) and retention time determined through hydrolysis of samples following Yuan & Chen, (1999). Since cultures were treated with HCl prior extraction (necessary for cell lysis), Chl *a* degaraded into phaeophytin a and phaeophorbide a and Chl *b* into phaeophorbide b. To correct for this, Chl *a* and *b* standards were subjected to the same acid treatment for the determination of corresponding response factors ($R_f$). Given that these degradation products have similar absorbance properties as the non-degraded pigments (Lorenzen, 1967), for clarity of interpretation, they are further designated as Chl *a* and Chl *b* in this study. Pigment concentrations were converted to intracellular pigment content by correcting for cell density and biovolume.

### 5.2.5. Statistics

The experiment was carried out with replications from four separately grown cultures. All values shown in the figures are expressed as mean ± SD. One way ANOVA’s were used to determine significant strain differences within individual irradiances for growth rate and pigment content. A factorial ANOVA, was used to test for main (G, E) and interaction effects (G x E) on cell type (flagellated, palmelloid, dead), biovolume, growth rate, $F_v/F_m$ ratio, cell

density, and pigment composition (free astaxanthin, esterified astaxanthin, canthaxanthin, echinenone, α-carotene, β-carotene, lutein violaxanthin, undefined carotenoids (UCA) group, Chl *a* and Ch *b*), total carotenoids, total chlorophylls and total pigments with irradiance (E) as a fixed factor and strain/genotype (G) as a random factor. All data were checked for normality and heteroscedasticity. Dependent variables were square root transformed if needed, to meet the assumptions of ANOVA. Since transformations did not remove non-normality, for the variables which were non normal, the raw data were used for the analysis of these variables yet using a more conservative significance level of $\leq 0.01$, as indicated in Table 2 (Fowler-Walker & Connell, 2002).

## 5.3. Results

Microscopy observations of all strains in stationary phase showed that upon increasing irradiance, several strains contained high numbers of palmelloids, which in some cases contained traces of astaxanthin accumulating from the cell center. Several strains showed high amounts of photo bleached cells upon increasing irradiance (Fig. 1A). As shown from the relative abundances of the cell types in stationary phase, strains exhibited pronounced differences in cell type composition upon exposure to irradiance (Fig. 1B). Generally, the relative percentage of palmelloid cells at irradiances between 2 and 15 µmol photons $m^{-2}$ $s^{-1}$ were between 0 and 22 %, and gradually increased reaching maxima of 40 - 75 % palmelloid cells of the total cell population between 52 -79 µmol photons $m^{-2}$ $s^{-1}$. Strain BE05_06 contained very little palmelloid cells (maximum 30 % of the total cell population) under all irradiances. Strain IT01_06 showed a particular pattern in its capacity to form palmelloids, which reached up to 60 % palmelloids of its total cell population at low irradiances (between 2-17 µmol photons $m^{-2}$ $s^{-1}$), almost no palmelloids at 30 µmol photons $m^{-2}$ $s^{-1}$, while reaching again up to 70 % palmelloids at irradiances above 79 µmol photons $m^{-2}$ $s^{-1}$. The ANOVA detected a highly significant irradiance and strain effect, but no significant strain x irradiance (G x E) interaction effect (Table 2) for the relative percentage of palmelloid cells. The reaction norms of the different cell types showed an increase of palmelloids and in parallel, a decrease in flagellated cells with irradiance, yet reaching maxima (for palmelloids) and minima (for flagellates) at irradiances above 79 µmol photons $m^{-2}$ $s^{-1}$ (Fig. 4). Although showing inverse tendencies with palmelloid cells upon increasing light (Fig. 4), the ANOVA with flagellated cells as factor detected significant irradiance effect and (G x E) interaction effects, but no significant strain effect (Table 2). This was probably due to the contribution of bleached cells in the total cell

population, which showed highly significant strain, irradiance and G x E interaction effects (Table 2). A relatively high amount of bleached cells (exceeding 46-50 % of the total cell population at 154 - 222 µmol photons $m^{-2}$ $s^{-1}$ respectively) was observed in strain BE05_06, indicating that this strain had poor adaptability to high irradiance (Fig. 1). Correspondingly, BE05_06 was also a poor producer of palmelloid cells. Strains NL01_04 and CCAP 34/14 also presented some bleached cells, particularly above 114 µmol photons $m^{-2}$ $s^{-1}$, yet generally not exceeding 30 % of the total cell population. Overall, the reaction norms of cell mortality displayed gradual increasing trend with irradiance (Fig. 4).

In terms of cell biovolume, the ANOVA showed significant strain effects (Table 2). Strains CCAP 34/14 and IT01_06 were the largest (47605 and 43564 µm³ respectively) while smallest average biovolume was recorded for BE05_06 and NIES 144 (17707 and 23729 µm³ respectively) (Fig. 1). However, no direct irradiance or G x E interaction effects were found for this variable (Table 2). This was also reflected in the norm of reaction for this trait (Fig. 4), showing no clear changing patterns of strain with irradiance, thus implying that cells remained within the range of “green cells” which unlike aplanospores do not vary considerably in size.

***Table 2*** *Results (F and p values) of the factorial ANOVA with strain=genotype (G) (n=6) as random and irradiance (E)(n=9) as fixed factors. Traits are grouped into categories, related to morphological changes, growth characteristics and pigment composition. NS P > 0.05; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.UCA= Unidentified carotenoid group.*

| Group | Variable | Irradiance | | Strain | | Irradiance X Strain | |
|---|---|---|---|---|---|---|---|
| | **Effect** | **E** | | **G** | | **G x E** | |
| | | *F* | *p* | *F* | *p* | *F* | *p* |
| Morphological changes | Palmelloid a | 57 | *** | 8 | *** | 2 | NS |
| | Flagellated a | 92 | *** | 3 | NS | 1 | NS |
| | Bleached | 59 | *** | 11 | *** | 19 | *** |
| | Cell biovolume | 1 | NS | 32 | *** | 0 | NS |
| Growth characteristics | Growth rate a | 193 | *** | 2 | NS | 1 | NS |
| | $F_v/F_m$ ratio a | 437 | *** | 9 | *** | 9 | *** |
| | Cell density a | 3 | NS | 6 | *** | 8 | *** |
| Pigment composition | Free astaxanthin a | 4 | NS | 0 | NS | 1 | NS |
| | Astaxanthin esters a | 34 | *** | 4 | ** | 4 | ** |
| | Canthaxanthin a | 15 | *** | 13 | *** | 6 | *** |
| | Echinenone a | 8 | ** | 2 | NS | 3 | NS |
| | α-carotene a | 0 | NS | 9 | *** | 0 | NS |
| | β-carotene a | 6 | NS | 18 | *** | 0 | NS |
| | Lutein a | 16 | *** | 26 | *** | 0 | NS |
| | Violaxanthin a | 11 | ** | 14 | *** | 1 | NS |
| | UCA a | 26 | *** | 10 | *** | 7 | *** |
| | Chl *a* a | 29 | *** | 15 | *** | 4 | ** |
| | Chl *b* a | 24 | *** | 8 | *** | 2 | NS |
| | Total carotenoids a | 25 | *** | 5 | *** | 4 | ** |
| | Total chlorophylls a | 31 | *** | 14 | *** | 4 | ** |
| | Total pigments | 10 | ** | 13 | *** | 4 | ** |

[a] Non normally distributed variables, more conservative level of significance was implemented *p* ≤ 0.01 is implemented

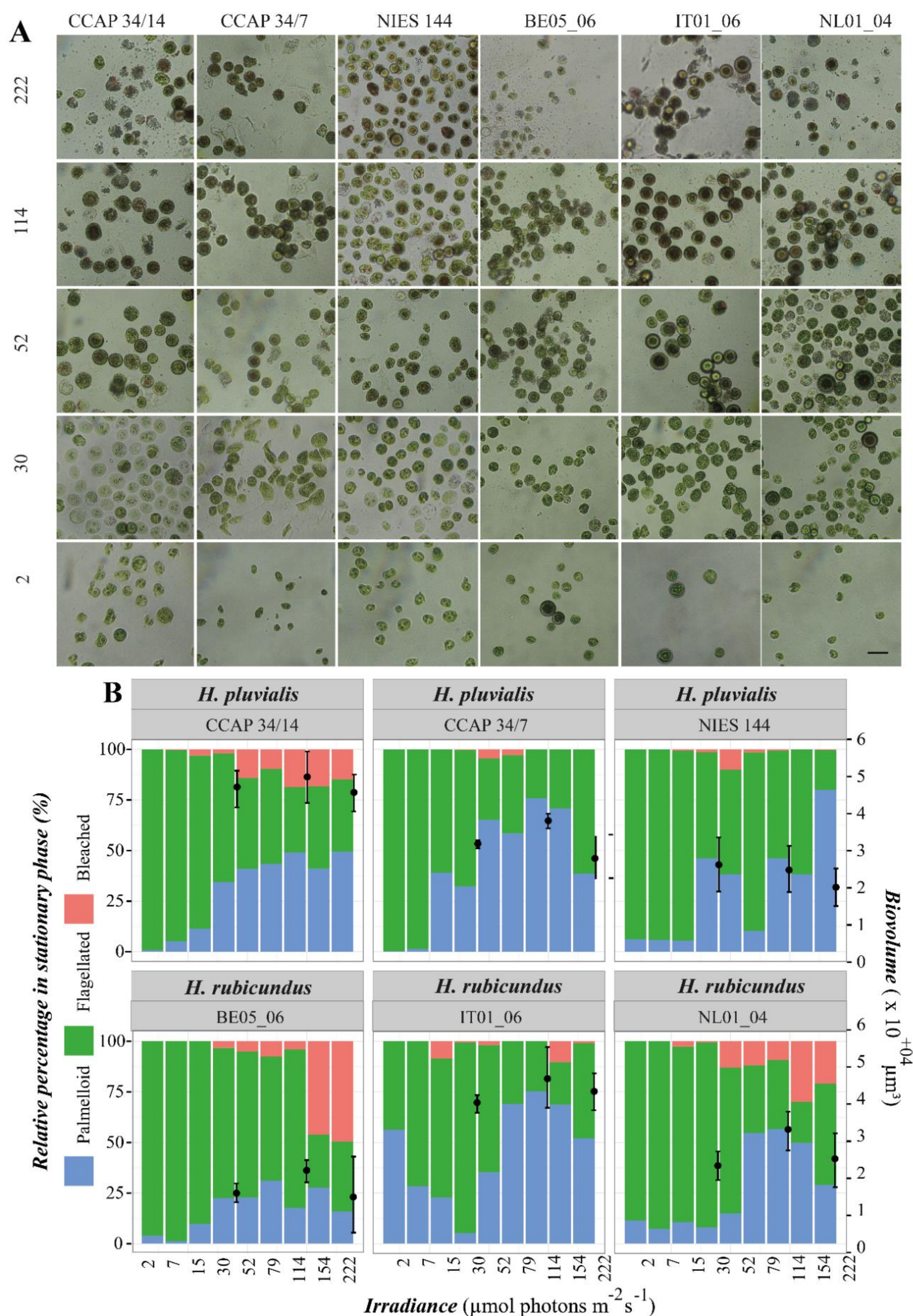



***Figure 1. A.*** *Stationary phase morphology of six Haematococcus strains grown under different irradiances (2,30,52,114,222 µmol photons $m^{-2}$ $s^{-1}$). Scale bar = 25µm* ***B.*** *Cell type (%) and average biovolume (µm³) in function of irradiance. Cell types are represented by the relative percentage (%)(n ≥ 250) of palmelloid (blue), flagellated (green) and bleached cells (red). Cell biovolume (µm³) is shown as black circles, error bars represent the standard deviations of the 3 replicates.*

As expected, the exponential growth rate of *Haematococcus* in response to increasing irradiance fitted a typical "P-I" (photosynthesis vs. irradiance) curve, namely, a linear increase followed by an asymptote as light became saturating (Figs. 2 & 4). The ANOVA detected a significant irradiance effect, but no direct strain effect or G x E interaction effect for growth rate (Table 2). The maximal growth rates for all strains (average 0.93 ± 0.04 divisions $d^{-1}$ for *H. rubicundus* and 1.01 ± 0.05 divisions $d^{-1}$ for *H. pluvialis*) was obtained at 154 µmol photons $m^{-2}$ $s^{-1}$ (Fig. 2, Table S1, Supplementary Data), yet as judged by the decreased variability, irradiance started to become saturating between 30 and 79 µmol photons $m^{-2}$ $s^{-1}$ for all strains. Noteworthy was the drop in growth rate at 114 µmol photons $m^{-2}$ $s^{-1}$ for NIES 144, NL01_04 and CCAP 34/7, anticipated and followed by a higher growth rate at lower and higher irradiances. The Monod equation allowed to better evaluate the irradiance preferences per strain. Maximal growth rate (µmax) was significantly different between strains ($P < 0.01$)(Fig. 2) and were highest for strains belonging to *H. pluvialis*: NIES 144 (1.13 ± 0.04 divisions $d^{-1}$), CCAP 34/14 (1.08 ± 0.03 divisions $d^{-1}$) and CCAP 34/7 (1.03 ± 0.03 divisions $d^{-1}$ ). Significant strain differences were found in the initial slope of the light-dependent growth rate ($\alpha_{\mu}$) ($P < 0.01$)(Fig. 2), which was highest for strain BE05_06 (0.14 ± 0.04).

Analysis of variance showed that unlike for growth rate, stationary phase cell density was not significantly affected by irradiance, yet a significant strain effect and interaction effect was found (Table 2). Unlike the growth rate reaction norm, the reaction norm of stationary cell densities showed a parabolic shape upon increasing irradiance for most strains (Figs. 2 & 4). These included CCAP 34/14, CCAP 34/7, IT01_06, and NL01_04 for which maximal cell densities were obtained at 30 (2.3 x $10^4$ ± 9.3 x $10^3$ cells $mL^{-1}$), 15 (5.7 x $10^4$ ± 6.5 x $10^3$ cells $mL^{-1}$), 30 (6.2 x $10^4$ ± 4.5 x $10^3$ cells $mL^{-1}$) and 30 (3.6 x $10^4$ ± 6.6 x $10^2$ cells $mL^{-1}$) µmol photons $m^{-2}$ $s^{-1}$ respectively. In contrast, cell densities were overall constant over irradiances for strain BE05_06, with a poor maximum at 52 µmol photons $m^{-2}$ $s^{-1}$ (5.4 x $10^4$ ± 7.4 x $10^3$ cells $mL^{-1}$). Strain NIES 144 showed a completely different pattern of cell density, increasing with irradiance and achieving highest densities (8.4 x $10^4$ ± 2.8 x $10^3$ cells $mL^{-1}$) at 222 µmol photons $m^{-2}$ $s^{-1}$.

Based on the factorial ANOVA, significant strain, irradiance and G x E interaction effects were found for the $F_v/F_m$ ratios (Table 2). The reaction norms (Fig. 4) showed constant $F_v/F_m$ ratios over irradiances up to 79 µmol photons $m^{-2}$ $s^{-1}$, after which they gradually decreased, indicating a redcuction of photosynthetic capacity (Fig. 3).

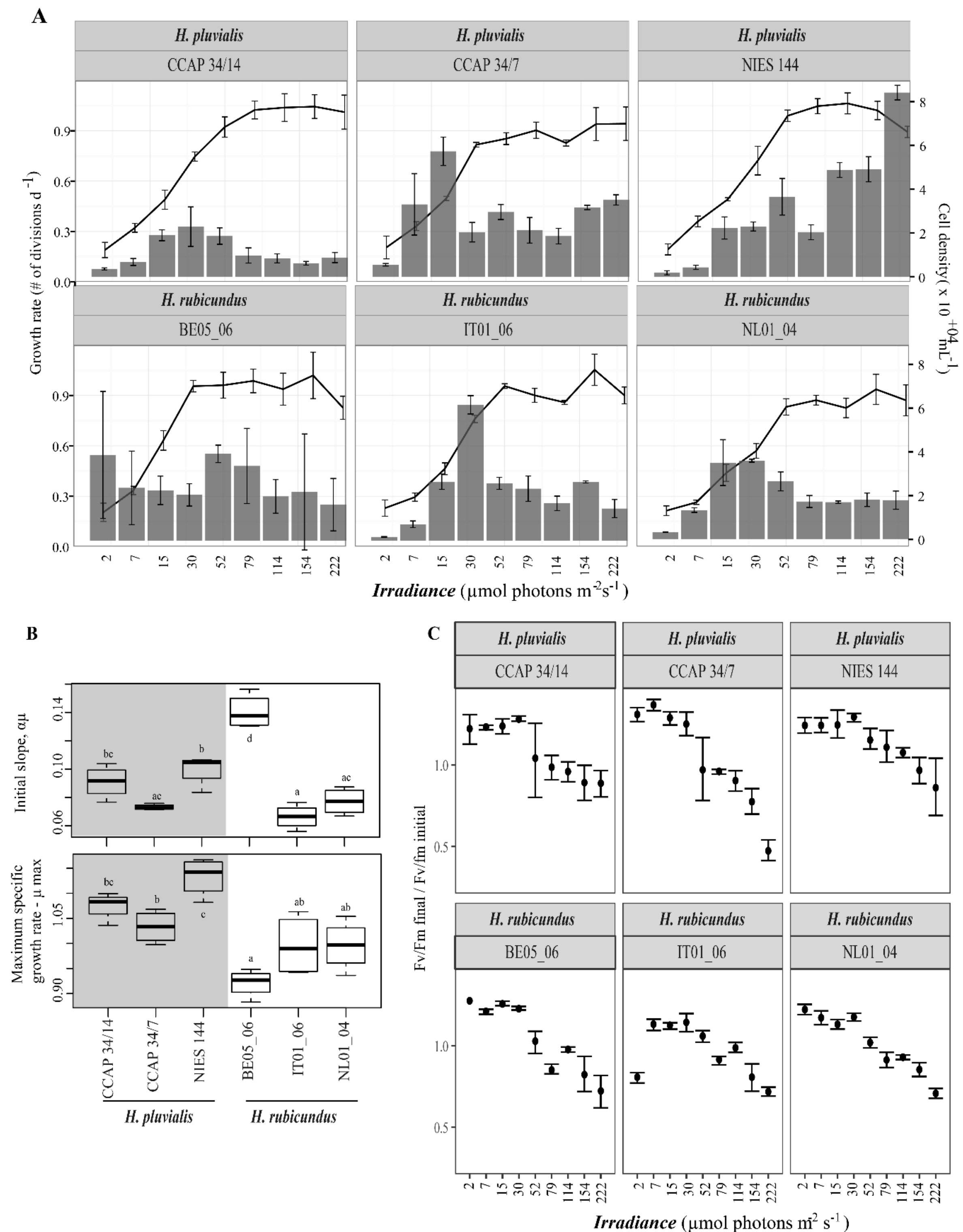



***Figure 2. A**. Growth rate (number of divisions $d^{-1}$)(line) and cell density (histogram) of the six strains of Haematococcus as function of irradiance. Each point is the average of four measurements and the standard deviation is shown by the error bars. **B.** Box plots of the initial slope (above) and maximum growth rate (number of divisions $d^{-1}$)(below) for each strain as calculated from the Monod equation. **C.** Ratios of the final photosynthetic efficiency $F_v/F_m$ over the initial $F_v/F_m$ for all six strains in function of irradiance. Each point is the average of four measurements and the standard deviation is shown by the error bars.*

The pigment composition of stationary cultures of *Haematococcus* strains (on cell biovolume basis) at irradiances of 30, 144 and 222 μmol photons $m^{-2}$ $s^{-1}$ is shown in Fig. 3, Table S2, Supplementary Data. The identified pigments included chlorophylls, Chl *a* and Chl *b*, as well as the following carotenoids: canthaxanthin, echinenone, α-carotene, β-carotene, lutein, violaxanthin, astaxanthin (free and esterified) and a group carotenoids (UCA) which could not be recognized based on the available standards. The ANOVA results showed significant strain effects for all pigments (except free astaxanthin and echinenone, which had no variance due to absence in these pigments in some strains) as well as for their (individual) sum (Table 2). Despite these differences, there were no consistent strain patterns for a particular pigment at a particular irradiance (Table S2). The total pigment content per cell biovolume was highest for strains CCAP 34/14, NIES 144 and IT01_06. Significant irradiance effects for all pigments (except α and β-carotenes and free astaxanthin) as well as their sum and the sum of individual carotenoids and chlorophylls were also found (Table 2). The reaction norms for total chlorophylls and total pigments complied with typical responses to irradiance within *H. pluvialis*, where characteristically, increases in irradiance result in increases in total carotenoids and subsequent decreases in chlorophylls (Fig. 4). On a cell biovolume basis, Chl *a* was the dominant pigment in all strains at all irradiances. Significant G x E interaction effects were found for astaxanthin esters, canthaxanthin echinenone, UCA and both chlorophylls as well as for their sum, the sum of carotenoids and total pigment (Table 2).

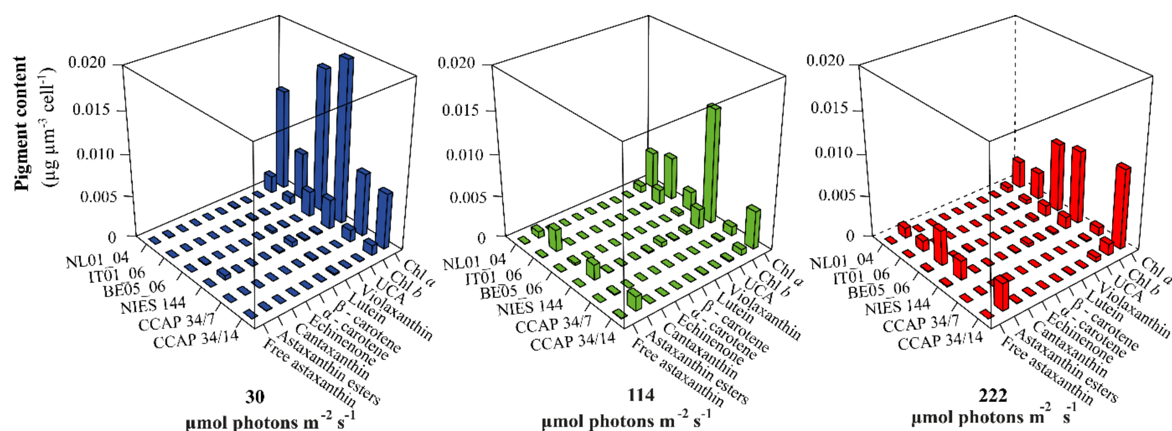



***Figure 3.*** *3D plots of the pigment content per strain at irradiances of 30 (blue), 114 (green) and 222 (red) μmol photons* $m^{-2}$ $s^{-1}$ *of the six strains under assessment.*

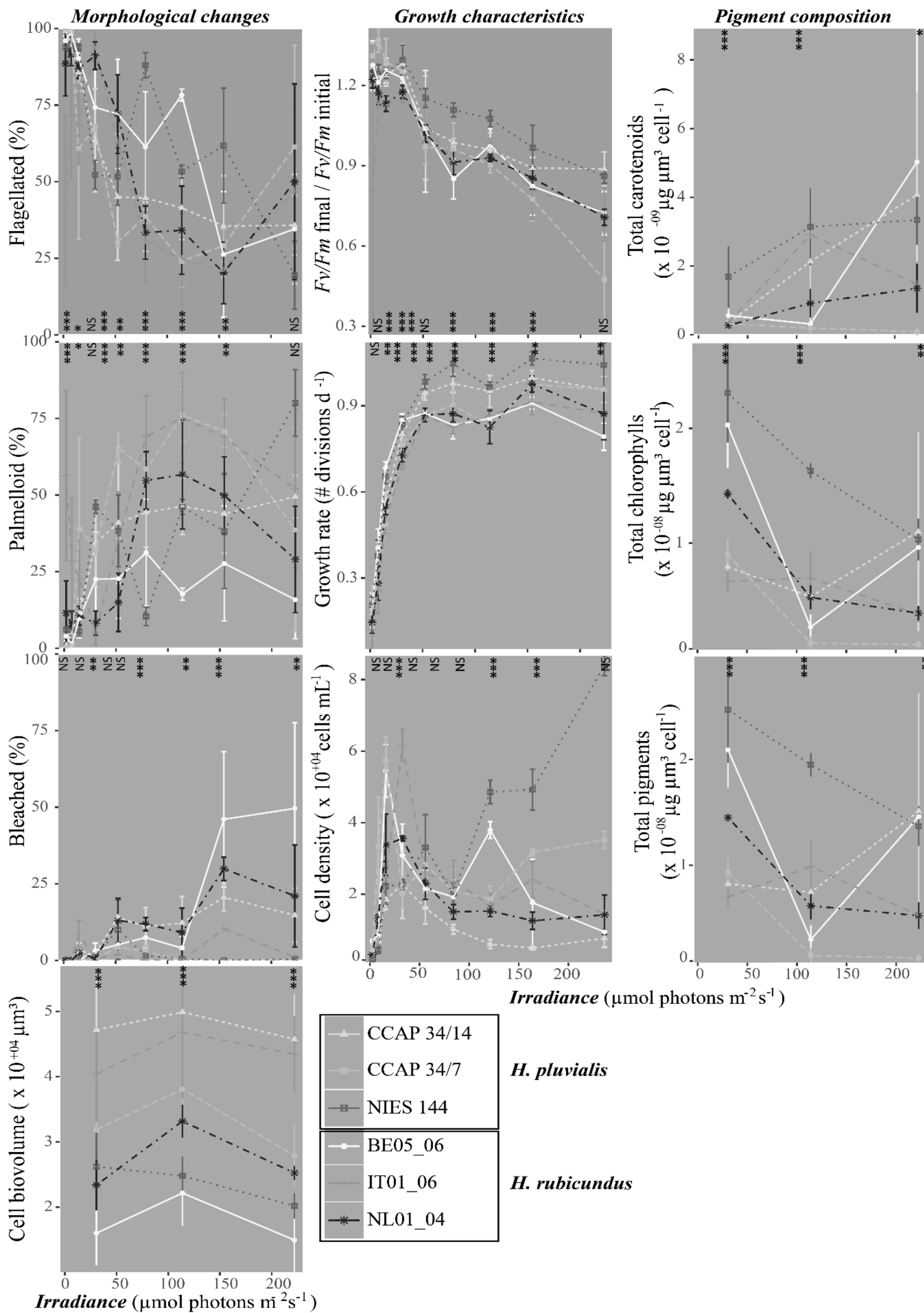



***Figure 4.*** *Reaction norms (morphological, growth behavioral and pigment composition) of six Haematococcus strains across an irradiance gradient. Each point represents the average value measured per strain (n=4, except for pigment composition where n=3). Results of the one way ANOVA are marked on top or below each graph per irradiance. NS P > 0.05; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001.*

## 5.4. Discussion

Since light stress is directly correlated with productivity, light responses of photosynthetic organisms have considerable economic and scientific interest. From a fundamental perspective, light responses represent a source of essential information to better understand the biology and distribution of natural populations, broadening the panorama of species/strain ecophysiology. While the effects of light intensity, photoperiod, duration and source have been extensively studied on the astaxanthin productivity capacity in *Haematococcus*, there is currently no consensus between the research community regarding preferred irradiance for productivity of green cells of *Haematococcus*.

***Strain effects*** - Although increases in light caused gross similar morphological, growth and pigment patterns amongst the six *Haematococcus* strains, each strain exhibited very divergent responses to irradiance specifically for the following steady state traits: cell- type, size, density, photosynthetic efficiency and pigment composition. In agreement with Allewaert *et al.,* (2017) and Han *et al.,* (2012), strains were found to differ in their capacity to form palmelloids. Strains which did not induce palmelloid cell formation upon increasing irradiance, were less protected, and suffered from photo bleaching (e.g. strain BE05_06, this study)(Han *et al.,* 2012). Highly significant strain differences were also found in cell biovolume. Strains CCAP 34/14 and IT01_06 had considerably larger cells under all irradiances. Therefore, it is important to take biovolume into account if irradiances are compared across studies, since it may significantly influence for instance biomass or pigment content on a dry weight basis. Unfortunately, cell size is hardly communicated in studies on *Haematococcus* physiology. Overall, growth rates in our experiment, were generally higher than the previously published growth rates for *H. pluvialis,* perhaps the result of high nitrogen content in the medium (Torzillo *et al.*, 2005; Allewaert *et al.*, 2017). Since we found no significant strain effects across irradiances in terms of exponential growth rate, we can conclude that there was no strain (amongst those studied) that "out-competed" all others consistently across all irradiances. However, the results from the Monod model, plead for a higher growth at high irradiances in strains NIES 144 > CCAP 34/14 > CCAP 34/7, which all belong to *H. pluvialis*. In terms of stationary cell density, strain NIES 144 showed significantly different behavior since it was the only strain showing increasing cell densities with irradiances up to 222 μmol photons $m^{-2}$ $s^{-1}$. This strain was already found as an outstanding producer of vegetative cells, at high rate when compared with sixteen other *H. pluvialis* strains under common garden conditions (Allewaert *et al.,* 2017).

Steady state photosynthetic stress, measured using the proxy $F_v/F_m$, and the resulting photosynthetic damage or photo bleaching (highly correlated with the relative amount of bleached cells, data not shown), also differed significantly between strains, implying that different photo protective strategies are adopted by different strains. This was confirmed by the highly specific responses in intracellular pigment composition upon increasing irradiance. Currently such differences in quantitative and qualitative pigment composition are lacking in available literature but are likely the result of strain specific expression patterns of genes responsible for pigment synthesis in *Haematococcus* as described by Gao *et al.*, (2015) in Australian strains of *H. pluvialis*. Metabolic pathways of astaxanthin biosynthesis are currently still not fully understood given the complexity in regulatory inputs and interplay with other pathways (Solovchenko, 2015). Studies such as these which demonstrate highly specific carotenogenesis machineries and photoacclimation strategies could potentially aid in better understanding gene regulation and targeting genes, leading to a better comprehension of the complex metabolic pathways in *Haematococcus*.

Given the limited amount of strains together with the heterogeneity in origin as well as phylogenetic background, no comparisons at species level are made in this work. A high degree of overlap in trait values was already reported in another study (Allewaert *et al.*, 2017). Although not statistically supported for all traits, strains with the most atypical patterns across the variables tested were NIES 144 and to some extent CCAP 34/14. Whether their different behavior may be linked to their origin (Japan and the USA respectively), their cultivation background (cryopreserved and sub-cultured respectively) or other stochastic factors remains subject of future investigation.

***Irradiance effects*** - Significant effects of irradiance for all strains were found in cell type, growth rate, photosynthetic efficiency, and pigment composition. Under replete conditions, *Haematococcus* cells are usually biflagellated with a thin cell wall. It has been demonstrated that when cultures become stationary, the relative amount of flagellated cells decreases, while palmelloid cells increase gradually until generally a maximum is reached (Allewaert *et al.*, 2017). In this respect, resource limitation may cause *Haematococcus* to transit to palmelloids, to which some strains would respond faster than others. Here, we showed that steady state populations of *Haematococcus* expressed an increasing share of palmelloids upon increasing irradiance. Conclusively, our results point out that the rate at which palmelloid cells are formed is increased under increasing irradiance. This finding is well in line with the prospect that the formation of palmelloids and concomitant thick amorphous multilayered cell walls in

*Haematococcus* may provide protection from increased irradiance (Han *et al.*, 2012). Although cells transited to palmelloids upon increasing irradiance, their biovolumes did not vary with irradiance, indicating that cell populations remained within the range of vegetative cells, promoting cell proliferation and little astaxanthin accumulation, as in Torzillo *et al.,* (2005) and Göksan and Ak (2006).

In accordance with previous findings, *Haematococcus* strains under assessment showed different optima for growth rate and for cell density and therefore, designating an optimum light intensity was theoretically impossible. Generally, maximal growth and minimal physiological stress was experienced by all strains between 30 and 79 µmol photons $m^{-2}$ $s^{-1}$, while maximal cell densities were obtained between 15 and 30 µmol photons $m^{-2}$ $s^{-1}$. Based on these two variables a "safe" irradiance range at which growth promotion and biomass production is possible, would be 15-79 µmol photons $m^{-2}$ $s^{-1}$, since most strains showed high growth, increased cell densities, while at the same time procuring little physiological stress. These ranges are well in line with Chekanov *et al.,* (2014) and Zhang *et al*., (2014). Under these conditions, stationary cultures were typically populated with a majority of flagellated cells, few palmelloids which were composed almost exclusively of chlorophylls (Chl *a* + *b*). Further changes in growth rate, $F_v/F_m$ ratios and cell densities as well as the pigment responses (viz. increase in carotenoids and subsequent decrease in chlorophylls) upon increasing irradiance were very common responses for *Haematococcus* as thouroughly described in Torzillo *et al.,* (2005).

The relatively low safe irradiance ranges for growth promotion and biomass production here found, raises the question on how *Haematococcus* cells manage to survive in its natural habitat, where typically, cells are exposed to irradiances which may reach up to 2000 µmol photons $m^{-2}$ $s^{-1}$. To date, the ecological strategies of *Haematococcus* are poorly understood, yet Proctor, 1957 and Genitsaris *et al*., (2016) agree that the success of *Haematococcus* populations to thrive in such habitats is highly related with its capacity to form aplanospores and subsequently astaxanthin. How and when astaxanthin catabolism and the switch from aplanospores to zoospores takes place is still an enigmatic process under natural circumstances.

***Irradiance reaction norms*** – The different reaction norms for each trait revealed considerable physiological diversity in the six genotypes assessed. Significant G x E interactions were found for mortality, $F_v/F_m$ ratio and cell density as well as several pigments, total pigments, chlorophylls and carotenoids. For these traits, strains showed significant differences in genetic rank (Fig. 4), implying that they are context dependent is such a way that it is not possible to

predict the ranking order of the responses from one light intensity to the next. Conlusively, to find optimal irradiances for these traits, it is mandatory to carry out studies under different culture conditions for each strain.

One main limitation of this study lies in the fact that only one single factor, light intensity, was varied across a range of interest while all other factors were set constant. Such commonly called "classical one-factor–at-a-time (OFAT)" approach presupposes that there are no interactions between independent factors, while there is current evidence that factors such as for example temperature and irradiance may interact (Evens *et al.*, 2008). Therefore, we should be careful upon interpretation of the results. Secondly, batch cultures were employed which although being convenient, present many sources of additional variation, such as nutrient depletion. All traits studied (excluding growht rate) were applied to steady state cultures, yet we do not know what causes the cultures to enter steady state. As the medium employed was enriched in nitrate, one may presume that cultures were not limited in nitrogen. Thus we do not know which factor actually caused reduced growth.

From a biotechnology view, the diverse niche specificities of *Haematococcus* strains may present an opportunity for large scale cultures through the use of mixed cultures instead of the single-strain or mono culture approaches currently used. Although this hypothesis remains to be validated, it is highly likely that strain consortia of *Haematococcus*, due to their resource-use complementarity may lead to increased yields and stability of cultures exposed to fluctuating environments (Kazamia *et al.*, 2014). From a more fundamental perspective, our data show that the relative success of each strain shifts in a temporally variable environment. Or viewed from another side, *Haematococcus* strain diversity is maintained due to changes in the abiotic and probably also the biotic environment. In fact, besides irradiance, the intensity and variation in many other actors such as temperature, nutrient availability, pH, salinity as well as biotic factors such as parasitism may interact and modulate genotypic fitness. Current knowledge on the nature and intensity of these factors in a natural context is essentially lacking in *Haematococcus* since the genus is more studied in artificial environments (bioreactors, cultivation vessels) than in its natural habitat.

## 5.5. Conclusions

The experimental data on G x E interactions in six *Haematococcus* strains from different origins showed different morphological, physiological and metabolic responses to irradiance. We have shown that most responses were highly influenced by irradiance and that each strain had

singular minima, optima and maxima for each factor. We suggest that at least one of the reasons why *Haematococcus* is capable of forming palmelloids is to cope with increased irradiance, illustrating the ability of *Haematococcus* species to track environmental changes and respond appropriately. This data and its implications will be valuable not only for understanding various photo adaptive strategies, but also for the improvement of photosynthetic ability of each strain via genetic engineering under environmental stress. We believe that all the irradiance responses mentioned above may contribute to the remarkable physiological plasticity within the *Haematococcus* genus and necessitate further attention.

## 5.6. Acknowledgements

The authors are indebted to the Belgian Coordinated Collection of Micro-organisms (http://bccm.belspo.be) for cryopreserving and providing all strains from this study.

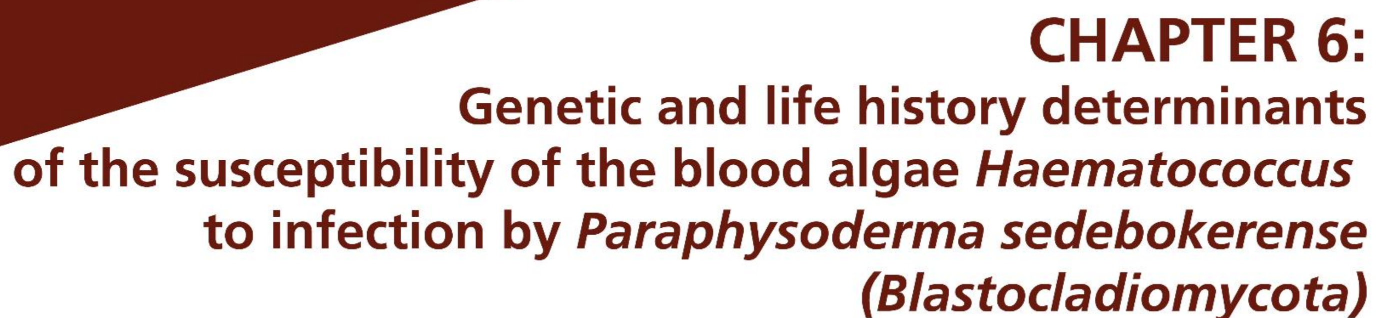
## 5.7. Supplementary Data

***Table S1.*** *Average ± SD of the growth rate (number of divisions $d^{-1}$) of the six different Haematococcus strains. P values indicate the strain difference*

| Species | Strain | 2 | 7 | 15 | 30 | 52 | 79 | 114 | 154 | 222 |
|---|---|---|---|---|---|---|---|---|---|---|
| | CCAP 34/14 | 0.17 ± 0.04 | 0.36 ± 0.06ab | 0.56 ± 0.03b | 0.8 ± 0.03a | 0.94 ± 0.02b | 0.97 ± 0.03bc | 0.95 ± 0.05b | 0.99 ± 0.02c | 0.95 ± 0.04ab |
| *H. pluvialis* | CCAP 34/7 | 0.17 ± 0.09 | 0.3 ± 0.03ab | 0.48 ± 0.02bc | 0.76 ± 0.01e | 0.86 ± 0.01a | 0.9 ± 0.04ac | 0.82 ± 0.01a | 0.97 ± 0.03bc | 0.95 ± 0.07ab |
| | NIES 144 | 0.2 ± 0.02 | 0.37 ± 0.05ab | 0.65 ± 0.02a | 0.83 ± 0.02a | 0.98 ± 0.02b | 1.04 ± 0.04b | 0.96 ± 0.01b | 1.06 ± 0.02ac | 1.04 ± 0.07a |
| **Average ± SD** | | **0.19 ± 0.06** | **0.35 ± 0.06** | **0.57 ± 0.08** | **0.80 ± 0.04** | **0.93 ± 0.05** | **0.98 ± 0.07** | **0.91 ± 0.07** | **1.01 ± 0.05** | **0.99 ± 0.07** |
| | IT01_06 | 0.12 ± 0.02 | 0.25 ± 0.05b | 0.5 ± 0.02b | 0.68 ± 0.02d | 0.86 ± 0.02a | 0.82 ± 0.04ac | 0.86 ± 0.01a | 0.91 ± 0.02b | 0.87 ± 0.07b |
| *H. rubicundus* | BE05_06 | 0.19 ± 0.05 | 0.4 ± 0.06a | 0.68 ± 0.02a | 0.84 ± 0.02a | 0.87 ± 0.01a | 0.83 ± 0.04a | 0.85 ± 0.02a | 0.9 ± 0.02b | 0.79 ± 0.04b |
| | NL01_04 | 0.14 ± 0.03 | 0.28 ± 0.05ab | 0.54 ± 0.02b | 0.72 ± 0.02bd | 0.86 ± 0.02a | 0.87 ± 0.02a | 0.82 ± 0.05a | 0.97 ± 0.02bc | 0.87 ± 0.09b |
| **Average ± SD** | | **0.16 ± 0.06** | **0.31 ± 0.09** | **0.58 ± 0.09** | **0.76 ± 0.07** | **0.87 ± 0.02** | **0.84 ± 0.04** | **0.85 ± 0.04** | **0.93 ± 0.04** | **0.85 ± 0.08** |
| Between strains *p values* | | *0.403* | *0.001* | *< 0.001* | *< 0.001* | *< 0.001* | *< 0.001* | *< 0.001* | *< 0.001* | *0.002* |
| Between species *p values* | | *0.245* | *0.539* | *0.991* | *0.441* | *0.143* | *0.039* | *0.224* | *0.082* | *0.023* |

***Table S2.*** *Specific pigment content (*$10^5 \mu g\ \mu m^{-3}\ cell^{-1}$) of each strain. UCA = Unidentified carotenoids*

| Species | Irradiance | Strain | Free astaxanthin | Astaxanthin likes | Canthaxanthin | Echinenone | α-carotene | β-carotene | Lutein | Violaxanthin | UCA | Chl a | Chl b |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *H. pluvialis* | 30 | CCAP 34/14 | 0 ± 0 | 5.49 ± 9.72 b | 1.12 ± 0.37 b | 0.15 ± 0.3 b | 0.23 ± 0.27 ab | 2.92 ± 1.6 b | 16.37 ± 6.24 b | 4.59 ± 1.14 b | 3.99 ± 1.23 b | 672.61 ± 167.24 b | 119.06 ± 29.01 b |
| | | CCAP 34/7 | 0 ± 0 | 3.01 ± 0.55 b | 0.42 ± 0.14 b | 0.18 ± 0.21 b | 0.1 ± 0.13 b | 3.78 ± 2.85 ab | 16.06 ± 7.94 b | 4.98 ± 3.63 b | 2.97 ± 1.75 b | 758.69 ± 108.27 bc | 141.4 ± 25.16 bc |
| *H. rubicundus* | | NIES 144 | 0 ± 0 | 49.95 ± 25.6 a | 4.84 ± 1.9 a | 2.73 ± 1.44 a | 1.36 ± 1.25 a | 17.17 ± 14.09 a | 57.79 ± 35.82 a | 16.56 ± 9.24 a | 18.2 ± 10.15 a | 1946.53 ± 372.31 a | 358.6 ± 61.26 a |
| | | BE05_06 | 0 ± 0 | 11.3 ± 3.17 b | 0.23 ± 0.46 b | 0.17 ± 0.34 b | 0.72 ± 0.27 ab | 5.36 ± 2.68 ab | 27.84 ± 13.01 ab | 7.83 ± 2.37 ab | 2.74 ± 1.8 b | 1725.12 ± 324.16 a | 308.11 ± 48.56 a |
| | | IT01_06 | 0 ± 0 | 2.92 ± 3.07 b | 0.26 ± 0.25 b | 0.24 ± 0.17 b | 0.37 ± 0.11 ab | 6.54 ± 1.66 ab | 15.75 ± 2.09 b | 4.54 ± 0.77 b | 3.68 ± 1.9 b | 576.91 ± 80.02 b | 88.11 ± 13.66 b |
| | | NL01_04 | 0 ± 0 | 6.19 ± 5.34 b | 0.57 ± 0.17 b | 0.35 ± 0.25 b | 0.38 ± 0.18 ab | 2.11 ± 0.86 b | 10.36 ± 4.01 b | 3.89 ± 1.79 b | 3.14 ± 1.08 b | 1219.69 ± 35.72 c | 206.05 ± 5.5 c |
| | ***p value*** | | ***no variance*** | ***<0.001*** | ***<0.001*** | ***<0.001*** | ***0.048*** | ***0.025*** | ***0.007*** | ***0.004*** | ***<0.001*** | ***<0.001*** | ***<0.001*** |
| *H. pluvialis* | 114 | CCAP 34/14 | 6.86 ± 8.38 | 170.97 ± 65.18 acd | 2.5 ± 0.73 b | 2.36 ± 1.58 ab | 0.37 ± 0.43 ab | 2.16 ± 1.62 b | 8.16 ± 5.51 b | 3.9 ± 2.61 b | 15.73 ± 11.67 b | 460.8 ± 78.88 b | 71.81 ± 13.8 ab |
| | | CCAP 34/7 | 0.35 ± 0.7 | 13.02 ± 8 b | 0.11 ± 0.13 c | 0.3 ± 0.29 b | 0.01 ± 0.03 b | 0.68 ± 0.78 b | 2.2 ± 1.89 b | 0.68 ± 0.63 b | 2.02 ± 2.15 b | 108.96 ± 49.26 c | 18.07 ± 7.85 b |
| *H. rubicundus* | | NIES 144 | 0 ± 0 | 181.56 ± 61.39 a | 6.63 ± 0.64 a | 4.77 ± 2.15 a | 1.58 ± 1.31 a | 16.17 ± 7.97 a | 53 ± 20.91 a | 12.22 ± 8.14 a | 37.51 ± 11.16 a | 1388.21 ± 53.99 a | 240.08 ± 8.69 a |
| | | BE05_06 | 3 ± 4.05 | 24.81 ± 20.24 b | 0.45 ± 0.42 c | 0 ± 0 b | 0 ± 0 b | 0.12 ± 0.25 b | 0.65 ± 0.91 b | 0.2 ± 0.27 b | 1.93 ± 2.03 b | 232.13 ± 93.81 c | 34.82 ± 17.34 b |
| | | IT01_06 | 5.17 ± 6.58 | 262.31 ± 46.27 d | 0.67 ± 0.78 c | 2.41 ± 0.71 ab | 0.32 ± 0.39 ab | 2.32 ± 0.77 b | 5.82 ± 2.28 b | 2.49 ± 0.97 b | 9.61 ± 4.75 b | 518.79 ± 72.82 b | 177.86 ± 209.12 ab |
| | | NL01_04 | 4.99 ± 6.06 | 76.7 ± 43.31 bc | 0.82 ± 0.46 c | 0.57 ± 0.47 b | 0.35 ± 0.56 ab | 0.38 ± 0.46 b | 2.75 ± 1.01 b | 0.58 ± 0.4 b | 4.38 ± 4.43 b | 443.78 ± 84.19 b | 81.67 ± 26.16 ab |
| | ***p value*** | | ***0.390*** | ***<0.001*** | ***<0.001*** | ***<0.001*** | ***0.024*** | ***<0.001*** | ***<0.001*** | ***0.001*** | ***<0.001*** | ***<0.001*** | ***0.013*** |
| *H. pluvialis* | 222 | CCAP 34/14 | 0 ± 0 | 325.23 ± 256.11 ab | 5.91 ± 4.25 ab | 4.8 ± 4.64 | 0.43 ± 0.5 ab | 3 ± 4.48 b | 8.86 ± 8.75 b | 4.67 ± 5.65 ab | 54.29 ± 22.96 a | 964.98 ± 761.92 b | 137.78 ± 105.02 b |
| | | CCAP 34/7 | 0 ± 0 | 6.56 ± 7.61 b | 0.22 ± 0.27 b | 0.36 ± 0.41 | 0 ± 0 b | 0.26 ± 0.3 b | 0.72 ± 0.98 b | 0.29 ± 0.33 b | 0.77 ± 0.94 b | 99.14 ± 27.1 a | 15.01 ± 3.99 a |
| *H. rubicundus* | | NIES 144 | 10.52 ± 21.05 | 228.14 ± 62.19 ab | 4.64 ± 0.26 ab | 6.66 ± 2.29 | 1.85 ± 1.26 a | 11.67 ± 3.72 a | 38.87 ± 10.23 a | 9.67 ± 6.68 a | 21.69 ± 16.08 ab | 887.45 ± 153.5 b | 142.31 ± 29.27 b |
| | | BE05_06 | 11.11 ± 22.22 | 418.74 ± 310.3 a | 7.22 ± 5.94 a | 16.27 ± 23.09 | 0.62 ± 1.25 ab | 3.03 ± 6.06 b | 9.57 ± 10.02 b | 2.85 ± 4.22 ab | 32.27 ± 22.83 ab | 831.91 ± 223.19 b | 129.87 ± 33.15 b |
| | | IT01_06 | 3.85 ± 7.7 | 126.49 ± 65.63 ab | 0.61 ± 0.62 ab | 0.86 ± 0.86 | 0.14 ± 0.28 ab | 0.26 ± 0.52 b | 1.13 ± 1.1 b | 0.67 ± 0.54 b | 12.88 ± 7.78 b | 328.68 ± 52.86 ab | 49.45 ± 17.15 ab |
| | | NL01_04 | 3.6 ± 4.5 | 120.79 ± 61.46 ab | 1.13 ± 0.79 ab | 1.06 ± 0.63 | 0 ± 0 b | 0 ± 0 b | 0.26 ± 0.43 b | 0.09 ± 0.18 b | 7.7 ± 5.09 b | 326.64 ± 50.26 ab | 62.61 ± 18.06 ab |
| | ***p value*** | | ***0.721*** | ***0.033*** | ***0.013*** | ***0.218*** | ***0.025*** | ***0.001*** | ***<0.001*** | ***0.023*** | ***0.001*** | ***0.006*** | ***0.004*** |



# CHAPTER 6:
# Genetic and life history determinants of the susceptibility of the blood algae *Haematococcus* to infection by *Paraphysoderma sedebokerense* (Blastocladiomycota)

Submitted as Allewaert CC., Hiegle N., Strittmatter M., de Blok R., Guerra T., Gachon CMM., Vyverman W. *Algal Research Special Issue on Crop protection* on 10/01/2017.

**Abstract**

*Haematococcus pluvialis* is currently cultivated at large scale for its ability to produce high amounts of the high value keto-carotenoid astaxanthin when encysted. Mass cultivation of this species is threatened by the parasite blastocladialean fungus, *Paraphysoderma sedebokerense* Boussiba, Zarka and James, responsible for the fast collapse of *Haematococcus* populations. Given the difficulty of maintaining pathogen-free production systems and the lack of treatment options, the selection and development of resistant *Haematococcus* strains could potentially present an efficient method to control infection.

In the present work, we examined the host specificity of *P. sedebokerense* (strain PS1) through quantitative phenotyping of 44 *Haematococcus* strains in a laboratory-controlled infectivity assay. We determined the growth and photosynthetic activity of strains in the presence and absence of PS1 over time (using Chl *a in vivo* fluorescence) and quantified the degree of infection through the intensity of fluorescence after staining with Wheat Germ Agglutinin (WGA)-Fluorescein, specific to zoospores and cysts of PS1. The measurements were converted into three infectivity proxies, allowing comparisons amongst strains. Eventually, microscopy was performed to check the life stage of *Haematococcus* upon infection.

Strains of *Haematococcus* clearly exhibited different levels of susceptibility against PS1 as determined by the three proxies. These were not related to phylogenetic background, nor the sampling origin of the strains. Among ten strains with low susceptibility, five occurred as flagellated state cultures, while others were palmelloid and/or aplanospore dominated. In addition, in a long term selection experiment, we showed that susceptibility to PS1 of a highly sensitive *H. pluvialis* strain decreased through the dominance of flagellated phenotypes over several generations of infection.

While providing considerable expansion of the relation between PS1 and *Haematococcus* our study opens the possibility for selection of resistant strains for large scale production.

## 6.1. Introduction

The green uni-cellular alga, *Haematococcus pluvialis* Flotow (Chlorophyceae), is bi-flagellated and motile under optimal growth conditions. When exposed to adverse conditions, it transforms into a non-motile palmelloid cell which further evolves into a resting cyst or aplanospore with a tough cell wall, accumulating large amounts of the oxygenated carotenoid astaxanthin. These different life stages exhibit radically different phenotypes (Gu *et al.*, 2013; Wayama *et al.*, 2013; Wang *et al.*, 2014). Aplanospores of *H. pluvialis* are so far the best known natural producers of astaxanthin, a highly valued carotenoid in cosmetic, nutraceutical and animal feed industries. Since astaxanthin is a powerful coloring agent with strong anti-oxidant capacity; *H. pluvialis* is highly demanded and cultivated at large scale currently by over sixteen international companies (Shah *et al.*, 2016). Although numerous grazers, pathogens and parasites are challenging mass culture of *H. pluvialis* worldwide (Gutman *et al.*, 2009), the blastocladialean fungus *Paraphysoderma sedebokerense* Boussiba, Zarka and James (James *et al.*, 2012) (hereafter called *PS*) is believed to be one of the most serious parasites (Han *et al.*, 2013). It has been discovered independently in several production facilities over the world, and was found responsible for reduced astaxanthin productivity as well as frequent culture collapses (Hoffman *et al.*, 2008; Gutman *et al.*, 2009; Gutman *et al.*, 2011). *PS* is noticeably a problem of persistent nature in culture facilities, since it may, like chytrid fungi, produce thick-walled cysts that withstand disinfection (Carney & Lane, 2015). Its complex life cycle is currently not fully understood (Letcher *et al.*, 2016; Strittmatter *et al.*, 2016).

Among all the disinfection methods tested so far (McBride *et al.*, 2012; Zhang *et al.*, 2013), only the use of $H_2O_2$ was proven to successfully eliminate *PS* (Carney & Sorensen, 2016). Nonetheless, the use of such stringent sterilization techniques may be expensive, labor intensive or have severe environmental impacts (Burridge *et al.*, 2010). Integrated pest management strategies have therefore been proposed as the most efficient long-term strategy for control of parasites in production facilities (Chandler *et al.*, 2011; Carney & Lane, 2015), where the cultivation of resistant strains, limited use of chemical agents, and the development of biological control systems are combined to limit the shortcomings of each individual strategy. In this respect, research has targeted the search and development (through classical mutagenesis or genetic engineering techniques) of disease-resistant strains (Carney & Lane, 2015).

Chytrids, close relatives of the Blastocladiales, have been widely studied for their interaction with phytoplankton. Species can be extremely variable in their host specificity (Ibelings *et al.*,

2004), with some being generalists while others are specialists, infecting one or several host species (Little *et al.*, 2006). In contrast; the host specificity of the Blastocladiales have been poorly studied. Species belonging to *Physoderma*, the sister genus of *Paraphysoderma,* are obligate parasites of phanerogams. In this genus, host specificity was long used as an important character to delineate taxa, resulting in the designation of generally one single host per species (Saunders & Sparrow, 1974), e.g. *Physoderma dulichii* specific to the threeway sedge, *Dilichium arundinaceum* (Johns, 1966). Only few cross inoculation trials were performed to study *Physoderma* host ranges, revealing either wide host ranges (Karling, 1956; Saunders & Sparrow, 1974) or limited host ranges (Lingappa, 1958). Being the only genus of this order infecting algae, *Paraphysoderma* also exhibits considerable variation in host specificity, at least based on the still rather limited available literature. So far, four strains of *PS* have been isolated from separate geographic locations. A strain of *PS* (TJ-2007a) from Israel was found to be highly specific to *Haematococcus,* given its capacity to infect twenty different strains of *H. pluvialis.* The same strain was able to infect other green algal genera yet without complete culture crashes (Hoffman *et al*., 2008; Gutman *et al*., 2009). Strain FD61 also identified as *PS*, was found in an outdoor biofuel production facility in New Mexico, US, infecting *Scenedesmus dimorphus*, where it resulted in complete population crashes (McBride *et al.*, 2014; Letcher *et al.*, 2016). Two additional strains, identified as *PS,* were discovered in open raceway ponds in Arizona, US (JEL821) (Carney *et al*., 2016) and outdoor cultures in Portugal (PS1) (Strittmatter *et al.*, 2016), in both cases infecting *H. pluvialis*. Although true host ranges of these strains remain unexplored, the heterogeneity in these reports suggest that strains of *PS,* though possessing identical SSU 18S ribosomal RNA sequences, might considerably differ in their infectivity behavior.

In most cases, host range studies are performed at the species level (Canter & Jaworski, 1981; Canter *et al.*, 1992; Doggett & Porter, 1995; Gromov *et al.*, 1999) yet specialization may become even more complex since it may also occur within species or populations. Intraspecific variation in infectivity is well described in pathogens affecting terrestrial plants but has been only poorly explored among algal lineages (Canter & Jaworski, 1979; Little *et al.*, 2006; West *et al.*, 2006; Gachon *et al.*, 2009; Van Wichelen *et al.*, 2012; Gsell *et al.*, 2013). Within the Blastocladiales, Eddins (1933) reported that varieties of corn could differ in their susceptibility to brown spot (*Physoderma zeae-maydis*) and succeeded in selecting and self-fertilizing plants resistant to the fungus. Intraspecific variation in susceptibility to *PS* amongst *Haematococcus* strains has never been reported yet a preference of *PS* for aplanospores and palmelloid cells of

*Haematococcus* was demonstrated while flagellated cells were not susceptible to infection (Gutman *et al.*, 2009). Given the lack of methodologies for *PS* detection and quantification, no comparative studies on *Haematococcus* has been performed thus far. Better understanding the causality of this specialism together with the identification of drivers of variation in susceptibility to *PS* are both key goals towards prediction and control of disease outbreaks in mass cultivation facilities.

In this study, we wanted to better comprehend the causality and drivers of specialism in the *Haematococcus* - PS1 interaction, through the establishment of a quantitative phenotyping method by which the relative susceptibility of strains of *Haematococcus* was tested, in an attempt to identify possibly resistant strains. Specifically, we examined *in vitro* infectivity profiles by *PS* on 44 *Haematococcus* strains (Allewaert *et al.*, 2015), which we graded for susceptibility to *PS* through the use of different infectivity proxies: maximal reduction percentage ($Red._{max.}$), the percentage of survival (*Survival*) and the density of infected cells ($Dens._{inf.}$). A parallel screening was performed to explore resistance in a large number of strains by categorizing infection through direct visual observation of 143 *Haematococcus* strains. Finally, a three-month selection experiment was set up to test the longer-term response of a highly susceptible *H. pluvialis* strain (C1) in co-culture with PS1. A selection towards flagellated phenotypes upon long term exposure to infection with *PS* was observed.

## 6.2. Material and methods

### 6.2.1. Biological material

The strain PS1 (described in Strittmatter *et al.* (2016)) was propagated from a clonal isolate of *H. pluvialis*, hereafter named C1, generated in-house from the strain SCCAP K-0084 (Scandinavian Culture Collection of Algae & Protozoa, http://www.sccap.dk). During the experimental period, PS1 was kept infective through the addition of infected PS1-C1 culture (5 mL) to uninfected exponential culture of C1 (10 mL), every four days. Infected cultures were placed in an incubator at 25 °C and continuously illuminated with 22 ± 4 µmol photons $m^{-2}$ $s^{-1}$ (Gutman *et al*., 2009; Strimatter *et al*., 2016). C1 was kept exponential by the addition of Bold Basal Medium modified with 3-fold nitrogen (BBM-3N, (Bischoff & Bold, 1963)) every four days, under following growth conditions: 23 °C, using a 16:8h light:dark regime and a light intensity of 20 ± 2 µmol photons $m^{-2}$ $s^{-1}$.

Details on the isolation and cultivation methods of the different *Haematococcus* strains used in this experiment can be found in Allewaert *et al*. (2015). In total, 60 *H. pluvialis* including nine

additional strains from four different culture collections (CCAP, NIES, SAG and SCCAP), 7 *H. rubens*, 40 *H. rubicundus* and 36 *H.* sp.(which were not assigned because not sequenced) strains were infected (Table S1, Supplementary Data). A selection of 44 strains was made by randomly selecting two strains per sampled location for the quantitative infectivity assay.

### 6.2.2. Algal growth conditions

All strains were grown from stock conditions (6 °C) in Bold Basal Medium modified with 3-fold nitrogen (BBM-3N, (Bischoff & Bold, 1963)) at 23 °C, using a 16:8h light:dark regime and a light intensity of 20 ± 2 µmol photons $m^{-2}$ $s^{-1}$ and further prepared and acclimated as in Allewaert *et al.* (2017).Where specified, 48 or 96 cell culture multiwell plates were used (Greiner Bio-One) with a working volume of 1 mL or 0.5 mL respectively.

For the infectivity assay, each strain was prepared in four replicates for infection (I, *n=4*) and two controls (C, *n=2*). The experiment was started after 15 days of cultivation, ensuring that all strains were stationary. Each replicate culture was harvested prior to setting up the experiment, washed twice with BBM-3N without phosphate and . brought to an initial $F_0$ value of 0.0043 (settings, 1-1-2 as measured by PAM fluorometer, see below) through the addition of BBM-3N without phosphate (1 mL, 48 multiwell plates), corresponding to a final cell density of approximately 1550 *Haematococcus* cells $mL^{-1}$.

The broader screening of strain susceptibility was performed on 143 strains of *Haematococcus*, in this case, strains were infected in 96 multiwell plates (0.5 mL working volume).

The long term selection experiment was performed using strain C1 cultured in industrial medium based on Algal medium, as in Fábregas *et al.* (1984) using 1 L balloon flasks (Normax, NormaLab) with a working volume of 500 mL.

### 6.2.3. Inoculation with *Paraphysoderma*

An aliquot (120 µL) of PS1 filtered through an 8 µm mesh size filter (Membrane filter Isopore, Millipore) was added to infect *Haematococcus* strains (I). To adjust for volume differences, the same volume (120 µL) yet this time filtered through a 0.22 µm sterile vacuum bottle top filter (EMD Millipore™ Steritop™) was added to the control cultures (C). The concentration of particles in the PS1 filtrate counted with a coulter counter (Beckman, 50 µm measuring tube), resulted in a total final number of particles of $9.91e^{+04} \pm 1.97e^{+04}$ particles $mL^{-1}$, sized between 2 and 8 µm, the current size ranges of PS1 life forms (Strittmatter *et al.*, 2016).

In the generalization experiment, 10 µL of PS1 filtrate was added to each strain. Host infected cultures were examined daily under an inverted light microscope to monitor the infection of the cells. In those wells showing no signs of PS1 infection after one first round, a second addition of PS1 filtrate was done to the same well.

After inoculation with PS1, plates from both experiments were randomly placed under experimental conditions: 25 °C (incubator) and continuously illuminated with 22 ± 4 µmol photons $m^{-2}$ $s^{-1}$ provided by cool white fluorescent tubes. Both experiments were followed during 19 days.

For the long term selection experiment, a selection scheme was followed (Fig. S6, Supplementary Data). A one week old culture of C1 (500 mL) was infected with 5 % of infective PS1-C1 culture. Culture conditions were as follow: 25 °C exposed to continuous light at 125 ± 10 µmol photons $m^{-2}$ $s^{-1}$. The infection was followed for 3 weeks until the culture had collapsed as confirmed microscopically. Although most of the cells in this culture had died, sporadically surviving C1 cells were observed that were flagellated and contained astaxanthin. Motile cells were collected through phototaxis after 24 h in a darkened 1 L graduated cylinder illuminated only on the culture surface, further transferred (100 mL) to a balloon flask, and enriched with nutrients of the industrial medium (cf. above). After 3 weeks of growth, the culture was scaled up (500 mL), and weekly subcultured to maintain the selective pressure, over a period of three months, through the transfer of both infected and non-infected cells (100 mL) to fresh medium (900 mL). A C1 control culture was generated from the source culture for this experiment, which had never been exposed to PS1. The control was further propagated following the same subcultivation routine as the infected culture. Life stages of C1 in both cultures were carefully monitored by subsampling weekly for observation under bright field microscopy.

### 6.2.4. Infection proxies

***Density of infected cells (Dens.*** $_{inf.}$***) -*** In order to obtain quantitative data on the 44 strains, counting of infected and uninfected cells using optical microscopy was unfeasible. Therefore, a novel spectrophotometric method to estimate the final number of infected cells was developed and validated. Infected cultures were treated with wheat germ agglutinin (WGA)-Fluorescein (FL-1021, Vector laboratories) which has a primary specificity to N-acetyl-D-glucosamine and was reported to bind PS1 zoospores and cysts without interfering with *Haematococcus* cells (Gutman *et al.*, 2011). The stability of WGA-Fluorescein upon staining of PS1-C1 cultures was

tested over time when cultures were treated with two different concentrations of stain (5 and 10 µg $mL^{-1}$) and revealed that the signal was stable after 80 min when stained with 5 µg $mL^{-1}$ (Fig. S1 A-B, Supplementary Data). Since the WGA-Fluorescein had to be washed out from the culture, the effect of diluting -due to washing- on the fluorescence signal was also tested. A calibration curve was built based on the density of infected cells (*Dens.* $_{inf.}$) versus the fluorescence intensity (FI). For this purpose, a dilution series was prepared (in a 48 well plate), with a mixture of C1-PS1 infected culture and uninfected C1 cells at various concentrations diluted in BBM-3N without phosphate (final volume of 1 mL). Each well was treated with 5 µg $mL^{-1}$ of WGA-Fluorescein, subsequently mixed by pipetting, and the plate was incubated for 80 min at 18 °C in the dark after which it was centrifuged (3000 rpm). Each well was washed before measurement of FI by replacing 90 % of the initial medium with fresh BBM-3N without phosphate. The washing step was repeated twice. After washing, well plates were measured with a Victor 3 multi-well plate reader, with 25 readings per well (Perkin Elmer - MTX Lab Systems) (Ex= 485 nm, Em=535 nm) (Fig. S1 C-D, Supplementary Data). For calibration, the amount of infected versus uninfected cells of *Haematococcus* were quantified under a fluorescence microscope (Zeiss Axioplan II, Filter set 01 shift free (F) EX BP 365/12) at 100 x magnification until a total of 250 specimens (infected+uninfected) was reached. The calibration curve of the infected cells counted versus FI signal (Fig.S1 E-F, Supplementary Data) showed significant correlation, without significant loss of signal after one washing step, ($R^2 = 0.9838$), demonstrating that FI could serve as proxy for *Dens.* $_{inf.}$ (cells $mL^{-1}$). For the infectivity assay, FI was measured at the end of the infectivity assay, after 19 days by adding 5 µg $mL^{-1}$ of WGA-Fluorescein to C and I cultures, incubating and washing once. To correct for background signal, the difference in final FI values between the infected (I) and the control cultures (C) of FI values previously normalized with their respective $F_0$ value for the last day of infection were calculated. These values were then translated into *Dens.* $_{inf}$. for each strain using the following equation: $Dens. inf. = (0.0175 \times FI) - 69.982$, obtained from the calibration curve based on average FI against number of infected cell (*n*=3) (Fig. S1 E, Supplementary Data).

***Maximal percentage reduction (Red.*** $_{max.}$***) -*** During the experimental period, Pulse Amplitude modulated fluorescence (PAM, Waltz MAXI Imaging PAM) was used to measure changes in $F_0$ and $F_v/F_m$ over time of the 44 strains: $F_0$, as proxy for Chl *a* to monitor changes in biomass and $F_v/F_m$, the maximum quantum yield to evaluate the culture health, or impairment of the photosystem II system and is a direct estimate of photosynthetic competency (Schreiber *et al.*, 1986; Kolber *et al.*, 1998). Daily measurements at a fixed time point were made using default

settings, intensity of 1, gain of 1 and damping of 2. Before each measurement, cultures were dark-adapted for 15 min after which a saturating pulse was fired to measure $F_0$, $F_m$ and $F_v/F_m$. Measurements were performed in complete darkness, after which, the plates were randomly placed back under experimental conditions. Based on measurements of $F_0$ trough time, the % reduction of uninfected cells were determined as in Gsell *et al.* (2013):

$$\%\,reduction = \frac{(1-(I)t)}{(C)t} \times 100$$

Where (I)= $F_0$ infected and (C)= $F_0$ control and $t$ the time point. The maximal % reduction, corresponding to the maximal value during the period of infection (*Red.* $_{max.}$) was used as proxy for infectivity, allowing strain comparisons.

***Survival*** **-**We quantified the survival ratio for each strain as follows:

$$Survival = \frac{(I)final}{(C)final} \times 100$$

Where (I) final = $F_0$ infected on day 19 and (C) final = $F_0$ control on day 19.

***Susceptibility classes*** **-** For the generalization experiment, susceptibility of 143 host strains was observed via light microscopy (Zeiss Axio Observer A1, 60 x magnification) and scored in three different classes: two different susceptibility classes, ranging from low (1), to high (2) and a resistant class (0) (Fig. S4, Supplementary Data).

### 6.2.5. Statistics

Statistics and graphics were performed using R Studio version (R Core Team, 2013). For the 44 strains, normality and equal variance tests were passed for all three infectivity proxies and one way ANOVAs were used to test for strains (using the entire data set), species (excluding uncharacterized strains) and location (excluding culture collection strains) effects on proxies: *Red.* $_{max.}$, *Survival, Dens.* $_{inf.}$. Correlation between these proxies was tested by calculating Pearson correlation coefficients (r) and the associated p-values, using the R lattice package in R version 3.02. (R Core Team, 2013). Correlations were considered statistically significant in $P \leq 0.05$. Principal component analysis (PCA) of the infectivity proxies was carried out to visualize patterns of infectivity among strains, using the devtools package in R version 3.0.2 (R Core Team, 2013). Due to the different units of measurements, all data were standardized before analysis (Z score transformation).

For the screening experiment, the Fisher exact test (given data contained counts <5) was used to test for differences between proportions in susceptibility classes for the 143 strains as function of species (excluding uncharacterized strains) or sampling location (excluding culture collection strains).

## 6.3. Results

The present study includes three experiments: (1) gauging sensitivity of 44 *Haematococcus* strains to PS1 using infectivity proxies (2) screening of 143 *Haematococcus* for PS1 susceptibility through categorization in susceptibility classes, as a generalization experiment and (3) screening for cells surviving PS1 infection in a culture of the highly susceptible C1 *Haematococcus* strain.

In the first experiment, a random selection of 44 strains of *Haematococcus* sampled from nineteen different localities throughout Europe was made, including generally two strains per sampled location. The growth and photosynthetic activity of *Haematococcus* cultures in stationary phase in a phosphate free medium was monitored upon infection with PS1 during 19 days. Under these circumstances, growth was limited and aplanospore formation initiated (Allewaert *et al.*, 2017). Biomass increase (based on $F_0$ as proxy) of infected cultures was suppressed (in no case enhanced), for almost all strains except the following six strains: BE03_05, BE10_09, CCAP 34/13, CZ01_08, NL03_06, SE02_11, which showed only slight variation in growth compared to the control (Fig. S2, Supplementary Data). The photosynthetic efficiency ($F_v/F_m$) of the 44 strains was also affected by the presence of PS1 (Fig. S3, Supplementary Data): while uninfected healthy cultures had an average $F_v/F_m$ between 0.6 and 0.7 which remained constant during the experimental period, infected cultures had varying patterns over time. Generally, sharp decreases in $F_v/F_m$ over the first 12 days of incubation were followed by increases of $F_v/F_m$, due to the death of successfully infected cells, and the selection of survivors, that seemed little or unaffected by PS1. These patterns of decreases in $F_v/F_m$ followed by a subsequent increase varied strongly between strains. Moreover, three strains did not show clear differences in $F_v/F_m$ with the control, including, BE03_05; BE04_17, BE10_09 indicating that PS1 presence did not affect their physiology.

Strain differences were also reflected in the patterns of reduction percentage (Fig. 1). Firstly, the starting point of infection (first positive difference between $F_0$ infected and $F_0$ control) varied amongst strains, with infection starting from day 3 in some strains, while first signs of

infection were only present from day 15 in others. Secondly, the progression of reduction through time varied between strains. Some strains exhibited sharp, abrupt increases over short time lapses of 1-3 days (e.g. BE05_10, BE08_04) while others showed a more gradual increase in reduction, spread over longer periods of time (e.g. NL02_08, CCAP 34/14).

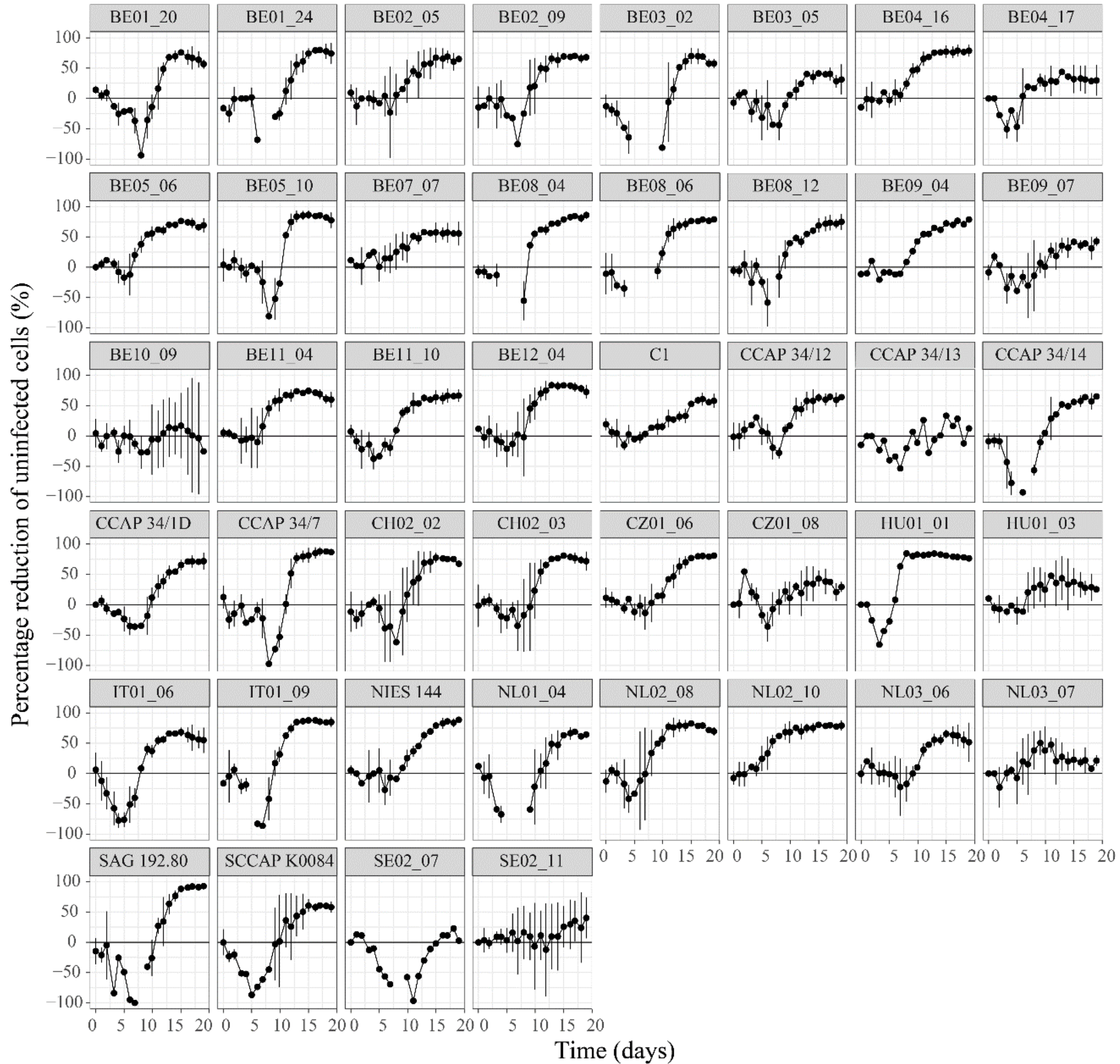



***Figure 1*** *Percentage reduction (%) in function of time (days) upon infection (started day 0) of the 44 strains tested with PS1. Each box represents a strain. Error bars represent the SD on the measurement. For clarity, the threshold for negative values was set at -100, leading to gaps in the graphs.*

Average values for each proxy and strains are given in Fig. 2. Average *Red.* $_{max.}$ for all strains comprised 70.87 ± 15.88 % and significantly differed between strains (Table 1), ranging from the lowest reduction observed in strain SE02_07 (22.69 %) to the highest reduction in strain SAG 192.80 (92.98 %). Next, significant strain differences were found for *Survival* (Table 1). The average *Survival* ratio of all strains was 40.58 ± 24.97, ranging from a minimum *Survival* ratio of 7.33 for strain SAG 192.80 to a maximum of 125.18 for strain BE10_09. Finally,

significant strain differences were found in the final *Dens.* $_{inf.}$ (Table 1). The average *Dens.* $_{inf.}$ was 1.47 x $10^4$ cells $mL^{-1}$ and varied from a minimum of 1.34 x $10^3$ cells $mL^{-1}$ (CCAP 34/13) to 5.81 x $10^4$ cells $mL^{-1}$ (SAG 192.80). For all three proxies, no significant differences between species or sample origin were found (Table 1).

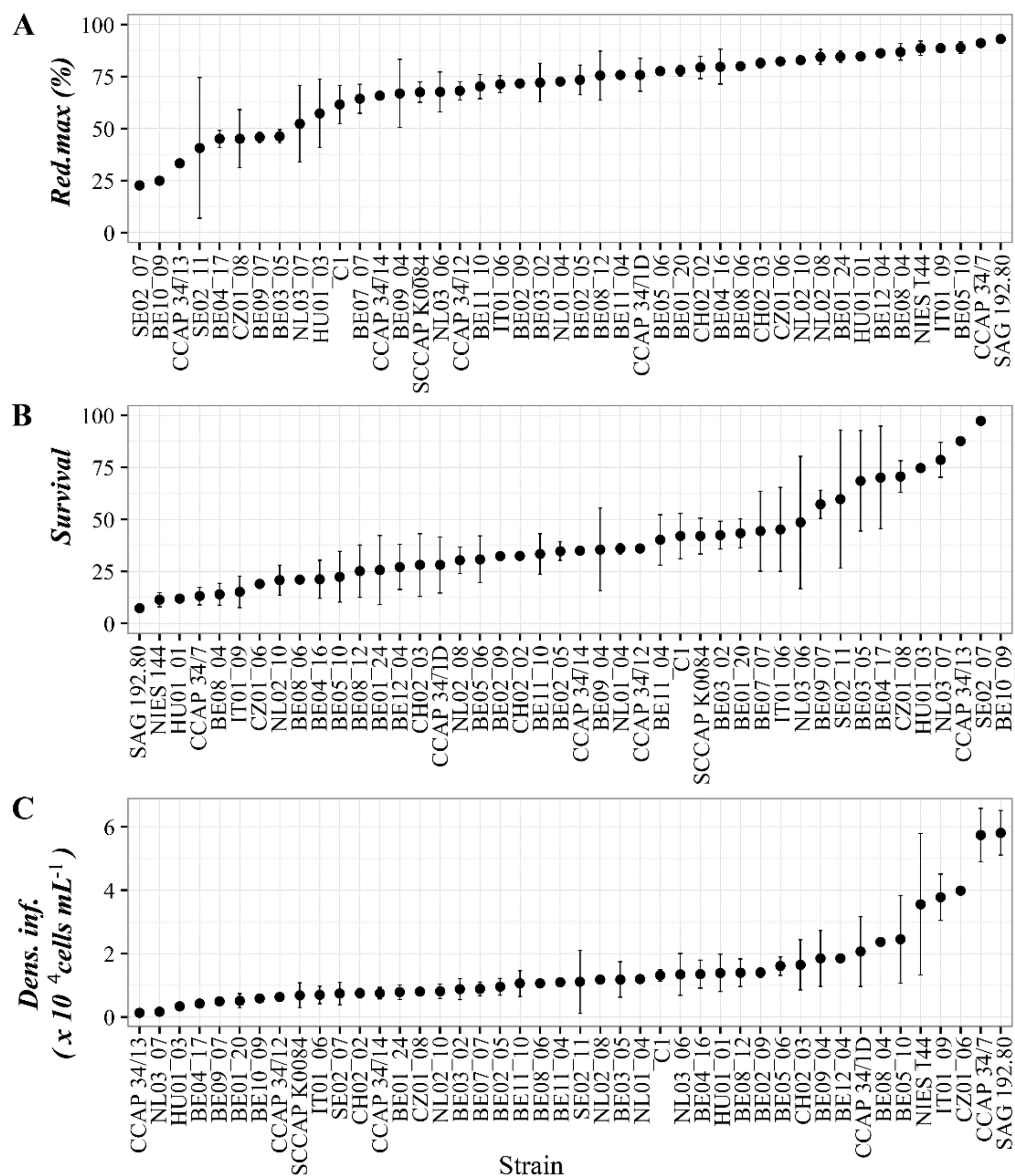



***Figure 2. A.*** *Means (± SD) of the maximum percentage reduction, Red.* $_{max.}$ *(%).* ***B.*** *Survival ratio and* ***C.*** *Density of infected cells (x $10^4$ cells $mL^{-1}$) Dens.* $_{inf.}$ *of 44 Haematococcus strains. Strains are ordered according to increasing mean for each variable.*

Correlation analysis between proxies (Fig. S4, Supplementary Data) showed that *Red.* $_{max}$. was significantly correlated with *Survival* and *Dens.* $_{inf.}$ (Pearson correlation, $r$ = -0.94, $P$ < 0.001

and $r = 0.56$, $P < 0.001$ respectively). *Survival* was also significantly correlated with *Dens.* $_{inf.}$ ($r = -0.58$, $P < 0.001$).

***Table 1.*** *Summary of the results of one-way ANOVA to test for differences at inter- and intra-species and population level for the proxies: maximum percentage reduction, Red.* $_{max.}$ *(%), Survival, and density of infected cells (cells* $mL^{-1}$*) Dens.* $_{inf.}$.

| Effects of | df | *Red.* $_{max}$. | *Survival* | *Dens.* $_{inf}$. |
|---|---|---|---|---|
| Strain | *43* | *P < 0.001* | *P = 0.010* | *P < 0.001* |
| | | F = 6.068 | F = 2.059 | F = 10.010 |
| Species | 2 | *P = 0.494* | *P = 0.489* | *P = 0.745* |
| | | F = 0.718 | F = 0.728 | F = 0.296 |
| Locality | 18 | *P = 0.035* | *P = 0.113* | *P = 0.868* |
| | | F = 2.508 | F = 1.841 | F = 0.579 |

Principal component analysis of the three proxies for infection (Fig. 3) showed that the first two dimensions of the PCA explained 98 % of the variation (first dimension: 80.1 %, second dimension: 17.9 %). Two groups were separated along the first axis: strains with a high susceptibility on the left side, determined by high *Red.* $_{max.}$, low *Survival* ratio and high *Dens.* $_{inf.}$, while less susceptible strains were concentrated on the right side, determined mainly by a high *Survival* ratio. No patterns of grouping were observed related to species, or sampling origin. The following ten strains were determined by a high survival ratio: HU01-03, CCAP 34/13, BE10_09, BE09_07, NL03_07, BE04_17, CZ01_08, BE03_05, SE02_07 and SE02_11. Microscopy observations performed at the end of the experiment, showed that strains BE01_20, BE03_05, BE09_07, BE11_04, CCAP 34/13, HU01_01, HU01_03 and SE02_07 of the infected cultures were substantially populated with flagellated or dividing cells, this in contrast with all other cultures which were either completely in palmelloid and/or aplanospore stages. Of these eight strains, five represented strains with low susceptibility to *PS*, determined by a low *Red.* $_{max.}$, a high *Survival* and a low *Dens.* $_{inf.}$

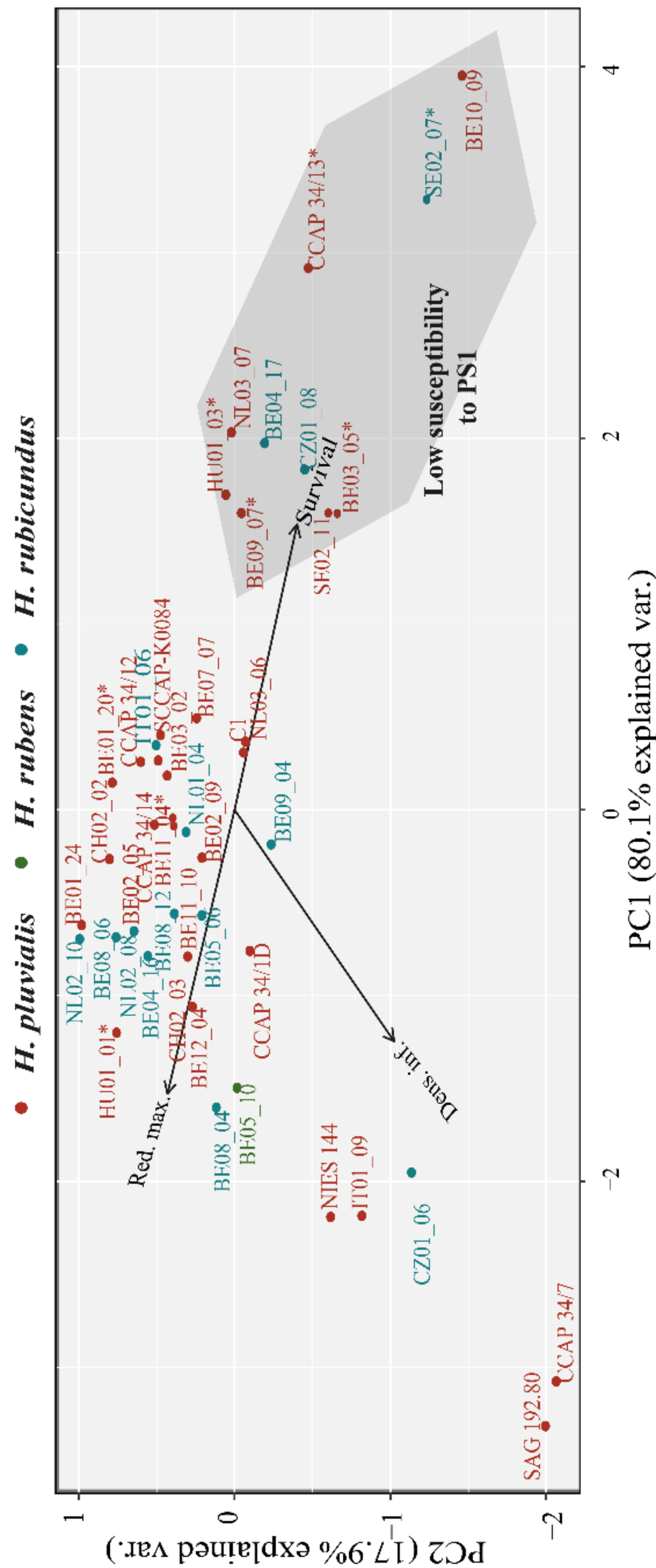
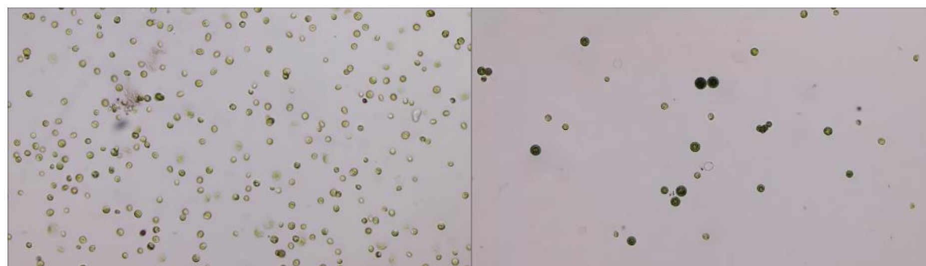



***Figure 3.*** *PCA of the proxies Red. $_{max}$, Survival and Dens. $_{inf}$ on H. pluvialis (red), H. rubicundus (turquoise), H. rubens (green). The direction of each arrow indicates increasing values of the corresponding infectivity proxy. The first two axes are shown, explaining 80.1 % and 17.9 % of total variation respectively. Strains with an asterisk, are strains that were dominantly flagellated at the end of the experiment.*

In the second experiment, a broader screening was performed to gain preliminary insights in the susceptibility of 143 strains of *Haematococcus* and one *Ettlia carotinosa* strain when exposed to PS1. Amongst these strains, 60 strains belonged to *H. pluvialis*, 40 to *H. rubicundus*, 7 to *H. rubens* and 36 to *H. sp.* (Table S1, Supplementary Data). In line with the above experiment, strains of *Haematococcus* showed different levels of susceptibility to *PS*. In total 83 strains were scored 2 (high susceptibility), 57 were scored 1 (low susceptibility), and 3 were scored 0 (no *PS* detected). Thus, three candidate strains were identified, which did not show any traces of PS1 after two subsequent infections: NL02_06; NL03_01; NL03_12, currently examined in more detail in order to confirm their resistant phenotype. We found no effect of phylogenetic background ($P = 0.076$, Fisher's exact test, excluding *H. sp.*), while a significant difference was found in strain origin ($P = 9.99 \times 10^{-04}$), Fisher's exact test, excluding culture collection strains (Fig. S5, Supplementary Data).

Finally, the long term experiment was designed to select for resistant cells within strain C1 (Fig. S6, Supplementary Data). This strain (the initial propagation host of PS1) clustered within the group of highly susceptible strains from the infectivity assay (Fig. 3). After being selected and collected from a crashed culture, the few surviving cells of C1 were scaled up and sub-cultivated, at regular time intervals. Based on the continuous presence of infected cell clumps upon subcultivation, clearly, the infection was ongoing, yet at very low level. In these cultures, the flagellated phenotype remained dominant throughout subcultivation with a minority of palmelloids and aplanospores, as presented in Video Data 1. In contrast, control cultures contained predominantly palmelloid and aplanospores while a minority of cells were flagellated, as can be observed from Video Data 2.



*Still image of Video Data 1 (left) and Video Data 2 (right)*

## 6.4.Discussion

Until today, studies on *Haematococcus-PS* interactions were entirely based on presence/absence scoring through visual assessments, sometimes using specific fluorescent stains to help identify and distinguish the pathogen (Gutman *et al.*, 2009; Gutman *et al.*, 2011). Microscopy, however, requiring trained personnel and much time, is as such largely impractical for a large number of strains and hence difficult to support with robust statistical analysis (Gachon *et al.*, 2009). Although qPCR assays are becoming readily available for detection and quantification of parasites (Gachon *et al.*, 2004; Gachon & Saindrenan, 2004; Gachon *et al.*, 2009), the chronic lack of rapid infectivity assays are impeding host-parasite studies. To the best of our knowledge, this study is the first to provide quantitative data on the susceptibility to fungal infection of a large number of algal strains through the use of several infectivity proxies. The high throughput nature of this study opens opportunities to perform successful phenotyping by simultaneously assessing multiple fitness traits related to infection. Theoretically, such information not only serves to better understand the causality of infectivity, but may also act as a basis for targeting resistant strains and subsequently genes responsible for resistance in the future. These targets may in the end, serve as framework for the development of strains with low or no susceptibility to *PS.*

Our results confirm the hypothesis that *Haematococcus* strains exhibit significantly different susceptibility to infection with PS1 as judged by the high variation in responses to the measured proxies $Red._{max}$, *Survival* and $Dens._{inf}$ amongst all 44 strains tested together with the variation in categories of infection from the generalization experiment. From all screened strains, three strains from the generalization experiment showed to be candidate resistant strains, currently data confirming their resistance are on their way.

These results represent strong evidence supporting a genetic determinism of *PS* resistance. Variations in host susceptibility within species was previously described for several chytrid strains infecting diatoms, dinoflagellates and cyanobacteria (Canter & Jaworski, 1986; Sonstebø & Rohrlack, 2011; Lepelletier *et al.*, 2014), but were limited to the above mentioned study on *Physoderma zeae-maydis* infecting several varieties and inbred lines of corn (Eddins, 1933) in the blastocladialean fungi. According to Gutman *et al.* (2011), mechanisms of chemotaxis are the primary determinant for host selection in *PS*. The recognition process between both organisms is mediated by D-galactose or N-acetyl-D-glucosamine moieties exposed at the surface of *Haematococcus* aplanospores and palmelloids, or the surface of the blastoclad (Gutman *et al.*, 2011). One explanation could be that, possibly, the intraspecific

variation in susceptibility observed here stems from modifications of the recognition oligosaccharides themselves, or from varying quantities or expression levels of the latter amongst *Haematococcus* isolates. In both cases, strains would differ in the regulatory machinery governing expression of the resistance molecule gene. The absence or modification of molecules impeding host-parasite reception is typical for bacteria to develop resistance to bacteriophages (Hyman & Abedon, 2010). Similar processes have not yet been reported in microalgae.

Parasitic infection applied by PS1 was not related to phylogenetic background, since amongst the three *Haematococcus* species tested (*H. pluvialis, H. rubicundus* and *H. rubens*), no significant differences in $Red._{max.}$, *Survival* and $Dens._{inf}$. were found. Similarly, no significant differences were found between sampling origin. Local adaptation which implies that the parasite population has higher performance to infect local vs. foreign host populations is a common result in microalgae ecology, but is certainly not universal (Kaltz & Shykoff, 1998) and may not be generalized to every trait (Eigemann *et al.*, 2013). The lack of local and species specific adaptations in the tested *Haematococcus* strains when exposed to PS1, together with the variations in measured strain-specific sensitivities of *Haematococcus* to PS1 (as judged by the correlation coefficients between parameters) imply that PS1 infectivity is the result of complex interactions between host and parasite, comprised of a genetic factor together with additional factors, which remain to be further clarified.

Since genetic correlations such as trade-offs between epidemiological and life-history traits are controlled by both opponents: the host and the parasites' genotype (Carius *et al.*, 2001; Lambrechts *et al.*, 2006), our study clearly cannot serve to make predictions on host parasite arms race given that only one single strain of *PS* was tested. Obviously, intraspecific variability in epidemic behavior and potential virulence between parasite strains may be as important as the intraspecific variation between hosts. Although some preliminary studies showed slightly variable epidemic behavior amongst all accessible *PS* strains (Hoffman *et al.*, 2008; McBride *et al.*, 2014; Carney & Sorensen, 2016; Strittmatter *et al.*, 2016), a systematic comparison of these strains remains to be completed. To date however, molecular data on *PS* facilitating such studies are virtually lacking from the available literature.

Microscopic observations of the less susceptible strains revealed that 50 % of the strains with potential low susceptibility were residing in flagellated state and still dividing. Two hypotheses have been proposed to account for the resistance of flagellated cells to *PS* attack (Hoffman *et*

*al.*, 2008). Firstly, their motility might allow physical escape from *PS* zoospores/amoebae and secondly, differences in the nature of cell cover between flagellated and non-motile *Haematococcus* cells may inhibit the chemically-mediated recognition between flagellated cells and *PS* (Gutman *et al.*, 2011). From a production perspective, however, flagellated cells are less desirable since they do accumulate little astaxanthin. In our infectivity assay, we did not investigate whether the amount of flagellated cells in infected cultures exceeded the amount in the control cultures, yet results from the long term selection experiment demonstrated that flagellated cell populations in the PS-selected culture largely exceeded those of the controls. This suggests that residing in flagellated stage is advantageous over aplanospore/palmelloid stages upon exposure to PS1. Furthermore, while high prevalence of flagellated cells in infected cultures with low susceptibility to PS1 infection occurred naturally, the recovery of highly susceptible C1 was only achieved by artificial enrichment of the flagellated population. The phenotype of the selected cells remained predominately flagellated in co-cultivation with PS1 during following generations throughout the cycles of subcultivation, protecting them from infection. At the end of the three-month experiment, the flagellated phenotype remained stable over several generations in the absence of PS. This observation suggests that flagellation results either of the selection of mutants expressing this phenotypes, or from long-lasting, heritable epigenetic modification triggered by the infection, which remains to be further investigated.

From our data it remains unclear whether the observed morphological plasticity is either induced or amplified by the presence of *PS*. In any case, this response implies that *Haematococcus* cells are able to perceive the presence of *PS,* suggesting possible reciprocal signaling between host and parasite. The formation of flagellated cells upon infection could possibly represent an escape strategy to infection. From the available literature, such flexible architecture has not yet been described as response to fungal infection, but is a commonly described reaction against predation or viruses across several taxonomic groups. In presence of *Amoebophrya spp.,* the red tide dinoflagellate *Scrippsiella trochoidiea* was able to speed up cyst production, the latter being able to resist infection (Chambouvet *et al.*, 2011). Similarly, in presence of the EhV virus, *Emiliania huxleyi* was capable of transiting from diploid to haploid phase, thereby altering the calcium metabolism, an essential element in virus – host recognition (Frada *et al.*, 2008). Similarly, transition from single flagellated cells to colonies in *Phaeocystis pouchettti* served as defense mechanism against the PpV virus (Jacobsen *et al.*, 2007). Phenotypic plasticity was also described for several green algal species in response to predation

(Lürling & Beekman, 2006), where algal colony or palmelloid cell formation was induced upon exposure to grazers, hampering as such their ingestion and digestion (Lürling, 2003).

We have shown that susceptibility or resistance of *Haematococcus* species to parasitic infection by PS1 is the product of a variety of factors that influence the host parasite relationship at many points. In parallel to these complex determinants, abiotic factors that modulate infection can also be presumed, which may in a natural context affect the susceptibility and resistance patterns found in this study. In fact, complex interactions of a spectrum of abiotic factors including light, temperature, nutrient, pH, oxygen and water turbulence may affect both the growth of the host and the parasite (Van Donk and Bruning 1995). Research on how abiotic factors affect *PS* growth and infectivity is still in its infancy. Hoffman *et al.* (2008) made some observations on the effects of light, oxygen and temperature, on the survival, growth and infectivity of *PS* in the absence and presence of *Haematococcus*. Yet, although rapidly progressing, many aspects of the ecology, life cycle and biology of *PS* (Letcher *et al.*, 2016; Strittmatter *et al.*, 2016) remain to be studied.

### 6.5. Conclusions

For the first time, strain-specific host reaction of *Haematococcus* in response to *PS* were quantified under laboratory settings. Considerable levels of susceptibility were measured as function of strain, undoubtedly reflecting the involvement of specific genes. Identifying the genes and molecules involved in this protection remains a challenge for the field, but current progress is being made. Given the destructive nature of this parasite, understanding genomic controls of pathogen resistance and related phenotypic differentiation in *Haematococcus* is imperative to improve assessment, prediction and probably remediation. Susceptibility also depended on the possession of flagellated cells. This finding emphasizes the need to determine the cellular mechanisms of these attributes. Our results support the hypothesis that *Haematococcus* is capable of sensing and responding to chemical cues from *PS*, suggesting a two-way communication between both species is present.

### 6.6.Acknowledgements

The authors are indebted to Sofie D'Hondt for obtaining sequences and the Belgian Coordinated Collection of Micro-organisms (http://bccm.belspo.be) for cryopreserving and providing strains from this study (accession numbers in Table A.1, Appendix). This work was supported by a Ph.D. grant (contract number: 121449) from the Agency for Innovation by Science and

Technology in Flanders (IWT-Flanders). MS and CMMG acknowledge the Genomia fund (project: AEID).

## 6.7. Supplementary Data

***Table S1.*** *Infection pattern of Paraphysoderma sedebokerense parasite strains, on the Haematococcus host strains. Susceptibility to parasite attack is indicated by three susceptibility classes ranging from low (1) to high (2) susceptibility and a resistant class (0). Strains in bold were used for the infectivity assay.ND Not determined.*

| Species | Strain number | Substrate type | Location | Geographical Coordinates | Collected by | Date | BOLD number | Genbank accession (ITS) | Genbank accession (*rbc* L) | Infected |
|---|---|---|---|---|---|---|---|---|---|---|
| *H. pluvialis* | **CCAP 34/7** | Freshwater | Ostpicken Island, Tvärminne, Finland | 59°50'35.4"N, 23°14'38.5"E | Droop M.R. | 1953 | HAEMA020-15 | KR914712 | FJ438476 | 2 |
| *H. pluvialis* | **SAG 34-1d = CCAP 34/1D** | Puddle in botanical garden | University of Basel, Zwitserland | 47°33'33.5"N, 7°34'56.9"E | Vischer W. | 1923 | HAEMA027-15 | KR914705 | ND | 2 |
| *H. pluvialis* | **NIES 144** | Freshwater, lake water | Sapporo Hokkaido, Japan | 43°3'43.5"N, 141°21'15.7"E | Ichimura T. | 16 Jul. 1964 | HAEMA023-15 | KR914709 | AB084336 | 1 |
| *H. pluvialis* | **SCCAP K-0084** | Small rock pool | Island of Trutbådan, Sweden | 58°42'0.0"N, 17°16'0.0"E | Christensen T. | 1950 | HAEMA021-15 | KR914711 | ND | 1 |
| *H. pluvialis* | **SAG 192.80** | Bog pool | Bruchberg/Harz Mts, Germany | 51°46'60.0"N, 10°31'0.0"E | Koch W. | 1959 | HAEMA022-15 | KR914710 | ND | 1 |
| *H. pluvialis* | **CCAP 34/12** | Freshwater | Ottowa, Kansas, USA | 38°36'56.8"N, 95°16'7.1"W | Ott F.D. | 1977 | HAEMA024-15 | KR914708 | ND | 2 |
| *H. pluvialis* | **SAG 49.94 = CCAP34/13** | Freshwater, birdbath | Ferrum, Virginia, USA | 36°55'22.5"N, 80°0'48.1"W | Ott F.D. | 1959 | HAEMA025-15 | KR914707 | ND | 2 |
| *H. pluvialis* | **CCAP 34/14** | Freshwater, cement urn | Cattonsville, Maryland, USA | 39°16'19.4"N, 76°43'54.9"W | Ott F.D. | 1989 | HAEMA026-15 | KR914706 | ND | 2 |
| *H. pluvialis* | **C1 strain = SCCAP K-0084** | Small rock pool | Island of Trutbådan, Sweden | 58°42'0.0"N, 17°16'0.0"E | Christensen T. | 1950 | HAEMA021-15 | KR914711 | ND | 2 |
| *H. pluvialis* | BE01_19 | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | ND | ND | ND | 2 |
| *H. pluvialis* | **BE01_20** | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | HAEMA029-15 | DGGE | ND | 2 |
| *H. pluvialis* | BE01_22 | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | ND | ND | ND | 1 |
| *H. pluvialis* | BE01_23 | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | ND | ND | ND | 2 |
| *H. pluvialis* | **BE01_24** | Dry crust on outdoor table | Ghent, Belgium | 51°01'55.5"N, 03°43'48.4"E | Allewaert C. | 2 Sep. 2012 | HAEMA029-15 | DGGE | ND | 1 |
| *H. pluvialis* | BE02_01 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE02_02 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE02_03 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE02_04 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | **BE02_05** | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | HAEMA030-15 | KR914734 | ND | 2 |
| *H. sp.* | BE02_07 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE02_08 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | **BE02_09** | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | HAEMA031-15 | KR914733 | ND | 2 |
| *H. pluvialis* | BE02_10 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 1 |
| *H. pluvialis* | BE02_11 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE02_14 | Water puddle in aluminum wheelbarrow | Munkzwalm, Belgium | 50°52'40.1"N, 03°43'59.0"E | Delbare D. | 11 Sep.2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE03_01 | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE03_02 | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | HAEMA032-15 | KR914732 | KR914694 | 2 |
| *H. pluvialis* | BE03_04 | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | ND | ND | ND | 2 |
| *H. sp.* | BE03_05 | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | HAEMA033-15 | KR914731 | KR914693 | 1 |
| *H. pluvialis* | **BE03_06** | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | ND | ND | ND | 1 |
| *H. pluvialis* | BE03_07 | Water puddle in concrete depression | Ghent, Belgium | 51°03'12.4"N, 03°42'26.9"E | Allewaert C. | 6 Aug. 2012 | ND | ND | ND | 1 |
| *H. rubicundus* | BE04_02 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_03 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_04 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_06 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | HAEMA041-15 | KR914739 | KR914698 | 2 |

***Table S1.*** Continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *H. rubicundus* | BE04_09 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | HAEMA042-15 | KR914738 | KR914697 | 2 |
| *H. rubens* | BE04_10 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_11 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 1 |
| *H. rubicundus* | BE04_12 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_13 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_14 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_15 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **BE04_16** | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **BE04_17** | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_18 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE04_19 | Water puddle in white porcelain sink | Aaigem, Belgium | 50°52'44.0"N, 03°55'58.9"E | Vyverman W. | 8 Feb. 2013 | ND | ND | ND | 1 |
| *H. rubicundus* | BE05_05 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | ND | ND | ND | 1 |
| *H. rubicundus* | **BE05_06** | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA044-15 | KR914737 | KR914696 | 1 |
| *H. rubicundus* | BE05_08 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | ND | ND | ND | 1 |
| *H. rubens* | **BE05_10** | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | ND | ND | ND | 2 |
| *H. rubens* | BE05_11 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | ND | ND | ND | 2 |
| *H. rubens* | BE05_12 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | ND | ND | ND | 2 |
| *H. rubens* | BE05_15 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | ND | ND | ND | 2 |
| *H. rubens* | BE05_16 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA060-15 | DGGE | ND | 2 |
| *H. rubens* | BE05_17 | Water puddle on white bucket | Ghent, Belgium | 51°01'27.5"N, 03°42'38.8"E | Allewaert C. | 6 May 2012 | HAEMA060-15 | DGGE | ND | 2 |
| *H. pluvialis* | BE07_05 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | ND | ND | ND | 2 |
| *H. pluvialis* | **BE07_07** | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | HAEMA068-15 | DGGE | ND | 2 |
| *H. pluvialis* | BE07_09 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | ND | ND | ND | 2 |
| *H. pluvialis* | BE07_10 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | ND | ND | ND | 1 |
| *H. pluvialis* | BE07_13 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | ND | ND | ND | 1 |
| *H. pluvialis* | BE07_14 | Water puddle on concrete floor | Ghent, Belgium | 51°01'04.3"N, 03°41'38.3"E | Allewaert C. | 25 Oct. 2012 | ND | ND | ND | 1 |
| *H. rubicundus* | BE08_01 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H.sp.* | BE08_02 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE08_03 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **BE08_04** | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA045-15 | KR914742 | KR914700 | 2 |
| *H. rubicundus* | BE08_05 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **BE08_06** | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA045-15 | KR914742 | KR914700 | 1 |
| *H. rubicundus* | BE08_07 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 1 |
| *H. rubicundus* | BE08_09 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 1 |
| *H. rubicundus* | BE08_10 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE08_11 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **BE08_12** | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | HAEMA046-15 | KR914741 | KR914699 | 2 |

***Table S1.*** Continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *H. rubicundus* | BE08_13 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | BE08_14 | Water puddle on green trash bin | Merelbeke, Belgium | 51°00'28.1"N, 03°45'38.5"E | Allewaert C. | 10 Feb. 2013 | ND | ND | ND | 2 |
| *H. sp.* | BE09_01 | Water puddle in a boat on the pond | Zilvermeer, Belgium | 51°13'47.22"N, 5°10'40.18"E | Allewaert C. | 17 Jul. 2013 | ND | ND | ND | 1 |
| *H. sp.* | BE09_02 | Water puddle in a boat on the pond | Zilvermeer, Belgium | 51°13'47.22"N, 5°10'40.18"E | Allewaert C. | 17 Jul. 2013 | ND | ND | ND | 1 |
| *H. sp.* | BE09_03 | Water puddle in a boat on the pond | Zilvermeer, Belgium | 51°13'47.22"N, 5°10'40.18"E | Allewaert C. | 17 Jul. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **BE09_04** | Water puddle in a boat on the pond | Zilvermeer, Belgium | 51°13'47.22"N, 5°10'40.18"E | Allewaert C. | 17 Jul. 2013 | ND | ND | ND | 2 |
| *H. sp.* | BE09_06 | Water puddle in a boat on the pond | Zilvermeer, Belgium | 51°13'47.22"N, 5°10'40.18"E | Allewaert C. | 17 Jul. 2013 | ND | ND | ND | 2 |
| *H. pluvialis* | **BE09_07** | Water puddle in a boat on the pond | Zilvermeer, Belgium | 51°13'47.22"N, 5°10'40.18"E | Allewaert C. | 17 Jul. 2013 | ND | ND | ND | 1 |
| *H. sp.* | BE09_12 | Water puddle in a boat on the pond | Zilvermeer, Belgium | 51°13'47.22"N, 5°10'40.18"E | Allewaert C. | 17 Jul. 2013 | ND | ND | ND | 1 |
| *H. sp.* | BE10_01 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct 2013 | ND | ND | ND | 2 |
| *H. sp.* | BE10_02 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct 2013 | ND | ND | ND | 1 |
| *H. rubicundus* | BE10_03 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct 2013 | HAEMA053-15 | KR914746 | ND | 1 |
| *H. sp.* | BE10_05 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct. 2013 | ND | ND | ND | 1 |
| *H. pluvialis* | BE10_08 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct. 2013 | ND | DGGE | ND | 1 |
| *H. pluvialis* | **BE10_09** | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct. 2013 | ND | ND | ND | 1 |
| *H. sp.* | BE10_10 | Rain water barrel | Ghent, Belgium | 51°04'29.6"N, 03°43'56.6"E | Vanormelingen P. | 17 Oct. 2013 | ND | ND | ND | 1 |
| *H. sp.* | BE11_03 | Black epiphyton bioreactor | Ghent, Belgium | 51°1'27.54"N, 3°42'38.85"E | Allewaert C. | 13 Jan. 2016 | ND | ND | ND | 1 |
| *H. pluvialis* | **BE11_04** | Black epiphyton bioreactor | Ghent, Belgium | 51°1'27.54"N, 3°42'38.85"E | Allewaert C. | 13 Jan. 2016 | ND | ND | ND | 1 |
| *H. pluvialis* | **BE11_10** | Black epiphyton bioreactor | Ghent, Belgium | 51°1'27.54"N, 3°42'38.85"E | Allewaert C. | 13 Jan. 2016 | ND | ND | ND | 1 |
| *H. sp.* | BE12_01 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 1 |
| *H. sp.* | BE12_03 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 1 |
| *H. pluvialis* | **BE12_04** | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. sp.* | BE12_05 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. sp.* | BE12_08 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. sp.* | BE12_10 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. sp.* | BE12_13 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. sp.* | BE12_16 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 1 |
| *H. sp.* | BE12_18 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. sp.* | BE12_22 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. sp.* | BE12_24 | Water puddle on plastic outdoor chair (white) | Drongen, Belgium | 51°2'21.89"N, 3°38'6, 38"E | Allewaert C. | 16 Jan. 2016 | ND | ND | ND | 2 |
| *H. rubicundus* | **CZ01_06** | Water puddle on white chair | Třeboň, Czech Republic | 49°0'18.3"N, 14°46'23.8"E | Strunecky O. | 12 Febr. 2013 | HAEMA071-15 | DGGE | ND | 2 |
| *H. rubicundus* | **CZ01_08** | Water puddle on white chair | Třeboň, Czech Republic | 49°0'18.3"N, 14°46'23.8"E | Strunecky O. | 12 Febr. 2013 | HAEMA047-15 | KR914751 | KR914703 | 1 |
| *H. pluvialis* | **HU01_01** | Rain water barrel, Ebes Hajdu-Bihar | Eastern Hungary | 47°28'15.27"N, 21°29'25.65"E | Basci I. | May 2012 | ND | ND | ND | 2 |
| *H. pluvialis* | HU01_02 | Rain water barrel, Ebes Hajdu-Bihar | Eastern Hungary | 47°28'15.27"N, 21°29'25.65"E | Basci I. | May 2012 | ND | ND | ND | 2 |
| *H. pluvialis* | **HU01_03** | Rain water barrel, Ebes Hajdu-Bihar | Eastern Hungary | 47°28'15.27"N, 21°29'25.65"E | Basci I. | May 2012 | ND | DGGE | ND | 2 |
| *H. pluvialis* | HU01_04 | Rain water barrel, Ebes Hajdu-Bihar | Eastern Hungary | 47°28'15.27"N, 21°29'25.65"E | Basci I. | May 2012 | ND | DGGE | ND | 2 |

***Table S1.*** Continued

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| *H. pluvialis* | HU01_05 | Rain water barrel, Ebes Hajdu-Bihar | Eastern Hungary | 47°28'15.27"N, 21°29'25.65"E | Basci I. | May 2012 | ND | ND | ND | 1 |
| *H. pluvialis* | IT01_03 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | HAEMA051-15 | DGGE | ND | 1 |
| *H. rubicundus* | **IT01_06** | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | HAEMA051-15 | DGGE | ND | 1 |
| *H. pluvialis* | IT01_07 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | ND | ND | ND | 1 |
| *H. pluvialis* | **IT01_09** | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | ND | DGGE | ND | 2 |
| *H. rubicundus* | IT01_18 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | ND | ND | ND | 2 |
| *H. pluvialis* | IT01_20 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | ND | ND | ND | 1 |
| *H. pluvialis* | IT01_21 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | ND | ND | ND | 1 |
| *H. pluvialis* | IT01_23 | Small water puddle in rock depression | Province of Pescara, Italy | 42°09'23.4"N, 14°00'11.4"E | Casteleyn G. | 1 Apr. 2013 | ND | ND | ND | 1 |
| *H. pluvialis* | **CH02_02 = CCAP34/1D** | Botanic garden, Univ. Basel | Zwitserland | 47°33'29.79"N, 7°35'0.36"E | Vischer | 1923 | ND | ND | ND | 2 |
| *H. pluvialis* | **CH02_03 = CCAP34/1D** | Botanic garden, Univ. Basel | Zwitserland | 47°33'29.79"N, 7°35'0.36"E | Vischer | 1923 | ND | ND | ND | 2 |
| *H. pluvialis* | CH02_04 = CCAP34/1D | Botanic garden, Univ. Basel | Zwitserland | 47°33'29.79"N, 7°35'0.36"E | Vischer | 1923 | ND | ND | ND | 2 |
| *H. pluvialis* | CH02_05 = CCAP34/1D | Botanic garden, Univ. Basel | Zwitserland | 47°33'29.79"N, 7°35'0.36"E | Vischer | 1923 | ND | DGGE | ND | 2 |
| *H. pluvialis* | CH02_08 = CCAP34/1D | Botanic garden, Univ. Basel | Zwitserland | 47°33'29.79"N, 7°35'0.36"E | Vischer | 1923 | ND | DGGE | ND | 2 |
| *H. rubicundus* | NL01_01 | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar 2013 | ND | ND | ND | 2 |
| *H. sp.* | NL01_03 | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **NL01_04** | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar 2013 | HAEMA049-15 | KR914749 | KR914701 | 1 |
| *H. rubicundus* | NL01_05 | Grey rain water barrel | Wageningen, The Netherlands | 51°59'15.5"N, 05°40'14.3"E | Wiezer S. | 6 Mar 2013 | HAEMA050-15 | KR914748 | ND | 1 |
| *H. sp.* | NL02_06 | Water puddle on white bioreactor | Wageningen, The Netherlands | 51°59'46.4"N, 05°39'29.4"E | Allewaert C. | 6 Jun. 2013 | ND | ND | ND | 0 |
| *H. rubicundus* | **NL02_08** | Water puddle on white bioreactor | Wageningen, The Netherlands | 51°59'46.4"N, 05°39'29.4"E | Allewaert C. | 6 Jun. 2013 | HAEMA052-15 | KR914747 | ND | 2 |
| *H. sp.* | **NL02_10** | Water puddle on white bioreactor | Wageningen, The Netherlands | 51°59'46.4"N, 05°39'29.4"E | Allewaert C. | 6 Jun. 2013 | HAEMA052-15 | KR914747 | ND | 2 |
| *H. sp.* | NL03_01 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 0 |
| *H. sp.* | NL03_02 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 1 |
| *H. sp.* | NL03_03 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 1 |
| *H. sp.* | NL03_04 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 1 |
| *H. sp.* | NL03_05 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 1 |
| *H. pluvialis* | **NL03_06** | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | HAEMA066-15 | KR914727 | ND | 1 |
| *H. pluvialis* | **NL03_07** | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | HAEMA066-15 | KR914727 | ND | 1 |
| *H. sp.* | NL03_08 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 1 |
| *H. sp.* | NL03_09 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 1 |
| *H. sp.* | NL03_10 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 1 |
| *H. sp.* | NL03_12 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 0 |
| *H. sp.* | NL03_16 | Water puddle on top of a container | Vlissingen, The Netherlands | 51°26'59.49"N, 3°35'19.25"E | Allewaert C. | 25 Jun. 2013 | ND | ND | ND | 2 |
| *H. rubicundus* | **SE02_07** | Rocky shoreline | Lake Erken, Sweden | 59°83'99.64"N, 18°63'87.53"E | Kahlert M. | 14 Oct 2013 | ND | ND | ND | 1 |
| *H. pluvialis* | **SE02_11** | Rocky shoreline | Lake Erken, Sweden | 59°83'99.64"N, 18°63'87.53"E | Kahlert M. | 14 Oct 2013 | ND | ND | ND | 1 |
| *Ettlia carotinosa* | 213/4, SAG 213-4; UTEX 113 | Freshwater | former Czechoslovakia | - | Mainx | - | GU292342 (SSU) | ND | ND | 2 |

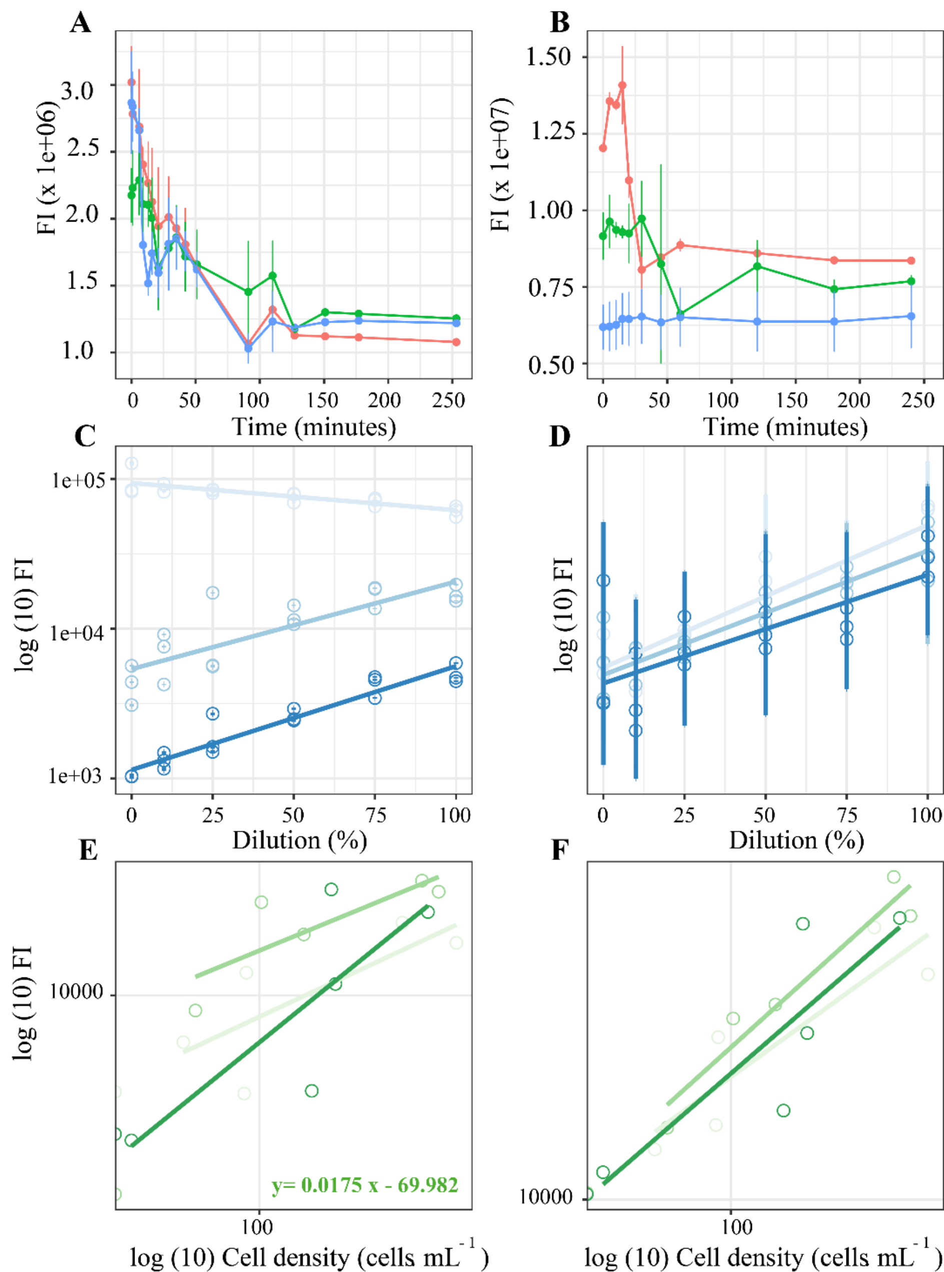



***Figure S1. A-B**. Stability of WGA-Fluorescein signal. Fluorescence intensity (FI) in function of time (minutes) upon addition of 5 µg mL$^{-1}$ (**A.**) and 10 µg mL$^{-1}$ (**B.**) of WGA-Fluorescein to cultures containing different concentrations of Haematococcus C1-PS1 (100 %= blue, 50 %= green and 0 %= red). **C-D.** Effect of WGA-Fluorescein washing on fluorescence signal. FI in function of dilution factor (100, 75, 50, 25, 12.5, 0 %) of PS1 serially diluted with Haematococcus C1 strain (total culture volume 1 mL), WGA-fluorescein stained cultures (**C.**) and non-stained cultures – or noise signal (**D.**). Measurements were performed 80 minutes after the addition of 5 µg mL$^{-1}$ WGA-Fluorescein, prior washing (light blue), after replacement of 90 % of the medium with fresh medium (medium blue) and after repeating the latter (dark blue). The three points correspond to three replicates. Error bars correspond to standard deviation on the measurement (n= 25). X and Y are log scales. **E-F.** Calibration series of FI with density of infected cells. FI in function of density of infected cells (cells mL$^{-1}$) measured after one wash step (**E.**) and two wash steps (**F.**) for three replicates (each one color). The density of infected and non-infected cells were quantified under a fluorescent microscope from an aliquot of centrifuged culture (10 µL)*

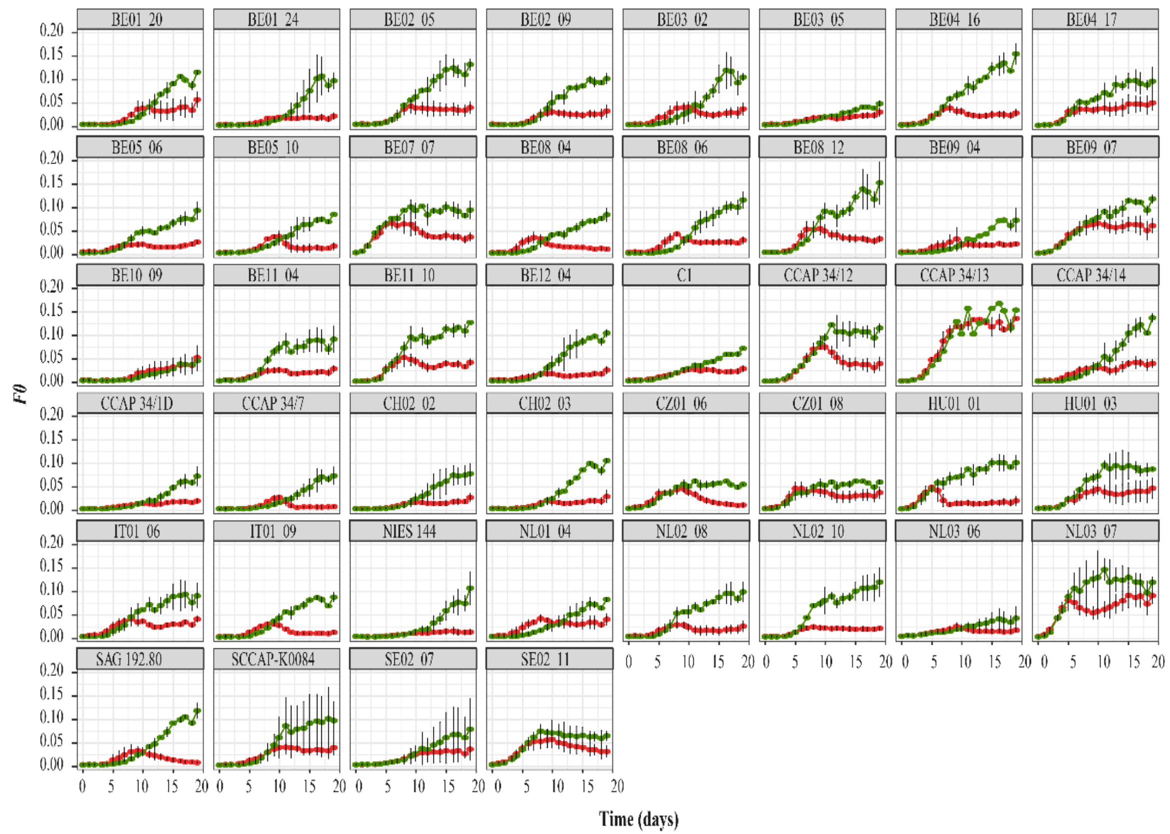



***Figure S2.*** *Growth curves based on $F_0$ in function of time (days) of infected (red) and control (green) cultures. Each box represents a strain. Error bars represent the SD on the measurement for the infected (n= 4) and control (n= 2) cultures.*

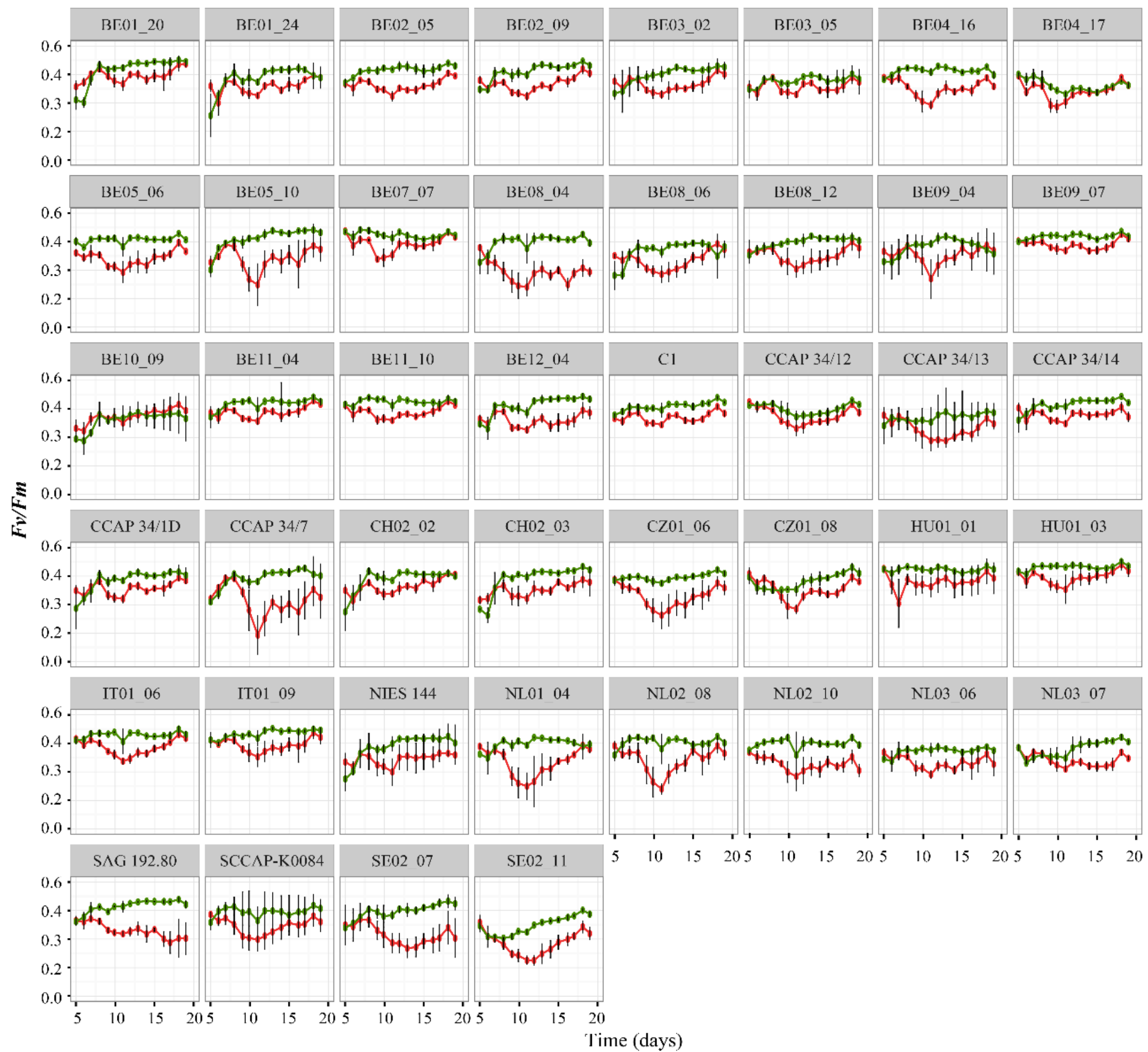



***Figure S3.*** *Evolution of fluorescence yield ($F_v/F_m$) in function of time (days) of infected (red) and control (green) cultures. Each box represents a strain. Error bars represent the SD on the measurement for the infected (n= 4) and control (n= 2) cultures. (nb day 0-5 are not shown)*

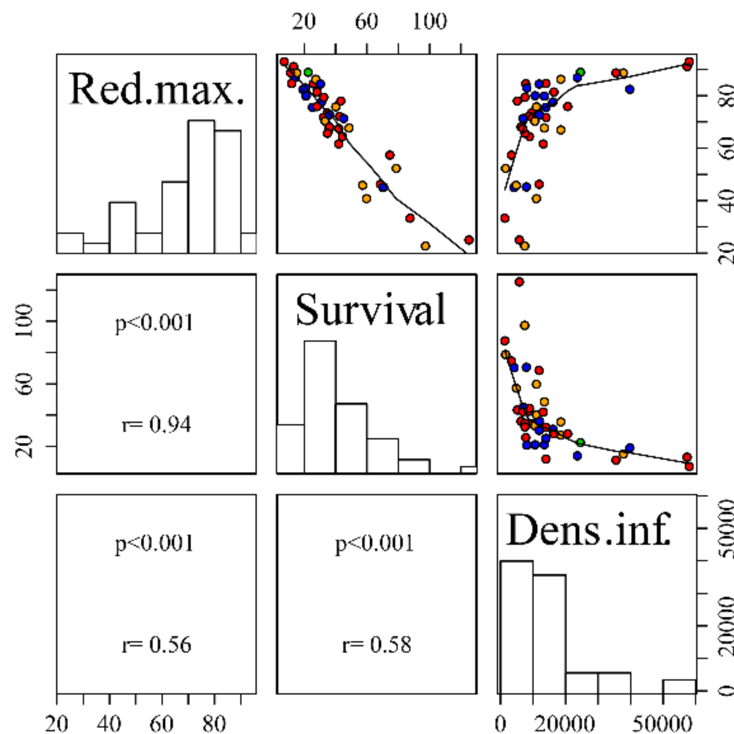



***Figure S4.*** *Correlation matrix between all pairs of infectivity proxies. In the left panel, the p-values and corresponding Pearson correlation factor are given. In the right diagonal the points, representing average value per strain (each different color representing a different species) and in the diagonal, the histograms, representing the distribution of values for each proxy are shown.*

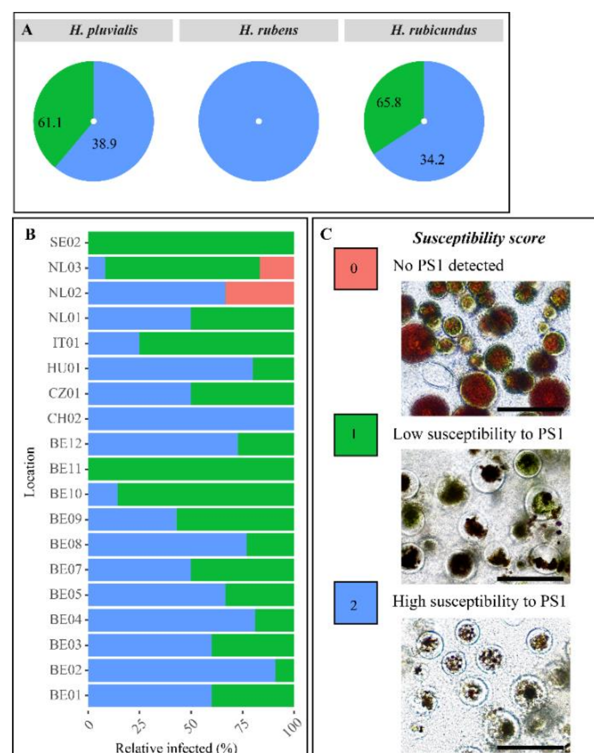



***Figure S5. A.*** *Pie charts of the frequency of scored for susceptibility to PS1 for the three species (H. sp. excluded).* ***B.*** *Frequency of scores for susceptibility to PS1 for the 19 locations as listed in Table S1.* ***C****. Susceptibility scores as graded by pictures. Scale bar= 100 µm*

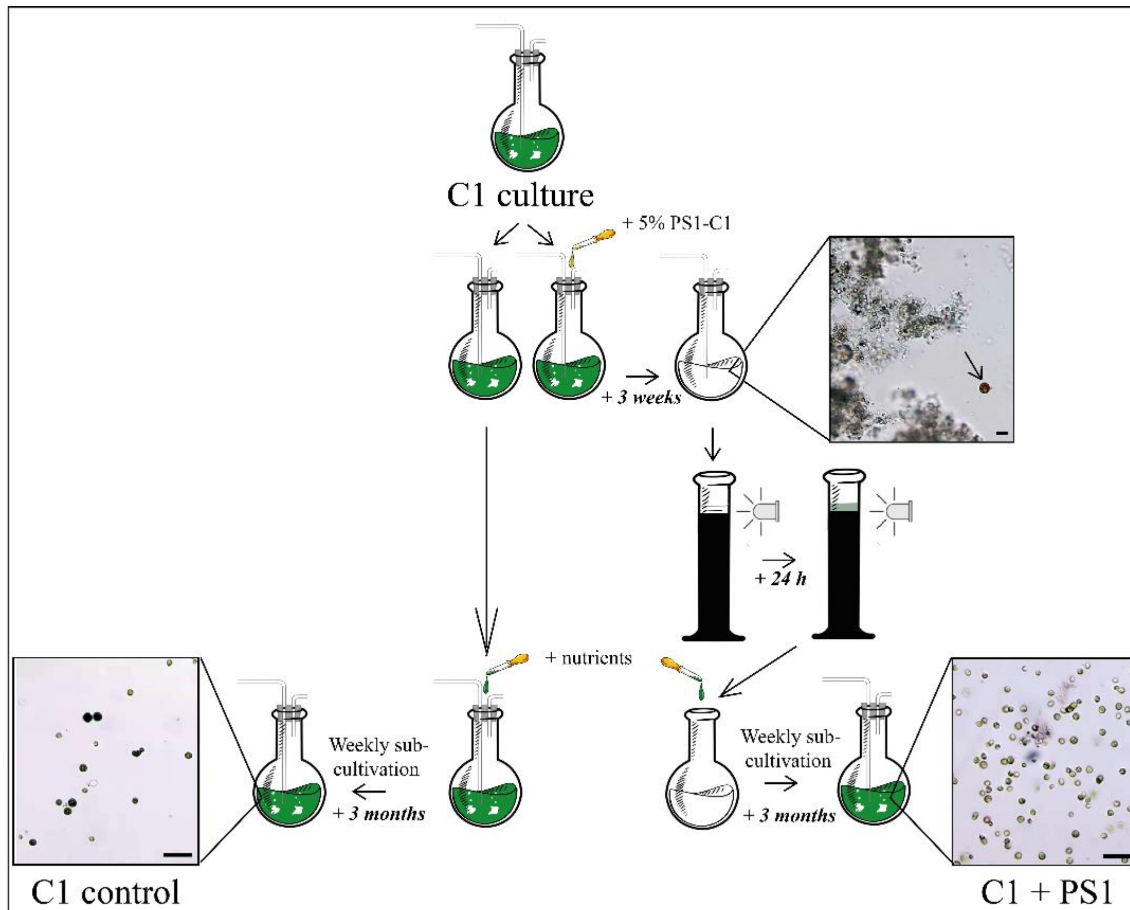
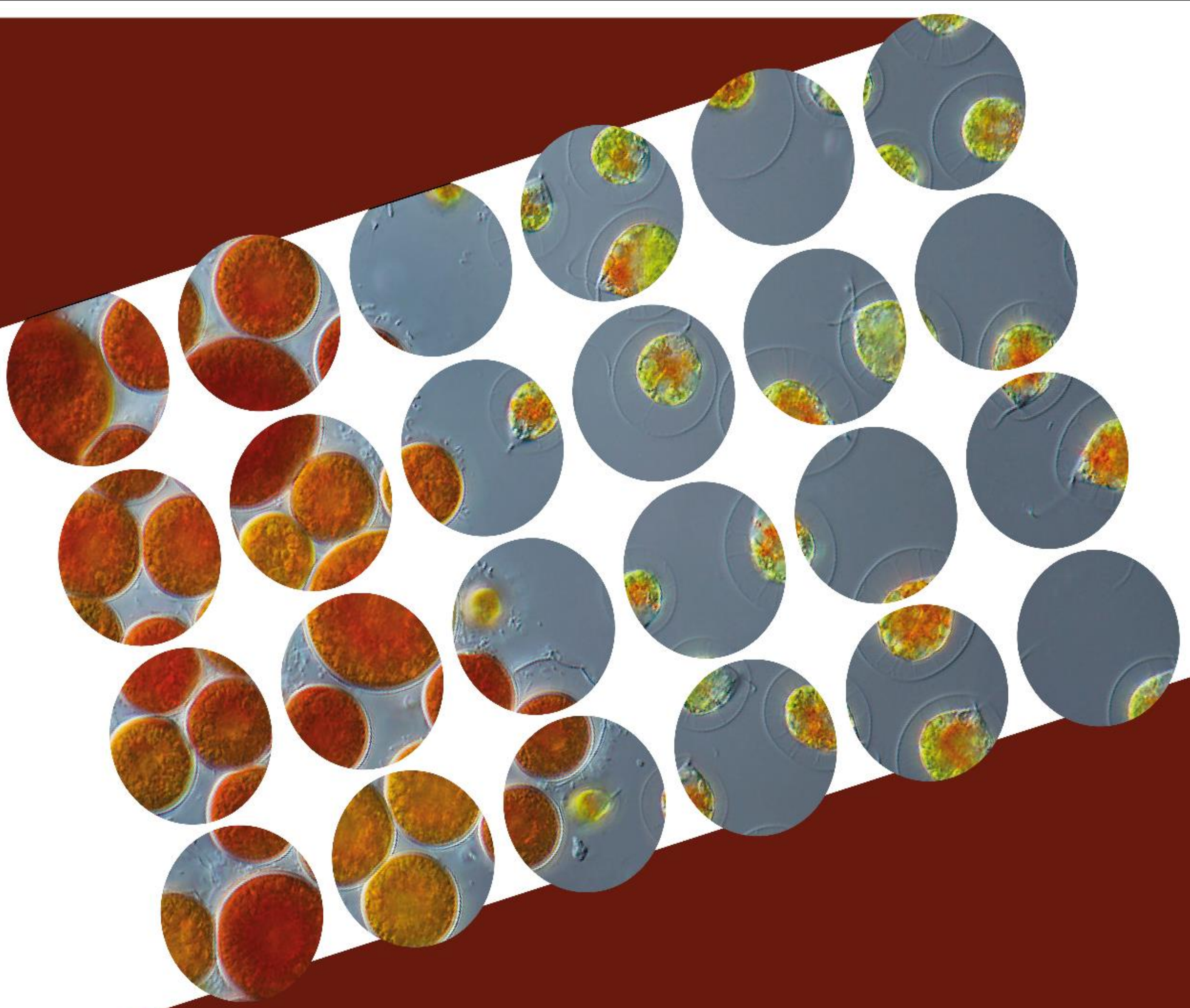



***Figure S6.*** *Schematic overview of the long term selection experiment with the procedure applied for the control on the left and the selected culture on the right. Scale bars correspond to 10 µm (figure above) and 100 µm (figures below). C1 = Haematococcus strain, PS1= Paraphysoderma sedebokerense strain from this study.*



# CHAPTER 7:
# General discussion and future perspectives

## 7.1.General discussion

The unicellular green microalgae *Haematococcus pluviali*s is able to produce up to 5 % astaxanthin of its dry weight. Therefore, the species is widely implemented as astaxanthin producer in the microalga biotechnology sector. However, there are many challenges for the development of large scale production of biomass and astaxanthin production from *H. pluvialis*. Unlike many other green algal taxa (e.g. *Chlorella, Chlamydomonas*), *H. pluvialis* is a slow grower, with a complex life cycle resulting in significant changes in morphology throughout production. Moreover, the species is highly vulnerable to contamination. In virtue of better understanding, and ultimately overcoming these constraints, this thesis explores the genotypic and phenotypic variation among clonal isolates of *Haematococcus* from various European source locations and assesses to which extent collections of natural strains could serve as a resource for strain selection and improvement. Furthermore, its unique morphogenetic response to environmental stress, the encystment process and the associated carotenoid synthesis, present important features of photosynthesis, motility and morphogenesis which can serve to better understand the functions of carotenoids in other microorganisms (Kobayashi *et al.*, 1997a). This last chapter will present a synthesis of the main findings of this thesis and will identify promising perspectives for future research.

### 7.1.1. Practical considerations for experimental manipulation of *Haematococcus* strains

Several of the physiological and biochemical challenges associated with the culture of *Haematococcus* were outlined in Chapter 1 & 2. These challenges, along with the great deal of experimental work on a large number of strains perfomed, required systematic and well controlled conditions for the manipulation of cultures and experimental design. Therefore, the first part of this discussion comprises some practical considerations for the experimental manipulation of *H. pluvialis*.

Given their different requirements, a two-step cultivation strategy, for which the biomass and astaxanthin accumulation stages were separated from one another was adopted for all experiments. To overcome the issue of having different morphotypes in one culture at the same time, strain **acclimatization** prior performing experiments was imperative. All strains were acclimatized under a constant set of conditions, followed by two re-inoculations every four days. The purpose of this acclimatization period was not only to assure homogenous cultures of exponential flagellated cells at the start of each experiment, but also served as a way to minimize the effect of history from previous culture conditions, necessary for comparative

physiological studies of strains from difference sources (Lakeman *et al.*, 2009)(Chapter 3 & 4 & 5).

The heterogeneity in approaches and methodologies reflected in the currently available literature does not allow to select optimal conditions promoting growth and astaxanthin production of *H. pluvialis*. Through the high variability in responses to temperature, nutrient and irradiance (Chapter 3 & 4 & 5) in different strains, together with the high variation in traits under common garden conditions (Chapter 4) we have validated the difficulty of establishing single optimal experimental conditions for promoting growth and carotenoid accumulation which are effective for all strains. Despite the fact that our experiments were all classical one-factor–at-a-time (OFAT) approaches (Chapter 5), where interactive effects of different environmental factors were not examined, the following standard **experimental ranges** were identified allowing successful biomass and astaxanthin production for all strains under assessment. For growth and biomass production of non-shaded cultures, “safe” irradiances (under which little photo inhibition occurred) ranged between 15 and 52 µmol photons $m^{-2}$ $s^{-1}$ (Chapter 5) and optimal growth temperatures between 17 and 23 °C (Chapter 3). While a systematic search for optimal stress factors promoting astaxanthin accumulation was beyond the scope of this work, amongst the nutrient stressors tested, phosphate starvation combined with increased irradiance resulted in highest astaxanthin content for four out of six strains without significant cellular damage as can be the case when nitrogen is omitted (Chapter 4)(Boussiba *et al.*, 1999).

The **evolution of cell types** (palmelloids and flagellates) versus time during the biomass accumulation phase was quantified for the first time (Chapter 4) and a **trigger** for increased transformation to palmelloid cells was identified (Chapter 5). Our findings support that the transition from flagellated cells to palmelloids was highly strain specific and a consequence of culture age (and subsequent probable decrease in nutrient composition), since cultures generally reached a maximum relative percentage of palmelloid cells when in stationary phase (Chapter 4). Moreover, in steady state cultures the relative percentages palmelloids increased together with the available irradiance (Chapter 5).

Finally, the bi-phasic strategy adopted in all experiments was a relatively long process from the beginning to the end (± 30 days) compared to other common fast growing taxa (e.g. *Chlorella, Nannochloropsis*) since the algae must first go through a stage of cell division, before entering a second stage where growth and motility is halted but astaxanthin is accumulated. Operating this long multistage culturing process, increases the risk of contamination. **Frequent**

**contaminations** were faced upon manipulation of *Haematococcus* strains, with high bacterial concentrations and fungi being the most common contaminants. Bacterial contaminations were reduced either through subsequent re-inoculations, serial centrifugation steps or in the worst case through the treatment with antibiotics overnight (Imipenem, up to 500 µg $mL^{-1}$). Treatment with Imipenem was also applied to strains prior to cryopreservation to guarantee a higher recovery post cryopreservation (cf. section 7.1.3.2.). Although the fungicide Carbendazim (Mahan *et al.*, 2005) showed promising results in the removal of fungal contaminants, the most secure and efficient - yet time consuming- way to get rid of fungi was to return to stock collections, which were preserved at 6 °C during the period of this thesis.

### 7.1.2. *Haematococcus pluvialis:* a complex of species

To date a huge body of literature reports on candidate *H. pluvialis* isolates with interesting properties, high productivities and industrial potential (Chapter 2 & 3). In most work, single strains are used of which the preference over another is never argumented. In the best case, these strains are standard laboratory strains obtained from common repositories, or culture collections providing the invaluable service of documenting them. In the worst case, strains are not linked to any formal species names, morphological description or DNA sequence and may therefore be falsely identified, resulting in potential misperceptions and loss of information in publications. The potential for exploration and eventual exploitation of additional *Haematococcus* strains has been limited by the lack of knowledge on diversity within this genus. Documenting this diversity, as was recently done for several model green algal taxa with biotechnology potential, including *Chlorella* (Krienitz *et al.*, 2015) and *Chlamydomonas* (Flowers *et al.*, 2015; Gallaher *et al.,* 2015) is essential for several reasons. Firstly, it may promote the detection of unsuspected biological functions in green protists in general (Krienitz *et al.*, 2015). Secondly, it may aid in optimization of biotechnological applications, specifically through the selection of future candidate taxa and strains for research on the genetics of – specifically - astaxanthin production (Georgianna & Mayfield, 2012; Gómez *et al.*, 2016). Finally, from a more fundamental perspective, knowledge on the diversity within these taxa may be crucial in advancing our understanding of green algal plant lineage relationships and ultimately their evolutionary background (Buchheim *et al.*, 2013; Krienitz *et al.*, 2015).

As exemplified in Chapter 2, a species concept that associates species with **entities in nature** while at the same time providing appropriate **operational criteria** for delimiting species does not exist at present. Alternatively, De Queiroz (2007) conceptualized species as "metapopulation-level evolutionary lineages, which because of their persistence may have

acquired different properties (morphological, ecological etc..)" (Chapter 2). From an practical aspect, De Queiroz's operational criteria encompass all lines of evidence or so-called "secondary properties" of species relevant to assessing the lineage divergence.

According to De Queiroz (2007), species delimitation or the assessment of species limits, comes down to the identification of separately evolving metapopulation lineages which may be further complemented with congruent lines of evidence. Such integrative approaches require the combination of carefully chosen phylogenetic methods, morphological, life cycle physiological and ecological performance data preferably under different conditions (phenotypic plasticity) and if possible ITS2/CBCs presence as unique possibility to predict the mating ability of sexual organisms (Darienko *et al.*, 2015). These kind of modern integrative algal taxonomy approaches involve multi-faceted research, knowledge on a large body of taxonomy and systematic data and require training in different methodologies, including e.g. culturing, molecular phylogenetic methods, microscopy (Pröschold *et al.*, 2001; Nakada *et al.*, 2008a; Nakada *et al.*, 2008b; Demchenko *et al.*, 2012). In line with De Quieroz, in this work, several lines of evidence or "secondary properties" including molecular, morphological, and physiological were integrated in support of the recognition of different species within the green algal genus *Haematococcus*.

Firstly, rDNA internal transcribed species (ITS) sequences of *Haematococcus* strains sampled across 15 different European locations, complemented with strains of South American origin were compared (Chapter 3). The phylogeny resolved **six separate lineages**, supported by statistical parsimony network and General Mixed Yule Coalescent (GMYC) analyses:. Three of these lineages were represented by our European strains including ***H. rubens*** and ***H. rubicundus***, which were formally described as new species, along with the well-established ***H. pluvialis***. Additional species diversity was found in *Haematococcus* yet their taxonomy requires to be dealt with in the future. The state of the art procedure is to base species species delimitations on multi-locus molecular data, using markers from different cell compartments (Leliaert *et al.*, 2014) or if possible to infer phylogenies based on entire organellar genomes (Krienitz *et al.*, 2015). Therfore, our ITS phylogeny was complemented with a genetic study of the *rbc*L. The *rbc*L phylogeny showed congruent patterns, but proved less variable than the ITS gene, a commonly reported issue in green algae (Kress *et al.*, 2009; Kazi *et al.*, 2013). The use of the ITS is therefore recommended as a suitable routine marker for strain identification within *Haematococcus*, at least until a candidate marker with similar resolution, convenient for routine

identification, is available. Besides varying at the species level, the ITS marker showed some variation below the species level.

Secondly, the presence of **compensatory base changes (CBCs)** in ITS rDNA secondary structures was assessed. Identification of CBCs in ITS2 secondary structures is commonly done in protists to support species delineations since their presence is strongly correlated with the biological species concept (Wolf *et al.*, 2013) as well as reproductive isolation (Coleman *et al.*, 1998; Coleman, 2000). We found one CBC in the ITS2, adding support to the separation of *H. rubicundus* from the *H. pluvialis* - *H. rubens* complex (Chapter 3), but the presence of CBCs in *Haematococcus* was rather limited. Important to note is that the absence of CBCs between species does not necessarily imply that species belong to the same biological species (Müller *et al.*, 2007; Alverson, 2008; Caisová *et al.*, 2011; Caisová *et al.*, 2013).

Thirdly, given the cryptic nature of all strains under assessment, no obvious **morphological** characters allowed to support delineation of the three genetic lineages. Overall, a high degree of intra- and interspecific overlap was found amongst strains in the chosen morphological traits. Nevertheless, modest separation between *H. rubicundus* and the *H. pluvialis* - *H. rubens* complex was possible based on the size of flagellated cells and aplanospores (Chapter 3 & 4), the aspect of cytoplasmic strains (Chapter 3), the shape of the protoplast apex (Chapter 3) and the rate at which palmelloid cells were formed (Chapter 4).

Finally, the phylogenetic framework set in Chapter 3 served as basis for **comparative physiological studies** throughout this thesis. Despite a high physiological versatility, given that all traits examined exhibited a considerable degree of intraspecific variation and overlap, some consistent species differences were found, adding evidence to the separation of *H. pluvialis* and *H. rubicundus,* in an informal sense. *H. rubicundus* strains had a tendency to grow faster (Chapter 4 & 5), while accumulating less DW during the stationary phase than *H. pluvialis* strains under favorable conditions (Chapter 4). *H. rubicundus* further accumulated less biomass when stressed (Chapter 4). No distinct species differences were found in temperature and irradiance optima, minima, or maxima, yet this could be attributable to the limited number of strains included for comparison and their similar geographic background (Chapter 3 & 5). In the same line, no species differences were found in vulnerability to the pathogen *P. sedebokerense* (Chapter 6).

Unfortunately, due to the still considerable uncertainty on the formation of gametes in *Haematococcus,* let alone on the process of conjugation, there is currently no repetitive procedure for gamete induction in *Haematococcus*. Sexual reproduction was not observed in experimental cultivation of our newly isolated accessions, consequently this line of evidence cannot be applied to the genus, at least not until its sexual life cycle is fully unraveled.

In conclusion, the *Haematococcus* genus remains highly promising for future species discovery. Extensive sampling efforts together with molecular phylogenies and taxonomic designations will allow to enlarge current *Haematococcus* collections. A profound knowledge on the existing diversity within this species may serve to reinterpret physiological and other experimental studies, also in applied research by linking differences in physiological and biochemical properties with genomic variation (Müller *et al.*, 2005).

### 7.1.3. Variation below the species level

In the above section, we have shown how different approaches support the delineation of species within *Haematococcus* based on findings throughout the different chapters of this work. Variation is commonly observed in a range of physiological, morphological, biochemical traits between species and subspecies complexes (Wood *et al*., 1992; Lakeman *et al*., 2009), but variation may also occur below the species level. One of Charles Darwin's greatest insights was recognizing that conspecific individuals differ in many traits, some more obvious than the other (Darwin, 1869). **Intraspecific trait variability** or **"individual variability"** is defined as the overall variability of trait values expressed by individuals/strains within a species (Albert *et al.*, 2011). Anywhere researchers have looked, they have found intraspecific variability in a myriad of traits such as, growth rates, cell size, photosynthesis rates, resource use, toxicity and temperature tolerance (Lundholm *et al.*, 2006; Wilson *et al.*, 2006; Zhang *et al.*, 2014; Harvey *et al.*, 2015; John *et al.*, 2015; Malcom *et al.*, 2015).

Increasing evidence of cases where intraspecific variation exceeded interspecific variation (Sayegh & Montagnes, 2011), has led to the thought that within-species diversity can be equally or even more important than between species diversity (Grettenberger & Tooker, 2015; Huber *et al.*, 2016) and that "species", through their classical taxonomic designation, may not be the ecological and evolutionary units that matter most. The observation of high, intraspecific variability in species traits suggest that approaches that try to distinguish species based on single characteristics (e.g. *H. pluvialis* are slow growers) need to first quantify the degree of within-species variability and determine if variation among strains of one species is sufficiently small

to permit distinction among species and higher taxonomic levels (Rynearson & Menden-Deuer, 2016). In this regard, shifting the level of research from that of a species to that of an individual/strain may bring new opportunities and challenges with respect to phenotyping (Rynearson & Menden-Deuer, 2016).

#### 7.1.3.1. Intraspecific trait variation in *Haematococcus*

##### 7.1.3.1.1. Genetically determined variation

Throughout this thesis, common garden experiments were recurrently employed as tool for comparative physiological studies between *Haematococcus* strains (Chapter 3 & 4 & 5 & 6). For this purpose, all strains were acclimatized, manipulated, cultivated, harvested and analyzed using strictly identical conditions. Such experiments enable to unravel the genetic basis of complex phenotypes across various strains and/or populations without the confounding effect of the environment (de Villemereuil *et al.*, 2016). Common garden experiments can also be used to study genotype-by-environment (G x E) interactions, by implementing the same design under different conditions, as was done in Chapters 3 & 4 & 5.

A tremendous reservoir of between-strain variability in morphological, physiological, and biochemical traits in *H. pluvialis* and *H. rubicundus* was found throughout this thesis (Chapters 3 & 4 & 5 & 6). Firstly, strains exhibited high **morphological plasticity** in several traits affecting the vegetative cells, including the number of pyrenoids, the shape of the protoplast apex, the presence and characteristics of cytoplasmic strains, the length and width of the cells and protoplast (Chapter 3). Also the aplanospore biovolume differed significantly from one strain to the other upon stress exposure (Chapter 4). **Physiological variation** in growth rate, biomass accumulation, capacity to form palmelloid cells was also highly significant between strains of both species grown and stressed under common garden conditions (Chapter 4). Combined, the effect of this trait variation was so high that it resulted in an up to fifteen fold variation in astaxanthin productivity between the poorest and the best astaxanthin producer (Chapter 4). Based on three different proxies for infectivity, strains further exhibited significant **different levels of susceptibility to the pathogen** *P. sedebokerense* (Chapter 6). Altogether, all traits assessed showed a high degree of genetically based phenotypic complexity which unequivocally imply that both the biotic (*Paraphysoderma*- *Haematococcus* interactions) and abiotic environment (temperature, irradiance, nutrients) play a role in shaping the diversity in *Haematococcus* genotypes.

The high broad sense heritability estimates of several traits related to astaxanthin productivity added support to the genetic determinism of variation amongst strains (Chapter 4). Another pattern emerging from the analysis of phenotypic variation showed that many traits co-varied, suggesting a common architecture. Astaxanthin productivity was largely determined by post stress traits such as the dry weight and astaxanthin accumulated as well as the aplanospore biovolume increases. The study of trait associations is important in this context, since they can tell more on an organisms ability to coordinate different cellular functions rather than functioning independently. Trait coordination may result in an increased performance and fitness in response to varying environmental conditions (Shurin *et al.*, 2013). Traits can also be negatively correlated in which case they are called trade-offs. Trade-offs arise from mechanistic constraints based on principles of cell biology and are largely embodied in available literature, e.g.. high growth rates are correlated with low lipid production (Smith *et al.*, 2010), unimodal relationships between cell size and growth rate (Maranon *et al.*, 2013). Trade-offs may further occur in competitive ability for different nutrients and light (Stomp *et al.*, 2004; Edwards *et al.*, 2011). Due to the absence of trade-offs in the investigated pre- and post-stress, we concluded that astaxanthin productivity was not limited by any of the traits examined. Conversely, significant positive trait correlations were found between astaxanthin productivity and several traits measured during the astaxanthin accumulation phase, including dry weight accumulation, astaxanthin accumulation and aplanospore biovolume increases. Our results further emphasized the importance of considering the right taxonomic level in the study of trait correlations. In fact, to avoid misperceptions of the true correlations, the study of trait associations should preferably be carried out at species level, since correlations found in individual species (*H. pluvialis* & *H. rubicundus*) may considerably differ from those found at the genus level (Chapter 4).

#### 7.1.3.1.2. G x E interactions

The replication of common garden experiments, though only on a limited number of *Haematococcus* genotypes, under a range of different temperatures (Chapter 3), nutrient limiting conditions (Chapter 4) and irradiances (Chapter 5), provided some preliminary insights into *Haematococcus* genotype - environment (G x E) interactions. Generally speaking, the different genotypes responded differently to the experimental conditions as can be seen from the selection of **reaction norms,** or, the response functions characteristic of different *Haematococcus* genotypes (Fig. 1). These reaction norms show in which way the environmental distribution is transformed into the phenotypic distribution and are differentiated for all

*Haematococcus* strains. It could be argued that the observed variation exaggerates the overall diversity within *Haematococcus*, since only a limited amount of strains were compared, which were deliberately chosen from distinctly different environments and had different culture backgrounds (culture collection versus wild strains).

Strains of *H. pluvialis* and *H. rubicundus* responded differently in their optimal, maximal temperature growth rate when exposed to a temperature gradient from 12 - 36 °C (Chapter 3). Similarly, high intraspecific variation was found in astaxanthin accumulation, aplanospore size, DW accumulated in response to four different nutrient limiting stress conditions/combinations (Chapter 4). Likewise, when exposed to an irradiance gradient between 2 - 222 µmol photons $m^{-2}$ $s^{-1}$, strains significantly differed in photosynthetic efficiency, cell density, biovolume, cell type and pigment composition (Chapter 5). Though many research efforts remain to be made in this domain, our results indicate that each strain is responding differently to the environmental cues imposed and therefore, highlighting (a) genotype(s) which performs best across all environmental ranges is theoretically impossible.

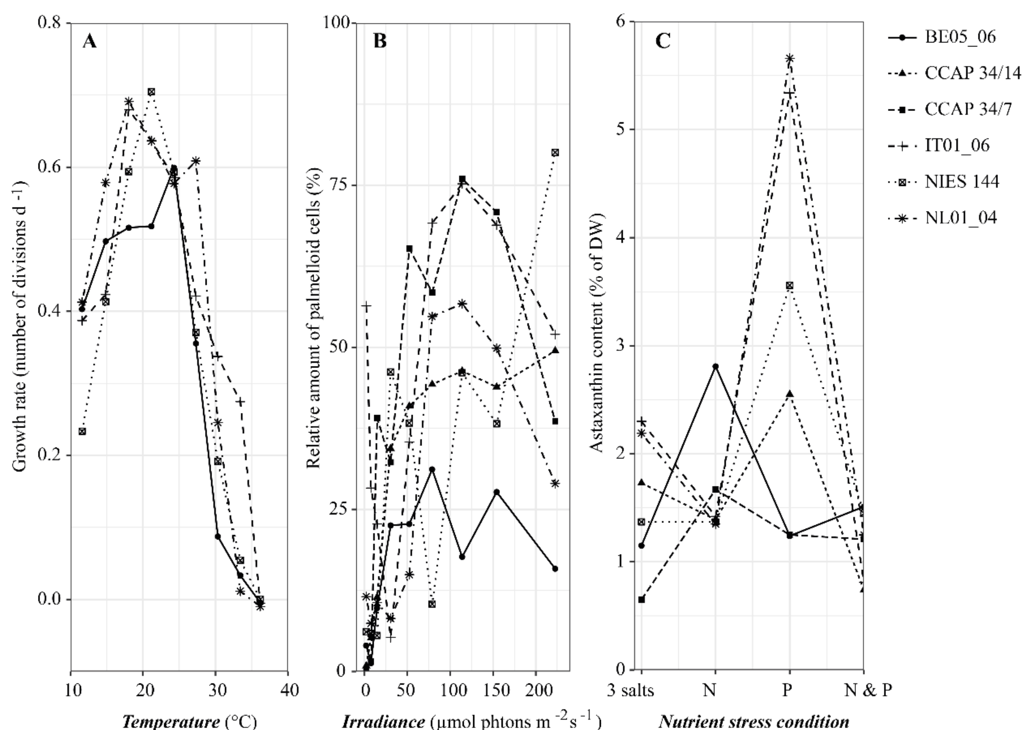



***Figure 1.*** *Examples of physiological, morphological and biochemical reaction norms of different Haematococcus strains upon different experimental conditions.* ***A.*** *Growth rate (number of divisions $d^{-1}$) in function of different temperatures (°C) (Chapter 3).* ***B.*** *Relative proportion of palmelloid cells (%) in stationary cultures under different irradiances (µmol photons $m^{-2}$ $s^{-1}$) (Chapter 5).* ***C.*** *Astaxanthin content (%) under different stress conditions (3 salts, nitrate (N), phosphate (P) and nitrate and phosphate (N&P) (Chapter 4).*

### 7.1.3.2. Population level variability in *Haematococcus*

Another organizational level of within species variation is at the population level. There is increasing evidence that genetically distinct populations harbor physiological variation since they have diverged and adapted to local conditions. Despite the potential for continuous dispersal and mixing between populations, strains living in a common area or near to each other may have genetic relationships and exchanges during sexual reproduction. Intra-population genotypic and phenotypic diversity was explored for a certain number of model phytoplankton species including diatoms (Rynearson *et al.*, 2006; Casteleyn *et al.*, 2010), dinoflagellates (Richlen *et al.*, 2012) and coccolithophores (Débora Iglesias-Rodríguez *et al.*, 2006; Gabler-Schwarz *et al.*, 2015).Within this thesis no strong signals suggesting population level variability in *Haematococcus* were observed neither from the genetic methods (Chapter 3) nor from the phenotypic data (Chapter 4 & 5 & 6), thus there is presently no evidence in favor of a hypothesis of local adaptation from the investigated traits. Nevertheless, it is still possible that strains are locally adapted to other environmental stressors. Compared to other aquatic green microalgae, the ecology of *Haematococcus*, its habitat, distribution, life style as well as population dynamics remain very much unexplored (Chapter 2). Population level studies are currently limited to the study of Noroozi *et al.,* (2012). The authors assessed the diversity of 10 *Haematococcus* strains based on both ISSR and RAPD molecular markers and showed that most strains from different countries grouped together, showing geographical discrimination. At the same time, no clear biogeographical patterns were found amongst strains, similar to our observations (Chapter 3) and those of Buchheim *et al.* (2013). Although possibly present in *Haematococcus*, there is currently no evidence that biogeographic structuring and local adaptation are common in populations of this widely distributed genus. Nevertheless, many questions remain unanswered with respect to its occurrence. What is its actual biogeographic spatial distribution pattern and range area? What are the different dispersion modes? Why do strains belonging to two different species co-occur in the same habitat ((Buchheim *et al.*, 2013), Chapter 3)? Future population genetic studies are needed on a large amount of genotypes with preferably highly heterogeneous ecological origins. Where possible, these studies should be combined with experiments for quantification of between and within population level variation. In the end, such studies will probably reveal more structured trait profiles, which will aid in the understanding of *Haematococcus* strategies to overcome adverse conditions and the mechanisms they use in their natural habitat, besides the importance of resting stages.

### 7.1.3.3. Sources of intraspecific trait variation

As shown in Chapter 5, intraspecific trait variation allowed different genotypes to respond differently to environmental changes. But how is this variation generated? The different sources of intraspecific trait variation are discussed in the following section.

Intraspecific trait variability is generally obtained through the combination and/or interaction of two mechanisms: **adaptation** or **acclimation** (Coleman *et al.*, 1994; Albert *et al.*, 2011) (Fig. 2). **Adaptation** is common when the phenotypic variability is caused by genetic variability between individual genotypes (Hughes *et al.*, 2008). This genetic variability is the result of evolutionary processes (genetic drift, mutation, selection and migration) and the raw material for species further evolution. **Acclimation** or phenotypic plasticity, is the potential of each individual genotype to produce multiple phenotypes under various environmental conditions. Multiple researchers further argumented that an additional **stochastic** factor is responsible for plasticity in trait expression (Albert *et al.*, 2011; Bolnick *et al.*, 2011). Obtaining data on phenotypic variability is time consuming (Houle *et al.*, 2010). Such data generally stem from databases, experiments under controlled conditions and/or from field sampling. The ease with which isolates are generated from natural samples and can be obtained from culture collections, along with the ability to perform phenotyping under controlled environmental conditions, have encouraged the use of experiments to study phenotypic variability in microalgae.

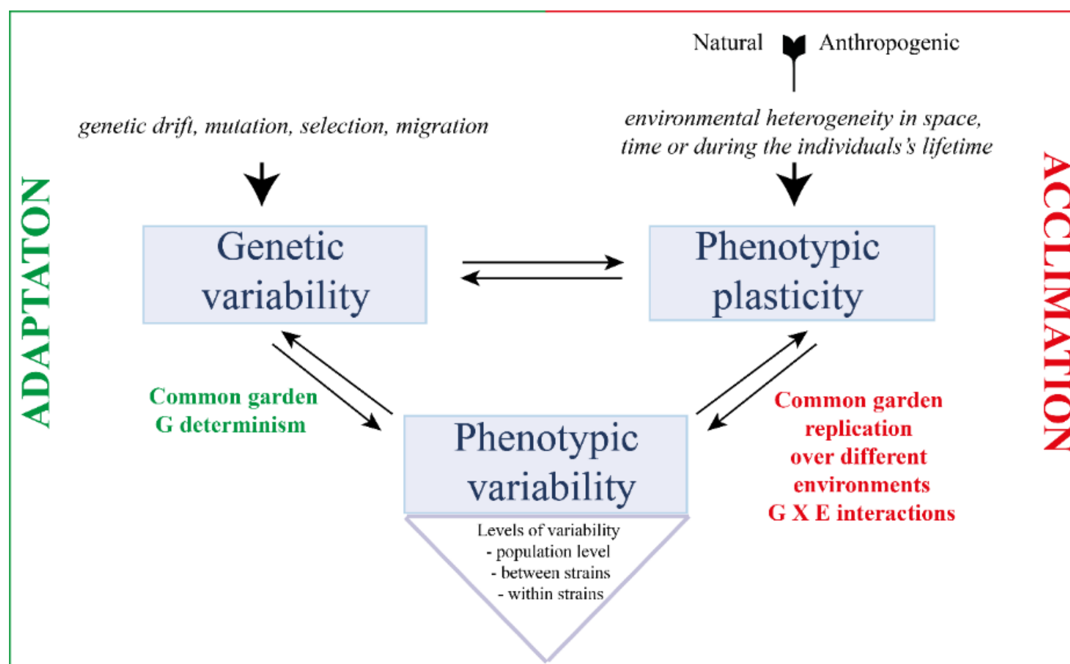



***Figure 2***. *Sources and levels of intraspecific trait variability or phenotypic variability*

The actual generators of diversity between *Haematococcus* strains remain an area of research to target, though some assumptions can be made. Firstly, diversity may be generated through its complicated life cycle which includes firstly, a sexual phase, (currently not possible to induce in the laboratory), which may be quite common under natural conditions. Secondly, the capacity of *Haematococcus* species to produce a dormant phase or aplanospores, which may represent true archives for existing variation, as reported in *Chaetoceros sp*. (Rynearson *et al.*, 2013) may potentially contribute to shaping diversity. Other common sources of diversity which are likely to play a role include *de novo* mutations as well as newly incorporated exogenous DNA through horizontal gene flow (Rynearson & Menden-Deuer, 2016).

Independent of which processes underlie the high diversity in *Haematococcus*, different selective pressures may play a role in maintaining, reducing or even eliminating this diversity. Understanding which selection pressures have played a role in shaping *Haematococcus* variation at intra- and interspecific level in the past is a very complex task which would start with a profound inventory of biotic and abiotic interactions shaping the temporal water bodies where *Haematococcus* species thrive.

In Chapter 4, we showed that anthropogenically-imposed **laboratory growth conditions** may have acted as a form of selection pressure shaping phenotypic diversity in *H. pluvialis*. An increasing body of evidence exists that algae do not remain genotypically and presumably functionally stable in culture over time. In fact, recently isolated *H. pluvialis* strains responded differently to the culture and stress conditions imposed than the culture collection strains (most commonly used in literature) for several traits. These differences added evidence to the fact that phenotypic changes, in the most cases, the loss in capacity to produce metabolites can be caused by long periods of culture in laboratory due to continuous serial transfers and cultivation under invariable conditions (Lakeman *et al.*, 2009; Day & Fleck, 2015; Gallaher *et al.*, 2015). Yet speculative, it is likely that contrasting responses between cultured and recent isolates were caused by mutations accumulating in the haploid cells of *Haematococcus* during growth and encystment in laboratory, which considerably differed from those in the natural habitat. One of the strategies of *Haematococcus* to survive harsh conditions imposed by small temporary water pools consists of transforming into aplanospores and accumulate astaxanthin, yet a low selective pressure on high astaxanthin content such as that is applied under standard growth conditions in the laboratory might have resulted in the accumulation of mutations lowering the astaxanthin content. From the cultured strains included in this thesis, all *H. pluvialis* (except NIES 144), were either kept in liquid medium or routinely grown on solid agar. It is impossible

to know how many cell divisions each *H. pluvialis* strain in culture has undergone since the initial isolation, but the oldest strain included in our study was isolated in the early 1950's. Hypothetically, if one doubling a day is considered over this time frame, cells could have had 24090 chances to mutate! In *Chlamydomonas*, a mean of 770 single nucleotide variants (SNVs) per strain was found, originating from mutations of the original *Chlamydomonas* genome through roughly 70 years of sub culturing (Gallaher *et al.*, 2015). The processes causing strains to change over time are thoroughly discussed by Lakeman *et al.,* (2009) and are beyond the scope of this thesis. Conclusively, the view of strains as static entities, or snapshots of a natural algal populations may need revision since increasing evidence shows that strains are actually ever changing laboratory populations.

In this view, this thesis highlights once more the importance of preserving strains in a metabolically inactive state through **cryopreservation**. At the time being, most *H. pluvialis* strains kept in renowned culture collections (e.g. CCAP, UTEX, SCCAP, SAG, SCCAP) are typically maintained by serial passaging on agar, which besides modifying genotypes, is a time and labor intensive practice. Cryopreservation has become a routine method for long-term storage favoring the maintenance of genetic content of a strain or cell type, but is not widely used for *Haematococcus*. We succeeded in the cryopreservation of all thirty strains included in Chapter 4 (available at http://bccm.belspo.be/) using dimethylsulfoxide (DMSO) as an effective cryoprotectant, when applied at low concentration (5 %). In this regard however, a common misconception is that a cryoprotocol successfully developed for one strain or species can be readily applied to other similar cells or organisms (Day & Fleck, 2015). *Haematococcus* strains exhibited high variability in survival post-thawing. In general, high densities of flagellated cells *Haematococcus* were necessary for successful cryopreservation, since resulting in higher survival rates post thawing compared to diluted cultures. This in contrast with *Chlamydomonas* cultures which, when attaining high densities suffered from the release of an unknown substance affecting post-thaw survival rates (Scarbrough & Wirschell, 2016).

### 7.1.4. Application potential

#### 7.1.4.1. Role of intraspecific trait variation in biotechnology

As discussed in detail in previous sections, tackling intraspecific trait variation is unavoidable if we aim to comprehend fundamental key research topics in biological sciences, such as evolution, biogeography and species responses to environmental stress. Moreover, elucidating the genetic underpinnings, the foundation of evolutionary change, of a trait is crucial to

understand the biological basis and maintenance of phenotypic variation. But how does this knowledge contribute to advancements in biotechnology and specifically in overcoming the major challenges for the improvement of *Haematococcus* biomass and astaxanthin production? The following points illustrate how a well-documented set of strains from a biotechnologically relevant genus, can add value to the optimization process of *Haematococcus* production.

First of all, if we reflect on the evolution of agriculture, plant breeders and farmers were able to make selection based on phenotypes, long before the discovery of DNA and molecular markers (Araus & Cairns, 2014). Several decades of crop selection, breeding, and testing of genotypes with desirable growth traits have contributed to substantial increases in resilience and productivity. Such selection and breeding strategies are currently poorly established in microalgae yet expected to emerge, given their capacity to rapidly divide, at a much faster rate (Georgianna and Mayfield, 2012). *Haematococcus* strains that exceed current astaxanthin yields in nature may exist hence it is presumed that achieving the efficiencies needed for competitive production might require improvement of strains through breeding or recombinant DNA technologies (Georgianna & Mayfield, 2012; Ratha & Prasanna, 2012). Understanding and quantifying the variation within natural accessions of a biotechnologically important species is essential in the establishment of an efficient program of genetic improvement either through classical breeding pathways or genetic engineering techniques. In this context, the development of improved strains may rely on the ability to identify the best genetic variation for advancement and is therefore essentially a numbers game: the more strains sampled, the greater the probability of identifying superior variation.

Secondly, currently metabolic processes of carotenogenesis in *Haematococcus* are still poorly understood, specifically the regulation and expression of carotenogenesis genes leading to astaxanthin formation (Li *et al.*, 2010; Solovchenko, 2015). There is increasing evidence that enzymatic reactions in the metabolic pathways of carotenogenesis in *H. pluvialis* (e.g. several forms of catalyzing agents in different *H. pluvialis* strains) (Grünewald *et al.*, 2001) as well as expression patterns of carotenogenesis genes (transcript patterns and induction ratios of the genes) are highly strain dependent (Gao *et al.*, 2015). In this respect, knowledge on the variation in natural accessions will assist in unravelling these complexities, to provide insights into underlying biological processes and mechanisms in future studies of gene function, the search of quantitative trait loci (QTL) and targeted selection experiments. Also in the domain of genome wide association studies (GWAS), which aim at uncovering the loci responsible for

phenotypic variation, screening of natural accessions will provide the opportunity for multiple-(population) GWAS (de Villemereuil *et al.*, 2016).

Thirdly, knowledge on trait co-variation, particularly, may be highly important for strain breeders and engineers, allowing a more targeted genetic improvement. Identifying trade-offs is essential in this optic, since they may severely limit the potential to engineer or select strains, that maximize a desired function (Shurin *et al.*, 2013; Shurin *et al.*, 2014). A recent example in *H. pluvialis* was the trade-off between increased transcript level of carotenoid genes and low levels of astaxanthin productivity (Gao *et al.,* 2015).

#### 7.1.4.2. Scaling-up: implications

The direct scale-up of the most performant strains from this study - strains with highest astaxanthin productivity - may not be the best procedure (Chapter 4) since translating laboratory findings to pilot or full scale commercial application is not just a matter of size but presents many challenges. Generally spoken, some of the imperatives that need to be optimized for large scale application include strain selection, seed culture preparation, biomass and astaxanthin yield optimization, bioreactor configuration, physico-chemical parameters and finally, post cultivation processing such as harvesting and bio compound extraction from the biomass. Clearly, strain selection is just the first step in the process.

When moving from laboratory to pilot/full scale systems two major complication-factors should be considered for strain selection:

1) First of all, one shifts from steady state laboratory standardized conditions to varying conditions outdoors. Since astaxanthin production is an expensive process, its economic viability can only be guaranteed if one would take advantage of outdoor available sunlight and atmospheric $CO_2$. Since outdoor culture conditions (e.g. temperature, light intensity and quality, photoperiod) are difficult to control, the ability of strains to perform well (=high astaxanthin productivity) in a changing environment needs to be considered as a factor in selection. In this respect, selecting strains from areas close to the site of production may present some advantages, allowing for a reduction in time required for acclimatization to local climatic conditions.
2) Once the choice of the operation mode has been made, many physico-chemical parameters such as light intensity, pH, temperature, salinity, conductivity, shear stress *(inter alia)* should be set before scaling up can be considered (Grobbelaar, 2009). Strains should be tested under the chosen circumstances with respect to astaxanthin

productivity. Finally, a range of different media from low to high nutrient concentrations, at which contamination is minimal, yet productivity maximal (without excessive nutrient supply) should be scanned.

While OFAT approaches may already provide valuable information on strain productivity (Chapter 5), other methods will become increasingly important for optimization studies such as response surface methodology, which explores the relationships between different variables in the search for optimal responses (Bilanovic *et al*., 2009). Such methods are fast and allow a large set of experiments to be done simultaneously. Also systems such as the 96-well twin-layer system designed by Nowack *et al*., (2005) may offer the possibility to screen large amounts of strains simultaneously upon exposure to specific culture conditions, bioactive compounds, pathogens, etc., particularly in light of the future development of one-phase approaches (instead of the current two stage phase approach employed) for immobilized growth and astaxanthin accumulation of *H. pluvialis* in twin layer photobioreactors (Kiperstok *et al*., 2017).

Once the choice of strain has been made, it remains imperative to do a full optimization study at lab scale before considering scaling up where the viability and robustness of the chosen strain upon scaling up is routinely checked (Rawat *et al*., 2013).

## 7.2. Final considerations and future perspectives

### 7.2.1 Final considerations

The results of this thesis provide new insights into the genetic, morphological and physiological diversity in *Haematococcus* strains at two levels: intra and interspecific. Our work is embedded within a conceptual framework or biotechnology pipeline, where the ultimate goal is to obtain high yielding strains with wide adaptability and increased resilience to pathogens. Our view is that the development of improved strains lies essentially in understanding how quantitative traits are linked to genes, which in turn requires integrated knowledge on **four important development pillars**: genetic diversity, environmental adaptation, phenotypic characterization and genetic information (Fig. 3). Throughout this work we have contributed to three of these pillars. Firstly, we documented the **genetic diversity** present in *Haematococcus* and showed that the genus constituted a complex of species with high intraspecific variation. We have further demonstrated that different genotypes had very different responses under varying **environmental conditions,** impeding as such the designation of (a) genotype(s) performing best across all environmental ranges. More comprehensive studies on the basic ecological features (habitat, occurrence) of this algae in a natural context, may contribute to this pillar in

the future. Next, we demonstrated that *Haematococcus* species and genotypes harbored an endless resource of complex **phenotypic variation** and **co-variation** in a whole set of morphological, structural, physiological, biochemical traits. This phenotypic variation was also reflected in responses to biotic interactions with a common threatening pathogen in culture of *Haematococcus*. Conclusively, this high phenomic-level variation, lead us to the understanding that physiological and biochemical processes in this genus are under the control of complex gene networks as well as aspects of the physical and environmental background, which need further investigation. Currently, the capacity of undertaking precision phenotyping, particularly under repeatable and representative growing conditions is limited, and lagging far behind the capacity to generate **genetic information**. Recent technological advances have led to increasing amounts of genomic, transcriptomic, methylomic and metabolomic data which will become more and more complete for *H. pluvialis.* Developments in high-throughput screening techniques for accurate and efficient phenotyping are therefore critical. The study of Flowers *et al.,* (2015) on *Chlamydomonas reinhardtii* is a great example of how large amounts of phenotypic variation observed in wild type isolates can quickly be correlated with genomic sequence data of the isolates, allowing to associate genomic DNA sequence changes with desirable phenotypes, or linking genes with traits, the ultimate goal for phenotype improvement.

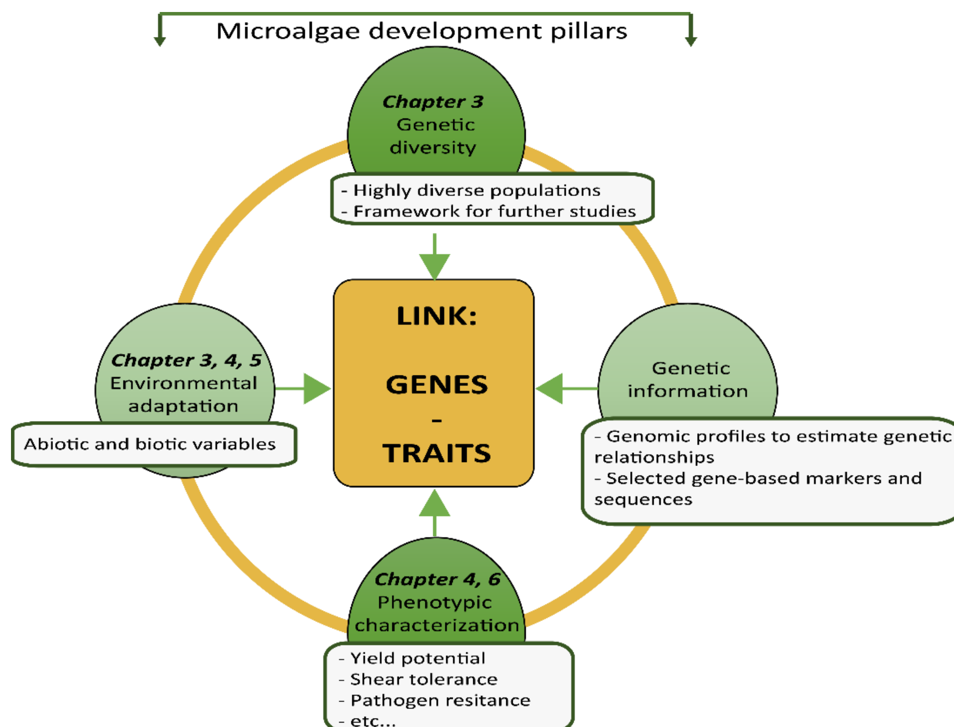



***Figure 3.*** *The four pillars of microalgae development (environmental adaptation, phenotypic characterization, genetic diversity, and genetic information) and the implications of phenotyping. Redrawn from Araus and Cairns (2014).*

### 7.2.2.Future perspectives

#### 7.2.2.1. Progress in cultivation strategies

Throughout this thesis, we have strongly focused on the organism itself, while technological developments as well as progress in culture strategies may also hold promise in the future development of large scale production. In this section, a recently emerging production strategy to which our findings may contribute in some way is proposed.

Modern algal culturing practices are dominated by monoculture systems, in which a single high-yielding strain is grown in a bioreactor. These systems are heavily dependent upon high levels of chemical inputs, for fast growth and productivity and are vulnerable to pests. In a world facing global warming and unpredictable ecological changes, monoculture systems may not be the future. A recently emerging concept in the sector is based on the notion that diversity can enhance productivity, since diverse communities, through niche differentiation and complementarity in resource use, are better at buffering against environmental stresses, pests, resulting in increased overall yields (Oncel *et al.*, 2011; Shurin *et al.*, 2013). Several case studies where microalgae were coupled with other microbial species, either algae, yeast of fungi (Magdouli *et al.*, 2016) resulted in for example increased lipid (Cheirsilp *et al.*, 2011; Zhao *et al.*, 2014) and polysaccharide (Angelis *et al.*, 2012) productivities.

So far, two co-cultivation cases were reported for *H. pluvialis.* Dong and Zhao (2004) showed that simultaneous cultivation of *H. pluvialis* with *Phaffia rhodozyma* (a natural producer of astaxanthin), facilitating gas exchange and fixation between both organisms, resulted in increased biomass and astaxanthin accumulation. In turn, Oncel *et al.* (2011), co-cultivated *H. pluvialis* with *Chlorella zofingiensis* hence the total carotenoid content of co-cultures did not exceed that of monocultures. Although their effectiveness remains to be validated, co-cultivation strategies definitely hold potential. Currently their development is limited due to concerns regarding heterogeneity of the end product together with the difficulty of tracing the composition of the community during cultivation (Magdouli *et al.*, 2016). It is in our opinion, that these issues could be overcome by the cultivation of different *Haematococcus* strains together, while at the same time benefiting a buffered and resilient consortium under varying culture conditions due to the high intraspecific variation. Clearly, experimental work is needed to validate this hypothesis. In this view, the difficulty of regulating and keeping track of intraspecific diversity in mixtures could be overcome by applying DGGE approaches such as

those developed for species characterization (Chapter 3), yet in a quantitative manner, using specific markers allowing better within species discrimination.

### 7.2.2.2. *Paraphysoderma – Haematococcus* interactions

This final section is dedicated to an area of research which is still in its infancy but deserves more attention. *P. sedebokerense* has a complex, only partially known life cycle and no efficient disease management method is currently available to control it in *Haematococcus* production facilities (Strittmatter *et al.*, 2016). Our understanding of *Paraphysoderma - Haematococcus* interactions is currently limited to the existence of complex determinants to resistance, with strains showing a genetic determinism with some strains responding through morphologic protection (Chapter 6). A limitation within this study was the occurrence of bacteria naturally associated with *Haematococcus* in our experiments. An increasing number of examples from the agricultural sector have shown that naturally co-occurring bacteria may help protect crops against fungal pathogens (Leveau & Preston, 2008; Hunziker *et al.*, 2015). The potential of naturally associated bacteria to slow down or inhibit infection is currently unexplored but may be a promising field or research to target, since these bacteria could serve as natural alternatives to the currently used stringent sterilization techniques.

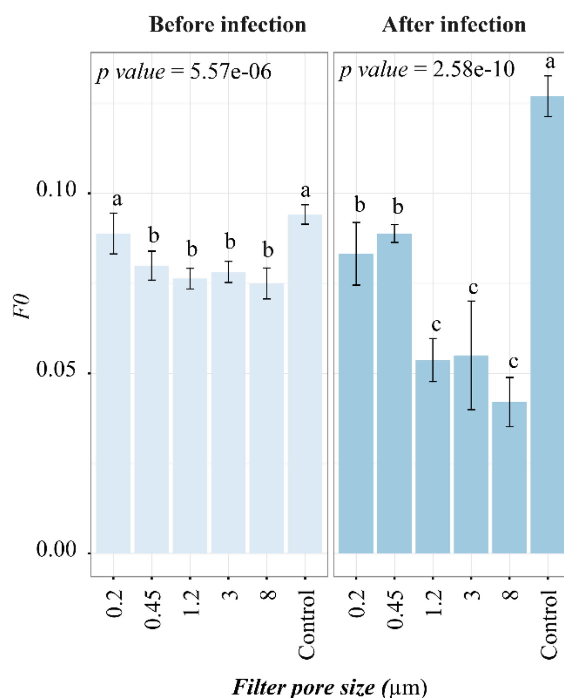
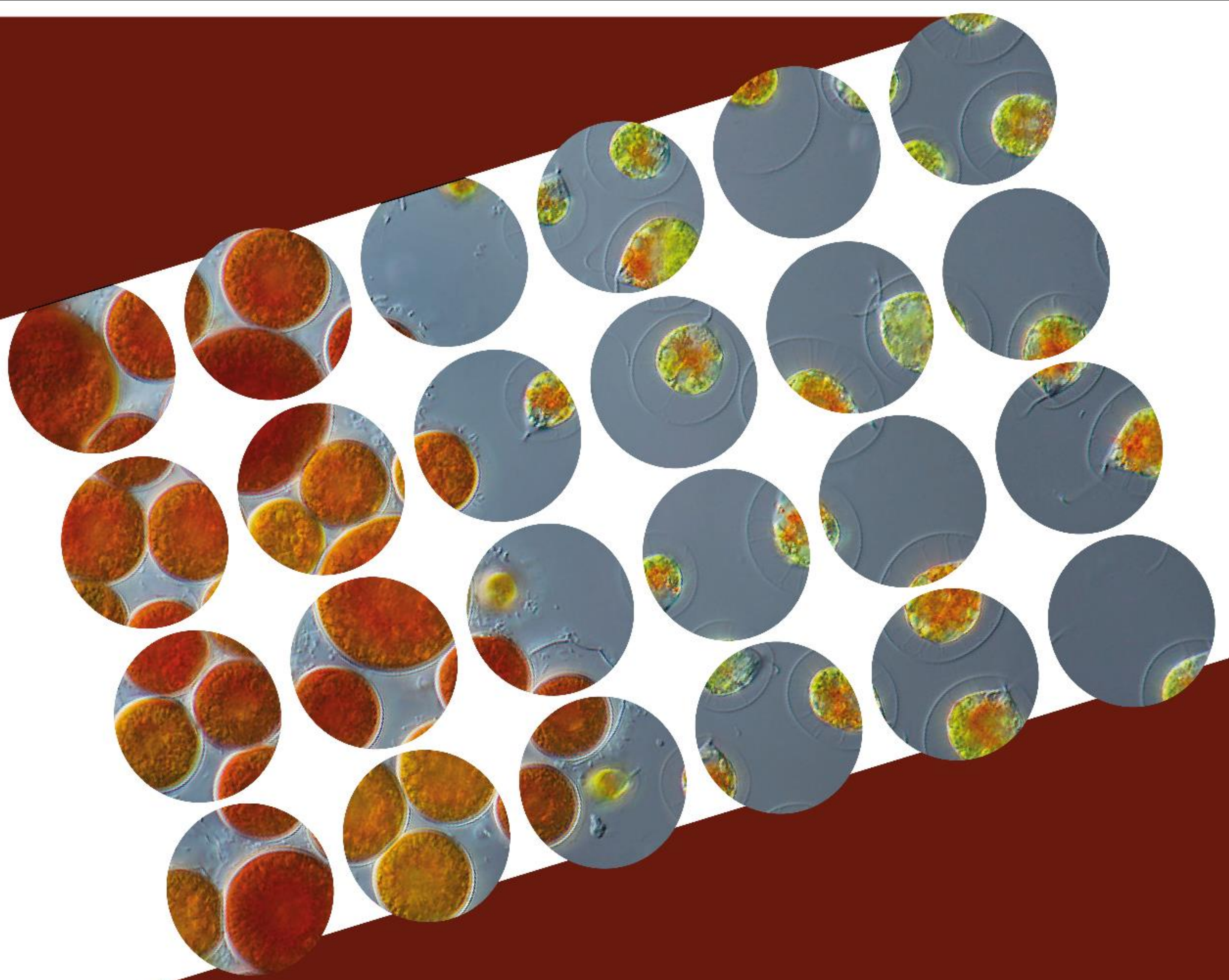



***Figure 3.*** *Effect of PS filtered through different pore sizes after 8 days of infection of the original host. Five different pore-sized membranes, 0.2 µm, 0.45 µm, 1.2 µm, 3 µm and 8 µm were used. Haematococcus C1 (original host) was inoculated with the 100 µm filtrate for 8 days, thereafter, the $F_0$ was determined. All data were mean ± SD (n= 4). p-values indicating significant differences as determined by ANOVA are given in the top left corner. Letters indicate differences between "treatments" as found performing post hoc Tukey tests.*

The further prospect of intercommunication between host and parasite is another potentially promising research area to target. In this context, in a simple pilot assay, we have established the involvement of chemical and/or bacterial cues associated with *P. sedebokerense* (comprised between 0.2 -0.45 µm), leading to significant biomass decreases in a highly vulnerable strain of *Haematococcus* (Fig. 3). Clearly, these pilot studies open perspective for future research into the fascinating interactions between *Haematococcus* - *Paraphysoderma* of which we have only “scratched the surface” so far.



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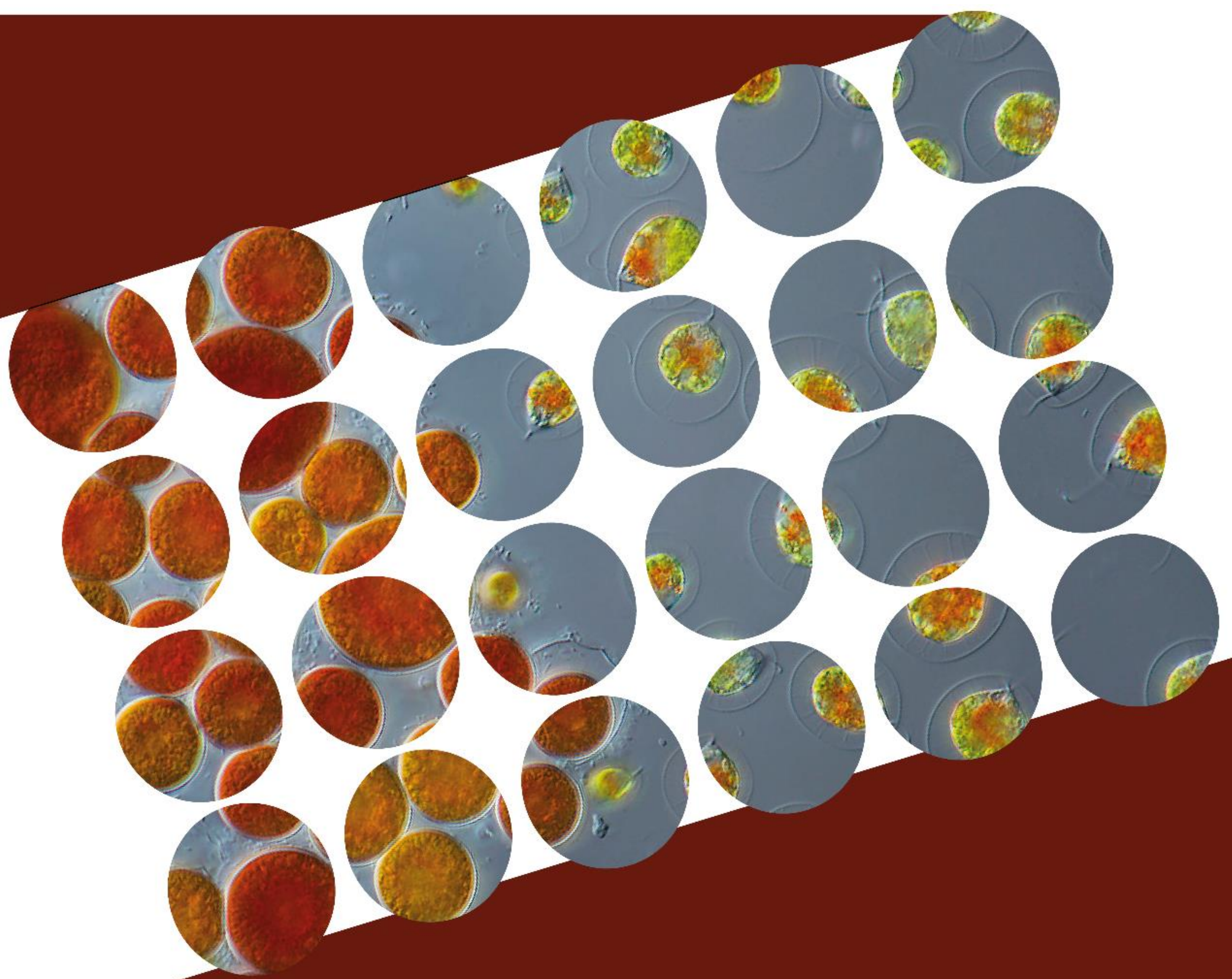

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*Richard Branson*

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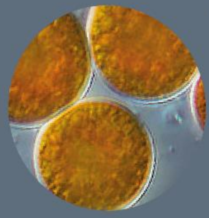

The results of this thesis provide new insights into the genetic, morphological and physiological diversity in Haematococcus strains at two levels: intra and interspecific. Our work is embedded within a conceptual framework or biotechnology pipeline, where the ultimate goal is to obtain high yielding strains with wide adapatbility and increased resilience to pathogens. Our view is that the development of improved strains lies essentially in understanding how quantitative traits are linked to genes, which in turn requires integrated knwoledge of four important developement pillars: genetic diversity, environmental adaptation, phenotypic characterization and genetic information. Throughout this work we have contributed to the three first pillars.