Accepted Manuscript

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Please cite this article as: Loris Fossier Marchan, Kim J. Lee Chang, Peter D. Nichols, Wilfrid J. Mitchell, Jane L. Polglase, Tony Gutierrez , Taxonomy, ecology and biotechnological applications of thraustochytrids: A review. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Jba(2017), doi: 10.1016/j.biotechadv.2017.09.003

Taxonomy, ecology and biotechnological applications of thraustochytrids: a review

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ABSTRACT

Thraustochytrids were first discovered in 1934, and since the 1960's they have been increasingly studied for their beneficial and deleterious effects. This review aims to provide an enhanced understanding of these protists with a particular emphasis on their taxonomy, ecology and biotechnology applications. Over the years, thraustochytrid taxonomy has improved with the development of modern molecular techniques and new biochemical markers, resulting in the isolation and description of new strains. In the present work, the taxonomic history of thraustochytrids is reviewed, while providing an up-to-date classification of these organisms. It also describes the various biomarkers that may be taken into consideration to support taxonomic characterisation of the thraustochytrids, together with a review of traditional and modern techniques for their isolation and molecular identification. The originality of this review lies in linking taxonomy and ecology of the thraustochytrids and their biotechnological applications as producers of docosahexaenoic acid (DHA), carotenoids, exopolysaccharides and other compounds of interest. The paper provides a summary of these aspects while also highlighting some of the most important recent studies in this field, which include the diversity of polyunsaturated fatty acid metabolism in thraustochytrids, some novel strategies for biomass production and recovery of compounds of interest. Furthermore, a detailed overview is provided of the direct and current applications of thraustochytrid-derived compounds in the food, fuel, cosmetic, pharmaceutical, and aquaculture industries and of some of the commercial products available. This review is intended to be a source of information and references on the thraustochytrids for both experts and those who are new to this field.

KEYWORDS

1. Introduction

There are numerous examples of case studies that initially started with the investigation of ecology of microorganisms but which eventually lead to biotechnological as a result of the discovery of new compounds with specific industrial or research applications. For example, numerous enzymes from extremophile microorganisms have found biotechnological applications of very high importance such as the Taq DNA polymerase which underpinned the polymerase chain reaction (de Champdoré et al., 2007). However, the importance of taxonomy is often overlooked in biotechnology as an indicator of potential novel innovations. Indeed, the close phylogenetic relationship between microorganisms can provide valuable information regarding general trends on growth requirements, production of compounds of interest, and ecological functions which collectively can lead to the discovery of a new group of functional microorganisms or functional bioactive compounds, or provide insight into process and method development in fermentation. In addition, while extensive efforts have been applied to the study of bacteria and fungi for uses in the food, chemical and pharmaceutical industries, heterotrophic protists (with the exception of photosynthetic microalgae) have been largely disregarded and left underexplored for many years (Kiy, 1998).

However, new tools in metagenomics analysis have shown the potential of protists in many biotechnology fields (Mackiewicz et al., 2010). In the last two decades, the drive for discovering new bioactive compounds, has lead an increasing number of scientists to tap into the little-known group of microorganisms that are the protists, with a particular interest in flagellates (e.g. *Leishmania*) and ciliates (e.g. *Tetrahymena*), notably as host organisms for the production of complex recombinant proteins (Breitling et al., 2002; Catalani et al., 2016; Cowan et al., 2014). Still, those working on these microorganisms face many future challenges as the mass culture of heterotrophic protists is reputed to be complex and difficult due to specific nutritional requirements and sensitivity to shear forces while being cultured, and longer generation times than many prokaryotes (Kiy, 1998).

In this study, we reviewed a group of heterotrophic marine biflagellate protists, the thraustochytrids, from the importance of their taxonomy to the current biotechnological applications, based on an appreciation of their ecology, growth requirements and metabolism. The thraustochytrids are well known in the field of single cell oil for their ability to produce the health-benefitting long-chain omega-3 oils, in particular DHA, in high absolute and relative content and are currently being exploited by several companies. While previous and recent reviews have provided very detailed analysis of specific aspects of the thraustochytrids, this review aims to provide a broad overview of these microorganisms from their first description to their current utilisation. By doing so, this review ensures the continuity of many studies on thraustochytrids, linking historical and modern taxonomy with growth and production patterns of certain compounds of interest, which directly feed into biotechnological applications. Hence, this is the first review with the aim of linking ecology and taxonomy to trends and production patterns among genera, in order to help maximise their uses in current and future biotechnological applications.

2. Taxonomy, phylogeny and morphological features of thraustochytrids

2.1. Taxonomy and phylogeny

Marine fungi, protists, heterokonts, chromists, or heterotrophic microalgae are the many common names, and sometimes misnomers, that have been used to describe thraustochytrids. As a result of this confusion, efforts have been made to establish the true taxonomic position of thraustochytrids and the related labyrinthulids and aplanochytrids within the domain Eukarya, but the classification of some doubtful strains and group members still remains unclear, and disagreements between taxonomists also still persist. The first description of a thraustochytrid was made in 1934. This was *Thraustochytrium proliferum,* which was isolated from the marine alga *Bryopsis plumose* in coastal waters near Woods Hole, Massachusetts (Sparrow, 1936). Initially, the family Thraustochytriaceae was incorrectly identified as belonging to the Phycomycetes, due to their ability to release biflagellate zoospores and form the rhizoid-like structures that are commonly referred to as the ectoplasmic net (EN) (Ellenbogen et al., 1969; Sparrow, 1960). Later, the thraustochytrids were assigned to the Oomycetes, (Sparrow, 1973), before Cavalier-Smith et al. (1994) showed that thraustochytrids were definitively not 'true' fungi or Oomycetes by analysis of their 5S and 18S rDNA. In parallel, other authors identified a series of common features between the thraustochytrids, the labyrinthulids and aplanochytrids, suggesting interrelationships (Alderman et al., 1974; Moss, 1986; Perkins, 1973a). With the advent of more studies supporting these interrelationships, the class Labyrinthulomycetes (International Code of Nomenclature for algae, fungi, and plants, ICN) (Von Arx, 1974) or Labyrinthulea (International Code of Zoological Nomenclature, ICZN) (Cavalier-Smith, 1986; Olive, 1975) were established in order to encompass all three groups (thraustochytrids, labyrinthulids, aplanochytrids) of microorganisms (Cavalier-Smith, 1989; Porter, 1990). However, difficulties persisted in positioning the Labyrinthulea (from now used as a synonym of Labyrinthulomycetes) in the tree of life and the relationship between the families it encompassed. Honda et al., (1999) definitively established the proximity of labyrinthulids and thraustochytrids as a monophyletic group within the stramenopiles using molecular techniques, but also distinguished two groups: the labyrinthulids phylogeny group (LPG) and the thraustochytrid phylogeny group (TPG). However, some strains initially belonging to the same genus were divided between the LPG and TPG groups, which generated more confusion, exposing the limit of taxonomy based only on morphology. This was further confirmed by experimental evidence, which also showed that in different nutritive environments, the same thraustochytrid can exhibit very different morphology, demonstrating the plasticity of these organisms. For instance, Raghukumar (1988a) and Honda et al., (1998) showed that *Schizochytrium mangrovei* and *Schizochytrium limacinum,* known for their characteristic successive bipartition mode of division (Goldstein and Belsky, 1964), also had an amoeboid stage (normally attributed to *Ulkenia* (Gaertner, 1977)), calling into question the features traditionally used to distinguish thraustochytrid genera.

Thanks to advancements in molecular techniques and the design of primers specifically for this group (Table 1), the paraphyletic taxon of the Labyrinthulea class and its further division into thraustochytrids, labyrinthulids and aplanochytrids was further supported and better understood. Tsui and Vrijmoed (2012) proposed a new classification of the Labyrinthulea, divided into two orders and four monophyletic groups. The first group contained most of the members of the thraustochytrids (which have cells which generally do not glide using the EN), the second and third groups encompassed the aplanochytrids and labyrinthulids

(which have cells which glide using the EN), while the fourth and last group contained the LPG thraustochytrids *Schizochytrium minuta* and *Thraustochytrium multirudimentale*, which were rearranged into a new genus *Oblongichytrium* (Yokoyama and Honda, 2007). The authors also established the Bicosoecida and not the Oomycetes, as initially supported by Oudot-Le Secq et al. (2006), as the direct sister group of Labyrinthulea. Over time, as old genera were amended or reclassified, and new genera discovered, the class Labyrinthulea grew bigger with the creation of five new genera (Yokoyama et al., 2007; Yokoyama and Honda, 2007), six new families and one super family, addition of three genera (Anderson and Cavalier-Smith, 2012), one superfamily and two associated genera (Gomaa et al., 2013) and most recently a further four novel genera (Doi and Honda, 2017; FioRito et al., 2016; Takahashi et al., 2016; Tice et al., 2016). The most up to date classification of the Labyrinthulea class based on these successive revisions is shown in Table 2.

In order to improve the classification of these organisms, several authors recommended including biochemical characteristics, such as lipid and carotenoid profiles (Ellenbogen et al., 1969; Findlay et al., 1986; Huang et al., 2003; Yokoyama et al., 2007; Yokoyama and Honda, 2007). Although Ellenbogen et al. (1969) and Findlay et al. (1986) recognised a potential phylogenetic implication of the polyunsaturated fatty acids (PUFA) profile in thraustochytrids, Huang et al., (2003) were the first to separate thraustochytrids based on their PUFA profiles into 5 major groups, assigning them each to a single cluster of strains based on their 18S rDNA gene. These strains formed predominantly DHA/DPAω6, DHA/DPAω6/EPA, DHA/EPA, DHA/DPAω6/EPA/ARA, and DHA/DPAω6/EPA/ARA/DTA[1]. However, as the work was mostly based on new isolates, this strategy failed to assign a PUFA profile for a particular genus, but established PUFA profile as a biochemical marker for thraustochytrids. This is particularly valuable, since PUFA profiles are little impacted with temperature ranging from 15 °C to 30 °C or different culture media (Fossier Marchan et al., 2017; Huang et al., 2003; Taoka et al., 2009a). Later, the correlation of a PUFA profile signature and the addition of carotenoid profile as a secondary biochemical signature, with the 18S rDNA signature, was further developed and refined, resulting in the recognition of new genera each with consistent biochemical markers and 18S rDNA signature and unique morphological characteristics within these new genera (Yokoyama et al., 2007; Yokoyama and Honda, 2007). The new profiling ultimately provided a more robust phylogeny for the class Labyrinthulea that comprised of monophylogenetic clades, with the exception of the genus *Thraustochytrium*.

At the present time, several taxonomies co-exist that encompass a mycological (Dick, 2001; Nakai and Naganuma, 2015) or a protistological approach (Anderson and Cavalier-Smith, 2012). This led to different terminologies are now in use to describe thraustochytrids as chromists or stramenopiles (Cavalier-Smith et al., 1994; Dick, 2001), while use of the term 'heterotrophic micro-algae' has also become common due to the close molecular phylogenetic relationship of the thraustochytrids to some photosynthetic micro-algae; the term is also understandable by the largest community of lay readers (Barclay et al., 1994). In this review, we will therefore focus primarily on microorganisms in the order

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[1] ARA: Arachidonic acid (20:4ω6), EPA: Eicosapentaenoic acid (20:5ω3), DPAω6: Docosapentaenoic acid (ω6 - Osbond acid, (22:5ω6)), DHA: Docosahexaenoic acid (22:6ω3), DTA: Docosatetraenoic acid (22:4ω6).

Thraustochytrida and exclude those constituting the superfamilies Amphitremida and Amphifiloidea.

2.2. The ultrastructure, morphology and cell division of thraustochytrids

Thraustochytrids are single cells, epi- and endobiotic, monocentric and eucarpic, with a branched or unbranched EN, and with a globose or sub-globose shape (Raghukumar, 2002; Sparrow, 1960). Commonly, zoospore cell size is in the range from 2.5–3.0 x 4.5–8.0 µm, while zoosporangium size is located between 15–35 µm when observed in seawater and pine pollen grain culture (Dick, 2001). The vegetative thalli of thraustochytrids are uni-nucleate, contain single dictyosomes (an individual stack of Golgi apparatus), and centrioles associated with shallow nuclear pockets, and are bound by discrete cell walls (Moss, 1986). Paranuclear bodies are characteristic of many thraustochytrids as regions of convoluted smooth endoplasmic reticulum enclosing ribosome-free cytoplasm frequently associated with the nucleus (Moss, 1986).

Mitochondria are numerous, polymorphic and characterized by tubular cristae. The cell cytoplasm is commonly granular and can sometimes be filled with multiple lipid bodies and contain other inclusions (Azevedo and Corral, 1997; Moss, 1980). Two main modes of cell division have been described for thraustochytrids, and these can sometimes co-exist within the same species. The first mode of cell division involves the formation of a zoosporangium by progressive cleavage, during which the cell wall remains intact after completion of karyokinesis, leading to multinuclear cells and proliferation bodies. The second mode of division is based on successive bipartition of a parent cell, immediately following karyokinesis, during which cytoplasmic schism occurs. Cytokinesis involves either invagination of the plasma membrane (presence of parental scales) or fusion with internal vesicle membranes (no scale membranes) (Moss, 1986). In both cases, an intermediate step can sometimes occur before or after cell division, described as an amoeboid stage, where a naked protoplast emerges from within the cell wall of a mature thallus (Raghukumar, 1982).

Although there remains a lack of unanimity amongst thraustochytrid taxonomists, there is a greater consensus on the presence of unique phenotypic features that thraustochytrids should share between them (Raghukumar, 1996). These specific features are: 1) a non-cellulosic cell wall that is composed of overlapping circular scales on a cellular membrane or plasmalemma; 2) the presence of an ectoplasmic net emerging from a subcellular organelle (the bothrosome or sagenogenetosome, SAG); and 3) biflagellate zoospores with a short posterior whiplash and a long anterior tinsel flagellum (Alderman et al., 1974; Moss, 1986). Further details for these three features follow.

2.2.1 Non-cellulosic cell wall

The circular scales are multilamellate and non-cellulosic, ranging from 2 to 3 nm thick with a circular diameter of 0.5 to 1 µm. They derive from dictyosome cisternae during thallus development (Moss, 1985) and consist of sulphated polysaccharides that are rich in galactose and xylose for the thraustochytrids, and in fucose for aplanochytrids and labyrinthulids (Bahnweg and Jackle, 1986). Scales are deposited onto the basal membrane through vesicles and merge together (Moss, 1985).

2.2.2 Ectoplasmic net:

The EN is an extension of the plasma membrane and emerges from each thallus from a unique sub-cellular organelle most often termed the SAG (Perkins, 1972) or sometimes the bothrosome (Porter, 1969). The exception to this is the genus *Althornia,* which does not produce EN. The SAG is made of a labyrinth of convergent and constricted lamellae of endoplasmic reticulum, connected on one side with the endoplasmic reticulum of the cell body, and on the other with the plasma membrane and the EN (Moss, 1980). One of the main differences between thraustochytrids and aplanochytrids, and of the former with the labyrinthulids is the distribution of SAG within the cell. With labyrinthulids, several organelles are distributed over the cell surface that results in an EN that entirely envelopes the cells in a colony and within which they can move. For the thraustochytrids and aplanochytrids, the EN has been reported to originate from a single trunk emerging from either a collection of many SAG (as for *Thraustochytrium motivum*) or from a single organelle (as for *Japonochytrium marinum* and *Schizochytrium aggregatum*) (Moss, 1985). The EN is believed to have multi-functional roles that include an adhesive function (allowing colonization of surfaces), a secretive and penetrative function (bringing digestive enzymes to food sources), and an absorptive function (for the assimilation of the products of digestion) (Nakai and Naganuma, 2015).

2.2.3 Biflagellate zoospores

The formation of biflagellate zoospores, with characteristic flagella, is directly related to their mode of division. Thraustochytrids divide in several ways, but commonly vegetative cells transform into a zoosporangium, either directly or after an amoeboid stage (release of a naked protoplast) (Gaertner, 1977). This then divides and the process ends by the release of zoospores. The zoospores can adopt various shapes – oblong, reniform, ovoid, elliptical, fusiform or elongate – but are always biflagellate, either directly after release or after a quiescent phase (Dick, 2001; Raghukumar, 1996). The long anterior flagellum (tinsel flagellum) possesses mastigonemes (hairs), while the shorter posterior flagellum (whiplash) is smooth (Aneja and Mehrotra, 2001) and confers the swimming motility of the zoospores. Aplanospores are often described in *Aplanochytrium* as crawling or gliding spores moving on the EN (Leander et al., 2004), but the term aplanospore was also used to described the non-motile spore (non-flagellated) in *Ulkenia visurgensis* and other organisms (Alderman et al., 1974; Moss, 1980).

2.3. Description of the genera

Currently, nine genera are recognised within the thraustochytrid family *sensu strico*, based on life cycle, morphology, ultrastructure, phylogenetic analysis and biochemical markers (PUFA and carotenoids profile): *Thraustochytrium* (Sparrow, 1936), *Japonochytrium* (Kobayashi and Ookubo, 1953), *Schizochytrium* (Goldstein and Belsky, 1964), *Ulkenia* (Gaertner, 1977), *Aurantiochytrium* (Yokoyama and Honda, 2007), *Sicyoidochytrium*, *Parietichytrium*, *Botryochytrium* (Yokoyama et al., 2007), and *Monorhizochytrium* (Doi and Honda, 2017). Two very closely related thraustochytrids have recently been removed from the *sensu stricto* family (Anderson and Cavalier-Smith, 2012), but are still considered by some scientists as belonging to the thraustochytrid group. These are *Oblongichytrium* (Yokoyama and Honda, 2007) and *Althornia* (Jones and Alderman, 1971). Additionally, a recent study defined *Stellarchytrium dubum* as belonging to a new genus within the Labyrinthulea, super family *Incertae sedis*, with cells that form an EN radiating outwards

from the colonies when grown on agar, very similar to thraustochytrids. This discovery assumes further revisions of this class in the future and potentially also of the Thraustochytrida (FioRito et al., 2016). The following section describes the morphological and biochemical characteristics (where possible) of each genus constituting the order Thraustochytrida. Table 3 shows a list of the different genera and species of thraustochytrids reported to date. An identification key in the appendix provides a list of the various morphological features and biochemical signatures used to identify thraustochytrid species.

2.3.1 Thraustochytrium

The genus *Thraustochytrium* currently includes 15 species, as listed in Table 3, which differ from each other by the absence or presence of one or several proliferation bodies and the mode of discharge of fully motile or quiescent zoospores from the 'parent' cell (by either partial or complete disintegration of the sporangial wall) (Dick, 2001). In non-proliferous forms (characterised by the absence of a proliferation body), the entire sporangium cleaves to form spores, as found in *T. roseum, T. arudimentale, T. pachydermum, T. striatum, T. aggregatum* and *T. caudivorum*. In the mono-proliferous forms (with a single proliferation body), a large basal protoplasmic unit is persistent and remains uncleaved with a wall deposited around it. This residual body persists after spore liberation, and then an internal proliferation occurs; the basal unit enlarges and becomes the new secondary zoosporangium, as occurs in *T. aureum, T. motivum, T. gaertnerium, T kinnei, T antarcticum, T. benthicola,* and *T. proliferum*. The newly persistent body of the secondary sporangium appears to be delimited during or prior to zoospore cleavage in the primary body. In multi-proliferous forms (two or more proliferation bodies), such as *T. kerguelense* and *T. rossii*, proliferation bodies remain and each of them give rise to a secondary sporangium. It has been observed under certain growth conditions that some species of *Thraustochytrium* can release amoeboid cells that give rise, after settlement, to a sporangium before the cleavage of zoospores occurs, as observed in *T. striatum* in the presence of bacteria (Raghukumar, 1992a) and *T. gaertnerium* (Bongiorni et al., 2005a). The genus *Thraustochytrium* may encompass sub-genera, because it does not form a monophylogenetic group based on 18S rDNA, and does not show a common PUFA or carotenoid profiles among species. Therefore, it is expected that new genera will be proposed in the future.

2.3.2 Japonochytrium

Japonochytrium is monotypic and is very similar to a non-proliferous form of *Thraustochytrium* (Kobayashi and Ookubo, 1953). It differs from other genera by having a subsporangial dilatation of the EN at the base of the thallus, termed the apophysis. *Japonochytrium marinum* liberates its zoospores through an apical pore in the sporangial wall (Moss, 1986). This strain may have been lost; the only isolate currently available is *Japonochytrium sp.* ATCC® 28207, but this is now believed to belong to *Ulkenia* (Yokoyama et al., 2007).

2.3.3 Schizochytrium

The only species recognised, *Schizochytrium aggregatum,* divides by successive bi-partitioning to form tetrads of zoosporangia (Goldstein and Belsky, 1964). Eventually, the vegetative cells undergo a progressive cleavage to form zoospores (Moss, 1986). *S.*

aggregatum, can produce up to 64 zoospores, reniform to ovoid in shape, while the zoosporangium can reach up to 140 μm (Dick, 2001). Yokoyama and Honda (2007) described *Schizochytrium* as showing large pale-yellow colonies, due to the production of β-carotene. The globose thallus possesses a thin wall, and the EN is well-developed. *Schizochytrium* is characterized by the production of ARA, which accounts for up to approximately 20% of the PUFA profile.

2.3.4 Oblongichytrium

Some *Oblongichytrium* strains were originally classified within the genus *Schizochytrium* and have a similar form and shape (Yokoyama and Honda, 2007). Similarly, they undergo continuous binary division, have a well-developed EN, and colonies are large and pale yellow, but the lipid profile is characterized by a high DPAω3:DPAω6 ratio and little production of ARA, while the carotenoid profile shows the production of β-carotene and canthaxanthin. The zoospores are narrow, elliptical to oblong in shape and are only released when the sporangia are transferred from agar to a broth medium. *T. multirudimentale*, *S. minutum* and *S. octosporum* are now classified under the respective names *O. multirudimentale, O. minutum* and *O. octosporum* (Yokoyama and Honda, 2007). However, *O. porteri*, a new species isolated from *Pisaster ochraceus* (sea star), was recently described to multiply through budding while lacking free-swimming zoospores (FioRito et al., 2016). It has recently been proposed that *Oblongichytrium* be considered as a new family, the Oblongychytriidae, which is distinguished from the Thraustochytriidae, *sensu stricto*, on the basis of the 18S rRNA gene sequence which clearly differentiates them (Anderson and Cavalier-Smith, 2012).

2.3.5 Aurantiochytrium

Aurantiochytrium is a sister genus of *Oblongichytrium* and *Schizochytrium*, and all were originally considered to form one genus (Yokoyama and Honda, 2007). Cells undergo continuous binary partition and are characterized by a thin-walled globose thallus. The zoospores released are similar in shape to those of *Schizochytrium*, and vegetative cells are generally dispersed as single cells. The EN is not very well developed and colonies on agar are small and pigmented orange due to the high production of astaxanthin, phoenicoxanthin, canthaxanthin and β-carotene. The fatty acid profile shows a presence, albeit low, of ARA, and a high level of DHA when expressed as % of PUFA. *S. limacinum* and *S. mangrovei* have both now been renamed as *A. limacinum* and *A. mangrovei* respectively. In these strains, under certain conditions, the discharge of an amoeboid cell has been observed prior to the cleavage of zoospores (Honda et al., 1998; Raghukumar, 1988a).

2.3.6 Ulkenia

Ulkenia strains are primarily characterized by an amoeboid stage, which appears after several classic binary divisions (Gaertner, 1977). The naked protoplast is either a uninucleated limax cell (*U. amoeboidea*) or multinucleated (*U. visurgensis* and *U. profunda*) (Moss, 1986, 1980; Raghukumar, 1982). The amoeboid cell settles and eventually rounds up to undergo division to form either a zoosporangium (*U. amoeboidea*) or an aplanosporangium (*U. visurgensis*) (Moss, 1980; Yokoyama et al., 2007). It may also divide directly to form zoospores and discharge its content in an amoeboid state (*U. profunda*) (Dick, 2001). Zoospores or aplanospores develop into trophic cells that divide through binary division before entering into a new naked protoplast stage at maturity. It is not clear

whether the naked protoplast is dispersed at the time of protoplasmic release as for *U. virsurgensis* and *U. profunda* (Dick, 2001), or persists as observed in *U. amoeboidea* SEK214 *and Ulkenia* sp. ATCC28207 (Yokoyama et al., 2007). The aplanospores observed by Moss (1980) could have been non-flagellated zoospores at the time of release that develop flagella at a later stage, as described by Yokoyama et al. (2007). *Ulkenia* is also characterized by an under-developed EN, and isolates grow as small colonies on agar. The thallus varies in size and shape (subglobose, globose or pear shape). The carotenoid profile includes the production of astaxanthin, phoenicoxanthin, echinenone and β-carotene, while the PUFA profile shows a high level of DHA (Yokoyama et al., 2007).

2.3.7 Sicyoidochytrium

The genus *Sicyoidochytrium* shows similar features to *Ulkenia* with small colonies and undeveloped unbranched EN and a comparable life cycle. The cell wall does not persist after the release of the uni-nucleated naked protoplast, which shows active motility (Dick, 2001; Moss, 1986). After undergoing a few divisions, the protoplast begins to form zoospores and develop into a zoosporangium. The distinct characteristic of the genus is underlined during the final division stage of zoospores, and shortly after their release, where some cells are still attached to each other showing a dumbbell-like organization. They complete their division by pinching and pulling the cytoplasm, eventually becoming zoospores with heterokont flagella that can swim away (Yokoyama et al., 2007). The carotenoid profile shows the production of canthaxanthin, echinenone and β-carotene, while high levels of DHA are produced with almost no production of ARA. *Ulkenia minuta* has been renamed *Sicyoidochytrium minutum* (Yokoyama et al., 2007).

2.3.8 Botryochytrium

Botryochytrium radiatum, formely known as *Ulkenia radiata,* shows comparatively larger colonies and a more developed EN compared to strains in the sister genus *Ulkenia* (Yokoyama et al., 2007). The life cycle is very similar to *Ulkenia* strains*;* but after the multinucleate protoplast is released (Moss, 1986), the cell wall disappears and the protoplast develops into a botryose (grape-shaped) zoosporangium by cleavage of early stage zoospores from a centripetal division. Eventually, the newly formed zoosporangium takes a star-like shape before zoospore formation (Dick, 2001; Yokoyama et al., 2007). Some of the released spores are not fully divided showing a dumbbell-like shape, but eventually they divide by means of pinching and pulling as described above. The fatty acid profile shows a high relative level of DPAω6, while the carotenoid profile shows the production of canthaxanthin, echinenone and β-carotene.

2.3.9 Parietichytrium

Parietichytrium sarkarianum, which was formerly known as *Ulkenia sarkariana* (Yokoyama et al., 2007), shows a similar life cycle to *Botryochytrium radiatum*, with a well-developed EN and a star-shaped multinucleate protoplast (Moss, 1986) forming before release of zoospores. Unlike *B. radiatum*, the cell wall persists after release of the protoplast, while the zoospores are fully divided at the time of discharge (Dick, 2001; Yokoyama et al., 2007). The fatty acid profile shows relatively high levels of DTA when compared to other genera (c.a. 10% of PUFA) while the carotenoid profile shows the production of β-carotene.

2.3.10 Monorhizochytrium

Monorhizochytrium is the latest genus described (Doi and Honda, 2017). Formerly known as *Thraustochytrium globosum*, *M. globosum* therefore has a similar life cycle to the non-proliferous forms of the species within the genus *Thraustochytrium*, that is the direct maturation of young vegetative cells into a zoosporangium without a residual basal body. However, the published study only presented a phylogenetic analysis and life cycle, and did not describe the PUFA and carotenoid profiles, or nor give additional information on the ultrastructure, which therefore allowed for only a partial characterisation of this new genus.

2.3.11 Althornia

Althornia crouchii (Jones and Alderman, 1971) is monotypic and is distinguished from other thraustochytrids by the absence of an EN and its associated SAG. This results in the cells existing in a free-floating, non-attached, form (Alderman et al., 1974). The cell wall is composed of scales and the mode of zoosporulation is somewhat similar to that of the genus *Thraustochytrium* (Moss, 1986). The genus was proposed based solely on morphological factors, but the type strain was lost before the 18S rDNA could be sequenced. Since the thraustochytrids have now been reclassified, the status of *Althornia* as a genus of the Thraustochytriidae *sensu stricto* must be considered doubtful.

2.3.12 Other genera within the order Thraustochytrida

Diplophrys strains have a scaly cell wall and an EN-like structure emerging from two extremities of the cell, which are referred to as filopodia (Dykstra and Porter, 1984). Recently, the genus was amended and *D. marina* has now been renamed as *Amphifila marina* (due to its occurrence in marine environments) (Anderson and Cavalier-Smith, 2012), while *D. stercorea* was moved to a separate genus, *Sorodiplophrys*, and renamed *S. stercorea* (due to its terrestrial habitat and sorocarpic aggregative behaviour) (Tice et al., 2016). Currently, the genus *Diplophrys* encompasses *D. archeri* (Costello et al., 2001) and *D. parva*, (Anderson and Cavalier-Smith, 2012) and the new species, *Diploprhys mutabilis* (Takahashi et al., 2014); all isolated from fresh water. However, due to lack of a defined SAG, the absence of zoospores and 18S rDNA molecular divergence, the genus *Diplophrys* is believed to belong to a separate family lineage *sensu stricto* from the Thraustochytriidae. Similarly, *Amphitrema wrightianum*, *Archerella flavum*, *Amphilila marina*, *Fibrophrys columna*, *Sorodiplophrys stercorea* and *Stellarchytrium dubum* all share common features with the thraustochytrids and are considered as closely related, but are classified under separate superfamilies (Anderson and Cavalier-Smith, 2012; FioRito et al., 2016; Gomaa et al., 2013; Takahashi et al., 2016; Tice et al., 2016). Little information is available on the genus *Elina* (Artemchuk, 1972), the type of member of which was historically classified as a thraustochytrid. *Elina* strains have a globose thallus and produce an EN, but its zoospores are reported to have only a single anterior flagellum, which has resulted in the genus also being associated with the Hyphochytridiomycetes (Fuller, 2001).

For the purpose of avoiding confusion, in the following sections below the newly accepted terminology for some of species formerly belonging to the genera *Thraustochytrium*, *Schizochytrium* and *Ulkenia* is used.

3. Ecology and environment

3.1. Ecology of thraustochytrids

Thraustochytrids have been isolated from marine habitats ranging from tropical waters close to coastal stations of the Indian and Pacific Oceans, and Northern Arabian sea, (Bongiorni et al., 2005a; Lee Chang et al., 2012; Ramaiah et al., 2005), through to temperate and cold waters of Australia, Argentina, the Mediterranean Sea and North Sea (Bongiorni and Dini, 2002; Lee Chang et al., 2012; Raghukumar and Schaumann, 1993; Rosa et al., 2011), to subantarctic or Antarctic waters (Bahnweg and Sparrow, 1974) and subarctic waters (Naganuma et al., 2006). The ocean water column is, in general, also rich in thraustochytrids, as they can be isolated in the photic zone of shallow coastal water (e.g. 35 m depth), in the euphotic zone (down to 200 m depth) or deeper in the aphotic zone (down to 2000 m depth) of open ocean waters (Bongiorni et al., 2005b; Li et al., 2013; Raghukumar et al., 2001). Cell counts of thraustochytrids according to the water column are shown in Table 4.

Thraustochytrids have an absolute requirement for sodium which cannot be replaced by potassium, and their preferred salinity range is from 20‰ to 34‰ – average seawater salinity is 30-35‰ (Raghukumar, 2002). Interestingly, these organisms have also been reported in hypersaline (Amon, 1978) and hyposaline environments (Jones and Harrison, 1976).

Due to their predominantly saprobic function, including mineralisation, scavenging, bacterivory and decomposition of highly recalcitrant organic matter (Raghukumar, 1992b; Raghukumar et al., 1994; Sharma et al., 1994), thraustochytrids are more commonly found in greater abundance in environments containing animal material detritus and/or decaying vegetable matter, such as surficial sediment layers, mangroves, salt marshes and river effluents. Any organic matter in a state of decomposition (including faeces) is a potential source of nutrients that can support the growth of thraustochytrids (Raghukumar, 2002). Hence, it is not surprising that thraustochytrids are likely to be found in polluted coastal waters or associated with marine snow particles (Lyons et al., 2005) that are rich in organic compounds, which has led to an interest in using these organisms for potential biotreatment of hydrocarbon contaminated seawater (Raikar et al., 2001) – a process patent from Raghukumar et al., (2008) to remove tar balls using thraustochytrids is one example of this.

Thraustochytrids are rarely found on living marine plants, such as macro- and micro-algae, and when they are present it is only in low numbers (Raghukumar, 2002). This is believed to be due to the secretion of antimicrobial compounds by the plants that inhibit colonisation of thraustochytrids and other microorganisms. For instance, Jensen et al., (1998) observed that the sea grass *Thalassia testudinum* inhibited the growth of *Schizochytrium aggregatum* by secretion of a flavone glycoside of the luteolin family.

There is no clear evidence of thraustochytrids causing any type of diseases on plants, but parasitic associations with invertebrates have been reported in many studies. Polglase (1980) was one of the first to report deleterious thraustochytrid-animal associations in the octopus *Eledone cirrhosa*, which suffered fatal ulcerative lesions that appeared to be passed from animal to animal, although it was not possible to confirm thraustochytrids as either the aetiological agent or as secondary opportunists. Similar observations were reported by Jones and O'Dor (1983) on squid gills, and similar infections with associated thraustochytrids have been reported in farmed rainbow trout, oysters, sponges, tunicates, cnidarians,

nudibranchs and free living flat worms (McLean and Porter, 1987; Polglase, 1981; Polglase et al., 1986; Schärer et al., 2007). The most studied thraustochytrid parasitic association is the Quahog Parasite Unknown (QPX) disease in the hard clam, *Mercenaria mercenaria* (Smolowitz et al., 1998; Whyte et al., 1994). Despite QPX having been characterised as belonging to the Thraustochytriidae and being closely related to *Thraustochytrium pachydermum*, no studies to-date have suggested a genus or species affiliation for this organism, thus it is still referred to as thraustochytrid QPX (Mass et al., 1999; Ragan et al., 2000; Stokes et al., 2002). Dahl and Allam (2007) established an experimental method of infecting by injecting QPX cells to *M. mercenaria*, but failed to mimic natural infection through the water in contact with the clams. This study could be considered as the first to demonstrate that a thraustochytrid is a pathogen using Koch's postulates. More recently, Burge et al. (2012) reported a new parasitic association between member of the Labyrinthulea and the sea fan, *Gorgonia ventalina*. Nevertheless, due to the presence of aplanochytrids and thraustochytrids cells in both unhealthy and healthy sea fans, and the inability to show reinfection after an inoculation, the authors could only posit an imbalanced or parasitic association between these organisms.

Taken collectively, these studies showed that associations between thraustochytrids and invertebrates define the thraustochytrids in question as either a direct causative agent of disease (a pathogen) or as parasitic opportunists, which may kill their hosts. Although it is still not yet clear what triggers thraustochytrid pathogenesis, it seems that environmental factors coupled with a compromised defence system in the host organisms could be contributing factors to infections involving thraustochytrids. It may be that similar associations are more common than have been detected, which may be an explanation for the repetitive infection of marine invertebrate cell lines, particularly from molluscs (Rinkevich, 1999). In other contexts, thraustochytrids have been shown to have beneficial symbiotic or mutualistic associations with invertebrates, particularly with corals, by possibly providing essential nutrient(s) (e.g. PUFA, carotenoids), allowing the host organism to survive stressful events (Arotsker et al., 2011). For further information on the parasitic and symbiotic associations of thraustochytrids with marine animals and plants, the reader is directed to a review on the topic by Raghukumar (2002).

It is also interesting to note that, the closely related labyrinthulids have been observed regularly on living macroalgae and, despite causing low level disease, they are believed to have a beneficial mutualistic relationship with algae and seagrasses, such as *Zostera marina,* under non-stress conditions (Brakel et al., 2014; Raghukumar, 2002). However, under some stress conditions, *Labyrinthula zosterae* was proven (by meeting Koch's postulates) to be the causative agents of disease outbreaks, the most prominent of which was "wasting disease", which caused the dieback of 90% of the *Z. marina* beds on both sides of the Atlantic Ocean in the 1930s (Muehlstein et al., 1991, 1988; Renn, 1935). In 2003, *Labyrinthula terrestris*, a novel labyrinthulid species, was also reported on land causing "rapid blight", a disease of turfgrass, although similar events reported as early as 1995 could be attributed to this organism (Bigelow et al., 2005; Olsen et al., 2003). At present, there is no evidence of a *Labyrinthula* species causing disease in other protists or in animals, although there is one report of a labyrinthulid as an endosymbiont of amoebae associated with fish gills (Dyková et al., 2008).

3.2. Isolation, preservation and detection of thraustochytrids

The development of methods for detection, isolation, cultivation and preservation of thraustochytrids has received increasing attention, although some long-standing established techniques remain in current use for these organisms. For instance, the isolation and/or detection of thraustochytrid-like microorganisms in an environmental sample is often performed by microscopy using sterile pine pollen grains as 'bait'. Indeed, pollen grains are an excellent enrichment component, as they possess a highly nutritive interior, within one of the most complex and recalcitrant outer walls in higher plants. This component of pollen is called the exine and is composed of a highly resistant biopolymer called sporopollenin (Domínguez et al., 1999). Sporopollenin is resistant to many biological, chemical and physical processes, making the study of its structural arrangement and chemical nature extremely difficult. Hence, only a few microorganisms, such as the thraustochytrids, are capable of colonising and penetrating the exterior surface matrix of the sporopollenin to reach the nutritive interior, making pollen an exceptionally selective agent for the isolation of these organisms. This technique was first established by Zopf in 1887 for the isolation of chytrids and other water fungi (Sparrow, 1960), and ultimately lead to the isolation of thraustochytrids. Colonized pollen grains are then transferred onto agar plates or into fresh broth medium, which is the first step of the isolation procedure (Goldstein and Belsky, 1964). Sporopollenin digestion by thraustochytrids was later confirmed by Perkins, (1973) who observed *Thraustochytrium movitum* and *Schizochytrium aggregatum* penetrating and digesting the sporopollenin layer of pollen grains to extract nutrients via the EN. Among pollen grains, pine pollen grain, such as from *Pinus radiata*, is traditionally the favoured 'bait' used for the isolation of thraustochytrids (Gupta et al., 2013b), but some authors report using sweet gum pollen of the genus *Liquidambor* (Rosa et al., 2011). Others have also used brine shrimp larvae (*Arternia* sp.) (Raghukumar, 1988b; Rosa et al., 2011), in particular for isolation of thraustochytrids from animal tissues.

Direct plating of fresh environmental samples on agar medium containing antibiotics and/or antifungal agents is another technique used for the isolation of thraustochytrids (Gupta et al., 2013b). Thraustochytrid-like colonies are then subcultured until axenic cultures are obtained. Rosa et al. (2011) tested six different media for isolation of thraustochytrid strains and determined the growth value (GV) based on a 5-point qualitative scale (0 to 4), where 0 indicated no cell growth and 4 indicated confluent cultures. Out of 28 evaluated thraustochytrid strains, 25 grew in all media tested, which included Mar Chiquita (MC), Mar Chiquita and Brain Heart Broth (MCBHB), Honda medium (H), modified Vishniac's medium (KMV), Serum Seawater Agar (SSA), and Glucose-Yeast-Peptone (GYP). All strains grew on SSA medium with a GV of 2, whereas on MC and MCBHB media 75% of the isolates grew with an average GV of 2.65. In GYP, H and KMV media, 43%, 82% and 96% of the isolates grew, with a GV of 1.86, 2.25 and 2.43 respectively.

To avoid bacterial and fungal contamination during the isolation procedure, antibiotics and antimycotics are generally used to suppress bacterial and fungal proliferation, many without causing any observable negative effects on most thraustochytrids. Wilkens and Maas (2012) studied antibiotic combinations during a first trial using three, or more, of eight antibiotics (streptomycin, penicillin, ampicillin, rifampicin, sodium nalidixic acid, tetracycline, gentamicin and nystatin) in three different media types (seawater complete (SWC), KMV

and By+ [2] agar). A second trial investigated different concentrations of the best antibiotic treatment identified to supress contaminants while enabling thraustochytrid growth. It was concluded that the best antibiotic treatment was a mixture of rifampicin (300 mg L^{-1}), streptomycin/penicillin (25 mg L^{-1}) and nystatin (10 mg L^{-1}) every 24 h for a minimum of 2 days. Similarly, another cocktail of antibiotics consisting of fluconazole (100 μg mL^{-1}), rifampicin (25 mg L^{-1}), ampicillin (20 mg L^{-1}), tetracycline (2 mg L^{-1}), penicillin (300 mg L^{-1}) and streptomycin (500 mg L^{-1}) resulted in the purification of 20 (63%) of the 32 isolates (Pandey and Bhathena, 2014). Sakaguchi et al. (2012) investigated the effect of 10 antibiotics and antifungal agents alone on *T. aureum* ATCC 34304, *Parietichytrium* sp. TA04Bb, *Schizochytrium* sp. SEK 579 and *A. limacinum* mh0186 when grown in Potato–Dextrose (PD) liquid medium. They found that G418 (2 mg mL^{-1}) and hygromycin B (2 mg mL^{-1}) inhibited the growth of all strains, whereas tetracycline (100 μg mL^{-1}), puromycin (100 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), streptomycin (500 μg mL^{-1}), kanamycin (50 μg mL^{-1}) and penicillin (500 μg mL^{-1}) exerted no effects. Furthermore, blasticidin (100 μg mL^{-1}) inhibited the growth of all the strains, except *Schizochytrium* sp., and zeomycin (1 mg mL^{-1}) inhibited *A. limacinum* only. Based on these results, the authors selected antibiotic resistant marker genes for transformation techniques with thraustochytrids, while providing at the same time a list of antibiotics that could be used during isolation procedures.
Limited information is available in mainstream literature on preservation of thraustochytrids. Cox et al. (2009) investigated the viability of 3 strains of *Thraustochytrium* sp. using 5 different cryoprotectant formulations. Samples were frozen at 1 °C min^{-1} to -80 °C overnight, and then transferred into liquid nitrogen and stored for 1 month prior to assessing their viability after rapid thawing (1 min at 50 °C in a water bath). Results showed that all strains were recovered successfully in a mixture of 10% (v/v) Me$_2$SO, 30% (v/v) horse serum, 50% (v/v) By-medium and 10% (v/v) inoculum. The use of glycerol, well known for its cryoprotectant properties, at 9% (v/v) and 18% (v/v) in By-medium and 10% (v/v) inoculum did not allow for similar recovery rates after the same storage conditions, although 15% (v/v) glycerol was previously used for the preservation of *Aurantiochytrium* sp. T66 (Jakobsen et al., 2008). It is clear, however, from the technical information available from collections, such as the American Type Culture Collection, that a range of methods of cryostorage are in use both in academia and industry.

Direct detection of thraustochytrids is essential to study the ecology of these organisms in their natural habitat, and also to identify them in environmental samples. For instance, a fast and reliable method for early detection of QPX thraustochytrids in aquaculture farms could help to reduce the number of disease outbreaks by the implementation of prompt preventive measures. To date, few techniques have been adapted and applied for the direct detection of thraustochytrids.

The early detection techniques for thraustochytrids were based on immunofluorescence methodology and were applied to *Ulkenia visurgensis* and *Aplanochytrium haliotidis* (Bower et al., 1989; Raghukumar, 1988b). However, immunoassays often require chemical treatments to fix the samples and to permeabilize the cell membrane, which is both laborious and introduces artifacts. In addition, each antibody must be raised against a

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[2] By-medium composition (percent w/v): (0.7%) glucose, (0.05%) yeast extract, (0.05%) peptone, (0.05%) gelatin hydrolysate, (70%) artificial seawater.

particular strain. As a result, Raghukumar and Schaumann (1993) developed an epifluorescence microscopy technique using acriflavine stain for the direct detection of thraustochytrid cells in natural water samples without prior cultivation or antibodies being required. Acriflavine hydrochloride stained all mature cell nuclei blue-green, while the sulphated polysaccharide cell wall was observed as red-orange under violet-blue excitation light; zoospores or young vegetative cells, lacking a sulphated polysaccharide cell wall, were only observed in blue-green. This technique was not specific since other protists present in the water sample also stained. Therefore, the authors recommended also distinguishing thraustochytrid cells by their morphology and lack of autofluorescence. As such, this technique may under-estimate the number of thraustochytrid cells as some strains adopt various shapes (including naked amoeboid protoplast), or have a thin cell wall at the early life cycle stage (zoospores or very young vegetative cells), which would make them difficult to detect by staining.

To overcome these limitations, Takao et al. (2007) suggested a new epifluorescence microscopy technique based on a fluorescence in situ hybridization (FISH) using an 18S rDNA targeted probe for the specific detection of thraustochytrids, which would stain all cells, including zoospores and amoeboid cells. The probe ThrFL1 (Table 1), labelled with fluorescein isothiocyanate (FITC), showed a strong reactivity with the TPG group (Honda et al., 1999), but not with diatoms, raphidophytes and dinoflagellates. However, this study did not evaluate the probe against any members belonging to the LPG group or QPX organisms. Currently, this technique is possibly the most accurate method for the detection and enumeration of thraustochytrids, but sensitivity could be increased by using brighter chromophores or coupling with a signal amplification procedure, such as CARD-FISH (Amann and Fuchs, 2008). In addition, this technique could be adapted with flow cytometry for direct counting of thraustochytrid populations, coupling it therefore with quantitative data. However, as the taxonomy and phylogeny of thraustochytrids remains unclear, a strong reactivity only with the TPG group can lead to overlooking the potential to identify other thraustochytrid strains, such as *Oblongichytrium multirudimentale* or the QPX organisms. A similar technique using in situ hydridization (ISH) was performed to detect either QPX organisms specifically, or the class Labyrinthulea in general in animal infected tissue using a QPX small-subunit ribosomal DNA (SSU rDNA) probe cocktail (QPX641 and QPX1318 probes) and the SSU rDNA probe LABY1336, respectively (Stokes et al., 2002). Other authors designed new methods based on real time quantitative PCR (Lyons et al., 2006) or denaturing gradient gel electrophoresis assays to detect the hard clam QPX pathogen (Gast et al., 2006). However, both techniques are laboratory based with limitations for rapid detection in the field.

Further work needs to be carried out in order to design better and more specific thraustochytrid detection assays based on new molecular probes or immunoassay approaches, such as a flow lateral test assay, similar to a pregnancy test, for the direct detection of QPX pathogens. The scope exists for consideration of use of a rapid fatty acid protocol for detection of thraustochytrids

4. Growth requirements and strategy for biomass production

Optimization of fermentation parameters to increase both biomass and high-value co-products, in particular DHA, has been a major topic of investigation for the biotechnological

exploitation of the thraustochytrids (Fan and Chen, 2007; Raghukumar, 2017). A recent publication reviewed in depth the influence of different parameters for DHA, carotenoid and squalene production with respect to thraustochytrid metabolism (Aasen et al., 2016). Hence, in this section we focus on the influence of temperature, dissolved oxygen, carbon and nitrogen sources and concentrations, as well as other additives, on biomass production, strain performance and production of certain value-added biomolecules synthesized by the thraustochytrids.

4.1. Temperature

Taoka et al. (2009) investigated the influence of temperature on the growth and fatty acid profile of *Aurantiochytrium* sp. mh0186. The authors found that temperatures between 15 °C and 30 °C did not cause significant changes in DHA concentration (determined as mg L^{-1}), nor on the final biomass (g L^{-1}). Nonetheless, temperature was found to alter the fatty acid profile at both ends of the range of the temperature studied, with relative levels of DHA (as % of total fatty acids, TFA) higher at 10 °C than at 35 °C, while little impact was observed between 15 °C and 30 °C. No growth of *Aurantiochytrium* sp. was recorded at 5 °C and 40 °C. Similar results were found with *Aurantiochytrium limacinum* strain mh0186 (Taoka et al., 2011a) and strain OUC88 (Zhu et al., 2008), with an optimal temperature for final DHA and biomass concentrations ranging from 16 °C to 23 °C. This was similarly reported with other genera, such as *Schizochytrium* sp. KF-1, *Aurantiochytrium mangrovei* KF-2, KF-7, KF-12, *Thraustochytrium striatum* KF-9 and *Ulkenia* sp. KF-13, with an optimal final biomass achieved between 15 °C – 30 °C (Fan et al., 2002). Similarly, Jain et al. (2004) and Taoka et al. (2011a) investigated the effect of cold shock stress on thraustochytrids by incubating strains in a 10 °C incubator for 48h and 72h, respectively, following growth. The results were shown to be strain dependent, with some strains showing an increased in DHA relative level, while *Aurantiochytrium limacinum* mh0186 showed only an increase in total lipid, but not in the relative level of DHA.

4.2. Dissolved oxygen

Thraustochytrids were originally believed to be obligate aerobes (Moss, 1986), meaning that oxygen is essential for their growth. However, it is now known that some have the potential to survive under microaerobic to anaerobic conditions, as these organisms have been detected in the oxygen minimum zone of the Arabian sea water column and sediment (Cathrine and Raghukumar, 2009; Raghukumar et al., 2001), but their ability to respire and grow under these conditions remains unsubstantiated. Excess oxygen can lead to the oxidation of unsaturated fatty acids due to the formation of reactive oxygen species. Indeed, when grown under high constant concentration of dissolved oxygen (20% of saturation), with an excess of carbon source and with nitrogen starvation, *Aurantiochytrium* sp. T66 showed high biomass yield (100 g L^{-1} DCW) (Jakobsen et al., 2008), but a low relative level of DHA (29% of TFA). However, under oxygen limitation, high levels of DHA were recorded (51% of TFA) together with a lower final cell concentration (26 g L^{-1} DCW). Hence, to obtain high biomass yield and high lipid content, Huang et al. (2012) applied intermittent oxygen feeding during the fed-batch fermentation of *Aurantiochytrium limacinum* SR21, while maintaining on average 50% saturation of dissolved oxygen (C:N ratio equal to 1.25 with feeding concentrations of 100 g L^{-1} glycerol, 40 g L^{-1} yeast extract, 40 g L^{-1} peptone at a feeding rate of 0.25 mL min^{-1} in 2 L fermentation broth). The study showed that an intermittent oxygen feeding strategy to maintain a 50% dissolved oxygen level, was

responsible for a dissolved oxygen fluctuation in the medium, enabling a high biomass to be achieved (62 g L^{-1}), while maintaining a high relative level of DHA (up to 73% of TFA) and DHA yield (20 g L^{-1}). Using *Schizochytrium* sp. S31, other authors showed similar results in a comparable fed-batch process when maintaining a constant high oxygen transfer coefficient ($K_L a = 1802$) through a rapid continuous supply of oxygen – biomass levels reached 96 g L^{-1} of DCW, with a DHA relative level of 42% of TFA and a DHA yield of 29 g L^{-1} (Chang et al., 2013). Therefore, although it is not yet clear whether a constant or intermittent level of oxygen is favourable for high DHA productivity yield, it has been shown across studies that oxygen is a vital and important parameter for the growth of thraustochytrids.

4.3. Carbon source and concentration

Although the PUFA profile can remain similar across genera and media composition, the lipid content of thraustochytrids can vary greatly depending on the strain, the medium composition and the growth conditions (Table 5), with TFA yield ranging from 8% up to 81.7% of DCW (Raghukumar, 2008). For instance, the same strain of *Schizochytrium* sp. SR21 showed variation in lipid content from 21 to 48% (Lewis et al., 1999) under different conditions as used by different researchers, while *Aurantiochytrium* sp. TC 20 also showed a variation in lipid content from 33-52% under different fed-batch regimes with varying medium compositions and carbon sources (Lee Chang et al., 2013a). Nonetheless, to qualify as oleaginous, a microorganism must accumulate at least 20-25 % of its weight as TFA (w/w% DCW) (Ratledge, 1991). In thraustochytrids, the lipid content is on average around 29% of DCW under non-optimised conditions (Raghukumar, 2008), but under optimised conditions, lipid content will on average reach 50% of DCW or higher (Table 6). Therefore, carbon source optimisation is often seen as a crucial step in developing a fermentation process for the production of DHA using thraustochytrids.

Many diverse carbon sources have been used to grow thraustochytrids as shown in Table 5. The most common have been glucose and glycerol, but other studies have used by-products from various industries, in particular the food and drink industry. However, exploiting by-products as ingredients in culture media for thraustochytrids in the production of DHA is generally yet to be optimized. Indeed, DHA productivity yields in these conditions are usually lower than when glucose or glycerol are used solely in culture media. In addition, using non-conventional feedstock such as by-products from another industry (in particular when these are not food-grade) can cause difficulties when seeking authorization for commercialization. This is because any food additive or ingredient, such as DHA, must be given a "generally recognized as safe" status which confirms the product is safe for human consumption, and gaining this could approval require further analysis, and/or other information (EFSA, 2012; FDA, 2004a; Ratledge, 2012).

The concentration of the carbon source is also an important factor, influencing significantly the growth of and DHA yields of some thraustochytrids. For instance, Yokochi et al. (1998) showed that the high tolerance of *Aurantiochytrium limacinum* SR21 to high concentrations of glucose and glycerol allowed this strain to produce a maximal biomass (ca. 35 g L^{-1}) and DHA yield (ca. 4 g L^{-1}), which was reached at a glucose concentration of 90 g L^{-1} or a glycerol concentration of 120 g L^{-1}. Similarly, *Thraustochytrium* sp. ONC-T18, when grown on a glucose concentration of 100 g L^{-1}, showed the highest DHA concentration at 4 g L^{-1} (Burja et al., 2006) and reached a biomass of 18 g $^{-1}$L, while *Aurantiochytrium* BL10 performed very well in 140 g $^{-1}$L of glucose, reaching 60 g $^{-1}$L of biomass, and 16.8 g L^{-1} of DHA (Yang et al.,

2010). However, despite tolerance to high glucose concentration, *Aurantiochytrium mangrovei* FB3 achieved maximal specific growth rates at lower glucose concentration (30 g L^{-1}) (Fan et al., 2010). Other authors found comparable results using a high glycerol concentration as the sole source of carbon instead of glucose. For example, *Aurantiochytrium limacinum* SR21 showed maximal biomass production (11 g L^{-1}) at 50 g L^{-1} of glycerol, but substrate inhibition was observed at higher concentrations (Huang et al., 2012). Gupta et al. (2013a), while investigating the influence of high glucose and glycerol concentrations with *Thraustochytrium* sp. AMCQS-5, showed that little effect on biomass production was observed across a range of glucose concentrations (from 5 g L^{-1} to 100 g L^{-1}), with a maximum biomass only reaching 1.44 g L^{-1} DCW, suggesting that glucose might not be metabolized by this strain. Conversely, an increasing concentration of glycerol greatly improved biomass production (8.32 g L^{-1} DCW at 40 g L^{-1} of glycerol), but growth inhibition was recorded at higher glycerol concentrations. Overall, many thraustochytrids can tolerate high glucose and glycerol concentrations, and either one or both sole carbon sources are often suitable for their growth, although optimal concentration is a strain dependent factor. As a result, and to minimize substrate inhibition, one strategy is to determine the highest carbon source concentration that gives the maximal specific growth rate, then to implement a fed-batch or continuous fermentation strategy with a feeding rate that would maintain the optimal carbon concentration as the sole carbon source that is consumed (Chang et al., 2013; Ethier et al., 2011; Huang et al., 2012; Lee Chang et al., 2013a).

4.4. Nitrogen source and C:N ratio

Nitrogen is also an important parameter for thraustochytrid growth. Several authors have used peptone and yeast extract (Huang et al., 2012), or corn steep liquor (CSL) (Yokochi et al., 1998), as nitrogen sources to increase both biomass production and TFA. Yokochi et al. (1998) investigated different nitrogen sources to maximize biomass and DHA yield in *Aurantiochytrium limacinum* SR21. The authors found that the use of CSL resulted in a final biomass concentration (ca. 15 g L^{-1}) comparable to that achieved with yeast extract used as a source of nitrogen, but with a DHA yield that was two-fold higher (1.5 g L^{-1}). Polypeptone and tryptone on the other hand supported production of very low biomass and DHA yields. Conversely, in an aim to increase squalene production and cell growth by *Aurantiochytrium* sp. BR-MP4-A1, Chen et al. (2010) showed that monosodium glutamate (MSG), yeast extract, peptone and tryptone were the best nitrogen sources for biomass production; they all yielded the maximal biomass concentration (ca. 8 g L^{-1}), while CSL resulted in little growth (below 4 g L^{-1}). The authors also showed that tryptone and yeast extract increased the squalene yield by a minimum of 6 to 7-fold when compared to defined nitrogen sources such as ammonium, MSG and urea. They therefore recommended using complex nitrogen sources, other than CSL, rather than their inorganic counterparts, except for MSG, for squalene production and cell growth.

A high carbon to nitrogen (C:N) ratio has been shown to improve lipid synthesis and DHA accumulation in flask fermentation with the thraustochytrid strain G13 (Bowles et al., 1999). These results were confirmed by Jakobsen et al. (2008) who provided evidence that TFA content increases in *Aurantiochytrium* sp. T66 when subjected to nitrogen starvation – i.e. a decrease in nitrogen content of the growth medium leads to lipid accumulation. Hence, a multi-phase fermentation strategy has been studied to optimize C:N ratio for biomass production in the first phases, and for DHA production and accumulation in later phases. T

Y. Huang et al. (2012) investigated a three phase fed-batch fermentation with three different C:N ratios of 0.5, 1.25 and 1.875, in lag, log and stationary phases respectively with *Aurantiochytrium limacinum* SR21. Results showed increasing DHA levels through the phases from 50% to 67% of TFA. After optimizing the system, the cultures reached a biomass of 62 g L^{-1}, while DHA yield reached 20 g L^{-1}. Similarly, a two-stage fermentation with an initial low C:N ratio (10:1), followed by a high C:N ratio (55:1) second stage was recommended by Rosa et al. (2010). Other authors have recommended altering the C:N ratio by decreasing the feeding rate of the sole carbon source at the end of fermentation (in the case of a fed-batch or continuous fermentation), resulting in higher final DHA concentration (Aasen et al., 2016).

4.5. Medium supplements

In their review, Fan and Chen (2007) reported the importance of phosphorus and sodium for thraustochytrid growth, as well as trace and micronutrients such as vitamins. For instance, optimal concentration of KH_2PO_4 was reported to be between 0.1 to 0.2 g L^{-1} for the genus *Thraustochytrium* while the genera *Schizochytrium* and *Ulkenia* required up to 3 g L^{-1}. Similarly, salts, and in particularly sodium salts such as sodium chloride and sulphate has been shown to be vital for thraustochytrids and many authors use artificial sea salts or half-strength seawater in their culture medium (Raghukumar, 2017). In addition, vitamins such as cobalamin, thiamine, biotin, nicotinic acid, riboflavin and pantothenic acid were also shown to be important as growth factors (Fan and Chen, 2007).

Other authors have been interested in supplementing the fermentation medium in order to target specific objectives. For example, polysorbate 80 was found to enhance both growth and total lipid accumulation in *Thraustochytrium aureum* ATCC 34304, but showed no effect on the relative level of DHA (Taoka et al., 2011b). Jasmonate and terbinafine were used to increase squalene content by either activating squalene synthase in *Schizochytrium mangrovei* (Yue and Jiang, 2009) or inhibiting squalene monooxygenase in *Aurantiochytrium mangrovei* FB3 (Fan et al., 2010), respectively, while the production of alkaline lipase was induced by the addition of olive oil (Kanchana et al., 2011). Another strategy investigated was to supplement the culture medium of a secondary fermentation with spent biomass or an enzymatic hydrolysate of the broth medium of the first fermentation (treated with an alcalase enzyme) in order to improve nutrient and resource efficiency through nutrient recycling (Lowrey et al., 2016a, 2016b). The results showed that supplementing secondary fermentation with an enzyme treated spent hydrolysate and fresh medium resulted in higher final biomass concentration (+55%) and final lipid concentration (+48%) when compared to the control (addition of fresh medium only). Similar investigation on nutrient recycling has been performed by supplementing culture medium with a 200 °C hydrothermal treated defatted thraustochytrid water soluble filtrate (WS) (Aida et al., 2017). The results showed that similar final biomass concentrations were achieved in the supplemented medium (20 g L^{-1}, 0.5 g L^{-1} yeast extract, 376 mg L^{-1} WS total nitrogen) when compared with the control (20 g L^{-1} glucose, 1 g L^{-1} tryptone, 0.5 g L^{-1} yeast extract), indicating that the WS filtrate could be effectively used as a recycled source of nutrients.

4.6. Light requirement

Thraustochytrids are often regarded as advantageous when compared to PUFA-producing phototrophic microorganisms such as microalgae, due to the absence of the requirement for a light source. This made thraustochytrids attractive for cultivation, because

photobioreactors are generally considerably more expensive to run than their stainless-steel counterparts, and light-dependent cultivation strategies are sometimes more complex to implement. Nonetheless, although light is not essential for thraustochytrid growth, an early study showed that thraustochytrids may have photoreceptors that could stimulate growth under light excitation (Goldstein, 1963). This was confirmed in a study by Yamaoka et al (2004) that showed the effect of near-infrared LED, blue, red and fluorescence (1,5 kLux) lights on *Thraustochytrium* sp. CHN-1 growth and carotenoid production. The authors showed that under 1,5kLux fluorescent light, maximal final biomass (c.a 2.5 g L^{-1}) was achieved under blue LED luminescence and also producing the highest carotenoid content (c.a 1 mg g^{-1} of DCW). In comparison, under dark conditions, the final biomass and total carotenoid content measured was 0.5 g L^{-1} and 0.2 μg g^{-1}, respectively This novel photo-cultivation strategy for thraustochytrids was later patented (Yamaoka, 2008). Another study showed that the final carotenoid concentration of mutant *Aurantiochytrium limacinum* BR.2.1.2 was more than double at 5 kLux compared to that in the absence of light (Chatdumrong et al., 2007). In addition, the French company Fermentalg recently patented a mixotrophic cultivation strategy for the production of astaxanthin and DHA using *Schizochytrium* sp. under discontinuous illumination (flash-mixotrophic) (Romari et al., 2015). Briefly, the microorganism was grown under classic heterotrophic conditions with the exception that the internal wall of the bioreactor was coated with light-emitting diodes providing intermittent flashes. Results showed an 18% increase in final biomass concentration, stable DHA relative content and hyper-production of astaxanthin from 0 mg g^{-1} (heterotrophic) to 2 mg g^{-1}.

5. Metabolism

With recent advances in molecular biology, extensive work has been conducted to improve understanding of different metabolic pathways and how they interconnect in thraustochytrids to produce a specific compound. With this new knowledge, researchers can optimize culture conditions more easily to enhance or inhibit the activity of key enzymes. This section gives a brief overview of the synthesis of PUFA, squalene, EPS and extracellular enzymes by thraustochytrids. An in-depth understanding of PUFA synthesis and formation of other products in thraustochytrids is provided in the recent reviews of Xie and Wang (2015) and Aasen et al. (2016)

5.1. Polyunsaturated fatty acid synthesis

Thraustochytrids are known to produce PUFA using two distinct pathways: the fatty acid synthase (FAS) aerobic pathway (also called the standard elongase-desaturase pathway), and the polyketide synthase-like (PKS) anaerobic pathway (also called the PUFA synthase pathway) (Qiu, 2003). Qiu (2003) suggested a potential FAS route in *Thraustochytrium* involving several elongase and desaturase enzymes in sequential order leading to biosynthesis of DHA. The "two pathway" hypothesis was developed and supported by the discovery of several elongase and desaturase enzymes in a number of strains of the genus *Thraustochytrium*, and these include: Δ4 desaturase (Kang et al., 2008; Nagano et al., 2011b; Qiu et al., 2001), Δ5 desaturase (Kang et al., 2008; Qiu et al., 2001; Sakaguchi et al., 2012), Δ5 elongase (Nagano et al., 2011b), Δ6 elongase (Wu et al., 2005), tauΔ12 desaturase, the functions of which were similar to a standard Δ12 desaturase (Matsuda et al., 2012) and ELO-like enzymes which act as elongases, but at multiple carbon positions – Δ9, Δ6, Δ5 (Kang et al., 2010; Ohara et al., 2013). As a result, this route is strongly suspected to be

used by *Thraustochytrium* species, but was reported to be incomplete or missing in *Schizochytrium* or *Aurantiochytrium* strains (Lippmeier et al., 2009; Nagano et al., 2011b).

Hence, it was shown that a second route, the PKS route, stood alongside the FAS route and might actually be the normal method of synthesis in some thraustochytrid strains (Metz et al., 2001). This route, to date only studied in thraustochytrids in *Schizochytrium*, is based on identification of open reading frames coding for proteins with homologous functions to FAS enzymes (elongation and desaturation of FA chains), but through different chemical reactions (Hauvermale et al., 2006; Lippmeier et al., 2009; Metz et al., 2009, 2001).

5.2. Squalene synthesis

The pathway for squalene synthesis in thraustochytrids has not yet been elucidated completely, although some studies have drawn together aspects of the pathway (Aasen et al., 2016). Yue and Jiang (2009) reported an increase in squalene content after addition of jasmonate, believed to be an activator of the squalene synthase in *Aurantiochytrium mangrovei*, but the mechanism for this is still poorly understood. This hypothesis was later confirmed by Hong et al. (2013) who characterised a squalene synthase in *Aurantiochytrium sp* KRS101 and showed its activity by the conversion of farnesyl diphosphate to squalene in the presence of NADPH and Mg^{2+}. Squalene synthesis in eukaryotes varies greatly between organisms, supporting the hypothesis that thraustochytrids may have their own squalene synthesis pathway (Spanova and Daum, 2011). Similarly, Fan et al. (2010) showed an increase of squalene by *Aurantiochytrium mangrovei* FB3 when terbinafine hydrochloride was added to the culture medium. Squalene is a precursor metabolite of numerous sterols, and terbinafine is known to inhibit sterol synthesis enzymes, such as squalene monooxygenase, which can lead to accumulation of squalene in the cell in the presence of terbinafine. Further work is required to better understand squalene metabolism in order to achieve increased yields in thraustochytrids.

5.3. Exopolysaccharide production

Thraustochytrids are also known to produce an exudate, commonly called exopolysaccharide (EPS) which could have commercial applications. Jain et al. (2005) showed that *Schizochytrium* sp. SC-1 and *Schizochytrium* sp. CW1 produce, respectively, 0.9 g L^{-1} and 1.1 g L^{-1} of EPS in M4 medium (containing 2% glucose) after 7-day incubation. After growing *Schizochytrium* sp. CW1 on ^{14}C-glucose, the authors showed that 7% of the labelled carbon was incorporated into EPS, 33% liberated as CO_2, and 60% used for other metabolic processes. The EPS exhibited a sugar content of 39% (compared to 53% for SC-1), of which 79% was glucose (compared to 75%) and 19% galactose (compared to 23%). Similar work was carried out on *Aurantiochytrium* sp. TC018, *Schizochytrium* sp. TC002, *Ulkenia* sp. TC010, and *Thraustochytrium* sp. TC004 (Lee Chang et al., 2014), but lower yields (around one-third) were achieved with maximal EPS concentrations of 0.25 g L^{-1}, 0.3 g L^{-1}, 0.01 g L^{-1} and 0.26 g L^{-1} of EPS, respectively; similar results were obtained in the study of Liu et al. (2014).

5.4. Extracellular enzymes

The biotechnological potential of enzymes has been mostly investigated in bacteria, in particular in extremophiles, but has been largely overlooked in heterotrophic protists such as thraustochytrids (Kiy, 1998). The suspected scavenging ecological role of

thraustochytrids triggered the hypothesis that interesting extracellular enzymes may be produced by these organisms (Bremer and Talbot, 1995; Raghukumar, 2008; Raghukumar et al., 1994; Sharma et al., 1994). Nagano et al. (2011a) detected cellulolytic activity in the genera *Botryochytrium*, *Oblongichytrium*, *Parietichytrium*, *Schizochytrium*, *Sicyoidochytrium*, *Thraustochytrium*, *Aplanochytrium* and *Ulkenia*, but not in *Aurantiochytrium*. These results were in contradiction with the previous study of Taoka et al. (2009b) who did not observe any cellulolytic activity in *Thraustochytrium*, *Schizochytrium* and *Aurantiochytrium*, sometimes using strains from the same culture collection. Other hydrolase activities have been detected in thraustochytrids, including agarase, amylase, proteinase, gelatinase, urease, lipase, α-glucosidase, phosphatase and xylanase, while chitinase, carrageenase, alginate lyase and pectinase are found occasionally (Devasia and Muraleedharan, 2012; Kanchana et al., 2011; Raghukumar et al., 1994; Sharma et al., 1994; Taoka et al., 2009b). Kanchana et al. (2011) discovered a lipase with optimum activity at alkaline pH showing biotechnological potential as an additive in detergent, while Brevnova et al. (2013) recently patented cellobiohydrolase type I from *Schizochytrium aggregatum.*

6. Current exploitation and further potential for thraustochytrid products

Omega-3 rich oils from thraustochytrids are now in production in a number of countries, and new thraustochytrid isolates are regularly being explored as possible improved or alternative sources of PUFA. DHA is an omega 3 long-chain (LC, $\geq C_{20}$) PUFA that plays a key role in cell signalling, cell interactions and membrane fluidity (Colomer et al., 2007). The consumption of DHA has been proven to have beneficial effects on human health at embryonic and post-natal life stage in the development of the neuronal, retinal and immune systems (Singh, 2005; Swanson et al., 2012), as well as in adulthood, in the prevention of cardiovascular disease, maintenance of the brain and learning functions, and in inflammation response systems (Horrocks and Yeo, 1999; Ruxton et al., 2004). The heterotrophic growth of thraustochytrids allows high biomass yield in a short space of time, and therefore high DHA productivity when compared to other oleaginous photoautotrophic microorganisms; such organisms have an obligate requirement for light, which directly affects their growth rate and the metabolic strategy adopted (Armenta and Valentine, 2013; Meng et al., 2009). To date, several thraustochytrid strains have shown great potential for industrial application (Table 6), with *Aurantiochytrium* sp. TC022 being one of the highest performing strains reported.

Several companies are now producing or using DHA-rich oil from thraustochytrids, particularly Royal DSM, which acquired both Martek Bioscience Corporation in 2010 and Ocean Nutrition Canada in 2012. DSM has become the leader in the production of omega-3 rich oil from thraustochytrids for human consumption and animal feeds, while Alltech is targeting animal feeds particularly with their product All-G-Rich™. As an example of a case study, DHASCO® is a bio-ingredient containing a minimum of 40% DHA sold by DSM as a nutritional oil for the fortification of infant formula products, omega-3 supplements, and products for pregnancy and nursing (DSM, 2015). This product was originally produced from *Crypthecodinium cohnii*, but is currently being replaced by DHA-rich oil from *Schizochytrium* sp. DSM incorporated DHASCO® in their trademark *life'sDHA*™ which is sold as a bio-ingredient in many food products (dairy, bakery, processed meat, beverage, etc.) in liquid or powder form, or as a soft gel capsule dietary supplement (Life'sDHA, 2012) or as a dietary supplement in DHAgold™ for animal feeds (DSM, 2012). Other products, such as Algamac™

(*Schizochytrium* sp.) as an aquaculture feed, Lonza's DHAid™ Algal Oil (*Ulkenia* sp.) as a bio-ingredient, Source-Omega's PureOne™ (*Schizochytrium* sp.) as a vegetarian nutraceutical, or Biotherm's Blue therapy (*Ulkenia* sp.) as a cosmetic, are also commercially available (Aquafauna Bio-Marine, 2000; Biotherm, 2013; Lonza, 2012; Source Omega, 2012).

The biotechnological potential of thraustochytrids as producers of LC-PUFA, carotenoids and other bioactive compounds, such as squalene and EPS, has been reviewed several times (Aasen et al., 2016; Gupta et al., 2012; Lewis et al., 1999; Raghukumar, 2008; Singh et al., 2014). Hence, this review will now focus on the current applications of thraustochytrids rather than their potential. An emphasis will be first given to risk assessment of thraustochytrid-derived oil and its uses as a food supplement in the food and aquaculture industries, and more recent advances in the development of thraustochytrid-derived oil and secondary compounds (squalene, EPS, carotenoids) for application in the biofuel, pharmaceutical, cosmetic and nutraceutical industries.

6.1. Thraustochytrid derived DHA rich oil as a bio-ingredient in food products: safety evaluation and formulation of novel food products.

An increasing number of functional food and cosmetic products using thraustochytrid oil have been developed in recent years following *Schizochytrium* sp. and *Ulkenia* sp. "algal oils" being given 'generally recognized as safe' status for human consumption by the American Food and Drugs Administration (FDA, 2004b, 2003), and by the European Commission (European Commission, 2009a, 2009b, 2003).

Numerous studies have evaluated the safety profile of thraustochytrid-derived oil in rats, mice, rabbits, swine and piglets (Abril et al., 2003; Blum et al., 2007a, 2007b, Fedorova-Dahms et al., 2014, 2011a, 2011b, Hammond et al., 2001a, 2001b, 2001c, 2001d; Kroes et al., 2003). None of the studies found any adverse effects of oil derived from *Schizochytrium* sp. or *Ulkenia* sp. in complementary diet treatments when compared to control diet-fed animals. Nor were any related effects found in terms of growth development (clinical observations, body weight, food consumption, mortality), pathology parameters (haematology, urinalysis, histopathology), genotoxicity, mutagenicity, or developmental and reproductive toxicity. Only during clinical inspection were increases in relative and absolute weight of the liver observed as treatment related effects, but this was considered as a physiological response to high intake levels of lipids and not as sufficiently adverse to warrant concern (Blum et al., 2007a, 2007b, Fedorova-Dahms et al., 2011a, 2011b).

In humans, Sanders et al. (2006) evaluated the effect of *Schizochytrium* sp. derived oil on cardiovascular risk factors in healthy subjects (39 men and 40 women). After a daily intake of 4 g of *Schizochytrium* sp. derived oil (1.5 g of DHA) or 4 g of refined olive oil (0 g of DHA as placebo), no adverse effects or pathological changes were reported. It was, however, observed that the DHA treatment significantly raised LDL-cholesterol and HDL-cholesterol levels. A high LDL-cholesterol level is often associated with a greater risk of cardiovascular diseases (CVD), whereas a high HDL level is regarded as a marker for low CVD risk. In this study, no change in the LDL:HDL ratio (1.82) was observed between the baseline and the end of the study, which is suggestive that the change in absolute LDL and HDL levels overall had a net neutral effect on CVD risk. Thus, the authors concluded that the intake of *Schizochytrium* sp. oil as a dietary supplement did not increase the risk of CVD in healthy men and women.

Harris and Von Schacky (2004) suggested taking the Omega-3 Index into consideration as a new risk factor for CVD. Indeed, they observed that a relative level of DHA + EPA above 8% in erythrocytes (red blood cells) is associated with greater cardiovascular protection. Data presented in the study carried out by Sanders et al. (2006), involving intake of a DHA supplement (1.5 g), can be used to calculate that the Omega-3 Index was equal to 8.4% compared to 6.6% for the control cohort, showing that beneficial effects can be associated with the consumption of thraustochytrid-derived oil. In addition, Ryan et al. (2010) reviewed, in depth, the safety evaluation of single cell oils, including thraustochytrid derived oils, together with the associated regulatory requirements in the USA, Canada, Australia, New Zealand and the European Union. Their study concluded that no adverse event associated with single cell oils (SCO) has been ever reported; neither have SCO been associated with allergic reactions, while satisfying safety requirements from all studied countries. The US Food and Drug Administration acknowledged a safe intake up to 3 g day^{-1} of DHA and EPA. Hence, SCO, which include thraustochytrid oil, are seen as the most promising fish oil alternatives for human consumption as a nutraceutical, or in animal feed where DHA enrichment can replace the use of fishmeal.

6.2. Developing new DHA enriched food products through ingredient substitution and omega-3 enriched animal feeds.

With successful risk assessments and accreditation given to thraustochytrid based oils, development of food products such as enriched omega-3 infant formula (DSM, 2015), eggs, milk and meat products (Ansorena and Astiasarán, 2013; Woods and Fearon, 2009) was made possible. For instance, Valencia et al. (2007) formulated a new type of dry, chorizo-type sausage by substituting pork fatback with 25% and 15% of *Schizochytrium* sp. oil. The authors concluded that a 15% substitution was sensorially acceptable, as no difference was noted by the panelists during a triangle sensory evaluation test. It also provided a healthier dry sausage with an ω6:ω3 ratio of 2.62 compared to 9.41 for the control (no substitution of fat). In addition, storage for 30 days under vacuum with the use of an antioxidant guaranteed oxidative stability.

Other authors directly fed lambs (Elmore et al., 2005; Meale et al., 2014), pigs (Marriott et al., 2002; Sardi et al., 2006), chickens (Rymer et al., 2010; Yan and Kim, 2013) or rabbits (Mordenti et al., 2010) a diet supplemented with *Schizochytrium* sp. derived DHA, and in all cases these studies showed an increase of DHA deposition into muscle lipids and adipose tissue without impairing growth and development. Similarly, Park et al. (2015), investigated the effect of a diet supplemented with dried *Schizochytrium* sp. (basal diet +0.5% or +1%) on laying hens' egg production, egg quality (yolk colour, eggshell thickness, egg weight) and the fatty acid profile of egg yolks. After 6 weeks on a 1% dietary supplement, not only were higher DHA levels observed in the fatty acid composition of the egg yolk, but also a better productivity and overall quality of the egg were achieved (thicker eggshell, stronger yolk colour). This study was in agreement with that of Chin et al. (2006) who reported an increase in the DHA level in egg yolk after feeding laying hens with a diet containing *Schizochytrium limacinum* SR-21 at +1% and +3% level over a period of 3 weeks.

6.3. Aquaculture feeds

DHA is an essential component for optimal growth and fish development. Hence, farmed fish diets require substantial amounts of DHA (Sargent, 1993; Sargent et al., 1999). Until recently, the DHA supply came from fish oil obtained from wild harvested fish, but this practice has come under scrutiny and been found environmentally unfriendly and unsustainable due to issues such as overfishing (Naylor et al., 2009). Thus, several fish feeding strategies are currently under investigation using thraustochytrids to replace fish oil. One strategy consists of enriching larval brine shrimps or rotifers with live thraustochytrid cells prior to feeding them to fish (Barclay and Zeller, 1996; Estudillo-del Castillo et al., 2009; Harel et al., 2002; Pacheco-Vega et al., 2015). The second strategy consists of directly feeding thraustochytrids (spray dried or freeze dried mix pellet) to fish (gilt-head bream larvae) (Ganuza et al., 2008) or molluscs (geoduck clam, abalone) (Arney et al., 2015; de la Peña et al., 2016). A third feeding approach involves the formulation of a fishmeal which includes thraustochytrid-derived oil or meal as an ingredient in the recipe. This strategy has been extensively studied with farmed fish in particular, such as salmon parr, catfish, Atlantic salmon post-smolt, juveniles of giant grouper and longfin yellowtail (Carter et al., 2003; Faukner et al., 2013; Garcia-Ortega et al., 2016; Kissinger et al., 2016; Kousoulaki et al., 2016; Miller et al., 2007; Sprague et al., 2015). These studies investigated growth and development as well as relative DHA levels and the $\omega3{:}\omega6$ ratio in fish or shellfish. As an example with cultured salmon, several studies found an increase of DHA level in fish fillet (parr and post-smolt) when using a diet supplemented with thraustochytrid oil (Kousoulaki et al., 2016; Miller et al., 2007) at 13% and 5% inclusion, respectively. In comparison, Sprague et al. (2015) recorded a lower relative DHA level (at 11% inclusion), and no significant difference was recorded in the study of Carter et al. (2003) at 10% inclusion when compared to control salmon post-smolt fed on fish oil at equivalent inclusion levels. All studies found normal growth and development of animals fed using the thraustochytrid oil when compared to diets based on fish oil, thus clearly confirming thraustochytrid oil as an adequate alternative to fish oil.

6.4. Biofuel production: from biodiesel to jet fuel

The first generation of biodiesel was produced from vegetable oils, such as rapeseed, soy, sunflower and palm oils (Ramos et al., 2009), but quickly showed several major limitations. In particular, the land required for crops and their water and fertilizer requirements were seen as environmentally unfriendly practices. As a result, SCO produced by oleaginous microorganisms were investigated to address these issues (Meng et al., 2009). Among them, "microalgae", and in particular heterotrophic microalgae, were regarded as good candidates to overcome the "food versus fuel" issues associated with using oils derived from food crops for biodiesel production. The benefits included their high lipid content accumulation, efficient production of biomass and simpler production processes (Mata et al., 2010; Meng et al., 2009; Miao and Wu, 2006). Hence, thraustochytrids were investigated as a promising subject for biodiesel production (Byreddy et al., 2015; Lee Chang et al., 2013b), with *Schizochytrium* sp. S056 (Chen et al., 2015) and *Aurantiochytrium limacinum* SR21 (ATCC MYA-1381) (Johnson and Wen, 2009) almost meeting ASTM standard requirements for intrinsic biodiesel properties. While assessing the life cycle of biodiesel production via hydro-processing from a thraustochytrid strain requiring 2.42 units of carbon per unit of biomass, Lee Chang et al. (2015) concluded that the energy return on energy invested value was lower than for fossil diesel when glycerol was used a sole carbon source. In comparison energy return was higher with molasses as the main carbon source, as the

latter contributed to a lower initial energy input. Thus, the authors advocated the need to develop further the use of low-cost carbon sources derived from agro-industrial wastes and to optimize further culture conditions to increase final biomass yields for the production of thraustochytrid-derived biodiesel.

Recently, a joint project involving Tohoku University, University of Tsukuba and the City of Sendai called "Next-generation energies for Tohoku recovery" has been seeking to produce hydrocarbon-rich liquid from domestic waste water (Tohji, 2012). The on-going project investigates the treatment of sludge water with *Aurantiochytrium* 18W-13a for the production of the highly unsaturated hydrocarbon squalene, which is then transformed into its saturated counterpart squalane and can be further converted into smaller alkane hydrocarbons, using ruthenium/cerium oxide as catalysts. These products are then convertible to gasoline or jet fuel (Oya et al., 2015; Phys.org, 2015).

6.5. Pharmaceutical and nutraceutical bioactive compounds from thraustochytrids

Squalene production by thraustochytrids for pharmaceutical and nutraceutical purposes is another promising biotechnological application (Lewis et al., 2001). Squalene is a polyunsaturated triterpenoid hydrocarbon which plays a key role in plants and animals as a precursor of many steroids, including cholesterol, and also bile acids, hormones, and vitamin D (Spanova and Daum, 2011). As a result, squalene is rarely accumulated in large quantities, but instead is converted into other bio-active molecules. Nonetheless, squalene is a powerful natural antioxidant that can provide protection against free radical and reactive oxygen species, and anti-tumorigenic, antimicrobial and cardio-protective activities have been reported, as well as increases in nonspecific immune functions (Spanova and Daum, 2011). Currently, the major source of squalene is the liver of deep sea sharks, which can contain between 50–80% of pure squalene (Bakes and Nichols, 1995). However, with shark populations generally decreasing, a sustainable alternative source of squalene is desirable and is being sought. Several studies have investigated the potential of thraustochytrids for squalene production, but yields initially ranged between 0.55 to 5.81 mg L^{-1} and were generally too low to provide a sustainable alternative (Chen et al., 2010; Jiang et al., 2004; Lewis et al., 2001; Li et al., 2009). More recently *Aurantiochytrium* sp.18W-13a was found to accumulate a high level of squalene (ca. 20% of DCW) with a maximal final concentration between 0.9 g L^{-1} and 1.29 g L^{-1} depending on the culture conditions (Kaya et al., 2011; Nakazawa et al., 2012). Recently, another thraustochytrid strain, Yonez5-1, was reported to reach an even higher final concentration (1.17 g L^{-1}) when compared to *Aurantiochytrium* sp.18W-13a (0.86 g L^{-1}) grown under the same conditions (Nakazawa et al., 2014). Some thraustochytrid strains do therefore have considerable potential as a sustainable and alternative source of squalene.

A substantial number of thraustochytrids also produce carotenoids, which can be used as food colourants or nutritional supplements because of their antioxidant, anti-free radical and apoptosis-inducing activities. The carotenoid pigments that thraustochytrids have been documented to produce include: astaxanthin, phoenicoxanthin, canthaxanthin, echienone and β-carotene (Aki et al., 2003; Yokoyama et al., 2007; Yokoyama and Honda, 2007). Total carotenoid content in thraustochytrids varies greatly between strains, from as low as 5.7 μg g^{-1} DCW for *Aurantiochytrium* sp. TC030 (Lee Chang et al., 2012) to 450 μg g^{-1} DCW after 8 days of incubation for *Thraustochytrium* sp. CHN-1 (Carmona et al., 2003). Other authors

found that the use of glycerol rather than glucose, as a sole carbon source resulted in greater carotenoid content for *Thraustochytrium* sp. AMCQS5-3, *Schizochytrium* sp. S31 and *Thraustochytrium* sp. S7 (Gupta et al., 2013a; Singh et al., 2015a). However, Singh et al. (2015b) showed greater yields of astaxanthin with *Schizochytrium* sp. S31 when glucose was used as a sole carbon source compared to glycerol. The authors also showed that the addition of propyl gallate or butylated hydroxytoluene increased astaxanthin levels under the glycerol regime. Petroleum ether/acetone/water (15:75:10) was shown to be the best solvent for extraction (Armenta et al., 2006), while ultrasonication was the best cell disruption method (Singh et al., 2015a) for obtaining maximal carotenoid yield.

Finally, thraustochytrids are being investigated for the production of pharmaceuticals through the diverse range of bioactive products they synthesize or as an innovative bio-engineering tool in the production of other compounds. For instance, Bayne et al. (2013) showed the potential of *Schizochytrium* sp. to be used as a tool for the production of recombinant antigens in a readily usable form for vaccination against influenza. Raghukumar et al. (2014) patented the production of antiviral EPS from thraustochytrids and aplanochytrids, which exhibited a broad-spectrum of antiviral activities. Others have studied the neuroprotective actions *in vivo* of an *Aurantiochytrium mangrovei* supplemented diet in *Drosophila melanogaster*, showing an up-regulation of mRNA of stress-defence genes involved in an anti-ageing effect (Huangfu et al., 2013). Similarly, the French company Roquette investigated the skin inflammation response and wound healing responses after oral administration and topical application of *Schizochytrium* sp. at different dosage concentration in mice for dermatological application (Hidalgo-Lucas et al., 2015). The study found that oral and cutaneous administration of the highest dosages (500 mg kg^{-1} or 10%) significantly improved skin reparation effects after an induced chronic skin inflammation; while the fastest and greatest effect during the wound healing after skin incision, was observed at lower dosage by the topical route (2.5%).

6.6. Genetic engineering approaches

A new approach for cost-effective production of omega-3 rich oils has been to transfer a set of genes involved in omega-3 and omega-6 synthesis into plants in order to produce high levels of PUFA in oilseed (Abbadi et al., 2004; Qi et al., 2004). Thus, the unique ability of thraustochytrids to produce high amounts of DHA and other LC-PUFA has resulted in several companies showing interest in metabolic engineering using thraustochytrid enzymes involved in PUFA synthesis. The first example of transfer of thraustochytrid genes into plants for the production of PUFA was patented by Kinney et al. (2004) from the US company Dupont using a Δ4 desaturase from *Schizochytrium aggregatum* or an elongase from *Thraustochytrium aureum*, among other genes. The authors showed maximum level of 19.6% of EPA and 3.3% of DHA in genetically modified *Glycine max* embryos and seeds. Similarly, Wu et al. (2005), from the Canadian company, Bioriginal Food and Science Corporation (now a division of the US Omega Protein Company), engineered a 9-gene construct, of which 4 genes came from *Thraustochytrium* sp. 26185, and showed a maximum level (wt%) of ARA, EPA and DHA (7.3%, 15% and 1.5%, respectively) in seeds of the transgenic *Brassica juncea* breeding line 1424. The Rothamsted Institute in the UK carried out extensive work on EPA and DHA biosynthesis in *Arabidopsis thaliana* and *Camelina sativa* using Δ4 and/or Δ5 desaturases from *Thraustochytrium* sp., in combination with genes from other microorganisms (Ruiz-Lopez et al., 2014, 2013). These studies led to

a field trial using *Camelina sativa* for EPA and DHA accumulation, in which the engineered plants showed as much total seed oil content as the wild type strain (ca. 30%), whilst also producing significant levels of omega-3 LC-PUFA (14.5%) in contrast to the wild type seeds that produced none at all. Specifically, levels of EPA and DHA ranged from 2.8% to 7.1% and 2.0% to 6.5%, respectively (Usher et al., 2015). This area of research is therefore promising, but all field crops have the disadvantage of using considerable land space, whereas direct culture of thraustochytrids and species of micro-algae can be done in a much smaller area. Another consideration is that the resulting seeds are genetically modified and as a result their use is presently restricted or banned in some countries.

Finally, other authors have developed transgene expression systems for transformation in *Schizochytrium* sp. through particle bombardment (Lippmeier et al., 2009; Metz et al., 2009), electroporation (Cheng et al., 2011), or using an *Agrobacterium tumefaciens* mediated transformation system (Cheng et al., 2012). Other researchers showed that direct mutagenesis using N-methyl-N'-nitrosoguanidine coupled with ultraviolet light (Chatdumrong et al., 2007; Lian et al., 2010), evolutionary engineering (Qi et al., 2017) or using low-energy ion implantation technology could improve the rate of generating hyper DHA-producing strains (Fu et al., 2016). For instance, Fu et al. (2016) successfully generated several mutants and, using the lipid stain Sudan Black B identified, a mutant strain in which DHA content 60% higher than the wild type strain, *Schizochytrium* sp. ATCC20888.

These new tools open possibilities for generating novel recombinant thraustochytrids with specifically designed pathways in order to tailor only for EPA and/or DHA production.

Conclusion

The complex taxonomy and widespread distribution of thraustochytrids in the marine water column and sediment, as well as their association with decaying plant and animal materials, diseased animals, and healthy corals, leads us to predict that an even greater biodiversity of thraustochytrids exists than is currently recognized. Hence, the taxon Labyrinthulea is likely to face more challenges in the near future as newly discovered organisms are classified. This is particularly because some doubtful affiliations and groupings remain and some historical type strains are believed to be lost (*Japonochytrium*, *Althornia*, etc.). An additional taxonomic criterion could be therefore considered based on protein sequences and/or conformation, in particular those involved in PUFA synthesis, creating a new proteomic signature. This could primarily focus on the main enzymes involved in the synthesis of PUFA because they appear to play key role in thraustochytrids, while being widely variable with two possible major pathways involved. The known biotechnological applications of DHA-rich oils and other compounds derived from thraustochytrids as bio-ingredients or nutraceuticals is only the tip of the iceberg for the future application of thraustochytrid biomass in animal feeds, particularly in the aquaculture industry, to alleviate environmental pressures from overfishing activities, and also to enhance the development of new food products. Further biotechnological applications and current projects also point to future prospects for exploitation of thraustochytrids in other domains, in particular in the biofuel and pharmaceutical industries. Further research is needed to investigate the potential of thraustochytrids for the bioremediation of wastewater, or of petrochemical pollutants, such

as in the event of an oil spill, due to their suspected ability to degrade hydrocarbons and potential to produce powerful enzymes and surfactants. To evolve these applications, more complete knowledge is needed to understand better the metabolic capabilities of thraustochytrids, as well as managing cultivation parameters and adopting optimal fermentation strategies for their biotechnological exploitation.

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Tables

Table 1 Nucleotide primers and probes used to amplify the 18S rDNA gene of Thraustochytrida for phylogenetic analysis and to detect their presence in environmental samples

Table 2 Taxonomic classification of Labyrinthulea (protistological taxon) adapted from Anderson and Cavalier-Smith (2012), Gomaa et al., (2013), and Beakes et al., (2014). Names in parentheses are the mycological nomenclature equivalents

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Table 6 Performance of the higher yielding thraustochytrid strains for DHA productivity

Table 1 Nucleotide primers and probes used to amplify the 18S rDNA gene of Thraustochytrida for phylogenetic analysis and to detect their presence in environmental samples

Primer for 18S rDNA amplification	Sequence (5' → 3')	Base Pair Position	References
Forward			
SR1	TACCTGGTTGATCCTGCCAG	1-20	Nakayama et al., (1996)
18S001	AACCTGGTTGATCCTGCCAGTA	1-22	Honda et al., (1999)
FA1	AAAGATTAAGCCATGCATGT	37-56	Mo et al., (2002)
NSF-370F	AGGGYTCGAYYCCGGAGA	370-387	Siboni et al., (2010)
F	GGGAGCCTGAGAGACGGC	387-404	Mo et al., (2002)
NS3	GCAAGTCTGGTGCCAGCAGCC	472-492	White et al., (1990)
FA2	GTCTGGTGCCAGCAGCCGCG	555-574	Mo et al., (2002)
F566	CAGCAGCCGCGGTAATTCC	566-585	Hadziavdic et al., (2014)
LABY-A	GGGATCGAAGATGATTAG	944-961	Stokes et al., (2002)
FA3	CTTAAAGGAATTGACGGAAG	1125-1144	Mo et al., (2002)
Reverse			
RA1	AGCTTTTTAACTGCAACAAC	605-624	Mo et al., (2002)
Lab-1017r	GACTACGATGGTATCTAATCATCTTCG	1017-1043	Siboni et al., (2010)
NS4	CTTCCGTCAATTCCTTTAAG	1101-1120	White et al., (1990)
RA2	CCCGTGTTGAGTCAAATTAAG	1171-1191	Mo et al., (2002)
RT1200	CCCGTGTTGAGTCAAATTAAGC	1200	Hadziavdic et al., (2014)
R	GGCCATGCACCACCACCC	1254-1271	Mo et al., (2002)
LABY-Y	CWCRAACTTCCTTCCGGT	1361-1378	Stokes et al., (2002)
RA3	CAATCGGTAGGTGCGACGGGCGG	1662-1684	Mo et al., (2002)
18S13	CCTTGTTACGACTTCACCTTCCTCT	1733-1757	Honda et al., (1999)
SR12	CCTTCCGCAGGTTCACCTAC	1781-1762	Nakayama et al., (1996)
NSR-1787	CYGCAGGTTCACCTACRG	1787-1804	Siboni et al., (2010)
Probes for detection			
ThrFL1	GTCGACAACTGATGGGGCAG	282-301	Takao et al., (2007)
LABY1336	AACCCGAAATGTCCCTCTAAGAAG	1336-1359	Stokes et al., (2002)

Table 2 Taxonomic classification of Labyrinthulea (protistological taxon) adapted from Anderson and Cavalier-Smith (2012), Gomaa et al., (2013), and Beakes et al., (2014). Names in parentheses are the mycological nomenclature equivalents. {kingdom Chromista, subkingdom Harosa, superphylum Heterokonta, phylum Bygira, subphylum Sagenista}

Order 1 Labyrinthulida (Labyrinthulales)

> **Family 1** Labyrinthulea (Labyrinthulaceae)
> *Labyrinthula*

> **Family 2** Aplanochytriidae (Aplanochytridiaceae)
> *Aplanochytrium*

Order 2 Thraustochytrida (Thraustochytridiales)

> **Family 1** Thraustochytriidae (Thraustochytridiacae)
> *Thraustochytrium, Japonochytrium, Schizochytrium, Aurantiochytrium, Ulkenia, Sicyoidochytrium, Parietichytrium, Botryochytrium, Monorhizochytrium*

> **Family 2** Oblongichytriidae (Oblongochytridiaceae)
> *Oblongichytrium*

> **Family 3** Althorniidae (Althornidiaceae)
> *Althornia*

Superfamily 1 Amphitremida

> **Family 1** Diplophryidae (Diplophryidaceae)
> *Diplophrys*

> **Family 2** Amphitremidae (Amphitremidaceae)
> *Amphitrema, Archella*

Superfamily 2 Amphifiloidea

> **Family 1** Amphifilidae (Amphifilaceae)
> *Amphifila, Fibrophrys*

> **Family 2** Sorodiplophryidae
> *Sorodiplophrys*

Superfamily 3 *Incertae sedis*

> *Stellarchytrium, Elina*

Table 3 Thraustochytriidae, Oblongichytriidae and Althorniidae genera and species names

Genus species(Basionym)	Isolated by	Amended
Thraustochytrium aureum	Goldstein, (1963)*	
Thraustochytrium motivum	Goldstein, (1963)*	
Thraustochytrium rossii	Bahnweg and Sparrow, (1974)	
Thraustochytrium kerguelense	Bahnweg and Sparrow, (1974)	
Thraustochytrium antarcticum	Bahnweg and Sparrow, (1974)	
Thraustochytrium kinnei	Gaertner, (1967)*	
Thraustochytrium benthicola	Raghu Kumar, (1980)	
Thraustochytrium proliferum	Sparrow, (1943)*	
Thraustochytrium pachydermum	Scholz, (1958)*	
Thraustochytrium arudimentale	Artemchuk, (1972)	
Thraustochytrium striatum	Schneider, (1967)*	
Thraustochytrium roseum	Goldstein, (1963)*	
Thraustochytrium aggregatum	Ulken, (1965)*	
Thraustochytrium gaertnerium	Bongiorni et al., (2005)	
Thraustochytrium caudivorum	Schärer et al., (2007)	
Monorhizochytrium globosum (*Thraustochytrium globosum*)	Kobayashi and Ookubo, (1953)	Doi and Honda (2017)
Japonochytrium marinum	Kobayashi and Ookubo, (1953)	
Schizochytrium aggregatum	Goldstein and Belsky, (1964)	
Oblongichytrium octosporum (*Schizochytrium octosporum*)	Raghukumar, (1988)*	Yokoyama and Honda, (2007)
Oblongichytrium minutum (*Schizochytrium minutum*)	Gaertner, (1972)*	Yokoyama and Honda, (2007)
Oblongichytrium multirudimentale (*Thraustochytrium multirudimentale*)	Goldstein, (1963)*	Yokoyama and Honda, (2007)
Oblongichytrium porteri sp. nov.	FioRito et al., (2016)	
Aurantiochytrium limacinum (*Schizochytrium limacinum*)	Honda et al., (1998)	Yokoyama and Honda, (2007)
Aurantiochytrium mangrovei (*Schizochytrium mangrovei*)	Raghukumar, (1988a)	(Yokoyama and Honda, 2007)
Ulkenia profunda	Gaertner, (1977)	
Ulkenia visurgensis	Ulken, (1965)*	Gaertner, (1977)
Ulkenia amoeboidea	Bahnweg and Sparrow, (1974)	Gaertner, (1977)

Sicyoidochytrium minutum *(Ulkenia minuta)*	Raghukumar, (1977)*	Yokoyama et al., (2007)
Botryochytrium radiatum *(Ulkenia radiata)*	Gaertner, (1977)	Yokoyama et al., (2007)
Parietichytrium sarkarianum *(Ulkenia sarkariana)*	Gaertner, (1977)	Yokoyama et al., (2007)
Althornia crouchii	Jones and Alderman, (1971)	

*information retrieved from the World Register of Marine Species (2016).

Table 4 Maximal cell count of thraustochytrids at different depth and regions of the world (cell L^{-1})

Region	0 - 30 m	100 m	>750 m	References
Arabian sea	1.3×10^6	1.8×10^5	3.6×10^4	Raghukumar et al., (2001)
Pachino Bay, Sicily	1.3×10^4	-	-	Bongiorni et al., (2005b)
Ligurian sea	6.1×10^4	-	-	Bongiorni and Dini, (2002)
Seto Inland Sea	1.0×10^5	1.0×10^2	-	Kimura et al., (1999)
Hawaiian waters	2.0×10^5	6.0×10^5	-	Li et al., (2013)
Norwegian sea	2.3×10^5	-	-	Naganuma et al., (2006)

Table 5 Biomass, lipid content and DHA yields for thraustochytrid strains grown on different carbon sources

Strain	Carbon source	DHA % TFA	TFA % DCW	Biomass g L⁻¹	DHA g L⁻¹	Fermentation volume	References
Schizochytrium mangrovei KF6	Bread crust (10 g L⁻¹)	11%	11%	6.5	0.08	1L	Fan et al., (2000)
Schizochytrium mangrovei KF6	Okara powder	4%	19%	7.5	0.05	1L	Fan et al., (2000)
Schizochytrium mangrovei KF6	Brewing grain waste from mash	6%	10%	10.0	0.06	1L	Fan et al., (2000)
Schizochytrium mangrovei SM3	Mixed food waste hydrolysate	24%	17%	20.0	0.80	2L	Pleissner et al., (2013)
Aurantiochytrium sp. KRS101	Spent yeast (200 g L⁻¹)	35%	4%	26.0	0.40	0.5L	Ryu et al., (2013)
Thraustochytriidae sp. M12-X1	Potato residual liquid (RP1-YE-MSG)	53%	14%	1.0	0.07	0.1L	Quilodrán et al., (2009)
Thraustochytriidae sp. M12-X1	"Cheeseclothed" residual beer liquid (RB-YE-MSG)	45%	16%	2.3	0.17	0.1L	Quilodrán et al., (2009)
Aurantiochytrium sp. KH105	Brown seaweed (60h)	45%	4%	3.0	0.05	6L	Arafiles et al., (2014)
Aurantiochytrium limacinum SR21	Crude glycerol	34%	51%	18.0	3.07	50mL	Chi et al., (2007b)
Aurantiochytrium limacinum SR21	Crude glycerol (continuous)	29%	50%	11.8	1.74	4.5L	Ethier et al., (2011)
Aurantiochytrium limacinum SR21	50% Sweet sorghum	34%	73%	9.4	2.35	100mL	Liang et al., (2010)
Aurantiochytrium mangrovei SK02	Coconut water	42%	50%	27.5	5.70	100mL	Unagul et al., (2007)
Aurantiochytrium limacinum SR21	Hydrolysed potato broth	32%	51%	16.3	2.69	50mL	Chi et al., (2007a)
Aurantiochytrium sp. TC20	Pure glycerol (fed batch)	48%	33%	55.9	8.93	2L	Lee Chang et al., (2013a)
Aurantiochytrium sp. TC20	Pure glucose (fed batch)	43%	34%	56.6	8.23	2L	Lee Chang et al., (2013a)
Schizochytrium sp. DT3	Hemp hydrolysate	38%	17%	1.8	0.11	200mL	Gupta et al., (2015)

Table 6 Performance of the higher yielding thraustochytrid strains for DHA productivity

Strains	Strategy	Biomass g L^{-1}	TFA % DCW	DHA			References
				%	g L^{-1}	mg L^{-1} h^{-1}	
Aurantiochytrium sp. T66	Fed excess C, limiting N and O2 limitation (fixed at 20 % saturation and decreased to 1 % at 49 h fermentation), 1.8 L bioreactor	100	58	30	15.6	93	Jakobsen et al., (2008)
Aurantiochytrium limacinum SR21	Fed-batch, excess glycerol (100 g L^{-1}), peptone (40 g L^{-1}), yeast extract (40 g L^{-1}), C:N ratio: 1.25	61.8	56	65	20.3	123	Huang et al., (2012)
Schizochytrium sp. CCTCC M209059	Intermittent glucose feeding kept above 15 g L^{-1}, 0.4-0.6 vvm, 1000 L bioreactor	71	35	49	15.8	119	Ren et al., (2010)
Schizochytrium sp. HX-308	35L, Intermittent glucose feeding kept above 15 g L^{-1}, two stage oxygen supply strategy	92.7	50	42	17.7	111	Qu et al., (2011)
Aurantiochytrium sp. TC22	Fed-batch, excess glycerol (40 g L^{-1}) + 26 h addition of addition 264 g gly+ nutrient (yeast extract) 1.6 L bioreactor)	70.8	52	39	14.3	207	Lee Chang et al., (2014)

APPENDIX

Key identification criteria for Labyrinthulea with a detailed identification for Thraustochytriidae and Oblongichytriidae. (adapted from Dick 2001; Bongiorni et al. 2005a; Yokoyama et al. 2007)

1. Spindle-shaped vegetative cells that glide inside tube-like anastomosing ectoplasmic nets. Trophic cells completely surrounded or embedded within the ectoplasmic nets……………………………………………………………………………Family: Labyrinthulidae

Single genus: *Labyrinthula*

1. Globose or subglobose vegetative cells not enrobed in ectoplasmic nets…………………………………………………………………………………………………2

2. Migration of vegetative cells observed in an extended period and aplanospores always formed……………………………………………………………………Family: Aplanochytriidae

Single genus: *Aplanochytrium*

2. Migration of vegetative cells, when present, at early stage………………………………………………………………………………………………3

3. Vegetative cells without ectoplasmic nets; associated *with* shells of *Ostrea* …………………………………………………………………………………Family: Althorniidae

Monotypic genus: *Althornia crouchii*

3. Vegetative cells with an ectoplasmic nets………………………………………………4

4. Vegetative cells with ectoplasmic nets emerging from two apical points on the cell (filopodia)………………………………………… ……………………………………Family: Diplophryidae

Single genus: *Diplophrys*

4. Vegetative cells with basal rhizoid-like ectoplasmic nets…………………………………5

5. Vegetative cells with apophysis in ectoplasmic nets; associated with red algae [on *Gracilaria;* sorus a zoosporangium; zoosporangium 16-30 × 10-20μm, thick-walled, wall persistent except for an apical pore; apophysis 10-13 × 5-10 μm; zoospores 4.0-5.0 μm diam., motile within the sporangium]……………………………………*Japonochytrium marinum*

5. Vegetative cells without apophysis in ectoplasmic net element………………………………6

6. Monoflagellate zoospores, one single anterior flagellum (doubtful taxa) …………………7

6. Biflagellate zoospores, long anterior flagella (tinsel flagellum) possesses mastigonemes hairs while the shorter posterior flagellum (whiplash) is smooth……………………8

7. Zoosporangial wall thick (>4 μm) [zoospores with a quiescent phase before swimming away]. Zoosporangia 8-20 μm diam …………………………………………*Elina sinorifica*

7. Zoosporangial wall thin (<2 μm) and completely dispersing at the time of zoospore release. Zoosporangia 20-30 μm diam.; zoospores 4.0-5.0 × 6.0-7.0μm…………………………………………… ………………………………………*Elina marisalba*

8.	Thallus occurring successive binary division (mitotic cellular division) of vegetative cells (thence developing secondary sporangia)...9

8.	Thallus developing into a single zoosporangium or amoeboid cell..............................15

9.	Individual cells > 6 µm diam...10

9.	Individual mature cells < 5 µm diam...13

10.	Large colony, well-developed ectoplasmic net. Ovoid zoospores 2.5-5.0 × 4.0-7.5 µm, trophic cells 6-12 µm diam. [cell masses (sori) of many sporocytes, 30-140 µm diam.; zoosporangial tetrads 13-29 µm diam.; each zoosporangium producing 32-64 zoospores; colony orange in mass and producing only ß-carotene and c.a 20% arachidonic acid.............................. ....*Schizochytrium aggregatum*

10.	Not well-developed ectoplasmic net..11

11.	No amoeboid stage. Cell masses (sori) of few sporocytes (<.12); trophic cells 6-9 µm diam.; each sporocyte becoming transformed into a single zoospore; [zoospore 2.6-3.3 × 4.1-4.8 µm]...*Aurantiochytrium mangrovei*

11.	Amoeboid stage sometime observed...12

12.	Cell masses (sori) of many sporocytes; trophic cells 6-15 µm diam.; each sporocyte undergoing mitosis to produce 2-8 zoospores. Zoospores 5.9-7.0 × 6.0-8.5 µm; trophic cells spherical, 7-15µm diam. (zoosporangia 12-24 µm diam. producing 16-64 zoospores; trophic cells forming limaciform binucleate amoeboid cells 12-20 × 5.0 8.0 µm with fine pseudopodia; rounded amoeboid cell forming 8 smaller zoospores, 3.5-5.0 × 4.5-6.0 µm)......................... ....*Aurantiochytrium limacinum*

12.	Trophic cells fusiform 2.1-4.0 × 2.2-6.5 µm to orbicular 2.3 × 7.8 µm. Circular large cell masses up to 60.3 µm diam. Subcentrally located nuclei and contractile vacuoles. Ectoplasmic nets 1.0 – 1.3 µm diam. Sporangium producing 10-55 spores but not free-swimming spores observed. Budding-like cell division with sometime amoeboid stages. Light yellow colonies....... ....*Oblongichytrium porter* sp. nov.

13.	Small compact sori 10-15 µm diam. releasing aggregates of small sporocytes [sporocytes producing <10 zoospores; zoospores 2.5-3.5 × 4.0-5.5 µm] ....
....*Thraustochytrium aggregatum*

13.	Oblong zoospores and producing canthaxanthin and ß-carotene, and ca. 20% docosapentaenoic acid. Well-developed ectoplasmic net and large colonies.....................14

14.	Cell masses (sori) of many sporocytes (ca 100); each sporocyte producing 8 zoospores [trophic cells 4-5µm diam.; zoospores 1.5-2.5× 3.5-5.0 µm.......................................
....*Oblongichytrium octosporum* [doubtful classification]

14.	Cell masses (sori) of few sporocytes (<8)..15

15.	Small cell masses (sori) (<8 µm diam.) composed up to 8 sporocytes (tetrads or octads of sporocytes); each sporocyte giving rise to 2 zoospores [zoospores 1.7 x 2.5 x 5.0 µm].. ....*Oblongichytrium minutum*

15.	Cell masses (sori) composed of 2 (rarely 3-4) sporocytes (initially considered as proliferation bodies), each with an ectoplasmic net (each rudiment monocarpic)

[zoosporangium 11-28 × 14-37 µm; wall persistent, zoospores released through ruptures motile; zoospores 2.0-3.5 ×4.5-7.5 µm]...... ....*Oblongichytrium multirudimentale*

16.   Multinucleate zoosporangium developing directly from a primary trophic cell. If amoeboid stage, it is not a prominent part of the life cycle……………………………………………………………………………………………………17
16.   Multinucleate zoosporangial contents released before cleavage as an amoeboid (undulate) protoplast………………………………………………………………………………30

17.   Trophic cell or thallus eucarpic with a persistent (apocytic) single or numerous fundaments (proliferation bodies) giving successive zoosporangial production………………18
17.   Trophic cell or thallus monocarpic and non-proliferous……………………………………25

18.   Single fundament or proliferation body produce……………………………………………19
18.   More than one fundaments or proliferation bodies……………………………………………24

19.   Proliferation body delineated prior to zoospore formation; prominent……………………20
19.   Proliferation body delineated during zoospore formation, not prominent……………22

20.   Zoospores non-motile at the time of release, lacking flagella; developing flagella subsequently; [zoosporangia 10-20 x 15-29 µm; zoospores 4.0 µm diam.]……………………………………
……………………………………………………………………………*Thraustochytrium proliferum*
20.   Zoospores motile even at the time of release, with fully motile flagella…………………21

21.   Zoosporangia globose, subglobose or obpyriform, most of the cell wall persistent after zoospore release. Zoosporangium large (7-28 ×15-35 µm); wall cracking open [zoospores 20.40 × 31-60 µm]………… ………………………………… …………*Thraustochytrium motivum*
21.   Zoosporangia obpyriform, most of the cell wall disintegrates during zoospore liberation, leaving just a collar around the zoosporangium. Cleavage planes primarily radial, central vacuole expanding to separate zoospores [zoosporangium 14-17 × 17-19 µm; rupturing in one or more places; [zoospores 2.5-3.0 x 4.5-4-8 µm]…..*Thraustochytrium kinnei*

22.   Zoosporangia small than 20 µm; not more than 50 zoospores per zoosporangium; no amoeboid stages produced during life cycle…………………………………………………………………23
22.   Zoosporangia often up to 30 µm diam; up to 75 zoospores produced: Amoeboid stages present during life cycle [zoosporangium diameter (15-30 µm); zoospores 5.5-7.3×3.0-4.5 µm; amoebae 25-40× 12.5-27.5 µm]…………………….*Thraustochytrium gaertnerium*

23.   Zoosporangia irregular in shape; zoospores ovoid; flagella apical and subapical; [zoosporangial diameter very variable (8-30µm); zoospores 3.0-3.5 ×3.5-6.3 µm]…………………………………………………… ………………………………*Thraustochytrium benthicola*
23.   Zoosporangia up to 17 µm diam; less than 50 zoospores produced; flagella lateral; wall thin, opening distally [colonies golden in mass; zoospores 2.0-4.0 x 3.0-6.0 µm]……………………………… ………………………… …………………………*Thraustochytrium aureum*

24.   Number of proliferation bodies 5-50; zoospores not motile at the time of discharge. Zoosporangia >35 µm diam.; rudiments numerous [zoosporangia 40-50 µm diam.; wall

completely dispersing; zoospores forming flagella after a quiescent state; zoospores 2.5-3.5 x 4.8-5.5 µm]…………… ..………………………….. .…….…………….……...……*Thraustochytrium rossii*

24.   Number of proliferation bodies 3-10; zoosporangia 20-30 µm diam.; zoospores discharged as flagellated bodies in a clump, almost instantly swimming away; [zoospores 2.5-30 × 5.0-7.0 µm]……….. ..……………….……….………………*Thraustochytrium kerguelensis*

25.   No zoosporangium cell wall. Direct partition in a synchronous way to form up to 12 zoospores. Not rupture of cell wall is observed. Small cell can exhibit an extension performing a snake-like movement [zoospores 4.0-5.0 × 2.4-3.7 µm; zoosporangia 10.4 × 26.7µm] Parasitic association with *Macrostomum lignano*……..*Thraustochytrium caudivorum*

25.   Zoosporangial wall present. Rupture of zoosporangial wall observed.…….……………26

26.   Zoosporangial wall thick (> 3 um)………………………. .……….……………………….……27
26.   Zoosporangial wall thin (< 2 µm).………….…………. .…………….…….……………….……28

27.   Zoosporangial wall thick (3-5 µm) zoosporangia 15-30 µm diam. [wall thick (3-5 µm); protoplast with large lipid globules; zoospores 5.0-7.0 µm) [zoospores with a quiescent phase before swimming away]………………………………………*Thraustochytrium pachydermum*

27.   Zoosporangium wall very thick (2-6 µm) Multinucleate zoosporangial contents released before cleavage as an amoeboid (undulate) protoplast. Zoosporoplasm released in a partially cleaved state [zoosporangium 14-46 µm diam.) [pink colonies, zoospores 2.5-4.0 × 4.5-7.5 µm]………………………………………………………………….*Thraustochytrium roseum*

28.   Zoosporangial wall partially persistent.……………………………………………………….29
28.   Zoosporangial wall completely dispersing at the time of zoospore release. sporangia 14-26 × 14.6-22 µm; [zoospores 2.0-3.7 × 3.4-6.7] Ameoboid stage in presence of bacteria .………………………………………………………………………….*Thraustochytrium striatum*

29.   Basal part of zoosporangial wall persistent (zoosporangia 5-10 × 5-20 µm; zoospores 2.5 × 3.0-4.0 µm; on marine *Ulothricales*) ……………………….…… *Monorhizochytrium globosum*

29.   Zoosporangium wall disintegrating at the apex only; zoosporangia 15 µm diam. (zoospores with a quiescent phase before swimming away)…*Thraustochytrium arudimentale*

30.   Large colony, well-developed ectoplasmic net………………………………………………31
30.   Small colony, not well-developed ectoplasmic net………………………………………….32

31.   Persistence of cell wall after releasing an amoeboid cell. [zoospores 2.9 × 4.2 µm]…………………………………………………………………..…....*Parietichytrium sarkarianum*

31.   Zoosporangia, 8-18 µm diam.; contents discharged in a star-like mass, dividing by centripetal cleavage to give zoospores; zoospores 3.0-4.0 × 5.0-8.0 µm. Cell wall dissolved after releasing an amoeboid cell………………………………………….….....*Botryochytrium radiatum*

32.   Secondary assimilative cells developing directly into secondary zoosporangia [zoosporangia 6-10 µm diam.; giving 4-20 secondary zoosporangia; secondary zoosporangia (sporocytes) producing 4-12 zoospores; zoospores forming by meaning of pinching and pulling with a quiescent phase………………………………………..…….*Sicyoidochytrium minutum*

32.   Zoospore forming without pinching and pulling division.………………………………….33

33. Zoosporangium wall persistent with apical pore. Zoospores abundantly produced [zoosporangia 20-25 µm diam]; zoosporangioplasm expelled and cleaving into ca64 cells 3.5-50 µm diam., which encyst and then behave as 'zoosporangia', each releasing a [zoospore 2.5-3.5 × 5.0-6.5 µm]………………………… ……………………………………………*Ulkenia amoeboidea*

33. Zoosporangium wall dispersing at time of naked protoplast release……………………………34

34. Zoosporangium cell wall thick (> 2 µm) Colour of colony in the mass hyaline; zoosporoplasm released in an amoeboid state [zoosporangium 15-20 µm diam.; zoospores 2.5-3.5 × 4.0-5.5 µm]…………………… ……………………………………………………… *Ulkenia visurgensis*

34. Zoosporangium cell wall thin (< 1 µm). Zoosporangia 16-22 µm diam.; contents discharged in an amoeboid state, dividing to give 1-5 rudiments and numerous zoospores; zoospores 2.9-4.0 × 3.5-4.8 µm…………………………………………………………………*Ulkenia profunda*