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DOTTORATO DI RICERCA IN
SCIENZE AGRARIE E AMBIENTALI
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Interactions in the phycosphere

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Summary

The interactions occurring in the phycosphere between algae and bacteria are of different kinds and may influence the growth of microalgae. In the past few years, the growing interest on microalgal industrial and biotechnological applications hijacked the attention of the scientific community on the study of microalgae-bacteria interactions. A deeper knowledge of the composition of the microbiota associated to microalgae and on the mechanisms ruling microalgae-bacteria interactions may improve the current mass-culture techniques and enhance the productivity. *Tetraselmis suecica* is a green marine alga with several biotechnological applications. It is mainly used for aquaculture and cosmetic applications and for the production of bioactive compounds. Nevertheless, still little is known about the microbiota associated to *T. suecica* and its effect on the microalga growth,

In this thesis the bacterial community associated with *T. suecica* (strain F&M M33) cultures, grown in different conditions, was described through a metagenomic approach, to evaluate the presence of a stably associated bacterial community. NGS metagenetic analysis were performed on four *T. suecica* cultures, derived from the same original culture but separated for years, and kept under different growing conditions. Despite the different characteristics of the cultures (seasons and cultures size), a “core” bacterial community was identified, accounting for the large part of the total bacterial community. Among the “core” OTUs it was possible to identify 7 different genera and at least 13 different families. However the relative abundance of the taxa forming the “core” community varied strongly, indicating that growing conditions and/or external contaminations influence the relative abundance of these microorganisms. Moreover, the isolation of strains belonging to the “core” community opened an interesting chance to evaluate the effects of these bacteria on *T. suecica* growth.

The understanding of which molecules are involved in the interactions is a fundamental part in the study of microalgal-bacteria interaction. The associated bacterial population, release several compounds which may act as microalgal growth promoting features (MPG) affecting different aspects of microalgae such the composition in terms of lipids, pigments or cell dimensions. However, to date only few studies have phenotypically characterized bacteria associated with microalgae for the selection of microalgal growth-promoting bacteria. Therefore, in this thesis 200 bacterial strains were isolated from different cultures of *Tetraselmis suecica*, identified through 16S rDNA sequencing and phenotypically characterized for MPG traits. 46 strains were been identified as producers of MPG compounds: either indol-3-acetic acid (IAA) and/or siderophores. 22 isolates were tested in co-cultures with the axenic microalga to their effects on *T. suecica* growth: 4 of them showed a positive effect on microalgal growth. In particular two strains: *Vitellibacter* sp. and *Sphyngopyxis flavimaris* were able to enhance considerably the growth of *T. suecica*, in the condition tested. To deepen the knowledge of the molecules involved in the interactions, the supernatants *T. suecica* cultures, axenic and in co-culture with *Vitellibacter* sp. or *Sphyngopyxis flavimaris,* were analyzed with a non-targeted metabolomics approach. The characterization of the exo-metabolome of *T. suecica*, was described for the first time. It was possible to identify culture-characteristics compounds and several compounds involved in the metabolism of vitamins. It was observed that *Sphyngopyxis flavimaris* produce IAA also in co-culture conditions.

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Chapter 1

Introduction

1.1 Interactions in the phycosphere

The most important ecological associations in aquatic environments are microalgal-bacterial relationships (Falkowski 2008), which play an important role in driving oceanic biogeochemical cycles. They strongly influence carbon and nutrient cycling, regulate the productivity and stability of aquatic food webs, and affect ocean–atmosphere fluxes (Azam & Malfatti 2007, Cole 1982, Yoch 2002). Microalgae with their photosynthetic activity regulate global carbon and oxygen fluxes in water environment, generating O_2 during photosynthesis and fixing about 40% of the total carbon (Azam & Ammerman 1984). They are also indirectly involved in climate regulation, since they synthesize the climatically active compound dimethylsulfoniopropionate (DMSP). DMSP oxidation products give acidic sulfate aerosols that act as cloud condensation nuclei and reflect sunlight (Falkowski 1994, Field *et al.,* 1998).

The nature and the consequences of microalgae-bacteria associations need to be deepened by research. They have been mostly studied at macro-scale level since the beginning, but more recent evidences indicate that such relationships are governed by microscale interactions, since they take place in the region immediately surrounding phytoplankton cells (Seymour *et al.,* 2017).

The zone rich in nutrients that surrounds microalgal cell is named “phycosphere” due to the several similarities existing with the more known “rhizosphere” (Bell & Mitchell, 1972) (**Fig.1**). Phytoplankton and plant roots both alter the chemical environment in their surroundings, changing the levels of oxygen and values of pH, and releasing a large array of organic compounds such as amino-acids, fatty acids, peptides, sugars, and vitamins (Grossart & Simon, 2007; Thornton, 2014; Azam & Malfatti, 2007; Philippot *et al.,* 2013). Phycosphere bacteria colonization is regulated by chemotaxis, a range of phytoplankton exudates attract marine bacteria, as happens within roots, where exudates stimulate the motility of soil bacteria, allowing the colonization of the rhizosphere (Bell, W. & Mitchell, 1972; Seymour *et al.,* 2009, Philippot *et al.,* 2013; Sonnenschein *et al.,* 2012). There is also a phylogenetic similarity between the microorganisms found in the two environments, for instance members of *Rhizobium* and *Sphingomonas* genera, which are known as Plant-growth-promoting bacteria, have been widely identified in green algae cultures (Ramanan *et al.,* 2015) (**Fig 1**).

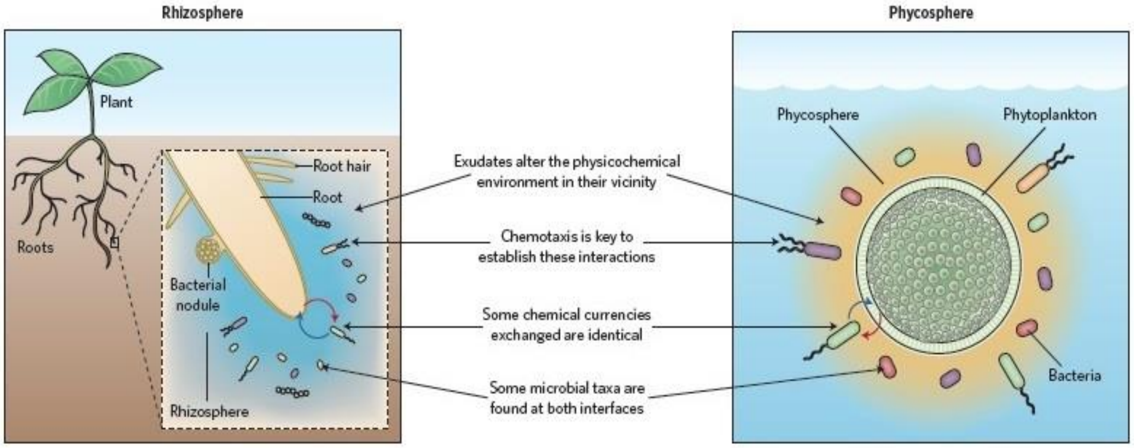

Figure 1. The similarities between the phycosphere and the rhyzosphere. The phycosphere is the aquatic analog of the rhyzosphere where several elements are condivided. The nature of the chemical exudates and of the compounds exchanged, the mechanism of chemotaxis that leads the colonization, the phylogeny of several bacterial taxa present in the two environments are the same (Seymour *et al.*, 2017)

Algae and bacteria have coexisted since the early stages of evolution, and such coevolution revolutionized the life on earth in many aspects (Ramanan *et al.*, 2016). The plastids are originated from an endosymbiosis event, definite as the "primary endosymbiosis", between an early eukaryotic cell and a cyanobacterium. The heterotrophic eukaryotic ancestor absorbed the cyanobacterium that through time transformed itself into an organelle, allowing eukaryotes to conquer the photosynthesis (Curtis *et al.*, 2012; Yoon *et al.*, 2004). From the primary endosymbiosis event the main groups of algae were originated: grenn algae, glaucophytes, and red algae (**Fig 2**). Afterwards the other existing algal groups were originated through the secondary and the tertiary endosymbiotic events (**Fig 2**) (Cooper and Smith 2015). The plastids of red and green algae were transferred to other eukaryotes giving birth to diatoms, giant kelp, bloom-forming haptophytes and toxic dinoflagellates, some of the most abundant and ecologically important aquatic photosynthesizers on Earth (Curtis *et al.*, 2012).

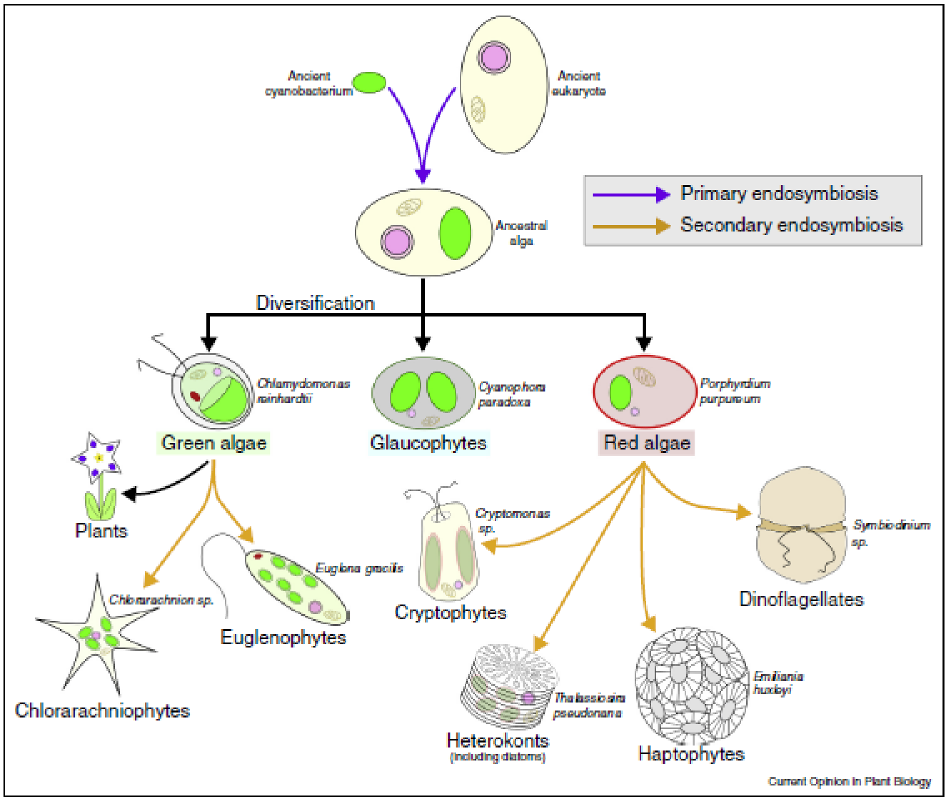

Figure 2. Scheme of the origin of algal major groups and their phylogeny. The ancestor of algae is thought to derive from a primary endosymbiotic event (blue line), between a heterotrophic eukaryote and a cyanobacterial partner. From this the three basal algal groups were originated: green algae, red algae and glaucophytes. From secondary (yellow line) and tertiary endosymbiotic events originated all the algal lineages (Cooper and Smith 2015).

Across time and through endosymbiosis events, algae evolved higher complexity till multicellularity (Herron *et al.*, 2009, Kirk, 2005). Heterotrophic bacteria associated with algae could have played a role during the secondary endosymbiosis events that led to multicellularity. A phylogenetic analysis on algae endosymbionts revealed that the endosymbionts found in the unicellular alga *Carteria cerasiformis* and those present in the multicellular algae *Pleodorina japonica* have a high similarity (Kawafune *et al.*, 2012). These endosymbionts belonging to the family Rickettsiaceae are known to be obligate intracellular bacteria mainly associated with arthropod, and considered the closest relatives of mitochondria. Several reviews have discussed the interactions between cyanobacteria, algae, bacteria and protists in a series of endosymbiotic events, which corroborate the theory of a role played by algal associated bacteria in the process of the origin of multicellularity (Decker and Holde, 2011; Keeling, 2009; Prechtl *et al.*, 2004; Thompson *et al.*, 2012; Tomitani *et al.*, 2006; Vaishnava and Striepen, 2006).

1.1.1 Microalgal-bacteria interactions

During their growth, microalgae release exudates that are utilized by bacteria for their growth. On the other hand, several studies have showed that the associated bacteria play a fundamental role in algal growth and fitness (Amin *et al.*, 2015; Gonzalez and Bashan, 2000; Kim *et al.*, 2014a; Seyedsayamdost *et al.*, 2011). However, it has been observed that nutrient availability has a key role in determining microalgae - bacteria relationships (Azam & Ammerman 1984, Legendre & Rassoulzadegan 1995). Indeed, bacteria can compete for inorganic nutrients, such as N or P, and release harmful compounds, such as algicidal molecules or exoenzymes, inducing microalgal death (**Fig 3**) (Joint *et al.*, 2002, Amin *et al.*, 2012; Natrah *et al.*, 2014; Cooper and Smith, 2015; Fuentes *et al.*, 2016; Ramanan *et al.*, 2016, Seyedsayamdost *et al.*, 2011). Although algae-bacteria interactions are represented by an array of possible relationships between two microoganisms, they could be summarized in four main types: mutualism, commensalism, competition, and parasitism.

Mutualism is a relationship, often obligate, between two or more partners of different species in which each one benefits from the other (Fuentes *et al.*, 2016, Seyedsayamdost *et al.*, 2011, Cooper & Smith 2015, Wang *et al.*, 2010). Mutualistic interactions between microalgae and bacteria are the more common interaction found in the phycosphere (Buchan *et al.*, 2014). An example of a mutualistic relationship is established between a bacterial species and an algal partner, i.e. the bacterium furnishes vitamins B1, B7 and B12 to microalgae that cannot synthesize them, in exchange for organic carbon (Croft *et al.*, 2005, Ramanan *et al.*, 2016, Croft *et al.*, 2006, Helliwell *et al.*, 2011) (**Fig 3**).

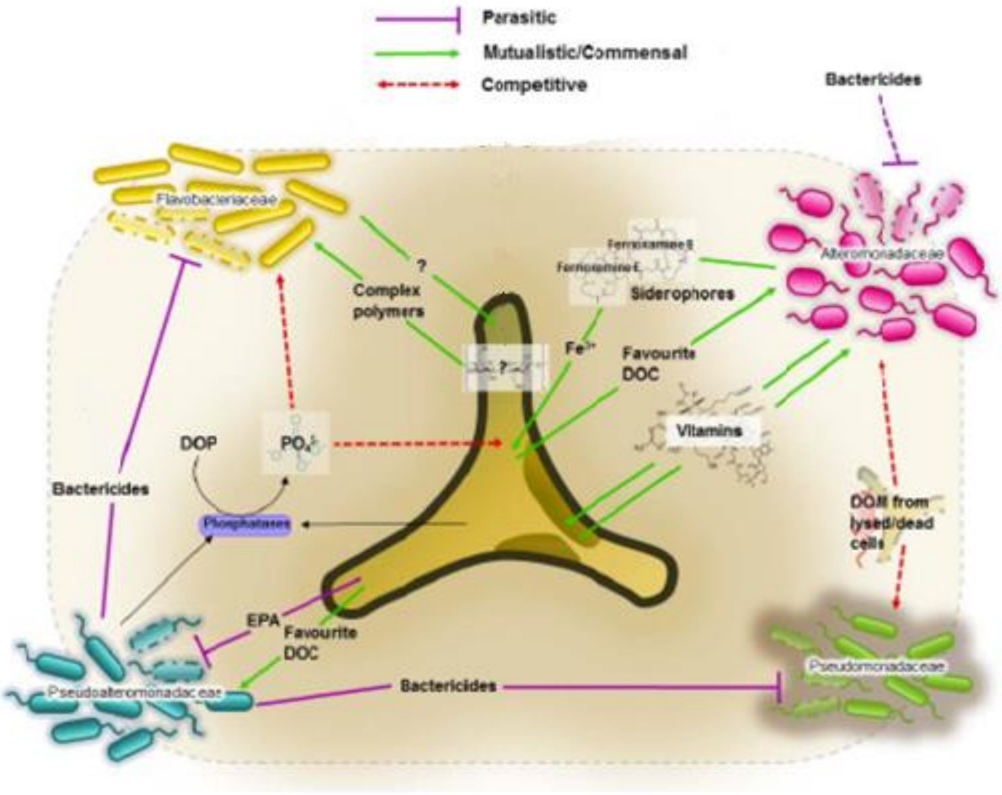

Figure 3. Network of putative interactions between *Phaeodactylum tricornutum* and identified bacterial families. The zone inside the dotted grey is the 'phycosphere'. Purple lines represent parasitic relationships ruled by the production of bactericidal compounds from certain species of bacteria against both other bacterial species and the microalga. Microalgae can excrete the fatty acid eicosapentaenoic acid (EPA) to protect themselves against opportunistic attack or pathogenic damage. Green lines represented mutualistic/commensal relationships. Compounds exchanged by both parts have different natures, from the microalga are released favourite dissolved organic carbon (favourite DOC), complex polymers and vitamins that are utilized by bacteria. In response from bacteria are produced other vitamins, enzymes phosphatases that help the solubilization of phosphate and siderophores that improve microalgal iron uptake (from Moejes *et al.*, 2017, modified).

The mutualism relationship is very complex and not fully described, and in many cases the molecules in exchange are still to be identified. Many studies pointed out the role of members of the genera *Azospirillum*, *Mesorhizobium* and *Rhizobium* in algal growth promotion (Gonzalez & Bashan 2000, Kim *et al.*, 2014a, Droop 2007, Hernandez *et al.*, 2009, Watanabe *et al.*, 2005). A *Rhizobium* strain, known to play a pivotal role in plant mutualistic interactions, when co-cultured with the green microalga *C. vulgaris* determined a marked increase in algal growth rate (**Table 1**) (Kim *et al.*, 2014a). Moreover, Cho *et al.*, (2015) obtained an enhanced growth by an artificial consortium of mutualistic bacteria cultured with *C. vulgaris,* indicating that the consortium might provide compounds that once metabolized by the alga, promote its growth (2015). A hypothesis is that these bacteria release growth promoting molecules such as phytohormones and receive amino acids and organic carbon (Kim *et al.,* 2014a). An example of how intricate and complicate may be microalgae-bacteria interactions is the relationship between *Emiliania huxleyi* and *Phaeobacter gallaeciensis* (**Table 1**). In the first phase of the algal growth, when *E. huxleyi* is healthy and provides dimethylsulfoniopropionate (DMSP), the bacterium establishes a mutualistic relationship and produces algal growth promoters and antibiotics to protect microalga from harmful bacteria. However, during the senescent phase of its life, the microalga releases P-coumaric acid that is interpreted by the bacterium as a signal of senescence, consequently the mutualist relationship turns into a negative parasitic interaction. *P. gallaeciensis* starts to produce potent algaecides that cause algal death and cell lysis (Seyedsayamdost *et al.,* 2011) (**Fig 4**).

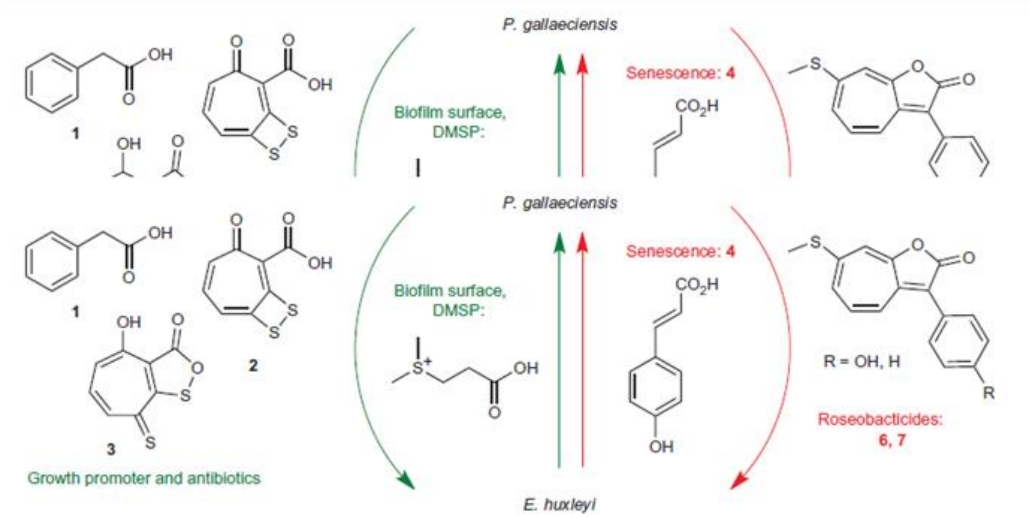

Figure 4. The shifting interaction between *Emiliana huxleyi* and *Phaeobacter gallaeciensis.* In the first phase of the algal life, there is a mutualistic relationship (green). *E. huxleyi* provduce DMSP and provides a biofilm surface to *P. gallaeciensis,* which produces algal growth promoters and antibiotics to protect the alga. When *E. huxleyi* senesces and releases the algal breakdown product P-coumaric acid, the interaction becomes parasitic (red) and *P. gallaeciensis* produce the potent algaecides (Seyedsayamdost *et al.,* 2017).

Table 1. Table summarizing the principal microalgae-bacteria interactions reported in literature, the molecules involved in the interactions ad and their effects on algal growth. Siderophores, indole-3-acetic acid and vitamin B12 are the first compounds produced by bacteria individuated to be involved in microalga-bacteria interactions. It is thought that

Microalga	**Mediatior from microalga**	**Bacterium**	**Mediatiors from bacteria**	**Type of interaction**	**Effect on microalga**	**Reference**
Ankistrodesmus sp.	DOM (?)*	*Rhizobium sp.*	Unknown	Synthetic mutualism	Chlorophyll, biomass and lipids accumulation	Do Nascimento *et al*.. 2013
Chlorella vulgaris	DOM (?)	*Rhyzobium sp*	Unknown	Synthetic mutualism	Growth enhanced	Cho *et al*.., 2015
Botrycoccus braunii	DOM (?)	*Rhyzobium sp*	acyl-homoserine lactones	Unknown	Growth enhanced	Rivas *et al*.. 2010
Chlorella vulgaris	DOM (?)	*Azospirillum brasilense*	Siderophore mediated nitrogen fixation	Unknown	Accumulation of carbohydrates/Accumulation of fatty acids and lipids	Choix *et al*.. 2012a,b Leyva *et al*.. 2014
Chlorella vulgaris	DOM (?)	*Rhizobium sp.*	Unknown	Mutualistic	Growth enhanced	Kim *et al*.., 2014a
Chlorella sorokiniana	DOM	*Azospirillum brasilense*	Nitrogen compounds	Synthetic mutualism	Accumulation of intra-cellular phosphate	de-Bashan *et al*.. 2016
Chlorella vulgaris	DOM (?)	*Azospirillum brasilense*	Indole-3-acetic acid	Unknown	Accumulation of intra-cellular phosphate	Meza *et al*.. 2015
Emiliana huxley	Dimethylsulphoniopropionate (DMSP)	*Phaeobacter gallaciensis*	Indole-3-acetic acid and antibiotics	Mutualistic	Growth enhanced	Seyedsayamdost *et al*.. 2011, Segev *et al*.. 2016
Emiliana huxley	p-coumaric acid	*Phaeobacter gallaciensis, Phaeobacter inhibens*	Roseobacticides	Opportunistic pathogen	Cell lysis adn death	Seyedsayamdost *et al*.. 2011, Segev *et al*.. 2016
Lobomonas rostata	DOM (?)	*Mezorhyzobium loti*	Vitamin B12	Mutualistic and facultative	Growth enhanced	Kazamia *et al*.. 2012
Neochloris oleoabundans , Scenedesmus sp.	DOM (?)	*Azotobacter vinelandii*	Siderophore azotobactin as a source of nitrogen	Commensalism	Heterotrophic accumulation of starch and carbohydrates	Villa *et al*.. 2014
Pseudo-nitzschia multiseries	Triptiophan	*Sulfitobacter sp.*	Indole-3-acetic acid	Mutualistic	Promoted cell division	Amin *et al*.. 2015
Thalassiosira pseudonana	Dimethylsulphoniopropionate (DMSP)	*Ruegeria pomeroyi*	Vitamin B12	Unknown	Growth enhanced	Durham *et al*.. 2015
Scrippsiella trochoidea	DOM	*Marinobacter sp, Roseobacter sp*	Siderophore Vibrioferrin	Unknown	Increased iron uptake	Amin *et al*.. 2009

(?) = indicates that is thought to be released by the micrgoalga but no evidences have been reported. Still a lot has to be understood in microalga-bacteria interactions

Commensalism is defined as a relationship in which only one partner benefits from the other. Although the differences between the interactions (commensalism/parasitism and commensalism/mutualism) may be hard to define, commensalism should be considered as a theoretical interval within the continuum of interactions (Zapalski *et al.,* 2011). There are studies that have been showed that in the phycosphere the role of nutrient availability, such as N and P, and light intensity, could be responsible of the shift from mutualism to parasitism and *vice versa via* commensalism (Ramanan *et al.,* 2016**,** Gurung *et al.,* 1999). A complex relationship can be established between the green algae *Neochloris oleoabundans* if co-cultured with *Azotobacter vinelandii* (**Table 1**). The microalga utilizes as a source of nitrogen the siderophore azotobactin produced by *A. vinelandii.* The bacterium is able to fix nitrogen under aerobic conditions and in the presence of reduced carbon sources such as sucrose or glycerol, furnished by the microalga. *A. vinelandii* produces also other siderophores to scavenge different metals from the environment, which are used by the microalga to grow (Villa *et al.,* 2014), indicating a commensalistic relationship and providing a proof of concept for developing a mutualistic relationship between the two microorganisms (Villa *et al.,* 2014).

Kazamia *et al.,* 2012 showed that in co-cultures of *Chlamydomonas reinhardtii* with the bacterium *Mesorhizobium loti,* the heterotrophic bacterium furnishes vitamin B12 to the microalga, consequently the microalga reduces the expression of its gene for the biosynthesis of such vitamin, whereas, in this case, the bacteria seems to not benefit from the organic carbon released by the alga (**Table 1**).

Competition is a microbial interaction that occurs between microorganisms inhabiting a common environment and contending the same nutrients and resources. An example can be found comparing the growth of both axenic and non-axenic *Scenedesmus ubliquus* cultures. When axenic and non-axenic *Scenedesmus ubliquus* cultures were grown in phosphate limited media, a significant decrease in the growth of non-axenic microalga culture was observed, suggesting a competition for nutrients by the bacteria (Danger *et al.* 2007). Moreover, Bratbak and Thingstad (1985) investigated a paradoxical ecological relationship between phytoplankton and bacteria, using a simple mathematical model. They simulate the algal growth in mineral nutrient limitation as a condition that stimulates the excretion of extracellular organic carbon by phytoplankton, which lead to an increase of bacterial growth. Bacterial growth requires additional uptake of mineral nutrients, originating a paradoxical situation where stressed algae by lack of mineral nutrients respond stimulating their competitors for nutrients lacking.

Parasitism can be defined as an interaction in which one species benefits at the expense of another one. Several bacteria are known to negatively affect microalgae, therefore, a growing number of studies propose them as microalgae and cyanobacterial bloom controlling microorganisms (Kim *et al.*,2008, Lee *et al.*,2010, Wang *et al.*,2010). As an example, seven algicidal bacteria were isolated in the phycosphere of the dinoflagellate *Cochlodinium polykrikoides* and were able to contain the microalgal blooms causing cell lysis (Kim *et al.*,2008). Moreover, phycospheric bacteria release enzymes such as *β*-glucosidase and chitinase that can directly cause microalgal cell lysis (Wang *et al.*,2010). Once the algal cell is lysed, the bacteria can use intracellular algal compounds as nutrients. On the other hand, some microalgae have developed a defense mechanism. Even if the mechanism it has still not been elucidated, the diatom *Chaetoceros didymus* resulted resistant to the attack of the bacterium *Kordia algicida* (Carsten & Pohnert 2011).

1.1.2 The role of specific substances in microalgal growth

Phycospheric bacteria produce a complex panel of compounds that influence the microalgal growth. Even if the identification and the individuation of the functions of several compounds are still a subject of study, a growing number of works identified siderophores, phytohormones, and vitamins as the main currency of exchange from phycospheric bacteria to microalgae.

The importance of iron on the growth and species composition of algal communities in the oceans is well documented (Amin *et al.*,2009). Iron represents an essential element for organisms due to its role in the photosynthesys, mitochondrial redox processes and in nitrate assimilation (Allen *et al.*,2008). However, in the marine environment iron is often present in concentrations limiting the growth of microorganims (estimated below 0.2 nM and on average 0.07 nM) (Johnson *et al.*,1997, Wells *et al.*,1995, Moore *et al.*,2012). In addition, most of the iron is bound to organic ligands and its bioavailability depends on ligand species (Naito *et al.*,2008, Gledhill & Buck 2012, Rue & Bruland 1995). Iron limitation induces chlorosis and reducing chlorophyll a synthesis. In particular photosystem

I (PSI) and ferredoxins can be affected (Richier *et al.*,2012, Jordan *et al.*,2001). To improve iron availability many microorganisms, such as marine bacteria, produce siderophores, small high-affinity iron chelating molecules needed to transport iron through cell membranes (Kraemer 2005) (**Fig 5**). The bioavailability of iron for many species of microalgae depends on their close interaction with some species of bacteria during blooms formation. Several isolates of the genus *Marinobacter*, ubiquitously found in close association with dinoflagellates and coccolithophores, were found to produce the siderophore vibrioferrin (**Fig 5a**), a member of the citric-acid siderophore class (Amin *et al.*,2009). The addition of a *Marinobacter* bacterium to a culture of *Scrippsiella trochoidea* lead to an increase of the growth rate and cell dimensions, indicating that the bacterium promotes algal assimilation of iron (Amin *et al.*,2009).

Siderophores are photoreactive when chelated to Fe(III), thus under natural sunlight they form the siderophores-Fe(III) complex and then release Fe(II) that may utilized by algae (Amin *et al.*,2009, Butler 2005) (**Fig 5b**).

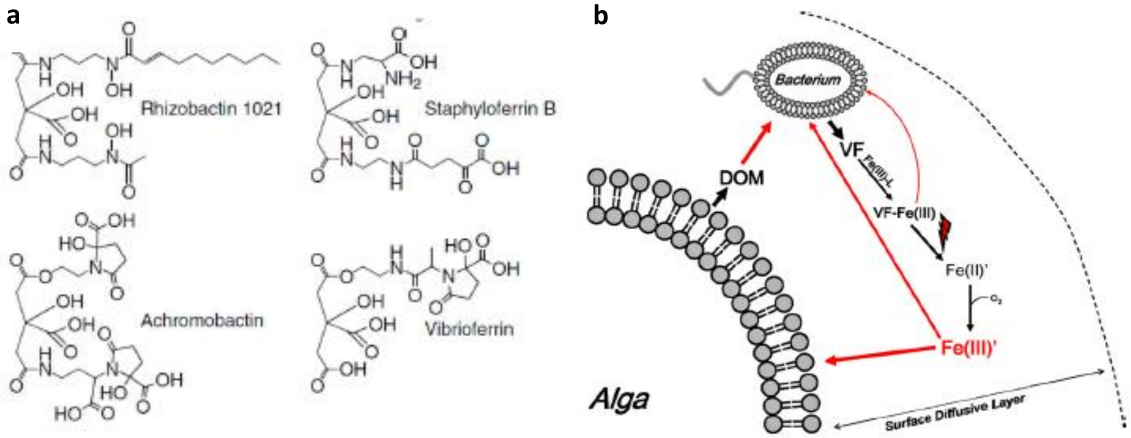

Figure 5. a) Citric-acid-containing siderophores, like Vibrioferrin: Rhizobactin, Staphiloferrin B, and Acromobactin (Modified from from Butler 2005); **b**) Mutualistic interaction between alga and bacteria based on the photoreductive dissociation of Fe(III)-Vibrioferrin (VF). The microalga secrete fixed carbon to support bacterial growth and VF production. Vibrioferrin, produced by bacteria binds iron in the dark, but is photolyzed with the light and release Fe(III) that is then assimilated by both organisms (Amin *et al.*,2009).

The indole-3-acetic acid (IAA) is the most common plant hormone of the auxin class. It regulates various aspects of microalgae and plant growth and development (Fu *et al.*,2015). IAA is also produced by bacterial pathogens and symbionts of plants and algae, to manipulate growth and development of their hosts by producing excess exogenous IAA or deviating the IAA biosynthesis pathway of their hosts (Labeeuw *et al.*., 2016).

IAA production can affect P metabolism of microalgae. A study on *Chlorella vulgaris* grown in alginate beads together with *Azospirillum brasilense* strains producing IAA or with their IAA deficient mutants, showed that higher amount of intra-cellular phosphate in *Chlorella* was accumulated when the microalga was grown in presence of wild type strains (de-Bashan *et al.,*2008, Meza *et al.*,2015). Algal intra-cellular phosphate accumulation was related with the amount of IAA produced (**Fig 5**). An important study by Amin *et al.,* (2015) showed an interaction between the coastal diatom *Pseudonitzschia multiseries* and a member of the genus *Sulfitobacter* (SA11) that can be defined as "species-specific". The bacterium SA11, using endogenous and diatom secreted tryptophan, promoted the diatom cell division through the secretion of synthetized IAA. These results demonstrated that IAA and tryptophan acted as signaling molecules in the diatom-bacteria relationship (Amin *et al.*,2015). These molecules are considered as part of a complex exchange of nutrients, including diatom-excreted organosulfur molecules and bacterial-excreted ammonia (**Fig 6**).

The role played by vitamins in microalgal metabolism has been widely demonstrated in literature. Vitamins of the B group, such as cobalamin (B12), biotin (B7) and thiamine (B1), have been shown to be fundamental for the growth of several species (Tang *et al.*, 2010, Croft *et al.*, 2005). Vitamin B1 acts as a co-factor for some enzymes in the citric acid cycle. Vitamin B1 it is also involved in the biosynthesis of branched-chain amino acids such as valine, isoleucine and leucine (Bertrand and Allen 2012).

Vitamin B12 is mainly part of enzymes methionine synthase, which is involved in DNA synthesis, and methylmalonyl CoA mutase, which is required for inorganic carbon assimilation (Panzeca *et al.,* 2006). Vitamin B7, also known as vitamin H plays a critical role as a co-factor in carbon dioxide metabolism for various carboxylases enzymes (Croft *et al.,* 2005), which are involved in numerous metabolic processes such as gluconeogenesis, citric acid cycle, regulation of gene expression, branched chain amino acid catabolism, and fatty acid biosynthesis (Cui *et al.*, 2012).

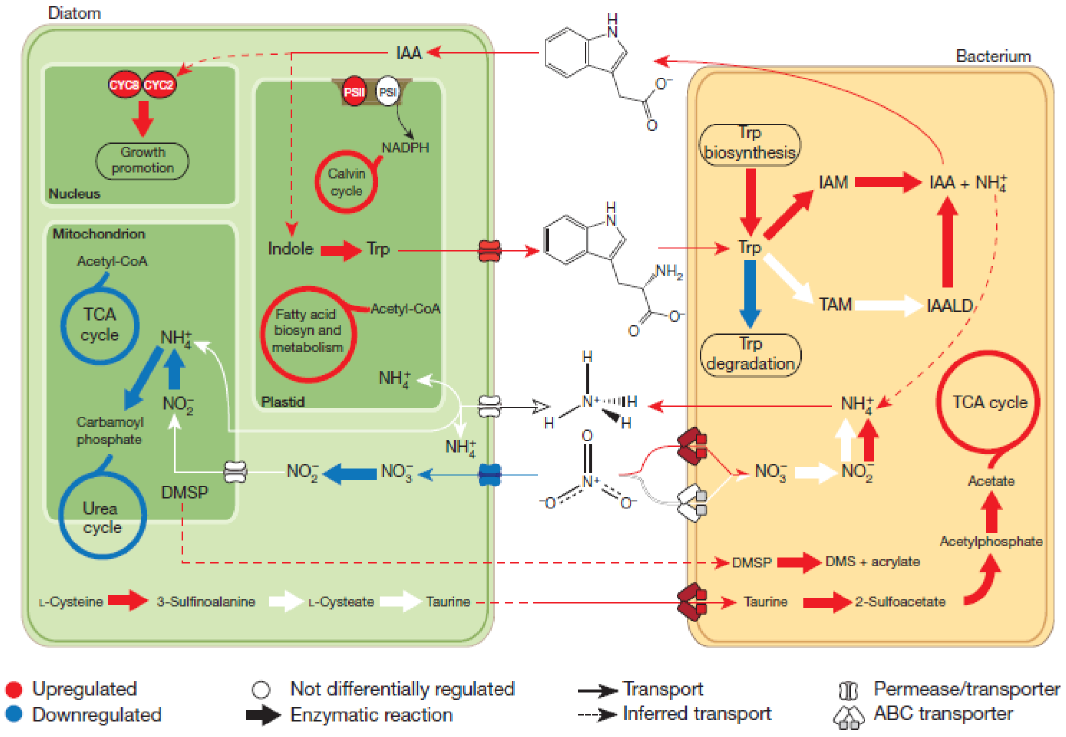

Figure 6. Model of *P. multiseries–Sulfitobacter* interactions based on transcriptomic and targeted metabolite analyses. Molecules with a structure indicate detection in the co-culture supernatant. Genes/transporters/metabolic cycles are shown as upregulated (red), downregulated (blue), or not differentially regulated (white) in co-culture relative to monocultures. Metabolic cycles were assigned an expression pattern if at least one gene specific for the cycle was differentially expressed and no others were regulated in the opposite direction. Trp, tryptophan; DMS, dimethyl sulfide; PSI, PSII, photosystem I, II; CYC2, CYC8, cyclins 2 and 8; IAALD, indole-3-acetaldehyde (Amin *et al.* 2015).

In a survey by Tang *et al.*, (2010) 27 species of phytoplankton involved in Harmful Algal Blooms (HAB's) were investigated by comparing the growth in vitamin free and complete media. 26 species of microalgae were vitamin B12 dependent, 20 species required B1, and 10 species required B7. In 2005 Croft *et al.*, compiled a survey of 326 species of phytoplankton vitamin needs, reporting 171 species as B12 auxotrophs. Moreover, to confirm literature data they tested in vitro a representative number of species from each phylum in minimal growth media for B12 dependency. Subsequently Croft *et al.*, (2006) reviewed the requirement for B1 and B7 for 306 algae: 61 species required vitamin B1, and 14 species vitamin B7. Notably, they found a correlation between the need of B7 and the need of B1 and/or B12.

Kazamia *et al.*, (2012) showed that not all B12 producing bacteria are able to support phytoplankton growth. For instance, when three different *Rhizobial* strains were added to an axenic culture of *Lobomonas rostrate*, only one was able to sustain algal growth in an extended sub-culture system. Xie *et al.,* (2013) found that the cobalamin-independent methionine synthase (METE) gene expression in *Chlamydomonas reinhardtii* is reduced by heat stress that lead to cell die, whether B12 is not available. However, the cobalamin-dependent methionine synthase METH-mediated methionine expression is activated both adding B12 in the media, or co-culturing with B12 producing bacteria, scavenging heat stress.

1.2 Microalgal-bacterial consortia applications

Besides their fundamental ecological role in biogeochemical cycles, microalgal-bacterial interactions are receiving increasing attention by scientific and industrial community for their environmental and biotechnological applications.

One of the first studies on microalgal-bacterial application regarded their use in the control of Harmful Algal Blooms (HABs) (González *et al.* 2000). HABs are due to an impressive algal growth with the production of toxic or harmful effects on fish, shellfish, marine mammals, birds and also humans, therefore it is of particular importance to find solutions, especially when concerning fresh waters for human use. Indeed bacteria living in the phycosphere can inhibit the growth of algae or even lyse algal cells (Lee *et al.*, 2000, Su *et al.*, 2007a). For algal lytic bacteria an important role in the control and elimination of the algal blooms was proposed (Mayali and Azam, 2004, Zheng *et al.*, 2005). The biomass of bacteria grows dramatically during the decline of the algal bloom, due to the increasing level of organic compounds released by the microalga. Therefore, keeping under a close surveillance for a long time microalgal-bacterial populations, could be meaningful for the control of the blooms (Srivastava *et al.*, 2014; Srivastava *et al.*, 2013). Another example of control bloom-forming microorganisms was represented by *Rhodococcus* strain, a typical bacterium found in eutrophic lakes, which suppressed bloom-forming cyanobacteria *Microcystis aeruginosa* producing cyanobactericidal compounds (Lee *et al.*, 2010).

Microalgal–bacterial consortia have been widely studied in the treatment of nutrient rich wastewater since the 1950s (Ramanan *et al.*,2016). One of the earliest descriptions of algal–bacterial interactions was presented by Oswald and Gotaas (1957). The mutually beneficial relationship established between microalgae and bacteria is employed to remove at the same time organic matter and mineral nutrients.

Heterotrophic bacteria use organic carbon for their growth, while microalgae fix carbon dioxide and immobilized mineral nutrients produced by the bacterial mineralization, producing oxygen that is required by aerobic bacteria (Mu˜noz and Guieysse, 2006). Together, algae and bacteria have enormous environmental potential in wastewater treatment. To date, several agro-industrial wastewaters can be treated with the system of high rate algal ponds (HRAP), that at the same time guarantee possibility for sewage treatment and biofuel production from microalgal biomasses (Craggs *et al.*, 2011; Kang *et al.*, 2014; Kim *et al.*, 2014b; Park *et al.*, 2011, 2013). Indeed, biomass generated in the process can be also used as a raw material for biofertilizer (Mulbry *et al.*, 2005, 2010). In addition to nutrients removal from the wastewater, several toxic metal ions may be immobilized and removed by microalgal growth. The role of microalgal-bacterial interactions has been proposed in the degradation of thiocyanate wastewater. Ryu *et al.*, (2015) demonstrated that a microalgal and bacterial consortium can effectively remove not only thiocyanate but oxidize nitrogen produced, extending the idea to use this method to degrade toxic substrates..

A metal deprivation results in stress responses, while a metal accumulation can cause toxicity (Glaesener *et al.*, 2013; Kropat *et al.*, 2015; Malasarn *et al.*, 2013). Algal–bacterial mutualistic relationship allows to detoxify and/or to assimilate metals from metal from rich environments, maintaining the right equilibrium. An algal–bacterial consortium formed by of *Chlorella sorokiniana* and *Azospirillum brasilensis* actively metabolized salicylate with a subsequent removal of heavy metals with different efficiency: copper more efficiently than nickel, cadmium and zinc (Muñoz and Guieysse, 2006).

Degradation of organic pollutants by microalgal–bacterial consortia has been investigated by many studies (Tang *et al.*, 2010b). In addition to the degradation of toxic pesticides such as DDT, atrazine and α-endosulfan, algal–bacterial interactions effectiveness in degrading organophosphate insecticides, was demonstrated (Subashchandrabose *et al.*, 2011, 2013). Biodegradation of toxic aromatic pollutants is photosynthesis-enhanced, and in the microalgal–bacterial microcosms the degradation of N-containing organic compounds can be more efficient (Subashchandrabose *et al.* 2011). As an example, the complete degradation of acetonitrile in a column photobioreactor was obtained by the consortium composed by *Chlorella sorokiniana* and the acetonitrile-degrading bacteria *Comamonas* (Muñoz *et al.*, 2005a). Black oil, acetonitrile, phenol, naphthalene, benzopyrene, dibenzofuran, are successfully degraded by bacteria/cyanobacteria and algae (Mahdavi *et al.*, 2015; Muñoz and Guieysse, 2006; Subashchandrabose *et al.*, 2013).

It is now accepted the idea that in all biotechnological applications, microalgae growth constantly with a microbial consortia associated, which influences microalgal productivity. Studies on co-cultivation between microalgal growth promoting bacteria (MGPB) and microalgae showed the ability of bacteria to enhance algal growth rate, and the utility of MGPB in mass cultivation to ameliorate the productivity (Patidar *et al.*, 2018). Furthermore, other studies demonstrated that when the microbial population is removed, the algal growth rate declined considerably (Cho *et al*, 2015b, Watanabe *et al.*, 2005). Microalgal-bacterial consortia are of particular commercial interest for their application in the pharmaceutical and nutraceutical industries. The multitude of high-value biomolecules, produced by microalgae, has several actual and potential applications (Padmaperuma *et al.* 2018). Moreover, microalgae have been individuated as ideal candidates for the production of new sources of renewable energy. During the past two decades, the interest in finding new sources of renewable energy has led to increasing research efforts to generate new and wide sources of biofuels (Wijffels *et al.*, 2010). Microalgae not only are capable of biofuel production, but are also simple microorganisms in terms of nutrient requirements and manipulation, facts that makes them particularly suitable for biofuel production (Machado & Atsumi 2012, Mata *et al.*, 2010). Bacterial communities associated with microalgae represent a key factor in microalgal biomass and lipid production and may stimulate or inhibit growth of biofuel-producing microalgae (Wang *et al.*, 2016). Recently a system using a mixed population of the microalgae *Chlamydomonas* sp. and *Scenedesmus* sp. and the mutualistic bacteria *Rhizobium* sp., was tested for coupled biohydrogen and biogas production (Wirth *et al.*,2015). Although it was possible to obtain from the microalgal-bacterial biomass hydrogen and biogas, the total production did not reach the levels obtained from biogas production from maize silage (Wirth *et al.* 2015). Notwithstanding all the recent progress and studies, to date there are no commercial biofuels derived from microalgae available, yet. Therefore, to obtain higher algal growth rates in mass cultivation, and improving current biotechonolgical applications of microalga-bacteria consortia, a constant monitoring of algae-bacteria community and a deeper knowledge of the mechanisms that rule the interactions, is needed (Cho *et al.*, 2015a).

1.3 *Tetraselmis suecica*

Tetraselmis (Stein 1878) is a green marine microalga belonging to the order of *Chlorodendrales*, of phylum *Chlorophyta* (**Fig 7**). Members of this genus are equally diffused in both marine and freshwater ecosystems, with several species. Their habitat range is mainly limited by water depth, which means nutrients and light availability (Norris *et al.*, 1980). Marine species are more known to occur in dense populations, causing blooms in tide pools or bays. Therefore, *Tetraselmis* species gained importance in studies for the understanding of dynamics of plankton growth. Moreover, even if the most part of the species are found as free living, *Tetraselmis* sp. may also occur as a symbiont of marine animals (Provasoli *et al.,* 1968). However, the main interest addressed to this microalga concerns its various applications in the market. It is actually commercialized by several companies as concentrated vital paste or lyophilized powder (Chini Zittelli *et al.*, 2006, Tredici *et al.*, 2009). Moreover, *Tetraselmis*, thanks to its high content in lipids, it has been proposed for biofuel production (Bondioli *et al.*, 2012, Yao *et al.*, 2012). The main current applications of *Tetraselmis* regard its use in aquaculture for the growth of young animals (Tredici *et al*,.

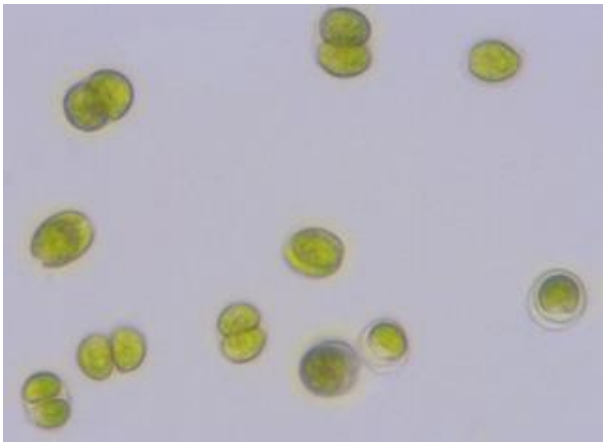

Figure 7 . *Tetraselmis suecica* culture viewed on a microscope showing cell in their common non-motile vegetative phase, some cells are performing cell division.

2009), as food for all the growth stages of bivalve molluscs, larval and juvenile stages of crustaceans and fishes, and to the growth of zooplankton, that consequently is used as food for fishes (Pulz&Gross 2004, Khatoon *et al.*, 2007, Banerjee *et al.*, 2010). It is also used for the cultivation of rotifers and *Artemia*, which are used as food for other animals. Indeed, *Tetraselmis* is actually used in "green water" technique, and is known for its antibacterial activity versus pathogens encountered in aquaculture (Regunathan and Wesley 2004, Makridis *et al.*, 2006) and for its potential probiotic action on fishes (Irianto and Austin 2002). *Tetraselmis* required for fishes nutritional purposes are grown in photobioreactors, but can also be cultivated directly in the same ponds of the animals. In aquaculture the "green water" technique consists of microalgae added directly to the water in the rearing tanks and co-cultured with the animals (Faulk & Holt 2005, Hargreaves 2006). In this way is obtained a co-culture where algae are used as nutriment by rotifers or by *Artemia* sp., which are consequently eaten by larvae, recreating the food chain present in the natural environment of fishes. Several studies demonstrated that this technique is able to increase the survival and growth rate of larvae (Rocha *et al.*, 2008, Lio-po 2005). Moreover, it has been demonstrated that several microalgae are able to interfere with quorum sensing, that regulates virulence of different aquaculture pathogens. This particular feature made the employement of *Tetraselmis suecica* in acquaculture of interest also for biocontrol of pathogen agents (Natrah *et al.*, 2011). Overall, these characteristics and the multitude of applications make the study of the bacterial population associated to *Tetraselmis suecica* of particular interest, not only to acquire more knowledge regarding this powerful microalga, but also for a possible improvement of biomass cultivation.

1.4 Omics approaches to study algal-microbial interactions

The phycosphere is a unique habitat characterized by a sophisticated network of interactions between bacteria and microalgae (Seymour *et al.*, 2017). Indeed, the complex formed by microalgae and it associated microbiota could be consider a holobiont. In the past decade, the study of this biome has gained attention but owing to its intrinsic complexity, it was hard to depict a clear picture of its structure and functions.

Thanks to the recent advancement of technology it is now possible to analyze complex ecosystems (such as phycosphere, rhizosphere, etc.) from different perspectives through multi-omics approaches (Fondi and Liò 2015, Segata *et al.*. 2013).

High-throughput sequencing costs are drastically decreased becoming affordable for most microbiological laboratories. This allowed a deep characterization of microbial communities increasing the amount of genetic information available for the scientific community. Targeted metagenomic allows describing the bacterial community structure reconstructing its taxonomic composition. Specific pipelines for bench experiments and bioinformatics analysis have been developed in the past years becoming one of the most popular approaches nowadays (Segata *et al.*, 2013). However to gain a complete description of microbiomes, taxonomic data need to be integrated with other multi-omics assays (**Fig 8**):

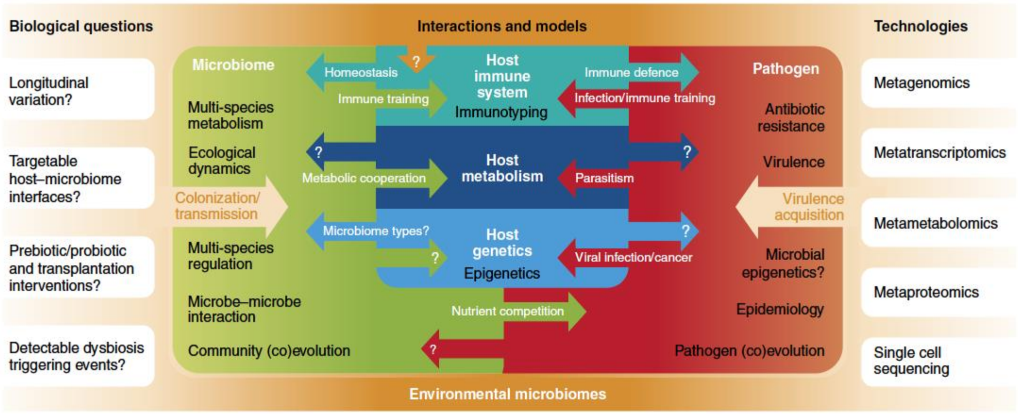

Figure 8. Open biological questions in microbial community biology, and emerging technologies and models for their exploration. Microbial communities are complex biological entities interacting with the environment, host organisms, and transient microbes. Predictive models for most of the interactions within these ecosystems are currently rare, but several studies have begun to provide key insights (Segata *et al.*, 2013)

- metagenomic, to evaluate the metabolic potentiality of a given community, depending on the sequencing coverage it is also possible to reconstruct entire genomes of microorganism present in the community (Segata *et al.*, 2013);

- metatrascriptomic, to investigate at the RNA level the metabolic process active in specific environmental conditions, even if there are some technical limitations due to RNA isolation especially in host associated communities (Bashiardes *et al.*, 2016);
- metabolomic, to analyze the metabolites produced by cellular processes, it is possible to obtain a chemical fingerprint of the community offering precise functional insights into the attendant static and dynamic physiological contexts (Rai *et al.,* 2016).

Combining the above approaches with metaproteomic, metaphenomics and other omics- approaches it is then possible to discover new findings on microalgae-bacteria interactions. Genome scale metabolic modeling and flux balance analysis, integrating data obtained by these different techniques allow the construction of metabolic models (Fondi and Liò 2015), helping to predict how the holobiont may react to different perturbations.

Nevertheless, we are still far from a complete comprehension of these complex biomes, and a deep characterization of all the partners involved in this process is still at the beginning (**Fig 9**).

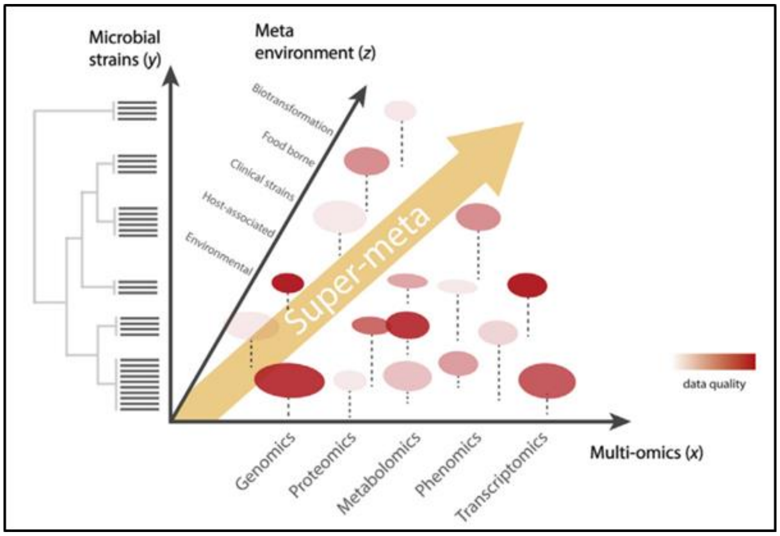

Figure 9. Super-meta and multi-parameter evidence synthesis approaches for –omics integration. Available technologies and public datasets allow approaching systems biology issues through a super-meta approach. Accordingly, different combinations of microbial ensembles (y axis), sampling environments (z axis) and/or -omics technologies (x axis) can be integrated and analyzed, exploiting, for example multievidence synthesis approaches. In this figure, the different circles represent different datasets, with hypothetical data quality represented in red-scale and the amount of data by circles size (Fondi and Liò 2015).

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Chapter 2

Aim

Several interactions happen within the phycosphere, between microalgae and bacteria. High-specificity interactions, such as symbiosis or parasitism, and low-specificity interactions, such as competitive or commensal relationships. Microalgal metabolism is influenced by the associated bacterial community, revealing that bacterial associations have a fundamental role for the ecological fitness of microalgal species. *Tetraselmis suecica* is a green marine microalga widely used in biotechnology. It is mainly employed in aquaculture, thanks to it has antibacterial activity against aquaculture pathogens and its potential probiotic action in fish, but is also gaining attention for biofuel production. Therefore, it is of interest to investigate the phycosphere of this microalga and to elucidate if there are bacterial species always associated, or if the associations are extremely various and strongly influenced by environmental conditions and by the quantity and quality of algal exudates.

The first aim of this thesis is to analyze the bacterial community associated with *T. suecica*, through a metagenomic approach to evaluate if it is possible to identify the presence of a bacterial "core" community in the *T. suecica* microbiome (**Chapter 3**).

Associated bacteria communities may affect microalgae growth and metabolism, influencing biomass yield and quality. The co-cultured bacterial population may release a broader array of substances such as vitamins, phytohormones (indole-3-acetic acid), amino acids, fatty acids, antibiotics, or siderophores, which may act as algal growth promoting factors. Nowadays only a few studies have addressed a characterization of bacteria associated with microalgae for the selection of microalgal growth-promoting bacteria. This is particularly important since the various applications of microalgae, therefore a deeper knowledge of the mechanisms that rule the interactions algae/bacteria could lead to an improvement in the current culturing methods.

The second aim of this thesis regards i) the characterization of the cultivable bacterial community associated with *T. suecica* to the isolation of strains able to enhance microalgal growth, and their characterization for Algal Growth Promoting features; ii) exo-metabolome analysis of co-cultures of *T. suecica* and growth promoting bacteria, to describe compounds that may be involved in the microalga/bacteria interactions (**Chapter 4**).

Chapter 3

Analysis of microbiota in cultures of the green microalga *Tetraselmis suecica*

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Keywords

Microalgae, bacteria, metagenomic analysis, microbiota, phycosphere, Tetraselmis suecica

Abstract

Bacteria associated with microalgae strongly affect algal biomass and derived products yield and quality. Nevertheless, only few studies have addressed a detailed phylogenetic characterization of bacterial communities associated with microalgae. In this study, the phycospheric bacterial communities associated with different *Tetraselmis suecica* F&M-M33 cultures, a green marine microalga with several industrial applications, were analyzed through a metagenomic approach. The *T. suecica* F&M-M33 cultures used were originated by the same ancestral microalgal non-axenic culture but were physically and geographically separated for years, and kept under different growing conditions. Despite the different history of cultures, a "core" bacterial community was identified, which accounts for 70% of the total bacterial community and it is formed by at least 13 families. Moreover, among the "core" OTUs, 7 different genera were identified. Nevertheless, a high variability in the relative proportions of the taxa forming the "core" community was encountered, indicating that growing conditions and/or external contaminations influence the relative abundance of these microorganisms. Our study allowed the identification of bacteria belonging to persistent taxa that will be used to deepen the knowledge on the complex relationship between *T. suecica* and its associated bacteria.

Introduction

The “phycosphere”, name given by analogy with the more known “rhizosphere”, is a zone rich in nutrients that surrounds microalgal cells (Bell & Mitchell, 1972). Microalgae, during growth produce and release several molecules (exudates), such as aminoacids, peptides, and sugars (Grossart & Simon, 2007; Thornton, 2014), which are utilized by bacteria for their growth. It has been observed that nutrient availability has a key role in determining the relationship between algae and bacteria. Bacteria may either stimulate or inhibit algal growth, and their interactions may range from parasitism to mutualism (Ramanan *et al.*, 2016). In this way, complex interactions are established between bacteria and microalgae (Segev *et al.*, 2016).

During the last years, several works attempted the description of the bacterial communities associated with different algal species (Hold *et al.*, 2001; Green *et al.*, 2004; Nicolas *et al.*, 2004; Makridis *et al.*, 2006; Sapp *et al.*, 2007; Lakaniemi *et al.*, 2012; Le Chevanton *et al.*, 2013). However, the rules driving the association mechanisms between bacteria and algae are still unclear, and there are more questions than answers (Ramanan *et al.*, 2016).

The study of microalgal bacterial communities, besides its importance in basic research, may also furnish new knowledge for microalgae biotechnological applications. Recently metagenomic studies have begun to describe the microbial communities associated with microalgae, (Wirth *et al.*, 2015; Krohn-Molt *et al.*, 2017; Sambles *et al.*, 2017) nevertheless scant information is available on their stability and resilience. Recently, it was shown that the succession of bacterial communities associated with *Nannochloropsis salina*, grown for one month in semicontinuous cultures in an open outdoor system, was influenced not only by environmental conditions but also by the seeded bacterial community (Geng *et al.*, 2016). Different microalgae select specifically their bacterial community composition; however, the level of specificity may vary (Krohn-Molt *et al.*, 2017). For example, it was shown that bacterial communities associated with *Chlorella* are more variable than those associated with *Scenedesmus* and *Micrasterias* (Krohn-Molt *et al.*, 2017). At the best of our knowledge, little information is available about *Tetraselmis suecica*/bacteria relationship (Nicolas *et al.*, 2004; Biondi *et al.*, 2017). *Tetraselmis* is a green marine microalga, suitable for various applications, mainly as food for bivalve mollusks or larval stages of crustaceans and in pseudo-green water technique (Tredici *et al.*, 2009). Because of its high protein and polyunsaturated fatty acid content, dried *T. suecica* inclusion in fish diet (particularly for European sea bass) represents an attractive potential application (Tulli *et al.*, 2012). *T. suecica* biomass cultivation has also gained attention for the production of biofuel, proteins and bioactive compounds such as polyunsaturated fatty acids, α-tocopherol, chlorophyll, β-carotene, and polyphenols (Schwenzfeier *et al.*, 2011; Bondioli *et al.*, 2012; Yao *et al.*, 2012; Perez-Lopez *et al.*, 2014). Considering that the microbiota associated with microalgae may influence both yield and quality of biomass, evaluating the structure and diversity of bacterial communities associated with *T. suecica* cultures is fundamental.

This study aimed to evaluate the structure and diversity of bacterial communities associated with *T. suecica* using a metagenomic approach, that is a useful tool for the study of complex microbial communities. Metagenomic analysis was applied to *T. suecica* F&M-M33 cultures, originated from an ancestral inoculum, and sub-cultured for years under different laboratory and growing conditions, to determine the potential presence of a “core” microbiota shared by all cultures.

Materials and Methods

Tetraselmis suecica *F&M-M33 cultures*

Tetraselmis suecica F&M-M33 was obtained from the Culture Collection of Microalgae and Cyanobacteria of Fotosintetica & Microbiolgica S.r.l., a spin-off of the University of Florence (Italy), and was used as inoculum for all cultures that were then grown under different conditions.

LAB1 and LAB2 samples were collected in November 2016 and in February 2017, respectively, from non-axenic laboratory flask cultures kept static and without CO_2 bubbling.

SES sample was collected from an outdoor culture in 40 L vertical GWP®-III photobioreactor (Chini Zittelli *et al.*, 2013a) at the experimental facilities of Fotosintetica & Microbiologica S.r.l. in Sesto Fiorentino (Florence, Italy).

CAM sample was obtained from a culture grown in the laboratory at the Archimede Ricerche S.r.l plant in Camporosso (Imperia, Italy) in a 0.5-l bubble tube inoculated using an outdoor culture performed in a GWP®-I photobioreactor (Rodolfi *et al.*, 2009) kept under greenhouse conditions. *T. suecica* F&M-M33 strain had been sent to the microalgae production plant of Archimede Ricerche S.r.l., for mass production about a decade ago.

All the cultures were grown in F medium (Guillard & Ryther, 1962). Artificial seawater (Adriatic Sea International, Rimini, Italy), used for medium preparation, was sterilized by autoclaving at 121 °C for 20 min for LAB1 and LAB2 cultures, whereas for SES and CAM cultures it was filtered through 1 µm filters (Domnick Hunter, Durham, UK). Stock nutrient solutions for LAB1, LAB2 and SES cultures were prepared in deionized water and autoclaved before addition to seawater, whereas for CAM stock solutions were prepared by dissolving salts in sterile water and were not autoclaved. After collection, the algal culture samples were stored at -80 °C.

Isolation and identification of bacterial strains

Bacterial strains were isolated from *T. suecica* F&M-M33 following the procedure described in Biondi *et al.* (2017). Colonies morphologically different (color, shape, edge, etc.) were picked and streaked on Marine Agar plates (Laboratorios CONDA, Madrid, Spain). Strains were then identified by 16S rDNA sequencing. 1 ml of a liquid culture, grown overnight in Marine Broth (Laboratorios CONDA) at 27 °C under 200 rpm agitation, was centrifuged at 16,000 g (Centrifuge 5415 D, Eppendorf) for 1 min. Bacterial pellets were frozen at -80°C for 20 min, then resuspended in 375 µl of a solution 30 mM NaCl, 2 mM EDTA (pH 8). The cell suspension was transferred into tubes containing glass beads and 125 µl of extraction buffer (500 mM NaCl, 50 mM Tris-HCl, 50 mM EDTA, 4% SDS, pH = 8). Samples were homogenized with a MM300 disrupter (Retsch, Haan Germany) for 2 min at 30 cycles/sec and then incubated 10 min at 70 °C. After 1 h incubation at 4°C, samples were centrifuged 1 min at 14,000 rpm, and the supernatant was recovered.

The DNA extracted was used as target for PCR amplification of the 16S rDNA gene using primers 63f and 1397r (Marchesi *et al.*, 1998). PCR reactions were carried out in 25 µl volume containing 1X Flexi PCR buffer (Promega, Madison, WI, USA), 1.5 mM $MgCl_2$, 250 µM deoxynucleotide triphosphates (dNTPs), 400 nM of each primer, 1U GoTaq®Flexi DNA polymerase (Promega) and 0.5% tween 20 (v/v). Amplifications were performed using a Tetrad 2 thermal cycler (Bio-Rad) under the following conditions: an initial denaturation for 4 min at 95 °C, 5 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s, 5 cycles of 95 °C for 30 s, 60 °C for 30 s (decreasing temperature by 1°C at each cycle), 72 °C for 45 s, 25 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and a final extension step at 72°C for 10 min. Amplicons were purified with PureLink^{TM}PCR purification kit (Invitrogen, Thermo Fisher Scientific) and subjected to sequencing at the Interdepartmental Centre for Agricultural, Chemical and Industrial Biotechnology (CIBIACI). The 16S rDNA sequence chromatograms were checked and edited to verify the absence of ambiguous peaks.

The 16S rRNA gene amplicon sequence data are available at GenBank database, accession numbers MH475102 to MH475135.

Total DNA extraction from* T. suecica *F&M-M33/bacteria microbiota

Total DNA was extracted following the CTAB buffer protocol (Pini *et al*.., 2012) combined with a mechanical disruption with glass beads. One ml of algal culture was centrifuged at 14,000 rpm for 1 min, the supernatant was discharged, and the collected algal biomass frozen at -80 °C for 20 min. The pellet was resuspended in 1 ml of CTAB buffer [2% (w/v) CTAB, 1.4 M NaCl, 0.2% (v/v) β-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0] and transferred in a 2 ml tube containing glass beads (0.75 – 1 mm diameter, Retsch), shacken 2 min at 30 cycles/sec using a MM300 disrupter (Retsch) and then incubated 1 h at 65 °C. After chloroform-isoamyl alcohol extraction, the aqueous phase was collected, and the nucleic acid was precipitated with isopropanol. DNA was washed twice with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). For each sample, DNA was extracted from three different aliquots and then mixed, and conserved at -20 °C.

PCR – Denaturing Gradient Gel Electrophoresis (DGGE)

Three primer sets were used to amplify three different variable regions of the 16S rDNA gene: V1-V3 region (63F-R518GC), V3-V5 region (357F-R907GC), and V6-V8 region

(F968GC-1401R) (Yu & Morrison, 2004). PCR reaction was carried out using a T100 Thermal Cycler (BioRad Laboratories, Hertfordshire, UK), in 25 µl final volume containing 1 X Flexi PCR buffer (Promega Italia, Milan, Italy), 1.5 mM MgCl2, 250 µM deoxynucleotide triphosphates (dNTPs), 400 nM each primer, and 1U GoTaq®Flexi DNA polymerase (Promega). Amplifications were performed under the following conditions: an initial denaturation for 4 min at 95 °C, then 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and a final extension step at 72 °C for 10 min. DGGE analysis was carried out by loading amplicons on a 6% polyacrylamide gel (acrylamide/bis 37.5:1), with a 47-60% denaturing gradient (100% denaturant consisting of 40% v/v deionized formamide, 7 M urea). Electrophoresis was performed on a Dcode DGGE System (BioRad) at 60 °C for 18 h and constant voltage of 85V. After electrophoresis gels were stained with SYBR®Gold (Molecular Probes, Eugene, OR, USA). The gel images were digitally captured under UV light using the ChemiDoc XRS apparatus (BioRad) and analyzed using the GelCompareII software v 4.6 (Applied Maths, Saint-Martens-Latem, Belgium). Each band was considered as representative of a single bacterial group and the band intensity as corresponding to its relative abundance. Richness was measured counting the number of bands in each DGGE profile, Shannon-Weiner diversity index was calculated as – ∑pi log2 (pi) where pi represents the relative abundance of a given fragment in the DGGE profile (Pastorelli et al., 2011).

Illumina MiSeq sequencing and data processing

For each sample the V3-V4 region of the 16S rDNA gene was amplified with primers Pro341f and Pro805R (Takahashi *et al.*, 2014), barcodes were added to the forward primer. Amplicons for each library were purified and mixed in equal proportion. Illumina MiSeq v3 chemistry 300 base paired-end (PE). Amplification and sequencing were performed at

BMR genomics (Padova, Italy). Reads were merged with FLASHv1.2.11 (Magoc & Salzberg, 2011) with the following parameters: -m 20, -M 280, Phred score default of 33. Sequences were then trimmed to discard primers with Prinseq-lite (Schmieder & Edwards, 2011) and sequences shorter than 200 bp were filtered out. Chimaeras were removed with USEARCH 6.1 (Edgar *et al.*, 2011). Open reference OTU picking was done with SUMACLUST within QIIME 1.9.1 (Caporaso *et al.*., 2010), using a similarity threshold of 0.97 and Greengenes 13.8 (DeSantis *et al.*., 2006) as reference database. OTUs representing less than 0.005% of total read abundance were discarded (Bokulich *et al.*., 2013). Sequences identified as chloroplasts, mitochondria and unassigned sequences were removed from further analysis. The 16S rRNA gene amplicon sequence data are available at the National Centre for Biotechnology Information Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra) bioproject number PRJNA474805, SRA accession SRP149916.

Results

Four cultures of the strain *T. suecica* F&M-M33 derived from the same original culture were investigated: LAB1 and LAB2, samples were obtained from flask maintenance cultures kept static under laboratory conditions; SES sample was taken from a culture in the vertical GWP®-III reactors, located at Fotosintetica & Microbiologica S.r.l.; CAM sample was taken from a culture grown in laboratory at the microalgae production plant of Archimede Ricerche S.r.l..

Isolation and identification of bacteria from* T. suecica *F&M-M33 cultures

On the basis of the different morphological characteristics of the colony (color, shape, edge, etc.) a total of 34 bacterial strains (Table 1) were isolated from *T. suecica* F&M-M33

cultures: 10, 12 and 12 bacterial strains were isolated from LAB1, SES and CAM cultures, respectively. LAB2 was not sampled because considered, in the design phase of experiments, similar to LAB1.

16S rDNA sequencing of isolates led to the identification of 23 different taxa (Table 1). The genera *Muricauda*, *Marinobacter* and *Roseivivax* were found in all cultures analyzed. *Mameliella*, *Nitratireductor* and *Labrenzia* were found in LAB1 and SES while *Nitratireductor* and *Labrenzia* were found in SES and CAM.

Selection of 16S rDNA primers to be used in microbiota analysis

The more promising 16S rDNA region for metagenomic analysis was selected on the basis of PCR-DGGE results. The diversity of bacterial communities, determined using Richness and Shannon-Weiner indices calculated in each DGGE profile, is reported in Table S1, for each primer set used. Richness ranged from 8 to 19 while Shannon-Weiner index from 2.048 to 2.891 (Table S1). The V3-V5 and V6-V8 regions allowed describing a higher diversity of the analyzed communities than V1-V3 region. With the exception of CAM samples, the V3-V5 region showed the highest values of Richness and Shannon-Weiner diversity indices (Table S1). Therefore, considering the results here obtained and those reported in literature (Sanchez *et al.*, 2007) the V3-V5 region of the 16S rRNA gene was selected for the metagenomic analysis of bacterial microbiota associated with *T. suecica* F&M-M33.

Metagenomic analysis of bacterial communities associated with laboratory and outdoor cultures of* T. suecica *F&M-M33

The composition of the microbial communities associated with four non-axenic *T. suecica* F&M-M33 cultures was analyzed at different taxonomic levels. Illumina MiSeq v3 sequencing, which was performed on the variable region V3-V4 of 16S rDNA produced

643,870 reads. A total of 122,341 sequences were obtained (ranging from around 17,000 to 45,000 sequences *per* library), which allowed the identification of 126 OTUs with a range from 64 to 93 *per* sample. Rarefaction curves showed a high sequencing coverage for all the samples (Fig. S2). The UPGMA dendrogram obtained with Dice coefficient similarity, which is based on presence-absence of OTUs, indicated samples LAB1 and LAB2 as the most similar, clustering with 82% of similarity, while SES was the most different, with 62% of similarity with all the others (Fig. 1). The bacterial community diversity descripted by the UPGMA dendrogram, which was obtained with Bray-Curtis index (based on OTUs relative abundances), showed a completely different tree-architecture in respect to that obtained using Dice coefficient. LAB1 and CAM were more similar in respect to the other samples, clustering together and sharing approximately 45% of similarity (Fig. 2). LAB2 shared roughly 20% of similarity with LAB1 and CAM, SES was the most different showing less than 15% of similarity with the other samples (Fig. 2).

Phylogenetic analysis of OTUs identified a total of 9 phyla which were observed in the SES sample while in the other samples were observed from 4 to 6 phyla. In all samples, *Proteobacteria* and *Bacteroidetes* were present accounting together for more than 97% of the total microbiota (Fig. 3). Relative abundance of *Proteobacteria* and *Bacteroidetes* highly varied in the samples. In LAB2 *Proteobacteria* accounted for the 87% of the total microbiota (Fig. 3); in LAB1 and CAM *Proteobacteria* and *Bacteroidetes* were both highly represented (45%-55% and 55-44% respectively); in SES *Bacteroidetes* was the most abundant phylum (68%).

At class level a total of 18 different groups were observed (16, 10, 7 and 8 in SES, LAB1, LAB2 and CAM, respectively). In the *Proteobacteria* phylum the most abundant class was *Alpha-proteobacteria* with a relative abundance varying between 29% (SES) and

82% (LAB2) of the total bacterial community. In the *Bacteroidetes* phylum, the class *Flavobacteriia* was the most represented (43%, 50%, 4.5% and 32.8% in LAB1, CAM, LAB2, and SES, respectively). In LAB2 and SES *Cytophagia* class had a relative abundance (3.7 and 31.1%, respectively) similar to that found for *Flavobacteriia*. Considering differences at genus level at least 58 different groups were detected: 17 genera (*Dyadobacter, Leadbetterella, Roseivirga, Fluviicola, Arenibacter, Muricauda,* and *Balneola* among *Bacteroidetes; Bacillus* among *Firmicutes; Pelagicoccus* among *Verrucomicrobia; Devosia, Nitratireductor, Hyphomonas, Anaerospora, Marivita, Alteromonas, Marinobacter, Alcanivorax,* and *Halomonas* among *Proteobacteria)* and 41 groups not identified at genus level. In SES sample was observed the highest number of groups (49) while in the other three samples were spanning from 34 to 39 (Fig. 3).

The overall analysis of the OTUs shared by all the 4 samples showed the presence of a “core” bacterial community. This stable bacterial community was formed by 37 OTUs out 126 (Fig. 4), which accounts for the 77, 82, 69 and 72% of the total sequences retrieved in LAB1, LAB2, SES, and CAM, respectively (Fig. 5). In addition, 18-26% of sequences are shared between two or three samples, while sequences from unique OTUs (belonging to only one sample) account only 0.01 - 6% of the sequences in LAB2 (2 OTUs), CAM (10 OTUs), and SES (25 OTUs). No unique OTUs were detected in LAB1 sample (Fig. 5).

The “core” bacterial community was represented by bacteria belonging to *Proteobacteria*, *Bacteroidetes* and *Planctomycetes* (Fig. 6). Nevertheless, the structure of the 4 bacterial “core” communities was highly different, reflecting what was observed for the total communities. LAB1 and CAM shared the most similar relative abundance of the two main phyla: *Bacteroidetes* accounted for 51.02% and 53.28% in LAB1 and CAM respectively, and *Proteobacteria* for 48.46, and 46.15% respectively. The most represented phylum in LAB2 was *Proteobacteria* with the 93% of “core” sequences, while in SES was the phylum *Bacteroidetes* with the 70% (Fig. 6). At lower taxonomic ranks, the “core” communities showed the presence of the orders *Cytophagales, Flavobacteriales, Pirellulales, Rhizobiales, Rhodobacterales, Sphingomonadales, Myxococcales* and *Alteromonadales* (Table S4). In the samples LAB1 and CAM, the order of *Flavobacteriales* represented the 49 and 47% of the total “core” community, respectively, while *Cytophagales* represented a slight part. On the contrary, in LAB2 and SES, *Flavobacteriales* and *Cytophagales* abundance were present at 6 and 26 %, respectively. SES was then the sample with the higher abundance of *Cytophagales* (Fig. 6).

The relative abundance of the different *Proteobacteria* orders shifted among samples in the “core” community. In LAB1 *Proteobacteria* were mainly represented by *Sphingomonadales* (24% of the “core” community), followed by *Rhodobacterales* (15%), while in CAM the most abundant order is represented by *Rhodobacterales* (31%) (Fig.6). The remaining four orders: *Rhizobiales, Sphingomonadales, Myxococcales* and *Alteromonadales*, varied between 0.5% and 7% (Fig. 6, Table S4). Notably, *Rhodobacterales* was the only order present by over the 15% in all the samples.

Relative abundance at family/genus level reflected the heterogeneity observed at phylum and order levels (Fig. 6, Table S5-6). At genus level *Marinobacter, Anaerospora, Hyphomonas, Devosia, Muricauda, Arenibacter, Leadbetterella* were identified as members of the “core” community, for 26 OTUs the identification at genus rank was not possible. In CAM and LAB1, the order *Flavobacteriales* is almost exclusively composed by genus *Muricauda*, while in SES is represented by the genus *Arenibacter* (Table S6). *Leadbetterella* genus was highly present only in the SES sample representing almost exclusively the order *Cytophagales.* For 22 OTUs was possible the identification only at family rank: *Cyclobacteriaceae*, *Pirellulaceae*, *Hyphomicrobiaceae*, *Phyllobacteriacea*, *Hyphomonadacea*, *Rhodobacteracea*, *Erythrobacteraceae*, *Sphingomonadaceae* and OM60. The remaining 4 OTUs were assigned at order level (*Rhizobiales*, *Sphingomonadales* and *Myxococcales*).

Discussion

In large-scale cultivation axenic conditions are unrealistic to achieve and maintain, thus algae are constantly co-cultivated with bacteria. Then, it is important to know which bacteria are associated with microalgae and their role on microalgal growth. Bacteria may be either derived from bacterial community associated with microalga or from contaminations in the culturing system (Tredici, 2003; Chini Zittelli *et al.*, 2013a; Chini Zittelli *et al.*, 2013b). Understanding which bacterial taxa are involved in resistance (insensitivity to disturbance) and resilience (the rate of recovery after disturbance) is important for predicting microalgae productivity (Shade *et al.*, 2012).

Therefore we aimed to evaluate the effects of different environmental/growth conditions on the structure of the bacterial communities associated with *T. suecica*. For this purpose we used different samples sub-cultivated from the same *T. suecica* F&M-M33 starter culture, but afterwards grown for a decade under different conditions (e.g. type of photo-bioreactor and production site).

Differences in *T. suecica* F&M-M33 in bacterial community structures, as observed in the UPGMA dendrograms, were mainly due to the relative abundances of the bacterial OTUs, and to a lesser extent to presence or absence of certain OTUs. Indeed, the laboratory maintenance cultures, which showed almost 85% of similarity in terms of OTUs presence/absence, have a highly different composition that may be related to the different sampling time. These results were further confirmed by analyzing the OTUs that form the "core" community, which may be more stably associated with *T. suecica* F&M-M33. The "core" represented roughly half of the OTUs of each culture in terms of presence/absence but more than 70% of the total number of sequences.

Among the OTUs retrieved in the "core" community it was possible to identify 11 OTUs at genus level, the other 26 were identified at family or order levels. Notably all the taxa identified are related to bacteria commonly found in marine environment, and all the OTUs fall in the classes *Proteobacteria* and *Bacteroidetes,* with the exception of one OTU belonging to the order *Pirellulaceae*. Among *Bacteroidetes,* the genus *Muricauda* was the predominant taxon within *Flavobacteriales* in three out of four *Tetraselmis* cultures. Therefore, *Muricauda* may play a key role in the bacterial community associated with *T. suecica* F&M-M33. The lowest presence of this genus was observed in SES that showed the highest relative frequency of the genus *Arenibacter*. Indeed, while *Muricauda* strains were isolated from all *T. suecica* F&M-M33 cultures, *Arenibacter* was isolated only in SES culture. Both *Muricauda* and *Arenibacter* are genus already described as associated to microalgae. *Muricauda* strains have been recently assessed to have a role in the enhancement of *Phaeodactylum tricornutum* growth. Their activity allowed up to 22% biomass augmentation thanks to the remineralization of organic nitrogen released by the microalgae (Le Chevanton *et al.*, 2013). Even if *Arenibacter* has been identified in the bacterial communities associated with *Chlorella*, *Gymnodinium,* and *Scrippsiella* (Green *et al.*, 2004; Green *et al.*, 2011; Makridis *et al.*, 2012), its role is still unclear.

The highly represented taxon in SES was *Leadbetterella,* which belongs to *Cytophagaceae*. Species of this family were found closely associated with, or attached to algal colonies of *Botrycoccus braunii* (Sambles *et al.*, 2017). Little evidences are reported regarding *Leadbetterella,* which to date was only found in association with the Prymnesiophyta *Chrysochromulina tobin* (Fixen *et al.*, 2016). The high frequency of *Leadbetterella* in SES sample may be due to different availability of nutrients in this culture respect to the other three samples. It is important to consider that the SES sample was taken from an outdoor culture. It is known that the availability of nutrient strongly affects *Cytophagaceae* growth in a water environment (Salis *et al.*, 2017).

Within *Proteobacteria* only few OTUs belonging to *Delta*- and *Gamma-proteobacteria* were identified. *Marinobacter* is the only genus identified belonging to *Gamma-proteobacteria*. *Marinobacter* strains were also isolated and identified from all the *T. suecica* F&M-M33 cultures. Several *Marinobacter* strains are siderophore producers (vibrioferrin), i.e., in *Scrippsiella trochoidea* they promote iron assimilation by facilitating its photochemical redox cycling (Amin *et al.*, 2009). *Marinobacter* is also able to enhance *Pseudo-nitzschia* growth (Sison-Mangus *et al.*, 2014), and was found to be the dominant group in different cultures of *Ostreococcus tauri* grown under different conditions (Lupette *et al.*, 2016).

Alpha-proteobacteria is one of the most represented classes in *T. suecica* F&M-M33 "core" bacterial community; nevertheless, it was not possible to identify bacteria below the family rank, with the exception of *Devosia, Hyphomonas* and *Anaerospora*. In all the cultures analyzed, a high presence of bacteria belonging to the family *Rhodobacteraceae* was observed. *Rhodobacteraceae* belongs to the *Roseobacter* group (Simon *et al.*, 2017), which is widely diffused in marine environment, often associated with microalgae and involved in important biogeochemical marine cycles (Buchan *et al.*, 2005). However, most of the strains here isolated were identified as members of *Rhodobacteraceae*: *Aestuariivita, Labrenzia, Mameliella, Marinovum, Oceanicola, Roseivivax, Roseovarius* and *Stappia*, indicating that *Rhodobacteraceae* was not only highly present but also showed a wide diversity. Members of the *Roseobacter* group have gained specific features related to the phycospheric environment: i.e., fucoidan (an antimicrobial polysaccharide produced by algae) desulphonation (Simon *et al.*, 2017) and indole-3-acetic acid production, a molecule enhancing microalgal growth (Amin *et al.*, 2015). *Stappia* strains showed a high growth promoting effect in *Tetraselmis striata* (Park *et al.*, 2017).

In summary the obtained results give a deep insight into *T. suecica* F&M-M33 associated bacterial community and support the hypothesis of the existence of an archetypal bacterial “core” community mainly formed by marine microorganisms. This “core” community was maintained for a decade in the four algal cultures analyzed, even if it was strongly influenced by environmental/culturing conditions in terms of relative abundance of the different groups. The restricted number of “core” OTUs described and the strains belonging to the “core” here isolated and identified may constitute a starting point to study their effects on *T. suecica* physiology in order to increase biomass production and/or quality. Indeed setting-up co-cultures assays where axenic microalgae and a single bacterial strain are grown together will allow investigating how the two organisms relate to each other in a simplified model.

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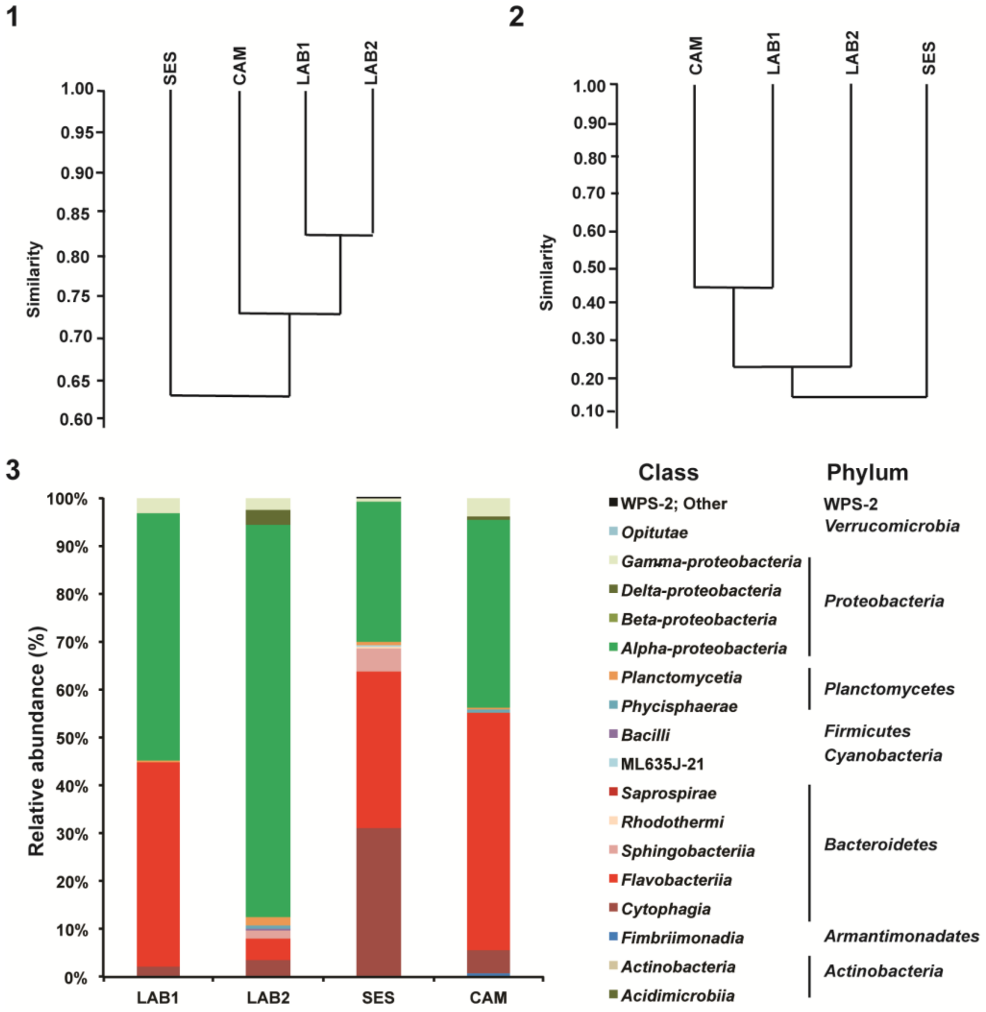
Figure legends

Figs 1-3. Bacterial communities associated with four different cultures of *Tetraselmis suecica* F&M-M33 deriving from the same starter culture. The same microalgal strain was grown in different conditions, for a decade: LAB1 and LAB2 derive from the same maintenance culture sampled at two different time; SES is a culture grown in a GWP®-III photobioreactor; CAM is a culture obtained from a 0.5-L bubble tube, inoculated with a culture grown in a GWP®-I photobioreactor. **Fig. 1.** Dendrogram showing the results of a hierarchical cluster analysis (UPGMA, Dice coefficient of similarity) based on the occurrences of OTUs in the four *T. suecica* F&M-M33 cultures; **Fig. 2.** Dendrogram showing the results of a hierarchical cluster analysis (UPGMA, Bray Curtis coefficient of similarity) based on the relative abundances of OTUs in the four *T. suecica* F&M-M33 cultures; **Fig. 3.** Stacked bar plot of class relative abundance of bacterial communities associated with four different cultures of *T. suecica* F&M-M33.

Figs 4-5. *Tetraselmis suecica* F&M-M33 “core” bacterial community. The same microalgal strain was grown in different conditions, for a decade: LAB1 and LAB2 derive from the same maintenance culture sampled at two different times; SES is a culture grown in a GWP®-III photobioreactor; CAM is a culture obtained from a 0.5-l bubble tube, inoculated with a culture grown in a GWP®-I photobioreactor. **Fig. 4.** Four-way Venn diagram of bacterial OTU distributions in the four samples; **Fig. 5.** Bar plot showing relative abundance (percentage) of unique, shared and “core” sequences retrieved.

Fig. 6. Pie charts of relative abundance of families forming the “core” bacterial community of *Tetraselmis suecica* F&M-M33 cultures. The same microalgal strain was grown in different conditions, for a decade: LAB1 is a laboratory maintenance culture sampled in autumn; LAB2 is a laboratory maintenance culture sampled in winter; SES is a culture grown in a GWP®-III photobioreactor; CAM is a culture obtained from a 0.5-l bubble tube, inoculated with a culture grown in a GWP®-I photobioreactor.



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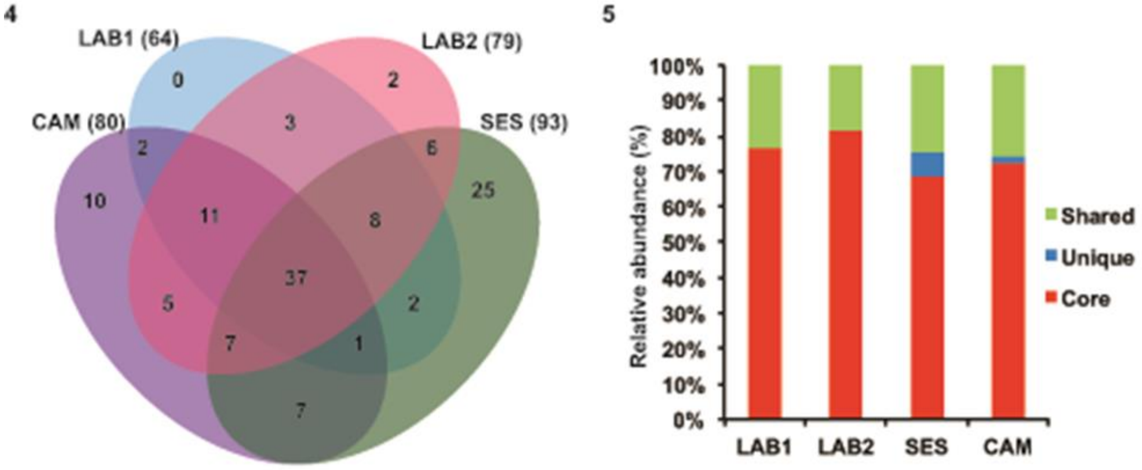

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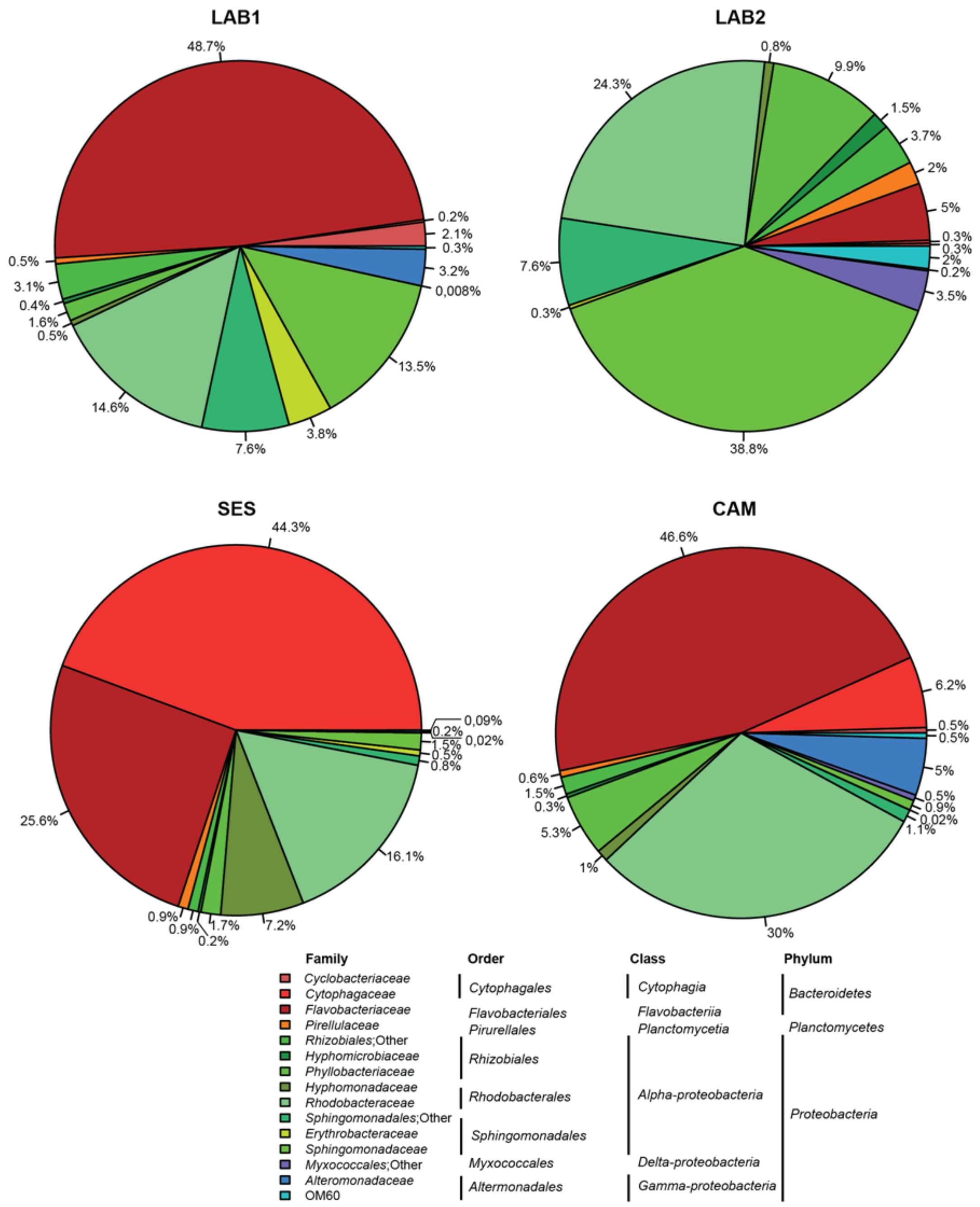

Fig. 6. Pie charts of relative abundance of families forming the “core” bacterial community of *Tetraselmis suecica* F&M-M33 cultures. The same microalgal strain was grown in different conditions, for a decade: LAB1 is a laboratory maintenance culture sampled in autumn; LAB2 is a laboratory maintenance culture sampled in winter; SES is a culture grown in a GWP®-III photobioreactor; CAM is a culture obtained from a 0.5-l bubble tube, inoculated with a culture grown in a GWP®-I photobioreactor.

Table 1. Identification of bacterial isolates through 16S rDNA sequencing. Strains were isolated from LAB1, SES and CAM *Tetraselmis suecica* F&M-M33 cultures. The same microalgal strain was grown in different conditions, for a decade: LAB1 derives from a laboratory maintenance culture; SES is a culture grown in a GWP®-III photobioreactors; CAM is a culture obtained from a 0.5-l bubble tube, inoculated with a culture grown in a GWP®-I photobioreactor.

Isolate	**Source**	**Closest match**	**Family**
M52B	LAB1	*Bacillus sp.*	*Bacillaceae*
14B51	CAM	*Bacillus idriensis*	“
RN41	LAB1	*Algoriphagus alophilus*	*Cyclobacteriaceae*
M62	LAB1	*Muricauda olearia*	*Flavobacteriaceae*
S12B1	SES	*Muricauda sp.*	“
24B2	CAM	*Muricauda sp.*	“
S11	SES	*Arenibacter algicola*	“
S59A2	SES	*Alcanivorax dieselolei*	*Alcanivoracaceae*
S102	SES	*Marinobacter flavimaris*	*Alteromonadaceae*
14C3	CAM	*Marinobacter flavimaris*	“
M42A	LAB1	*Marinobacter flavimaris*	“
14A5	CAM	*Amorphus coralli*	*Rhodobiaceae*
14C7	CAM	*Amorphus coralli*	“
RN32	LAB1	*Hoeflea sp.*	*Phyllobacteriaceae*
34C3	CAM	*Mesorhizobium thiogangeticum*	“
S69	SES	*Nitratireductor sp.*	“
24A6	CAM	*Nitratireductor sp.*	“
S09	SES	*Rhodobacteraceae bacterium*	*Rhodobacteraceae*
S90	SES	*Labrenzia aggregata*	“
23B3	CAM	*Labrenzia aggregata*	“
M61	LAB1	*Mameliella sp.*	“
S16	SES	*Mameliella sp.*	“
S105	SES	*Mameliella sp.*	“
S02	SES	*Marinovum algicola*	“
N41	LAB1	*Pseudooceanicola sp.*	“

S88	SES	*Roseivivax sp.*	“
14B2	CAM	*Roseivivax sp.*	“
M43B	LAB1	*Roseivivax sp.*	“
14B8	CAM	*Roseovarius sp.*	“
23B5	CAM	*Stappia indica*	“
14A3	CAM	*Stappia stellulata*	“
14C4	CAM	*Stappia stellulata*	“
M58	LAB1	*Rhodobacteraceae bacterium*	“
M54A3	LAB1	*Porphyrobacter sanguineus*	*Erythrobacteraceae*

Supplementary material

Table S1. Richness (number of bands) and diversity (Shannon-Weiner index) of DGGE banding profiles of 16S rDNA from bacterial communities associated with *Tetraselmis suecica* F&M-M33. V1-V3, V3-V5 and V6-V8 indicate the 16s rDNA regions amplified by primer pairs employed in the analysis. The same microalgal strain was grown in different conditions, for a decade: LAB1 and LAB2 derive from the same maintenance culture sampled at two different time (autumn and winter respectively); SES is a culture grown in a GWP®-III photobioreactors; CAM is a culture obtained from a 0.5-l bubble tube, inoculated with a culture grown in a GWP®-I photobioreactor.

Culture	**Richness index**			**Shannon-Weiner index**		
	V1-V3[a]	**V3-V5** [a]	**V6-V8** [a]	**V1-V3** [a]	**V3-V5** [a]	**V6-V8** [a]
LAB1	8	15	14	2.048	2.656	2.588
LAB2	14	16	15	2.615	2.756	2.649
CAM	11	13	19	2.368	2.504	2.891
SES	13	14	11	2.521	2.583	2.360

[a] 16S rRNA hypervariable regions targeted

Table S2. QIIME taxa table of *Tetraselmis suecica* F&M-M33 microbiota composition (total and core) for each sample at phylum level.

Total community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Actinobacteria	0.000117647	0.000235294	5.88E-05	0.001470588
k__Bacteria;p__Armatimonadetes	0	0	0.000117647	0.005470588
k__Bacteria;p__Bacteroidetes	0.448058824	0.098882353	0.688235294	0.544529412
k__Bacteria;p__Cyanobacteria	0	0	0.004352941	0
k__Bacteria;p__Firmicutes	0	0.000470588	5.88E-05	0.000294118
k__Bacteria;p__Planctomycetes	0.004	0.024705882	0.006058824	0.009764706
k__Bacteria;p__Proteobacteria	0.547823529	0.875705882	0.299411765	0.438470588
k__Bacteria;p__Verrucomicrobia	0	0	0.000823529	0
k__Bacteria;p__WPS-2	0	0	0.000882353	0

Core community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Bacteroidetes	0.510186887	0.054944265	0.699444682	0.532823558
k__Bacteria;p__Planctomycetes	0.005208333	0.019633225	0.008799658	0.005694298
k__Bacteria;p__Proteobacteria	0.484604779	0.92542251	0.29175566	0.461482144

Table S3. QIIME taxa table of *Tetraselmis suecica* F&M-M33 microbiota composition (total and core) for each sample at class level.

Total community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia	0.0001176	0.0002353	0	0.0009412
k__Bacteria;p__Actinobacteria;c__Actinobacteria	0	0	5.88E-05	0.0005294
k__Bacteria;p__Armatimonadetes;c__[Fimbriimonadia]	0	0	0.0001176	0.0054706
k__Bacteria;p__Bacteroidetes;c__Cytophagia	0.0206471	0.0368235	0.3110588	0.0486471
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia	0.4271765	0.0448824	0.3275882	0.4958824
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia	0.0002353	0.0171765	0.0483529	0
k__Bacteria;p__Bacteroidetes;c__[Rhodothermi]	0	0	0.0005294	0
k__Bacteria;p__Bacteroidetes;c__[Saprospirae]	0	0	0.0007059	0
k__Bacteria;p__Cyanobacteria;c__ML635J-21	0	0	0.0043529	0
k__Bacteria;p__Firmicutes;c__Bacilli	0	0.0004706	5.88E-05	0.0002941
k__Bacteria;p__Planctomycetes;c__Phycisphaerae	0	0.0086471	0	0.0056471
k__Bacteria;p__Planctomycetes;c__Planctomycetia	0.004	0.0160588	0.0060588	0.0041176
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	0.5151765	0.8195882	0.2928824	0.3945294
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria	0	0	0.0008824	0
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria	5.88E-05	0.0316471	0.0002353	0.0037647
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	0.0325882	0.0244706	0.0054118	0.0401765
k__Bacteria;p__Verrucomicrobia;c__Opitutae	0	0	0.0008235	0
k__Bacteria;p__WPS-2;c__	0	0	0.0008824	0

Core community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Bacteroidetes;c__Cytophagia	0.0231311	0.005178	0.4432294	0.066786
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia	0.4870558	0.0497663	0.2562153	0.4660376
k__Bacteria;p__Planctomycetes;c__Planctomycetia	0.0052083	0.0196332	0.0087997	0.0056943
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria	0.4501379	0.8686084	0.2895344	0.4020174
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria	7.66E-05	0.0354549	0.0002563	0.0050435
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria	0.0343903	0.0213592	0.001965	0.0544212

Table S4. QIIME taxa table of *Tetraselmis suecica* F&M-M33 microbiota composition (total and core) for each sample at order level.

Total community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales	0.0001176	0.0002353	0	0.0009412
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales	0	0	5.88E-05	0.0005294
k__Bacteria;p__Armatimonadetes;c__[Fimbriimonadia];o__[Fimbriimonadales]	0	0	0.0001176	0.0054706
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales	0.0206471	0.0368235	0.3110588	0.0486471
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales	0.4271765	0.0448824	0.3275882	0.4958824
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales	0.0002353	0.0171765	0.0483529	0
k__Bacteria;p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales]	0	0	0.0005294	0
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales]	0	0	0.0007059	0
k__Bacteria;p__Cyanobacteria;c__ML635J-21;o__	0	0	0.0043529	0

k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales	0	0.0004706	5.88E-05	0.0002941
k__Bacteria;p__Planctomycetes;c__Phycisphaerae;o__Phycisphaerales	0	0.0086471	0	0.0056471
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales	0.004	0.0160588	0.0060588	0.0041176
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other	0.0064706	0.0405882	0.0055882	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__	5.88E-05	0.0007059	0.0002941	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Kiloniellales	0	0	0	0.0004118
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales	0.0410588	0.1471765	0.0205882	0.1425882
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales	0.2727059	0.2465882	0.2372941	0.2368235
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales	0	0	0.0005294	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales	0	0	0.0089412	0.0001176
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales	0.1948824	0.3845294	0.0196471	0.0145882
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales	0	0	0.0008824	0
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales	5.88E-05	0.0316471	0.0002353	0.0037647
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales	0.0264118	0.0174706	0.0037647	0.0393529
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__HTCC2188	0	0	0.0014118	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales	0.0044118	0.0050588	0.0001765	0.0008235
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales	0	0	5.88E-05	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales	0.0017647	0.0019412	0	0
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Pelagicoccales]	0	0	0.0008235	0
k__Bacteria;p__WPS-2;c__;o__	0	0	0.0008824	0

Core community	LAB1	LAB2	SES	CAM

k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales	0.0231311	0.005178	0.4432294	0.066786
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales	0.4870558	0.0497663	0.2562153	0.4660376
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales	0.0052083	0.0196332	0.0087997	0.0056943
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales	0.0504749	0.1503056	0.0285348	0.0716668
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales	0.1507353	0.2509169	0.2328919	0.3104206
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales	0.2489277	0.4673858	0.0281076	0.01993
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales	7.66E-05	0.0354549	0.0002563	0.0050435
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales	0.0343903	0.0213592	0.001965	0.0544212

Table S5. QIIME taxa table of *Tetraselmis suecica* F&M-M33 microbiota composition (total and core) for each sample at family level.

Total community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__C111	0.000117647	0.000235294	0	0.000941176
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae	0	0	5.88E-05	0.000529412
k__Bacteria;p__Armatimonadetes;c__[Fimbriimonadia];o__[Fimbriimonadales];f__	0	0	0.000117647	0.005470588
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cyclobacteriaceae	0.016058824	0.002117647	0.005352941	0.003411765
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae	0.001705882	0.002117647	0.305705882	0.044941176
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Flammeovirgaceae	0.002882353	0.032588235	0	0.000294118
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__	0.039235294	0	0.000411765	0.003705882
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae	0	0.000647059	0.051117647	0
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae	0.387941176	0.044235294	0.276058824	0.492176471
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobact	0.0002	0.017176471	0.04835	0

eriales;f__	35294		2941	
k__Bacteria;p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__[Balneolaceae]	0	0	0.000529412	0
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Saprospiraceae	0	0	0.000705882	0
k__Bacteria;p__Cyanobacteria;c__ML635J-21;o__;f__	0	0	0.004352941	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae	0	0.000470588	5.88E-05	0.000294118
k__Bacteria;p__Planctomycetes;c__Phycisphaerae;o__Phycisphaerales;f__	0	0.008647059	0	0.005647059
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae	0.004	0.016058824	0.006058824	0.004117647
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other	0.006470588	0.040588235	0.005588235	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__;f__	5.88E-05	0.000705882	0.000294118	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Kiloniellales;f__	0	0	0	0.000411765
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other	0.001176471	0.001529412	0.000764706	0.040294118
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__	0.024823529	0.047647059	0.005352941	0.043882353

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae	0.002941176	0.012705882	0.001823529	0.018647059
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae	0.012117647	0.085294118	0.012647059	0.039764706
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae	0.003647059	0.007117647	0.071764706	0.007294118
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae	0.269058824	0.239470588	0.165529412	0.229529412
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae	0	0	0.000529412	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__	0	0	0.008941176	0.000117647
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;Other	0.060176471	0.062529412	0.005647059	0.008058824
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__	5.88E-05	0.000529412	0	5.88E-05
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae	0.031117647	0.002705882	0.003823529	0.000117647
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae	0.103529412	0.318764706	0.010176471	0.006352941
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae	0	0	0.000882353	0
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxoco	5.88E-	0.031647059	0.00023	0.00376

ccales;f__	05		5294	4706
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae	0.024411765	0.001470588	0.002588235	0.036
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__HTCC2188	0	0	0.001117647	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__OM60	0.002	0.016	5.88E-05	0.003352941
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__HTCC2188;f__	0	0	0.001411765	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Alcanivoracaceae	0.004411765	0.005058824	0	0.000823529
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae	0	0	0.000176471	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Pseudoalteromonadaceae	0	0	5.88E-05	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae	0.001764706	0.001941176	0	0
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Pelagicoccales];f__[Pelagicoccaceae]	0	0	0.000823529	0
k__Bacteria;p__WPS-2;c__;o__;f__	0	0	0.000882353	0

Core community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cyclobacteriaceae	0.020909926	0.002588997	8.54E-05	0.004718132
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae	0.002221201	0.002588997	0.443143956	0.062067843
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae	0.48705576	0.049766271	0.256215293	0.466037582
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae	0.005208333	0.019633225	0.008799658	0.005694298
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other	0.00145527	0.001654081	0.001110636	0.003416579
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__	0.029488358	0.035239123	0.007774455	0.011713984
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae	0.003829657	0.014742898	0.002477574	0.003172537
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae	0.015701593	0.098669543	0.017172149	0.053363703
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae	0.004748775	0.007838907	0.072276805	0.010005694
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae	0.14598652	0.243078029	0.160615122	0.30041487
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;Other	0.075903799	0.076015822	0.00811619	0.011063207

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae	0.038296569	0.003236246	0.005211448	0.000162694
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae	0.134727328	0.388133765	0.014780009	0.008704141
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__	7.66E-05	0.035454872	0.000256301	0.005043521
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae	0.031786152	0.001797914	0.001879539	0.04978443
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__OM60	0.002604167	0.019561309	8.54E-05	0.004636785

Table S6. QIIME taxa table of *Tetraselmis suecica* F&M-M33 microbiota composition (total and core) for each sample at genus level.

Total community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Actinobacteria;c__Acidimicrobiia;o__Acidimicrobiales;f__C111;g__	0.0001176	0.0002353	0	0.0009412
k__Bacteria;p__Actinobacteria;c__Actinobacteria;o__Actinomycetales;f__Microbacteriaceae;g__	0	0	5.88E-05	0.0005294
k__Bacteria;p__Armatimonadetes;c__[Fimbriimonadia];o__[Fimbriimonadales];f__;g__	0	0	0.0001176	0.0054706
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cyclobacteriaceae;g__	0.0160588	0.0021176	0.0053529	0.0034118
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Dyadobacter	0	0	0.0003529	0
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Leadbetterella	0.0017059	0.0021176	0.3053529	0.0449412
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Flammeovirgaceae;g__Roseivirga	0.0028824	0.0325882	0	0.0002941
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__;g__	0.0392353	0	0.0004118	0.0037059
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Cryomorphaceae;g__Fluviicola	0	0.0006471	0.0511176	0
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;Other	0.0136471	0.0004118	0.0944118	0
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__	0	0.0026471	0.0006471	0.1537647

k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Arenibacter	0.0001765	0.0197059	0.1780588	5.88E-05
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Muricauda	0.3741176	0.0214706	0.0029412	0.3383529
k__Bacteria;p__Bacteroidetes;c__Sphingobacteriia;o__Sphingobacteriales;f__;g__	0.0002353	0.0171765	0.0483529	0
k__Bacteria;p__Bacteroidetes;c__[Rhodothermi];o__[Rhodothermales];f__[Balneolaceae];g__Balneola	0	0	0.0005294	0
k__Bacteria;p__Bacteroidetes;c__[Saprospirae];o__[Saprospirales];f__Saprospiraceae;g__	0	0	0.0007059	0
k__Bacteria;p__Cyanobacteria;c__ML635J-21;o__;f__;g__	0	0	0.0043529	0
k__Bacteria;p__Firmicutes;c__Bacilli;o__Bacillales;f__Bacillaceae;g__Bacillus	0	0.0004706	5.88E-05	0.0002941
k__Bacteria;p__Planctomycetes;c__Phycisphaerae;o__Phycisphaerales;f__;g__	0	0.0086471	0	0.0056471
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__	0.004	0.0160588	0.0060588	0.0041176
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;Other;Other;Other	0.0064706	0.0405882	0.0055882	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__;f__;g__	5.88E-05	0.0007059	0.0002941	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Kiloniellales;f__;g__	0	0	0	0.0004118
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other;Other	0.0011765	0.0015294	0.0007647	0.0402941
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__;g__	0.0248235	0.0476471	0.0053529	0.0438824

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;Other	0.0024118	0.0117059	0.0004706	0.0001765
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__	0	0.0006471	0.0001176	0.0158824
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia	0.0005294	0.0003529	0.0012353	0.0025882
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;Other	0	0.0001765	0.0008235	5.88E-05
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__	0.0120588	0.0811765	0.0118235	0.0392941
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__Nitratireductor	5.88E-05	0.0039412	0	0.0004118
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae;g__	0.0031176	0.0012941	0.0704118	0.0049412
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae;g__Hyphomonas	0.0005294	0.0058235	0.0013529	0.0023529
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;Other	0.0095882	0.0901765	0.0091176	0.0257647
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__	0.2589412	0.1410588	0.1313529	0.2011176
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Anaerospora	0.0005294	0.0082353	0.0008824	0.0026471
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Marivita	0	0	0.0241765	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodospirillales;f__Rhodospirillaceae;g__	0	0	0.0005294	0
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rickettsiales;f__;g__	0	0	0.0089412	0.0001176

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;Other;Other	0.0601765	0.0625294	0.0056471	0.0080588
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__;g__	5.88E-05	0.0005294	0	5.88E-05
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;Other	0.0288235	0.0025294	0.0035294	5.88E-05
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__	0.0022941	0.0001765	0.0002941	5.88E-05
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;Other	0.1034706	0.3174706	0.0101765	0.0062941
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;g__	5.88E-05	0.0012941	0	5.88E-05
k__Bacteria;p__Proteobacteria;c__Betaproteobacteria;o__Burkholderiales;f__Alcaligenaceae;g__	0	0	0.0008824	0
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__;g__	5.88E-05	0.0316471	0.0002353	0.0037647
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Alteromonas	0	0	0.0012941	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Marinobacter	0.0244118	0.0014706	0.0012941	0.036
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__HTCC2188;g__HTCC	0	0	0.0011176	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__OM60;Other	0.002	0.016	5.88E-05	0.0033529
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__HTCC2188;f__;g__	0	0	0.0014118	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Alcanivoracaceae;g__Alcanivorax	0.0044118	0.0050588	0	0.0008235

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales;f__Halomonadaceae;g__Halomonas	0	0	0.0001765	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales;f__Pseudoalteromonadaceae;g__	0	0	5.88E-05	0
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Xanthomonadales;f__Sinobacteraceae;g__	0.0017647	0.0019412	0	0
k__Bacteria;p__Verrucomicrobia;c__Opitutae;o__[Pelagicoccales];f__[Pelagicoccaceae];g__Pelagicoccus	0	0	0.0008235	0
k__Bacteria;p__WPS-2;c__;o__;f__;g__	0	0	0.0008824	0

Core community	LAB1	LAB2	SES	CAM
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cyclobacteriaceae;g__	0.0209099	0.002589	8.54E-05	0.0047181
k__Bacteria;p__Bacteroidetes;c__Cytophagia;o__Cytophagales;f__Cytophagaceae;g__Leadbetterella	0.0022212	0.002589	0.443144	0.0620678
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Arenibacter	0.0002298	0.0235167	0.2519436	8.13E-05
k__Bacteria;p__Bacteroidetes;c__Flavobacteriia;o__Flavobacteriales;f__Flavobacteriaceae;g__Muricauda	0.486826	0.0262496	0.0042717	0.4659562
k__Bacteria;p__Planctomycetes;c__Planctomycetia;o__Pirellulales;f__Pirellulaceae;g__	0.0052083	0.0196332	0.0087997	0.0056943
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;Other;Other	0.0014553	0.0016541	0.0011106	0.0034166
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__;g__	0.0294884	0.0352391	0.0077745	0.011714

k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;Other	0.0031403	0.0143114	0.0006835	0.000244
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Hyphomicrobiaceae;g__Devosia	0.0006893	0.0004315	0.0017941	0.0029285
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales;f__Phyllobacteriaceae;g__	0.0157016	0.0986695	0.0171721	0.0533637
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae;g__	0.0040594	0.0015822	0.0704827	0.0067518
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Hyphomonadaceae;g__Hyphomonas	0.0006893	0.0062567	0.0017941	0.0032539
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;Other	0.0124847	0.1102481	0.0120461	0.0352233
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__	0.1328125	0.1250629	0.1475438	0.2630765
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales;f__Rhodobacteraceae;g__Anaerospora	0.0006893	0.007767	0.0010252	0.002115
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;Other;Other	0.0759038	0.0760158	0.0081162	0.0110632
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;Other	0.0375306	0.0030924	0.005126	8.13E-05
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Erythrobacteraceae;g__	0.0007659	0.0001438	8.54E-05	8.13E-05
k__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales;f__Sphingomonadaceae;Other	0.1347273	0.3881338	0.01478	0.0087041
k__Bacteria;p__Proteobacteria;c__Deltaproteobacteria;o__Myxococcales;f__;g__	7.66E-05	0.0354549	0.0002563	0.0050435

k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__Alteromonadaceae;g__Marinobacter	0.0317862	0.0017979	0.0018795	0.0497844
k__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales;f__OM60;Other	0.0026042	0.0195613	8.54E-05	0.0046368

Chapter 4

Tetraselmis suecica and associated bacteria interactions

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Personal contribution: conceived and designed the experiments, performed the experiments, analyzed the an wrote the paper

Tetraselmis suecica and associated bacteria interactions

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Keywords: Microalgae, phycosphere, exo-metabolome, *Tetraselmis suecica*, co-culture.

Abstract

Algae and bacteria establish complex relationships influencing their growth. Associated bacterial communities affect microalgae physiology and metabolism, altering biomass yield and quality. In this study, 226 bacterial strains, isolated from *Tetraselmis suecica* F&M-M33, were identified and phenotypically characterized for microalgal growth promoting characteristics. Selected strains were employed in co-culture assays to evaluate the effect of single strains on *T. suecica* growth. Co-cultures with *Vitellibacter* sp. strain AAD2 and *Sphyngopyxis flavimaris* strain AG5 showed an increase in microalgal growth. The exo-metabolome of *T. suecica* cultures, axenic and co-cultured with these strains, was determined using a non-targeted metabolomic approach. This allowed the detection of 131 entities and the identification of 103 compounds. Among them, 18 molecules were attributed as possibly related to microalgae-bacteria interactions.

INTRODUCTION

During last years the study of microalgae-bacteria interactions increased drastically due also to the possible role of associated bacteria on the modulation of algal production (Subashchandrabose *et al.*, 2011; Fouilland, 2012). Ranging over food industry, animal feed, cosmetics energy and phycoremediation, microalgae are always cultivated with an associated bacterial community, due to the difficulties needed to maintain axenic conditions (Fuentes *et al.* 2015, Borowitzka *et al.* 2013, Wan-Loy 2012). Bacteria may have strong influence on productivity and cell density of different microalgae species. For example, the addition of *Bacillus* sp. to different microalgal cultures showed significant growth-promoting effects on seven algal species (Liu *et al.*, 2008). Furthermore, bacteria may affect other aspects of microalgal morphology and metabolism, such as cell size, pigment, lipid content, and fatty acids composition (de-Bashan *et al.*, 2002; Do Nascimento *et al.*, 2013). Recently it has been shown that *Azospirillum brasilense* and *Bacillus pumilus* enhanced up to six-fold the growth of the microalga *Chlorella sorokiniana* UTEX 2714 and its cell volume by about three-fold (Amavizca *et al.*, 2017).

The effect of bacterial population on microalgal growth is mediated by a broad array of substances such as vitamins, phytohormones (e.g. indole-3-acetic acid), amino acids, fatty acids, antibiotics, or siderophores, which may act as algal growth promoting molecules (Ramanan *et al.*, 2016; Tandon *et al.*, 2017; Patidar *et al.*, 2018). In analogy to plant rhizosphere, the area surrounding the algal cell, where interactions between bacteria and microalgal cells take place, has been termed ‘phycosphere’ (Bell and Mitchell, 1972), thus similarly the bacteria enhancing microalgal growth may be indicated as microalgae growth-promoting bacteria (MGPB) (de-Bashan *et al.*, 2004). Modification of the phycosphere, like the addition of selected bacterial strains, may represent a fundamental strategy for improving microalgae production reliability and may lead to cost reduction in biomass production and harvesting (Fukami *et al.*, 1997). Despite this, there are only few studies that have addressed a characterization of bacteria associated with microalgae for the selection of MGPB.

Tetraselmis species gained importance in studies for the understanding of dynamics of plankton growth and the main interest addressed to this microalga concerns its various applications in the market including aquaculture, where it is employed in the diet of bivalve mollusks and larval stages of crustaceans (Tredici *et al.*, 2009), and cosmetic applications (Pertile *et al.*, 2010). Therefore *Tetraselmis suecica* could be a valuable model to investigate microalgal bacterial interactions. Therefore, the aim of this study was to investigate the relationships between *T. suecica* and its phycosperic bacteria. More than 220 bacterial strains from *T. suecica* F&M-M33 cultures were phenotypically characterized for MGP traits (i.e. IAA and siderophore production), and some selected strains were tested in co-cultures to evaluate their promoting effects on axenic *T. suecica* growth. The exo-metabolome of *T. suecica* cultures, axenic and co-cultured with two selected bacteria strains were determined using a non-targeted metabolomics approach.

MATERIALS AND METHODS

Bacterial strains

Bacterial strains were isolated from a laboratory and a photobioreactor *T. suecica* F&M-M33 culture, following the procedure described in Biondi *et al.* (Biondi *et al.*, 2017). Colonies were picked and streaked on Marine Agar plates incubated at 27° C. Strains were identified by 16S rDNA sequencing as described in Piampiano *et al.* (submitted). Strains, previously isolated from *T. suecica* F&M-M33-M33 and identified (61 strains), were also included (Biondi *et al.* 2017).

Screening for MGPB features

Indole-3-acetic acid (IAA) production was determined using a modified protocol of the method described by Bric (Bric *et al.*, 1991). Bacteria were streaked on Marine Agar (Laboratorios CONDA, Madrid, Spain) plates in the presence of tryptophan (0.5 mM) and incubated for 24 h at 27°C. After bacterial growth, a sterilized nylon membrane was placed over the medium, and plates were further incubated for 24 h at 27°C. Nylon membranes were then removed from agar plates and placed on a Whatman paper disk saturated with 2.5 mL Salkowsky reagent (0.01 M $FeCl_3$ in 35% $HClO_4$) for 30 min at room temperature. The detection of IAA was determined by the development of pink color on nylon membranes.

Siderophore production was assessed modifying the O-CAS assay (Perez-Miranda *et al.*, 2007). Bacterial isolates were grown on iron-deprived sea salts agar medium, implemented with peptone and yeast extract (Sea salts 36 g/L, Peptone 7.5 g/L, yeast extract 1.5 g/L, agar 1.6 g/L). Iron deprived medium was prepared as follow: iron was removed from a peptone (75 g/L) /yeast extract (15 g/L) solution using 3% 8-hydroxyquinoline in chloroform; the peptone/yeast extract solution was filter sterilized and 100 mL were mixed with 900 mL of sea salts agar medium. Cutures were grown at 27 °C for 72 h. Then the plates were covered with the CAS-agar overlay gel (Perez-Miranda *et al.*, 2007). Strains were considered positive for siderophore production when, after 2 h incubation, a change in color was observed in the overlaid medium (from blue to orange).

Co-culture assays

The axenic culture of the green marine alga *Tetraselmis suecica* F&M-M33 used as inoculum in co-culture assays, were grown in 100-mL flasks (50 mL of culture) using F medium without vitamin. Axenity of cultures were checked by microscopic inspections and by incubating aliquots in F medium (Guillard and Ryther, 1962) supplemented with glucose (2 g l^{-1}) and yeast extract (2 g l^{-1}) and in Marine Broth, for 72 h at 27 °C. The flask cultures capped with SILIOCOSEN® stoppers were kept under continuous illumination (80 µmol photons m^{-2} s^{-1}) in static conditions to avoid moist formation and thus reduce the possibility of contamination. Temperature of incubation was 23±2 °C.

Co-culture assays were performed in 6-multiwell plates. For each co-culture experiment three wells of a 6-multiwell plate were inoculated with 4.5 mL of a 0.2 g/l (dry weight) *T. suecica* F&M-M33 axenic culture (corresponding to 0.6×10^6 cells/mL) in F medium without vitamin addition and 500 µl of a bacterial suspension with OD_{600}=0.05. One well was inoculated with the axenic microalgae to determine possible external contaminations, and two wells with NaCl 0.8%, to control evaporation rate. Plates were incubated for 10 days at 27 °C, under continuous illumination of about 90 µmol photons m^{-2} s^{-1} and orbital shaking (85 rpm).

The growth of alga was determined by biomass productivity measured as dry weight according to Guccione *et al.* (Guccione *et al.*, 2014), and through cell counting using a Thoma haemocytometer. Productivity was calculated both in terms of biomass concentration and cell number. The biomass concentration (or cell number) at the start of the experiment was subtracted from that determined at the end of the experiment and the difference was divided by the duration of the experiment. Bacterial cell concentration was measured at the end of the experiment by standard plate count on Marine Agar plates incubated for 72 h at 27°C.

Metabolomics

Samples for metabolomics analyses were prepared in erlenmeyer flasks (250 mL) with 47.5 mL of an axenic culture of *T. suecica* F&M-M33 and 2.5 mL (OD_{600}= 0.05) of *Vitellibacter* sp. AAD2 or *Sphyngopixis flavimaris* AG5) liquid culture grown in agitation for 24h at 27°C on Marine Broth. Erlenmeyer flasks with an axenic culture of *T. suecica* F&M-M33 was used as control. Co-cultures were incubated 60 days at 23±2°C and illumination (ca 60 µmol photons m^{-2} s^{-1}). After 60 days each culture was centrifuged 15 min at 938 g (ALC PK121R). Supernatant was aliquoted and concentrated 1.2 X at 45 °C for 90 min (Concentrator 5301, Eppendorf, Hamburg). Samples were filter sterilized (0.2 µm) and stored at -80°C.

The samples were minimally processed before injection in the analysis system. Samples were thawed and distributed in 15 mL tubesmL. After vortexing (1 min), 1 mL of each sample was filtered through a 0.22 μm PVDF filter. Separation was performed on an UPLC system (Agilent 1290 Infinity, Agilent Technologies, Waldbronn, Germany) coupled to a Mass QTOF (6550 Accurate) with electrospray ionization via Jet Stream Technology and a C18 column (Poroshell 120, 3 × 100 mm, 2.7 μm pore size) (UPLC-ESI-QTOF-MS). Chromatographic and mass spectrometry conditions were established to detect differences on the general metabolomic profile. Briefly, 200 μL of extracts was injected and separated on the column with a flow rate of 0.4 mL/min and mobile phases of water with 0.1% formic acid (v/v) (A) and acetonitrile (ACN) with 0.1% formic acid (v/v) (B). Compounds were separated using the following gradient conditions: 0–10 min, 1–18 % phase-B; 10–16 min, 18–38 % phase- B; 16–22 min, 38–95 % phase-B. Finally, the phase B content was returned to the initial conditions (1 %) for 1 min and the column re-equilibrated for 5 min. Nitrogen was used as nebulizer (35 psi, 9 L/min) and drying gas (280 °C, 9 L/min, sheath gas temperature 400 °C, and sheath gas flow 12 L/min). Spectra were acquired in the range of *m/z* 100−1100 in negative mode, with a fragmentor voltage of 100 V and an acquisition rate 1.5 spectra/s. Samples were also analyzed in the positive mode, applying the same conditions. To assure mass accuracy during the MS analysis external calibration of the instrument was performed at the beginning of the batch, introducing a mixture of reference compounds (Tuning Mix).

Metabolomics data treatment

The metabolomics data acquisition system stored the raw data set of chromatographic and mass spectral data at profile and centroid data format to guarantee the complete collection of necessary information. Agilent metabolomics workstation software was used to process the raw data. Mass Hunter Qualitative Analysis (version B.06.00 Agilent Technologies) and Profinder (version B.06.00 Agilent Technologies) were used to perform the metabolomics treatment analysis. Mass Hunter Qualitative Analysis software was used to examine the chromatographic profile and the mass spectrum of the samples. Profinder software was used to data processing. Data processing was performed by the Molecular Feature Extraction (MFE) including specific features: i) Peak picking, the peaks were detected and extracted by retention time restriction, *m/z* restriction, peak counts filter and peak spacing tolerance; ii) compounds filter by mass filter; iii) compounds binning and alignment; iv) post-processing filter by the score of the molecular feature extraction.

A total of 1631 entities were retained and aligned across all the samples after applying all the filters. After that, the most relevant entities were launched against databases to the identification.

RESULTS

Identification and phenotypic characterization of phycospheric bacterial strains

A total of 226 bacterial strains isolated from *T. suecica* F&M-M33 cultures were characterized: 165 were isolated and identified through 16S rDNA sequencing in this work (Table S1), 61 strains were previously isolated and identified [Piampiano *et al.* (submitted); Biondi *et al.* 2017](Table S2).

The 165 isolates belong to 21 different taxa, spanning three phyla: *Firmicutes*, *Bacteroidetes* and *Proteobacteria* (Table 1). 51% of the isolates was represented by members of the family *Rhodobacteraceae*, which accounted for the highest number of genera detected (1 out of 21). Notably 22 isolates (13.3% of the total) were affiliated with the *Gammaproteobacteria* and in this group 18 isolates belonged to *Marinobacter* genus, in particular three of them were identified as member of the species *Marinobacter flavimaris* (strain 14C3, M42A and S102). *Muricauda* genus represented alone 23% (38 isolates) of the total strains isolated and the large majority of *Bacteroidetes* (49 isolates) retrieved in this work (Table 1).

The strains were screened for the ability to produce algae growth promoting molecules, such as indol-3-acetic acid (IAA) and siderophores. Only 31 isolates out of 226 were able to produce IAA (Table 2). Among the bacteria producing IAA, 18 strains belonged to genus *Marinobacter*, seven IAA-producer strains were identified as members of the family *Rhodobacteraceae: Stappia* sp., *Mameliella* sp., *Marinovum* sp., *Labrenzia* sp., *Roseobacter* sp., *Ruegeria* sp. and *Sagittula* sp. Six other strains able to produce IAA belonged to the genera *Planococcus* sp., *Muricauda* sp., *Pseudomonas* sp., *Amorphus* sp. and *Spingopyxis* sp. (Table 2).

The production of siderophores was assessed through the employment of a modified CAS-Agar assay the “O-CAS assay” (Perez-Miranda *et al.* 2007). Four isolates identified as siderophore producers were members of the species *Bacillus indicus*, two were members of the species *Stappia indica.* Five of the detected strains, belonged to the genera *Labrenzia* sp., *Roseivivax* sp, and Stappia sp., which are among the *Rhodobacteraceae* family (Table 2). Notably, *Amorphous coralli* strain 14C7 and strain 14A5, and *Stappia stellulata* strain 14C4 and strain 23B5 showed both MGP traits.

Effect of single bacterial strain on the productivity of *Tetraselmis suecica* F&M-M33

22 strains, selected on the basis of their phylogeny and MGP traits, were further analyzed to test their effect on *T. suecica* F&M-M33 growth (Table S3) The strains belong to different species, (except for *Marinobacter* sp. and *Amorpus coralli*); eight strains were IAA producers, eight strains were siderophores producers and six strains did not resulted MGP (Table S4). Co-cultures of axenic *T. suecica* and single bacterial strain were performed. *Vitellibacter* sp. strain AAD2, *Sphyngopyxis flavimaris* strain AG5, *Stappia indica* strain 23B5 and *Pseudomonas knackmussii* strain PR1 were able to enhance ($p < 0.05$) the productivity of microalgal in terms of biomass production (Figure 1). The higher value of biomass increase (more than 25%) was achieved with *Vitellibacter* sp. AAD2. None of the strain tested was able to enhance significantly ($p < 0.05$) the productivity calculated as number of cells, while in five co-cultures the number of algal cells decreased (Figure 1). In co-cultures with *Amorphus coralli* strain 14C7 and *S. indica* strain 23B5 the number of algal cells decreased by more than 25% (28% and 27% respectively) ($p < 0.05$).

Metabolomic analysis of the co-cultures

Vitellibacter AAD2 and *S. flavimaris* AG5 were selected for metabolomics analysis co-cultures assay since were the two strains inducing the highest and significant ($p < 0.05$) increase in *T. suecica* F&M-M33 biomasses production. After 60 days of growth, the supernatant of *T. suecica* samples axenic and in co-culture were analyzed through UPLC-ESI QTOF-MS performed in positive and negative mode to determine metabolites with different chemical properties. Complex UPLC-ESI QTOF-MS metabolic profiles were obtained and a total of 131 entities were retrieved, considering both positive and negative ionization mode analysis data (Tables S4 and S5). A similar number of entities were retrieved for the axenic culture (Axe) and co-cultures (Axe+AAD2 and Axe+AG5) in both ionization modes. For Axe 63 and 57 entities were obtained for positive and negative mode, respectively. In the co-culture Axe+AAD2, 58 and 54 entities were detected, and in Axe+AG5 63 and 52 (positive and negative mode respectively) (Tables S4 and S5). A similar number of entities shared by the samples were retrieved in both the ionization modes analysis, 48 in positive and 46 in negative (Figure 2). Entities in common between the two co-cultures were respectively 2 in the positive mode analysis and 3 in the negative.

Out of 131 entities obtained a total of 103 compounds were identified on databases (Figure 3, Tables S4 and S5). Among the identified compounds, tri- and tetra-peptides, accounted for the 13% of the total. Terpenes were the second main category of molecules detected (8.26%). Other two important classes of compounds detected were aromatic compounds and fatty acids, which accounted for 8.21 and 6.72 %, respectively. Compounds with a structure formula similar to prostaglandine (PG) were identified, which were named as prostaglandine-like compounds (PG-like). PG-like were identified in both positive and negative analysis, and all the co-culture. Compounds involved in the triptophan/IAA metabolism (3.73 %) and in the metabolism of various vitamins of B group (2.99%) (Figure 3), such as pantothenic acid (or vitamin B5), 5-piridoxolactone (vitamin B6 metabolism) and 8-Amino-7-oxononanoate (vitamin B1 metabolism) (Tables S4 and S5) were identified. In each co-culture it was possible to identify culture-characteristic entities. A total of 7 entities were present only in Axe, 6 were identified as: di-tert-butyl pentasulphide, succinic acid, 4-hydroxy-L-threonine, guanosine, L-phenylalanine and a PGE-like compound (Table 3). Polypeptides as Ser-Met-Arg, Asp-Asn-Ar-Ser and Phe-Phe-Ile-Ala were retrieved only in Axe+AAD2 co-culture. Moreover the compounds N-alpha-acetyllysine and indole-3-acetic acid were found only in the supernatant of Axe+AG5 co-culture. Notably, in both co-culture supernatant the presence of 8-amino-7-oxononanoate, a compound involved in the metabolism of biotine, was noticed (Table 3).

DISCUSSION

T. suecica in all its industrial applications is usually grown with its associated bacterial community, it is then important to elucidate the role of the different microorganisms present in the phycosphere, to promote its gowth. More than 200 bacterial strains were isolated from *T. suecica* F&M-M33 cultures and screened for microalgal growth promoting (MGP) traits such as IAA and siderophore production. IAA is produced by many different bacteria and it influences microalgae growth in terms of cell dimension and number (de-Bashan *et al.* 2008, Do Nascimento *et al.*, 2013; Meza *et al.,* 2015; Labeeuw *et al.*, 2016). Most of the isolates producing IAA belong to the genus *Marinobacter*, a characteristic never reported associated to this genus, to our best knowledge. Nevertheless, the strains tested in co-cultures had negative effects on *T. suecica* growth, indicating that the IAA producing trait is not always associated to a beneficial role in *T. suecica* growth.

Microbial siderophores are often produced by bacteria, widely diffused in the marine environment and found in association with several species of microalgae (Amin *et al.*, 2009b; Lupette *et al.*, 2016). Siderophores, as Fe-Cathecol, stimulate the growth of the diatoms and play an important role in controlling the uptake of iron complexed with organic materials (Naito *et al.*, 2008; Amin *et al.*, 2009a; Moejes *et al.*, 2017). A well-studied genus in relation to siderophore production is *Marinobacter*, which was frequently found in association with microalgae (Amin *et al.*, 2009b; Amin *et al.*, 2012). Only one *Marinobacter* isolated in this work was able to produce siderophores. In literature has been reported that *Marinobacter* microalgal-associated did not produce the siderophores commonly produced by free-living members of the *Marinobacter* genus (Amin *et al.*, 2009). Two isolates belonging to *Stappia* genus were able to produce siderophores. Recently, a *Stappia* strain isolated from the phycosphere of *Tetraselmis striata* (Park *et al.*, 2017) showed to increase *T. striata* growth, although it was not clarified which factors were responsible of this effect. Out of 22 strains were tested in co-cultures and, 16 strains were able to produce MGP molecules. An increase in *T. suecica* growth was observed only for two strains producing IAA (*Sphyngopyxis flavimaris* strain AG5 and *Pseudomonas knackmussii* strain PR1) and two strains producing siderophores (*Vitellibacter* strain AAD2 and *Stappia* sp. strain 23B5). The other 10 isolates did not induce any measurable effect on the microalga suggesting that the MGP molecules are not produced by these strains in the co-culture conditions used or that their effect is not enough to elicit a significant response. Therefore, the global analysis of our data suggested that the IAA and siderophore production was not linked to *T. suecica* growth.

The two strains showing the best boosting effect on the growth of *T. suecica* were *Sphyngopyxis flavimaris* strain AG5, and *Vitellibacter* strain AAD2. To date, there are no evidences of a marked growth enhancing effect on *T. suecica* by bacteria belonging to these genera. Therefore to better understand role of the two growth enhancing strains in *T. suecica* phycosphere, the exo-metabolome of the axenic culture and of co-cultures was analyzed. As expected, the supernatant of *T. suecica* co-cultured with *S. flavimaris*, contained IAA. *S. flavimaris* strain AG5 belongs to the S*phingomonadaceae* family. Members of this family have been already described in association with several microalgae species e.g. *Micrasterias crux-melitensis, Chlorella saccharophila*, *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Botryococcus braunii*, (Krohn-Molt *et al.*, 2017). Members of the genus *Sphyngopyxis* sp. were found in association with *Gymnodium catenatum* and *Scenedesmus quadricauda* (Green *et al.*, 2004; Krohn-Molt *et al.*, 2017). However more compounds exclusively present in the Axe+AG5 co-culture were retrieved: such as 8-amino-7-oxononanoic acid, and other compounds involved in the metabolism of vitamin B5, vitamin B1 and vitamin B6. *T. suecica* is not auxotrophic for vitamins, however an increase of vitamins in its phycosphere may have a growth enhancement effect (Dammak *et al* 2017, Biondi *et al* 2018).

Vitellibacter sp. strain AAD2 was identified as one of the strains producing siderophores. *Vitellibacter* is a genus belonging to the *Flavobacteriaceae* family, of the *Cytophaga-Flavobacterium* cluster, which has been shown to account for more than 10% of the total bacterial community in coastal and offshore waters (Glockner *et al.*, 1999; Abell and Bowman, 2005; DeLong *et al.*, 2006). The metabolomics analysis of the supernatant of the Axe+AAD2 revealed the presence of several polypeptides (tri- and tetrapeptides), indeed genome analysis of *Vitellibacter flavimaris* and *Vitellibacter vlavostokensis* revealed the presence of 12 genes encoding for protease (Thevarajoo *et al.*, 2015; 2018), that could actively depolymerize the protein released by *T. suecica*.

The non-target metabolomics allowed the description of the exo-metabolome of *T. suecica,* after a long period of growth, and allowed to identify some molecules present only in the axenic condition. These molecules (like succinic acid, guanosine, L-phenylalanine), which cannot be found in the exo-metabolome of the co-cultures, are probably metabolized by bacteria. Among the compounds found in exo-metabolome two groups are not usually related with microalgae: PG-like compounds and cucurbitacin E. PG-like compounds were retrieved in both xenic and axenic cultures, indeed it was recently reported that in the microalga *Skeletonema marinoi* were found animal-like prostaglandins, which may act cell-to-cell signaling (Di Dato *et al.*, 2017). Cucurbitacin E is usually found in *Cucurbitaceae* plants which function as a defense against herbivores, but it was also isolated from mushrooms *Russula* and *Hebeloma*, and from shell-less marine molluscs (Jian *et al*., 2005). Cucurbitacin E was found in both co-cultures and, if these results will be confirmed, its role should be investigated because cucurbitacins have cytotoxic properties and are under study for their potential biological activities (Duncan et al., 1996).

In conclusion, several strains able to produce MGP compounds were isolated from the phycosphere of *T. suecica*, however a clear increase in biomass production was observed just for four of them. We may then hypothesize that the production of these molecules is not enough in the other strains or a more sophisticated interaction is required to achieve an increase in microalgae growth. Indeed the exo-metabolome of *T. suecica* revealed the presence of many molecules whose ecological role is not clear. Therefore, still a lot has to be understood regarding molecules involved in microalgal-bacteria relationships.

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Figures

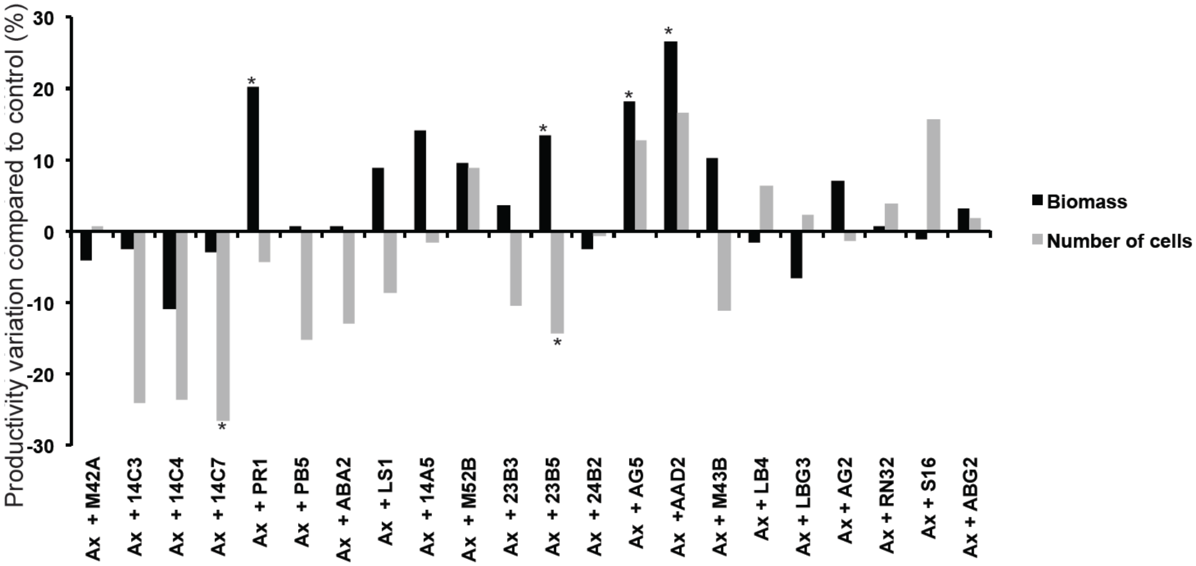

Figure 1. Variation of biomass (black) and number of cells (grey) productivity of co-cultures with respect to that of the axenic *T. suecica* culture. Co-cultures were obtained using an axenic culture of *Tetraselmis suecica* F&M-M33, inoculated with a pure culture of a selected bacterial strain (Ax+strain name). Co-cultures and axenic cultures were grown for 10 days at 27°C and under continuous illumination of about 90 µmol photons m^{-2} s^{-1} and shaking (85 rpm).

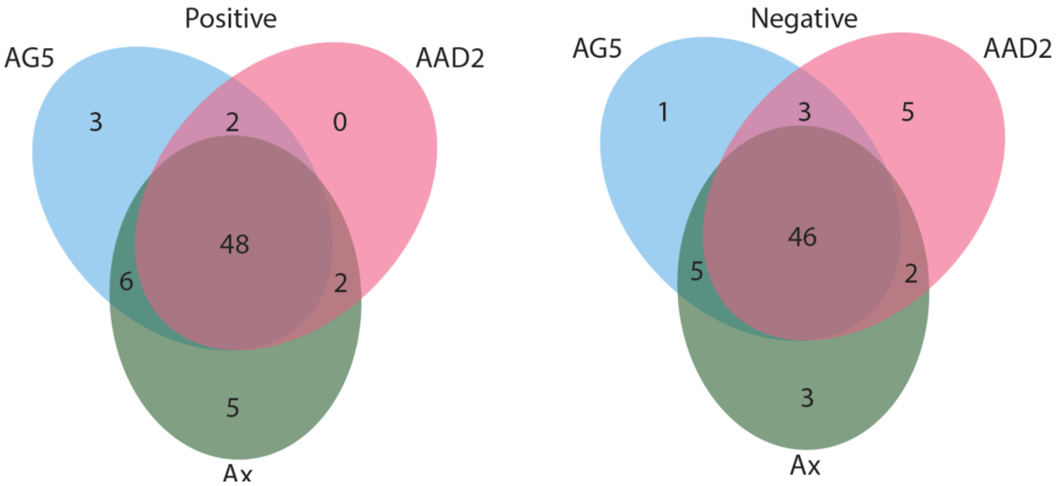

Figure 2. Venn diagrams showing unique entities, entities shared by all or by two culture supernatants of the axenic *Tetraselmis suecica* F&M-M33 (Axe), the co-culture obtained from the axenic *T. suecica* F&M-M33 and *Sphyngopyxis flavimaris* strain AG5 (Axe+AG5), and the axenic *T. suecica* F&M-M33 and *Vitellibacter* sp. strain AAD2. A non-targeted metabolomic analysis was performed through UPLC-ESI QTOF-MS. Data are referring to A) Positive ionization mode and B) Negative ionization mode.

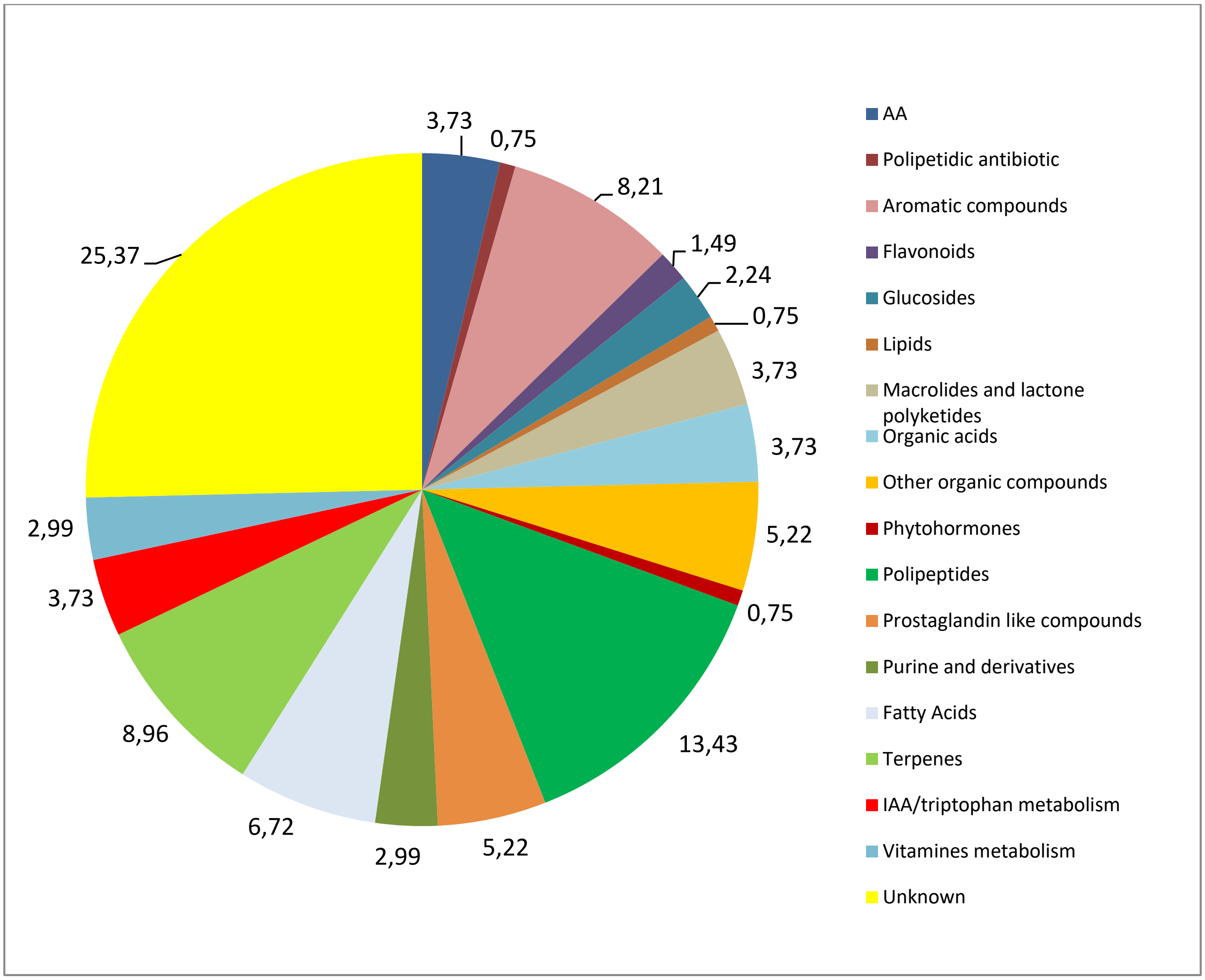

Figure 3. Circle diagram of the metabolites classification by compounds family. A set of 135 metabolites were classified from the total of entities detected and aligned after data processing. A non-targeted metabolomic analysis was performed through UPLC-ESI QTOF-MS,on the supernatant of axenic and co-cultures of *T. suecica*.

Tables

Table 1. Number of isolates from cultures of *Tetraselmis suecica* (strain F&M-M33) and phylogenetic attribution based on 16S rDNA sequencing.

Closest match	**Family**	**Class**	**N° of Isolates**
Bacillus sp.	Bacillaceae	Bacilli	4
Planococcus sp.	Planococcaceae	“	2
Algoriphagus sp.	Cyclobacteriaceae	Cytophagia	4
Muricauda sp.	Flavobacteriaceae	Flavobacteriia	38
Arenibacter sp.	“	“	7
Alcanivorax sp.	Alcanivoracaceae	Gammaproteobacteria	4
Marinobacter sp	Alteromonadaceae	“	18
Pseudomonas sp	Pseudomonadaceae	“	1
Amorphus coralli	Rhodobiaceae	Alphaproteobacteria	2
Nitratireductor sp	“	“	1
Rhodobacteraceae bacterium	Rhodobacteraceae	“	4
Aestuariivita sp .	“	“	1
Labrenzia sp	“	“	1
Mameliella sp .	“	“	26
Maribius sp	“	“	3
Marinovum sp	“	“	5
Pseudooceanicola sp.	“	“	2
Roseivivax sp.	“	“	16
Roseovarius sp.	“	“	8
Stappia sp.	“	“	16
Sulfitobacter sp.	“	“	2

Table 2. Identification of isolated bacteria producing microalgal growth promoting features (MGP feature). The production of Indole-3-acetic acid (IAA) was determined trhough a modification of the method described by Bric (Bric *et al.*., 1991); the production of siderophores (sid).

Strain	**Closest match**	**Family**	**MGP characteristic**	
			IAA	**Sid**
S109A1	*Planococcus sp*	Planococcaceae	+*	-**
S109A2	*Planococcus sp*	Planococcaceae	+	-
14A5G	*Bacillus indicus*	Bacillaceae	-	+
14CBOH1	*Bacillus indicus*	Bacillaceae	-	+
14C51	*Bacillus indicus*	Bacillaceae	-	+
14C52	*Bacillus indicus*	Bacillaceae	-	+
34B5	*Bacillus indicus*	Bacillaceae	-	+
331R	*Marinobacter sp*	Alteromonadaceae	-	+
13A13	*Marinobacter sp*	Alteromonadaceae	+	-
13A1B	*Marinobacter sp*	Alteromonadaceae	+	-
14C3	*Marinobacter flavimaris*	Alteromonadaceae	+	-
23A2	*Marinobacter sp*	Alteromonadaceae	+	-
23A6	*Marinobacter sp*	Alteromonadaceae	+	-
23B6	*Marinobacter sp*	Alteromonadaceae	+	-
23B9	*Marinobacter sp*	Alteromonadaceae	+	-
24A3	*Marinobacter sp*	Alteromonadaceae	+	-
24B3	*Marinobacter sp*	Alteromonadaceae	+	-
24C2	*Marinobacter sp*	Alteromonadaceae	+	-
24C2B	*Marinobacter sp*	Alteromonadaceae	+	-
24C3R	*Marinobacter sp*	Alteromonadaceae	+	-
ABA1	*Marinobacter sp. Set72*	Alteromonadaceae	+	-
M42A	*Marinobacter flavimaris*	Alteromonadaceae	+	-
M51	*Marinobacter sp*	Alteromonadaceae	+	-
M57A	*Marinobacter sp*	Alteromonadaceae	+	-
S102	*Marinobacter flavimaris*	Alteromonadaceae	+	-
S32B1	*Marinobacter sp*	Alteromonadaceae	+	-
24B2	*Muricauda aquimarina*	Flavobacteriaceae	-	+
S32A	*Muricauda sp.*	Flavobacteriaceae	+	-
AAD2	*Vitellibacter sp. 2-4*	Flavobacteriaceae	-	+
LS1	*Algoriphagus halophilus HME7998*	Cyclobacteriaceae	-	+

PR1	*Pseudomonas knackmussii*	Pseudomonadaceae	+	-
14A5	*Amorphus coralli*	Rhodobiaceae	-	+
14C7	*Amorphus coralli*	Rhodobiaceae	+	-
14C4	*Stappia stellulata*	Rhodobacteraceae	+	-
S105	*Mameliella sp*	Rhodobacteraceae	+	-
S108	*Marinovum algicola*	Rhodobacteraceae	+	-
23B3	*Labrenzia sp*	Rhodobacteraceae	-	+
S90	*Labrenzia sp*	Rhodobacteraceae	-	+
S78	*Labrenzia sp*	Rhodobacteraceae	+	-
ABA2	*Roseobacter sp. 49Xb1*	Rhodobacteraceae	+	-
M43B	*Roseivivax sp.*	Rhodobacteraceae	-	+
PB5	*Ruegeria sp. DG1292*	Rhodobacteraceae	+	-
AB1	*Sagittula sp. SBW235b*	Rhodobacteraceae	+	-
23B5	*Stappia indica*	Rhodobacteraceae	-	+
23B8	*Stappia indica*	Rhodobacteraceae	-	+
AG5	*Sphingopyxis flavimaris R-36742*	Sphingomonadaceae	+	-

* = strain positive to the MGP characteristic tested; ** = strain negative to the MGP characteristic tested

Table 3. Compounds identified on Metlin database in the supernatant of *T. suecica* cultures. The supernatant of cultures of *T. suecica* F&M-M33 axenic (Axe), *T. suecica* F&M-M33 co-cultured with *Vitellibacter* sp 2-4 strain AAD2 (Axe+AAD2) and *T. suecica* F&M-M33 co-cultured with *Spyngopyxix flavimaris* strain AG5 (Axe+AG5) were analyzed through a non-targeted metabolomic. Data are referring to the analysis conducted in positive (pos) ionization mode and in negative (neg) ionization mode.

Mass	**Ionization Mode**	**Sample**			**Formula**	**Identification on Database**
		Axe	**Axe +**	**Axe + AAD2**		
301,9998	neg	+[a]	-[b]	-	NA[c]	NF[d]
274,0011	neg	+	-	-	C6H10O10S	di-tert-butyl pentasulphide
118,0272	pos	+	-	-	C4H6O4	succinic acid
135,0544	pos	+	-	-	C4H9NO4	4-hydroxy-l-threonine
283,0917	pos	+	-	-	C10H13N5O5	guanosine
165,0791	pos	+	-	-	C9H11NO2	L-phenylalanine
280,1162	pos	-	+	-	NA	NF
188,1168	pos	-	+	-	C8H16N2O3	n-alpha-acetyllysine
392,181	neg	-	-	+	C21H28O7	Ser Met Arg
247,1473	neg	-	+	-	NA	NF
187,1209	pos	-	+	+	C9H17NO3	8-amino-7-oxononanoic acid
556,3016	neg	-	+	+	C25H49O11P	PI(P-16:0/0:0)/cucurbitacin E
272,1635	pos	-	+	+	NA	NF
394,2349	neg	-	-	+	NA	NF
490,2148	neg	-	-	+	C17H30N8O9	Asp Asn Arg Ser
352,2258	neg	+	-	-	C20H32O5	PGE2[e]
496,2674	neg	-	-	+	C27H36N4O5	Phe Phe Ile Ala
250,1934	neg	-	-	+	C26H42O8	4,7,10-hexadecatrienoic acid

[a]= entitie present in the sample; [b]= entitie not present in the sample; [c]= entities not assigned; [d]= molecular formula not found in the Database; [e]=the compounds shown are Prostaglandine Like compound, named as PG-like compounds in the text;

Supplemetary material

Table S1. Identification of bacterial isolates through 16S rDNA sequencing. Strains were isolated from different cultures of *T. suecica* F&M-M33

Phylum	Class	Order	Family	Genus	Specie	Isolate
Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	*Algoriphagus*	*A. alophilus*	M42B
Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	*Algoriphagus*	*A. alophilus*	M55
Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	*Algoriphagus*	*A. alophilus*	N42
Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	*Algoriphagus*	*A. alophilus*	S71
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	13A1R
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	13A21
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	14A1
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	14C1
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	24A1
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	24A2
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	24B1
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	24B5G
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	33B2G
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	34A2
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	34B1G
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	34B2
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	34C2
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	M30A1
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	M30C
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	M52BG

Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S07
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S14
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S17B
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S22
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S24
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S27A2
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S32A
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S35A
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S36
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S37B
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S54
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S62
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S64A
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S64B2
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S70
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S76
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S79
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S83A
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S85
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S86
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S97
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Muricauda*	*M.* sp	S109B
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	*A.* sp	S40
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	*A.* sp	S41B1
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	*A.* sp	S41B2
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	*A.* sp	S66
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	*A.* sp	S67A1
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	*A.* sp	S92
Bacteroidetes	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Arenibacter*	*A.* sp	S96
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. idriensis*	13A22

Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B. idriensis*	34B5
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B.* sp	14CBOH1
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	*B.* sp	14A5G
Firmicutes	Bacilli	Bacillales	Planococcaceae	*Planococcus*	*P.* sp	S109A
Firmicutes	Bacilli	Bacillales	Planococcaceae	*Planococcus*	*P.* sp	S109A2
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	*Alcanivorax*	*A. dieselolei*	S59B
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	*Alcanivorax*	*A. dieselolei*	S08A
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	*Alcanivorax*	*A. dieselolei*	S56B2
Proteobacteria	Gammaproteobacteria	Oceanospirillales	Alcanivoracaceae	*Alcanivorax*	*A. xenumutants*	S93
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	S32B1
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	S103
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	13A1B
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	13A13
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	23A1
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	23A2
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	23A6
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	23B6
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	23B9
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	24A3
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	24B3
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	24B5B
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	24C2
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	24C2B
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	24C3R
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	33A1R
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	M51
Proteobacteria	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Marinobacter*	*M. flavimaris*	M57
Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	*Pseudomonas*	*P. kymnyogenensis*	M54A7
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhodobiaceae	*Amorphus*	*Amorphus coralli*	33A5
Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhodobiaceae	*Amorphus*	*Amorphus coralli*	34B4

Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	*Nitratireductor*	*N.* sp	S68
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	-	*Rhodobac. Bacterium*	S09
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	-	*Rhodobac. Bacterium*	S37
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	-	*Rhodobac. Bacterium*	S60
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	-	*Rhodobac. Bacterium*	S61
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Aestuariivita*	*A. boseongensis*	S94B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Labrenzia*	*L.* sp	S78
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S03B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S12A
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S18
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S19
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S21
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S23
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S27A
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S27B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S32B2
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S39
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S43
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S45
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S50B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S51
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S63
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S65
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S67B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S73
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S74
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S80
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S81
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S82
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S83B

Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M.*sp	S87
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Mameliella*	*M. atlantica*	S98
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Marinovum*	*M.* sp	S77
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Marinovum*	*M. algicola*	S84
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Marinovum*	*M. algicola*	S101
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Marinovum*	*M. algicola*	S106
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Marinovum*	*M. algicola*	S108
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Maribius*	*M.* sp	S50A
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Maribius*	*M.* sp	S107
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Maribius*	*M.* sp	S111
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Pseudoceanicola*	*P. nanhaiensis*	M30B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Pseudoceanicola*	*P. nanhaiensis*	RN42
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	S06
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	S29
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	S35B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	S52
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	S89
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	S99
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	23C6
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	24A2R
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	24A4
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	24B4
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	M43A
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	M53A
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	M53B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	M53A2
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	M68
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseivivax*	*R.* sp	M69
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R. algicolus*	14B7
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	14B BHO1

Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	S01
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	S15
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	S17
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	S28A1
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	S56B1
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	S104
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Roseovarius*	*R.* sp	S104
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S.indica*	23B8
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S.indica*	33A2
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. sp.*	14B1
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. sp.*	14C6
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	13B2
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	14C2
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	14C8
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	23B2
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	23B7
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	23B8
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	23C8
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	33A2
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	33A6
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	33A7
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	34B1B
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Stappia*	*S. stellulata*	34C1
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Sulfitobacter*	*S.* sp	S75A
Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	*Sulfitobacter*	*S.* sp	S75B2

Table S2. Bacterial collection of strains previous isolates from *Tetraselmis suecica* F&M-M33 cultures and published in Biondi *et al*. 2017 and Piampiano *et al* 2018.

Strain	Closest match	Family	Reference
M52B	*Bacillus* sp.	Bacillaceae	Piampiano *et al* 2018
14B51	*Bacillus idriensis*	Bacillaceae	Piampiano *et al* 2018
LS1	*Algoriphagus halophilus* HME7998	Cyclobacteriaceae	Biondi *et al* 2017
RN41	*Algoriphagus alophilus*	Cyclobacteriaceae	Piampiano *et al* 2018
AR5	*Cyclobacterium marinum* VBW098	Cyclobacteriaceae	Biondi *et al* 2017
PRS2	*Cyclobacterium marinum* VBW098	Cyclobacteriaceae	Biondi *et al* 2017
AG2	*Leeuwenhoekiella* sp. M56-8	Flavobacteriaceae	Biondi *et al* 2017
PG1	*Salegentibacter mishustinae strain* NBRC 100592	Flavobacteriaceae	Biondi *et al* 2017
AAD2	*Vitellibacter* sp. 2-4	Flavobacteriaceae	Biondi *et al* 2017
M62	*Muricauda olearia*	Flavobacteriaceae	Piampiano *et al* 2018
LG3	*Muricauda aquimarina strain* 97A	Flavobacteriaceae	Biondi *et al* 2017
S12B1	*Muricauda* sp.	Flavobacteriaceae	Piampiano *et al* 2018
24B2	*Muricauda* sp.	Flavobacteriaceae	Piampiano *et al* 2018
S11	*Arenibacter algicola*	Flavobacteriaceae	Piampiano *et al* 2018
AA1	*Arenibacter* sp. MGE_SAT_544_1	Flavobacteriaceae	Biondi *et al* 2017
S59A2	*Alcanivorax dieselolei*	Alcanivoracaceae	Piampiano *et al* 2018
S102	*Marinobacter flavimaris*	Alteromonadaceae	Piampiano *et al* 2018
14C3	*Marinobacter flavimaris*	Alteromonadaceae	Piampiano *et al* 2018
M42A	*Marinobacter flavimaris*	Alteromonadaceae	Piampiano *et al* 2018
ABA1	*Marinobacter* sp. Set72	Alteromonadaceae	Biondi *et al* 2017
14A5	*Amorphus coralli*	Rhodobiaceae	Piampiano *et al* 2018
14C7	*Amorphus coralli*	Rhodobiaceae	Piampiano *et al* 2018
ABP3	*Caulobacter bacterium* MOLA 378	Caulobacteraceae	Biondi *et al* 2017

RN32	*Hoeflea* sp.	Phyllobacteriaceae	Piampiano *et al* 2018
IF3	*Mesorhizobium* sp. DG943	Phyllobacteriaceae	Biondi *et al* 2017
LB4	*Mesorhizobium* sp. VBW011	Phyllobacteriaceae	Biondi *et al* 2017
34C3	*Mesorhizobium thiogangeticum*	Phyllobacteriaceae	Piampiano *et al* 2018
S69	*Nitratireductor* sp.	Phyllobacteriaceae	Piampiano *et al* 2018
24A6	*Nitratireductor* sp.	Phyllobacteriaceae	Piampiano *et al* 2018
PR2	*Robiginosimarina momoshimaensis* NRBC 101843	Hyphomicrobiaceae	Biondi *et al* 2017
S09	*Rhodobacteraceae bacterium*	Rhodobacteraceae	Piampiano *et al* 2018
M58	*Rhodobacteraceae bacterium*	Rhodobacteraceae	Piampiano *et al* 2018
ABA2	*Roseobacter* sp. 49Xb1	Rhodobacteraceae	Biondi *et al* 2017
S90	*Labrenzia aggregata*	Rhodobacteraceae	Piampiano *et al* 2018
M61	*Mameliella* sp.	Rhodobacteraceae	Piampiano *et al* 2018
S16	*Mameliella* sp.	Rhodobacteraceae	Piampiano *et al* 2018
S105	*Mameliella* sp.	Rhodobacteraceae	Piampiano *et al* 2018
ABG2	*Ponticoccus* sp. *MBTDCMFRIMab06*	Rhodobacteraceae	Biondi *et al* 2017
S02	*Marinovum algicola*	Rhodobacteraceae	Piampiano *et al* 2018
PB5	*Marinovum algicola strain LPB0094*	Rhodobacteraceae	Biondi *et al* 2017
N41	*Pseudooceanicola* sp.	Rhodobacteraceae	Piampiano *et al* 2018
AB1	*Sagittula stellata strain SBW235a*	Rhodobacteraceae	Biondi *et al* 2017
LBG3	*Roseivivax halotolerans* NBRC16686	Rhodobacteraceae	Biondi *et al* 2017
S88	*Roseivivax* sp.	Rhodobacteraceae	Piampiano *et al* 2018
14B2	*Roseivivax* sp.	Rhodobacteraceae	Piampiano *et al* 2018
M43B	*Roseivivax* sp.	Rhodobacteraceae	Piampiano *et al* 2018
IB3	*Roseovarius mucosus type strain DFL-24*	Rhodobacteraceae	Biondi *et al* 2017
LB2	*Roseovarius indicus strain B108*	Rhodobacteraceae	Biondi *et al* 2017
14B8	*Roseovarius* sp.	Rhodobacteraceae	Piampiano *et al* 2018
ABP2	*Stappia* sp. *FG-4*	Rhodobacteraceae	Biondi *et al* 2017

23B5	*Stappia indica*	Rhodobacteraceae	Piampiano *et al* 2018
14A3	*Stappia stellulata*	Rhodobacteraceae	Piampiano *et al* 2018
14C4	*Stappia stellulata*	Rhodobacteraceae	Piampiano *et al* 2018
M54A3	*Porphyrobacter sanguineus*	Erythrobacteraceae	Piampiano *et al* 2018
AAD3	*Porphyrobacter* sp. MBIC3897	Erythrobacteraceae	Biondi *et al* 2017
ARS1	*Porphyrobacter sanguineus* NBRC 15763 *type strain*	Erythrobacteraceae	Biondi *et al* 2017
AG5	*Sphingopyxis flavimaris* R-36742	Sphingomonadaceae	Biondi *et al* 2017
PR1	*Pseudomonas knackmussii strain B13*	Pseudomonadaceae	Biondi *et al* 2017
IB4	*Halomonas* sp. MA-9A	Halomonadaceae	Biondi *et al* 2017
AT1	*Pelagicoccus albus* YM14-201	Puniceicoccaceae	Biondi *et al* 2017
EA1	*Microbacterium aurantiacum strain* CIP 105730	Microbacteriaceae	Biondi *et al* 2017

Table S3. Strain tested in co-colture assays to evaluate the effects on the growth of *T. suecica* F&M-M33. Strains were selected for their MGP traits, Indole-3-acetic acid (IAA) and siderophore production (SID).

Strain	**Reference**	**Closest relative**	**Family**	**MGP Caract.**
14C3	Piampiano *et al*	*Marinobacter flavimaris*	Alteromonadaceae	IAA
M42A	Piampiano *et al*	*Marinobacter flavimaris*	Alteromonadaceae	IAA
14C4	Piampiano *et al*	*Stappia Stellulata*	Rhodobacteriaceae	IAA
14C7	Piampiano *et al*	*Amorphus Coralli*	Rhodobiaceae	IAA
ABA2	Biondi *et al* 2017	*Roseobacter* sp. 49Xb1	Rhodobacteriaceae	IAA
PB5	Biondi *et al* 2017	*Ruegeria* sp. DG1292	Rhodobacteriaceae	IAA
AG5	Biondi *et al* 2017	*Sphyngopyxis flavimaris* R-36742	Sphyngomonadaceae	IAA
PR1	Biondi *et al* 2017	*Pseudomonas xantomarina*	Pseudomonadaceae	IAA
23B3	Piampiano *et al*	*Labrenzia* sp	Rhodobacteriaceae	SID
23B5	Piampiano *et al*	*Stappia indica*	Rhodobacteriaceae	SID
M43B	Piampiano *et al*	*Roseivivax* sp	Rhodobacteriaceae	SID
24B2	Piampiano *et al*	*Muricauda* sp	Flavobacteriaceae	SID
14A5	Piampiano *et al*	*Amorphus coralli*	Rhodobiaceae	SID
M52B	Piampiano *et al*	*Bacillus indicus*	Bacillaceae	SID
AAD2	Biondi *et al* 2017	*Vitellibacter* sp. *2-4*	Flavobacteriaceae	SID
LS1	Biondi *et al* 2017	*Algoriphagus halophilus* HME7998	Cyclobacteriaceae	SID
RN32	Piampiano *et al*	*Hoeflea* sp.	Phyllobacteriaceae	-*
S16	Piampiano *et al*	*Mameliella* sp.	Rhodobacteriaceae	-
ABG2	Biondi *et al* 2017	*Ponticoccus* sp. MBTDCMFRIMab06	Rhodobacteriaceae	-

LBG3	Biondi *et al* 2017	*Roseivivax halotolerans* NBRC16686	Rhodobacteriaceae	-
AG2	Biondi *et al* 2017	*Leeuwenhokiella marinoflava* P80	Flavobacteriaceae	-
LB4	Biondi *et al* 2017	*Mesorhizobium* sp. VBW011	Phyllobacteriaceae	-

*=no MGP charachteristics were associated to the strains used in this test

Table S4. Entities identified in the surnatant of *T. suecica* cultures. The surnatant of cultures of *T. suecica* F&M-M33 axenic (Axe), *T. suecica* F&M-M33 co-cultured with *Vitellibacter sp* 2-4 strain AAD2 (Axe+AAD2) and *T. suecica* F&M-M33 co-cultured with *Spyngopyxix flavimaris* strain AG5 (Axe+AG5) were analyzed through a non-targeted metabolomic. Data are referring to the analysis conducted in positive ionization mode. Entities were identified on Metlin, Metlin metab and Lipids databases.

Mass	**RT**	**Sample**			**Molecular formula**	**Databases**		
		Axe	**Axe + AG5**	**Axe + AAD2**		**Metlin**	**Metlin metab**	**Lipids**
134.0581	2.25	+[a]	+	+	C5H10O4	2,3-dihydroxy-3-methylbutyric acid	2-deoxy-d-ribose	2,3-dihydroxy-valeric acid
290.0986	2.25	+	+	+	NA[c]	NF[d]	NF	NF
258.0363	2.63	+	-[b]	-	NA	NF	NF	NF
118.0272	2.63	+	-	-	C4H6O4	succinic acid	succinic acid	succinic acid
135.0544	3.18	+	-	-	C4H9NO4	4-hydroxy-l-threonine	adenine	NF
283.0917	3.42	+	-	-	C10H13N5O5	guanosine	guanosine	NF
326.1156	3.48	+	+	-	C19H18O5	2-methyl-5,7,8-trimetroxoflavone	NF	NF
318.1302	3.79	+	+	+	C19H18O5	NF	methylophiopogonone b	eucalyptin
148.0736	3.79	+	+	+	C6H12O4	mevalonic acid	(r)-mevalonic acid	(r)-mevalonic acid
204.0094	4	+	+	+	C7H8O5S	2-hydroxy-5-methoxybenzene-1-sulfonic acid	NF	NF
165.0791	4.32	+	-	-	C9H11NO2	l-phenylalanine	l-phenylalanine	NF
280.1162	4.41	-	+	-	NA	NF	NF	NF
342.0919	4.56	+	+	-	C15H18O9	NF	caffeic acid 3-glucoside	NF
209.0723	4.6	+	+	+	C7H15NO4S	NF	NF	NF
219.1109	4.94	+	+	-	C9H17NO5	pantothenic acid	pantothenic acid	NF
188.1168	5.21	+	+	-	C8H16N2O3	n-alpha-acetyllysine	glycyl-l-leucine	NF
221.0688	5.28	+	+	+	C11H11NO4	6-hydroxyindolelactate	6-hydroxyindolelactate	NF
248.1267	5.33	+	+	+	C12H16N4O2	NF	histidylproline diketopiperazine	NF

217.9889	5.9	+	+	+	$C_7H_6O_6S$	5-sulfosalicylic acid	5-sulfosalicylic acid	NF
172.0196	6.02	+	-	+	$C_7H_8O_4S$	NF	p-cresol sulfate	NF
173.9987	6.22	+	+	+	$C_6H_6O_4S$	4-hydroxybenzenesulfonic acid	phenyl sulfate	NF
218.116	6.85	+	+	+	$C_{10}H_{18}O_5$	NF	2-hydroxy-decanedioic acid	NF
130.063	7.45	+	+	-	$C_6H_{10}O_3$	ketoleucine	ketoleucine	NF
221.0693	8.13	+	+	+	$C_{11}H_{11}NO_4$	6-hydroxyindolelactate	6-hydroxyindolelactate	NF
132.079	9.3	+	+	-	$C_6H_{12}O_3$	2-hydroxy-4-methylvaleric acid	d-leucic acid	NF
316.1431	9.45	+	+	+	NA	NF	NF	NF
187.1209	10.41	-	+	+	$C_9H_{17}NO_3$	KAPA	8-amino-7-oxononanoate	NF
165.0425	11.89	+	+	-	$C_8H_7NO_3$	5-pyridoxolactone	4-pyridoxolactone	NF
159.0689	12.45	+	+	+	$C_{10}H_9NO$	indoleacetaldehyde	indoleacetaldehyde	NF
374.2667	12.58	+	+	+	NA	NF	NF	NF
374.2672	12.758	+	+	+	NA	NF	NF	NF
330.122	13	+	+	+	$C_{17}H_{18}N_2O_5$	miraxanthin-III	miraxanthin-iii	NF
582.2815	13.47	+	+	+	$C_{26}H_{47}O_{12}P$	PI(17:2(9z,12z)/0:0)	aralionine A	PI(17:2(9z,12z)/0:0)
272.1635	13.53	-	+	+	NA	NF	NF	NF
532.2899	13.73	+	+	+	$C_{20}H_{40}N_{10}O_5S$	arg cys val arg	NF	NF
188.105	13.81	+	+	+	$C_9H_{16}O_4$	3-methylsuberic acid	methyl n-(a-methylbutyryl)glycine	NF
402.2622	13.86	+	+	+	$C_{21}H_{38}O_7$	triisopentyl citrate	erythronolide B	NF
532.2898	13.91	+	+	+	$C_{20}H_{40}N_{10}O_5S$	arg cys val arg	NF	NF
404.2789	14.48	+	+	+	NA	NF	NF	NF
516.2956	14.908	+	+	+	$C_{26}H_{44}O_{10}$	NF	l-olivosyl-oleandolide	l-olivosyl-oleandolide
502.3149	15.03	+	+	+	$C_{33}H_{42}O_4$	NF	kolanone	NF
516.2953	15.1	+	+	+	$C_{26}H_{44}O_{10}$	NF	l-olivosyl-oleandolide	l-olivosyl-oleandolide
516.2956	15.27	+	+	+	$C_{26}H_{44}O_{10}$	NF	l-olivosyl-oleandolide	l-olivosyl-oleandolide
298.1791	15.56	+	+	+	$C_{16}H_{26}O_5$	NF	NF	tetranor-pge1
354.2416	16.66	+	+	+	$C_{20}H_{34}O_5$	PGE1[e]	PGF2α[e]	PGF2α
498.2842	16.76	+	+	+	NA	NF	NF	NF
354.2413	16.89	+	-	+	$C_{20}H_{34}O_5$	PGE1	PGF2α	PGF2α

498.2839	17.32	+	+	+	NA	NF	NF	NF
326.211	17.65	+	+	+	NA	NF	NF	NF
328.2265	18.155	+	+	+	C19H28N4O	4-(butylamino)-n,8-dipropylcinnoline-3-carboxamide	9s,11r,15s-trihydroxy-2,3-dinor-13e-prostaenoic acid-cyclo[8s,12r]	9-hydroperoxy-12,13-epoxy-10-octadecenoic acid
514.2781	18.55	+	+	+	C26H42O10	cinncassiol d1 glucoside	NF	NF
500.2991	18.65	+	+	+	C26H44O9	mupirocin	mupirocin	NF
352.2255	18.7	+	+	+	C20H32O5	PGE2[e]	PGH2[e]	5,14,15-trihydroxy-6,8,10,12-eicosatetraenoic acid
496.2681	18.91	+	+	+	C27H36N4O5	phe ala phe ile	fexaramine	NF
482.2892	19.05	+	+	+	C26H42O8	fusicoccin h		NF
496.2673	19.14	-	+	+	C27H36N4O5	phe phe ala leu	fexaramine	NF
482.289	19.26	+	+	+	C26H42O8	fusicoccin h		NF
294.1843	19.56	+	+	+	C17H26O4	NF	phytuberin	NF
310.179	19.84	+	+	+	C18H30S2	4-(dodecylsulfanyl)benzene-1-thiol	botrydial	methyl 8-[2-(2-formyl-vinyl)-3-hydroxy-5-oxo-cyclopentyl]-octanoate
383.0781	20.27	+	+	+	NA	NF	NF	NF
195.0901	20.35	+	+	+	NA	NF	NF	NF
267.1117	20.51	+	+	+	NA	NF	NF	NF
316.2626	20.74	+	+	+	C18H36O4	2,3-dihydroxy stearic acid	(9s,10s)-9,10-dihydroxyoctadecanoic acid	2,3-dihydroxy stearic acid
266.1565	21.03	+	+	+	C15H22O4	NF	mukaadial	(+)-blennin d
234.1623	21.59	+	+	+	C15H22O2	NF	bakkenolide a	alcyopterosins o

[a]= entitie present in the sample; [b]= entitie not present in the sample; [c]= entities not assigned; [d]= molecular formula not found in the Database; [e]=the compounds shown are Prostaglandine Like compound, named as PG-like compounds in the text;

Table S5. Entities identified in the surnatant of *T. suecica* cultures. The surnatant of cultures of *T. suecica* F&M-M33 axenic (Axe), *T. suecica* F&M-M33 co-cultured with *Vitellibacter sp* 2-4 strain AAD2 (Axe+AAD2) and *T. suecica* F&M-M33 co-cultured with *Spyngopyxix flavimaris* strain AG5 (Axe+AG5) were analyzed through a non-targeted metabolomic. Data are referring to the analysis conducted in negative ionization mode. Entities were identified on Metlin, Metlin metab and Lipids databases.

Mass	RT (min)	Sample			Molecular Formula	Databases		
		AXE	AXE + AG5	AXE + AAD2		Metlin	Metlin metabolites	Lipids
210,9567	1,134	+[a]	-[b]	+	C8H5NS3	2H-[1,3] Dithiolo[4,5-f] [1,3] benzothiazole	NF[d]	NF
290,0987	2,247	+	+	+	C16H18O3S	2-(3-Methoxybenzene-1-sulfonyl)-1,3,5-trimethylbenzene	NF	NF
134,0585	2,249	+	+	+	C5H10O4	2,3-dihydroxy-3-methylbutyric acid	2-Deoxy-D-Ribose	2,3-dihydroxy-valeric acid
424,1432	2,249	+	-	+	C15H28N4O6S2	Met Ser Gly Met	Eprosartan	NF
301,9998	2,624	+	-	-	NA[c]	NF	NF	NF
274,0011	2,6300	+	-	-	C6H10O10S	Di-tert-butyl pentasulphide	L-Iduronate 2-sulfate	NF
267,0967	3,1820	+	+	-	C10H13N5O4	Adenosine	NF	NF
135,0546	3,1830	+	+	-	C5H5N5	Adenine	NF	NF
303,0735	3,183	+	+	-	NA	NF	NF	NF
381,09	3,183	+	+	-	NA	NF	NF	NF
318,1296	3,787	+	+	+	NA	NF	NF	NF
278,032	3,7880	+	-	+	C6H16O8P2	1,6-Hexanediol bisphosphate	NF	NF
284,0487	3,7890	+	+	+	C8H21ClO3Si3	2-(3-Chloropropyl)-2,4,4,6,6-pentamethyl-1,3,5,2,4,6-trioxatrisilinane	NF	NF
170,0552	3,789	+	-	+	C8H10O4	3,4-bis(methylene)-Hexanedioic acid	DL-3,4-Dihydroxyphenyl glycol	NF
268,0193	3,789	+	-	+	C12H10Cl2N2O	NF	3,3'-Dichloro-4,4'-diaminodiphenyl ether	NF
148,0737	3,7900	+	+	+	C6H12O4	Mevalonic acid	(R)-Mevalonic acid	NF

209,0622	4,56	+	+	+	C9H11N3OS	N-(4-Thioureido-phenyl)-acetamide	NF	NF
370,0551	6,8400	+	+	+	C15H14O11	2-O-Caffeoylhydroxycitric acid	NF	NF
218,1156	6,845	+	+	+	C10H18O5	2-Hydroxydecanedioic acid	3-hydroxy-sebacic acid	NF
458,2129	6,8450	+	+	+	C18H30N6O8	Ser Pro Gln Gln	NF	NF
392,181	7,415	-	-	+	C21H28O7	Ser Met Arg	Viguiestenin	NF
221,069	8,133	+	+	+	C11H11NO4	6-Hydroxyindolelactate	NF	NF
289,056	8,134	+	+	+	C8H12N5O5P	NF	9-((2-Phosphonylmethoxy)ethyl)guanine	NF
392,277	8,193	+	+	+	NA	NF	NF	NF
316,1428	9,448	+	+	+	NA	NF	NF	NF
352,1189	9,448	+	+	+	NA	NF	NF	NF
247,1473	10,322	-	+	-	C14H18FN3	4-[4-(2-Fluorophenyl)piperazin-1-yl]butanenitrile	NF	NF
209,1032	10,414	-	+	+	NA	NF	NF	NF
187,121	10,416	-	+	+	C9H17NO3	8-Amino-7-oxononanoate	NF	NF
289,0269	12,459	+	+	+	C10H12ClN3O3S	NF	Benzenesulfonamide, 3-chloro-4-(3-methyl-5-oxo-1-imidazolidinyl)-	NF
556,3016	13,277	-	+	+	C25H49O11P	PI(P-16:0/0:0)/cucurbitacin E	NF	NF
520,3257	13,277	+	+	+	C27H44N4O6	Leu Tyr Leu Ile	NF	NF
262,1624	13,529	-	+	+	C12H30SSi2	C12H30SSi2	NF	NF
404,2776	14,476	+	+	+	NA	NF	NF	NF
394,2349	14,649	-	-	+	NA	NF	NF	NF
516,2939	14,908	+	+	+	C26H42O11	L-Olivosyl-oleandolide	NF	NF
530,2727	14,924	+	+	+	C26H44O10	C26H42O11	NF	NF
516,2942	15,271	+	+	+	C26H44O10	C26H44O10	NF	NF
298,1788	15,551	+	+	+	NA	NF	NF	NF
514,278	16,072	+	+	+	NA	NF	NF	NF
422,1945	16,298	+	+	+	C23H26N4O4	Trp Ala Phe	NF	NF
490,2148	16,66	-	-	+	C17H30N8O9	Asp Asn Arg Ser	NF	NF
354,2408	16,66	+	+	+	C20H34O5	PGE1[e]	PGF2α [e]	NF
368,2199	16,66	+	+	+	C20H32O6	19(R)-hydroxy-PGE2 [e]	PGG2 [e]	NF

326,2096	17,65	+	+	+	C18H30O5	2R-hydroperoxy-9Z,12Z,15Z-octadecatrienoic acid	2,3-dinor-11b-PGF2α [e]	2R-hydroperoxy-9Z,12Z,15Z-octadecatrienoic acid
440,202	17,65	+	+	+	C22H32O9	Phe Gln Phe	10-Deacetyl-2-debenzoylbaccatin III	NF
764,3687	17,65	+	+	+	C34H52N8O12	L-Tyrosyl-L-threonyl-L-seryl-L-asparaginyl-L-leucyl-L-alanyl-L-proline	NF	NF
328,2252	18,152	+	+	+	C18H32O5	9-hydroperoxy-12,13-epoxy-10-octadecenoic acid	9S,11R,15S-trihydroxy-2,3-dinor-13E-prostaenoic acid-cyclo[8S,12R]	NF
352,2258	18,7000	+	-	-	C20H32O5	PGE2 [e]	NF	NF
496,2675	18,917	+	+	+	C27H36N4O5	Phe Phe Ile Ala	NF	NF
518,2491	18,917	+	+	+	C24H34N6O7	Trp Gln Val Ser	NF	NF
482,2879	19,047	+	+	+	C26H42O8	Fusicoccin H	NF	NF
496,2674	19,133	-	-	+	C27H36N4O5	Phe Phe Ile Ala	NF	NF
312,1931	19,239	+	+	+	NA	NF	NF	NF
222,1619	19,558	+	+	+	C14H22O2	Rishitin	NF	7E,9E,11-Dodecatrienyl acetate
362,1703	19,558	+	+	+	C20H26O6	NF	NF	gibberellin A19
237,1128	19,559	+	+	+	C9H20NO4P	15175-91-4	NF	NF
294,1834	19,559	+	+	+	C14H22O2	6-Gingerol	Phytuberin	NF
208,1465	19,577	+	+	+	C13H20O2	4-Heptyloxyphenol	NF	NF
482,2882	19,579	+	+	+	C16H22N6O4	Fusicoccin H	NF	NF
352,0583	19,624	+	+	+	C19H12O7	NF	Daphnoretin	12a-Hydroxydolineone
250,1934	19,855	-	-	+	C26H42O8	4,7,10-hexadecatrienoic acid	Methyl farnesoate	C16:3n-6,9,12
195,0898	20,348	+	+	+	C10H13NO3	L-Tyrosine methyl ester	L-Tyrosine methyl ester	NF
267,111	20,505	+	+	+	NA	NF	NF	NF
240,0749	21,455	+	+	+	C10H12N2O5	NF	Dinoseb	NF
234,1624	21,59	+	+	+	C15H22O2	Confertifoline	Bakkenolide A	Alcyopterosins O

[a]= entitie present in the sample; [b]= entitie not present in the sample; [c]= entities not assigned; [d]= molecular formula not found in the Database; [e]=the compounds shown are Prostaglandine Like compound, named as PG-like compounds in the text;

Chapter 5

Concluding remarks

The “phycosphere” is the zone rich in nutrients that surrounds microalgal cells and it is thought to be responsible for the formation of an associated bacterial community. Several microalgal-bacterial interactions can occur in this micro-environment including symbiosis, parasitism, competition or commensalism. It has been demonstrated that the establishment of these relationships depends on the exchange of diverse chemicals that include both growth resources and molecules involved in metabolism modulation such as vitamins and hormones. A deeper knowledge of the mechanisms that rule microalgae-bacteria interactions should useful not only for basic research but also to improve microalgal productivity.

The structure of the bacterial community associate to *T. suecica* was evaluated through metagenetic analysis on four microalgal cultures derived from the same original non-axenic culture, but physically and geographically separated for years, and kept under different growing conditions. It was found a “core” of microbial taxa maintained for a decade in the four cultures analyzed. The identified bacteria at genus level were *Marinobacter, Anaerospora, Hyphomonas, Devosia, Muricauda, Arenibacter, Leadbetterella,* which are commonly found in marine environments. Therefore, we may suppose that they were originally associated to *T. suecica* F&M-M33 at the sampling time. Bacteria belonging to the “core” community represent the major part of the associated microbiota (70-80%). However, the relative abundance of the different groups was strongly influenced by environmental/culturing conditions.

Substances released by the associated bacteria community may affect microalgal growth and metabolism, influencing biomass yield and quality. Therefore strains with Microalgal Growth Promoting (MGP) characteristics [indol-3-acetic acid (IAA) and siderophore production], isolated from the different cultures, were tested in co-cultures to evaluate their effects on *T. suecica* growth. Among all the strains tested we identified two microorganisms able to enhance microalgal biomass: *Vitellibacter sp.* and *Sphingopyxis flavimaris.* Non-targeted metabolomic analysis on *T. suecica* exo-metabolome (axenic and in co-culture) confirmed that *Sphingopyxis flavimaris* produces IAA in presence of the microalga while for *Vitellibacter sp.* co-culture it is not clear which mechanism induces an increase of *T. suecica* growth. For the first time the exo-metabolome of *T. suecica* in xenic and axenic conditions was studied. It was possible to identify culture-characteristic compounds, especially compounds present only in the axenic supernatant, such as di-tert-butyl pentasulphide, succinic acid, 4-hydroxy-l-threonine, guanosine and L-phenylalanine, that should be metabolized by bacteria once co-cultured. Moreover, the presence of many molecules whose ecological role is not clear was observed, however several compounds involved in metabolism of vitamins and phytohormones were identified.

Overall, the data obtained in this thesis, that have combined metagenomics and metabolomics approach, constitute the first base to open the black box *Tetraselmis suecica* /associated bacteria holobiont. More studies are needed to deepen the role of isolate bacteria on microalgal metabolism and *vice versa*.

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