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FUNCTIONAL EFFECTS OF *POLYSIPHONIA* SP. EPIPHYTISM ON THE FARMED *KAPPAPHYCUS ALVAREZII* (DOTY) LIAO: COMPETITION FOR THE RESOURCE, PARASITISM OR BOTH?

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Keywords

Stable isotopes · Nutrient incorporation · Nitrogen · Carbon · Madagascar · Carrageenan · Rhodophyta · Seaweed farming · Western Indian Ocean

Abstracts

Seaweed farming for the production of carrageenan is a growing economic activity. Like everywhere in the marine environment, farmed algae such as *Kappaphycus alvarezii* can host algal organisms as epiphytes. Epiphytes ensure important functions in natural ecosystems, but these organisms can have negative impacts on their hosts and, in aquaculture be considered a plague responsible for significant economic losses. The mechanisms by which epiphytes act functionally on their hosts are multiple: shading effects, competition for nutrients or parasitism. Parasitism is characterised by the epiphyte diverting a proportion of the host's resources. The objective of our work was to assess the impact of the epiphyte *Polysiphonia* sp. on the N and C acquisition of its farmed host *K. alvarezii* using two isotopic experiments with ^{13}C and ^{15}N as tracers. Our results demonstrated a double cumulative action: epiphytes could be capable of quickly outcompeting their hosts in terms of nutrient acquisition because of their better efficiency in C and N uptake, while also functionally qualifying as true parasites, as they divert some of the N resources acquired by their host. In terms of biocontrol, we suggest that the choice of nutrient-rich areas to practice *Kappaphycus* farming is likely to favour the epiphytes rather than their hosts, considering their relative needs and abilities to incorporate nutrients.

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Introduction

Originating from East Asia, the 'cottonii', *Kappaphycus alvarezii* (Liao 1996), has been farmed for its carrageenan content since the 1970s in the Philippines (Doty and Alvarez 1975; Hurtado et al. 2015). Becoming an alternative to traditional fishing for local communities, the farming activities of *K. alvarezii* has increased rapidly in coastal areas of the Western Indian Ocean. Madagascar is one of the first regions, along with Tanzania, which introduced *K. alvarezii* for seaweed farming (Ateweberhan et al. 2015). However, production demonstrates significant inter-annual variability. In 2009, Madagascar exported 1861 t of dry mass of *K. alvarezii*, but only 196 t in 2011. In 2020 this had returned to the level of 2009 production (i.e. 1708 t) (Frédéric Pascal, personal communication). This variability is partly related to the occurrence of algal diseases, such as the epiphytic algae outbreak (Tsiresy et al. 2016; Ndawala et al. 2022); accordingly, several companies have ceased their activities following major and recurrent losses of their production (Ateweberhan et al. 2015).

In ecology, epiphytism is considered as a form of symbiosis characterised by an organism living fixed on a plant substrate (i.e. the host or basiphyte) (Steel and Bastow Wilson 2003; Parmentier and Michel 2013). In marine ecosystems, epiphytic organisms are common and diverse. They serve many ecological functions, including being consumed by many invertebrates at the bottom of associated food webs (Karez et al. 2000; Lepoint et al. 2000; Borowitzka et al. 2006). They can be microalgae or other unicellular eukaryotes but also sessile invertebrates or macroalgae (Behera et al. 2022).

In seaweed farming, the sudden and massive development of certain epiphytes on the surface of cultivated algae leads to altered carrageenan quality, with significant economic consequences (Vairappan et al. 2014; Ward et al. 2020; Behera et al. 2022). Ultimately, such blooms of epiphytic algae represent a major threat to tropical seaweed farming socio-economic systems (Ingle et al. 2020) and have been termed 'epiphytic disease' or 'epiphytic filamentous algae disease' (EFAD). Epiphytic outbreaks can even contribute to the additional development of opportunistic diseases, such as ice-ice disease (Ward et al. 2020; 2022). Heavy infections of epiphytic algae are known to cause damage to the cortex of *Kappaphycus*, thus leaving the host vulnerable to infection by opportunistic bacteria (Vairappan et al. 2008; Tsiresy et al. 2016). For *Kappaphycus* farming, EFAD consists mostly of filamentous algal species attaching to the cortical layers of the host algal thallus, thus roughening it (Doty and Alvarez 1975; Tsiresy et al. 2016). These filamentous algae have been described as belonging to the genera *Polysiphonia* (Greville) or *Neosiphonia* Kim and Lee (Masuda et al. 2001 ; Hurtado et al. 2006; Tsiresy 2016). In Madagascar, Tsiresy (2016) showed that epiphytes form a monophyletic clade well separated from the rest of the existing *Neosiphonia* and *Polysiphonia* species. Their results support the view that the EFAD in Madagascar is caused by a single new species of *Polysiphonia* which is probably undescribed (Tsiresy 2016).

The first symptom of an EFAD infection is the appearance of small black spots (Vairappan et al. 2008; Tsiresy et al. 2016). The epiphyte attaches to the host through a primary rhizoid, and small lesions may appear. Thereafter, development takes place and the vegetative form appears after 2-4 weeks, depending on the temperature and salinity conditions of the sea water (Tsiresy et al. 2016). The epiphyte can be a solitary filament with several secondary rhizoids or several epiphytes appearing

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from a single opening (of a wart) (Vairappan et al. 2008; Tsiresy et al. 2016). This vegetative form has a size of about 0.5 mm and a density of less than 25.0 epiphytes per cm^2, and it persists throughout the dry season. Upon maturation—that is, after 4-6 weeks—the epiphyte looks more like a hairy

tuft (Vairappan et al. 2008; Tsiresy et al. 2016). This creates an effect called 'goosebumps' (Doty and Alvarez 1975; Hurtado et al. 2006). Tsiresy et al. (2016) demonstrated with transmission electron microscopy that these epiphytes penetrate deeply into their hosts, raising the hypothesis of parasitic symbiosis. There are five types of morphological relationships between an epiphyte and its host that have been described in detail by Leonardi et al. (2006) and Ingle et al. (2018): Type I, epiphytes are weakly attached to the host surface so there is no tissue damage; Type II, epiphytes are strongly attached to the host surface, but there is still no tissue damage; Type III, epiphytes penetrate the outer layer of the host cell wall without damaging the cortical cells; Type IV, epiphytes penetrate the outer layer of the host cell wall and cause cellular disorganisation; and Type V, epiphytes invade deeper host tissues by growing between the cells, associated with the destruction of cortical and medullary cells (Tsiresy et al. 2016). In *K. alvarezii*, *Polysiphonia* epiphytism belongs to Type V.

Epiphytic macroalgae have received particular attention for the negative effects they exert on their hosts, potentially reducing their fitness, most notably for epiphytic-seagrass interactions (Yamamoto et al. 2013). Competition for light and nutrients is most significant and specific to epiphytic macroalgae (Sand-Jensen 1977; Silberstein et al. 1986; Berger et al. 2003). Regardless of the stage of epiphytism, the presence of macroscopic organisms on the surface of algae or seagrass can potentially affect the functioning of the host plant (Behera et al. 2022). In particular, when the biomass (or size) of the epiphytes is high, shading effects are observed (Sand- Jensen 1977; Orth and Van Montfrans 1984; Buschmann and Gómez 1993). These effects result in the reduced availability of light for the host plant and, consequently, reduced photosynthetic rates and primary production. Nevertheless, shading is not the only effect on epiphytes on their host.

Another possible effect is competition for the acquisition of nutrients and inorganic carbon between epiphytic algae and their hosts (Lepoint et al. 2007). Algal epiphytes and host plants need to acquire nutrient salts and inorganic carbon from the environment. Many environments are nutrient deficient (i.e. oligotrophic) and this can lead to growth limitations of algae and competition between epiphytes and hosts (Lepoint et al. 2004). For morpho-functional reasons, the nutrient needs and incorporation capacities of the host and the epiphytes are generally different (Hurd et al. 2014). Epiphytic algae, in particular filamentous algae like the *Polysiphonia* type, have shorter lifespan and have a high need for nutrients that they must acquire quickly (Campbell 2001). Host like *Kappaphycus* have longer lifespans and more perennial structures and are generally less rich in nitrogen or phosphorus. Their incorporation rates are relatively lower than their epiphytes (Pedersen 1995; Pedersen and Borum 1997). Under these conditions, the host cannot compete with their

epiphytes, especially during periods of strong growth. Leal et al. (2020) demonstrated that the interaction in co-culture between both *Gracilaria chilensis* (syn. *Agarophyton chilense*) and its green

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algal epiphyte *Rhizoclonium* sp. seems to be regulated by nutrient availability as well as it demands utilization rather than light availability.

When epiphytes invade the inner part of the host, we might hypothesise that epiphytic algae can potentially divert organic and inorganic nutrients from their host to their advantage, thus adopting a partially or totally parasitic way of life (Ingle et al. 2018; Behera et al. 2022). Depending on the epiphyte biomass, this could represent a significant loss for the host plant. Nevertheless, in the absence of experimental assessment, it is currently difficult to prove that EFAD functionally qualifies as a parasite. Therefore, the impact of a general effect of epiphytism (e.g. shading, competition for resources) must be distinguished from parasitism (e.g. diverting host resources), a particular effect restricted to epiphytes with morphological structures invading their hosts.

The main objectives of this work were to assess the effects of a filamentous algal disease on resource acquisition in *K. alvarezii* and to assess whether EFAD qualifies as a parasite from a functional point of view. These objectives were achieved with two consecutive mesocosm experiments using

isotopic labelling with ^{13}C, added in the form of sodium bicarbonate and ^{15}N, added in the form of ammonium chloride. The first experiment aimed to measure the relative incorporation of carbon and nitrogen by *K. alvarezii* and its epiphytes to estimate the 'competition' effect of the presence of epiphytes. In the second experiment, the transfers of carbon and nitrogen from algae to epiphytes were measured to assess the parasitic effect of the epiphytes.

Materials and methods

BIOLOGICAL MATERIAL

Kappaphycus alvarezii (n = 30, 20 infected and 10 uninfected algae), farmed in situ for four weeks, was collected during a low spring tide on 18 November 2016 in Sarodrano (Madagascar; coordinates -23.51'S, 43° 74 'E) (Fig. 1a). Infected and uninfected algae were collected in the same area but not on the same culture line. Tsiresy (2016), using morphology and genetic approaches, has identified these epiphytes as a species of *Polysiphonia*. The absence of epiphytes on uninfected algae was assessed in the field using a field magnifier. Hosts were infected with EFAD at stage 3 of their development (Tsiresy et al. 2016); this is an intermediate stage in which the epiphytes have a feathery appearance and are visible externally but not present throughout the thallus. At this stage, the host seem only slightly infected by the presence of epiphytes (i.e. no 'goosebumps').

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EXPERIMENTAL PROTOCOLS

EXPERIMENT 1: COMPETITIVE EFFECT

Two tanks with dimensions of 350 × 70 × 20 cm (490 L) were filled with filtered seawater. Sea water taken from local tidal area come first in a decantation tank, then pass on a sand filter and then is filtered through 0.2 µm filter. A pump and bubbler ensured water flow and oxygenation (Fig. 1b). Both infected and uninfected algae were acclimated for 36 h in tank 1, and then five uninfected algae and five infected algae were sampled (= T_0) to determine their natural isotopic compositions and the C and N contents at the start of the experimentation.

The labelled reagents were added as $NaH^{13}CO_3$ (15 g, 99 ^{13}C%, Eurisotop) and $^{15}NH_4Cl$ (40 mg, 99 ^{15}N%, Eurisotop) in seawater were added to tank 1 at 10 a.m. and 3 p.m., respectively. A preliminary experiment demonstrated that the incorporation of nitrogen was extremely rapid and required much lower tracer concentrations (Tsiresy 2016). Samples (n = 5 infected and n = 5 uninfected) were taken at 5:00 p.m. (=$T_{1,}$), which closed the first experiment aiming to compare the incorporation of C and N by infected and uninfected algae and by the epiphytes of infected algae.

EXPERIMENT 2: PARASITISM EFFECT

In the second part of the experiment, we aimed to measure the transfer of C and N between epiphytes and hosts. Accordingly, tracing was stopped by transferring the remaining labelled algae (n = 10) to tank 2 with renewed seawater (i.e. no more label in the aquarium). Aeration and water flow were identical to tank 1. The algae were maintained alive in the tank for two days. Then, samples of 5 infected algae were taken on November 21 (T_2) and 5 on November 22 (T_3).

TREATMENT OF SAMPLES

All samples were dried in open air according to the method used by the farmers (Fig. 1c) and labelled and packaged separately to avoid any inter-algae contamination by isotopic tracers. A complete and rigorous cleaning of the instruments, tanks and laboratory locations in contact with the marked samples was carried out.

Seaweed contains a large amount of salt, which is exuded during drying. This salt was removed by rinsing to correctly measure the biomasses of algae and epiphytes and to perform the isotopic and elemental measurements. Each piece of seaweed was washed individually in demineralised water 3 times for 3 min and then placed in an oven at 50 °C until completely dried.

The infected samples were at an early stage of the disease; consequently, the epiphytes present were microscopic and difficult to separate from their host. Scraping was carried out under a binocular magnifying glass (Leica MS 5, using × 32 magnification) using toothbrushes, candle brushes, paintbrushes and scalpels. Between every sample, the scraping instruments were cleaned twice with Milli-Q water and then once with acetone to avoid any contamination between samples.

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The collected epiphytes were placed in preweighed tin cups, returned to the oven and weighed (Mettler Toledo microbalance, precision 1 µg). For weighing, the following protocol was adopted: the cups were placed in an oven at 50 °C for 2 h to dry and allowed to cool before they were weighed (3 weighings, non-consecutive, averaged). The host were placed in glass flasks, returned to the oven and weighed (analytical balance, Mettler Toledo, 0.1 mg).

The *Kappaphycus* were ground using a Retsch MM 301 micro-ball mill (2 min, 25 Hz) to obtain a homogeneous powder. Between each grinding, the containers were cleaned twice with Milli-Q water, then once with acetone to avoid contamination between samples.

Figure 1 a *Kappaphycus alvarezii* cultivation on bottom line (Sarodrano, Madagascar, -23.51'S, 43° 74'E); **(b)** experimental aquarium (490 L) at Fisheries and Marine Science Institute (IH.SM) (Toliara University, Madagascar) equipped with water current pump and *Kappaphycus* samples; **(c)** *Kappaphycus* samples drying after their collection at the end of experiment 2

ISOTOPIC AND ELEMENTAL MEASUREMENTS

Isotope measurements of ^{13}C and ^{15}N were performed with an isotope ratio mass spectrometer (Isoprime 100, Isoprime, UK) coupled to a C-N-S elemental analyser (VarioMicro, Elementar, Germany). Samples were weighed in tin cups. IAEA-N1 and IAEA C6 were used as certified substances. The isotopic results are expressed in atom%, which represents the proportions of an isotope (ex: ^{15}N) compared to the total quantity of stable isotopes of the element (ex: $^{15}N + ^{14}N$) and

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expressed as ^{15}N atom% or ^{15}N%. The elemental compositions are expressed as a percentage of the dry weight for the element considered (%C, %N) in the sample.

CALCULATIONS AND STATISTICS

For experiment 1 (competition hypothesis), incorporation rates were calculated following these steps:

Step 1: Calculate the atom percentages of ^{13}C and ^{15}N in excess in the sample according to the following formula:

$$atom\%\ in\ excess = measured\ atom\% - natural\ atom\%$$

The natural atom% was determined on the T_0 samples (i.e. pre-incorporation).

Step 2: Calculate the elemental quantities of C and N in the sample, expressed in mgC and mgN, according to the following formula:

$$Elemental\ quantities = sample\ dry\ mass\ \times\ \%Elem$$

where %Elem is the measured percentage of carbon or nitrogen in the sample.

Step 3: Calculate the quantities of ^{13}C and ^{15}N incorporated into the sample, expressed in mg^{13}C and mg^{15}N, according to the following formula (here for ^{13}C):

$$^{13}C\ quantity\ = \frac{C\ quantity}{100} \times atom\%\ excess$$

Step 4: Calculate the quantities of ^{13}C and ^{15}N incorporated into the sample per hour (i.e. absolute incorporation), expressed in µg^{13}C h^{-1} and µg^{15}N h^{-1}, according to the following formula (here for carbon):

$$^{13}C\ absolute\ incorporation = \frac{^{13}C\ quantity \times 1000}{Experiment\ duration}$$

Step 5: Calculate the levels of ^{13}C and ^{15}N incorporated into the sample per milligram of C and N per hour (i.e. relative incorporation or incorporation rate), expressed in µg^{13}C $mg^{-1}C$ h^{-1} and µg^{15}N $mg^{-1}N$ h^{-1}, according to the following formula (here for carbon):

$$^{13}C\ incorporation\ rate = \frac{^{13}C\ absolute\ incorporation}{quantity\ of\ C\ in\ the\ sample}$$

For experiment 2 (parasitism hypothesis), the transfer of carbon or nitrogen was calculated in the same way as in steps 1-3. The quantity of ^{13}C or ^{15}N transferred was divided by the quantity of total carbon or nitrogen (i.e. µg^{15}N $mg^{-1}N$) to relativise it with the biomass of individuals.

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Kruskal-Wallis non-parametric tests, followed by Dunn's post hoc test in cases of significant differences, were used to compare the absolute quantities of ^{13}C and ^{15}N and the rates incorporated and transferred in the samples. All test results were considered significant at p-value ≤ 0.05. These tests were performed using Past 4.02 software (Hammer et al. 2001).

Results

The average dry mass (DM) of the hosts and their epiphytes was 4153.5 ± 1600.6 mg and 3.3 ± 1.5 mg, respectively. Epiphytes therefore represented less than 0.1% of the total biomass (i.e. epiphytes plus *Kappaphycus*). No difference in dry mass was observed between infected and uninfected *Kappaphycus*. Average (± s.d.) %N and %C measured for *Kappaphycus alvarezii* were 29.9 ± 0.7% DM; 0.5 ± 0.1% DM, respectively, resulting in average C/N (w:w) ratios of 50 ± 7.0. There were no significant differences between infected and uninfected *Kappaphycus* in terms of C and N composition. Average %N, %C and C/N (w:w) ratios measured for epiphytes were 19.0 ± 5.2% DM, 3.2 ± 1.9% DM, and 6.5 ± 2.3, respectively.

EXPERIMENT 1: TEST OF COMPETITION HYPOTHESIS

During the first experiment, uninfected *Kappaphycus* incorporated per hour between 53.2 and 244.5 $\mu g^{13}C\ h^{-1}$ (mean 113.9 ± 70.0 $\mu g^{13}C\ h^{-1}$), while infected *Kappaphycus* incorporated per hour between 56.5 and 166.3 $\mu g^{13}C\ h^{-1}$ (mean 106.9 ± 35.2 $\mu g^{13}C\ h^{-1}$) (Fig. 2a). Epiphytes incorporated per hour between 0.0 and 0.2 $\mu g^{13}C\ h^{-1}$ (average 0.1 ± 0.1 $\mu g^{13}C\ h^{-1}$). The Kruskal-Wallis test indicated significant differences ($\chi^2 = 17.82$, $p \leq 0.001$) between these three groups. There was no significant difference in the rate of carbon incorporation between infected and uninfected *Kappaphycus* hosts (Dunn's post hoc test, $p > 0.05$). However, there was a significant difference between the infected *K. alvarezii* and its epiphytes (Dunn's post hoc test, $p \leq 0.001$) as *Kappaphycus* incorporated per hour more carbon than its epiphytes.

Concerning nitrogen, uninfected *Kappaphycus* incorporated per hour between 3.5 and 17.3 $\mu g^{15}N\ h^{-1}$ (mean 7.6 ± 5.1 $\mu g^{15}N\ h^{-1}$) while infected *Kappaphycus* had an incorporation per hour between 2.9 and 8.5 $\mu g^{15}N\ h^{-1}$ (mean 6.0 ± 1.9 $\mu g^{15}N\ h^{-1}$) (Fig. 2). Epiphytes incorporated between 0.0 and 0.1 $\mu g^{15}N\ h^{-1}$ (mean 0.1 ± 0.0 $\mu g^{15}N\ h^{-1}$) (Fig. 2c). The Kruskal-Wallis test revealed a significant difference ($\chi^2 = 17.81$, $p \leq 0.001$) between the groups. No significant difference was observed between infected and uninfected *Kappaphycus* (Dunn's post hoc test, $p > 0.05$). In contrast, epiphytes had a significantly lower rate of nitrogen incorporation than their host (Dunn's post hoc test, $p \leq 0.001$).

The relative carbon incorporation of infected and uninfected *Kappaphycus* varied between 0.0 and 0.2 $\mu g^{13}C\ mg^{-1}C\ h^{-1}$ (Fig. 2b), while epiphytic algae incorporated between 0.1 and 0.4 $\mu g^{13}C\ mg^{-1}C\ h^{-1}$ which was significantly higher than in *Kappaphycus* (Kruskal-Wallis test, $\chi^2 = 7.2$, $p \leq 0.05$, Dunn's post hoc test, $p \leq 0.001$). There were no significant differences between the hosts, regardless of whether they were infected (Dunn's post hoc test, $p > 0.05$).

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Similar results were observed for relative nitrogen incorporation (Fig. 2d) with no significant differences between uninfected and infected *Kappaphycus* (mean 0.3 ± 0.2 and 0.3 ± 0.1 $\mu g^{15}N\ mg^{-1}Nh^{-1}$, respectively) (Fig. 2d). Epiphytes incorporated between 0.4 and 1.4 $\mu g^{15}N\ mg^{-1}N\ h^{-1}$ (mean 0.7 ± 0.4 $\mu g^{15}N\ mg^{-1}N\ h^{-1}$) which was significantly higher than in *Kappaphycus* host (Kruskal-Wallis test, $\chi^2 = 9.72$, $p \leq 0.005$ Dunn's post hoc test, $p \leq 0.001$) (Fig. 2d).

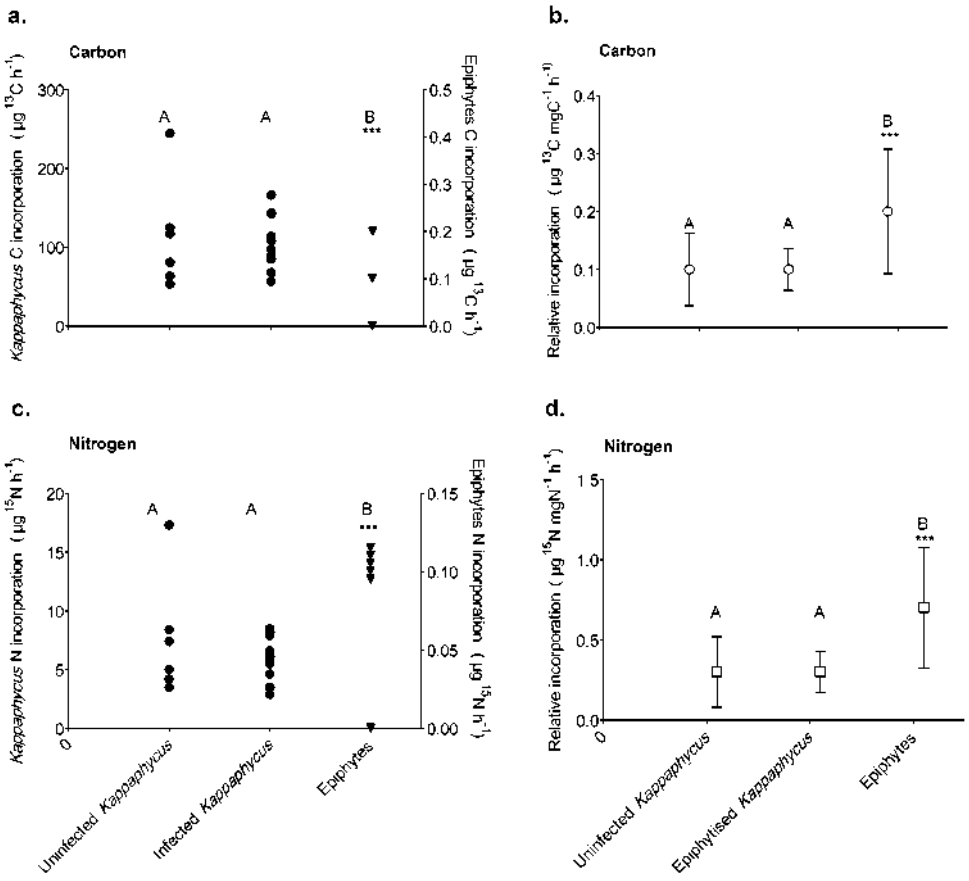

Figure 2. Absolute (a, c) and relative (b, d) incorporations of ^{13}C and ^{15}N by infected and uninfected *Kappaphycus alvarezii* and their epiphytes. Capital letters refer to statistical test significance based on Kruskal-Wallis non-parametric tests, followed by Dunn's post hoc test in cases of significant differences. Shared letters indicate no significant differences. *** $p \leq 0.001$

EXPERIMENT 2: TEST OF PARASITISM HYPOTHESIS

During the second experiment, *Kappaphycus* showed quantities of ^{13}C in the tissues varying from 0.3 to 1.2 $\mu g^{13}C\ mg^{-1}C$ (averages: 0.6 ± 0.3 $\mu g^{13}C\ mg^{-1}C$ for T_0, 0.6 ± 0.2 $\mu g^{13}C\ mg^{-1}C$ for T_1 and 0.6 ± 0.3 $\mu g^{13}C\ mg^{-1}C$ for T_2) (Fig. 3a). No significant variation was observed across sampling times (Kruskal-Wallis test, $\chi^2 = 0.06$, test $p > 0.05$).

Epiphytes showed quantities of ^{13}C in the tissues varying from 0.6 to 2.7 $\mu g^{13}C\ mg^{-1}C$ (averages: 1.3 ± 0.7 $\mu g^{13}C\ mg^{-1}C$ for T_0, 1.2 ± 0.5 $\mu g^{13}C\ mg^{-1}C$ for T_1 and 1.7 ± 0.8 $\mu g^{13}C\ mg^{-1}C$ for T_2) (Fig. 3a). Like

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Kappaphycus, no significant variation was observed across sampling times (Kruskal-Wallis test, χ^2 = 0.58, p > 0.05).

Kappaphycus showed quantities of ^{15}N in the tissues varying from 2.1 to 8.8 $\mu g^{15}N\ mg^{-1}N$ (averages: 2.1 ± 0.0 $\mu g^{15}N\ mg^{-1}N$ for T_0, 6.4 ± 0.0 $\mu g^{15}N\ mg^{-1}N$ for T_1 and 7.8 ± 0.9 $\mu g^{15}N\ mg^{-1}N$ for T_2) (Fig. 3b) and these quantities varied across time (Kruskal-Wallis test, χ^2 = 14.93, p ≤ 0.001). Between T_0 and T_1, a significant increase in the proportion of ^{15}N in the tissues was observed (Dunn's post hoc test p ≤ 0.01) when no significant variation was detected between T_1 and T_2 (Dunn's post hoc test p > 0.05).

Epiphytes presented quantities of ^{15}N in the tissues varying between 0.8 and 9.3 $\mu g^{15}N\ mg^{-1}N$ (averages: 0.8 ± 0.0 $\mu g^{15}N$

$mg^{-1}N$ for T_0, 2.2 ± 0.8 $\mu g^{15}N\ mg^{-1}N$ for T_1 and 5.4 ± 2.3 $\mu g^{15}N\ mg^{-1}N$ for T_2) (Fig. 3b) and these levels of ^{15}N in the tissues varied across time (Kruskal-Wallis test, χ^2 = 16.1, p ≤ 0.001). A significant increase was detected between every sampling period (Dunn's post hoc test, p ≤ 0.01).

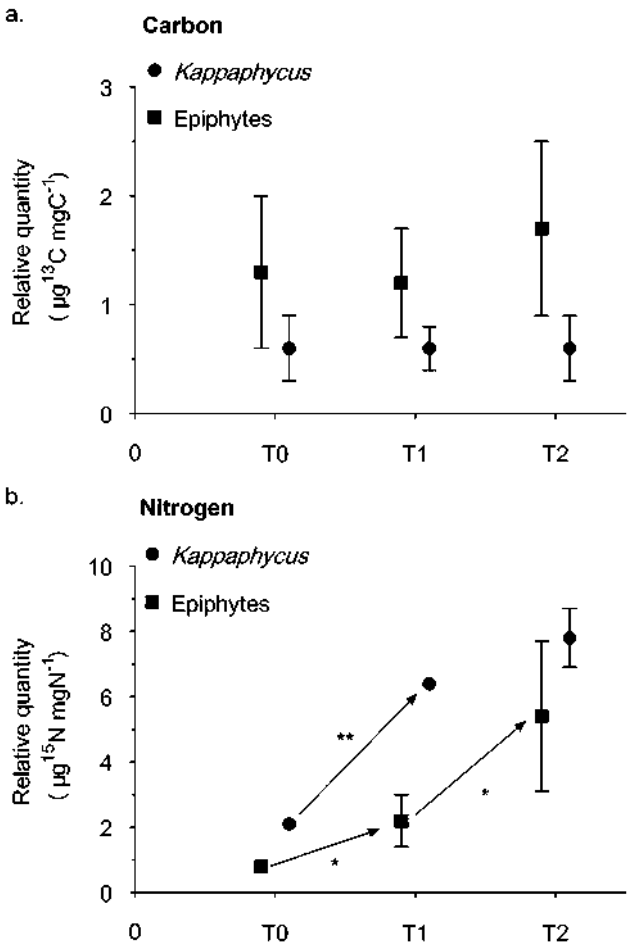

Figure 3. ^{13}C and ^{15}N quantities measured after 24 h (T1) and 48 h (T2) in infected *Kappaphycus alvarezii* and their epiphytes. Arrow indicates statistical significant differences between two samples. * p ≤ 0.05; ** p ≤ 0.01 (Kruskal-Wallis non-parametric tests, followed by Dunn's post hoc test in cases of significant differences). Absence of arrow indicates absence of significant difference

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Discussion

Our experiments showed that, at the early stage of EFAD, epiphytic algae have few effects on the acquisition of carbon and nitrogen by the host macroalga *Kappaphycus alvarezii*. Indeed, there were no significant differences between the C and N quantities incorporated by infected or uninfected *Kappaphycus*. This is related to the fact that the quantities of carbon and nitrogen incorporated by epiphytes were very low, of the order of a microgram, which was explained by their small size. The epiphytes sampled on a portion of *Kappaphycus* incorporated, on average, 1000 times less carbon and 60 times less nitrogen than the sampled portion of *K. alvarezii*. At this step of infection, which was stage 3 (as defined by Tsiresy et al. 2016), there was therefore no measurable effect of epiphytes on their hosts for C and N incorporation. Nevertheless, it is important to note that it was only possible to assess incorporation by external epiphyte biomass, as it was impossible to separate the host and internal epiphytic tissues. Internal biomass develops in the same time as external biomass (Tsiresy et al. 2016) and could be as important as external biomass at this early infesting stage. Incorporation—but also transfer—must be considered minimal values here, as the contribution of internal epiphytic biomass was not assessed.

As epiphytes grow and multiply, their effects on N and C incorporation would certainly increase (Behera et al. 2022). When these incorporated quantities were calculated proportionally to the biomass (i.e. relative incorporation), the epiphytes, per unit of biomass, incorporated twice the quantity of carbon compared to the infected algae and a little more than twice the amount of nitrogen. This difference between epiphytic algae and their hosts has been measured in the epiphytes-seagrass community (Lepoint et al. 2007). Incorporation differences may be related to the life history traits of algae (Pedersen and Borum 1997; Padilla and Allen 2000; Hurd et al. 2014). Ephemeral algae display higher growth rates and higher nutrient incorporation rates than algae with longer lifespans (Pedersen and Borum 1997; Leal et al. 2020). Consequently, epiphytic algae, which have generally a shorter life span, tend to have a higher growth rate and N requirement than their hosts (Lepoint et al. 2007; Leal et al. 2020). In ephemeral algae, these nutrient are used directly for growth needs. In more perennial ones, nutrient can be stored or used directly for growth. In Pedersen and Borum (1997), the high N requirements of ephemeral algae were caused by up to 13-fold higher growth rates and 2- to threefold higher N content at maximum growth. Here, the N needs of *Polysiphonia* epiphytes were greater than the relative need of *Kappaphycus*, as demonstrated by C/N ratios (6 vs. 59 for *Polysiphonia* epiphytes and *Kappaphycus*, respectively). This means that, per C unit, the N needs of epiphytes are ten times higher than those of their hosts. To reach these needs, epiphytes display higher incorporation rate (Pedersen and Borum 1997). Despite the differential use of N (i.e. direct use for growth vs. growth and storage), higher incorporation rate gives probably a competitive advantage to epiphytes compared to their host.

C incorporation is also related to life history traits and morphology. A higher growth rate is linked to higher photosynthetic activity and inorganic carbon fixation. Additionally, species with fine tube morphology, like those displayed by *Polysiphonia* sp., are known to have a higher maximal

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photosynthetic rate (i.e. P_{max}) than coarse branched algae like *Kappaphycus* and a better capacity to exploit high light intensity (Saco and Ganzon-Fortes 2022).

Due to the faster growth of epiphytes, the competition effect could increase over time. Fully developed epiphytes (e.g. phase IV to V of infestation) could outcompete their host because of their higher relative incorporation rate and the reverse biomass effect observed at the start of infection. This phenomenon could be accentuated by two other processes. First, epiphytes could reduce the intensity of light reaching the host surface as demonstrated for seagrass and their epiphytes (Orth and Van Monfrans 1984) or the rhodophyte *Gracilaria chilensis* and its *Ulva* epiphytes in situ (Buschmann and Gómez 1993). Larger and more numerous epiphytes can shade, preventing sunlight from reaching *Kappaphycus* to carry out photosynthesis. Nevertheless, in the relation between *G. chilensis* and its epiphytes *Rhizoclonium* sp., this shading effect was not significant in in vitro experiment (Leal et al. 2020). Nutrient effect was far more important in the *G. chilensis/Rhizoclonium* relationship with observed consequences on physiological status when both algae grew together (Leal et al. 2020). Nutrient effect could be probably also more important than shading in our case study considering the smaller size of *Polysiphonia* on *Kappaphycus* than *Rhizoclonium* on *Gracilaria*.

We hypothesise that nutrient and inorganic carbon effect include "competition" effect (see above) but also "accessibility" effect. Indeed, during EFA development, as epiphytes grow in size, they have easier access to the nutrient and inorganic carbon of the boundary layer and could therefore partially limit the quantity of nutrients and inorganic carbon available to the host (Dodds 1991). All of these processes—shading, facilitated access to nutrients and competition for inorganic elements—are to the disadvantage of the algal host and could reduce its growth possibilities.

The second experiment aimed to assess whether epiphytes growing on *Kappaphycus* are functionally parasites and to quantify the transfer of N and C between the host and its epiphytes. Concerning carbon, the experiment showed that there was very little variation in the quantity of ^{13}C for infected *K. alvarezii* and their epiphytes during the experiment. It seems to indicate that C transfer does not occur as much between epiphytes and their hosts. Nevertheless, it is possible that experiment duration was too short to show such transfer. In addition, as already noted, it was not possible to decipher the internal epiphyte biomass from its host biomass. It is therefore possible that our experiment under-evaluated the transfer of C.

Regarding nitrogen, in T_1, the results were quite surprising because in the absence of a label in the water, the amount of total tracer increased in both the host and the epiphytes. This probably means that some tracer remained on the surface of the organisms during their transfer to the second basin (i.e. the transition between experiments 1 and 2). The incorporation of nutrients inside the algae is not instantaneous, and as there was no rinsing of the fresh algae before their transfer to the second aquarium to avoid an alteration of the fragile epiphytes on their surface, unincorporated material remained on the host surface. The tracer was therefore incorporated during T_0 and T_1, despite the change of algae from tank 1 (labelled) to tank 2 (unlabelled).

In the last sampling (T_2, two days after experiment 2 started), the amount of nitrogen in the host remained stable, but it increased significantly (doubling) in epiphytes. This demonstrates without

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ambiguity that there was a transfer of nitrogen between the host and its epiphytes. These results demonstrate that *Polysiphonia* can be qualified here as a functional parasite insofar as it diverts part of its host's resources to its benefit. The cost of this diversion of resources is two-fold for the host: first, the host loses some of the N it needs, and second, the metabolic cost linked to the incorporation and assimilation of these elements are expended by the host.

Such transfers between hosts and parasitic epiphytes have also been demonstrated for the red alga *Vertebrata lanosa* (*Polysiphonia lanosa*) growing as epiphytes on *Ascophyllum nodosum* for both phosphorus and organic

carbon (Penot et al. 1993; Ciciotte and Thomas 1997). Interestingly, Ciciotte and Thomas (1997) demonstrated that reverse transfer (i.e. from parasite to host) was also possible for carbon compounds and hypothesised that it could play a role in the host-parasite relationship (i.e. chemical communication). This is a hypothesis to test with our model in a longer experiment. In contrast, phosphorus transfer is only unidirectional, from the host to the epiphytes, which supports their classification as functionally parasitic (Penot et al. 1993). This shows that the relationship between a host and its epiphytes is complex.

Another example is the cultivated red alga *G. chilensis*, which can be heavily epiphytised by diverse species (from type I to type V) (Leonardi et al. 2006). Among the most common species, the green alga *Ulva* lactuca penetrates the host cell wall and causes intracellular disorganisation (epiphytism type IV) (Dawes et al. 2000). *Polysiphonia harveyi* (now *Melanothamnus harveyi*) causes compressed cells, chloroplast disorganisation and digested areas in the host wall (epiphytism type V) (Leonardi et al. 2006). It is therefore probable that in this case, *Polysiphonia* also qualifies as a functional parasite of *G. chilensis*. Buschmann and Gómez (1993) showed that *Ulva* epiphytes act negatively on *Gracilaria* production in two main ways: an increased drag effect on the host due to increasing mass and a decreased photosynthetic rate due to shading. For the epiphytism of *Gracilaria* by the green alga *Rhizoclonium* sp., nutrient competition is involved and shading probably do not influence G. chilense growth. In all cases, different epiphytic species could have different (and sometimes cumulative) effects on their hosts.

Conclusion

It is well known that *Polysiphonia* developing on farmed *Kappaphycus* have negative action on their growth and on their carrageenan production and quality (e.g. Vairappan et al. 2014). Our work demonstrates that the negative action of epiphytes on its host could follow several potential mechanisms, including (a) competing for nutrients, as in the case of all epiphytisms and (b) becoming functionally parasitic by diverting increasingly large amounts of the resources acquired by the host over time. Our data demonstrated *Polysiphonia* diverted nitrogen incorporated by its host and, later in its development, could compete with its host for both nutrient and inorganic carbon. This later hypothesis is related to measured higher relative incorporation rates for epiphytes

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for both C and N and from other examples found in the literature (e.g. Pedersen and Borum 1997; Leal et al. 2020). Nevertheless, a longer experiment is necessary to better explore this last hypothesis.

In terms of biocontrol, we suggest that the choice of nutrient-rich areas to practice *Kappaphycus* farming is likely to favour the epiphytes rather than the hosts, considering their relative needs and abilities to incorporate nutrients. In the natural environment, epiphytes can be controlled by grazing invertebrates (Michel et al. 2015). The presence of grazers could be boosted on the production lines of algal farms, particularly amphipod crustaceans, herbivores that prefer epiphytes over host plants. This could bring other benefits, such as attracting invertivorous fish that can be caught by local fishermen. However, the most economical and secure solution is probably to remove the lines at an early stage of infestation, as is currently practiced (Ndawala et al. 2022).

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Authors' contributions

G.L., G.T. and I.E. contributed to the study's conception and design. Experiments were conducted by G.L., G.T. and B.F. Biological material was provided by F.P. Material preparation and analyses were performed by M.D., G.T. and G.L. The first draft of the manuscript was written by G.L., and all authors commented on previous versions of the manuscript. Financial resources were secured by I.E. All authors read and approved the final manuscript.

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