Chemosymbiotic bivalves contribute to the nitrogen budget of seagrass ecosystems

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Abstract
In many seagrass sediments, lucinid bivalves and their sulfur-oxidizing symbionts are thought to underpin key ecosystem functions, but little is known about their role in nutrient cycles, particularly nitrogen. We used natural stable isotopes, elemental analyses, and stable isotope probing to study the ecological stoichiometry of a lucinid symbiosis in spring and fall. Chemoautotrophy appeared to dominate in fall, when chemoautotrophic carbon fixation rates were up to one order of magnitude higher as compared with the spring, suggesting a flexible nutritional mutualism. In fall, an isotope pool dilution experiment revealed carbon limitation of the symbiosis and ammonium excretion rates up to tenfold higher compared with fluxes reported for nonsymbiotic marine bivalves. These results provide evidence that lucinid bivalves can contribute substantial amounts of ammonium to the ecosystem. Given the preference of seagrasses for this nitrogen source, lucinid bivalves’ contribution may boost productivity of these important blue carbon ecosystems.

Introduction
Shallow-water chemosynthetic symbioses are widespread where decomposition of organic matter produces sulfide [1]. However, their relevance for ecosystem functioning has received limited attention due to the assumption that chemosynthesis plays a minor role in shallow-water ecosystems. Recent studies are challenging this assumption [2–4].

In seagrass sediments, bivalves of the family Lucinidae consume sulfide through their chemosynthetic symbionts, allowing more plant growth while relying on the seagrass to stimulate sulfide production by free-living sulfate-reducing microorganisms [3]. Still, we know little about nutrient cycling in lucinid bivalves at both the organism and the ecosystem scale. Most studies to date have focused on carbon (C) fixation by the symbionts and transfer to the host [5, 6] or on the additional contribution of filter feeding to host nutrition [7]. Nitrogen (N) metabolism has received far less attention until recently, when dinitrogen (N2) fixation by chemosynthetic symbionts was shown to be possible in...
two lucinid species [8, 9]. Concurrently, chemosynthetic
symbioses can, to varying degrees, gain their N from
ammonium (NH₄⁺), nitrate, or dissolved free amino acids in
their environment [10–12], with the symbionts being able to
recycle N waste compounds within the symbiosis [13].
Surprisingly, although these studies attest to the expanded N
metabolic versatility of chemosynthetic symbioses, the
significance of lucinid bivalves in contributing to their
ecosystem N budget has been largely overlooked. Since the
lucinid symbionts have a versatile C and N metabolic
repertoire, being able to
fix inorganic C or grow hetero-
trophically, and to take up various nitrogen forms [8],
they might provide lucinid bivalves with a distinct advan-
tage over nonsymbiotic filter-feeding bivalves, while
boosting their role in the biogeochemistry of seagrass
ecosystems.

Methods

We studied a lucinid bivalve (Loripes orbiculatus) in the
seagrass (Posidonia oceanica) sediments of Elba Island
(Italy) during two field expeditions in April (spring) and
October (fall) 2016. P. oceanica tends to consume porewater
nutrients (particularly nitrogen) during the growth phase
(spring and summer), which are therefore depleted in fall, while sulfide accumulates as a result of leaf burial and
decomposition. To check if this was true for our study site,
we analyzed porewater inorganic nutrient concentrations
(dissolved inorganic nitrogen—DIN and dissolved inorganic
phosphorus—DIP) down to 60 cm below the sediment sur-
face, with a resolution of 5 cm. Stable isotope probing with
¹³C–NaHCO₃ and ¹⁵N–N₂ was used to quantify C and N₂
fixation by the chemosynthetic symbionts. Exogenous sulfide
was not added to the incubation seawater as our primary goal
was to investigate environmentally driven differences in
physiology in both seasons. An isotope pool dilution (IPD)
experiment with ¹⁵N–NH₄Cl, was conducted in October to
quantify gross and net NH₄⁺ fluxes by the bivalve symbiosis.
The IPD technique has not yet been applied in marine
symbiosis research. This technique involves labeling the
nutrient pool of interest (in our case by adding ¹⁵NH₄⁺). By
quantifying the relative proportion of heavy and light iso-
topes in the nutrient pool, and the change in concentration
over time, gross production (i.e., mineralization) and con-
sumption (i.e., immobilization) rates can be calculated.
Finally, elemental and natural stable isotope analyses (δ¹³C
and δ¹⁵N, C:N ratio; symbiotic tissue mass index, SMI; and
gill total S content) were carried out to study the stoichio-
metric and isotopic niche (as proxies of the ecological niche)
of host and symbiont under the two contrasting seasons.
Individual δ¹³C and δ¹⁵N values of symbiont-bearing and
nonsymbiotic tissues were analyzed to compare isotopic

Results and discussion

The biogeochemistry of P. oceanica sediments is highly
influenced by the seagrass seasonal growth, leaf burial, and
decay by microorganisms. P. oceanica growth shows a late
spring maximum and a fall minimum [14]. The plant tends to
consume porewater nutrients (particularly nitrogen) during the
growth phase (spring and summer), which are therefore
depleted in fall, while sulfide accumulates as a result of leaf
burial and decomposition [15]. Our porewater profiles con-
firm this pattern, with higher DIN concentrations and DIN:
DIP ratios in April compared with October (p < 0.01; Fig. S1).
L. orbiculatus is able to supplement its diet with filter
feeding on a seasonal basis [7]. Here we show that not only the
host, but also the chemoautotrophic symbionts may modulate
their metabolic activities according to the availability of
external (or recycled) resources. C fixation by the symbionts
was roughly 10-fold higher in October compared with April
(p < 0.001; Fig. 1a). N₂ fixation, measured for the first time
here in a chemosynthetic symbiosis using the ¹⁵N–N₂ method,
also increased in October, although not significantly (Fig. 1b).
The boost in autotrophy was potentially mediated by higher
sulfur energy storage within the symbionts (p < 0.01; Fig. S4).

The increased C fixation rates drove the C:N ratio of the
symbionts higher, but not of the host (p > 0.001; Fig. 2a),
attesting to the stoichiometric flexibility of the autotrophic

Fig. 1 Results from ¹³C–HCO₃⁻ and ¹⁵N–N₂ isotope probing experi-
ments: a Carbon and b dinitrogen fixation by the microbial
symbionts (nmol C (or N) g gill tissue⁻¹ h⁻¹ ± SE, n = 5). Sampling points are
color-coded in purple (April) and cyan (October). Different lowercase
letters indicate significant differences (p < 0.05, PERMANOVA)
partner in the symbiosis and the homeostasis of the het-
erotrophic host [16]. However, the distribution of bi-variate
Bayesian ellipses shows that the natural isotopic niche of
the sul-
fi
de-oxidizing symbionts was signi-
ficantly larger in
the samples collected in October (Fig. S2; i.e., lower trophic
specialization), which may indicate a history of mixotrophic
metabolism of the endosymbionts, consistent with the pre-

The proportion of symbiont-hosting gill biomass (SMI)
was lower in October ($p < 0.05$; Fig. 2b). At the same
time, there was a strong overlap in C isotopic niche of host and
symbionts, indicating a match in their C source, while there
was a mismatch in April (Fig. 2c). These results could be
explained by a flexible nutritional mutualism. Under nutrient
rich/high productivity conditions in April, when labile
organic matter in seagrass sediments is highest [17], the host
relies more on mixotrophy through filter feeding. Under nutrient depleted/low productivity but sulfide-rich conditions
in October, the symbiosis shifts toward relying more on the
symbionts as a source of energy. Our observation that sym-
biont C fixation rates were ten times higher in October
compared with April is consistent with this theory.

Filter-feeding bivalves that do not host chemosynthetic
symbionts enter a “dormant” state in summer, possibly due
to food limitation [18 and references therein]. The ability to
harvest energy throughout the summer and fall by relying
on symbiont primary production when food availability is
low would provide lucinid bivalves with a distinct advan-
tage over nonsymbiotic filter-feeding bivalves. While more
targeted approaches will be needed to conclusively verify
this hypothesis, gross NH$_4^+$ production and consumption measured in October using IPD indicated that the symbiosis
was indeed C limited, as bivalves consumed NH$_4^+$ only
when exposed to a source of labile organic C (Fig. S3).

The same experiments, using IPD on an invertebrate
symbiotic animal for the first time to our knowledge, allowed
us to quantify gross and net excretion rates contributed by the
symbiosis to its surroundings. Net excretion by the bivalves
was ~15 µmol NH$_4^+$ gSFDW$^{-1}$ h$^{-1}$ (Fig. S3), which is up to
tenfold higher compared with NH$_4^+$ excretion rates reported
for other nonsymbiotic marine bivalves [19] and testifies to
the potential of these chemosynthetic symbioses to underpin
ecosystem functioning by nitrogen provisioning.

**Conclusions**

In this study, we show that _L. orbiculatus_ likely has a flexible
nutritional mutualism, in which host and symbionts cycle
between a looser trophic association and a tight chemoauto-
trophic partnership, changing nutritional strategy according to
the environmental conditions. Further, we report that under C-
limiting conditions these chemosymbiotic bivalves can excrete
substantial amounts of NH$_4^+$ to the environment. In seagrass
sediments, lucinids and their endosymbionts are not only

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**Fig. 2** Results from freshly sampled bivalve specimens: **a** C:N ratio ($\pm$ SE, $n = 10$) of symbiont-free (Symb-) and symbiont-hosting (Symb+) animal tissues; **b** Symbiotic tissue mass index—SMI. The SMI indicates the proportion of symbiont-hosting gill biomass (mg mm$^{-1}$ $\pm$ SE, $n = 10$; see Supplementary Methods for details on how this index was calculated); **c** Biplot of the natural abundance of $^{13}$C and $^{15}$N isotopes showing the total amount of niche space occupied (total area, dashed polygons) and the isotopic niche width (standard ellipse area, solid ellipses) as proxies of trophic specialization of symbiont-free (squares) and symbiont-hosting (triangles) animal tissues. Sampling points are color-coded in purple (April) and cyan (October). Different lowercase letters indicate significant differences ($p < 0.05$, PERMANOVA)
relevant for their role in sulfide detoxification [3], but can also provide the plant’s preferred N form [20], thus contributing to the productivity of these important blue carbon ecosystems.

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**Author contributions** UC contributed to the design of the research project, conducted the fieldwork and performed all the experiments, analyzed the data, and wrote the manuscript. MB conducted 15N- NH4+ measurements at the membrane inlet mass spectrometer, and provided critical input for data interpretation. RL conducted bulk stable isotope measurements on bivalve tissues and provided critical input for data interpretation. SL provided guidance for GC-MS measurements of 15N enrichments in seawater samples. MM assisted in designing the IPD experiment and provided critical input for data interpretation. JP contributed to the project during fieldwork and sample analyses. VM and TH conducted gill total S measurements of bivalve specimens. MW assisted with the organization and conduct of all fieldwork activities. JMP contributed to the design of the research project, data interpretation, and manuscript production.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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