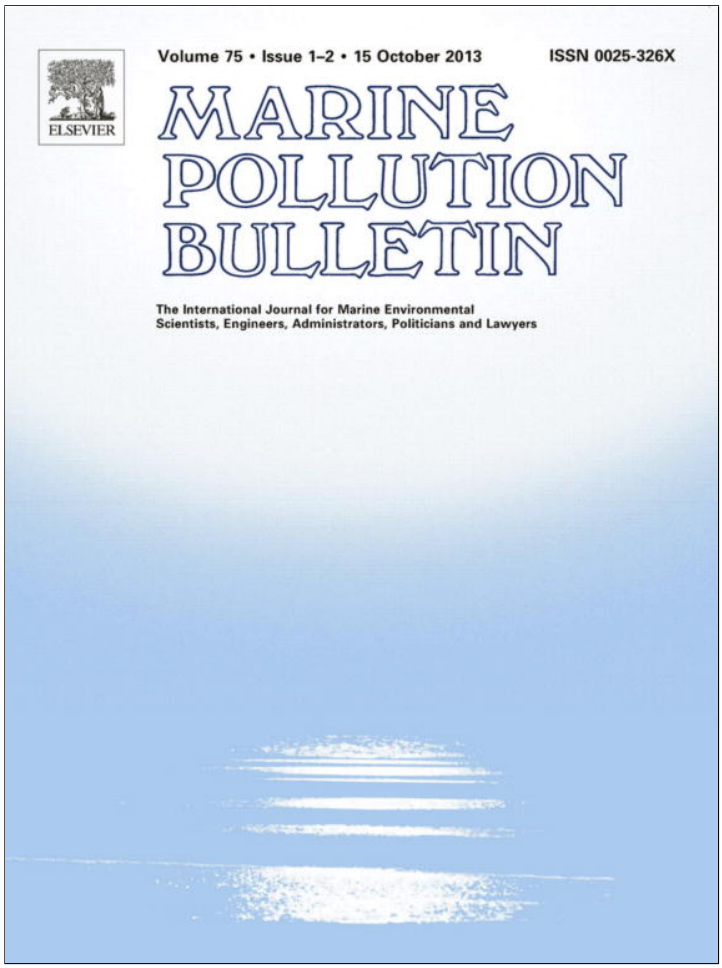

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Interception of nutrient rich submarine groundwater discharge seepage on European temperate beaches by the acoel flatworm, *Symsagittifera roscoffensis*

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ABSTRACT

Submarine groundwater discharge (SGD) occurs in intertidal areas, representing a largely unquantified source of solute fluxes to adjacent coastal zones, with nitrogen being constantly the keynote chemical of concern. In *Olhos de Água* SGD is present as groundwater springs or merely sub-aerial runoff. The occurrence of the flatworm *Symsagittifera roscoffensis* is described for the first time in *Olhos de Água* in connection to seepage flows. To assess the impact of this symbiotic flatworm on the nitrogen associated to groundwater discharge flow at the beach, nitrate uptake experiments were conducted in laboratory microcosms. Our results show that *S. roscoffensis* actively uptakes nitrate at different rates depending on light availability, with rates ~10 times higher than that of its symbiotic microalgae alone. This supports the hypothesis that*S. roscoffensis* could be an important *in situ* nitrate interceptor, potentially playing a biological role on the transformation of groundwater-borne nitrate loads at the land–ocean boundary.

1. Introduction

Over the past century, humans have more than doubled the rate of nitrogen (N) input into terrestrial ecosystems, mostly through the increased use of agricultural fertilizers, leading to worldwide water quality degradation (Cardinale, 2011). The main sources of nutrient enrichment of coastal waters are domestic waste effluents, nutrient-rich effluents from aquaculture, and direct loading from upland contaminated rivers (EEA, 2010). Despite less attention given to submarine groundwater discharge (SGD), it has also been shown to be a potential major contributor of dissolved constituents to coastal areas (Burnett et al., 2003; Cable et al., 1997; Corbett et al., 1999; Niencheski et al., 2007; Slomp and Van Cappellen, 2004).

More specifically, since the 1970s, nitrate (NO_3–N) contamination of groundwater has become a significant environmental problem, with many parts of the world now reporting groundwater nitrate pollution (Beeson and Cook, 2004; Burden, 1982; EEA, 2000; Rao, 2006; Rivett et al., 2007; Roy et al., 2007; Spalding et al., 1993). These increasing nitrate concentrations in groundwater, when not degraded within the aquifer's transit pathway to sea, may emerge within groundwater discharge areas within rivers, lakes and costal marine water bodies (Andersen et al., 2007; Rivett et al., 2008). Particularly in permeable coastal sediments, groundwater is commonly a major route of transport from land to sea for freshwater and associated land-derived nutrient loads (Valiela et al., 2000). Accordingly, the control of nitrogen input into watersheds and its removal became part of environmental priorities (Cardinale, 2011).

In the Algarve region (southern Portugal), groundwater from local aquifers is no exception to the high concentrations of nitrate originated from agricultural fertilizer (Almeida and Silva, 1987; Stigter et al., 1998, 2006). Among the regional carbonated aquifers, groundwater nitrate concentrations can reach values above 50 mg/l of NO_3–N (Almeida and Silva, 1987; INAG, 2001). Most of these aquifers discharge in the coastal area either through diffuse flow at the intertidal zone, as reported in the Ria Formosa Lagoon (Leote et al., 2008) or through localized springs in beaches such as the ones found in front of the "Olhos de Água" village – the village owes its name to the presence of several groundwater springs, in the intertidal and subtidal zone ('Olhos de Água' in Portuguese refers to groundwater springs).

In the present paper, we report the presence of the acoel flatworm *Symsagittifera roscoffensis* for the first time in Southern Portugal, in beaches from coastal areas known for their groundwater discharges, such as in *Olhos de Água* and *Praia da Galé*.

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S.roscoffensis Graff, 1891 (Worms AphiaID 484585) is an acoel flatworm that lives in symbiosis with the unicellular microalgae *Tetraselmis convolutae* Parke and Manton, 1967 (Algaebase taxon LSID: urn:lsid:algaebase.org:taxname:47269). During high tide they live interstitially in sandy beaches (Doonan and Gooday, 1982) surfacing during low tide and becoming visible in the foreshore as a thin green patch (Adam and Balzer, 2004). The flatworm was referred as *Convoluta roscoffensis* until the 90s and occurs naturally on north-eastern Atlantic sandy shores, including northwestern France and the Channel Islands (Doonan and Gooday, 1982; Serôdio et al., 2011). It forms colonies that can reach millions of individuals per square meter on sandy beaches with run-off water (Dupont et al., 2012). These flatworms can reach a size of 2–4 mm, exhibiting a green color because of the endosymbiont algae, with densities of up to $2–7 \times 10^4$ per animal (Doonan and Gooday, 1982; Serôdio et al., 2011). While the algae supply sugars, amino acids and oxygen to the worm, the animal provides shelter against predation and optimal photosynthetic conditions for the algae by actively seeking optimal light exposure (Doonan and Gooday, 1982; McCoy and Balzer, 2002; Serôdio et al., 2011).

The main goals of the present study were thus to (i) describe and quantify the presence of flatworm *S. roscoffensis* in SGD impacted sites, (ii) test its ability to uptake NO_3–N as a nitrogen source under strictly controlled conditions, and finally (iii) carry out a preliminary evaluation of the impact of its uptake rates on nutrient delivery via SGD to the coastal environment. Our working hypothesis is that *S. roscoffensis* chooses SGD impacted sites due to the high nutrient availability associated to these waters and therefore can be a potential natural bioremediator.

2. Materials and methods

2.1. Characterization and quantification of natural occurrence of the acoel flatworm

Specimens of *S. roscoffensis* were collected at *Praia de Olhos de Água*, Albufeira, Southern Portugal in July 2012 (Fig. 1).

Besides the presence of the flatworm, this location was chosen because it has long been known to be a site impacted by SGD – the Phoenicians based themselves there (700 B.C.) because of the springs. Differences in density distribution of the flatworm were observed during several visual surveys throughout the year and varied from very dense (Fig. 2) to non-observable, through the seasons.

When present, flatworms appeared most of the time as small green clusters on the sand surface in the upper intertidal zone. To evaluate the surface coverage density of flatworms, during July 2012 we sampled 1 cm in depth of sediment using mini cores (10 ml syringe, 1.5 cm diameter) during low tide, when the flatworms were on the sand surface. Because the distribution of the flatworms during this period was confined in very small patches, three different locations were sampled (not randomly, in triplicates according to visual observations of the flatworm's density: high density, low density and non observable. Samples were transported to the laboratory within the hour in centrifuge tubes topped up with seawater from the site. In the laboratory, samples were placed in petri dishes and flatworms were anesthetized with isotonic $MgCl_2$ (Salvenmoser et al., 2010). When movement was no longer observed, 5 ml of rose Bengal dye ($2\ g\ l^{-1}$) was added until all the flatworms became pink. The excess of dye was withdrawn and seawater was added. Flatworms were then counted under a dissection microscope, density calculated per unit area and values reported as the number of flatworms per cm^2. The density of the flatworms was then classified according to four density range groups defined by a combination of the visual observations at the beach and the count results: very high density, high density (dark green areas), low density (light green areas) and non-observable (absence of green areas). Because visual observations during other visits to the field site revealed a density of flatworms much higher (based on the intensity of the green color) than the one observed within the previously defined high density category, another category for the flatworm's density distribution (very high density) was considered.

2.2. Characterization of SGD collected from different locations in Olhos de Água

To assess the possible contribution of the flatworms to nutrient interception at the SGD impacted sites with high nitrate concentrations, water samples were collected and nitrate concentrations and salinity measured at four different sites of known groundwater discharge: a small groundwater spring (GS1) near the cliffs, a big groundwater spring (GS2) that is only exposed during low tide, at a location of water insurgency from the limestone platforms (RP) and finally, from the sites where the flatworms were collected for density counts (PW – porewater) – Fig. 1. Water was collected directly into 500 ml PE bottles at all locations, with the exception of the PW samples; for these, 10 cm wells were deployed in the sediment and the accumulated interstitial water within was directly filtered through a Rhizon™ membrane with 0.1 μm pore size (Rhizosphere) into sterile vacuum vials (BD Vacutainer®). All other water samples were filtered similarly, and the sample set was then stored at 4 °C until nutrient analysis.

2.3. Nitrate assimilation experiment

In order to evaluate nitrate uptake rates by *S. roscoffensis*, flatworm specimens were collected at the same location. Individuals were collected from the wet sand surface using a plastic Pasteur pipette with care employed to minimize the concurrent collection of sand grains, and were transported to the laboratory, within 1 h from collection, in seawater collected at the same location.

In the laboratory, flatworms were counted under a dissection microscope and separated into individual petri dishes (300 individuals per petri dish approximately), containing seawater collected during field sampling. Prior to use, the seawater used in the petri dishes was filtered through 0.45 μm cellulose acetate filters and autoclaved for 15 min at 105 °C. Glass flasks (Schott) with 1 L capacity were then filled with filtered and autoclaved seawater and henceforth used as microcosms. $NaNO_3$ was employed as an NO_3–N source to prepare two incubation solutions of different final concentrations, 50 μM and 800 μM NO_3–N. All the incubation experiments were executed in triplicate. A phytoclimatic chamber was programmed for 100% light intensity (approximately 112 $\mu mol\ m^{-2}\ s^{-1}$, measured with a Li–250A, Li–COR®) radiometer on a 12 h alternating light–dark cycle, at 20 °C. In preparation for the actual experiment, microcosms and petri dishes with flatworms were initially acclimatized to these conditions for 15 h (2 h light period followed by 12 h dark, then 1 h light). Subsequently, the acclimatized set-ups were subject to the experimental conditions as described, following the natural light cycle from the time and day of collection. After acclimatization and immediately before adding the flatworms, a 25 ml water aliquot was taken from each of the microcosms, to represent initial conditions (Time zero, T_0). Water samples were then taken every hour for the next 23 h, immediately filtered through fiberglass filters (Whatman® GF/F 25 mm) directly into sterile vacuum vials (BD Vacutainer®) and stored at 4 °C until nutrient analysis. All microcosms were kept under a continuous flow of air, bubbled into the solutions throughout the experiment.

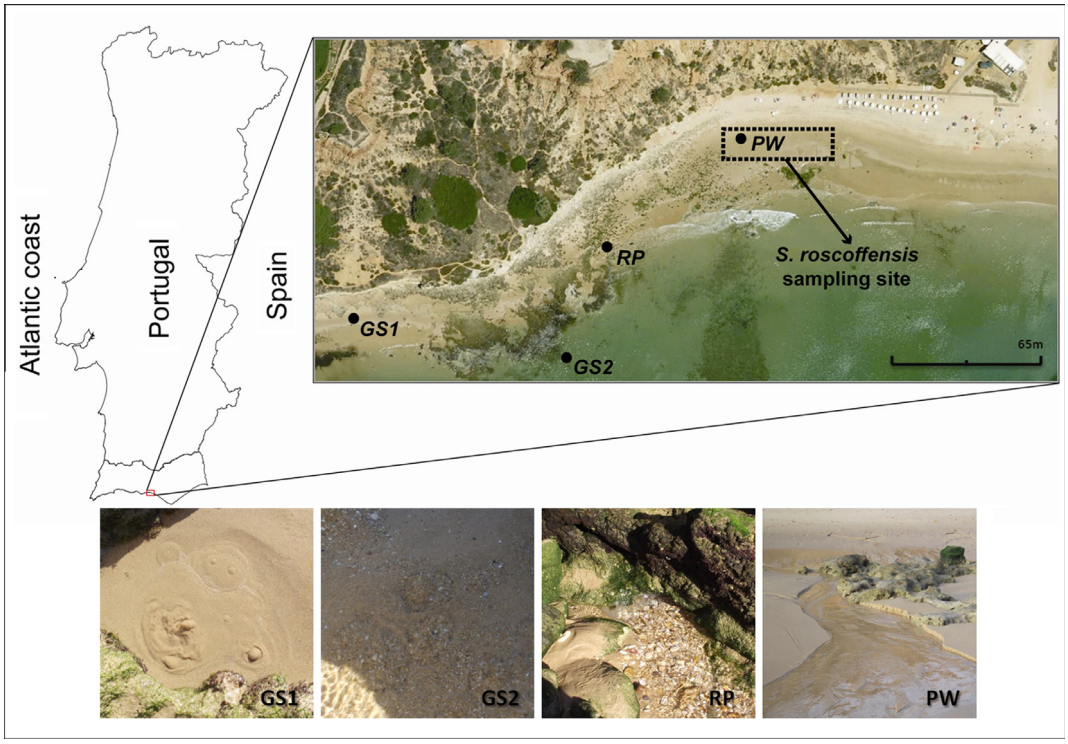

Fig. 1. Location of the *Symsagittifera roscofensis* and water sampling sites in Olhos de Água, southern Portugal. PW – porewater sampled on flatworm dense areas, RP – Rockpool, GS1 – Groundwater spring 1 and GS2 – Groundwater spring 2.

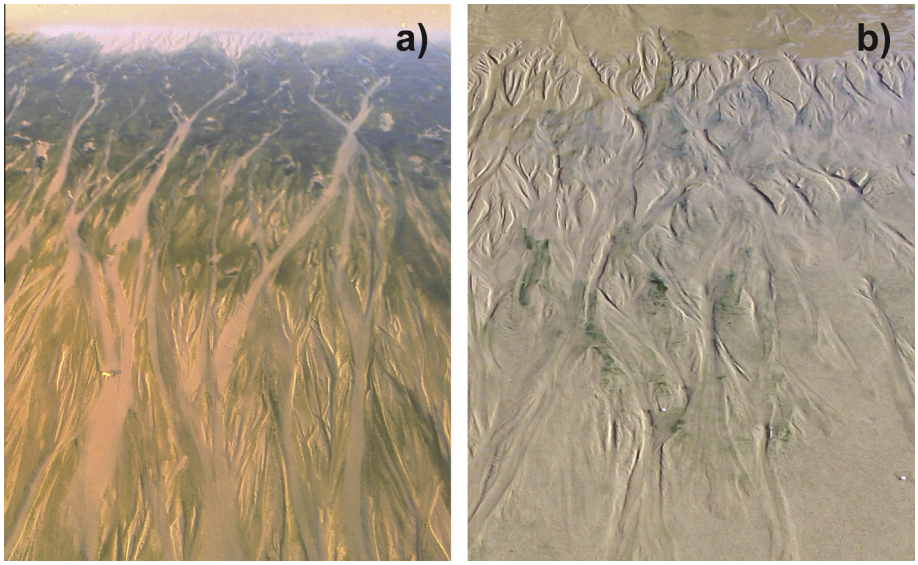

Fig. 2. *Symsagittifera roscoffensis* density in different times of the year. (a) January 2012 – very high density (up to 1000 flatworms/cm^2) and (b) July 2012 – High density (±250 flatworms/cm^2).

2.4. Nutrient analysis

Collected samples were analysed for nitrate + nitrite, nitrite and ammonia in a FOSS Flow Injection Analyser (FIAstar 5000). Nitrite determination was colorimetric, based on the development of red azo dye with a detection limit of 0.007 μM, while nitrate + nitrite determination was based on prior reduction of nitrate using cadmium–copper reduction (detection limit 0.036 μM). The amount of nitrate present in a sample was then calculated by subtracting the amount of nitrite from the total oxidized nitrogen as determined via Cd–Cu reduction (Hansen and Koroleff, 1999). Ammonium determination followed a photometrical method (FIAstar 5000 – Application Notes (2008), Version 1.80, FOSS). However NH_4–N results are not shown here because all the samples showed concentrations below the detection limit of the equipment (0.07 μM NH_4^+).

2.5. Data analysis

Concentrations measured throughout the experiment were then employed to calculate two parameters needed to characterize N uptake metabolism: the net nitrate uptake rate (NUR_a) and the

net nitrite excess rate (NER_i) within each replicate microcosm. Both were determined from the slopes of linear regression of the nitrate and nitrite concentrations in the solution as a function of time. Assuming that the temporal change in N-concentration within the solution were only due to the activity of *S. roscoffensis*, the uptake rate can be considered as the symmetric value of the slopes, i.e., negative slope values correspond to positive values and therefore to a N-uptake and positive slope values to negative values and therefore to a N-excretion. Nitrate assimilation rates (NAR_a), corresponding to the absolute value of the sum ofNUR_a and NER_i, were then calculated and converted to assimilation rate per individual (nmol h^{-1} ind^{-1}). NAR_a therefore represents the rate at which nitrate was taken up and in fact used by the endosymbiont microalgae. Effects of initial NO_3–N concentration on nitrate uptake and nitrite excretion were investigated during light and dark conditions separately. ANOVA and MANOVA tests were employed to statistically evaluate the differences between different sets of results.

3. Results

3.1. Quantification and characterization of the occurrence of the acoel flatworm and SGD's nitrate concentrations in Olhos de Água

During July 2012, the density of *S. roscoffensis* observed on-site corresponded to 253 ± 26 flatworms per cm^2 of sediment surface area (Fig. 2b). However, both the distribution and density of flatworms in *Olhos de Água* varied through the whole year; from non observable (up to 5 flatworms cm^{-2}) to small random green patches in the sand (less dense – ±100 flatworms cm^{-2} to dense −250 flatworms cm^{-2}) or even large longitudinal green areas with up to 6 m in length (very dense more than 1000 flatworms cm^{-2} – Fig. 2a) along the whole intertidal cross-section of the beach (approximately 150 m). The intertidal area of the beach was mainly composed of fine to medium sand (data not shown). However, there were no apparent indications of any direct relation between the presence of flatworms and sand grain size.

On the other hand, these areas where the flatworms can be found are known sites of SGD associated with high nitrate concentrations. The highest nitrate concentration found was 521.65 ± 3.89 μM of NO_3–N in GS1, corresponding to where the lowest salinity was found whereas the lowest nitrate concentration (17.00 ± 0.13 μM of NO_3–N) and highest salinity (35.6) were found in PW site (Table 1).

3.2. Inorganic nitrogen uptake rates

Effects of initial NO_3–N concentration on nitrate assimilation and nitrite excess were investigated for the two contrasting light availability periods. Fig. 3 shows the changes in nitrate concentrations with incubation time under two initial NO_3–N concentrations: 50 μM and 800 μM.

The incubation experiments show a decrease in NO_3–N concentration in the solution containing the ~300 individuals from 61.19 ± 0.86 to 50.97 ± 1.10 μM and from 793.66 ± 23.74 to 746.95 ± 24.05 μM for the 50 μM and 800 μM concentrations, respectively, during a period of 23 h. This decrease in NO_3–N concentration occurred during the light period. In contrast, during the dark period, NO_3–N concentrations in the microcosms remained stable. As mentioned before, nitrate uptake rates correspond to the symmetric value of the slope of the linear changes in concentration in the solution reported to nmol h^{-1} ind^{-1}. However, since nitrite excretion was observed during part of the experiment, it is fair to assume that only part of this nitrate was actually used in the nitrate reduction pathway. Consequently, nitrate assimilation rates (NAR_a) were calculated separately to represent the real nitrate assimilation by the microalgae. The calculated nitrate assimilation rates were significantly dependent of initial NO_3–N concentration during light period ($p < 0.05$): 0.31 ± 0.19 nmol h^{-1} ind^{-1} for 50 μM initial concentrations and 1.97 ± 0.78 nmol h^{-1} ind^{-1} for 800 μM (each individual corresponds to a flatworm and its endosymbiont algae population). In opposition, during the dark period the rate of nitrate concentration change measured within the microcosms were not significantly different (0.04 ± 0.06 and 0.71 ± 0.30 nmol h^{-1} ind^{-1} for 50 and 800 μM incubations, respectively – Table 2).

Although nitrite was not added to the incubation medium, its concentration in the supernatant varied from ~0.5 to ~3.5 μM during the whole experiment for both treatments, with an increase of NO_2–N concentration during light period and stable readings during dark period (Fig. 4).

The rate of nitrite concentration changes measured were not significantly different between the two microcosms neither during light nor dark periods (−0.15 ± 0.02 and −0.23 ± 0.02 nmol h^{-1} - ind^{-1} for 50 μM incubation and 0.02 ± 0.01 and −0.03 ± 0.02 nmol h^{-1} ind^{-1} for and 800 μM incubation during light and dark periods, respectively) (Table 2).

Ammonium (NH4–N) concentrations remained below detection limit for all treatments throughout the entire experiment.

4. Discussion

4.1. Nitrate uptake rates and nitrite excess rates

The magnitude of the assimilation rate of NO_3–N by *S. roscoffensis* co-varies with the initial availability of nitrate in the incubation medium: the higher the incubation concentrations, the higher the uptake rates. Although this has already been reported for other microalgae (for e.g. the green microalgae *Chlorella vulgaris*, Jeanfils et al., 1993), this is the first time that is described for *S. roscoffensis*. Besides the initial NO_3–N concentration, light was one of the parameters that most influenced nitrate behavior within the microcosms. A decrease of NO_3–N concentration can be observed during light period (at different rates according to the initial NO_3–N concentration), whilst during the dark period, NO_3–N in the incubations seems to stabilize (Fig. 3). This behavior is coincident with the photosynthetic process that only occurs in the presence of light, confirming that it is the symbiotic microalgae that uptakes nutrients from the water and not the flatworm itself. For that reason, even when great amounts of nitrate (or nitrite) are present as substrate, which usually induces high activities of nitrate and nitrite reductase, the induction of these enzymes additionally requires light (Ullrich et al., 1998).

Under light availability, nitrite exhibits the opposite behavior of nitrate assimilation. At the beginning of the experiments, only a residual amount of nitrite was present in the incubation medium, but its concentration started to rise during the incubation under light (reaching ~3.5 μM). Conversely, during the dark period its concentration stabilized, similarly to nitrate (Fig. 4). In contrast with nitrate concentrations, the rate of change observed in nitrite

Table 1
Nitrate concentrations and salinity in different locations of submarine groundwater discharge in Olhos de Água. GS1 – near the cliff walls, GS2 – near the low tide shoreface, RP – groundwater coming out directly from limestone, PW – porewater on the *S. roscoffensis* sampling sites.

	NO_3–N (μM)	Salinity (ppt)
Groundwater spring (GS1)	521.65 ± 3.89	2.1
Groundwater spring (GS2)	507.48 ± 13.46	2.4
Rockpool (RP)	478.40 ± 14.26	4.4
Porewater (PW)	17.00 ± 0.13	35.6

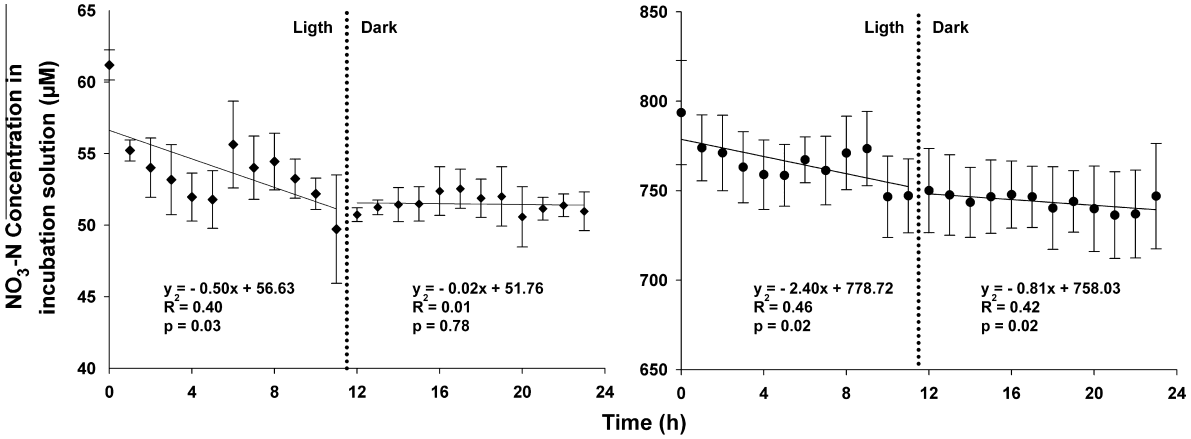

Fig. 3. Nitrate (NO_3–N) concentration variation in the incubation solution. Initial nitrate concentrations were 50 and 800 µM. Calculated nitrate uptake rates during the light period were 0.46 ± 0.18 and 2.20 ± 0.76 nmol h^{-1} ind^{-1} and during the dark period 0.02 ± 0.05 and 0.74 ± 0.28 nmol h^{-1} ind^{-1}, respectively for (♦) 50 and (●) 800 µM. Standard deviation is represented as error bars and *p*-values represent the significance of the slope.

Table 2
Nitrate and nitrite uptake rates and nitrate assimilation rates based on nutrient measurement of the incubation solution for initial nitrate concentrations of 50 and 800 µM during light and dark period (*p*-values indicate the significance of the calculated rates differences between the two initial NO_3–N concentration).

		Light			Dark		
		50 µM	800 µM	*ANOVA p-value*	50 µM	800 µM	*ANOVA p-value*
Uptake rate (nmol h^{-1} ind^{-1})	NO_3–N	0.46 ± 0.18	2.20 ± 0.76	0.005***	0.02 ± 0.05	0.74 ± 0.28	0.363
	NO_2–N	−0.15 ± 0.02	−0.23 ± 0.02	0.154	0.02 ± 0.01	−0.03 ± 0.02	0.052*
Assimilation rate (nmol h^{-1} ind^{-1})	NO_3–N	0.31 ± 0.19	1.97 ± 0.78	0.009**	0.04 ± 0.06	0.71 ± 0.30	0.392

Statistical significance levels:
*** <0.001
** <0.01
* <0.05

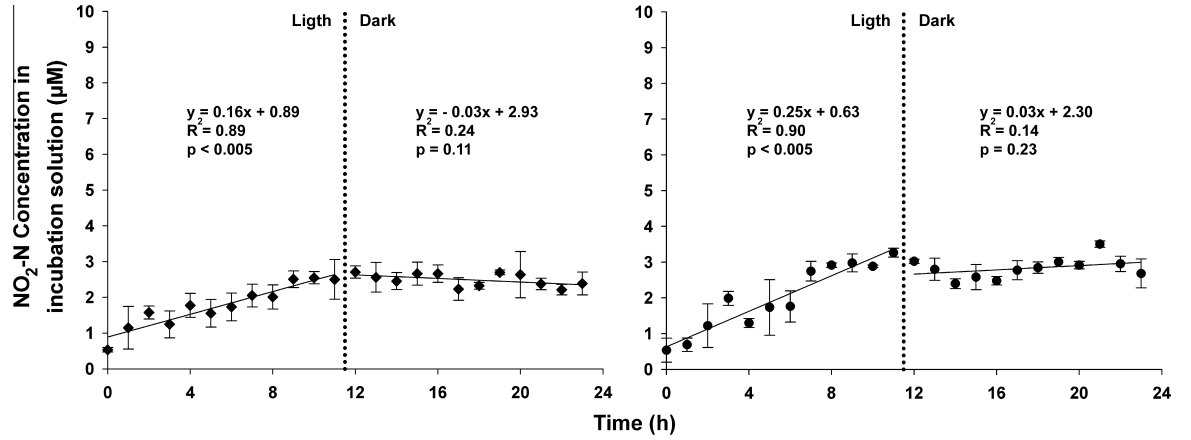

Fig. 4. Nitrite (NO_2–N) concentration variation in the incubation solution. Initial nitrate concentrations were 50 and 800 µM. Calculated nitrite excess rates during the light period were 0.15 ± 0.02 and −0.23 ± 0.02 nmol h^{-1} ind^{-1} and during the dark period 0.02 ± 0.01 and −0.03 ± 0.02 nmol h^{-1} ind^{-1}, respectively for (♦) 50 and (●) 800 µM. Standard deviation is represented as error bars and *p*-values represent the significance of the slope.

concentrations was not dependent of initial nitrate concentrations, and can be considered to be the same for both treatments (50 and 800 µM of NO_3–N – Table 2). Nitrite release by phytoplankton during nitrate assimilation has been reported by several authors (Al-Qutob et al., 2002; Collos, 1998; Vaccaro and Ryther, 1960; Wada and Hattori, 1971). As nitrite concentrations initially increased and then stabilized, one can consider that there was a moment during which nitrite was excreted. Accordingly, we can only assume that: (1) nitrite was only excreted during light periods (2) nitrite was excreted during both light and dark periods but also, subsequently taken up during the dark period and (3) nitrite was excreted and taken up during light and dark period but at different rates. Here it is assumed that the observed behavior of nitrite is due to its excretion during light period only. Indeed, the excretion of nitrite suggests very high initial nitrate concentrations, probably because the endosymbiotic microalgae cells weren't able to successfully use the entire nitrate pool absorbed at the same high rate as that of absorption. Therefore, nitrite excretion is an indication of the uncoupling which can exist between the transport and assimilation of nitrate, implying that the rate of nitrate reductase activity is greater than that of nitrite reductase. If true, this means that nitrite reduction is the most limiting factor in the transport and

reduction process, an observation that is consistent with other studies (for e.g. Sciandra and Amara, 1994). However, other authors argue that during nitrate spike experiments, where phytoplankton cells were not pre-acclimated to assimilate nitrates rapidly, the release of nitrite is a transient response to the abrupt increase in NO_3–N uptake rate (Laws and Wong, 1978; Serra et al., 1978a). Our data is not consistent with this explanation, however. Rather, it shows that nitrite excretion occurs at similar rates independently of the initial nitrate concentration, and furthermore, that the released concentration is approximately the same during light period. This might suggest that accumulation of external nitrite inhibits further nitrite excretion, as proposed by Kiefer et al. (1976).

Even the incubation carried out in the presence of 50 μM NO_3–N showed nitrite excretion, suggesting that the microalgae enzymes are already saturated. Therefore, it was expected that in 800 μM incubation the nitrate assimilation rate couldn't exceed the one obtained for the 50 μM incubation (0.31 ± 0.19 nmol h^{-1} - ind^{-1}). However, nitrate assimilation rate for 800 μM treatment was much higher (1.97 ± 0.78 nmol h^{-1} ind^{-1}), suggesting that the *T. convolutae* cells are able to adapt their enzyme production according to the availability of substrate in the surrounding environment.

Although high nitrate uptake/assimilation rates were measured during this experiment, it is still not clear if these corresponded to the absolute maximum uptake rates by *S. roscoffensis*. This is due to possible dissolved inorganic carbon (DIC) limitation during the experiment, owing to experimental design. As one of the objectives of this experiment was to measure only *S. roscoffensis* nitrate uptake rates, without the potentially co-influence of DIC (groundwater in Karst systems has a very large range of DIC content reported), the seawater used as incubation solution was autoclaved to ensure that any microplankton present in the seawater was inactivated. Although one could also argue that degassing during the process could be potentially important in limiting the amount of total dissolved CO_2 in the incubation solution available for photosynthesis during the experiment. Some authors argue that most of the N-sufficient green algae and cyanobacteria studied so far do not uptake or reduce NO_3–N in the absence of CO_2 due to the lack of stored carbohydrates (Huertas et al., 2000; Huppe and Turpin, 1994; Turpin, 1991). In the present work, because DIC was not measured, it can only be assumed that the presented uptake rates are under dissolved inorganic carbon limitation. Future experiments are planned to account for co-limiting factors.

4.2. S. roscoffensis as an interceptor for SGD-borne nitrate loading

The high nitrate concentration employed in one of the treatments (800 μM) was chosen due to its rare occurrence in natural marine environments, even in very polluted areas (for hypereutrophic waters, TN > ~145 μM; Yang et al., 2008). However, nitrate concentrations of this magnitude have been reported for SGD plumes, seepage through beach sands and in sediment porewaters (Leote et al., 2008; Usui et al., 1998).

Assuming that only the microalgae *T. convolutae* inside the flatworm uptake nitrate, we can compare the obtained uptake rates in this experiment with uptake rates of other *Tetraselmis* species already described in literature. When incubated in conditions similar to the ones in the present experiment (12:12 light/dark cycle, ~880 μM of NO_3–N initial concentration), *Tetraselmis chuii* showed assimilation rates of 0.06–0.20 nmol h^{-1} ind^{-1} (Meseck et al., 2005). The uptake rates are converted assuming that each flatworm could shelter between 2–7 × 10^4 endosymbiotic algae cells of *T. Chuii* in agreement with descriptions of endosymbiotic algae *T.convoluta* (Doonan and Gooday, 1982). Comparatively, *S. roscoffensis* NO_3–N assimilation rates were ~1.97 ± 0.78 nmol h^{-1} ind^{-1}. This means that this symbiotic relationship presents great advantage in what concerns *T. Convolutae* nitrate uptake as it showed a NO_3–N assimilation rate ~10 times higher than *T. Chuii*, implying that the acoel flatworm shows greater potential as a natural nutrient interceptor. This difference in assimilation rates can be due to several factors: (1) the loss of *T. convolutae* theca in the course of the symbiosis with the flatworm (Lee, 2008); (2) the lightstimulation of nitrate uptake and (3) the species difference with *T. chuii which* is not the symbiotic microalgae. In the first hypothesis, the microalgae loss of its theca would somehow facilitate the transport of extracellular nitrate to the microalgae cells (Lobban and Harrison, 1997). On the other hand, nitrate uptake is stimulated by light because it increases the rate at which cell nitrogen is formed (Grant and Turner, 1969). During the experiments presented here the flatworms moved to the top of the incubator flasks so they could be nearer to the light source, seeking to provide optimal photosynthetic conditions to the microalgae. Therefore, light-dependent assimilation enzymatic systems (like nitrate reductase) could probably be magnified, turning nitrate assimilations rate higher. Notwithstanding, here we compare flatworm assimilation rates of*T. convolutae* with *T. chuii* that, despite being from the same genus, is not the same species. Because nitrate uptake rates can differ significantly between species, it would have been more correct to use the number of microalgae per flatworm from our study area to do the extrapolation. Indeed, the assimilation rates described for *T. chuii* (Doonan and Gooday, 1982) were obtained from flatworms collected in a very different area and probably with a very different microalgae content, specially due to different climatic conditions (e.g., light intensity). Unfortunately, we did not manage to extract the microalgae *T. convolutae* from the flatworm even using methods described before (Boyle and Smith, 1975; Doonan and Gooday, 1982).

According to our data from July 2012 (Table 2), groundwater springs presented a very low salinity even when located very near the shoreface during low tide. This indicates that minimum or no biological reactions occur in the groundwater emerging from the sand and rock, explaining why such high nitrate concentrations are found in these sites. On the other hand, porewater salinity on the sites where the flatworms were collected was much higher (due to the presence of recirculated seawater and dissolution of salts in the sand) and nitrate concentrations much lower, suggesting that, in addition to mixing, the flatworms might be one of the possible benthic organisms intercepting nitrate in these seepage areas.

Assuming that *S. roscoffensis* was the only organism mitigating nitrate in the beach and that ±500 μM is the concentration of nitrate in groundwater emerging and ~17 μM is the concentration of nitrate after flatworm uptake (Table 1), this would mean that approximately 97% of nitrate associated to the groundwater discharge was recycled by *S. roscoffensis*.

Furthermore, it is possible to estimate the amount of this contaminant that can be taken up by the flatworm per unit of area. As discussed before, when maximum density of the flatworm is present in the beach around 10^7 flatworms per m^2 can be found in an area of 150 × 3 m (very high density area) plus 2.5 × 10^6 flatworms per m^2 in an area of 150 × 6 m (high density area – Fig. 2). According to the acoel flatworm uptake rates achieved in the microcosm experiments during light period (0.46 ± 0.18 and 2.20 ± 0.76 nmol h^{-1} ind^{-1} for 50 μM and 800 μM of initial NO_3–N concentration, respectively) and considering the number of flatworms per square meter, the expected uptake rates in the natural environment would be between 4.6 × 10^{-3} and 0.02 mol h^{-1} m^{-2} of nitrate in the very high density areas and between 1.15 × 10^{-3} and 5.5 × 10^{-3} mol h^{-1} m^{-2} in a high density area.

Further data is needed (e.g., SGD flow rate) to achieve the real impact of *S. roscoffensis* in intercepting nitrate from SGDs in *Olhos de Água* beach, but the presented nitrate uptake rates and

percentage of nitrate removal show that the acoel flatworm can have a crucial role in intercepting N fluxes from beaches into the adjoining coastal waters. Being already part of the ecosystem, *S. roscoffensis* can be considered a natural bioremediator with an extremely high capacity to remove the excess of nitrate derived mostly from anthropogenic pressures in the local aquifer.

5. Conclusions

Despite experimental challenges and limitations during the experiments, *S. roscoffensis* showed intrinsic characteristics that support a great potential to be a nutrient interceptor in SGD areas. Because no toxic or harmful effects associated with the presence of the flatworm in natural ecosystems are known, great benefits are potentially seen with the use of *S. roscoffensis* as a potential bioremediator. Augmenting studies can be recommended for further validation and additional data to the present study.

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