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Nitrogen uptake kinetics of freshly isolated zooxanthellae

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Zooxanthellae freshly isolated from the coral host *Pocillopora damicornis* exhibited substrate-saturable uptake kinetics for ammonium, nitrate and urea. Maximum uptake velocity for ammonium [10.1 nmol. (μg chl-*a*).$^{-1}$h^{-1}] was greater than that for nitrate [2.8 nmol.(μg chl-*a*).$^{-1}$h^{-1}] and urea [0.37 nmol. (μg chl-*a*).$^{-1}$h^{-1}]. Half-saturation constants for uptake of the three nitrogen compounds were in the range of 10-15 μmol.l^{-1}. Generally, uptake of any one nitrogen substrate appears to be inhibited by the other 2 substrates when the latter are present in higher concentrations. The observed interactions can be explained by the uncompetitive model. These results are interpreted as indicative of a simultaneous uptake of all 3 nitrogen compounds by the zooxanthellae.

Freshly isolated zooxanthellae (FIZ), as well as whole corals (with the symbiont) can take up nitrate, ammonium and urea, and these three nitrogenous compounds are supplied to them continuously by polyp (ammonium and urea), and by nitrification on the coral colony (nitrate)[1]. Though the studies have shown[1] that zooxanthellae can derive a major portion of their N requirement from coral excretion alone, the N uptake in these studies might have been a nutrient-limited one, since the experiments were carried out at ambient seawater concentrations, whereas the supply from various external and internal sources may enhance the N locally available near a coral colony. Some such sources are terrestrial runoff[2], ground waters[3], upwelling near the reef[4], excretion by migrating fish schools[5], mineralisation within the "interstitial" waters of the branches of corals[6] and by the endolithic community in the coral heads[7]. These diverse regenerative sources are likely to supply N in one or more forms, and at varying concentrations. Since the zooxanthellae have a potential for uptake of ammonium, nitrate and urea, this study has been undertaken to investigate uptake kinetics of FIZ for these 3 N sources and the effects, at different concentrations, of one or the other N source, on the uptake of the third. Uptake kinetics for nitrate and ammonium are known earlier, both in FIZ[8], and in cultured zooxanthellae[9], but not for urea. The interactions among these 3 N sources on N uptake by FIZ are also not known. Again, previous studies on N uptake kinetics of FIZ were by following the depletion of substrate over a time course, whereas this study is the first to use ^{15}N for measuring directly the kinetics of N uptake.

Materials and Methods

The present study was carried out during the 189th cruise of *R.V. Gaveshani* to the Lakshadweep atolls (Dec. 1987). Colonies of *Pocillopora damicornis* were freshly collected from the lagoon and crushed in 10-20 ml of Millipore (0.45 μm porosity) filtered seawater (MFSW). The homogenate was strained through a 100 μm Nitex net and centrifuged (2000 rpm for 5 min). Zooxanthellae pellet was resuspended in MFSW, washed several times until it was free from polyp debris and made up to a known volume with MFSW.

Aliquots of FIZ obtained as above were distributed into 20 glass bottles, each containing 250 ml of MFSW. Stock solutions of ^{15}N-labelled (99% enrichment) ammonium chloride, sodium nitrate and urea were prepared and added (in each case) to the bottles so as to give final concentrations of 1-60 μmol.l^{-1}, with increments of 1 μmol.l^{-1} up to 10 μmol.l^{-1} and subsequently with increments of 5 μmol.l^{-1} up to 60 μmol.l^{-1} (urea concentrations used were half of those of ammonium and nitrate, since unlike the latter two, 1 μmol of urea contains two N atoms). Uptake measurements were carried out in an aquarium placed inside a constant light chamber, with the ambient seawater circulating through the tank to provide cooling. The light source was a bank of cool day-light flourescent tubes and the incident light intensity at the surface of the bottles was 60 μE.m.$^{-2}$s^{-1}. After an incubation for 2 h, the FIZ were harvested on GF/F filter pads (preignited at 400°C). Samples for zero-time uptake were obtained by adding ^{15}N to a subsample and filtering immediately. Subsamples of FIZ were analyzed for chl-*a*.

In experiments designed to test the effects of any one of the two N sources on the uptake of the third by FIZ, the N source for uptake was given as ^{15}N, and the other N was given in unlabelled form. For example, when uptake of ammonium was to be tested against nitrate, ammonium was given as ^{15}N-labelled ammonium chloride at final concentrations of 10, 20, 30, 40 and 50 µmol.l^{-1}, and unlabelled nitrate was added at three concentrations of 10, 20 and 30 µmol.l^{-1}. Similar procedures (and concentrations) were adopted in the cases of ammonium uptake in the presence of urea, nitrate uptake in the presence of ammonium/urea, and urea uptake in the presence of ammonium/nitrate. Experimental conditions were the same as those followed for uptake kinetic studies, and appropriate subsamples for zero-time ^{15}N uptake and chl-*a* were taken for each series of experiments.

Nutrients and chl-*a* were measured following standard procedures[10-12]. Particulate organic N content was measured by Kjeldahl digestion, and ^{15}N enrichment by emission spectrometry in a JASCO N-150 heavy nitrogen analyser.

Kinetics of uptake were analysed by plotting the data for uptake velocity V [nmol.(µg chl-*a*).$^{-1}$h^{-1}] against the substrate concentration S (µmol.l^{-1}), and the kinetic constants (V_{max} and K_s) were calculated by linear regressions of the reciprocals of V and S (Lineweaver-Burk plots).

Results

Plots of uptake *vs* substrate concentrations for ammonium, nitrate and urea (Fig. 1) demonstrate that the uptake kinetics for the three N compounds are well described by the Michaelis-Menten hyperbolae. The kinetic constants calculated from reciprocal plots of V and S are given in Table 1.

Nitrate uptake in presence of ammonium and urea —Nitrate uptake by FIZ was significantly less in the presence of ammonium and urea (Fig. 2 a, c). With ammonium at 10, 20 and 30 µmol.l^{-1} levels, this decrease was from a V_{max} of 2.8 nmol.(µg chl-*a*).$^{-1}$h^{-1} in the control down to 0.84, 0.25 and 0.5 nmol.(µg chl-*a*).$^{-1}$h^{-1} respectively. In fact, though ammonium at 20 µmol.l^{-1} level appears to suppress nitrate uptake much more than at 30 µmol.l^{-1} level, the uptake rates in both the instances were not significantly different. Similarly, urea at concentrations of 5, 10 and 15 µmol.l^{-1} reduced the V_{max} to 1.3, 0.45 and 0.4 nmol.(µg chl-*a*).$^{-1}$h^{-1} respectively. Transformation of nitrate uptake curves with ammonium and urea addition to

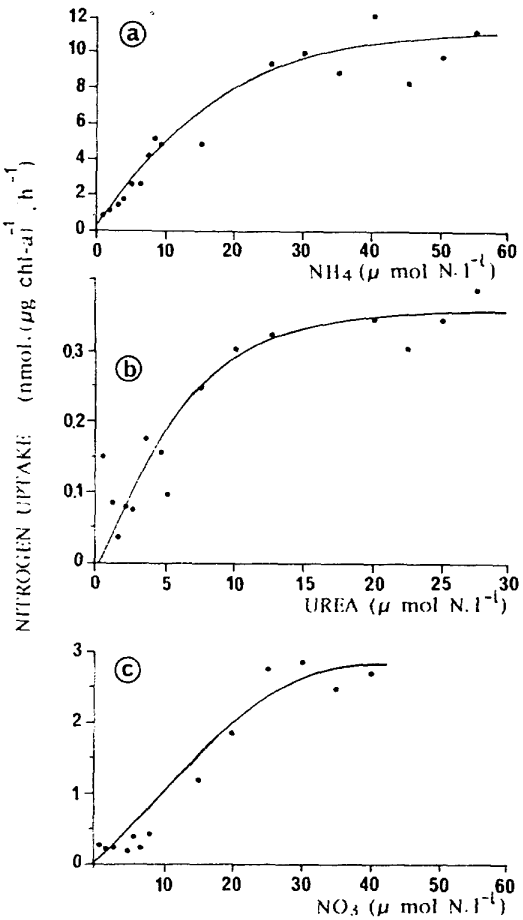

Fig. 1—Uptake rate *vs* substrate concentration for ammonium, nitrate and urea

Table 1—Kinetic constants for ammonium, nitrate and urea uptake by FIZ and the supply rate of the three N sources to them

N source	V_{max} [nmol. (µg chl-*a*).$^{-1}$h^{-1}]	K_s (µmol.l^{-1})	N supply (in nmol h^{-1}) to one µg chl-*a*	Supply source
Ammonium	10.1	10	11.2-38.8	Excretion
Nitrate	2.8	15	9.5-21.6	Nitrification
Urea	0.37	10	0.6-1.0	Excretion

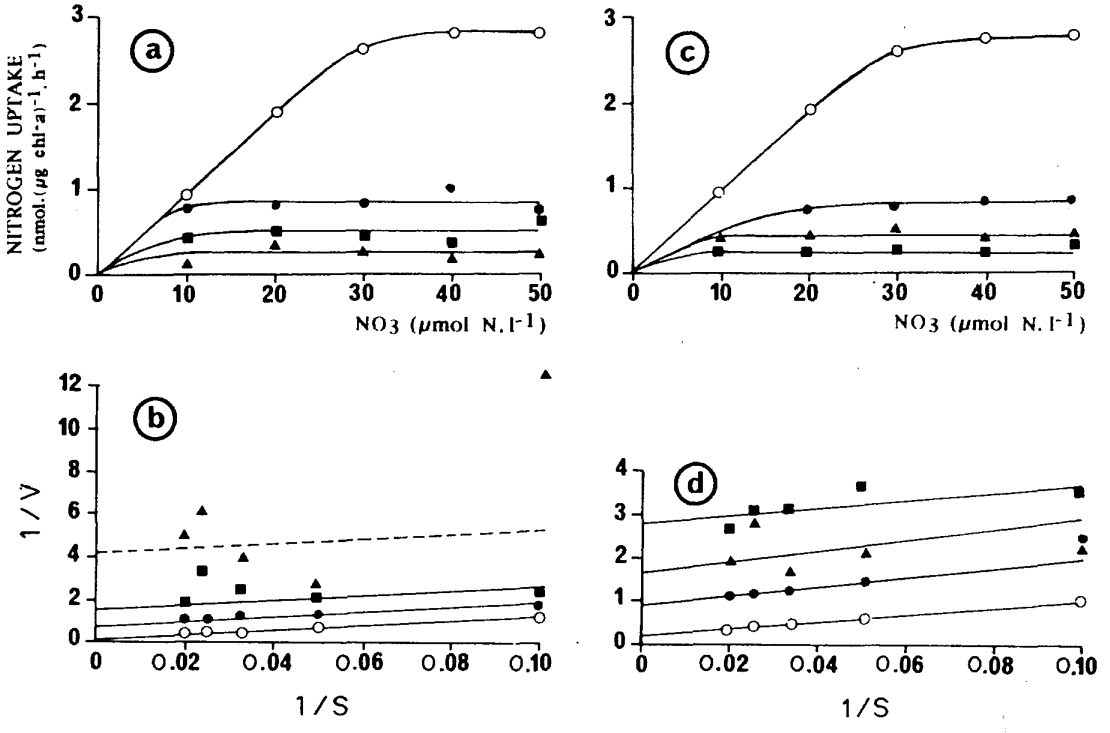

Fig. 2—Nitrate uptake by FIZ in the presence of ammonium (a) and urea (c), and the corresponding Lineweaver-Burk plots (b and d). Ammonium addition was at 0 (open circles), 10 (closed circles), 20 (triangles) and 30 (squares) µmol.l^{-1} levels. Urea addition was at 0 (open circles), 5 (closed circles), 10 (triangles) and 15 (squares) µmol.l^{-1} levels

Lineweaver-Burk plots (Fig. 2 b, d) shows that the inhibition in both cases follows the uncompetitive model.

Ammonium uptake in presence of nitrate and urea—Interestingly, ammonium uptake also appears to be inhibited in the presence of nitrate and urea (Fig. 3 a, c). With nitrate from 10 to 30 µmol.l^{-1}, V_{max} for ammonium uptake decreased from 10.1 nmol.(µg chl-*a*).$^{-1}$h^{-1} in the control through 8.2 and 4.6 to 2.3 nmol.(µg chl-*a*).$^{-1}$h^{-1}. With urea at 5 and 10 µmol.l^{-1} the decrease in V_{max} was down to 8 and 6.6 nmol. (µg chl-*a*).$^{-1}$h^{-1}, though at 15 µmol.l^{-1} urea did not appear to influence ammonium uptake. Lineweaver-Burk plots of ammonium uptake curves with nitrate and urea addition (Fig. 3 b, d) shows that, as with nitrate uptake, the inhibition was of the uncompetitive type.

Urea uptake in presence of ammonium and nitrate—Presence of ammonium and nitrate reduced urea uptake by FIZ (Fig. 4 a, c). With ammonium, the reduction was from a control V_{max} of 0.37 to around 0.1 nmol. (µg chl-*a*).$^{-1}$h^{-1} and with nitrate, it was down to 0.12-0.25 nmol. (µg chl-*a*).$^{-1}$h^{-1}. Reciprocal plots of uptake *vs* substrate (Fig. 4 b, d) shows that, as with ammonium and nitrate uptake, the inhibition was of the uncompetitive model.

Discussion

Zooxanthellae, as isolates or in symbiotic association with corals (and other hosts), can take up ammonium and nitrate from ambient waters[13], and this uptake follows substrate-saturable kinetics in either case[8,9,14]. The V_{max} for ammonium uptake calculated in this study is in the range known for zooxanthellae freshly isolated [6.6-43 nmol. (µg chl-*a*).$^{-1}$h^{-1}] or cultured [16-41 nmol.(µg chl-*a*).$^{-1}$h^{-1}]. So is the K_s in comparison with those for FIZ (5-22 µmol.l^{-1}) from other studies, though K_s for cultured zooxanthellae are in general much lower (0.4-2 µmol.l^{-1}). V_{max} for nitrate uptake is also in the range known for FIZ from several hosts [10-5.5 nmol. (µg chl-*a*).$^{-1}$h^{-1}] and is closer to those from coral hosts [0.6-1.48 nmol. (µg chl-*a*).$^{-1}$h^{-1}], though in cultured zooxanthellae it is much higher [14 nmol. (µg chl-*a*).$^{-1}$h^{-1}], probably because they were grown on nitrate. The K_s for nitrate uptake is, however, higher than those in other studies for freshly isolated (0.2-3.14 µmol.l^{-1}) or cultured

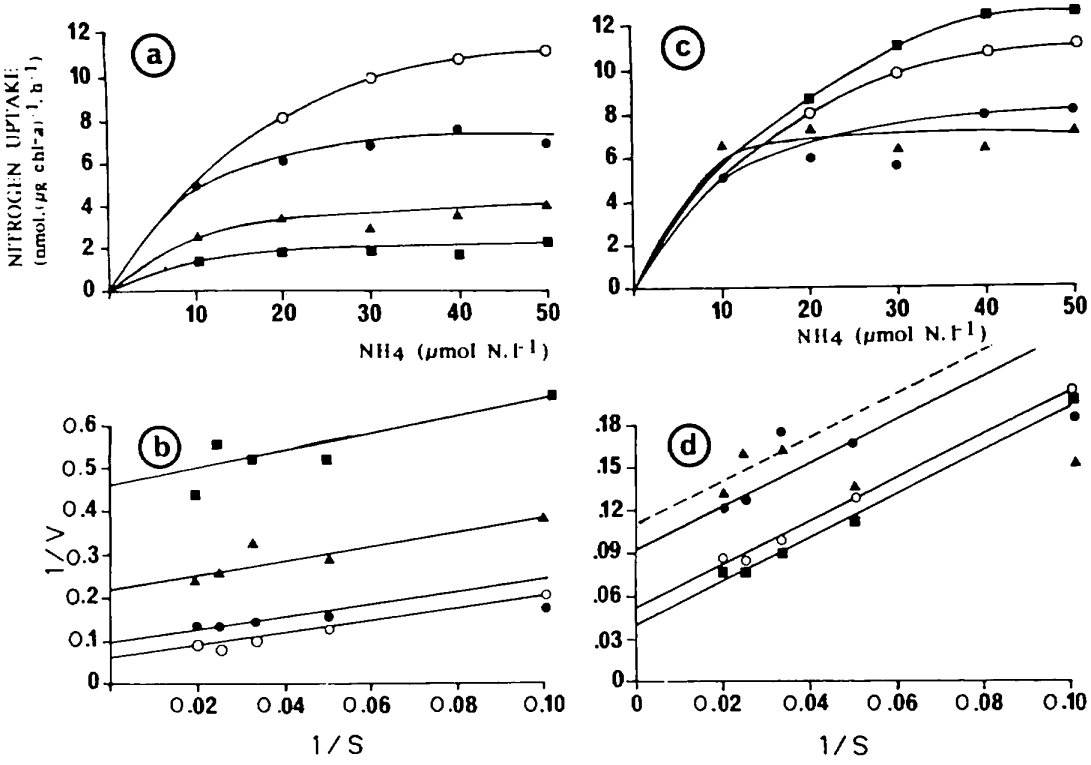

Fig. 3--Ammonium uptake by FIZ in the presence of nitrate (a) and urea (c), and the corresponding Lineweaver-Burk plots (b and d). Addition of nitrate was at 0, 10, 20 and 30 µmol l^{-1} levels and that of urea was at 0, 5, 10 and 15 µmol l^{-1} levels. Symbols are as for Fig. 2

(0.5-0.8 µmol l^{-1}) zooxanthellae. V_{max} and K_s for ammonium[14] and nitrate[15] in intact corals are much lower than with FIZ. While these differences may reflect variations in experimental conditions, particularly light, and may possibly also the genetic differences in the same species (several strains of zooxanthellae exist[16]), it becomes nevertheless evident that uptake of both ammonium and nitrate is a normal feature of N nutrition of zooxanthellae.

Urea is definitely a source of N accepted by the FIZ and intact corals[1], and its uptake also follows substrate-saturable kinetics, with the kinetic constants comparable to those of microalgae in culture[17,18]. This is the only study to the authors' knowledge to have tested urea as a N source for uptake by zooxanthellae, and comparable data are lacking.

The results suggest that zooxanthellae *in vivo* are N-limited, a conclusion that agrees with that of Cook & D'Elia[19] and D'Elia & Cook[20]. The evidence lies in the ability of zooxanthellae in intact symbiosis[1] as well as when freshly isolated to take up all the 3 forms of N from a wide range of ambient concentrations. This implies that the enzyme systems for uptake of nitrate and urea are probably not repressed as would be expected when there is a continuous production of catabolically-derived ammonium in the host intracellular environment, a situation that can only happen when N from ammonium is not adequate enough to satisfy the total N requirements of the algae. This can further be supported by comparing the rates of supply of the three N sources to unit biomass of zooxanthellae, and the maximum rates at which these are removed (Table 1). Average supply of ammonium to algal biomass of 1µg.chl-*a* was 24 nmol.h^{-1}, and that of urea was 0.8 nmol.h^{-1}. These exceed V_{max} by a factor of 2. In the present experiments the incubations were at a light intensity of 60 µE.$m^{-2}s^{-1}$, whereas photosynthesis by FIZ peaks at 120-130 µE.$m^{-2}s^{-1}$ (ref. 21). Given the greater transparency of reef waters, it is quite conceivable that the zooxanthellae in corals from shallow waters photosynthesize at saturating light intensities, in which event there would be a doubling

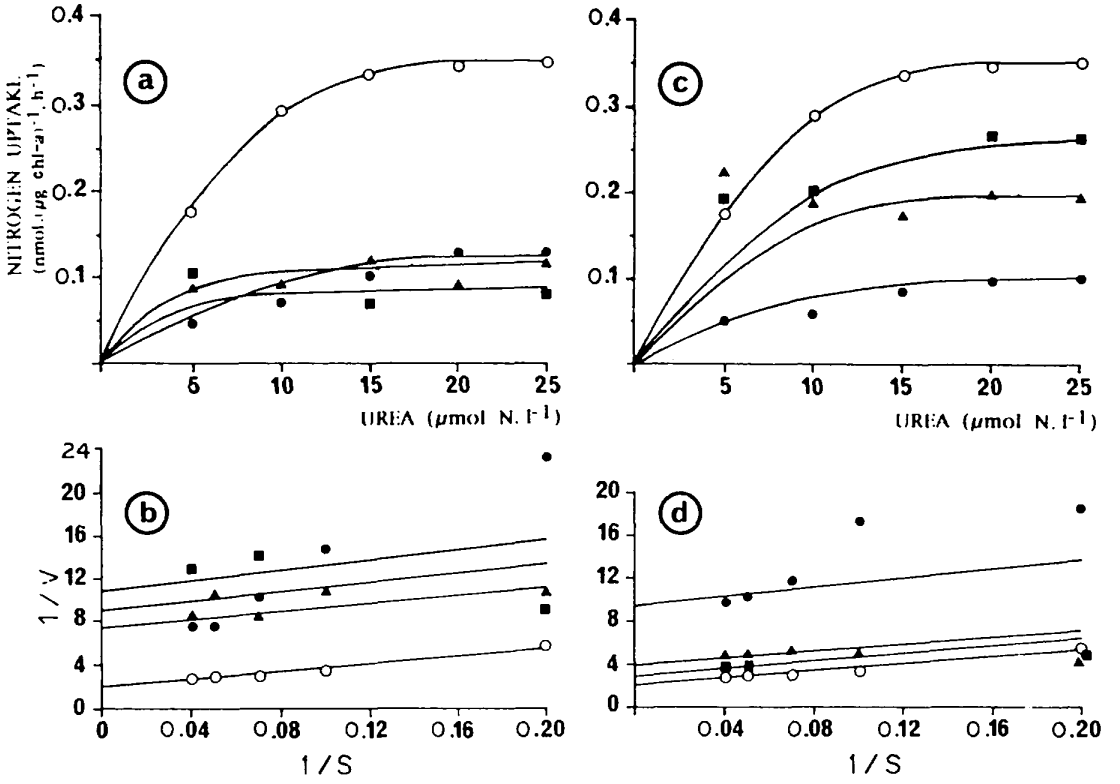

Fig. 4.--Urea uptake by FIZ in the presence of ammonium (a) and nitrate (c), and the corresponding Lineweaver-Burk plots (b and d). Addition of ammonium and nitrate was at 0, 10, 20 and 30 µmol.l^{-1} levels. Symbols are as for Fig. 2

of V_{max}, with N supply/uptake ratios consequently approaching unity. Under these conditions, there would be a N limitation in the host intracellular environment, a conclusion supported by the findings that intact corals still remove ammonium from ambient waters as much in light as in dark[14].

Presence of ammonium at higher concentrations significantly reduced the uptake of nitrate and urea. This was expected, and is consistent with the findings that phytoplankton from cultures as well as natural populations take up ammonium preferentially, and that the presence of ammonium may even suppress nitrate uptake[22,23] in these algae. But what was not expected was that the uptake of ammonium itself should be significantly affected by the presence of high concentrations of nitrate and urea, or that the nitrate uptake be affected by urea as much as by ammonium.

The possibility that the 3 N compounds should mutually suppress the uptake of one another in zooxanthellae does not seem to be an attractive hypothesis viewed from the perspective that the algae, in the first place, may not at all have adequate N supplied to them for a maximum uptake. Instead, it is quite likely that the uptake of the three N compounds is simultaneous, and that the proportion of the labelled N taken up from one N source becomes smaller consequent to a simultaneous uptake of unlabelled N from the other (a dilution of the fixed ^{15}N), thus presenting an impression of an apparent reduction in uptake. Some arguments can be advanced in support of this:

To begin with, there was no total suppression of uptake of any one N compound in the presence of the other two, and the fixed ^{15}N measured in the algae are still significant over a range of substrate concentrations but decrease in proportion with increase in the concentration of unlabelled N in the medium. This is unlike the case in some situations where a total suppression occurs; for *e.g.* addition of ammonium totally suppresses nitrate uptake in the free-living algae within the first few minutes[24,25].

Secondly, the ambient concentrations of nitrate, ammonium and urea in reef waters do not indicate a possibility of total inhibition of uptake of any one substrate. Typically, ammonium is the form in which N is predominantly regenerated, exceeding by several times the urea release by heterotrophs such as

zooplankton[26] and corals[1] or nitrification[27] in and around coral colonies. If ammonium by virtue of its greater production were to suppress the uptake of nitrate or urea, the latter two should occur in concentrations relatively greater than that of ammonium in reef waters. This is not the case and the mean ambient concentrations of urea and nitrate were only 1.4 and 0.5 µmol.l^{-1} respectively in spite of the greater coral biomass on the reef, nor were they much different from ammonium levels either in the reef (1.7 µmol.l^{-1}) or oceanic waters[27].

Thirdly, simultaneous uptake of N from two substrates is not new with free-living algae, and there is no reason why it should not be so with algae in symbiosis. This has been shown for ammonium and nitrate in algae growing on limited N supply[23,28], for ammonium and urea in natural populations but at different rates according to their relative concentrations[29], and for ammonium, urea, nitrate and nitrite when ambient concentrations were very low[30]. Simultaneous uptake of multiple N sources - ammonium, nitrate and urea—has also been shown recently in cultures of *Skeletonema costatum*[31]. In FIZ itself, nitrate uptake continued even when ammonium was present up to 20 µmol.l^{-1} (ref. 8).

Finally, in all cases the effect of one N compound on the uptake of the other follows the uncompetitive inhibition kinetics where both V_{max} and K_s decrease. This can also be interpreted to mean a simultaneous uptake, since uncompetitive inhibition is rare in one-substrate reactions but common in two-substrate reactions, and the parallel double-reciprocal plots are characteristics of double displacement reactions where the free enzyme alternatively combines with two substrates[32].

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