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Macroalgae $\delta^{15}N$ values in well-mixed estuaries: Indicator of anthropogenic nitrogen input or macroalgae metabolism ?

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Abstract:

Although nitrogen stable isotope ratio ($\delta^{15}N$) in macroalgae is widely used as a bioindicator of anthropogenic nitrogen inputs to the coastal zone, recent studies suggest the possible role of macroalgae metabolism in $\delta^{15}N$ variability. Simultaneous determinations of $\delta^{15}N$ of dissolved inorganic nitrogen (DIN) along the land–sea continuum, inter-species variability of $\delta^{15}N$ and its sensitivity to environmental factors are necessary to confirm the efficiency of macroalgae $\delta^{15}N$ in monitoring nitrogen origin in mixed-use watersheds. In this study, $\delta^{15}N$ of annual and perennial macroalgae (*Ulva* sp., *Enteromorpha* sp., *Fucus vesiculosus* and *Fucus serratus*) are compared to $\delta^{15}N$-DIN along the Charente Estuary, after characterizing $\delta^{15}N$ of the three main DIN sources (*i.e.* cultivated area, pasture, sewage treatment plant outlet). During late winter and spring, when human activities produce high DIN inputs, DIN sources exhibit distinct $\delta^{15}N$ signals in nitrate ($-_3NONO3-$) and ammonium ($^+_4NHNH4+$): cultivated area (+6.5 ± 0.6‰ and +9.0 ± 11.0‰), pasture (+9.2 ± 1.8‰ and +12.4‰) and sewage treatment plant discharge (+16.9 ± 8.7‰ and +25.4 ± 5.9‰). While sources show distinct $-\delta15_3N-NO\delta15N-NO3-$ in this multiple source catchment, the overall mixture of $-_3NONO3-$ sources – generally >95% DIN – leads to low variations of $\delta15N-{-_3}NO\delta15N-NO3-$ at the mouth of the estuary (+7.7 to +8.4‰). Even if estuarine $\delta15N-{-_3}NO\delta15N-NO3-$ values are not significantly different from pristine continental and oceanic site (+7.3‰ and +7.4‰), macroalgae $\delta^{15}N$ values are generally higher at the mouth of the estuary. This highlights high anthropogenic DIN inputs in the estuary, and enhanced contribution of ^{15}N-depleted $^+_4NHNH4+$ in oceanic waters. Although seasonal variations in $\delta15N-{-_3}NO\delta15N-NO3-$ are low, the same temporal trends in macroalgae $\delta^{15}N$ values at estuarine and oceanic sites, and inter-species differences in $\delta^{15}N$ values, suggest that macroalgae $\delta^{15}N$ values might be modified by the metabolic response of macroalgae to environmental parameters (*e.g.*, temperature, light, DIN concentrations). Differences between annual and perennial macroalgae indicate both a higher integration time of perennial compared to annual macroalgae and the possible role of passive *versus* active uptake mechanisms. Further studies are required to characterize the sensitivity of macroalgae fractionation to variable environmental conditions and uptake mechanisms.

Keywords: nitrogen isotopes ; Nitrate ; Ammonium ; primary producers ; indicators ; land–sea continuum

1. INTRODUCTION

The intensification of urbanization, together with agricultural development, have worldwide increased dissolved inorganic nitrogen (DIN) entering estuarine and coastal waters (Nixon, 1995; Middelburg and Nieuwenhuize, 2001). These high DIN supply enhance the total production of ecosystems (Fujita, 1985; Cloern, 2001; Savage et al., 2002), which often lead to environmental disturbances such as ephemeral algal blooms and anoxic events (Valiela et al., 1992; Conley et al., 2009; Howarth et al., 2011). Estuaries are one of the ecosystems most heavily affected by human activities taking place either into estuaries themselves (*e.g.*, fisheries, recreation, introduction of exotic species) or on watersheds (*e.g.*, agriculture, urbanization). They are thus very sensitive to eutrophication (Vitousek et al., 1997; Cloern, 2001; Halpern et al., 2008).

While DIN concentration is commonly used to monitor the spatio-temporal extent of anthropogenic perturbations (*e.g.* eutrophication) in coastal areas, this chemical indicator does not permit identifying DIN origins (*e.g.*, agriculture, sewage, industry). This information may however be important for restricting or eliminating future nitrogen loading. In the last years, nitrogen stable isotope ratios ($\delta^{15}N$) have been used to identify the origin of anthropogenic nitrogen loads to aquatic ecosystems (Jones et al., 2001; Curt et al., 2004; Costanzo et al., 2005). The success of this tool is based on different isotopic signature of DIN sources. The $\delta^{15}N$-DIN of fertilizers - which derive from industrial fixation of atmospheric N_2 - is usually lower (-4 to +4 ‰) than $\delta^{15}N$-DIN resulting from soil N mineralization (+4 to +10 ‰; Heaton, 1986; Lindau et al., 1989). Sewage effluents are often more ^{15}N-enriched due to the enzymatic preference of bacteria towards ^{14}N over ^{15}N during ammonia volatilization and denitrification processes (Heaton, 1986; McClelland and Valiela, 1998; Horrigan et al., 1990).

Most studies devoted to trace anthropogenic inputs generally deal with the characterization of only one dominant load from one watershed or sub-watershed – which often

concern urban inputs (Costanzo et al., 2005; McClelland et al., 1997; Valiela et al., 1992) or agricultural inputs (Anderson and Cabana, 2005; Howarth, 2008) – or two different N sources (Costanzo et al., 2003; Strauch et al., 2008). Most aquatic systems however receive N from multiple sources: intensive culture, pasture, urban waste, atmospheric and/or natural inputs. Interpretation of the N pools is consequently often difficult and requires first a characterization of each source, including natural ones.

The $\delta^{15}N$ values of ammonium (NH_4^+) and nitrate (NO_3^-) can moreover differ from $\delta^{15}N$ of DIN sources in estuarine and coastal ecosystems. Estuaries are indeed well known to modify and attenuate DIN transfer from rivers to the coastal sea in response to estuarine processes, such as nitrification, denitrification, volatilization or algal uptake, and mixing of riverine and oceanic waters. It is thus essential to take into account these estuarine processes, which can significantly modify $\delta^{15}N$ values of NH_4^+ and NO_3^- during the transport of DIN along the land-sea continuum (Cifuentes et al., 1989; Middelburg and Nieuwenhuize, 2001; Sebilo et al., 2006).

The concentration and the $\delta^{15}N$ value of DIN can also vary at small temporal scales (hours, days), which is only resolved by high frequency and heavy surveys. Measuring $\delta^{15}N$ in benthic sessile species is advantageous as these species integrate spatial and temporal variability of $\delta^{15}N$-DIN (Gartner et al., 2002; Savage and Elmgren, 2004). The $\delta^{15}N$ values in producers are thus broadly used to monitor anthropogenic inputs of DIN to aquatic ecosystems, such as the $\delta^{15}N$ values in consumers that are commonly used to monitor particulate organic nitrogen (PON) inputs (McClelland et al., 1997; Costanzo et al., 2001; Cole et al., 2004; Cohen and Fong, 2005; Costanzo et al., 2005; Fertig et al., 2010; García-Sanz et al., 2011).

While producers and consumers are efficient integrators, $\delta^{15}N$ values in consumers have been shown to vary with species diet and even more with tissue types (Lorrain et al., 2002). This suggests that the choice of tissues and species is not trivial and must be performed with caution, as recently highlighted for filter feeders along an inshore-offshore gradient on the

continental shelf of the Bay of Biscay (Nerot et al., 2011). Compared to studies on consumers, few studies have investigated variations between tissues (Savage and Elmgren, 2004) and species of primary producers, and especially macroalgae (*e.g.* Cole et al., 2005; Grall et al., 2006). Savage and Elmgren (2004) showed that decreasing $\delta^{15}N$ values along the frond of a perennial macroalgae *Fucus vesiculosus* was related to changes in sewage DIN loads, while similarity and differences between species is generally related to changes in $\delta^{15}N$-DIN and available DIN forms.

Such as for consumers, environmental and/or metabolic factors can also induce variations of $\delta^{15}N$ values in macroalgae. Several studies have shown the role of environmental factors such as light, temperature or nutrient concentrations in modifying $\delta^{15}N$ values in macroalgae (Pedersen et al., 2004; Dudley et al., 2010). Even if the role of environmental factors (*e.g.* light, growth rate, nutrient availability) on fractionation has been shown for microalgae (Pennock et al., 1996; Needoba and Harrison, 2004), little is however known about fractionation in macroalgae (Dudley et al., 2010). The main hypotheses to explain fractionation in macroalgae are the control of environmental conditions on uptake and growth rates of macroalgae (Taylor et al., 1998; Cohen and Fong, 2004; Pederson et al., 2004; Dudley et al., 2010) and/or the combination of passive and/or active transport mechanisms of DIN that varies among macroalgae species (Taylor et al., 1998). The comparison between $\delta^{15}N$ of DIN and macroalgae might permit to verify if $\delta^{15}N$ values in macroalgae indicate spatial and seasonal variations of $\delta^{15}N$-DIN only, or also changes in environmental conditions and/or fractionation. Simultaneous investigations of $\delta^{15}N$ of sources (DIN or PON) and biological integrators are however not frequent (Deutsch and Voss, 2006).

The Charente River and Estuary - that flows into Marennes-Oléron Bay - is a typical example of temperate macrotidal ecosystem impacted by multiple anthropogenic activities. While the watershed is one of the most nitrate (NO_3^-)-polluted watersheds of the South West of France due to intensive agriculture (http://www.eau-adour-garonne.fr), the coastal bay is the

main European shellfish production area. Water management is therefore essential in this area (Bry and Hoflack, 2004; http://www.eau-adour-garonne.fr), as human activity changes (*e.g.* DIN loads) control the functioning of downstream ecosystems, and as the good ecological status of surface waters required by the European community may not be reached in 2015 (http://www.observatoire-environnement.org).

The aim of this study is to investigate if $\delta^{15}N$ values of macroalgae growing at the mouth of a well-mixed estuary receiving high DIN loads from multiple sources are efficient indicators of anthropogenic DIN loads, or if changes in environmental conditions and/or macroalgae metabolism can alter the signal. We especially answer and discuss the following questions: (1) Is $\delta^{15}N$-DIN efficient to distinguish the main DIN sources? (2) Is $\delta^{15}N$-DIN modified by estuarine processes during the transport of DIN to the coastal zone? (3) Do macroalgae $\delta^{15}N$ values record $\delta^{15}N$ values of anthropogenic DIN inputs at the month of the estuary? Do species characterized by different uptake rates, *i.e.* annual and perennial species, show similar or different $\delta^{15}N$? Do differences between $\delta^{15}N$ values in DIN and macroalgae highlight the possible alteration of $\delta^{15}N$ values by macroalgae metabolism?

2. MATERIALS AND METHODS

2.1. Study area

The drainage area of the Charente River extends over 10,000 km^2 (Fig. 1) and represents the main freshwater input to the Marennes-Oléron Bay (Ravail et al., 1988). Regardless of river flow, the plume of the Charente River is generally limited to the coastal zone (Stanisière et al., 2006). This leads to a mean flushing time of waters at the mouth of the estuary higher than the 11 days calculated in the Bay (Stanisière et al., 2006). Riverine nutrients sustain high primary production in the bay (185 gC m^{-2} yr^{-1}; Struski and Bacher, 2006), supporting half of the French shellfish production. NO_3^- concentrations are particularly high, often above 400 µmol l^{-1} at the mouth of the estuary by the end of winter, mainly due to the application of fertilizers within the watershed*. Agriculture activity covers around 79 % of the surface of the watershed, while forests cover only 13 % and are mainly located in its eastern part (Corinne Land Cover 2006, http://sd1878-2.sivit.org/). A few small cities, totaling 260,000 inhabitants, equipped with sewage treatment plants, are mainly located along the 360 km of river. The climate is oceanic temperate with episodic and intense rainfall in spring that lead to a rapid increase in stream flow (up to 800 m^3 s^{-1}) and dryness during summer (1 m^3 s^{-1}) at Saint-Savinien (Bry and Hoflack, 2004).

2.2. Sample collection

Water samples were collected monthly from January to May 2006, a period typically associated with the highest flow conditions of the year (Fig. 2). Three sites were sampled to

* In 2006, the use of fertilizers on spring cultures was allowed from mid-February to the end of June (http://www.observatoire-environnement.org) and high spreading activity was observed during our sampling period, even in February.

characterize DIN anthropogenic sources: i) “Culture”, a cultivated area receiving mainly chemical fertilizers at Saint-Coutant, ii) “Pasture”, an extensive pasture fertilized by animal manure at Candé, and iii) “STP”, the outlet of the secondary sewage treatment plant of Saint-Savinien city, characterized by activated muds and extended aeration (http://assainissement.developpement-durable.gouv.fr) that enhance nitrification processes (Fig. 1). Water samples were collected in a small stream (at Culture and Pasture stations) or directly in the effluent of the STP to the river. Samples were not available at Culture in May due to the stream dryness induced by culture irrigation. Three other sites were sampled along the Charente Estuary: “St. 1” at Saint-Savinien, “St. 2” at Rochefort, and “St. 4” at the mouth of the estuary. Riverine and oceanic reference sites, more preserved from human activities, were also sampled: i) “ref_R”, a riverine reference site located upstream of the Echelle River, a tributary of the Charente River characterized by a forested sub-watershed, and ii) “ref_O”, an oceanic reference site located on the most oceanic part of Ré island. The latter is considered not to be influenced by the Charente inputs as currents tend to deviate riverine waters to the south of the bay (Dechambenoy et al., 1977; Stanisière et al., 2006). DIN inputs from the Gironde River - which plume extends sometimes to the Marennes-Oléron bay during flood events (Ravail et al., 1988) – and/or from the Sèvre Niortaise River might have been low at the oceanic reference site because of oceanic current influence. No samples were collected at ref_O in January. At all stations, duplicate water samples were collected in acid-washed polypropylene dark 5 l bottles, brought back to the laboratory in a cooler and filtered on GF-C filters (Whatman®). Aliquots were directly analyzed for DIN and the remainder of the samples was frozen at -20°C before DIN extraction for isotope analyses. No replicates were performed for DIN sources, but duplicates were sampled at St. 1, 2 and 4.

Two annual species (*Ulva sp.*, *Enteromorpha sp.**) and two perennial species (*Fucus*

* The common denomination *Enteromorpha sp.* was used to distinguish this specimen from *Ulva sp.* although *Enteromorpha sp.* was recently showed to belong to the genera Ulva (Hayden et al., 2003).

vesiculosus, *Fucus serratus*) were sampled. For each species, triplicate fronds of three individuals were collected monthly on intertidal rocky banks at two sites at the mouth of the estuary (St. 3 and 4a), and at ref_O (Fig. 1). Macroalgae were first washed in filtered seawater, then dipped in HCl 0.1 M for a few minutes and thoroughly rinsed with deionized water to clean macroalgae fronds of calcareous organisms. The whole individual (for annual algae) or the 2 cm of the apex of the longest vegetative fronds, corresponding to the new grown tips (for perennial algae) were stored at -20°C for $\delta^{15}N$ analyses. Intra-individual variations of $\delta^{15}N$ were investigated along two *Fucus serratus* fronds (1 cm resolution) collected at ref_O and St. 4a on March 1st, 2006. No replicates were taken and the inter-individual variability of $\delta^{15}N$ along the fronds was estimated from the variability measured monthly in the 2 cm of the apex.

2.3. *In situ* measurements of *Fucus* growth rates

In order to evaluate the time period integrated in each cm of perennial species fronds, field growth measurements were performed at St. 4a. Nine individuals of both *Fucus vesiculosus* and *Fucus serratus* were tagged with color plastic collars and with a small notch made at about 2 cm from the frond apex. Measurements of the maximum frond length were performed from January to May 2006, along with the measurement of the distance from the notch to the frond tip, in order to check which part of the plant was actively growing.

2.4. Laboratory measurements of NO_3^- uptake rates

As NO_3^- is the dominant DIN form available for macroalgae at the mouth of the estuary (generally > 95 %), NO_3^- uptake experiments were carried out in the concentration range measured at the mouth of the estuary, 50 and 500 µmol l^{-1}. In order to compare the uptake rates of annual and perennial species, these experiments were performed on both annual and

perennial macroalgae species (*Ulva sp.*, *Enteromorpha sp.*, *Fucus serratus*[*]) collected at station St. 4a on March 1st, 2006. Macroalgae were thoroughly cleaned and stored in filtered offshore, nutrient-poor seawater during 8 days in order to measure the initial NO_3^- uptake rate (Lartigue and Sherman, 2005). Macroalgae were incubated in 250 ml of seawater enriched with NO_3^-, at 12°C and a PAR light level of 200 µmol photons m^{-2} s^{-1}. For each species, incubations were performed at 50 and 500 µmol NO_3^- l^{-1}, respectively. 10 ml of water was taken in each experimental tank at the start of the experiment and after 1h, 24h and 48h to analyze NO_3^- concentrations. NO_3^- uptake rates were calculated as the decrease of NO_3^- with time reported to initial macroalgae biomass (µmol N g DW^{-1} h^{-1}).

2.5. Nutrient and macroalgae sample analyses

Nitrate (NO_3^-, including nitrite) and ammonium (NH_4^+) concentrations were analyzed using a Skalar continuous flow analyzer SA 40, following the methods developed by Strickland and Parsons (1972), and Koroleff (1969), respectively. DIN is the sum of NO_3^- and NH_4^+ concentrations.

Analyses of $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ were carried out by adapting a two-step ammonia microdiffusion method developed by Sigman et al. (1997) and Holmes et al. (1998). The microdiffusion method permits the successive and total recovery of NO_3^- and NH_4^+. The method consists in adding diffusion packets (containing acidified glassfiber disks), MgO (first step, transformation of NH_4^+ to ammonia) and Devarda alloy (second step, transformation of NO_3^- to NH_4^+, transformed then to ammonia by MgO) in sealed bottles. As DIN concentrations and salinity varied among a large range in our study, water samples were first adjusted with (1) deionized water to a final volume of 50 ml in a 100 ml screwcap bottle to get around 30 µmol

* As growth rates of *Fucus vesiculosus* and *Fucus serratus* were similar, uptake rates were only measured on *Fucus serratus*.

N l^{-1} necessary for $\delta^{15}N$ analyses, and (2) NaCl to obtain a final salinity of 50. Note that only few samples were analyzed for $\delta^{15}N$-NH_4^+ because of low NH_4^+ concentrations†. Two-week incubations and addition of increasing MgO and Devarda's alloy quantities permitted to determine that 4 days and 0.2 mg of MgO and of Devarda's alloy were necessary for the total recovery NO_3^- and NH_4^+ in a shaking water bath at 33°C. Our method allowed the total recovery of NH_4^+ (97 ± 3 %; first step) and NO_3^- (98 ± 3 %; second step). All precautions were taken to avoid contaminations: glassware and plasticware were acid-washed, diffusion packets were prepared a few hours before the start of the experiments and stored in a desiccator, diffusion packets were removed from sample bottles after each step and stored in a desiccator over P_2O_5 and concentrated H_2SO_4 beakers to trap any trace of water and ammonia. Glass fiber disks used to trap NO_3^- and NH_4^+ were taken out from diffusion packets, placed in tin capsules and immediately analyzed to determine $\delta^{15}N$ and avoid any reaction between tin and acidified disks.

Frozen macroalgae tissues were freeze-dried, ground using a ball mill and stored in a desiccator chamber before stable isotope analyses. Around 0.750 ± 0.001 mg DW of macroalgae powder were weighed in tin capsules for $\delta^{15}N$ measurements.

For both glass fiber disk and macroalgae powder, N isotope analyses were carried out using an Isoprime IRMS (Micromass, UK) after sample combustion in an elemental analyzer Eurovector EA3024 (Eurovector, Milan, Italy). Results at natural abundance level were expressed in delta notation, using atmospheric nitrogen as a standard, according to the following equation:

$$\delta^{15}N\ ‰ = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where $R = {}^{15}N/{}^{14}N$

The standard deviation of triplicate measurements of $\delta^{15}N$ was lower than 0.2 ‰ for

† A new method recently developed by Zhang et al. (2007), but absent when analyses were performed, allow to measure reliable $\delta^{15}N$ values over an NH_4^+ concentration range of 0.5-10 µM.

macroalgae tissues and lower than 0.3 ‰ for extracted ammonia. The low standard deviation allowed studying seasonal variations of $\delta^{15}N\text{-}NO_3^-$ without replicate analyses.

2.6. Statistics

All statistical tests were performed using R software (cran.r-project.org). Before each statistical analysis, Shapiro and Bartlett tests were performed to test dataset normality and homoscedasticity, respectively. One-way and two-way ANOVA tests were applied on datasets following normal distributions and showing homogeneous variances. When necessary, inverse or squared transformation of datasets was first performed to obtain normal distributions and homogeneous variances, or non-parametric Kruskal-Wallis test was applied. ANOVA tests were used to determine the difference in NO_3^- concentrations depending on DIN source type* (one-way ANOVA) and on DIN source type and station (two-way ANOVA). A one-way ANOVA was performed to investigate the station effect on $\delta^{15}N\text{-}NO_3^-$ of sources and on $\delta^{15}N\text{-}NO_3^-$ of estuary and reference stations, respectively. A two-way ANOVA permitted to determine the effect of DIN source type and station on the $\delta^{15}N\text{-}NO_3^-$ dataset. Statistical tests were not possible on $\delta^{15}N\text{-}NH_4^+$ dataset because of the low number of values. The effect of site, time and species on macroalgae $\delta^{15}N$ was investigated by a three-way ANOVA, while a two-way ANOVA was used to study the effect of site and time on $\delta^{15}N$-macroalgae for each species. Variations of $\delta^{15}N$ along macroalgae fronds were investigated with a Kruskal-Wallis test. Significant differences of growth rates were tested for the factors species and time (two-way ANOVA), and uptake rates for the factor NO_3^- concentration for each species (Kruskal-Wallis). Parametric Pearson or non-parametric Spearman tests were performed to identify significant correlations between (1) NO_3^- concentrations, $\delta^{15}N\text{-}NO_3^-$, $\delta^{15}N$-macroalgae datasets and Charente River flow, (2) temporal $\delta^{15}N$-macroalgae at ref_O, St. 3 and 4a for each species,

* The factor type is either source, estuary or reference.

and (3) δ^{15}N-macroalgae along the frond of *Fucus serratus* at ref_O and St. 4a. The two latter statistic results were not presented in figures or tables but only in the text. For all statistical tests, a probability (p) of 0.05 was used to determine statistical significance.

3. RESULTS

3.1. Environmental data

During our study, high river flow occurred from mid-February to mid-April 2006, with a maximum of ca. 400 $m^3 s^{-1}$ by the end of March (Fig. 2A). The river flow in 2006 was higher than 2004-2011 averaged flow, and even more than the river flow of the two previous years of dryness (< 100 $m^3 s^{-1}$; http://www.fleuve-charente.net). At the mouth of the estuary (St. 4), mean temperatures ranged from 5 °C in February to 15 °C in April and May while mean salinity strongly decreased from 28 in February to 13 in April and reached a maximum of 31 in May (Fig. 2B). The highest mean turbidity (~350 NTU) was observed in March and the lowest (0-10 NTU) in February and May. At the oceanic reference site (ref_O), waters are generally characterized by similar and slightly less variable temperature (7-14 °C) and high salinity (32-35; PREVIMER, http://www.previmer.org), but lower turbidity (<10 NTU; Gohin, 2010) than at the mouth of the Charente Estuary, from January to June.

NH_4^+ concentrations were generally low and < 5% of DIN concentrations. NH_4^+ concentrations were < 6 µmol l^{-1} at Pasture, St. 1, St. 2, St. 4 and ref_O, except nine values comprised between 11 and 31 µmol l^{-1} (Fig. 3A, B, C). Conversely, NH_4^+ concentrations reached 47 µmol l^{-1} in February and 92 µmol l^{-1} in March (25-50 % of DIN concentrations) at ref_R, and increased from 30 µmol l^{-1} in January to more than 300 µmol l^{-1} in May at STP.

NO_3^- concentrations were much higher than NH_4^+ concentrations. NO_3^- concentrations were significantly different between sources (one-way ANOVA, F=53.4, p<0.0001) and between all stations, *i.e.*, sources, estuary and reference stations (one-way ANOVA, F=35.2, p<0.0001). NO_3^- concentrations were significantly higher at Culture (1200-1300 µmol l^{-1}, Fig. 3D), than at Pasture (240-500 µmol l^{-1}, Tukey, p<0.0001) and STP (80-650 µmol l^{-1}, Tukey, p<0.0001). In the Charente Estuary (Fig. 3E), NO_3^- concentrations at St. 4 (50-120 µmol l^{-1},

with a maximum of 310 µmol l^{-1} in March) were significantly lower than at St. 1 and St. 2 (~ 400 µmol l^{-1}, Tukey, $p<0.001$) but not significantly different from those measured at riverine and oceanic reference sites (Tukey, $p=0.999$ and $p=0.935$). NO_3^- concentrations were close to 75-140 µmol l^{-1} at ref_R and 3-20 µmol l^{-1} (with a maximum of 110 µmol l^{-1} in February) at ref_O (Fig. 3F). Temporal variations of NO_3^- concentrations of each source were generally low.

3.2. δ^{15}N-DIN of anthropogenic sources

The few $\delta^{15}N\text{-}NH_4^+$ values available were generally higher than $\delta^{15}N\text{-}NO_3^-$ for the three DIN sources (Fig. 3G). $\delta^{15}N\text{-}NH_4^+$ was +1.1 ‰ in January and +16.8 ‰ in May at Culture, +12.4 ‰ in January at Pasture, and varied from +18.8 to +31.2 ‰ from January to March at STP.

$\delta^{15}N\text{-}NO_3^-$ values were significantly different depending on sources (one-way ANOVA, $F=4.72$, $p<0.05$). $\delta^{15}N\text{-}NO_3^-$ at Culture (5.7-7 ‰) were significantly lower than at STP (10.1-31.8 ‰, Tukey, $p<0.05$) but not significantly different from Pasture (7.3-11.2 ‰, Tukey, $p=0.755$). Temporal variation of $\delta^{15}N\text{-}NO_3^-$ of each source was generally low, excepted at STP where $\delta^{15}N\text{-}NO_3^-$ increased from 10.1 ‰ in February to 31.8 ‰ in May.

3.3. δ^{15}N-DIN along the estuary

The few $\delta^{15}N\text{-}NH_4^+$ values available varied from 1.7-1.8 ‰ at St. 1 to 6.6 ‰ at St. 2 in March, and 2.5 ‰ at St. 4 in January (Fig. 3H). Even if only few data were available and prevented statistical analyses, these estuarine $\delta^{15}N\text{-}NH_4^+$ values were similar or higher than those measured at reference sites (1.8 ‰ at ref_O and 0.3-1.2 ‰ at ref_R; Fig. 3I).

$\delta^{15}N\text{-}NO_3^-$ were not significantly different along the Charente Estuary and at reference stations (one-way ANOVA, $F=0.88$, $p=0.486$). The values measured along the estuary (6.4-7.8 ‰ at St. 1, 7.0-7.9 ‰ at St. 2, 7.2-9 ‰ at St. 4) were in the range of values measured at

riverine and oceanic reference sites (6.9-7.6 ‰ at ref_R and 6.8-7.9 ‰ at ref_O). $\delta^{15}N\text{-}NO_3^-$ of each estuarine station were not significantly different from $\delta^{15}N\text{-}NO_3^-$ measured at Culture and Pasture, but significantly lower than at STP (Tukey, $p<0.05$).

3.4. $\delta^{15}N$ of annual and perennial macroalgae

Significant differences of $\delta^{15}N$ were observed between species, time, site and combinations of these factors (Table 1, three-way ANOVA). The factors time and site explained > 50 % of the variability. Tukey post-hoc tests showed that each macroalgae species had significantly different $\delta^{15}N$ ($p<0.01$), excepted E and FV ($p=0.985$). For each species, $\delta^{15}N$ were significantly different depending on factors time and site, excepted FS (Table 2, two-way ANOVA). All $\delta^{15}N$ values were represented in Fig. 4.

Whereas $\delta^{15}N$ values were not significantly different between St. 3 and St. 4a for *Ulva sp.* (10.1 +/- 1.2 ‰ *versus* 9.8 +/- 1.8 ‰; Tukey, $p=0.215$), *Enteromorpha sp.* (8.5 +/- 1.9 ‰ *versus* 7.1 +/- 2.2 ‰; Tukey, $p=0.283$) and *Fucus vesiculosus* (9.5 +/- 1.0 ‰ *versus* 9.8 +/- 1.3 ‰; Tukey, $p=0.994$), $\delta^{15}N$ of *Fucus serratus* were higher at St. 3 than at St. 4a (10.5 +/- 0.3 ‰ *versus* 8.0 +/- 2.1 ‰; Tukey, $p<0.001$). $\delta^{15}N$ values were significantly lower at ref_O than at St. 4a for *Ulva sp.* (6.2 +/- 1.8 ‰; Tukey, $p<0.0001$) and *Fucus serratus* (2.1 +/- 3.3 ‰; Tukey, $p<0.0001$), higher for *Enteromorpha sp.* (7.7 +/- 0.5 ‰; Tukey, $p<0.05$) and similar for *Fucus vesiculosus* (6.4 +/- 2.3 ‰; Tukey, $p=0.128$).

As similar trends were observed at St. 4a and St. 3 (see above), trends at ref_O were only compared to those of St. 4a. Similar trends were observed at ref_O compared to St. 4a, especially for *Ulva sp.* (Pearson, cor=0.70, $p<0.01$) and *Fucus serratus* (Pearson, cor=0.58, $p<0.05$).

$\delta^{15}N$ significantly decreased from January to April for *Ulva sp.,* were similar from January to April and higher in May for *Enteromorpha sp.*, were similar over the overall period for *Fucus vesiculosus*, and significantly increased from February to May for *Fucus serratus*.

As no differences were observed between the two *Fucus* species and between St. 3 and St. 4a, $\delta^{15}N$ along the frond were only analyzed for *Fucus serratus* at St. 4a and compared to ref_O. As observed during the overall study (Fig. 4), $\delta^{15}N$ values of *Fucus serratus* apex were significantly higher at St. 4a than at ref_O (+7.1 ‰ *versus* -0.2 ‰; Tukey, $p<0.01$) in March (Fig. 5). The difference observed at the apex disappeared quickly after 4 cm. $\delta^{15}N$ of *Fucus serratus* fronds were correlated (Spearman, cor=0.76, $p<0.01$) and not significantly different at St. 4a and ref_O (Kruskal-Wallis, $K^2=0.936$, $p=0.11$).

3.5. Growth and uptake rates of annual *versus* perennial macroalgae

Growth rates were not significantly different between the two perennial species *Fucus vesiculosus* and *Fucus serratus* (Fig. 6; two-way ANOVA, F=0.103, $p=0.750$), but significantly different with time (two-way ANOVA, F=0.33, $p<0.001$). Tukey post-hoc tests showed lower growth rates in February than March and May ($p<0.01$). Growth rates of *Fucus serratus* and *Fucus vesiculosus* increased from 1.8 +/- 1.3 and 1.2 +/- 0.9 cm $month^{-1}$ in February to 3.9 +/- 2.0 and 4.6 +/- 1.6 cm $month^{-1}$ in May.

As growth rates were identical for both *Fucus* species (Fig. 6), uptake rates were only measured for *Fucus serratus*. Mean hourly uptake rates (at NO_3^- concentrations of 500 µmol l^{-1} and 50 µmol l^{-1}, respectively) were 6.5 +/- 0.2 and 3.9 +/- 1.0 µmol N g DW^{-1} h^{-1} for *Ulva sp.*, 4.1 +/- 1.1 and 2.3 +/- 0.4 µmol N g DW^{-1} h^{-1} for *Enteromorpha sp.* and 1.6 +/- 0.4 and 0.5 +/- 0.3 µmol N g DW^{-1} h^{-1} for *Fucus* sp. (Fig. 7). Uptake rates were significantly higher for annual species (*Ulva sp.* and *Enteromorpha sp.*) than for perennial species *Fucus serratus* (Kruskal-Wallis, $K^2=9.72$, $p<0.01$). Uptake rates were significantly higher at NO_3^- concentrations of 500 µmol l^{-1} than 50 µmol l^{-1} for *Enteromorpha sp.* (Kruskal-Wallis, $K^2=3.86$, $p<0.05$), but no statistical tests were performed for *Ulva sp*. and *Fucus serratus* because only duplicates were available at 500 µmol l^{-1} and 50 µmol l^{-1}, respectively.

4. DISCUSSION

4.1. Characterization of DIN sources in a multiple source watershed

Studying the natural isotopic signature of DIN ($\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$, respectively) in streams flowing into the Charente Estuary proves successful in discriminating the three main DIN sources in a multiple source watershed (Table 3): Culture (+6.5 ± 0.6 ‰ and +9 ± 11 ‰), Pasture (+9.2 ± 1.8 ‰ and +12.4 ‰) and STP (+16.9 ± 8.7 ‰ and +25.4 ± 5.9 ‰).

The lower $\delta^{15}N\text{-}NO_3^-$ associated to high NO_3^- concentrations at the cultivated site indicates the anthropogenic delivery of fertilizers. Synthetic fertilizers have usually low $\delta^{15}N\text{-}NO_3^-$ (-7.5 to +6.6 ‰; Vitoria et al., 2004), as they are made by industrial fixation of atmospheric N_2 (Heaton, 1986; Lindau et al., 1989). In this study, $\delta^{15}N\text{-}NO_3^-$ measured at the cultivated site is higher than the median $\delta^{15}N\text{-}NO_3^-$ of synthetic fertilizers (+1.8 ‰; Vitoria et al., 2004), and only slightly lower than at riverine and oceanic reference sites. This confirms that fields receiving synthetic fertilizers do not always show low $\delta^{15}N\text{-}NO_3^-$ (Vitoria et al., 2004) and that measurement must be performed at the local scale. The high $\delta^{15}N\text{-}NO_3^-$ at the cultivated site compared to the lower median value of fertilizers (+1.8 ‰) might be explained by (1) synthetic fertilizers characterized by the upper range of $\delta^{15}N\text{-}NO_3^-$ values, possibly reaching +6.6 ‰ (Vitoria et al., 2004), (2) inputs of generally $\delta^{15}N$-enriched NO_3^- originated from manure (> +5.9 ‰; Curt et al., 2004), and/or (3) inputs of N stored in lands during the two previous years of dryness and characterized by higher $\delta^{15}N\text{-}NO_3^-$ due to denitrification - which process increases $\delta^{15}N\text{-}NO_3^-$ values (+15 to +30 ‰; Kendall, 1998).

While the cultivated site shows distinct $\delta^{15}N$-DIN values compared to riverine and oceanic reference sites, $\delta^{15}N\text{-}NO_3^-$ measured at the pasture site is not significantly different from riverine and oceanic reference sites and from the cultivated site, which prevented the

distinction between pasture and ambient NO_3^-. δ^{15}N-DIN at the pasture site (> +9 ‰) is in the highly variable range of values found in manure (+5.9 to +36.7 ‰; Curt et al., 2004), generally ^{15}N-enriched by ammonia volatilization (Mantilla Morales, 1995). In addition to denitrification indicated by slightly higher δ^{15}N-NO_3^- compared to reference sites, the higher δ^{15}N-NH_4^+ compared to δ^{15}N-NO_3^- at cultivated and pasture sites indicates that nitrification probably happens in these small streams as observed in the Scheldt Estuary (Middelburg and Nieuwenhuize, 2001). We assume however that denitrification and nitrification are limited by the low temperatures in winter, which explain the low differences between the cultivated, pasture and reference sites. Discrimination of the different sources may however be much higher in summer when bacterial processes are enhanced by higher temperatures.

Contrary to cultivated and pasture areas, the high δ^{15}N-NO_3^- and δ^{15}N-NH_4^+ values observed at STP outlets agree with values commonly > +10 ‰ in used and sewage waters or in highly urbanized watersheds (Curt et al., 2004; McClelland and Valiela, 1998). Such high δ^{15}N-DIN are mostly explained by sewage treatments (*e.g.*, water aeration and bacterial enhancement), which enhance PON degradation and bacterial processes (*e.g.*, ammonification, nitrification, denitrification). Bacteria metabolize preferentially light isotopes ^{14}N and leave heavy isotopes ^{15}N in the environment (Cifuentes et al., 1989; Cline and Kaplan, 1975; Owens, 1987), which leads to a ^{15}N-enrichment of DIN in the system (Kellman and Hillaire-Marcel, 1998). The increase of δ^{15}N-NO_3^- during spring in the STP outlet results from increasing temperature and decreasing river flow conditions, which enhances bacterial activity and water residence time, and decreases sludge dilution. The concurrent increase of δ^{15}N-NO_3^- with temperature and the decrease of river discharge, precipitation and soil leaching in summer suggest that the contribution of STP outlets to estuarine waters may be more significant in summer in temperate ecosystems, and show even more visible δ^{15}N-DIN increase.

The main anthropogenic inputs of DIN to the Charente Estuary, *i.e.*, fertilizers (Culture), animal manure (Pasture) and sewage treatment plant outlets (STP), are mainly

constituted of NO_3^- (> 95 %). Additionally to the high quantitative NO_3^- delivery associated to human activities, NO_3^- is also the dominant DIN form because of its higher turnover time compared to NH_4^+ and NO_2^- (Middelburg and Nieuwenhuize, 2000). In the Charente Estuary, such as in any system receiving high riverine DIN fluxes (high flow and DIN concentrations), the atmospheric contribution to DIN - whose $\delta^{15}N\text{-}NO_3^-$ and $\delta^{15}N\text{-}NH_4^+$ generally vary in a wide range of -15 to +15 ‰ (Heaton, 1986; Kendall, 1998; Paerl and Fogel, 1994) - is negligible. The main NO_3^- sources to the Charente River and Estuary originate from the use of fertilizers on agricultural fields that cover ~ 79 % of the watershed area. The strong contribution of fertilizers is highlighted by high NO_3^- concentrations in small streams of cultivated sub-watersheds (1200-1300 µmol l^{-1}, this study), and along the Charente River and at the mouth of the estuary (50-500 µmol l^{-1}, this study).

4.2. Mixing of DIN sources in a well-mixed estuary

As the Charente watershed is dominated by agriculture activities (*e.g.*, fertilizer use) and as we found that $\delta^{15}N\text{-}NO_3^-$ was lower at the cultivated site compared to the riverine and oceanic reference sites, we expected that estuarine $\delta^{15}N\text{-}NO_3^-$ decreased in response to enhanced synthetic fertilizer use or soil leaching during high precipitation and river discharge. The significant correlations between NO_3^- concentrations and the river flow at the upper and mid estuary (St. 1 and 2; Fig. 8A) confirm the increase of NO_3^- concentrations due to soil leaching under high precipitation regime. The lack of correlation at the mouth of the estuary (St. 4; Fig. 8A) emphasizes however the dilution and mixing of NO_3^--enriched riverine waters with oceanic waters, which phenomenon is commonly observed downward of estuaries (Fry, 2002). Correlations between NO_3^- concentrations (indicated by macroalgae N content) and $\delta^{15}N\text{-}NO_3^-$ have already been observed in agriculture watersheds dominated by ^{15}N-depleted fertilizers or by ^{15}N-enriched sewage (Costanzo et al., 2003). In our study, the absence of significant

correlation between NO_3^- concentrations and $\delta^{15}N\text{-}NO_3^-$ along the estuary (St. 1, 2 and 4; Fig. 8B) highlights that increases of river flow and NO_3^- concentrations are not always associated with a decrease of $\delta^{15}N\text{-}NO_3^-$, which has already been linked to the complexity and fragmentation of land uses in the drainage basin of the Mississippi River (Chang et al., 2002). The absence of seasonal variations of $\delta^{15}N\text{-}NO_3^-$ in the Charente Estuary highlights that temporal variations are not always observed in a well-mixed system submitted to multiple DIN sources. This might be explained by (1) the absence of drastic $\delta^{15}N\text{-}NO_3^-$ differences between the dominant NO_3^- source (*e.g.* fertilizers) and reference sites, and (2) a strong signature of residual terrestrial DIN stored during the previous years of dryness.

The temporal variability of $\delta^{15}N$-DIN at shorter time scales (Cifuentes et al., 1989; Horrigan et al., 1990) may have been low in our study because of large NO_3^- stocks and low NO_3^- turnover compared to NH_4^+. We compared then monthly $\delta^{15}N$-DIN and $\delta^{15}N$ of biological DIN integrators - *i.e.*, macroalgae - at the mouth of the Charente Estuary and at an oceanic site.

4.3. Macroalgae, efficient integrator of $\delta^{15}N$-DIN in a well-mixed estuary?

4.3.1. Signature of anthropogenic DIN loads from the estuary

If we only consider $\delta^{15}N$ of macroalgae but not $\delta^{15}N$-DIN, the generally higher $\delta^{15}N$ at the mouth of the Charente Estuary compared to the oceanic site in the tissues of all macroalgae species (excepted *Enteromorpha sp.*, Fig. 4) could be first attributed to higher anthropogenic DIN inputs flowing from the drainage basin. The $\delta^{15}N$ enrichment in macroalgae tissues have often been observed along urbanized estuaries receiving enriched $\delta^{15}N\text{-}NO_3^-$ (Cole et al., 2004, 2005; Costanzo et al., 2005; Fertig et al., 2009). In such human-impacted ecosystems, macroalgae are broadly used to trace spatio-temporal distribution of anthropogenic inputs (Costanzo et al., 2001), such as marine dilution or improvement of sewage treatment plant efficiency (Costanzo et al., 2005). The absence of $\delta^{15}N$ differences between macroalgae

collected at the two stations of the Charente Estuary mouth highlights that both stations receive similar δ^{15}N-DIN inputs. For *Fucus serratus*, the lower δ^{15}N at the outer than at the inner station of the estuary mouth however suggests a small decreasing influence of anthropogenic DIN sources from the Charente Estuary to the ocean due to dilution, as already observed for benthic filter feeders in this ecosystem (Fry, 2002). Nevertheless, these arguments are not enough to explain ^{15}N-enriched macroalgae tissues at the estuary mouth compared to the oceanic site, as similar δ^{15}N of NO_3^- - the dominant DIN form - is observed at both sites. Similar absence of evident relationship between δ^{15}N of estuarine primary producers and riverine nutrient loads has already been highlighted in Australian estuarine lagoons (Scanes et al., 2007). Our results highlight thus that studying δ^{15}N-DIN is essential to confirm the observed trends of macroalgae δ^{15}N. The physiological response of annual and perennial macroalgae to environmental conditions (*e.g.*, other N forms, abiotic parameters) is thus discussed below.

4.3.2. Physiological response of macroalgae to environmental conditions

The lower δ^{15}N of macroalgae (excepted *Enteromorpha sp.*) at the oceanic site is expected to reflect the lower δ^{15}N-DIN generally observed in marine waters (+4 to +7‰; Altabet et al., 1999; Minagawa and Wada, 1986; Owens, 1987). In this study, δ^{15}N-NO_3^- is however similar at the oceanic and estuarine sites and cannot explain these differences. On the contrary, δ^{15}N-NH_4^+ is lower at the oceanic site (+1.8 ‰) and might contribute to the lower δ^{15}N of macroalgae. NH_4^+ can indeed constitute a significant source of DIN as (1) NH_4^+ is preferentially assimilated by macroalgae compared to NO_3^- (Cohen and Fong, 2004), and (2) the absence of visible variations of NH_4^+ concentrations can be hidden by balanced sources and sinks of NH_4^+ (Middelburg and Nieuwenhuize, 2001; Sebilo et al., 2006). The uptake of NH_4^+ is even expected to be higher at oceanic sites where NO_3^- and NH_4^+ concentrations are respectively lower and slightly higher than at the estuarine mouth (this study).

At the mouth of the Charente Estuary, the higher $\delta^{15}N$ of macroalgae compared to the oceanic reference site rather reflects $\delta^{15}N\text{-}NO_3^-$ and not $\delta^{15}N\text{-}NH_4^+$. NH_4^+ concentrations were always low at the estuary mouth (1-7 % DIN) and its contribution to macroalgae uptake might have been insignificant compared to NO_3^- concentrations (50-350 µmol l^{-1}). As for the oceanic site, the low concentrations and small temporal variations of NH_4^+ can have hidden significant NH_4^+ uptake due to a balance between NH_4^+ uptake and production, as already observed in the Loire and Seine estuaries (Middelburg and Nieuwenhuize, 2001; Sebilo et al., 2006). The absence of spatial variations of $\delta^{15}N\text{-}NO_3^-$ along the estuary suggests however that mineralization and nitrification processes might have been low along the estuary, reinforcing the low uptake of NH_4^+ by macroalgae.

Two other processes can increase $\delta^{15}N$ values in macroalgae tissues. The bacterial processes at the surface of macroalgae frond, *e.g.*, organic N mineralization and N fixation (Goecke et al., 2010), might increase $\delta^{15}N$-DIN at the vicinity of the frond. Benthic NH_4^+ fluxes - which might be ^{15}N-enriched due to sedimentary nitrification (Brandes and Devol, 1997) - are also hypothesized to explain the higher $\delta^{15}N$ in macroalgae compared to $\delta^{15}N$-DIN, as extended intertidal mudflats are present at the mouth of the estuary (Fig. 1). Both bacterial processes on the frond and benthic NH_4^+ fluxes are however expected to be low in this study because of the low winter temperatures (Feuillet-Girard et al., 1997). As explained above, the absence of variations along the estuary is rather explained by the intense and rapid mixing of riverine and marine waters by tidal currents and waves in the Charente Estuary, which was also highlighted for POM in this estuary (Modéran et al., 2012). The role of benthic fluxes and bacterial processes might significantly modify macroalgae $\delta^{15}N$ at higher summer temperatures, especially in shallow and productive ecosystems characterized by high residence time.

Another factor potentially controlling $\delta^{15}N$ values in macroalgae tissues is the possible uptake of dissolved organic N (DON) by macroalgae. Macroalgae uptake of urea and amino

acid was recently emphasized by Tyler et al. (2005). Van Engeland et al. (2011) even showed that DON uptake could be equivalent to DIN uptake for macrophytes. Even if urea is increasingly spread on the Charente watershed, concentrations in urea and amino acids were still low in estuarine waters in 2002-2005 (<8 µmol l^{-1} and < 4 µmol l^{-1}, respectively; Bechemin, 2008). These low DON concentrations compared to NO_3^- concentrations emphasize the negligible contribution of DON to macroalgae $\delta^{15}N$ values. In spite of low concentrations, the increasing trend in the Charente Estuary suggests that this source of N could become significant in the next years and should be monitored. DON is rarely measured in estuarine studies, as it is quickly mineralized during its transport along estuaries, and as DIN is often the dominant N form in highly fertilized watersheds. Quantitative DON loads can however account for 20 to 90 % of estuarine N loads (Seitzinger and Sanders, 1997). The role of DON on macroalgae growth should be more investigated, especially in estuaries receiving urea-fertilized waters. The few available values of estuarine $\delta^{15}N$-DON generally range between +3 and +10.8 ‰ (review in Guo et al., 2003). Further quantifications of spatio-temporal variations of $\delta^{15}N$-DON, in addition to DON concentrations, would help in estimating the role of DON in modifying macroalgae $\delta^{15}N$ values, and especially its temporal variations in estuarine and coastal ecosystems.

The same monthly trends of macroalgae $\delta^{15}N$ at estuarine and oceanic sites suggests either (1) anthropogenic NO_3^- inputs to the oceanic site, (2) similar temporal variations of $\delta^{15}N$-NO_3^- in estuarine and oceanic waters, or (3) physiological processes. The input of anthropogenic NO_3^- to the oceanic site is discarded due to water currents in the Marennes-Oléron Bay (Stanisière et al., 2006), and the temporal variations of estuarine and oceanic $\delta^{15}N$-NO_3^- are dismissed due to constant monthly $\delta^{15}N$-NO_3^- at both sites (this study). The physiological response of macroalgae to local and regional changes of environmental parameters, *e.g.*, temperature and light, might more reliably explain the similar seasonal variations of macroalgae $\delta^{15}N$ at both estuarine and oceanic sites. Although fractionation

studies are numerous on phytoplankton species (*e.g.* Pennock et al., 1996; Needoba and Harrison, 2004), much fewer exist on macroalgae (Cohen and Fong, 2004; Dudley et al., 2010). It is reliable from these studies that variations of environmental parameters could modify algae uptake and growth rates, which might influence fractionation. Temperature and light similarly increased from winter to summer at both sites, and especially after March, leading to the enhancement of macroalgae growth rates (this study). The enhancement of macroalgae metabolism might have increased fractionation, as already observed for plants (McKee et al., 2002) and macroalgae (Dudley et al., 2010). This might have led to a lower selection of light isotopes, explaining the increase of $\delta^{15}N$ values from April (for annual species) or March (for perennial species) to May (this study). Seasonal variations of metabolism have also been reported to lead to higher temporal variations of $\delta^{15}N$ values in consumers than diet changes (*i.e.* pectinidae, Lorrain et al., 2002). As reported for consumers, our study suggests that the physiological response of primary producers (*i.e.* macroalgae) to changing environmental conditions might alter macroalgae fractionation and, thus, $\delta^{15}N$ values. More studies on macroalgae are however needed to confirm the hypothesis that increases of light and temperature as well as nutrient concentrations could enhance macroalgae metabolism and led to increasing $\delta^{15}N$ in both estuarine and oceanic waters.

4.3.3. Annual *versus* perennial species

The decrease of $\delta^{15}N$ values from January to April followed by an increase in May in annual species is not observed in perennial species, which might be explained by differences in growth and uptake rates and/or uptake mechanisms between annual and perennial species. These variations of macroalgae $\delta^{15}N$ values might have resulted from the faster metabolic response of annual species to environmental changes compared to perennial species. The annual and r-selected species *Ulva sp.* and *Enteromorpha sp.* (Raven and Taylor, 2002) are indeed characterized by high N demand and uptake rates (Raven and Taylor, 2003; Pedersen

and Borum, 1997; Teichberg et al., 2008). The high uptake rates - even faster than those of perennial species (this study) - often lead to higher productivity compared to perennial *Fucus sp*. (North America shores; Littler, 1980). Transplantation experiments showed that this rapid turnover of *Ulva sp.* tissues explains that $\delta^{15}N$ values quickly reflects external DIN loads and not the internal initial content in *Ulva sp.* (Aguiar et al., 2003; Teichberg et al., 2008). The $\delta^{15}N$ values in annual species tissues consequently integrate shorter time periods and more transient $\delta^{15}N$-DIN variations than perennial species (Aguiar et al., 2003; Teichberg et al., 2008). Finally, the low fractionation during DIN uptake in annual species can be related to the dominance of diffusive DIN transport, which process has already been shown to prevail in *Ulva sp.*, in particular in DIN-enriched environments (Taylor et al., 1998).

The two perennial macroalgae *Fucus serratus* and *Fucus vesiculosus* growing at the mouth of the Charente Estuary do not show large monthly variations of $\delta^{15}N$. These results agree with the constant $\delta^{15}N\text{-}NO_3^-$ in estuarine waters (this study) and confirm that monthly measurements of $\delta^{15}N\text{-}NO_3^-$ are representative of monthly trends in systems submitted to low $\delta^{15}N\text{-}NO_3^-$ variations. Even not significant, the slightly positive correlation between $\delta^{15}N$ of *Fucus* species and $\delta^{15}N\text{-}NO_3^-$ in estuarine waters (Fig. 8C) suggested that perennial maroalgae tissues have integrated the slight increase in estuarine $\delta^{15}N\text{-}NO_3^-$. The similar $\delta^{15}N$ of *Fucus serratus* and *Fucus vesiculosus* is consistent with the similar *in situ* growth rates of both species (2-4 cm $month^{-1}$, this study). These growth rates are in the range of the maximal growth rates of *Fucus serratus* measured in the Great Bay Estuary in northeastern America (2.5-3.6 cm $month^{-1}$; Mathieson et al., 1976), and confirm that these two species integrate the same time period. Sampling of the first 2 cm (this study) integrate thus a time period ranging from 2 weeks (in March and May) to 4 weeks (in February and April). Even if this study confirms that perennial species integrate longer temporal changes in DIN loads, due to the slower uptake and tissue turnover compared to annual macroalgae, the difference in uptake rates might also impact fractionation which potentially explains the differences in seasonal

changes of $\delta^{15}N$ in annual *versus* perennial macroalgae.

As passive and active transport mechanisms might have an impact on macroalgae fractionation (Taylor et al., 1998; Dudley et al., 2010), we also expect the role of uptake mechanisms, as well as the loss of ^{14}N subsequent to uptake (Dudley et al., 2010), to explain $\delta^{15}N$ enrichments that are not linked to increases in $\delta^{15}N$-DIN. Further work is however required to elucidate the role of metabolic processes in controlling macroalgae $\delta^{15}N$ values, and especially to quantify the net fractionation in macroalgae, its control by environmental parameters, and the relative contribution of transport mechanisms in macroalgae $\delta^{15}N$ values.

4.3.4. Perennial macroalgae, retroactive indicators of DIN inputs?

As growing apex of perennial macroalgae could integrate weekly-monthly changes of $\delta^{15}N$-DIN, we have investigated the integration of annual and pluri-annual $\delta^{15}N$-DIN variations along macroalgae fronds at estuarine and oceanic sites. In this study, $\delta^{15}N$ values increases from the apex to the basis of *Fucus serratus* at both sites (Fig. 5). This trend has already been observed in *Fucus vesiculosus* and attributed to a decrease of sewage loads (Savage and Elmgren, 2004). In the Charente Estuary, anthropogenic DIN inputs, mostly due to fertilizers, has increased over the last 25 years (http://www.eau-adour-garonne.fr) and might not have drastically changed over the previous years, while at the oceanic site, the low DIN inputs are little influenced by anthropogenic activities and characterized by low DIN concentrations (Fig. 3C, F). At both sites, the role of drastic $\delta^{15}N$-DIN changes over the past years might probably not explain the observed increase of $\delta^{15}N$ along the frond. Additionally, $\delta^{15}N$ differences between *Fucus serratus* fronds at estuarine and oceanic sites quickly disappear after the 4^{th} cm from the apex (corresponding to 1 to 2 months from measured *in situ* growth rates). The similarity of $\delta^{15}N$ values in the oldest parts of the frond was not expected because environmental conditions (*e.g.*, DIN concentrations, turbidity and salinity) are usually different at these sites, and different $\delta^{15}N$ values are observed in macroalgae apex over the sampled

period (Fig. 4). The variations of $\delta^{15}N$ values along the frond might more likely be due to physiological processes, *e.g.*, growth rate variations, as suggested above for seasonal variations of $\delta^{15}N$ at the apex. The similar $\delta^{15}N$ values along the frond (except the apex) at both sites are rather explained by the biochemical processes involved in the formation of the frond than by variations of DIN inputs, *e.g.* the loss of ^{14}N subsequent to uptake (Dudley et al., 2010). The biochemical processes and the growth rate of *Fucus* fronds may be more important than $\delta^{15}N$-DIN in determining $\delta^{15}N$ of perennial macroalgae tissues (Deutsch and Voss, 2006). From our results, the use of macroalgae as indicators of N input variations over a long time should be completed by studies of the variability of macroalgae fractionation in response to environmental parameters and growth rates, and by comparison with reference sites.

5. CONCLUSIONS

This study in the Charente watershed confirms that the main DIN sources (*i.e.*, cultivated area, pasture, STP outlet) are characterized by distinct $\delta^{15}N$ values. Although the main sources of anthropogenic DIN, mostly NO_3^-, have distinct $\delta^{15}N$, their mixture lead to estuarine $\delta^{15}N$-NO_3^- close to riverine and oceanic reference sites. The high NO_3^- concentrations measured in streams of cultivated areas associated to relatively high $\delta^{15}N$ values compared to synthetic fertilizers suggest that heavy rains do not only flush out freshly spread fertilizers, but also DIN formed by the degradation of organic matter stored during the previous dry years. The integration of $\delta^{15}N$-DIN in macroalgae tissues leads to generally higher $\delta^{15}N$ in macroalgae growing at the mouth of the estuary than at the oceanic site. Our results suggest that, even if macroalgae (partially or totally) integrate $\delta^{15}N$-DIN over time, the metabolic response of macroalgae to environmental parameters (*e.g.*, turbidity, temperature and DIN concentrations) might strongly modify macroalgae $\delta^{15}N$ values. A better understanding of the control of environmental parameters on macroalgae fractionation is thus needed, which have strong implications for the use of macroalgae $\delta^{15}N$ to monitor anthropogenic loads. Coupling these results with quantitative and qualitative database of nutrient runoffs for each landuse would help to move from a bioindicative to a more quantitative and predictive tool.

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Tables

Table 1. Results of three-way ANOVA on macroalgae $\delta^{15}N$ depending on interacting factors time, site and species. df = degree of freedom; *%SS = % Sum Square; F* = statistical values; *p* = *p* value.

Factors	df	%SS	*F*	*p*
Time	4	20.6	92.54	<0.0001
Site	4	32.2	154.74	<0.0001
Species	4	2.0	22.75	<0.0001
Time:site	13	11.1	12.56	<0.0001
Time:species	12	6.6	12.23	<0.0001
Site:species	8	17.1	49.08	<0.0001
Time:site:species	25	10.4	3.04	<0.0001

Table 2. Results of repeated two-way ANOVA for each macroalgae species depending on interacting factors time and site. df = degree of freedom; *F* = statistical values; *p* = *p* value.

Species	Factor	df	*F*	*p*
Ulva sp.	Time	4	28.36	<0.0001
	Site	2	104.37	<0.0001
	Time:site	7	6.78	<0.001
Enteromorpha sp.	Time	4	9.97	<0.001
	Site	2	5.19	<0.05
	Time:site	7	1.94	0.130
Fucus serratus	Time	4	0.76	0.560
	Site	2	2.74	0.080
	Time:site	7	0.92	0.508
Fucus vesiculosus	Time	4	23.51	<0.0001
	Site	2	90.82	<0.0001
	Time:site	7	25.26	<0.0001

Table 3. Mean δ15N-+$_4$NHδ15N-NH4+ and δ15N-−$_3$NOδ15N-NO3− values of DIN sources, estuarine and reference stations for the sampled period (January–May 2006). The number of values (*n*) depended on stations.

Type	Site	$\delta 15N-+_4NH$ (‰)			$\delta 15N--_3NO$ (‰)		
		Mean	SD	*n*	Mean	SD	*n*
Source	Culture	9.0	11.0	2	6.5	0.6	4
	Pasture	12.4	–	1	9.2	1.8	4
	STP	25.4	5.9	4	16.9	8.7	5
Estuary	St. 1	1.8	1.8	2	7.5	1.2	10
	St. 2	6.6	–	1	7.5	0.3	8
	St. 4	2.5	–	1	8.5	3.9	10
Reference	ref_0	1.8	–	1	7.4	0.8	2
	ref_R	0.8	0.6	2	7.3	0.3	8

Figures

Figure 1. Study area and location of the stations sampled along the Charente River and Estuary: DIN sources (Culture: cultivated area, Pasture: pasture zone, STP: Sewage Treatment Plant of Saint-Savinien), estuarine stations (St. 1, St. 2, St. 3, St. 4), and reference stations (ref_R: riverine reference, ref_O: oceanic reference). Water samples were collected at all stations, and macroalgae were sampled at St. 3, 4a and ref_O. Are also indicated the major cities (black circles), the limit of dynamic tidal influence (grey line), the river flow measurement station at Beillant (black star).

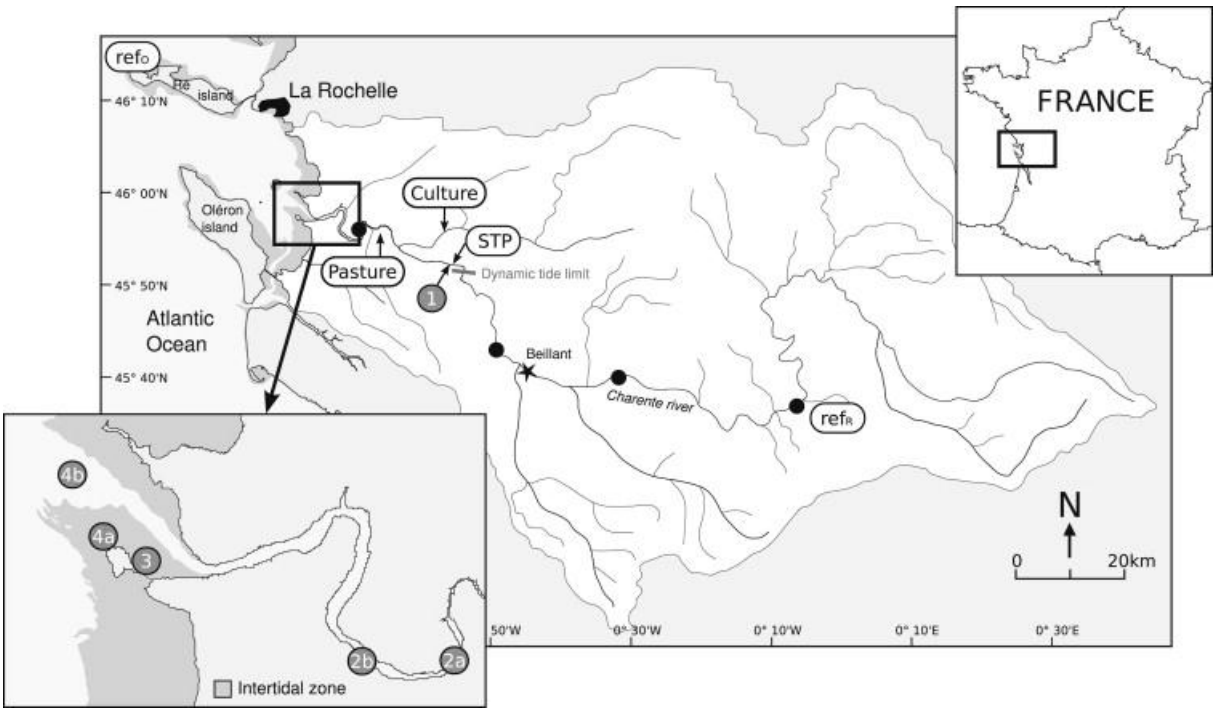

Figure 2. (A) Charente River flow measured at Beillant in 2006 compared to the 2004–2011 and 2004–2005 averaged river flows (source RPDE, http://info.eau-poitou-charente.org), and sampling dates of water (black arrows) and macroalgae (grey arrows); (B) Temperature (black points), salinity (grey points) and turbidity (white points) measured at St. 4b located at the mouth of the Charente Estuary (source Ifremer, http://archimer.ifremer.fr/doc/00041/15210/12538.pdf).

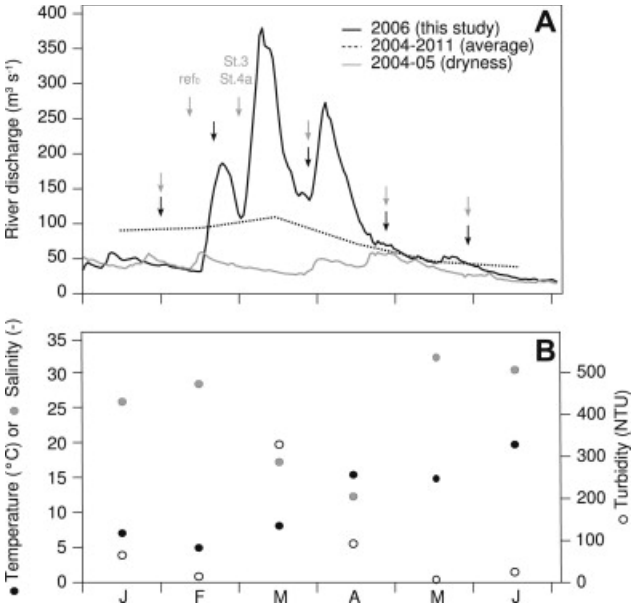

Figure 3. Temporal evolution of $+_4NHNH4+$ concentrations (A, B, C), $-_3NONO3-$ concentrations (D, E, F), $\delta15N-{-}_3NO\delta15N\text{-}NO3-$ and $\delta15N-{+}_4NH\delta15N\text{-}NH4+$ (G, H, I) from January to May 2006 for DIN sources (left panels), estuarine stations (middle panels) and reference stations (right panels). Open and full symbols represent $+_4NHNH4+$ and $-_3NONO3-$, respectively. Post-hoc statistical differences between temporal trends at each station are indicated by letters (a, b) on the right of each plot.

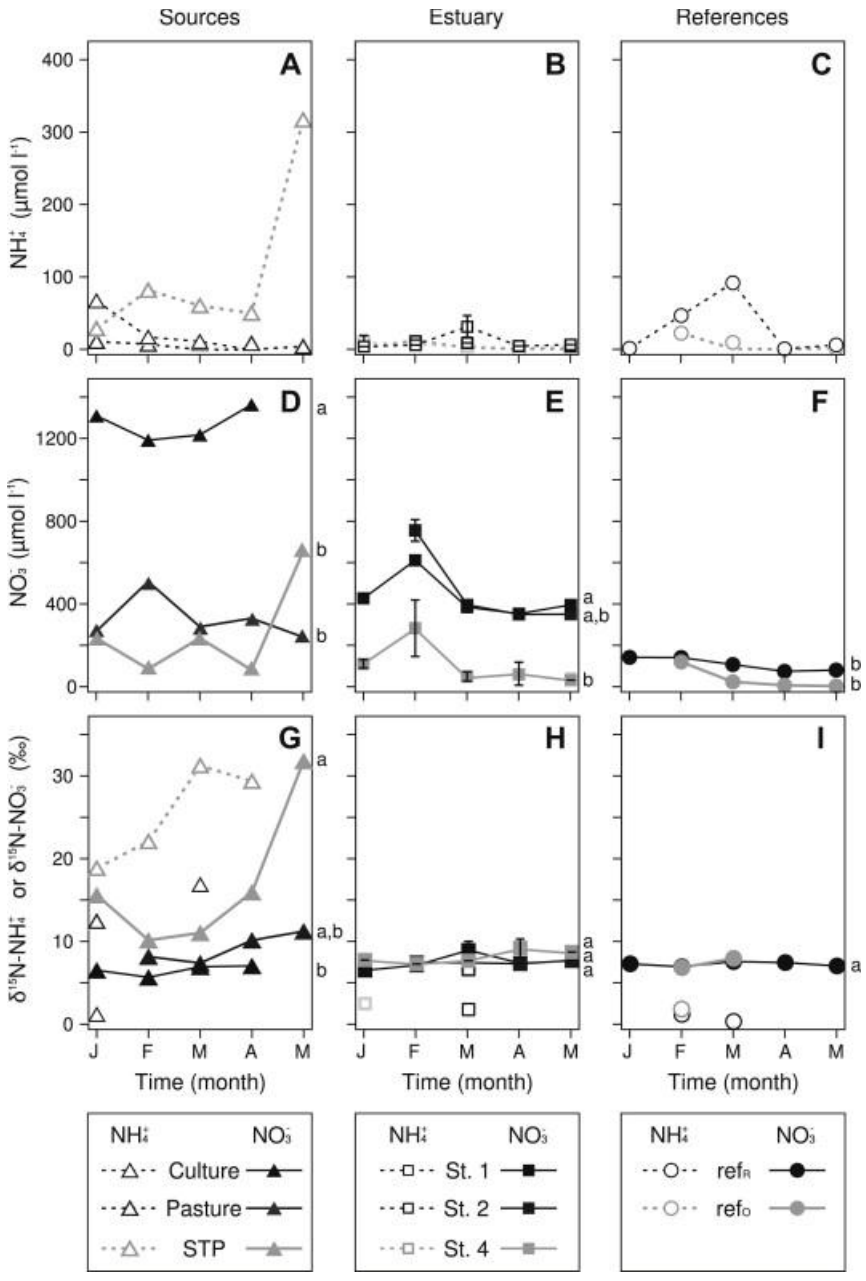

Figure 4. Temporal evolution of $\delta^{15}N$ values in macroalgae tissues of *Ulva* sp. (A), *Enteromorpha* sp. (B), *Fucus serratus* (C) and *Fucus vesiculosus* (D) at St. 3 (inner estuary mouth, white circles), St. 4a (outer estuary mouth, black circles) and ref_O (oceanic reference, grey circles). Post-hoc statistical differences between $\delta^{15}N$ values at each station and at each season are indicated by letters (a, b, c) on the right and on the top of each plot, respectively.

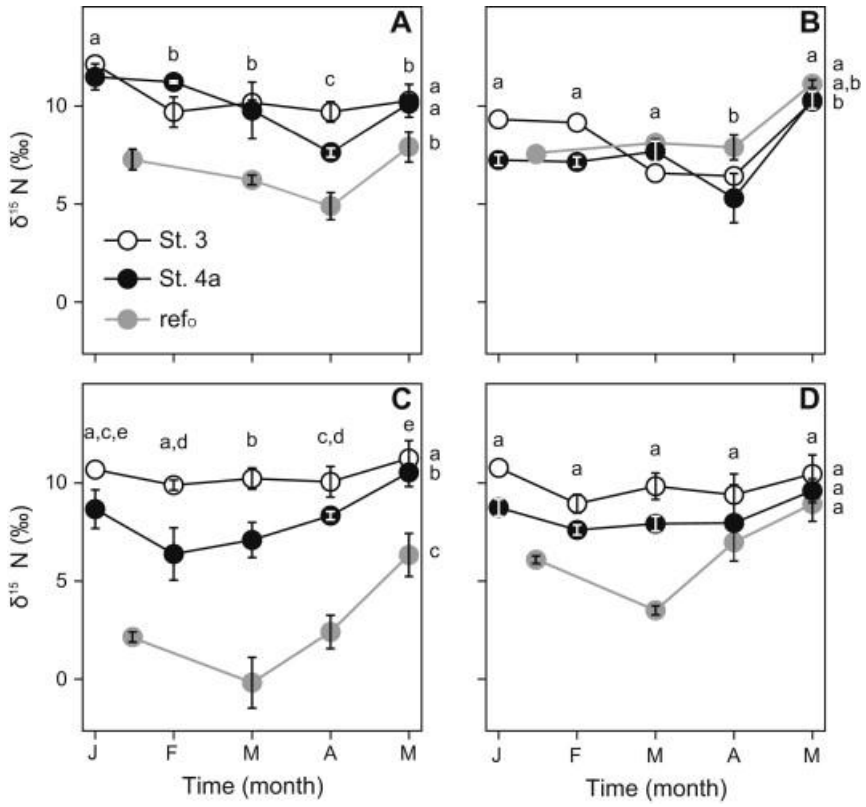

Figure 5. Evolution of $\delta^{15}N$ values along *Fucus serratus* fronds sampled at St. 4a (estuary, black circles) and ref_O (ocean, grey circles). The grey area corresponds to the winter 2005–2006.

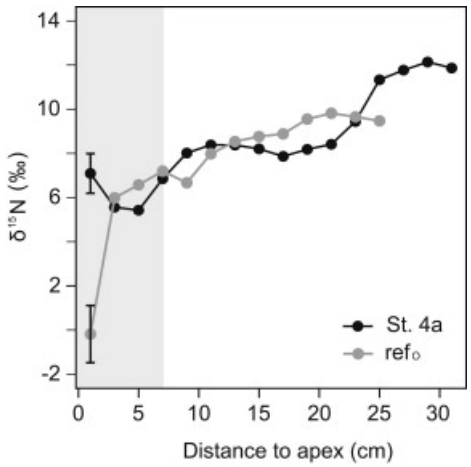

Figure 6. *In situ* growth rate of *Fucus serratus* (black circles) and *Fucus vesiculosus* (white circles) at St. 4a. *n* = 9. Post-hoc statistical differences between growth rates for the different seasons are indicated by letters (a, b).

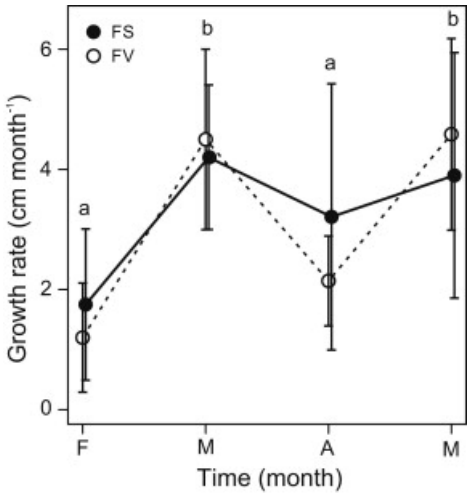

Figure 7. $-_3$NONO3− uptake rates determined in controlled conditions for *Ulva* sp. (U), *Enteromorpha* sp. (E) and *Fucus serratus* (FS) as a function of $-_3$NONO3− concentrations: 500 µmol l^{-1} (dark grey bar) and 50 µmol l^{-1} (light grey bar). *n* = 3. Post-hoc statistical differences between uptake rates for the different species are indicated by letters (a, b).

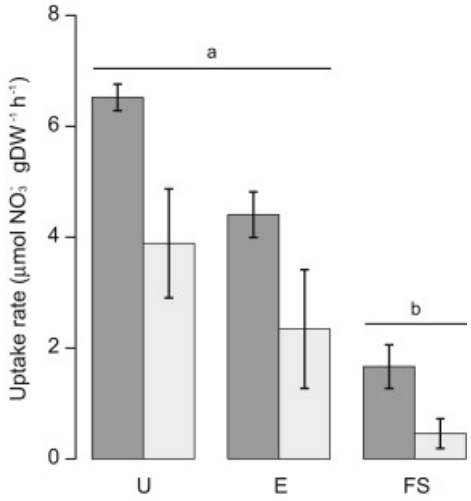

Figure 8. (A) −3NONO3− concentrations as a function of river discharge, (B) δ15N--3NOδ15N-NO3− as a function of −3NONO3− concentrations, at St. 1, St. 2 and St. 4 over the sampled period, and (C) δ^{15}N of *Ulva* sp. (U), *Enteromorpha* sp. (E), *Fucus serratus* (FS) and *Fucus vesiculosus* (FV) as a function of δ15N--3NOδ15N-NO3− at St. 4a over the sampled period. The correlation coefficient (*r*) is indicated for each station, and a black star highlights the significant correlations.