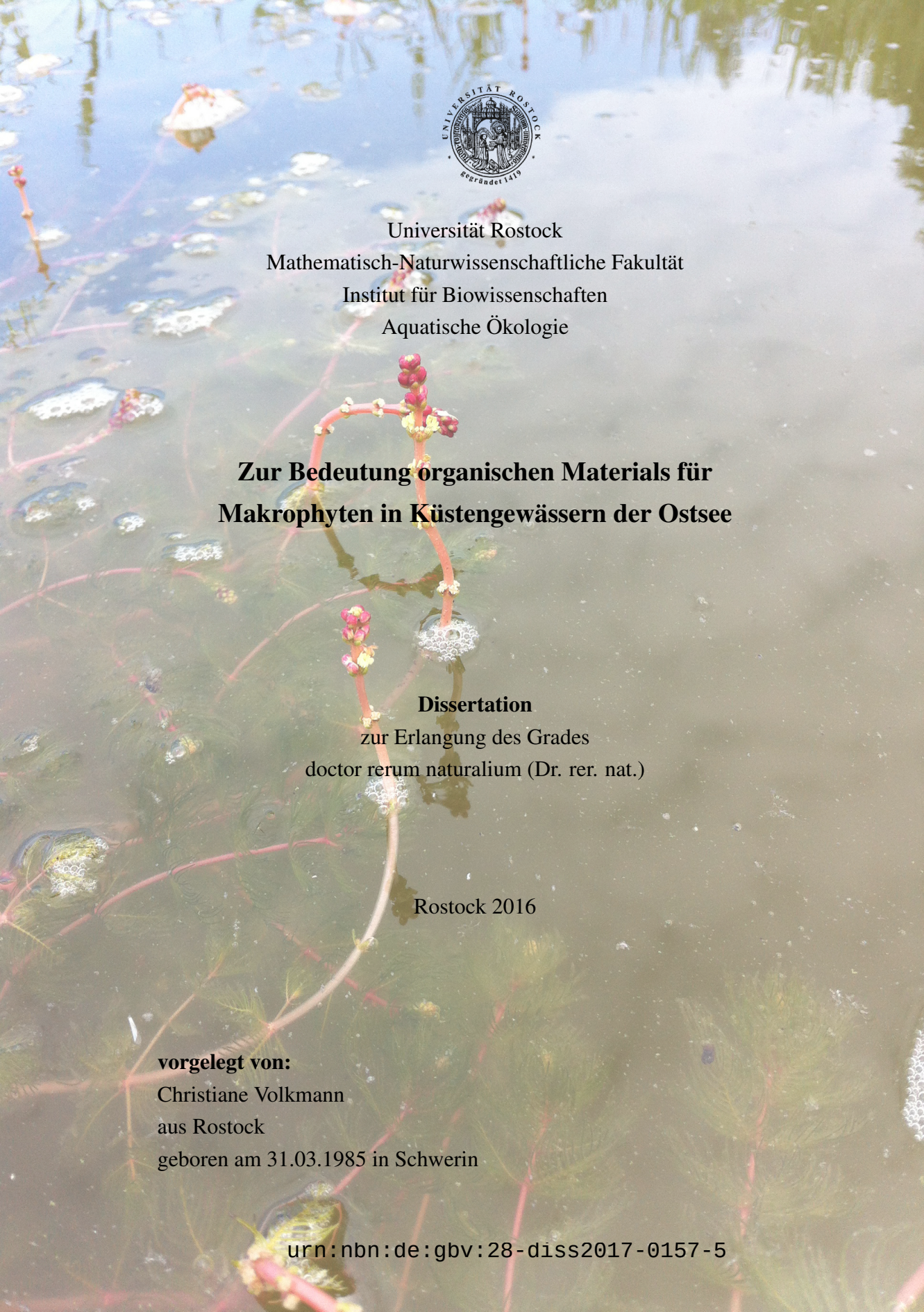

Universität Rostock
Mathematisch-Naturwissenschaftliche Fakultät
Institut für Biowissenschaften
Aquatische Ökologie

Zur Bedeutung organischen Materials für Makrophyten in Küstengewässern der Ostsee

Dissertation
zur Erlangung des Grades
doctor rerum naturalium (Dr. rer. nat.)

Rostock 2016

vorgelegt von:
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urn:nbn:de:gbv:28-diss2017-0157-5

Universität Rostock
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Datum der Einreichung: 04.05.2016
Datum der Verteidigung: 30.09.2016

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The impact of organic material for macrophytes in coastal waters of the Baltic Sea

CHRISTIANE VOLKMANN

ROSTOCK 2016

'The truth isn't easily pinned to a page. In the bathtub of history the truth is harder to hold than the soap and much more difficult to find.'

Terry Pratchett

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Summary

Increasing nitrogen delivery into aquatic ecosystems has become a serious problem worldwide and resulted in changes in habitat, food web structure and nutrient cycling. Coastal waters are mainly affected by the high nutrient delivery because they are an interface between terrestrial and marine ecosystems. Increasing blooms of phytoplankton stimulated by eutrophication and loss of macrophytes are often observed. The latter lose their habitat due to less light penetration. Macrophytes, however, are important components of coastal ecosystems and directly influence sediment dynamics, hydrology and biochemical cycles. These key functions are essential for a good ecological status as it is stated in the European Water Frame Directive. To protect and promote such macrophyte communities a profound knowledge on factors controlling distribution and growth are required. Variables like light, sedimentation and water motion have been investigated in relation to macrophyte abundance but could not explain the patchy occurrence. This is also the case for the Darss-Zingst Bodden Chain (DZBC), an inner coastal water at the southern coast of the Baltic Sea. Despite high N inputs, the demand on ammonium and nitrate can be higher than the supply, resulting in N-limitation and competition for primary nutrients. Hence, the first aim of this study was to investigate the uptake and translocation of DON as possible alternative N source for macrophytes by means of N isotopes as tracer addition. DON was long believed to be only accessible by bacteria, but the labile part of DON was found to be assimilated also by phytoplankton and seagrasses. Experiments were designed to test the DON uptake and it was shown that the macrophytes *Stuckenia pectinata*, *Chara aspera* and *Chara tomentosa* were able to incorporate DON, which was preferred over nitrate but not over ammonium. Moreover the uptake of nutrients was studied for both, the shoot/phylloids and roots/rhiziods, which often comprise $\leq$ 10 % of the total biomass and were therefore assumed to have only anchor function in the sediment. In charophytes also translocation between rhizoids and phylloids could be detected. Macrophytes seemed to have an advantage over phytoplankton by their ability to use the nutrient pool of sediments and the water column.

To understand the growth of macrophytes over a seasonal cycle and how they rely on available nutrients from different sources, the isotopic signatures of macrophytes in comparison to nitrate and DON were measured at six stations along the DZBC. As the major nitrate source the river Recknitz flows into the western end of the DZBC delivering nitrate with high δ^{15}N values. The salinity gradient along the DZBC was expected to also dilute the nutrient concentrations and impact the δ^{15}N values. First it was ruled out that the stable isotope signature in different segments of one specimen impacts the overall stable isotope values of a plant. However, a spatial heterogeneity

among sampling sites exists and isotope ratios of macrophytes seem to reflect multiple site specific factors like nutrient availability, land-use and hydrology.
DON concentrations and isotopic values do not change over an annual cycle or along the salinity gradient of the DZBC and higher time-resolution may be required, since it is known that the labile fraction of DON is utilized within hours to days. In the field, the consumption of DON by macrophytes has been indirectly confirmed by a significant relationship between δ^{15}N of DON in the water and δ^{15}N in the macrophytes. Also the signature of nitrate and suspended PON were analysed to seek relations between those N pools. In contrary nitrate isotopic ratios were much lower and thus indicate to be less a N source for macrophytes. Simultaneously occurring processes in the highly variable inner coastal water lead to ambiguous signals and hamper to identify the sources of DON. Similarly, δ^{15}N-PON and δ^{15}N-DON values can be explained by the assimilation of DON by phytoplankton or by degradation of PON to DON. Overall it is strongly recommended that DON should be integrated into N loading budgets to coastal systems not only for macrophytes but for the whole primary producer community.

Zusammenfassung

Zunehmende Stickstoffeinträge in aquatische Ökosysteme sind ein ernst zunehmendes, weltweites Problem geworden und resultieren in Habitat- und Nahrungsnetzänderung als auch Nährstoffkreisläufen. Vor allem Küstengewässer sind besonders betroffen, da sie als Übergangszone sowohl von terrestrischen als auch marinen Ökosystemen be- einflusst werden. Ansteigende Phytoplanktonblüten, angeregt durch die Eutrophierung, und der Verlust von Makrophyten wurde oft beobachtet. Letztere werden durch geringe Lichttiefen verdrängt. Makrophyten in Küstengewässern sind jedoch wichtige Komponenten und beeinflussen Sedimentdynamiken, Hydrologie und biochemische Kreisläufe direkt. Derartige Schlüsselfunktionen sind für das Erlangen des guten ökologischen Zustandes, wie er von der Europäischen Wasserrahmenrichtlinie gefordert ist, essentiell. Um solche Gemeinschaften zu schützen und unterstützen sind Grundkenntnisse zu Faktoren, die das Wachstum und die Verbreitung kontrollieren, notwendig. Licht, Sedimentationsprozesse und Wasserbewegungen als Variablen wurden untersucht und konnten das inhomogene Vorkommen von Makrophyten nicht erklären. In der Darss-Zingster-Boddenkette (DZBK), einem inneren Küstengewässer im Süden der Ostsee, ist dieser Fall eingetreten. Trotz hoher Nährstoffeinträge kann der Bedarf an Nährstoffen, wie Ammonium und Nitrat das Angebot übersteigen, und resultiert in N-Limitation und damit Konkurrenz um Nährstoffe. Erstes Ziel dieser Studie war Aufnahme und Transport von gelösten organischen Stickstoff (DON) als mögliche, alternative N-Quelle für Makrophyten mit Stickstoffisotopen als Tracer zu untersuchen. Lange wurde geglaubt, dass DON der bakteriellen Gemeinschaft vorbehalten ist, aber es konnte gezeigt werden, dass die labile Fraktion von DON auch von Seegräsern und Phytoplankton assimiliert werden kann. Experiment zur DON-Aufnahme wurden durchge- führt und konnten nachweisen, dass die Makrophyten *Stuckenia pectinata*, *Chara aspera* und *Chara tomentosa* in der Lage waren, DON, welches Nitrat nicht jedoch Ammonium bevorzugt wurde, zu inkorporieren. Zusätzlich wurde gezeigt, dass die Nährstoffaufnahme der untersuchten Makrophyten sowohl über Blätter als auch Wurzeln stattfindet, wobei letzere meist weniger als 10 % Biomasse ausmachen und ihnen demzufolge nur Ankerfunktion zugesprochen wurde. In Characeen wurden Translokationsprozesse zwischen Phylloiden und Rhizoiden detektiert. Makrophyten haben dem Phytoplankton gegenüber einen Vorteil, da sowohl der Pool der Sedimente als auch der Wassersäule genutzt werden kann.
Um das Wachstum von Makrophyten und deren Bedarf an verfügbaren Nährstoffen, verschiedener Quellen zu verstehen, wurden die Isotopensignaturen von Makrophyten, Nitrat und DON an sechs Stationen der DZBK verglichen. Der Fluss Recknitz als Hauptquelle für Nitrat mit hohen δ^{15}N Werten, transportiert dieses in die westliche

DZBK. Entlang des Salinitätsgradienten der DZBK wurde erwartet, dass die Nährstoffkonzentrationen und auch die Beiträge an hohen δ^{15}N Werten verdünnt werden. Es kam heraus, dass sich die stabile Isotopensignatur verschiedener Abschnitte einer einzelnen Pflanze, nicht unterscheidet. Dagegen existieren räumliche Unterschiede und die Isotopenverhältnisse von Makrophyten reflektieren scheinbar multiple Standort-spezifische Faktoren wie die Nährstoffverfügbarkeit, Landnutzung und Hydrologie. Konzentrationen und Isotopenwerte von DON verändern sich nicht über einen Jahresgang oder entlang des Salinitätsgradienten der DZBK und höhere Zeitauflösungen sind notwendig, denn es ist bekannt, dass die labile Fraktion von DON innerhalb von Stunden bis Tagen assimiliert wird. Im Freiland konnte die DON Aufnahme von Makrophyten indirekt nachgewiesen werden, da die δ^{15}N Werte von DON im Wasser und in den Makrophyten direkt in Beziehung zueinander standen. Zusätzlich wurden die Isotopenwerte von Nitrat und suspendierten PON analysiert, um Verbindungen zwischen den N-pools aufzudecken. Nitrat dagegen ist deutlich niedriger und spielt weniger eine Rolle als N-Quelle für Makrophyten. Gleichzeitig ablaufende Prozesse in dem hoch dynamisch Küstengewässer führen zu mehrdeutigen Signalen und erschweren die Identifikation von DON Quellen. Ähnlich Isotopenverhältnisse in DON und PON können durch die Assimilation von DON von Phytoplankton oder der Zersetzung PON zu DON erklärt werden. Zusammenfassend wird daher empfohlen, DON bei N-Einträgen in Küstengewässer für alle Primärproduzenten mit zu berücksichtigen.

1 Introduction

1.1 Eutrophication and its consequences for coastal waters and primary producers

Eutrophication is a global, increasing phenomenon in freshwater and marine systems (Pearl, 1997), caused by nutrient enrichment from anthropogenic point and diffuse sources (agriculture, fertilizers, combustion, industry, sewage). Elevated human activity, population density and land-use of the surrounding catchment areas induce nutrient increases which lead to symptoms like anoxia (Diaz and Rosenberg, 2008; Karlson *et al.*, 2002), phytoplankton blooms, decreasing water clarity, developments of HAB's (harmful algal blooms) and shifts of pelagic and benthic communities and abundances (Baastrup-Spohr *et al.*, 2013; Blindow, 2000; Carpenter and Lodge, 1986). These effects of eutrophication are responsible for loss or degradation of habitats with consequences to marine biodiversity and changes in ecosystem structure and function.
Especially coastal water, which are of economic and tourism interest, are concerned. The resulting great pressure on coastal regions worldwide makes the development of sustainable strategies of these sensitive ecosystems necessary. The Water Framework Directive calls for an effort to reach and maintain the ecological good status and protection of coastal waters. The implementation of these aims is difficult, because coastal systems are extremely dynamic, highly productive areas with biogeochemical gradients in salinity, temperature and primary productivity. As interface between land and sea they are influenced by the marine and the freshwater inputs. Those transition zones often have buffer capacity which means that terrestrial inputs will be stored and retained in the systems and entering the open sea indirectly and with reduced intensity.
Due to the human activity, nitrogen and phosphorous contents via river inputs increased (Galloway *et al.*, 1996; Laznik *et al.*, 1999; Nixon *et al.*, 1996) and the limits of filter effects are nearly exhausted. Nutrients are one of the main factors initiating the growth and production of primary producers and the mechanisms of nutrient cycles are fundamental for the understanding of coastal waters processes and their management. Primary producers (phytoplankton and macrophytobenthos) competing for nutrients and the development of massive biomass production in the vegetation period can lead to nutrient limitations. Since N and P-cycles have been altered by human activity, it is important to understand the complex driving factors of primary production and possible deficiencies. To stop massive biomass production, especially phytoplankton blooms, and associated limitations the nutrient input has to be diminish. Selig *et al.* (2006) demonstrated that a reduction in the nutrient load from rivers did not reduce the nutrient content in the systems, because high internal nutrient loads and release

of nutrients from the sediments can trigger phytoplankton blooms again. Phosphor, which is mainly reported as limiting factor in freshwater systems, is well investigated as a nutrient in aquatic systems. However, less well attention has been paid to nitrogen which can represent a limiting factor for primary producers especially in marine systems. Other studies prove that freshwater, marine and coastal water can be both, N- and P-limited (Elser *et al.*, 2007; Guildford and Hecky, 2000; Schumann *et al.*, 2009). Over the past few decades, many studies have used concentrations and the stable isotopic composition of nitrate (Nestler *et al.*, 2011) and ammonium e.g. in estuarine systems to investigate sources and development of N pollution (Kroeger *et al.*, 2006; McClelland and Valiela, 1998). Other studies have shown that primary producers (phytoplankton rather than macrophytes) as consumers of nitrogen are also good indicators (Cole *et al.*, 2004; Costanzo *et al.*, 2001). Changes in abundances and diversity of sensitive species like seagrass meadows, macrophytes and reefs are good indicators for coastal zones and estuaries. The question here is, how nitrogen is influencing the growth of macrophytobenthos and which sanctions are necessary to reach phytoplankton-poor and macrophytobenthos-rich systems, which are able to buffer nutrients.

1.2 Macrophytes

Macrophytes are aquatic, photosynthetic and conspicuous plants. They are a functional rather than phylogenetic group and are represented in two kingdoms and span at least six major divisions (Rhodophyta, Phaeophyta, Chlorophyta, Cyanophyta, Bryophyta and Spermatophyta) with worldwide distribution (wetlands, shallow lakes, streams, estuaries and coastal zones). Diverse forms exist from simple chains of prokaryotic cells, single-celled yet multinucleate thalli and members with complex internal structures analogous to vascular plants. Limiting factors for growth and geographic distribution of submerged rooted vegetation are salinity (Bonis *et al.*, 1993; Grillas, 1990), temperature, light (Blindow, 1992), herbivory, epiphytes (Gross *et al.*, 2003) and physical disturbance (Kovtun *et al.*, 2011; Zhang *et al.*, 2014). Bornette and Puijalon (2011) reviewed the influence of environmental parameters of aquatic systems on species occurrence, life-history traits and community dynamics among aquatic plants. Biotic interaction and competition among phototrophs (macrophytes, phytoplankton, periphyton) with respect to turbidity and nutrients were studied in estuarine systems by Sand-Jensen and Borum (1991). A short overview of nutrients regulating the macrophytobenthos dispersal is given in Fig. 1.

The importance of N in supporting coastal primary production has been established (citations in Fong (2008)). Sources of nitrogen to macrophytes are in general new (al-

lochthonous = terrestrial inputs via rivers, upwelling, N-fixation, groundwater, aerial deposition) or *in situ* sources (autochhtonous = regeneration of N from primary producers during decomposition, recycling of N from sediments and biota) (Fong, 2008). O'Brien *et al.* (2014) found that the demand of nutrients for macrophytes is much lower than the supply available from the water column and consequently their metabolism has limited influence on water nutrient concentrations. But it was shown also that stocks of rooted submerged macrophytes can affect chemical, physical and biological mechanisms contributing to the removal and degradation of nutrients (Carpenter and Lodge, 1986; O'Brien *et al.*, 2014) and large oscillations of macrophyte biomass can alter the system directly or indirectly. Caffrey and Kemp (1990) reported indirect positive effects for the N cycle and their associated microbial community. Microbes were responsible for increasing ammonification and nitrification rates, whereas macrophytes supported these processes by release of oxygen. Denitrification and thus the loss of nitrogen out of the system, were found to be significantly greater in vegetated (with *P. perfoliatus*) than bare sediments (Caffrey and Kemp, 1992). Charophytes, a class of green algae with the habitus of higher plants, restrict sediment resuspension (Kufel and Kufel, 2002).

Macrophytes diversity regulate metabolism, nutrient cycling and turbidity and enhance the functioning and associated services (Engelhardt and Ritchie, 2001; Evrard *et al.*, 2005; Fonseca *et al.*, 1982). The potential of macrohytes play an important role by ensuring a favourable environment, better ecology and general constitution of the aquatic systems (Gumbricht, 1993). Not only the abilities of macrophytes are of interest for science and practical management. Submerged marine vegetation, especially charophytes, sensitively react to elevated nutrient contents and were early identified as indicators for good ecological status and low nutrient levels (Krause, 1981). Most charophyte species were found to be limited to concentrations of < 0.02 mg L^{-1} for orthophosphate (Forsberg, 1965) and < 0.2 mg L^{-1} for ammonium. Higher concentrations are not toxic (Blindow, 1988) but but they usually coincide with an increased phytoplankton growth, decreasing light availability and nutrient limitation for the macrophytes, thereby hampering their growth. The application of macrophytes as indicator for eutrophication, were unfortunately first integrated at the beginning of 20th century in coastal waters (Selig *et al.*, 2007; Selig and Sagert, 2008). This delayed implementation is explained due to the missing knowledge of relations among macrophytes and their environmental factors.

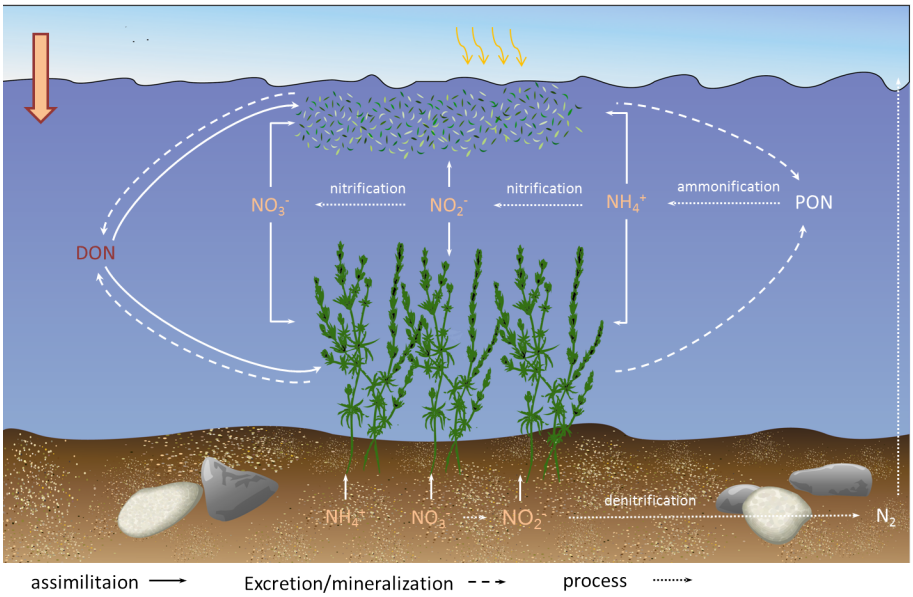

Figure 1: Factors influencing the distribution and growth of macrophytobenthos in coastal water.

1.3 The Darss-Zingst Bodden Chain (DZBC) - an inner coastal water of the Baltic Sea

The Baltic Sea is one of the worlds most eutrophied aquatic system and the anthropogenic eutrophication effects are well studied (Elmgren, 2001). Nitrogen enters the Baltic Sea via rivers (Oder, Vista, Neva, Neman, Daugava, 265-979 kt N year^{-1}), atmospheric deposition (185 kt N year^{-1}), N_2-fixation (926 kt N year^{-1}) and point sources (Grimvall and Stålnacke, 2001). Especially concerned are the coastal zones which are affected by direct or diffuse nutrient loads from the surrounding drainage systems. Consequences of eutrophication are hypoxia, occurrence of cyanobacteria and other phytoplankton blooms as well as shifts in abundance and diversity of benthic flora and fauna. The DZBC is an inner coastal water of Germany and one of many estuaries of the Baltic Sea affected and altered in the last decades by eutrophication. The DZBC is classified as a typical shallow water estuary with pronounced spatio-temporal variabilities in salinity, primary production and nutrient contents (Schlungbaum *et al.*, 1994). Investigations of the DZBC started 1968 (Schiewer, 2004) and raising nutrient content confirms strong eutrophication (Schlungbaum *et al.*, 1994). First changes began 1972 with a massive reduction of submerged macrophytes in the western parts of the bodden chain and 10–15 years later in the eastern parts (Schiewer, 1998). Since then phyto-

plankton became dominant and dead biomass led to higher denitrifaction rates. The absence of rooted vegetation increased turbidity and resuspension of sediments. At the beginning of the 90ies with sewage and waste-water management, remesotrophication could be achieved with a reduction of P load of 60 % and N of 20-30 %. Nitrogen concentrations did not reach the goal of a reduction of 50 % which is because of re-mobilized sediments and internal recycling processes(Bachor, 2005). Until today the system is phytoplankton dominated (Fig. 2) and light and nutrients are in bad conditions. Even though the submerged vegetation starts to recover in the DZBC, although with lower depth limit as result of light competition (Porsche *et al.*, 2008). Selig *et al.* (2009) studied the annual and spatial distribution of macrophytobenthos from 2001–2007 and found strongly varying vegetation communities within this investigation period. This patchiness could not be explained and there is still a gap of knowledge about the distribution and success of macrophytobenthos.

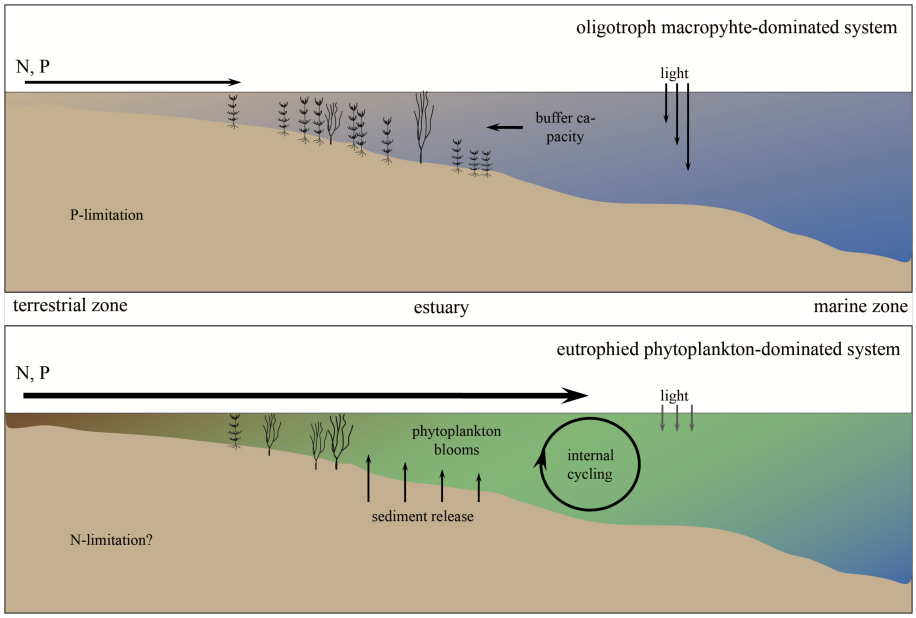

Figure 2: Development of an inner coastal water of the Baltic Sea, the DZBC, during nutrient enrichment in the last decades. First stage was macrophyte-dominated system with great light penetration, clear-water and high buffer capacity for entering nutrients from rivers. Primary producers were mainly charophytes and some vascular macrophytes. With increasing nutrient enrichment not only charophytes were replaced by *Stuckenia pectinata* but also the whole macrophytobenthos nearly disappeared to the benefit of phytoplankton blooms. The light penetration and buffer capacity drastically decrease. Internal processes (biomass into dead material, bacterial decomposition) and release of nutrients from sediments amplify the water pollution.

1.4 Dissolved organic nitrogen as part of the N-cycle

Nitrogen in aquatic systems exists in the particulate and dissolved form where the latter one is defined as the fraction passing through a filter with a pore size of 0.7 µm (typically glass fibre) (Fong, 2008). Dissolved nitrogen consists of inorganic (ammonium, nitrate and nitrite) and organic compartments. The first ones are easy to measure and well investigated for different aquatic environments (Boynton and Kemp, 2008; Gruber, 2008). Until today the quantification and determination of chemical composition of DON is a great challenge. This is due to the complex and heterogeneous composition of DON containing biologically labile and refractory components like urea (LMW = low molecular weight organic compound), DCAA (dissolved combined amino acids including proteins, oligopeptides, amino acids), DFAA (dissolved free amino acids), DPA (dissolved primary amines), nuclein acids (DNA and RNA), methylamines, and humic and fulvic substances (humic acids, fulvic acids) (Alluhihare and Meador, 2008; Benner, 2002; McCarthy *et al.*, 1997). The turnover rate of single molecules vary strongly from hours and days of the labile fraction to month and hundreds of years of the refractory fraction, which comprise in general the bulk of DON (Bronk, 1997). Determination of concentrations of DON and isotopic values are not easy (Antia *et al.*, 1991; Bronk *et al.*, 2000; Sharp, 2002) because direct measurements are not possible. Concentrations of DON at coastal zones are around 10 µM, at estuaries around 25 µM and in rivers around 35 µM (Bronk, 1997). In estuaries and coastal zones the DON pool amount to 13 and 18 %, respectively in contrast, 83 % DON has been determined for the surface waters of the open ocean and thus representing the biggest pool (Berman and Bronk, 2003). For example, in the Golf of Riga DON concentrations of 5–20 µM were found by Jørgensen *et al.* (1999) and 29.8–34.8 µM in 5 rivers entering the Baltic Sea (Stepanauskas *et al.*, 2002). The seasonality of DON concentrations is poorly investigated (Thomas, 1997) and seems to be very individual for different aquatic systems. DON exceeds dissolved inorganic nitrogen (DIN) concentrations frequently in marine and freshwaters even in N-limited systems (Berman and Bronk, 2003; Bronk *et al.*, 2007). Those concentrations of DON, which were persistently high, led to the erroneous view that the DON pool was largely refractory and only utilised for bacterial production. But in the last decades there were raising evidences for DON uptake by bacteria and phytoplankton (Berman and Bronk, 2003; Bronk and Glibert, 1993; Korth *et al.*, 2012). Since these findings, many suggested to include DON in N loading budgets to coastal zones and estuaries, particularly as they may contribute to growth of the primary producers (Bronk *et al.*, 2007). Sources of DON come from allochthonous (terrestrial run-off, leaching from plant detritus and soils into streams and rivers, sediments, groundwater and atmospheric deposition) or autochthonous (release by primary

producers and bacteria, excretion from micro- and mesozooplankton, viral lysis of bacteria and eukaryotic cells and particle solubilisation) (Berman and Bronk, 2003). The bulk of DON undergoes chemical processes like phototransformation by sunlight and biological processes (assimilation, release, bacterial degradation) (Fig. 3).
For coastal areas terrestrial inputs via rivers dominate and highest concentrations of DON are found close to the mouth of rivers (Bronk, 1997; Stepanauskas *et al.*, 2002). Primary producers can use the labile fraction of the additional nitrogen sources. For example, bioassay experiments in nine US rivers indicated that up to 23 % of DON is bioavailable (Wiegner *et al.*, 2006). In general, the bioavailability of terrestrially derived DON is variable between 2–70 % (Seitzinger and Sanders, 1997; Stepanauskas *et al.*, 2002; Wiegner *et al.*, 2006; Wiegner and Seitzinger, 2004). It is speculated that the bioavailability and the composition of DON may depend on the source (McCallister *et al.*, 2006). DON from anthropogenic sources seems to be more bioavailable than DON exported from forested regions and wetlands (Seitzinger, 2002). Especially in summer DON may significantly enhance primary production and its impact on the primary producers community and contribution to eutrophication seems to be greater than thought(Berman and Bronk, 2003; Stepanauskas *et al.*, 2002, 1999).

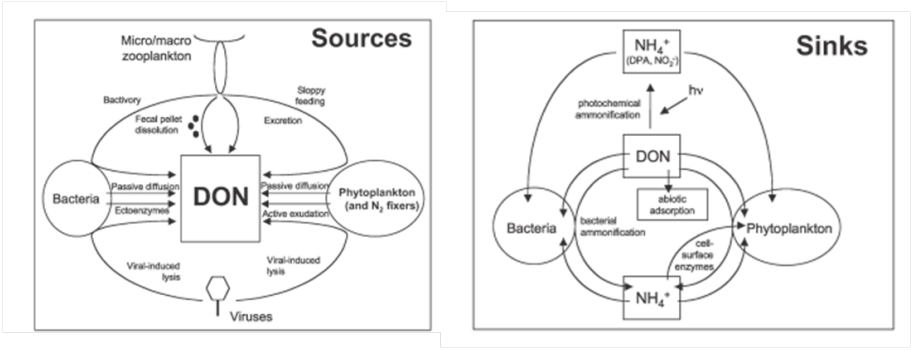

Figure 3: Dissolved organic nitrogen in the N-cycle (by Berman and Bronk (2003))

1.5 Stable isotopes

Stable isotopes and their application in ecological studies
Isotopes are variations of elements with the same atomic number (protons), but differ in the mass number due to different numbers of neutrons. The term is deduced from the greek words ”iso” (equal) and ”topos” (place) and refer to the periodic table of elements. Around 1500 isotopes exist, whereas only around 300 are stable. That means decaying processes are accomplished on geological time-scale in contrary to

non-stable isotope, which decay in extremely shorter periods based on their instability and therefore, are radioactive (Hoefs, 1997). Exceedingly small differences in mass number (with one neutron more or less) can be detected as ratio between the heavy to light isotopes. These ratios are measured against international accepted reference standard gases in the isotope ratio mass spectrometer (IRMS). So stable isotopes are not expressed as absolute number but relative to a standard as delta notation with ‰ as unit:

$$\delta^{15}N = \left(\frac{^{15}N/^{14}N_{sample}}{^{15}N/^{14}N_{standard}} - 1 \right) x1000 \tag{1}$$

This equation is often used for naturally occurring stable isotope ratios, but it should be noted that isotopes are also used as tracers. With tracers isotopes are at elevated levels, and so are often expressed in atom %:

$$Atom\% = 100x \left(\frac{R_{sample}}{(1 + R_{sample})} \right) \tag{2}$$

where R is the ratio of heavy to light isotopes. In ecological research stable isotope data of nitrogen, carbon and oxygen are most important to understand biological and associated processes. In Tab. 1 the stable isotopes, abundances and international standards are presented. Nitrogen isotopes are useful to trace nitrogen in biogeochemical processes (Bedard-Haughn *et al.*, 2003), to identify sources of nitrogen (Costanzo *et al.*, 2001), to map gradients and give informations on trophic positions (Minagawa and Wada, 1984). Also carbon isotopes were used in food web analyses (DeNiro and Epstein, 1978). In general in elements with low atomic number like carbon and nitrogen isotope effects are possible. Fractionation is a process changing the ratios of stable isotopes in substrate and product. Mechanism behind this effect are the physical reaction velocity (heavier isotopes react slower), strength between chemical bonds in molecules and equilibrium or kinetic processes (diffusion, biological processes e.g. assimilation or other enzymatic effects). For example, during uptake processes the lighter isotopes are preferred over the heavier ones, leading to an enrichment of heavy isotopes in the remaining substrate and the organisms becomes progressively lighter. This fractionation is reported as isotope fractionation factor ε and can be described by the 'Rayleigh equation':

$$\delta_{reactant} = \delta_{initial} - \varepsilon[ln(f)] \tag{3}$$

where f is the fraction of reactant remaining (nitrate/nitrate$_{initial}$) and $\delta_{initial}$ is the isotope value of initial reactant pool. In practice ε is the negative slope of the linear relation of the heavy isotope vs. the natural logarithm of the fraction of the reactant

remaining.

Table 1: Stable isotopes common in ecological research, their relative abundances and international standards in order of increasing mass.

Element	Isotope	Abundance [%]	International standard	Absolute abundance of the standard ($R_{standard}$)
Carbon	^{12}C	98.892	Vienna Pee Dee	^{13}C:^{12}C = 0.0112372
	^{13}C	1.108	Belemnite (VPDB)	
Nitrogen	^{14}N	99.635	Atmospheric	^{15}N:^{14}N = 0.0036765
	^{15}N	0.365	nitrogen	
Oxygen	^{16}O	99.759	VSMOW in water	VSMOW = 0.0020052
	^{17}O	0.037	generally VPDB in	VPDB = 0.0020672
	^{18}O	0.204	CO_2 or carbonate	

Stable isotope signatures of aquatic plants

Nutrient sources of primary producers can be estimated with stable isotope data assuming that stable isotope values reflect the sources in a predictable manner and with little variations (Bunn *et al.*, 2013; Cohen and Fong, 2005). $\delta^{13}C$ in aquatic plants (Tab. 2) ranges widely from -47 to -8 ‰ with a typical range of -30 to -20 ‰. Ranges of $\delta^{15}N$ are -15 to 20 ‰ (Cloern *et al.*, 2002; Fry and Sherr, 1984; Kendall *et al.*, 2001). This great variability results from isotopic fractionation of ^{13}C and ^{15}N in aquatic plants, which is mainly dependent on their photosynthetic pathway (C_3- vs. C_4-plants and crassulacean acid metabolism (CAM)), the isotopic composition of the source (DIN and dissolved inorganic carbon (DIC)) and the intracellular concentrations (Evans, 2001; Fry and Sherr, 1984; Handley and Raven, 1992; Högberg, 1997; O'Leary, 1981). The carbon isotopic fractionation during photosynthesis is dependent on several factors, e.g. the concentrations of DIC, the form (hydrogen carbonate (HCO_3) or carbon dioxide (CO_2)), differents (all 3 exist in aquatic plants), associated enzymes, the pH (regulate carbonate system), temperature and respiratory quotient (Carvalho and Eyre, 2011; Fogel and Cifuentes, 1993; Fry and Sherr, 1984; Hecky and Hesslein, 1995).

In general C_4-plants fix CO_2 via the enzyme phosphoenolpyruvate (PEP) carboxylase which lead to a fractionation factor from -17 to -9 ‰ in terrestrial plants. In contrary, Ribulose-1,5-bisphosphate carboxylase/oxygenase (RUBISCO) is the responsible enzyme for CO_2 fixation during the Calvin-cycle in C_3-plants and is responsible for a fractionation factor of -32 to -20 ‰ in terrestrial plants. The third photosynthetic path-

way - the CAM separate the uptake and fixation of CO_2 temporally. CO_2 is fixed at night in form of malate and stored in the vacuoles. At day malate is transformed to CO_2 and released out of the vacuoles in chloroplasts, where the Calvin cycle proceeded with RUBISCO. Due to their similarity carbon stable isotope data can not be used to distinguish between CAM and C_4.

Table 2: General range of isotopic values [‰] in coastal ecosystem (adapted from Fry and Sherr (1984)).

Source	δ^{13}C [‰]	δ^{15}N [‰]
terrestrial C_3 plants	-23 to -30	-10 to 10
terrestrial C_4 plants	-10 to -14	-10 to 10
river seston (POM)	-25 to -27	
peat deposits	-12 to -28	
C_3 marsh plants	-23 to -26	
C_4 marsh plants	-12 to -14	
seagrasses	-3 to -15	
macroalgae	-8 to -27	
macrophytes	-20 to -30	-15 to +20
benthic unicellular algae	-10 to -20	
temperate marine phytoplankton	-18 to -24	
river-estuarine phytoplankton	-24 to -30	
autotrophic sulfur bacteria	-20 to -38	
methane-oxidizing bacteria	-62	

Fractionation factors in aquatic plants vary more widely from -11 to -50 ‰ (Keeley and Sandquist, 1992). Also in contrast to terrestrial plants, aquatic primary producers derive inorganic carbon from DIC in form of HCO_3, which can be -7 to -11 less negative than CO_2 (Keeley and Sandquist, 1992). Keeley *et al.* (1986) tested 22 aquatic plants and could not find different photosynthetic pathways based on δ_{13}C and suggested to use hydrogen isotopes for separating CAM- and non-CAM-plants. Aquatic CAM species accumulate greater levels of deuterium than non-CAM species. Benedict *et al.* (1980) found similar δ_{13}C values in seagrasses and C_4-plants.

Nitrogen stable isotope in plants are not so well established like carbon stable isotopes due to the complexity of the N-cycle and the analytical challenges in measuring nitrogen stable isotopes. Robinson (2001) reviewed the application of nitrogen stable isotopes in ecology and predict that isotopic values of plants reflect something more than the nitrogen source. Fogel and Cifuentes (1993) found that fractionation during

ammonium assimilation in aquatic algae is dependent on whether algae cells are nitrogen limited, enzyme limited, or diffusion limited, respectively (-4, -17, -27 ‰). Table 3 gives a short overview of fractionation factors during N assimilation of aquatic plants. In general fractionation in macrophytes is much lower compared to phytoplankton.

Table 3: Nitrogen isotopic fractionation during assimilation in aquatic primary producers. Fractionation of NO_3^- assimilation or NH_4^+ assimilation was investigated for species marked with ∘ and •, respectively. For species without a symbol, authors provide no information.

Species	Isotope effect (ε)	Source
macrophytes:		
Spartina alterinfolia	1.1–3.5	(Martinetto *et al.*, 2006)
Zostera marina	-0.2	
Ulva sp.	~ 3.0	(Carvalho and Eyre, 2011)
Ulva lactuca	3–4	(Teichberg *et al.*, 2006)
Potamogeton, Vallisneria and *Myriophyllum* ∘	1.9–3.6	(Brabandere *et al.*, 2007)
Enteromorpha intestinalis	no fractionation	(Cohen and Fong, 2005)
phytoplankton:		
different phytoplankton taxa	2.2–6.2	(Needoba *et al.*, 2003)
Skeletonema costatum ∘	-9.0 ± 0.7	(Pennock *et al.*, 1996)
Skeletonema costatum •	-7.8 - -27.2	
Emiliania huxleyi ∘	4-5	(Waser *et al.*, 1998)
Emiliania huxleyi •	15-19	
Thalassiosira weissflogii	1.4 ± 0.4	(Karsh *et al.*, 2014)
periphyton	0.7-2.5	
phytoplankton •	-14 to -20	(Cifuentes *et al.*, 1989)

As with carbon, nitrogen fractionation is dependent on the substrate (DIN), which derives from different sources (allochthonous vs autochthonous) and can be changed during nitrogen cycle (e.g. nitrification, denitrification). The responsible enzyme for nitrogen assimilation is nitrate-reductase. Little isotopic discrimination of DIC and DIN occurs, when pool sizes are low and growth rate/supply is high and plants assimilate all the available C and N (Kendall *et al.*, 2001). *Vice versa* when pool sizes are large and the supply low isotopic fractionation is greatest. More details can be found in the review of of Kendall *et al.* (2007), Montoya (2007) and Finlay and Kendall (2007).

1.6 Framework

The goal of this dissertation is an evaluation of the impact of DON as possible nitrogen source for macrophytes in coastal waters. Also the isotopic signature of macrophytes and DON were investigated to test their application as index for water management. The discussion is divided into three parts. Chapter 3 to 5 are presented in a manuscript-like structure and a statement on my contribution to the manuscript in detail can be found at the end of the thesis. Material and methods descriptions were explained in Chapter 2 to avoid repetition. Chapter 6 summarizes the results of this dissertation and gives an outlook for future research. Chapter 3 focus on the ability of macrophytes to use dissolved organic nitrogen as nitrogen source. Laboratory δ^{15}N tracer experiment were carried out to examine and compare the uptake and translocation of nitrate, ammonium and DON (an amino acid mixture as labile fraction of DON was applied) of three macrophytes (*Chara aspera*, *Chara tomentosa* and *Stuckenia pectinata*). Species were selected and cultivated according to their abundance at the study site. Moreover, the contribution of roots/rhizoids and shoots/phylloids on nitrogen uptake was identified. This part provides fundamental findings for macrophytes signatures and DON as a relevant part of the nitrogen cycle will be discussed in chapter 4 and 5. The chapter is published 2016 in "Journal of Experimental Marine Biology and Ecology, 477:23–30" and co-authored by Susanne Halbedel, Maren Voss and Hendrik Schubert. Chapter 4 addresses the identifications of stable isotope signals as well as C:N ratios of macrophytes and their spatio-temporal pattern. Also the intra-variability and segments of single plants are investigated. Natural abundances of δ^{15}N and δ^{13}C and the C:N ratio of macrophytes of the DZBC were analysed over an annual cycle. Chapter 5 combines the natural abundance of signatures of macrophytes with the signature of DON, nitrate and particulate organic matter (POM) to identify the source of nitrogen and the impact of DON to the growth of aquatic primary producers in an eutrophied inner coastal water. Also δ^{15}N, δ^{13}C and C:N ratio of POM were investigated. Isotope signatures and concentrations of DON and nitrate were analysed at five dates over an annual cycle. Concentrations of nutrients and water parameters were investigated in order to provide an interpretation of isotopic values.

2 Material and Methods

2.1 Application of methods

The following subsections one by one desribe the methods or equipment used in this investigations. Methods used for one single chapter are listed in particular in the Experimental Setup section.

2.2 Sampling site - Darss-Zingst bodden chain

Studying inner coastal waters, the Darss-Zingst Bodden Chain (DZBC) in the Southern Baltic Sea has been selected, which is located in Mecklenburg-Vorpommern (North Eastern Germany). Four bodden (Saaler bodden, Bodstedter bodden, Barther bodden and Grabow), connected via narrow channels, provide a surface area of 197 km^2 (Fig. 4). The water body affected by the rivers Recknitz (= 42 %), Barthe (= 19 %) (Schlungbaum *et al.*, 1994) and other small rivers and brooks, constitute a mainly agricultural- and forestry-, as well as touristic-influenced catchment area of 1578 km^2.

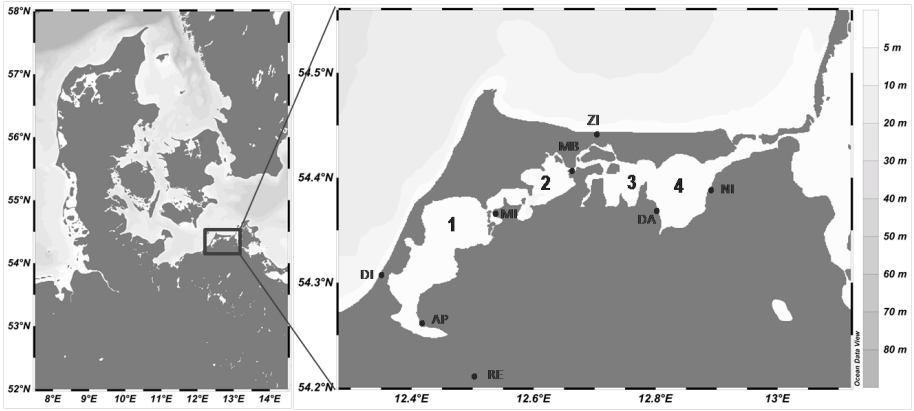

Figure 4: Map of the DZBC at the south-west of the Baltic Sea with four basins: 1-Saaler bodden, 2-Bodstedter bodden, 3-Barther bodden and 4-Grabow and the sampling stations: Recknitz (R), Pütnitz (P), Michaelsdorf (MI), Meiningerbrücke (MB), Dabitz (DA) and Nisdorf (NI). Dierhagen and Zingst were taken as reference stations from the Baltic Sea outside of the DZBC.The map was modified from Ocean Data View Version 4.7.4 (Schlitzer, 2004).

Today the only access to the Baltic Sea is provided by the Gellenstrom, which is a narrow and shallow passage. That is why the inflow of saltier and nutrient-poor water from the Baltic Sea is minimized. The exchange between the marine and fresh water resulted in an increasing salinity range from 0–10 and decreasing eutrophication-gradient (hyper-eutroph) (Bachor, 2005) from west to east. The DZBC is a highly eutrophied

shallow water estuary with an average depth of 2 m. Increasing urbanization 35 years ago led to a change from clear water with large stocks of macrophytes to phytoplankton dominated system with Secchi depth < 0.5 m and increasing nutrient loads (Schiewer *et al.*, 1999). In the period from 1993–1997 the nitrogen load of the Recknitz were 1234.7 t N a^{-1} and 37.4 t P a^{-1} whereby 97.7 % and 91.8 %, respectively were diffuse (Mewes, 2004). The annual average value for nutrients in Barther bodden from 2000–2010 were 116 µmol L^{-1} TN and 3 µmol L^{-1} TP (Blindow and Meyer, 2015; LUNG, 2013).

2.3 Nutrient measurements

2.3.1 Photometric measurements and sample preparation

Concentrations of dissolved nutrients ammonium (NH_4^+), NO_3^- and nitrite (NO_2^-)) were measured colorimetrically with a spectrophotometer (UVmini-1240, Shimadzu) based on Lambert-Beer's law:

$$E = \lg\left(\frac{I_0}{I_1}\right) = \varepsilon \cdot c \cdot d \tag{4}$$

where E is the extinction, I_0 the initial light intensity, I_1 the intensity after passage through the sample solution, c and ε are the concentration and the molar absorptivity, respectively, of the absorbing compound and d the optical path length (cuvette length). All measurements were performed with blanks and standards. After sampling water samples were quickly transported to the laboratory where they were filtered (combusted GF/F-filters, 25 mm ∅, 0.7 µm) and frozen at -20 °C until measurements. In the presence of oxygen bacterial transformation processes of NH_4^+ start very fast. In this case reagents were added on-site to the unfiltered sample.

2.3.2 Nitrate

NO_3^- and NO_2^- concentrations were measured simultaneously using the method with spongy cadmium after Jones (1984). It is based on the reduction of nitrate to nitrite with spongy cadmium as reducing agent. Because of its toxicity, this method was replaced by the method after Doane and Horwáth (2003) and Miranda *et al.* (2001), where vanadium chloride (VCl_3) acts as the reducing agent. No differences between accuracy and detections limits of the two methods existed. All water samples taken after February 2014 from field and samples of the uptake-experiment were measured with the new method. Except samples from persulfate oxidation were still, measured with the spongy cadmium method. Extinction at 543 nm and potassium nitrate (KNO_3) as stan-

dard solution were used in both cases. Accuracy for the method was ± 0.011 µmol L^{-1} and the detection limit was ± 0.033 µmol L^{-1}.

2.3.3 Nitrite

Standard method for the determination of NO_2^- concentrations based on the reaction of NO_2^- and an aromatic amine to a diazonium salt, which react with another aromatic amine. Product is a red-coloured azo dye (adapted for sea water by Bendschneider and Robinson (1952)). Extinction at 543 nm and sodium nitrite ($NaNO_2$) as standard solution were used. Limits of detection for this method was 0–10 µmol L^{-1} and accuracy was 0.1 µmol L^{-1}.

2.3.4 Ammonium

Measurements of ammonia independent of method were reported always as sum of ammonia (NH_3) and NH_4^+. Reactions are complex and cannot be explained fully, but principal based on the creation of the blue color of indophenol by phenol and hypochlorite (or Trichloroisocyanuric acid) in presence of NH_4^+ ((Berthelot, 1859) as cited in Grasshoff and Johannsen (1972)). Extinction at 630 nm and ammonium chloride (NH_4Cl) as standard solution were used. Accuracy and detection limit was ± 0.2 µmol L^{-1} and ± 0.01 µmol L^{-1}, respectively.

2.3.5 Total dissolved nitrogen

The oxidation of organic nitrogen compounds with potassium peroxodisulphate to NO_3^- is described in Grasshoff and Johannsen (1972). 40 ml of a filtered sample was filled in a Teflon-tube. 10 ml of new oxidation solution - potassium peroxodisulphate ($K_2S_2O_8$) with low nitrogen contents (Merck Milipore 105091) - was added to the sample and the tube was closed immediately to avoid leakage of developing gases. Samples, standards and blinds run trough a program in a microwave(CEM), which was navigated via a PC-program (Synprep.). Ethylenediaminetetraacetic acid (EDTA) was used as standard solution. Detection limit was ± 0.2 µmol L^{-1}. After treatment NO_3^- in samples was analysed with the spongy-cadmium method.

2.4 Isotope measurements

Measurements of isotope ratios (heavy to light) were conducted in an isotope ratio mass spectrometer (IRMS). Solid samples, wrapped in capsules, were converted into gases by combustion in the elemental analyser (EA). Oxidation were done at 1020 °C in a helium gas stream while adding oxygen. The resulting gases (CO_2, N_2, nitrous oxides

and SO_2) were transported with a helium gas stream to a reduction tube (650 °C) where nitrous oxides were reduced to elemental nitrogen (N_2). Water from the combustion step is retained by a water-trap filled with a desiccant and the remaining gases (CO_2 and N_2) were separated in a GC-column at 50 °C. Amounts of CO_2 and N_2 were reduced in the split interface, so < 1 % reach the IRMS. In the IRMS ionisation of the gases may be achieved using electron ionisation. The ionised gases were accelerated, focused as a beam and separated in a single magnetic sector analyser by their mass. Detected ions were used to calculate the final stable isotope ratio which was calculated relative to a standard of known isotopic composition (Fig. 5). Isotope analyses were done with the IRMS Delta plus (Thermo Finnigan), where an EA Flash (Thermo 1112) was connected. Standards were injected via an Interface Conflow III. A second system include an IRMS Delta V Advantage (Thermo Scientific) with an Flash 2000 and an Interface Conflow IV (Finnigan) was also used. At last the GCPal Autosampler and Gasbench II were used for denitrified samples. The precision of the analyses was ± 0.2 ‰ for isotope ratios and 1 % for the elemental analysis. Isotope values were reported relative to reference gases (N_2, CO_2, CO), which were calibrated against international standards and were measured along with every sample. Furthermore, measurements of internal lab standards after every fifth sample allowed a correction of small equipment specific variations. The standard used for measurements are shown in Tab. 4.

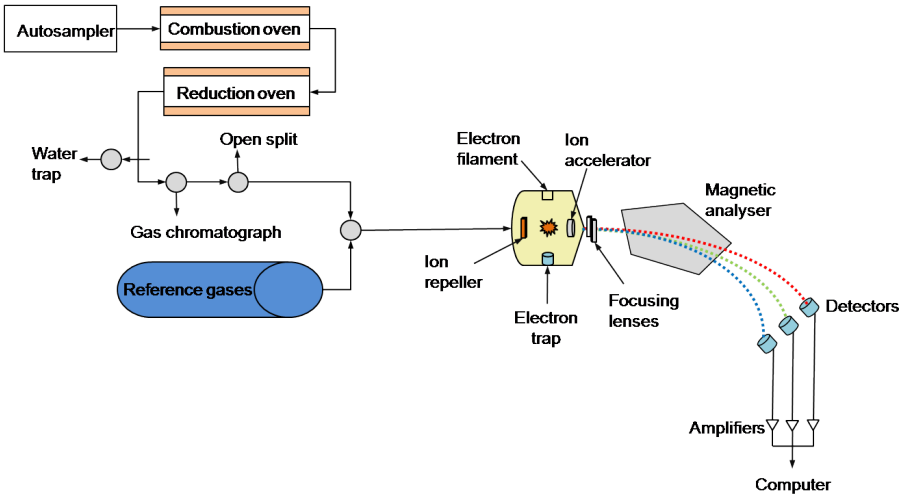

Figure 5: Schematic analysis in an isotope ratio mass spectrometer (redrawn after Muccio and Jackson (2009)).

Table 4: International and internal standards for the measurements of stable isotopes (from Deutsch (2005)).

Standards	δ^{15}N [‰]	δ^{13}C [‰]	δ^{18}O [‰]
IAEA-N1	0.4 ± 0.07		
IAEA-N2	20.3 ± 0.09		
IAEA C6		-10.43 ± 0.13	
NBS 22		-29.74 ± 0.12	
USGS 24		-15.99 ± 0.11	
IAEA KNO_3			25.1 ± 0.6
IAEA C3			32.2 ± 0.2
USGS 34			-27.9 ± 0.75
Peptone	5.8 ± 0.02	-22.11 ± 0.17	
Acetanilide	-1.7 ± 0.2	-29.81 ± 0.19	
Merck-KNO_3	-0.4 ± 0.16		24.6 ± 0.7

2.4.1 POM and macrophytes

To measure the isotopic composition (δ^{15}N and δ^{13}C), particulate organic carbon (POC)- and particulate organic nitrogen (PON)-content as well as CN-ratios, a defined volume of water samples was filtrated on combusted (450 °C, 4 h) GF/F-filters (Whatman®, 25 mm ∅, 0.7 µm). Filters were dried in an oven overnight at 60 °C, wrapped in tin capsules and pressed to a pellet. Samples of macrophytes were rinsed with deionized water and dried in a furnace overnight at 60 °C. For measurements, the macrophytes were ground to a fine powder, weighed, wrapped into tin capsules and pressed to a pellet. Tin capsules were combusted in the EA-analyser and isotopes of gases determined in the IRMS.

2.4.2 N_2O from NO_3^-

δ^{15}N and δ^{18}O in dissolved nitrogen (NO_3^- and NO_2^-) of seawater were measured with the denitrifier method after Sigman *et al.* (2001) and Casciotti *et al.* (2002). This method based on the conversion of NO_3^- to nitrite oxid (N_2O) by the denitrifying bacteria *Pseudomonas aureofaciens*, which is missing the NO_2^- reductase and conduct only an incomplete denitrification until N_2O. Colonies of bacteria were transferred to medium. After two days of incubation the culture was divided into 50 ml aliquots and centrifuged (8 min, 5000 rpm). The supernatant medium was decanted and then each

cell pellet was resuspended in spent medium. Concentrated cells were aliquotted into 20 ml headspace vials (2 ml per vial) with each vial presenting one analysis. Vials were capped with Teflon-backed silicone septa and crimp seals. Remaining NO_2^- was removed and anaerobic conditions were guaranteed by purging at 10–20 ml min^{-1} for 2 h with N_2 or helium. Purging gas was introduced through a 26-gauge needle. At the end of purging time the vent needle was removed. Sample addition was adjusted to a final sample size of 10–20 nmoles N. Incubations over night allowed the complete conversion of NO_3^- to N_2O. Vials were stored inverted to reduce leakage of N_2O. After the incubation, 0.1–0.2 ml of 10 N sodium hydroxide solution (NaOH) was injected into each headspace, which led to a pH > 12, lysed bacteria and the termination of the reaction. Measurements were proceeded in the IRMS. The precision of this method is ± 0.2 ‰ at concentration ≤ 0.1 µM.

2.4.3 Isotope composition of DON

To determine δ^{15}N-DON it was necessary to remove the inorganic nitrogen compounds like ammonium and nitrate (Sigman *et al.*, 1997), whose concentrations in comparison to the concentrations of DON were to high to neglect them. The sample volume was dependent on NO_3^- concentration per applied volume (1–7 µmol). Hence, a defined volume of a filtered sample was transferred into a 250 ml DURAN® flask. In two steps ammonium and nitrate were trapped in combusted (450 °C, 4 h) GF/D filters (Whatman®, ∅ 1 cm). These filters were sandwiched between two prepared Teflon-filters, which were washed with 10 % HCl and deionised water and dried in furnace at 60 °C overnight. To remove NH_4^+ in the first step the samples were treated to a salinity of 30. 1 g combusted magnesium oxide (MgO) (450 °C, 4 h) was added to each sample. pH had to be adjusted with 10 %-NaOH at least to 10.5. Finally filter-packages were transferred to DURAN flask, which were closed with a screw cap (including Teflon lip seal). The DURAN flasks were shaken (60 rpm) at 30 °C in a water bath for a week. During this time dissolved ammonium is formed to gaseous NH_3, which could diffuse into the Teflon-package. In this package the acidified GF-F filter shifted the NH_4^+-NH_3 equilibrium back to NH_4^+, which was trapped in the filter. In a second step, the ammonium-filter-package was replaced by a new one. pH was controlled before and after the addition of 150 mg combusted (450 °C, 4 h) Dervadas Alloy (DA) and adjusted again, if necessary. After another week of shaking the DURAN flasks, the pH was finally recorded. After every treatment all filter-packages were dried for two days in a desiccator with open sulphuric acid (H_2SO_4) and then in a furnace at 60 °C overnight. Teflon-filters were rejected and GF-D filters were wrapped into silver capsules, pressed to pellets and δ^{15}N-NH_4^+ and δ^{15}N-NO_3^- were measured in IRMS.

Resulting filtrates, just remaining DON, were filtered to reject chemicals and frozen at -20 °C until further measurements.

2.5 Water parameters

Temperature [°C], salinity, pH and dissolved oxygen [mg L^{-1}] were measured with the multiparameter meter HQ40d (Hach) in the field. Oxygen saturation [%] was calculated allowing for temperature-depending solubility of oxygen (Weiss, 1970).

2.6 Cultivation of macrophytes

Representative species of the three most abundant communities of macrophytes were collected at the DZBC, an inner coastal basin of the southern Baltic Sea: *Chara aspera* C.L.Willdenow, 1809 (small Characeae community), *Chara tomentosa* Linnaeus, 1753 (large Characeae community), and *Stuckenia pectinata* (L.) Börner, 1912 (syn. *Potamogeton pectinatus*) (Potamogeton-Myriophyllum community) (Schubert *et al.*, 2003). The plants were collected in spring, when the presence of undesirable epiphyton and bacterial biofilm is relatively low. These macrophytes were grown in the laboratory under controlled conditions (light:dark 12:12 h, temperature 15.5 °C). Habitat water instead of medium, was used and filtered (mesh width 55 µm) at least every 2nd week in all treatments. Species were planted in sediment in a cylindrical glass vessel. A fragment of Characeae, which is less demanding for nutrients, was embedded in artificial, autoclaved pure sea sand (AppliChem). Only phosphate was added to the sand (12 g P kg^{-1} sand). A thin layer of phosphate-free sand was spread on top as a barrier to inhibit the development of phytoplankton and bacteria in the water column (Wüstenberg *et al.*, 2011). A minimum of two knots of Characeae were planted to ensure growth. Rhizoid development required the removal of the apical part above the first developed ring of branchlets. *S. pectinata* was cultivated using roots, which were planted in natural sediment, because it is impossible to grow the plants on pure nutrient-free sediment.

2.7 Bacterial biofilm on macrophytes

The plants were placed in sterile 15-ml polypropylene tubes with 9 ml of sodium pyrophosphate (0.1 M $Na_4P_2O_7 \times 10\ H_2O$, $NaPP_i$). The associated biofilm was detached by ultrasonication for 60 s, followed by 15 min of vigorous shaking (110 rpm) and a second round of 60-s ultrasonication (Hempel *et al.*, 2008). The samples were fixed with of 1 ml formaldehyde (37 % final concentration in samples) and bacterial abundance (cells cm^{-2}) was determined using DAPI-fluorescence-microscopy. The surface

area of the macrophytes, necessary for calculations, was determined gravimetrically, as described by Steubing and Fangmeier (1992).

2.8 Statistical analysis

When necessary, the data set was constituted by logarithmical transformation to accomplish normality(Kolmogorov-Smirnov test) and equality of variances for a variable (Levene's test). Differences between group means were tested with the t-test or with analysis of variance (ANOVA). Post-hoc tests for multivariate analysis were done with Tukey's HSD or Bonferoni. All tests were done at the 5 % significance level. Pearson correlation coefficient was calculated to consider a linear relation between two variables. All statistical analyses were done with IBM SPSS Statistics 22.

2.9 EOF analysis

Empirical orthogonal function (EOF) analysis is a multivariate method for data compressions noise and dimensionality reduction broadly used in meteorology and oceanography. EOF are looking for pattern that explain the maximum amount of variance in a dimensional data set. Variables which are a function of space (r - number of stations) and time (t - number of observations) are expanded into a finite series of EOFs and can be expressed as:

$$G(r,t) = \sum_{i=1}^{K} \vec{m}_i(r)\alpha_i(t) + noise \tag{5}$$

where $\vec{m}_i$(r) is the pattern fixed in space and α_i(t) is the time coefficient. K is the number of EOFs and G are the variables. Canonical correlation analysis (CCA) is a way of measuring the linear relationship between two multidimensional variables and were also performed with the EOF analysis.

3 DON - a potential nitrogen source for macrophytes

3.1 Introduction

The nutritional pollution through human activity and agriculture in catchment areas of coastal zones and estuaries have led to the increasing eutrophication of coastal waters and thus to a shift in plant communities, from macrophytes to phytoplankton dominated systems (Gocke *et al.*, 2003; Kovtun *et al.*, 2009; Munkes, 2005; Schumann *et al.*, 2006). Macrophytes can therefore, be used as an indicator species in assessments of the good environmental status of a water body. However, to do so requires a detailed understanding of the mechanisms underlying nutrient uptake and the growth of these key species.

Abiotic factors such as light limitation and sedimentation were shown to indirectly influence growth (Angelstein *et al.*, 2009; Kovtun-Kante *et al.*, 2014; Schaible and Schubert, 2008). Another important aspect is the competition for nutrients between macrophytes and phytoplankton. A number of studies have examined the role of phosphorus as a limiting factor (Angelstein and Schubert, 2009; Reid *et al.*, 2000; Rip *et al.*, 2007). Although nitrogen limitation is less well explored, it has been documented in freshwater and marine environments (Bianchi *et al.*, 2000). In examining the mechanisms of nitrogen limitation, both the sources (sediment vs. water column) of the different nitrogen species and the ability of primary producers to assimilate them must be considered.

The two forms of nitrogen, dissolved organic nitrogen (DON) and dissolved inorganic nitrogen (DIN), differ in their availability (Stepanauskas *et al.*, 2000). DON accounts for anywhere between 20 and 90 % of the total nitrogen pool (Petrone *et al.*, 2009; Seitzinger and Sanders, 1997). However, its concentration was previously thought to be small and was usually not included in studies of the nitrogen uptake by phototrophic organism. In addition, 20–30 years ago DON was considered to be largely refractory and was thus ignored as a nutrient source. This erroneous conclusion was based on the complex composition of DON (Bronk, 1997), which includes the poorly decomposable humic and fulvic fractions. However, once DON was identified as a nutrient source the conversion of DON into biomass by phytoplankton and microorganisms was demonstrated on short time by several groups (Andersson *et al.*, 2006; Berg *et al.*, 1997; Berman and Chava, 1999; Bronk *et al.*, 1994; Fiedler *et al.*, 2015). The uptake of DON by macrophytes is of particular interest given that Tyler *et al.* (2005) showed that the nitrogen requirement of non-rooted red and green algae can be satisfied to a significant extent by DON. Subsequently, the uptake of DON was also demonstrated in sea grasses (La Nafie *et al.*, 2014; Van Engeland *et al.*, 2011; Vonk *et al.*, 2008), but

whether it also occurs in other rooted macrophytes is unknown.

Unlike phytoplankton, which derives their nutrients only from the water column, rooted submerged macrophytes are also able to use nutrients from the sediments. Thus, studies of the uptake of nutrients by macrophytes must consider both the roots and shoots. Nutrient uptake by the roots of submerged aquatic plants and the mechanism of nutrient transport has been often discussed, but are still, subjects of debate in the literature (Agami and Waisel, 1986; Takayanagi *et al.*, 2012; Wilson *et al.*, 1988). In comparative terms, the rhizoids of rooted macrophytes often comprise ≤ 10 % of the total algal biomass (Brenkert and Amundsen, 1982). The low biomass of rhizoids compared to phylloids suggests that their main function is to anchor plants in the sediments, with nutrient acquisition playing only a minor role (Sutcliffe, 1959). Many studies have provided support for this hypothesis, by showing that the nitrogen requirement of macrophytes can be fulfilled solely by uptake via the shoots (Madsen and Cedergreen, 2002). In contrast, others have shown that both shoots and roots substantially contribute to the nutrient supply (Carignan and Kalff, 1980; Nichols and Keeney, 1976), albeit in different, species-specific proportions. Hence, both the role of rhizoids and the mechanisms of nutrient transport are subjects of debate in the literature (Agami and Waisel, 1986; Takayanagi *et al.*, 2012).

Due to the lack of transpiration in submerged plants, nutrient transport must rely on alternative mechanisms (Raven, 1981) as, e. g., cytoplasmatic streaming. Most submerged macrophytes have vascular bundles, which in rooted macrophytes allow the transport of phosphorus e. g. in both directions, downwards (basipetal) and upwards (acropetal) (Angelstein and Schubert, 2009; Littlefield and Forsberg, 1965). In plants such as Characeae, which lack vascular bundles, nutrient acquisition remains to be explained. Previous studies on uptake of nutrients have either disregarded or at least tried to remove the biofilm before the experiments. To our knowledge, there is no possibility to obtain the macrophytes axenic. In this study, naturally occurring biofilm is quantitatively contributed to nutrient uptake. A phenomenon that also happens in nature, however, we did not aim to determine the proportions of the bacterial biofilm (belong to the bacteria, diatoms and attached algae) involved in the uptake of nitrogen compounds. Macrophytes and its bacterial biofilm were considered together. We hypothesized that: (1) DON provides an alternative to DIN as a nitrogen source that allows the successful growth of macrophytes, as suggested in other studies (Mozdzer *et al.*, 2010). (2) The uptake of each nitrogen source via the roots is as important as via the shoots. Thus, the aims of this study were (1) to demonstrate the uptake of DON vs. DIN (nitrate and ammonium) by rooted submerged macrophytes and (2) to determine whether shoots and roots are largely responsible for nutrient uptake and whether transport in fact, occurs. We therefore, used ^{15}N-labelled ammonium, nitrate, and an amino-acid mixture

(as DON) and examined the uptake and translocation of these nitrogen sources in three common macrophytes found in inner coastal, heavily eutrophied waters. In addition, the microbial biofilm and its role in nutrient uptake was considered.

3.2 Experimental set-up

The uptake and translocation of nutrients were investigated using three different ^{15}N-labelled substrates. Sodium nitrate and ammonium chloride (both 99 % ^{15}N) were added as inorganic compounds. An amino-acid solution (Sigma Aldrich, 17 amino acids, 99 % ^{15}N) represented easily accessible DON (Fig. 6). In the following, we refer to the upper and lower compartments of all species as the shoots and roots, even though in charophytes they are correctly denoted as phylloids and rhizoids. To compare the different substrates directly, the same concentrations were used in all treatments. In the highly eutrophied Darss-Zingst Bodden Chain (DZBC), the typical ammonium concentration in the water column during the growing season is $\backsim$10 µmol L^{-1}, which was therefore, the concentration used in all treatments, with 10 % of each solution enriched with ^{15}N. Incubation time was based on uptake kinetics determined in preliminary experiments and were set to 5 h. Subsamples of macrophytes were taken at the beginning of the experiment (natural abundance) and after 5 h of incubation (enrichment determinations).

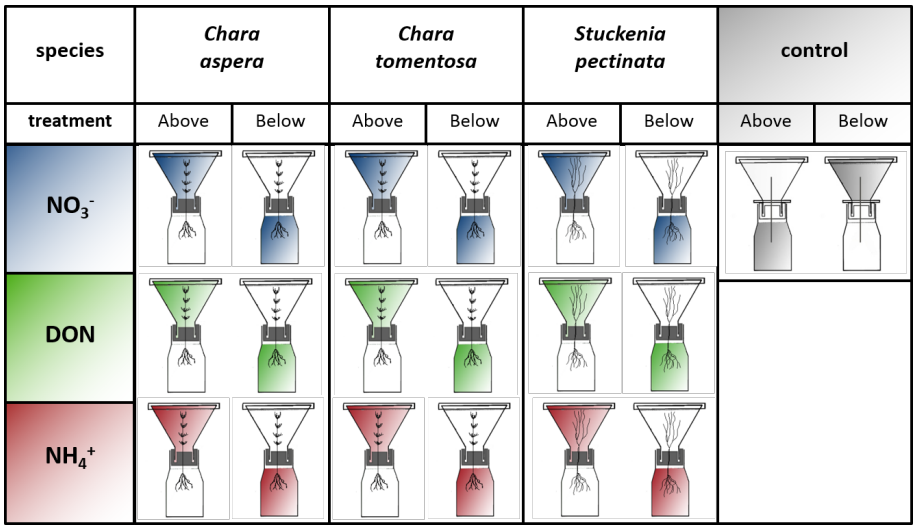

Figure 6: Overview of substrates, species and treatments used for the experiment.

The samples were briefly rinsed with deionized water and dried in an oven overnight

at 60 °C. For measurements, the macrophytes were ground to a fine powder, weighed, and wrapped into tin caps. Nitrogen stable isotope measurements were done with Thermo Scientific instruments. The isotope ratio mass spectrometer (IRMS) (Delta V Advantage) was connected to an elemental analyser (Flash 2000) via an open split interface (Interface Conflow IV). N-contents were determined along with the $\delta\ ^{15}$N analysis (EA-IRMS). The standard substance acetanilide (Merck) was used for calibration of particulate nitrogen measurements. N_2 as standard gas was calibrated against the IAEA standard substances (N1, N2, N3). The precision of the analyses was better than 0.2 ‰ $\delta\ ^{15}$N and 1 % for the elemental analysis.

To compare the uptake and translocation of roots and shoots, a two-compartment apparatus (Frank and Hodgson, 1964) made up of an Erlenmeyer flask and glass funnel with an impermeable plug (Fig. 7), was assembled. The macrophytes were carefully inserted into the plug. We used two different treatments, mimicking above- and below-ground. For the former, ^{15}N-labelled substrates were added to the upper (shoot) compartment at a concentration of 10 µmol L^{-1}. To get a defined diffusion gradient, non-labelled substrates were added to the roots at a concentration of 1 µmol L^{-1}. The opposite was done for the below-ground (roots) treatment. Substrate uptake was determined by measurements of the labelled tissues (above-ground: shoots, below-ground: roots), and translocation by analysing the non-labelled compartments (above-ground: roots, below-ground: shoots). Controls were performed by inserting glass rods, instead of plants, into the setup.

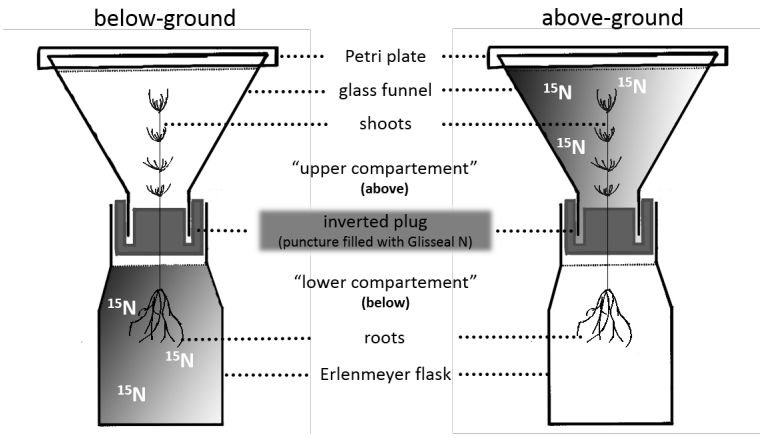

Figure 7: Experimental set-up for the below-ground (left) and above-ground (right) treatments. An Erlenmeyer flask (lower compartment) containing the roots and a glass funnel (upper compartment) containing the shoots were separated with an inverted plug. The macrophytes were inserted into the plug, which was then sealed with the fat Glisseal N to ensure its impermeability. A Petri plate was used to protect against evaporation. Image adapted from Angelstein and Schubert (2009).

3.2.1 Preliminary test

Before the actual experiment, several test runs were performed to determine the appropriate incubation time and to confirm the complete separation of the two reservoirs (above and below the plug).

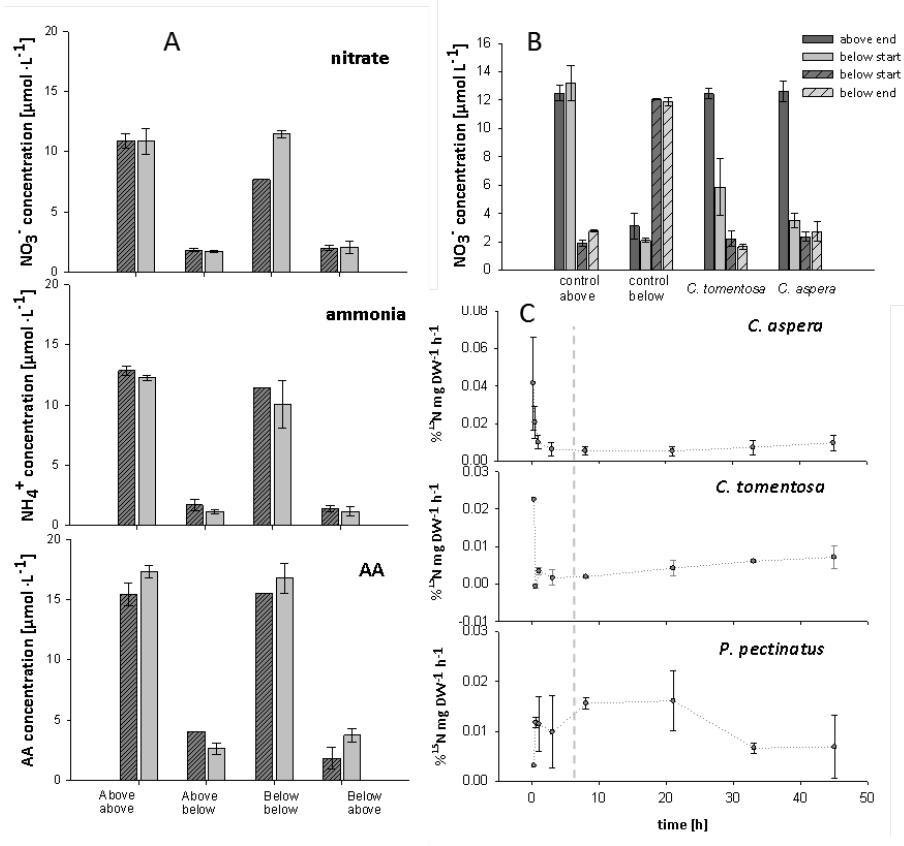

Figure 8: Control treatments were carried out using nitrate (upper panel), ammonium (middle panel), amino acid (lower panel). The impermeability of the plugs was confirmed by inserting glass rods instead of macrophytes. The start (striped, dark gray bars) and end (solid, light gray bars) values determined after 5 h of incubation are shown for above-ground (samples from upper and below part) and below-ground (samples from upper and below part) treatments. B - Preliminary test of the impermeability of the plug over a 2-week period (start values: dark gray bars, end values: light gray bars). Above-ground (nitrate added to the funnel) were performed with control, *C. tomentosa* and *C. aspera* and below-ground treatment only with control (nitrate added below to the flask). Control treatments were performed using a glass rod rather than the plants. Samples were taken from the upper (solid bars) and lower (striped bars) compartments. C - Uptake kinetics of the species *Chara aspera*, *Chara tomentosa*, and *Stuckenia pectinata*. The dotted line marks the end of the 5-h incubation time.

The incubation time was set based on the uptake kinetics of the three species. It needed to be long enough to obtain a clear signal while avoiding the risk of damaging the

plants. In accordance with the results of the test runs, the incubation time was set at 5 h, which allowed comparisons among the tested species (Fig. 8C). In preliminary tests of the permeability of the plug, nitrate was added to the upper (above-ground) or lower (below-ground) compartment at a concentration of 10 µmol L^{-1} and the apparatus was monitored for two weeks. No exchange between the two compartments occurred in flasks where glass rods were inserted. A decrease in nitrate concentrations occurred only in *C. tomentosa* and *C. aspera*. As there was no increase in nitrate in the lower compartment, its consumption by shoots of Characeae as a nutrient was assumed (Fig. 8B). Additional controls during the experiment were carried out as described above, by placing glass rods instead of the macrophytes, into the apparatus. Nitrate, ammonium, and the amino-acid mixture were added to the above-ground and below-ground compartments at concentrations of 10 µmol L^{-1}. Samples were taken from upper and lower compartment in both treatments. In all tests, the results showed no statistically significant exchange (Fig. 8A).

3.2.2 Data treatment

Isotopic fractions, instead of δ-values, were used according to Van Engeland *et al.* (2011). Isotope enrichment (E_{sample}) [atom %] was based on the difference between the end (F_{end}) and start (F_{start}) values of subsamples of the macrophyte, where F_{start} is the natural abundance of ^{15}N:

$$E_{sample} = F_{end} - F_{start} \tag{6}$$

Due to the adsorption of NH_4^+ onto any surfaces slight enrichment of ^{15}N were revealed, which is a well-known but often neglected experimental problem. Tests to minimize the enrichment of $^{15}NH_4^+$ on plant surfaces and to reduce the resulting errors in calculations of the uptake rates were performed (Fig. 9). Based on these measurements a correction factor for every species was determined 3 min after ammonium addition:

$$E_{sample} = F_{end}–F_{corr} - F_{start} \tag{7}$$

where F_{corr} is the isotope fraction of plant tissue after 3 min or more specifically the assumed absorption of $^{15}NH_4^+$ onto macrophyte surfaces. Specific nitrogen uptake rates, defined as V_{sample} [µmol ^{15}N mg DW^{-1} h^{-1}], were calculated as:

$$V_{sample} = \frac{PON \cdot sample}{time \cdot dryweight} \tag{8}$$

where PON is the nitrogen content of combusted macrophyte sample (µmol), the incubation time is 5 h, and the dry weight (DW) is the biomass of the plant tissue (mg).

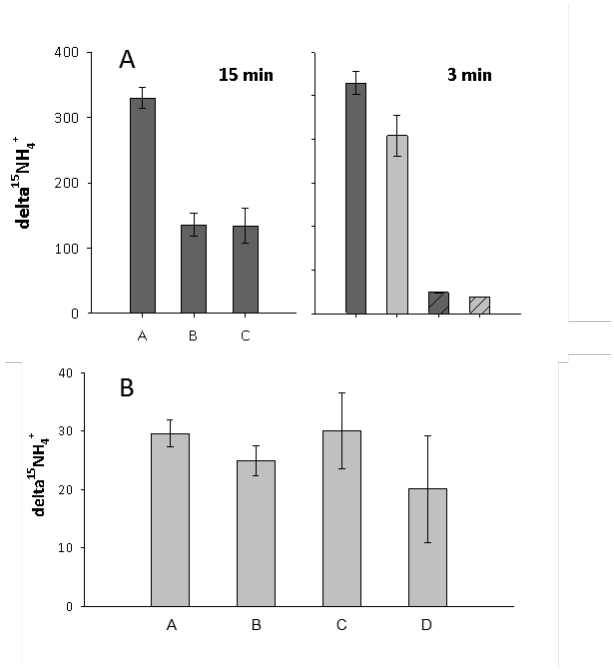

Figure 9: A – Preliminary test of the adsorption capacity of labelled ^{15}N-NH_4^+ after 15 min (left) and 3 min (right) of incubation. The macrophytes were incubated in 100 % $^{15}NH_4^+$(solid bars) or 10 % $^{15}NH_4^+$ (striped bars) solutions. Then the macrophytes were rinsed with hydrochloric acid (HCl) (dark gray bars) or deionized water (light gray bars). After a 15-min incubation, the macrophytes were rinsed immediately (A) or stored for 10 (B) or 20 (C) min in HCl solution and then rinsed. B - The change in ^{15}N after a 3-min incubation as a correction factor for ammonium adsorption by the shoots of *Chara aspera* (A), *Chara tomentosa* (B) and *Stuckenia pectinata* (C) and by the roots of *S. pectinata* (D). Error bars indicate the standard error.

Specific uptake rates were compared between different species and individuals with varying biomass. Corrections for available substrate concentration at the beginning of the experiment (t = 0) were achieved as follows:

$$\%V_{sample} = \frac{100 \cdot V_{sample}}{N_{added}} \tag{9}$$

where N_{added} is the available substrate and $\%V_{sample}$ [% mg DW^{-1} h^{-1}] represents the uptake rate per amount of available substrate (henceforth termed as uptake rate).

3.2.3 Statistical analyses

^{15}N enrichment in subsamples of macrophytes was calculated as the difference between the natural abundance of ^{15}N and the abundance after the incubation (paired t-test). Nitrogen uptake and translocation (presence of ^{15}N in non-labelled tissue) were determined based on comparisons of the start and end values of ^{15}N (t-test) of subsamples for the tested macrophytes. Translocation was detected based on the accumulation of ^{15}N at the start vs. the end of the incubation in the non-labelled compartment (roots in the above-ground and shoots in the below-ground experiments). Translocation rates were calculated just as uptake rates. Positive translocation rates per amount evidenced an enrichment of ^{15}N in tissues not directly in contact with the labelled substrate. Negative translocation rates per amount, were defined as those in which the starting value of ^{15}N in the tissues was greater than the final value after incubation, defined as no translocation. Normality (Kolmogorov-Smirnov test) was constituted by logarithmical transformation of ^{15}N enrichments and uptake/translocation rates. Significant differences in the uptake/translocation rate ($\%V_{sample}$) of the different substrates by the macrophyte species and their compartments (roots vs. shoots) were investigated using an analysis of variance (ANOVA). Differences between group means within the ANOVA were tested using Tukey's HSD test (species × treatment × substrate). All tests were done at the 5 % significance level. All statistical analyses were done with IBM SPSS Statistics 22.

3.3 Results

3.3.1 Uptake of nitrogen components

The results showed that ^{15}N enrichment occurred in all treatments except in the case of nitrate in the roots of *C. aspera* and of ammonium in roots of *C. tomentosa* ($p > 0.05$, t-test). For all other treatments ^{15}N enrichment occurred and based on these values, uptake rates could be calculated. These uptake rates (with respect to biomass, incubation time, available substrate concentrations) ranged from 0.07 to 0.3 $\%^{15}N$ mg DW^{-1} h^{-1}, representing the uptake rate per amount of available substrate (henceforth termed as uptake rate) (Fig. 10). In general, ammonium showed the highest uptake rates with a mean of 0.116 $\%^{15}N$ mg DW^{-1} h^{-1} ($p < 0.05$ vs. nitrate and AA, ANOVA, Tukey's HSD). Amino acids with a mean of 0.024 $\%^{15}N$ mg DW^{-1} h^{-1} were preferred over nitrate with a mean of 0.007 $\%^{15}N$ mg DW^{-1} h^{-1} ($p < 0.001$, ANOVA, Tukey's HSD).

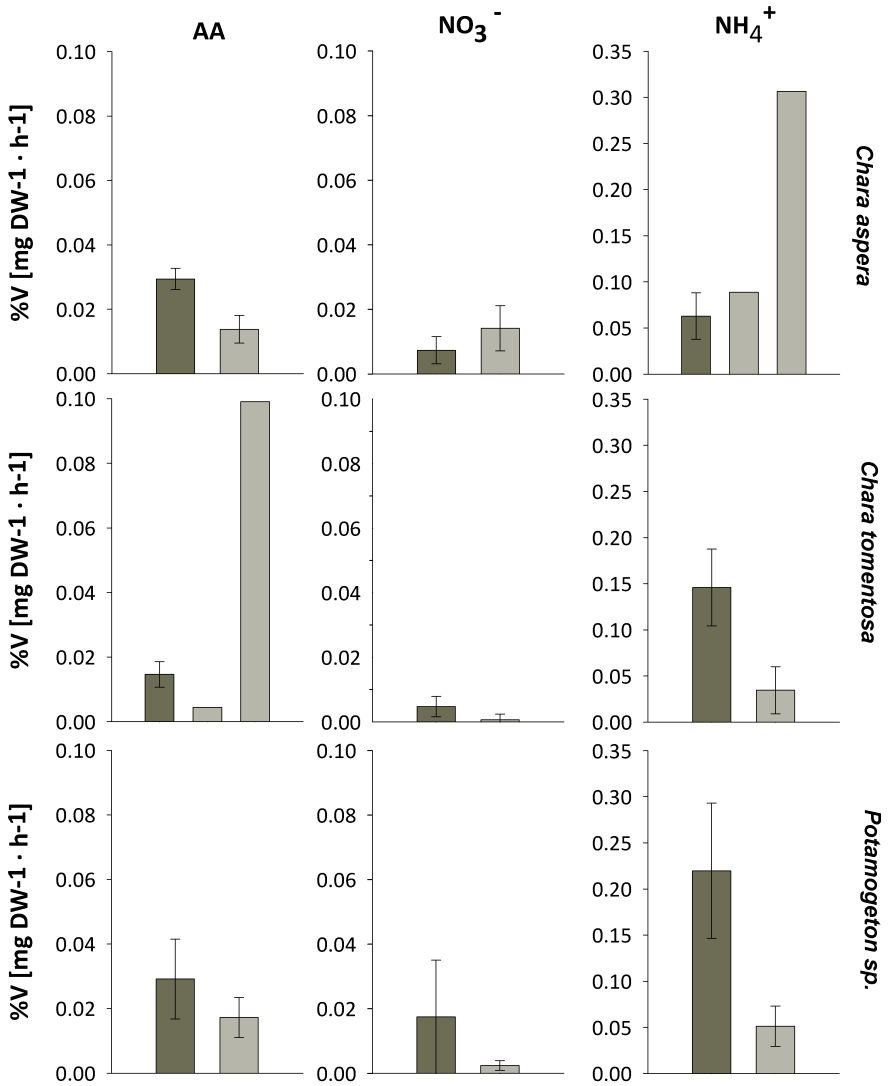

Figure 10: Normalized uptake rates (%V) of shoots (dark gray bars) and roots (light gray bars) of *Chara aspera* (top; n = 4), *Chara tomentosa* (middle; n = 4), and *Stuckenia pectinata* (bottom; n = 5). Three different ^{15}N-labelled substrates, an amino acids mixture (AA), nitrate (NO_3^-) and ammonium (NH_4^+) were tested. Note the different scale of the y-axis on the right side for the uptake rates of ammonium. Error bars indicate the standard error.

Only in *C. aspera* preferences for nitrate and amino acids were determined ($p > 0.05$, 1-way ANOVA, Tukey's HSD). As shown in Tab. 5, the preferential uptake of different substrates depended on the species and the tissue (roots or shoots). There were no

significant differences in uptake rates by the roots (above-ground) and shoots (below-ground). The exceptions were nitrate and ammonium ($p < 0.05$, t-test) uptake in *S. pectinata* and amino acids ($p < 0.05$, t-test) uptake in *C. aspera*, in which the rates were always higher in the shoots than in the roots. A comparison of the three species showed that uptake rates were lowest in *C. tomentosa* (Fig. 10) whereas those of *C. aspera* and *S. pectinata* were in the same range ($p > 0.05$, 3-way ANOVA, Tukey's HSD). Two bars for uptake rates and no standard deviation of ammonium in *C. aspera* and amino acids in *C. tomentosa*, are shown in Fig. 10, because we were forced to minimize the number of replicates to $n = 2$. The biomass of each replicate ($n > 2$) was too low, to get a clear signal from IRMS so we combined some subsamples to one sample. Although these data were excluded from statistical analysis, they are presented to demonstrate the wide variation in the responses of the same species to the same substrate.

Table 5: Results of the univariate ANOVA using the factors species, substrate, and treatment (above- and below-ground).

	df	F	Significance
Species	2	3.985	.260
Treatment	1	9.816	.003
Substrate	2	84.819	.000
Species + treatment	2	6.919	.003
Species + substrate	4	1.375	.259
Treatment + substrate	2	0.541	.586
Species + treatment + substrate	4	3.691	.012

3.3.2 Translocation of nitrogen components

^{15}N enrichment was determined in the roots of *C. aspera* for ammonium and nitrate ($p < 0.05$, t-test) and in the roots of *C. tomentosa* also for ammonium and nitrate ($p < 0.05$, t-test). The shoots of *C. tomentosa* did not show significant ammonium enrichment ($p > 0.05$), in contrast to the translocation rates (presence of ^{15}N), which were was statistically significant.

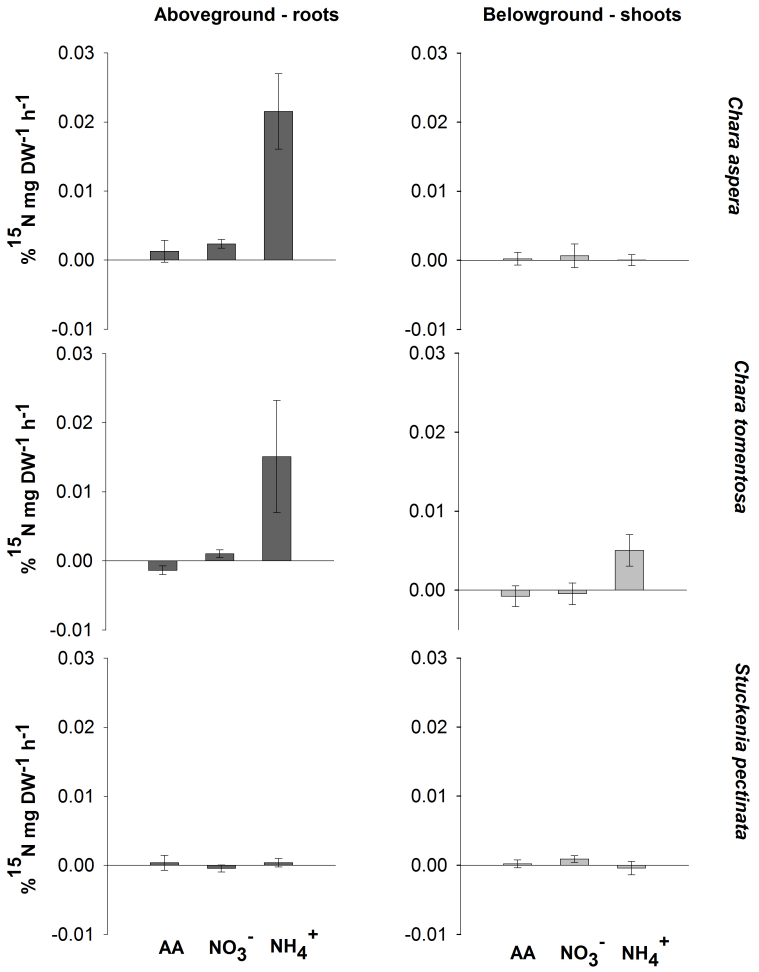

Figure 11: Normalized uptake rates (%V) of *Chara aspera* (top), *Chara tomentosa* (middle), and *Stuckenia pectinata* (bottom). Three different ^{15}N-labelled substrates, an amino acids mixture (AA), nitrate (NO_3^-) and ammonium (NH_4^+) were added to the above-ground (dark gray bars) and below-ground compartments (light gray bars). Macrophyte samples from above-ground were taken from the roots vs. below-ground from the shoots. Uptake in this form represented the detection of ^{15}N in the non labelled part of the plants. Error bars indicate the standard error.

Accordingly, in *C. aspera* positive translocation for ammonium ($21.5{\cdot}10^{-3} \pm 5.5{\cdot}10^{-3}$ µmol^{15}N mg DW^{-1} h^{-1}) and nitrate ($2.3{\cdot}10^{-3} \pm 0.6{\cdot}10^{-3}$ µmol^{15}N mg DW^{-1} h^{-1}) were determined only in the roots (Fig. 11), whereas in *C. tomentosa* ammonium translocation was positive in both the roots ($15.1{\cdot}10^{-3} \pm 8.1{\cdot}10^{-3}$ µmol^{15}N mg DW^{-1} h^{-1}) and the shoots ($5.3{\cdot}10^{-3} \pm 2.0{\cdot}10^{-3}$ µmol^{15}N mg DW^{-1} h^{-1}) (Fig. 11). For *S. pectinata*, the translocation rates in both compartments were below the detection limit for

all of the tested substrates (Fig. 11). Also no translocation could be detected for amino acids in any species.

3.3.3 Quantification of the bacterial biofilm

The biofilm was quantified as bacterial counts per unit cm^{-2} (Fig. 12A+B), with one count equal to one bacterial cell. The bacterial abundances of *C. tomentosa* and *C. aspera* were $7.0 \cdot 10^6 \pm 3.4 \cdot 10^6$ and $7.1 \cdot 10^6 \pm 2.6 \cdot 10^6$ cells cm^{-2}, respectively (n = 6 each). *S. pectinata* seems to have much lower bacterial abundances with only $3.2 \cdot 10^6 \pm 2.6 \cdot 10^6$ cells cm^{-2}, but differences between species were not significant ($p > 0.05$, df = 2, F = 3.209, 1-way ANOVA).

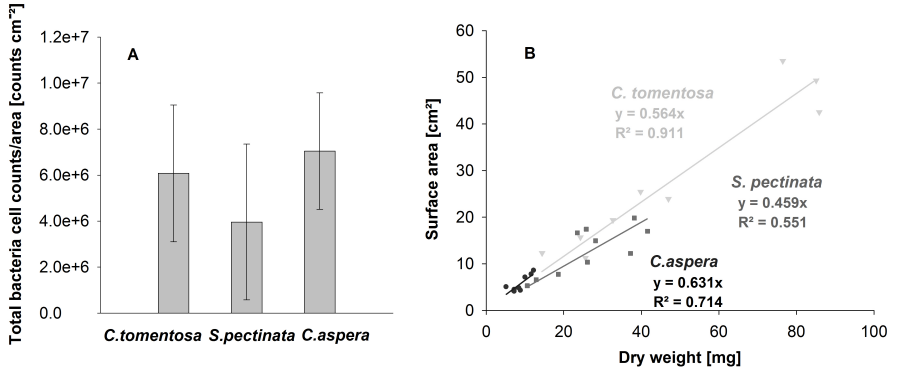

Figure 12: A - Bacterial abundance of *Chara tomentosa*, *Stuckenia pectinata*, and *Chara aspera*. Error bars indicate the standard error (n = 6). B - Surface area (in cm^2) of *C. tomentosa*, *C. aspera*, and *S. pectinata*, showing the regression line and equation (n = 10).

3.4 Discussion

3.4.1 Uptake rates and translocation of DIN and DON

Nitrogen is an essential macronutrient and potentially a limiting factor for the growth and distribution of submerged rooted aquatic plants (Kosten *et al.*, 2009; Meyer *et al.*, 2013). This study examined the uptake and translocation of dissolved organic and inorganic nitrogen compounds by the roots and shoots of three common species of the Baltic Sea and quantified for the first time the microbial biofilm of non-vascular rooting charophytes.

All three species of rooted macrophytes were able to take up organic and inorganic substrates, as determined by the enrichment of ^{15}N-labelled compounds. However,

the uptake rates differed for the three substrates and ammonium was the preferred nitrogen source, as shown in previous studies (Bornette and Puijalon, 2011; Ozimek *et al.*, 1993), followed by the amino-acid mixture, representing DON sources. The lowest uptake rates were those of nitrate. Overall, the uptake rates determined in our study are similar to those reported by other authors. For example, Van Engeland *et al.* (2011) found uptake rates of up to 0.25 $\%^{15}$N mg DW^{-1} h^{-1} ammonium and 0.05 $\%^{15}$N mg DW^{-1} h^{-1} amino acids. Nonetheless, the amino-acid mixture reflected an essential but only minor fraction of DON, which is more heterogeneous in natural ecosystems and dominated by refractory compounds (Bronk, 1997). Therefore, the uptake rates for amino acids in comparison with uptake under natural conditions may have been overestimated.

Uptake rates differed not only between substrates but also between species and were significantly lower for *C. tomentosa* than for *C. aspera* and *S. pectinata*. By no means this should be interpreted as a difference in their potential capability for DON uptake. The results should be interpreted with care because the rates might not represent the potential DON uptake capabilities. The differences in uptake rates between species could also be explained by the absence of a nutrient depletion. Retained nutrients originating from the habitat water despite the long cultivation period and the low nutrient demand of charophytes (O'Brien *et al.*, 2014) might have contributed to a sufficient nutrient supply, suppressing labelled DON uptake.

Our study demonstrated that the uptake of nitrogen by shoots and roots was, in most cases, statistically significant, as also shown in other experiments investigating inorganic nitrogen (Box, 1987; Shardendu and Ambasht, 1991) and phosphate (Littlefield and Forsberg, 1965). The two compartments are in same ratios capable for nutrient uptake, consistent with the nutritional independence of the roots. Andrews *et al.* (1984) showed that the shoots of *C. hispida* are able to assimilate 3–4 times more ^{14}C than the roots (both compartments were illuminated). They also found that the uptake of phosphate by shoots or roots depends on its external concentration. At external concentrations of up to 100 mmol m^{-3} phosphate uptake by the roots exceeded that by the shoots (Andrews, 1987). Thus, in general, while both roots and shoots are responsible for satisfying nutrient requirements, in our experiment there was little tendency of greater uptake by shoots than by roots. For *S. pectinata* (nitrate and ammonium) and *C. aspera* (AA), root and shoot uptake rates differed significantly, suggesting the existence of translocation mechanisms in these species.

In macrophytes, acropetal translocation may give these plants a clear advantage over phytoplankton, which can only access nutrients in the water column. The translocation values in our experiments were ten times lower than the uptake rates. This can be attributed to the absence of nutrient depletion during the cultivation period and the

short incubation time, such that the maximum rate for transport could not be achieved. However, translocation was not detected in *S. pectinata* although translocation should be physiologically possible as it is a vascular plant. By contrast, in both *C. tomentosa* and *C. aspera* the basipetal translocation of nitrate and ammonium was detected. In addition, a signal indicating the acropetal transport of ammonium was seen in *C. tomentosa*. Translocation in both directions was shown in other studies for Characeae (Box *et al.*, 1984; Littlefield and Forsberg, 1965). For example, Vermeer *et al.* (2003) reported acropetal rather than basipetal transport in *Chara spp.*. However, translocation of complex nitrogen substances in these species was not expected because the mechanism in charophytes has yet not been understood completely. Characeae, as non-vascular plants, differ from higher macrophytes in that they lack vascular bundles for translocation. The translocation mechanism of N compounds in charophytes is poorly understood, although diffusion is known to be involved in intercellular transport, and cytoplasmic streaming in the axial direction in intracellular transport (Bostrom and Walker, 1976; Goldstein and van de Meent, 2015; Mimura *et al.*, 1998; Raven, 2013; Smith, 1966). The latter has been well-documented in plants with very large cells and sufficient differentiation (Raven, 2003). Nutrient transfer takes place between internodal cells (which can be several centimetres long); these are linked with one another by small nodular cells and plasmodesmata. The driving force for nutrient transport via cytoplasmic streaming is the myosin-actin system (Shimmen and Yokota, 2004).
While our study did not investigate the mechanism of translocation, it did confirm the translocation of DIN by Characeae. Moreover, our results demonstrated a potential compensation of a nutrient deficiency in one of the two possible nutrient pools, the sediment or the water column, with the other. Macrophytes thus possess a growth advantage over phytoplankton.

3.4.2 Impact of bacterial biofilm

Whether DON is taken up directly or indirectly after bacterial conversion to inorganic nitrogen could not be determined in our experiments. Bacterial growth is a well-known problem in macrophyte experiments and it complicates the handling of sensitive and fragile species like those of the Characeae. Despite the controlled conditions in the laboratory during the macrophyte growth period, there was considerable bacterial growth as well. However, observations in the field of optically dense biofilm on macrophytes indicate a far higher number of bacteria per surface area of the plant under natural conditions. Despite our efforts to keep bacterial growth as low as possible during the study, the presence of bacteria on the plants may have facilitated the conversion of amino acids to NH_4^+; however, this should have been equal in all experiments and for

all species, allowing comparisons of the uptake rates. Cotner and Gardner (1993) found bacterial ammonium regeneration rates of 17–112 nM h^{-1} and assumed that dissolved free amino acids were the main substrate for ammonium regeneration. In the study of Kirchman *et al.* (1989), the direct metabolism of ^{15}N-labelled amino acids accounted for 50 % of total ammonium production. The association between macrophytes and bacterial biofilm and their mutual influence have long been recognized (Prowse, 1959). Caffrey and Kemp (1990) demonstrated that microbial communities regulate nitrogen availability for plant growth. Other studies followed the transfer of nitrogen from the sediment (roots of macrophytes) to shoot-colonized epiphytes (McRoy and Goering, 1974). According to Riis *et al.* (2012), 30 % of the available ammonium is taken up by epiphytes (including diatoms). The experimental conditions used in our study did not allow us to determine whether the biofilm on the three macrophyte species exerted positive (remineralization of DON to DIN) or negative (competition) effects.

3.5 Conclusion

This study demonstrates the uptake of DON originating from the water column and also the sediment in charophytes and the angiosperm macrophyte *S. pectinata*. Moreover, the ability to translocate inorganic nitrogen up- and downwards is important in a limiting system and leads to a competitive advantage over phytoplankton. Bacterial biofilm was considered qualitatively, but its negative or positive impact on uptake and hence growth of macrophytes has to be investigated further. However, the fact that amino acids as part of DON can be used directly or indirectly by the three tested macrophytes as a nitrogen source prove that DON compounds can significantly contribute to the overall nitrogen demand of the tested macrophytes.

4 Mapping macrophyte isotopic composition and C:N ratios in the Darss-Zingst Bodden Chain

4.1 Motivation

An increase in human population and associated changes in land use have caused increased nutrient loads to estuarine environments. In regions impacted by eutrophication, abundances of aquatic plants often decreased (Blindow, 2000; Castro and Freitas, 2006; Costanzo *et al.*, 2001; Kovtun *et al.*, 2009; Munkes, 2005). Macrophytes, especially charophytes, were found to be sensitive species and disappear first when the nutrient balance is disturbed. Their relevance as key species of polluted waters were precociously recognized (Krause, 1981).

Submerged macrophyte vegetation nearly vanished around 30–40 years ago in the DZBC (Schiewer, 2006) due to increasing nutrient inputs, decreasing water clarity and competition of shading and fast-growing phytoplankton blooms. The pressure on macrophytes is great and many studies were conducted to explain the relation regarding the light conditions and inorganic nutrients (Blindow *et al.*, 2003, 2002; Küster *et al.*, 2004; Schaible and Schubert, 2008; Selig *et al.*, 2009). Today, macrophytes reoccur and abundances irregularly increase again (Selig *et al.*, 2009). However, the water quality, despite great efforts, is still not ideal for macrophytes and the trigger explaining the patchy distribution and occurrence of macrophytes in the DZBC could still not be identified.

Physicochemical data like nutrient concentrations in the water column, salinity and phytoplankton biomass are authorized parameters, which were usually used to quantify levels of eutrophication. In many cases, relationships between the aforementioned parameters and the productivity or abundance of primary producers are not clear (Castro *et al.*, 2007). Another disadvantage of these techniques is that e. g. nutrient concentrations are rapidly lost as the pollutant is diluted and at best, provide only an instantaneous view of nutrient input (Gartner *et al.*, 2002). Additionally, the analysis of nutrients and phytoplankton biomass are laborious, time-consuming and costly and often only provide a pattern of effluent dispersal.

Modern techniques use carbon and nitrogen stable isotope compositions of DIC and DIN (like nitrate or ammonium), POM and primary producers to identify biological processes and environmental changes. Furthermore, stable isotope composition of aquatic plants where shown to reflect the origin of nutrients and their alterations (Chang *et al.*, 2009; Deutsch and Voss, 2006; Fry and Sherr, 1984; Kendall *et al.*, 2001). Since then stable isotope signatures were investigated in various aquatic systems for a numerous of taxonomic groups (e. g. phytoplankton, macroalgae, seagrasses

and macrophytes) in order to reveal photosynthetic or physiological pathways (Bronk and Glibert, 1991; Maberly *et al.*, 1992; McMillan *et al.*, 1980) which cause high variations in δ^{13}C and δ^{15}N composition. Other studies evaluate whether stable isotopes of primary producers were useful indicators of environmental changes (Chang *et al.*, 2009; Cole *et al.*, 2004; Costanzo *et al.*, 2003, 2001), determine food web dynamics (Bode *et al.*, 2006; Deegan and Garritt, 1997) or identify sources of POM (Kendall *et al.*, 2001; Nakatsuka *et al.*, 1992).

At the coast of Galicia (Spain) δ^{15}N of stored samples of the macroalgae *Fucus sp.* as an indicator of eutrophication were measured to monitor contamination of coastal areas from 1990–2007. Results of this investigation showed that δ^{15}N in macroalgae could be used for evaluating success of environmental policies, such as those outlined in the European Water Framework Directives (Water Framework Directive 2000/60/EC) and aimed at supporting the creation of programmes that determine the state of aquatic environments (Viana *et al.*, 2011). Also Costanzo *et al.* (2001) used nitrogen isotope signatures of macrophytes within an estuary to localize anthropogenic impacts. They established maps of stable isotope compositions of macrophytes as bioindicators for nitrogen sewage distribution for protection of aquatic systems in Australia. Such contoured results are important tools for nutrient reduction strategies and help to understand the wide range of stable isotopic variation in macrophytes. The range of δ^{15}N is –15 to 20 ‰ (Bode *et al.*, 2006) and of δ^{13}C -30 to -20 ‰ (Kendall *et al.*, 2001). This great variability in isotope ratios of nitrogen and carbon in macrophytes are based on (1) different sources of dissolved inorganic carbon and dissolved inorganic nitrogen, (2) possible fractionation processes and (3) intracellular concentrations (Fry and Sherr, 1984). These factors can lead to a great variation in isotope ratios in space, time and species.

Based on this background the goal of this work was analyse isotopic signatures and also the C:N ratio of existing macrophytes in the DZBC in detail over a year to find pattern. Possible variations and obvious pattern must be considered when relating isotopic composition of macrophytes to environmental variables and δ^{13} and δ^{15} values in carbon and nitrogen sources. Macrophytes isotope composition were analysed to evaluate whether they are additional indicators of eutrophication to the existing indexes like chlorophyll, nutrient concentrations or Secchi depth. The following patterns were investigated:

A. Different parts of individual plants:

- Younger plant parts are expected to discriminate more against ^{15}N and ^{13}C compared to older parts or the roots.

B. Annual pattern in relation with primary production:

- A seasonal pattern (similar to nutrient concentrations and phytoplankton development) is expected for stable isotope composition in macrophytes.
- During summer when macrophytes grow and assimilate isotopic values were expected to be higher than in winter, because they reflect the sources.

C. Changes across a salinity gradient:

- Along the salinity gradient of the estuary the impact of anthropogenic nutrient inputs and also isotope composition were expected to decrease gradually.
- Stations with high nutrient inputs like the river Recknitz are expected to be enriched in heavy isotopes.
- Marine, nutrient-poor sites were expected to have lower isotopic ratios.

D. Intra-specific physiological pathways:

- Differences between species of one genus are expected to have small, if any differences in isotopic composition, because they use the same physiological pathways.
- Greater differences in isotopic composition are expected between different genera or groups like the non-vascular and vascular plants, where different translocation or uptake mechanism can lead to isotopic discrimination.

4.2 Sampling

Macrophyte samples were collected bi-weekly (June–October 2013 and March–June 2014) to monthly (November 2013–February 2014) over a whole year and analysed for nitrogen and carbon stable isotopic compositions as well as C:N ratios. Four sites along the salinity gradient of the Darss-Zingst Bodden Chain were investigated: Pütnitz, Michaelsdorf, Dabitz and Nisdorf, which represent every single bodden. Station Pütnitz is a decommissioned airport around 5 km remote from the city Pütnitz. Samples were taken near shore in a water depth < 1 m. The angiosperm, vascular plants *Stuckenia pectinata* and *Myriophyllum spicatum* and also the non-vascular Characeae - *Chara aspera*, *Chara baltica*, *Chara tomentosa* and *Chara canescens* were species found in the DZBC and collected for analysis (Fig. 13).

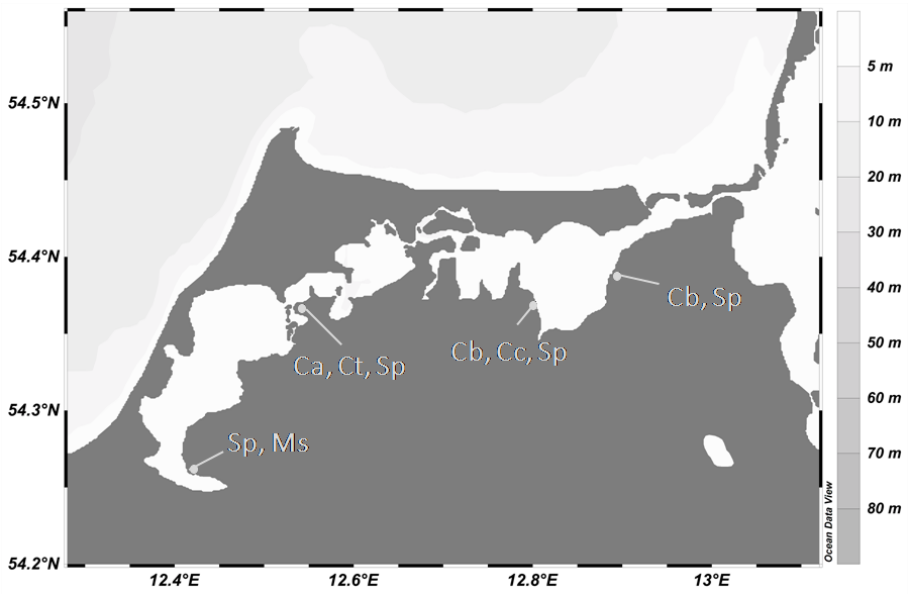

Figure 13: Distribution of *Chara aspera* (Ca), *Chara baltica* (Cb), *Chara canescens* (Cc), *Chara tomentosa* (Ca), *Myriophyllum spicatum* (Ms) and *Stuckenia pectinata* (Sp) in the Darss-Zingst Bodden Chain. The map was modified from Ocean Data View Version 4.7.4 (Schlitzer, 2004).

Species determination was done morphologically by identification keys of Krause (1997) for charophytes and Van de Weyer and Schmidt (2011) for vascular plants. Analysis of nitrogen and carbon stable isotopic compositions and C:N ratios of dried macrophytes were conducted in the IRMS. For further details see section 'Material and Methods'.

4.3 Results

4.3.1 Variability among plant parts

Differences in isotopic composition and C:N ratios among individual plants were studied. Therefore, macrophytes were cut in four nearly identical parts: the apical top, two segments under the apical top and the root/rhizoid. Distinct differences were expected between segments. To see pattern, measured values were given as a ratio of one part to the mean of all four parts. In general the δ^{15}N, δ^{13}C and C:N ratio of the apical top, second part, third part and root did not differ significantly to the mean of all parts ($p > 0.05$, ANOVA). Fig. 14 illustrates this exemplary for the δ^{15}N values in *S. pectinata* at the station Dabitz.

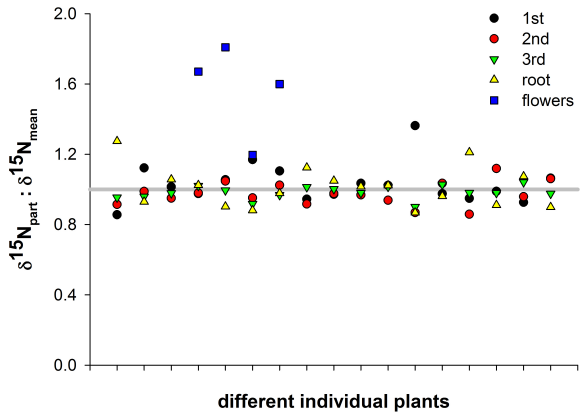

Figure 14: Comparison of parts from individual macrophyte plants with 1st = apical tip, 2nd = segment under apical tip, 3rd = segment under 2nd, roots and flowers for *S. pectinata* at station Dabitz. Grey bar indicates a ratio of part to mean of all parts of 1. Values are given as ratio of a part to the mean of all parts.

With a few exceptions significant differences among plant parts ($p < 0.05$) existed for δ^{13}C and C:N ratio for some species at certain stations (Tab. 6). Based on the total data set, these exceptions are negligible and for further analysis the average of all parts will be used.

Additionally samples of flowers, if available, were compared to the other plant parts. Flowers were only found in summer for *S.pecitnata* at station Pütnitz, Dabitz and Nisdorf and for *M. spicatum* (species only at Pütnitz). Flowers of *M. spicatum* were significantly different compared to the mean of other plants for δ^{15}N, δ^{13}C and C:N ratio. Results for *S. pectinata* were non-homogeneous. For δ^{13}C no significant differences could be shown. δ^{15}N values in flowers differ from other plant parts at station Dabitz (Fig. 14) and Nisdorf, but not at the Pütnitz. C:N ratio in flowers differ from other plant

parts at station Dabitz and Pütnitz, but not at Michaelsdorf.

Table 6: Exceptions for significant differences in segments of macrophytes.

Station	Species	variable	segments	p
Dabitz	*Stuckenia sp.*	root vs. apical top	δ^{13}C	0.011
Nisdorf	*Stuckenia sp.*	root vs. second part	δ^{13}C	0.037
Pütnitz	*M. spicatum*	root vs. second part	δ^{13}C	0.048
	M. spicatum	root vs. apical top	C:N	0.037
	M. spicatum	root vs. second part	C:N	0.000
	M. spicatum	root vs. third part	C:N	0.001
Michaelsdorf	*Chara aspera*	root vs. second part	C:N	0.002
		root vs. third part	C:N	0.003

4.3.2 Seasonal pattern

To study the seasonal pattern of isotopic and C:N ratios in macrophytes, samples (n = 147) were taken bi-weekly to monthly from June 2013 to July 2014. Fig. 15 shows the development of isotopic and elemental values of different species, but for further analysis, the whole community of macrophytes and not single species were considered.
The δ^{15}N and δ^{13}C values in macrophytes over an annual cycle ranged from -4.2 to 13.5 ‰ and from -17.6 to -6.7 ‰, respectively. With a range of 18.9 ‰ the variability of δ^{15}N was greater than δ^{13}C with only 10.91 ‰. Due to the high variability of isotopic ratios over the year, data were pooled to test for seasons (summer versus winter). Samples collected from June to September 2013 represent the summer period (n = 73) and December 2013 to March 2014 the winter period (n = 22). Independent of species and stations the δ^{15}N ratio in summer ranged from 0.4 to 13.5 ‰ and in winter from -0.3 to 9.5 ‰. The mean of δ^{15}N values in winter was 6.3 ± 2.2 ‰ and in summer 6.4 ± 2.7 ‰. A high variation but no seasonal patterns between summer and winter could be found for δ^{15}N ($p \geq 0.05$; F = 0.936; independent t-test). The δ^{13}C values of macrophytes were also not significantly different in summer and winter ($p \geq 0.05$; F = 0.041; independent t-test). The mean in summer was -12.3 ± 2.0 ‰ and in winter -13.6 ± 2.0 ‰. δ^{13}C values in summer ranged from -6.7 to -17.4 ‰ and in winter from -10.4 to -17.5 ‰. In contrast, the C:N ratios were significantly higher in summer with a mean of 30.5 ± 1.3 compared to winter with a mean of 20.9 ± 4.7 ($p \leq 0.05$; F = 8.469; independent t-test). The C:N ratios in summer ranged from 9.0 to 65.8 and in winter from 16.0 to 33.2 (Fig. 15).

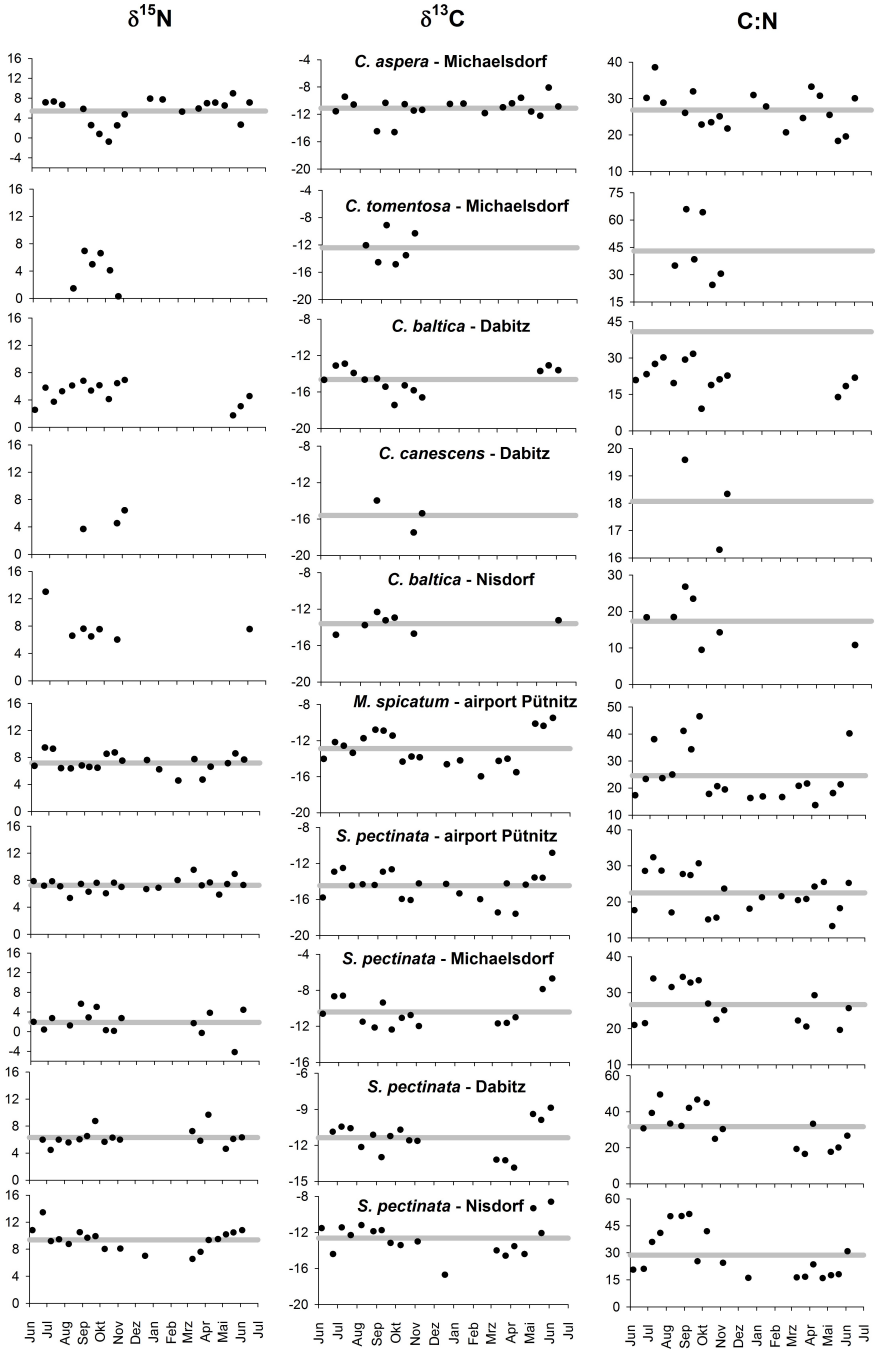

Figure 15: Seasonal changes of δ^{15}N, δ^{13}C and C:N ratios over an annual cycle (June 2013–July 2014) collected along a salinity gradient in six species at four sites of the Darss-Zingst Bodden Chain. Grey bars show the average. Beware of different scale in ordinates for δ^{13}C and C:N ratios.

4.3.3 Spatial pattern

Samples of macrophytes at four sites along the increasing salinity and decreasing eutrophication gradient of the DZBC were compared: Pütnitz (n = 42), Michaelsdorf (n = 43), Dabitz (n = 35) and Nisdorf (n = 27) (Fig. 16). Differences were expected due to different influences of inputs (nutrient-rich freshwater from catchment area vs. relatively nutrient-poor marine water from the Baltic Sea).
Averages of δ^{15}N values were at Pütnitz 7.2 ± 1.1 ‰ and at Dabitz 5.6 ± 1.6 ‰. The lowest average of δ^{15}N was found at station Michaelsdorf with 3.8 ± 2.9 ‰ and highest at station Nisdorf with 8.8 ± 2.2 ‰. With -4.2 ‰ the lowest δ^{15}N value was found at station Michaelsdorf and the highest with 14.7 ‰ at station Nisdorf. All stations were significantly different ($p < 0.005$, Kruskal-Wallis-test) and no spatial pattern could be found for δ^{15}N or δ^{13}C. Averages of δ^{13}C were at Pütnitz -13.76 ± 2.0 ‰, at Dabitz -13.1 ± 2.2 ‰ and at Nisdorf -13.0 ± 1.7 ‰. No distinct differences existed between these stations ($p > 0.05$; ANOVA; post-hoc Bonferroni).

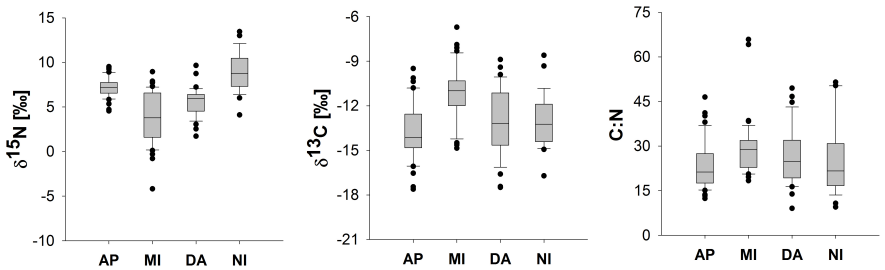

Figure 16: Box-Whisker-Plots showing the median (horizontal line inside boxes) and variability (box ends show interquartile range, vertical lines the full range, dots the outliers) of δ^{15} N, δ^{13}C and C:N ratio measured at four stations in the Darss-Zingst Bodden Chain. Stations are arranged by salinity gradient (left to right increasing) with P - Pütnitz, MI - Michaelsdorf, DA - Dabitz and NI - Nisdorf.

In contrary, the station Michaelsdorf was enriched compared to other stations with a δ^{13}C ratio of -11.0 ± 1.9 ‰ ($p \leq 0.005$; ANOVA; post-hoc Bonferroni). Lowest δ^{13}C ratio was found at station Pütnitz with -17.6 ‰ and highest at station Michaelsdorf with -6.7 ‰. Also the C:N ratios showed no pattern with means at Pütnitz of 23.3 ± 7.9, at Michaelsdorf of 29.3 ± 9.5, at Dabitz of 26.6 ± 9.8 and at Nisdorf of 25.5 ± 23.0. A significant difference existed between the stations Pütnitz and Michaelsdorf ($p \leq 0.05$; ANOVA; Bonferroni post-hoc).

Table 7: Overview of δ^{15}N, δ^{13}C and C:N ratios for all collected species from the DZBC at the stations P - Pütnitz, MI - Michaelsdorf, DA - Dabitz and NI - Nisdorf. Samples were collected regularly over a whole year. Data are arranged by species and their locations.

Species	Station	δ^{15}N				δ^{13}C				CN			
		Min	Max	Mean	SD	Min	Max	Mean	SD	Min	Max	Mean	SD
C. aspera	MI	-0.8	9.0	5.3	2.6	-14.6	-8.1	-11.2	1.6	18.3	38.5	27.0	5.1
C. baltica	DA	1.7	6.9	4.9	1.7	-17.4	-12.9	-14.6	1.3	9.0	31.7	22.0	6.2
C. baltica	NI	6.0	13.0	7.6	2.4	-14.8	-12.3	-13.6	0.9	9.5	26.8	17.9	6.4
C. canescens	MI	3.7	6.4	4.7	1.4	-17.5	-14.0	-15.5	1.8	16.3	26.2	20.1	1.7
C. tomentosa	MI	0.3	6.9	3.6	2.6	-14.9	-9.1	-11.9	2.3	30.5	65.8	44.1	15.6
M. spicatum	AP	4.6	9.5	7.2	1.4	-16.0	-9.5	-12.9	1.9	13.6	46.5	24.6	13.5
S. pectinata	AP	5.3	9.5	7.3	1.0	-17.6	-10.8	-14.6	1.7	12.4	32.3	22.0	6.1
S. pectinata	MI	-4.2	5.6	1.9	2.7	-12.3	-6.7	-10.4	1.8	19.6	34.3	26.7	6.1
S. pectinata	DA	4.5	9.7	6.3	2.0	-13.9	-8.9	-11.4	1.4	16.5	49.5	31.7	11.6
S. pectinata	NI	6.5	13.5	9.5	2.0	-16.7	-8.6	-12.6	1.9	15.9	51.5	28.8	12.7
total		-4.2	13.5	6.1		-17.6	-6.7	-12.7		9.0	65.8	26.2	

4.3.4 Intra-variability of species

Differences among six species were tested: *C. aspera* (n = 20), *C. baltica* (n = 23), *C. canescens* (n = 4), *C. tomentosa* (n = 6), *M. spicatum* (n = 22) and *S. pectinata* (n = 72). It was expected that there are differences between vascular and non-vascular species. Independent of stations and time δ^{15}N values ranged from -4.2 ‰ to 13.5 ‰ (both lowest and highest value in *S. pectinata*). Mean per species ranged from 3.6 to 6.9 ‰ (*C. tomentosa* and *M. spicatum*) (Fig. 17).

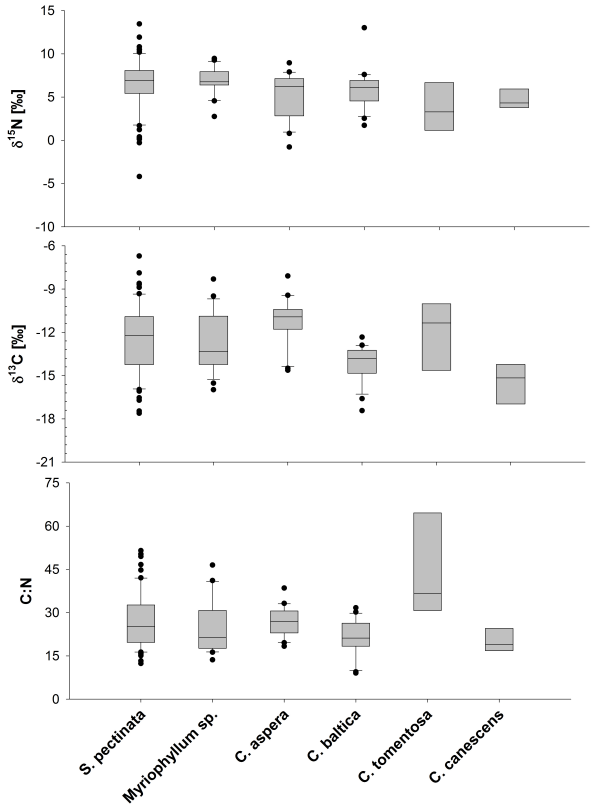

Figure 17: Box-Whisker-Plots showing the median (horizontal line inside boxes) and variability (box ends show interquartile range, vertical lines the full range, dots the outliers) of δ^{15}N, δ^{13}C and C:N ratio measured in six species of vascular and non-vascular plant collected from the Darss-Zingst Bodden Chain over an annual cycle.

No significant differences between species existed for δ^{15}N ratios ($p \geq 0.05$; ANOVA; Bonferroni post-hoc). But there was a significant difference between the vascular (6.6 ± 2.8 ‰) and non-vascular (5.3 ± 2.5 ‰) species ($p \leq 0.05$; independent t-

test). δ^{13}C values ranged from -6.7 ‰ to -17.6 ‰. Mean per species ranged from -11.2 to -15.5 ‰ (*C. aspera* and *C. canescens*), respectively. Significant differences existed between *C. aspera* vs. *C. baltica*/*C. cansecens* and *C. baltica* vs. *S. pectinata* ($p < 0.05$; ANOVA; Bonferroni). No significant difference existed between the vascular (-12.5 ± 2.3 ‰) and non-vascular (-12.9 ± 2.2 ‰) species ($p \geq 0.05$; independent t-test). Independent of stations and time C:N ratio ranged from 9.0 - 65.8. Mean per species ranged from 20.1 - 44.1 (*C. canescens* and *C. tomentosa*), respectively. *C. tomentosa* was significantly different compared to all other species ($p \leq 0.05$; ANOVA; Bonferroni). Variability between the vascular (26.5 ± 9.6 ‰) and non-vascular (25.7 ± 10.3 ‰) species ($p = 0.619$; independent t-test) do not exist.

4.4 Discussion

4.4.1 Factors influencing isotope composition in macrophytes

Isotope-based approaches have been shown to be powerful tools to describe biochemical processes and to link nutrient inputs to primary producers in aquatic ecosystems. Such conclusions require distinct isotopic signals, but the high variability of stable isotope values in macrophytes can complicate the interpretation. The high range of isotopic signatures of macrophytes is not only result of spatio-temporal pattern (Matuszak *et al.*, 2011; Mayr *et al.*, 2011), but are also affected by a number of abiotic and biotic factors. Cooper (1989) found that the internal use of CO_2 and the ability to use atmospheric CO_2 for emersed plants can decrease δ^{13}C values. Higher diffuse boundary layer resistance due to decreased water turbulence lead to enriched δ^{13}C ratios in periphyton (France, 1995) and *Chara fibrosa* Ellawala *et al.* (2012). Also a relation was detected between the macrophyte δ^{15}N values and nutrient limitation in tarns (King *et al.*, 2009) and lakes (Jones *et al.*, 2004). Highly alkaline pH resulting from photosynthesis may alter the δ^{13}C in encrustations of *Chara hispida* (Pentecost *et al.*, 2006). This wide range of isotopic signatures in primary producers should be characterized in more detail and the analysis of bigger datasets rather than single measurements can help to understand linkages between biogeochemical processes and macrophytes in aquatic systems.

In this study the nitrogen and carbon isotopic composition and C:N ratios of submerged vegetation of the DZBC were analysed to consider distinct C-N isotopic signatures (1) in different segments of individual plants and (2) among species with possible different physiological pathways. (3) Spatio-temporal patterns of stable isotope values in macrophytes were investigated at four stations over an annual cycle since there are a limited number of studies.

4.4.2 Patterns among parts of individual aquatic plants

Plant isotope ratios were often used to identify the nutrient source with similar isotopic signature (Costanzo *et al.*, 2001). Requirements for such application are that no fractionation occurs during uptake, transport or other physiological pathways of nitrogen or carbon. If different plant parts have the same isotope value no fractionation occurs within them. To test this, four different segments of individual macrophytes were analysed on δ^{15}N, δ^{13}C and C:N ratio. No significant difference in isotopic composition and C:N ratios could be found in the four segments although elevated isotope values in roots as storage organ and enrichment during ageing in older (lower) plant segments were assumed. Pardo *et al.* (2013) analysed δ^{15}N in root, stem and leaf tissues of individual terrestrial *Acer sacchrum* (sugar maple) and *Fagus grandifolia* (American beech) plants in New Hampshire (USA). Based on differences of δ^{15}N in plant parts, they suggested that fractionation occurs during transport processes and assimilation of nitrogen. Zhou *et al.* (2006) investigated the water associated sedges *Scripus sp.* and *Carex scabrifolia* and found differences in C:N ratios in stems and roots, but not in the C-N isotopic composition. Since we could not find distinct differences between the four segments (apical tip to root), we suggest that fractionation of N and C did not occur during translocation or processes. Whether macrophytes fractionate during assimilation still remains an open question as many different sources of nutrients exist in the water column and sediment. For further analysis the mean of the four segments was used and discussed.

Only the flowers of *M. spicatum* and *S. pectinata* showed enriched δ^{15}N values compared to the other plant parts. Muzuka and Shunula (2006) found high nitrogen contents in flowers of individual mangrove species, but no changes in isotope composition compared to other plant parts (bark, root, leaves, and fruits). McMillan *et al.* (1980) found a range in δ^{13}C values from 1.9–4.7 ‰ among individual seagrass leaves of the same species at one site. Stephenson *et al.* (1984) detected similar variations of up to 8.0 ‰ of δ^{13}C in individual seagrasses. Other comparable studies investigating different parts in aquatic plants do not exist to our knowledge.

Another possible factor influencing isotopic signature could be ageing processes responsible for modulated fractionation during ontogenesis. The study of Cloern *et al.* (2002) compared newly produced living foliage with one year old decomposing dead foliage of macrophytes and could not find distinct differences in δ^{15}N and δ^{13}C. They assumed that senescence is more depending on time and species and not on ageing of parts. Decomposing processes occur together with dense mats of biofilm. Stephenson *et al.* (1984) tested the carbon isotopic composition of *Zostera marina* and *Laminaria lingicruris* and their epibionts and could not find differences. Thus, regarding δ^{13}C

epibionts are neglectable. Fellerhoff *et al.* (2003) came to nearly the same conclusion and did not find changes of δ^{13}C during decomposition. But δ^{15}N was variable (± 6.0 ‰) due to microbial activity. The C:N ratio decreased from 17-50 in macrophytes to 7-12 in POM which was on the same level as the biofilm. Thus, δ^{15}N values of macrophytes vary quantitatively and qualitatively due to microbial activity. The study of Currin *et al.* (1995) describes a shift of 2–3 ‰ in δ^{15}N ratios for live and standing dead *Spartina alterniflora*. As influencing factor they also quote the microbial activity. There is still a lack of knowledge about different parts in aquatic plants, but different segments do not seem to alter isotopic or C:N ratios which are rather depending on microbial activity. Heterotrophic, bacterial transformation (during assimilation and release) of nutrients, which in turn can be assimilated by macrophytes, may alter the isotopic composition. Since there are no investigations to the impact of biofilms on macrophytes those conclusions remain speculative.

4.4.3 Seasonal variability of macrophytes over an annual cycle

Differences over time in the isotopic values of macrophytes have been shown in other studies (Fry and Sherr (1984); Kendall *et al.* (2001) and references therein). δ^{15}N ratios of macrophytes of the DZBC had a high variability with values ranging from -4.2 to 13.5 ‰ but seasonal patterns do not exist. δ^{15}N in charophytes of Lake Constance also showed a wide variability and no temporal pattern (Matuszak *et al.*, 2011). The variability of δ^{15}N in time still remains unexplained. Cifuentes *et al.* (1989) found a relation between the reactions of nitrogen cycles at different rates throughout the year and the isotopic composition of NH_4^+. Such changes may be explained by the different isotopic signature of dissolved inorganic nitrogen (ammonium, nitrate, nitrite as part of the nitrogen cycle) over time and will be reflected in macrophytes when they consume it. For example, biological fractionation caused by assimilation of nitrate can lead to distinct patterns in the remaining substrate with decreasing concentrations and increasing δ^{15}N values (Fig. 18). It is possible that these theoretical developments can not be detected in the field, because mixing and overlapping processes change the signal. Also δ^{13}C values in macrophytes of the DZBC did not show obvious pattern over time. In contrary, δ^{13}C values for phytoplankton and other aquatic vegetation (Matuszak *et al.*, 2011) were shown to be higher in winter and lower in summer and indicate increasing photosynthesis during biomass development. Cloern *et al.* (2002) investigated the stable isotopic composition of aquatic and terrestrial plants in the estuarine San Francisco Bay and found high variability of δ^{13}C and δ^{15}N in ten functional groups (e. g. terrestrial plants, phytoplankton, salt marsh). Also the seasonal pattern of carbon and nitrogen isotopic composition varied among single species (gradual decline

during growth season, low seasonal oscillation or no periodicity). Despite senescence they could not give an explanation for this variability over time and concluded that isotopic signatures of aquatic plants are not predictable.
Only the C:N ratios had distinct seasonal differences, where the summer period showed elevated values with an average of ∽30 compared to winter the period with ∽20. This development of C:N ratios was also indicated before and could be shown for phytoplankton (Mariotti *et al.*, 1984; Nakatsuka *et al.*, 1992) and also macrophytes (Cloern *et al.*, 2002). Due to the biomass development of macrophytes with beginning of growing season C:N ratios are increasing until reaching the maximum productivity and following rapidly decreases when plants die. In field rather smooth than rapid declines are found, which can be explained by the slow decomposition processes of macrophytes.

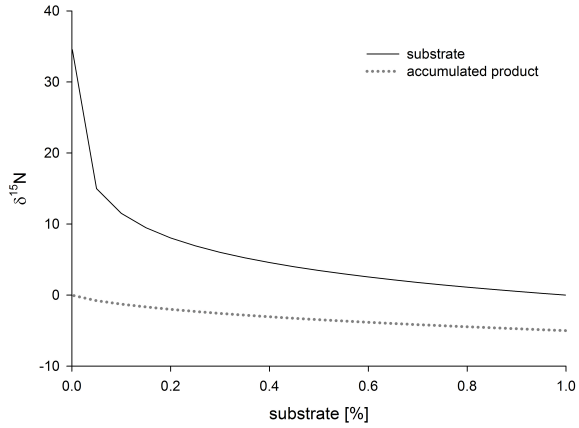

Figure 18: Theoretical development $\delta^{15}NO_3^-$ over nitrate concentration with a fractionation factor of 5 ‰ typical for nitrate assimilation by phytoplankton.

4.4.4 Spatial variability of macrophytes in the DZBC

Estuarine gradients from freshwater to sea in general reflect the gradient from nutrient rich to nutrient poor conditions and their isotopic signature (Brabandere *et al.*, 2007; Costanzo *et al.*, 2001; Fry, 2002; Rogers, 1999). Savage and Elmgren (2004) studied the δ^{15}N ratios in *Fucus vesiculosus* to contour the spatial extent of sewage-derived N and found decreasing δ^{15}N with distance from anthropogenic inputs (8–9 ‰ and 4 ‰, respectively). Spatial pattern in stable isotopic composition of macrophytes were not found in the way we expected. Due to the high nutrient load of the river Recknitz compared to the less-influenced regions like Nisdorf, we expected a gradual decrease of stable isotope values in macrophytes. Significant differences between all stations were detected, even when the range in δ^{15}N was not high with a maximal difference on average by $\backsim$ 5 ‰. Surprisingly we could not find a gradual pattern correlated to riverine sources of nutrients. Michaelsdorf had lowest δ^{15}N ratios, which could be correlated with direct catchment area. It is a protected national park, where extensive managed grassland is the sole agricultural technique applied. In contrary, Nisdorf, with highest mean of δ^{15}N, is surrounded by intensively used fields and enriched values may be caused by excessive use of fertilizers. Decreasing δ^{15}N ratios in macrophytes with distance to the high anthropogenic loading were found within the Warnow River Estuary (Deutsch, 2005), in a coastal Baltic embayment (Savage and Elmgren, 2004) and in the Gulf of California (Pinón-Gimate *et al.*, 2009). Dissolved inorganic nitrogen from rivers and local sewage treatment in general have elevated δ^{15}N ratios, thus altering the δ^{15}N ratios of macrophytes when they assimilate them as nutrients (Gartner *et al.*, 2002; Peipoch *et al.*, 2012). Also King *et al.* (2009) explained the high variability of δ^{15}N (–9.9 - 10.6 ‰) for macrophyte samples from 30 U. K. upland tarns by differences of N- or P-limitations on sites. Site-specific differences in δ^{15}N and δ^{13}C of charophytes in Lake Constance were found by Matuszak *et al.* (2011), too. They could not find pattern for the variability of δ^{15}N at different sites, but found enriched δ^{13}C at shallow, nutrient-rich sites and depleted δ^{13}C in deeper and at nutrient-poor sites. δ^{13}C in macrophytes of the DZBC also showed no gradual decline. All stations were similar, except Michaelsdorf with heavier δ^{13}C. The temperature controls the pH which in turn regulates the bicarbonate-system. The pH leads to shifts in equilibrium of bicarbonate, atmospheric and dissolved forms of CO_2 which have distinct isotopic variability. The use of bicarbonate and free carbon dioxide in different portions could explain the pattern in macrophytes isotopic ratios (Cooper, 1989). The pH and temperature at station Michaelsdorf did not differ from other stations and could not explain the elevated δ^{13}C. The variability of δ^{15}N and δ^{13}C at a single station is not a result of distinct signatures from different species but can be rather a natural variability of

microhabitats. With the expectation at station Dabitz, where δ^{13}C ranges of *Chara sp.* and *S. pectinata* are different, at all other stations species signatures are in the same range (Fig. 19). Other studies found gradual gradients in isotopic composition, but nitrogen and carbon isotope ratios of the DZBC at different locations are very individual. A possible explanation for this unexpected result is the nearly closed morphology of this inner coastal water themselves, which leads to complications in water exchange. The C:N ratios were all nearly identical at the stations, which was not surprising, since the macrophytes development is independent of the stations and more dependent on the season.

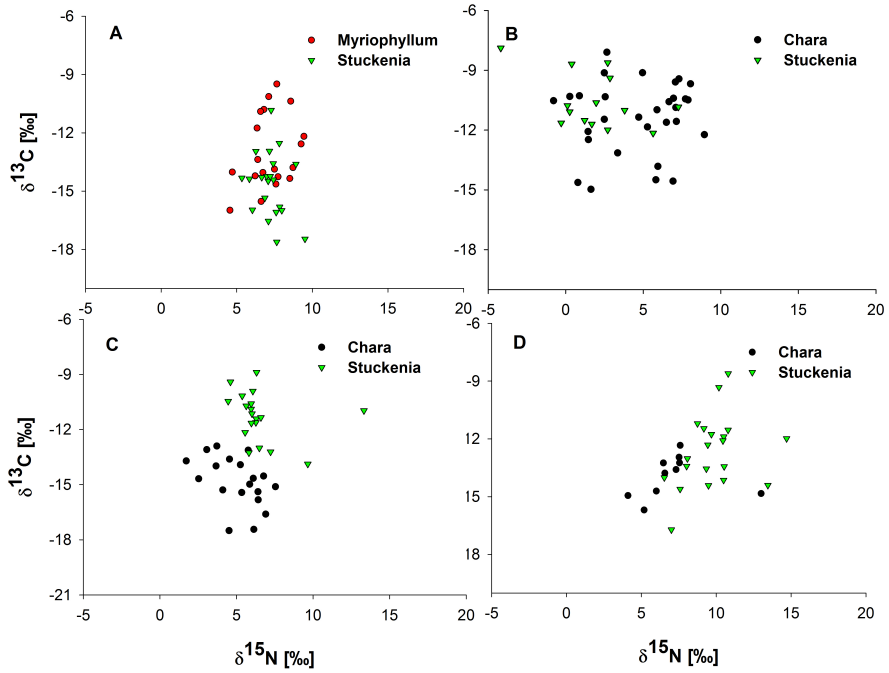

Figure 19: Plots of δ^{15} N and δ^{13}C measured at four stations of the DZBC and existing species over an annual cycle with A - Pütnitz, B - Michaelsdorf, C - Dabitz and D - Nisdorf.

4.4.5 Intra-specific variability

Isotopic composition of plant groups reflect not only the source of nutrients DIN and DIC but are also dependent on fraction processes and internal nutrient contents. Fractionation factors vary in metabolism mechanism and the associated enzymes and they can also be changed by factors like diffusion rates, growth rates, degree of mixing and the carbon and nitrogen pool size (Fogel and Cifuentes, 1993; Fry and Sherr, 1984;

Kendall *et al.*, 2001).

Different photosynthesis exists and thus divided the plant into three groups: C_3-, C_4- and CAM-plants. C_3 plants has a distinct $\delta^{13}C$ signature (-32 to -20 ‰) from C_4- and CAM-plants, which can not be distinguished by their $\delta^{13}C$ values (-17 to -9 ‰).The responsible enzyme during nitrogen assimilation in land plants is the nitrogenase-reductase (reduction of nitrate to nitrite) and the nitrite reductase (reduction of nitrite to ammonia). Ammonia (both absorbed and synthesized) is incorporated into amino acids via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Crawford, 1995). Nitrogen metabolism in macrophytes is not investigated in detail. Nevertheless, the variability of $\delta^{15}N$ during assimilation and translocation were expected to be low, since the same metabolism mechanism should be performed. Differences in isotope ratios were only expected between the genera *Chara sp.*, *Myriophyllum sp.* and *Stuckenia sp.* but not within the species of one genera.

The different species of the DZBC did not show distinct signatures for $\delta^{15}N$ as well as $\delta^{13}C$. But we could detect a difference with a mean of 1.2 ‰ for $\delta^{15}N$ between vascular and non-vascular species. Cloern *et al.* (2002) analysed nitrogen and isotopic composition of different plant groups (based on plant habitat, and photosynthetic pathway) along the San Francisco Bay and found higher $\delta^{15}N$ in salt-marsh and submerged vascular plants than in freshwater marsh plants and estuarine microalgae which again were higher than terrestrial plants. They detected lowest $\delta^{13}C$ values in terrestrial and salt-marsh plants followed by the filamentous algae, phytoplankton and submerged vascular plants and highest in C_4 salt-marsh plants. They explained the occurring variability of species in $\delta^{15}N$ and $\delta^{13}C$ by a high within-habitat variability, which is caused by genetic differences in assimilation, biosynthesis and isotopic fractionation and by microscale gradients in abiotic factors that can change the isotopic ratio of nutrients. In contrary, in a greater approach Marconi *et al.* (2011) could not separate reliably the $\delta^{15}N$ in phylogenetic plant groups but they could find greater $\delta^{13}C$ ratios in Ulvophyceae than in Floridiophyceae and explained this with the mainly CO_2 use of Rhodophyta in light limited habitats. Keeley *et al.* (1986) concluded that a differentiation for CAM and non-CAM species, due to similar $\delta^{13}C$ ratios, in aquatic systems is not possible. In contrary, natural abundances of organic matter from macroalgae species unable to use HCO_3 (-29.9 to -34.5 ‰) were distinct from those who can use HCO_3 and CO_2 (-8.8 to -22.6 ‰) (Maberly *et al.*, 1992). Kohzu *et al.* (2008) investigated isotopic composition of riparian plants in relation to nutrient inputs and suggested that inter-specific differences were caused by hydrological differences in habitats, inter-specific differences in N nutrition and metabolism. The variable nitrogen-availability relative to demand and the source of nutrients from sediment, water or both can lead to different $\delta^{15}N$ ratios in macrophytes (3.0 ‰, 0.3 ‰ and 1.6 ‰, respectively)

(King *et al.*, 2009). Gartner *et al.* (2002) concluded that differences in isotopic signatures resulted from functional forms of low and fast nutrient uptake rates. They could prove that macro-algal species with fast uptake rates reflected recent sewage dispersal while species with slower rates provided signals over a longer season. The difference of δ^{15}N between vascular and non-vascular macrophytes with a mean of 1.2 ‰ was not significant (Fig. 20).

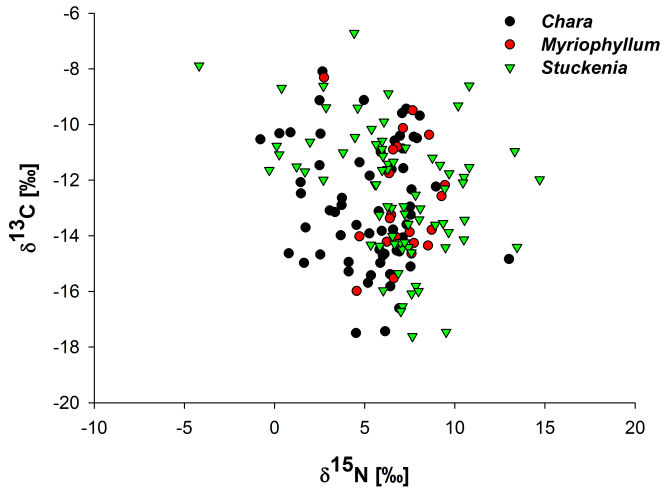

Figure 20: Plot of δ^{15} N and δ^{13}C measured in the vascular *S. pecitnata* (red dots) and *M. spicatum* (green triangle) and the non-vascular *Chara*-species (black dots) of the DZBC over an annual cycle at four stations.

Neither the nitrogen metabolism nor the fractionation factor during assimilation are investigated for the macrophytes species of the DZBC further distinctions based on δ^{15} N and δ^{13}C between species or functional groups are hampered. Differences of single species at different sites did not show distinct difference, but for *S. pectinata*, which has lighter ratios at station Michaelsdorf and heavier ratios at station Nisdorf (Fig. 21). The δ^{13}C values of *S. pectinata* at Pütnitz are lower compared to the other station. Possible explanation for this natural variability are microhabitats. C:N ratios among species also did not differ with a mean of 26.2 (independent of season) and those ratios are located in the range described for macrophytes (Duarte, 1992; Kendall *et al.*, 2001).

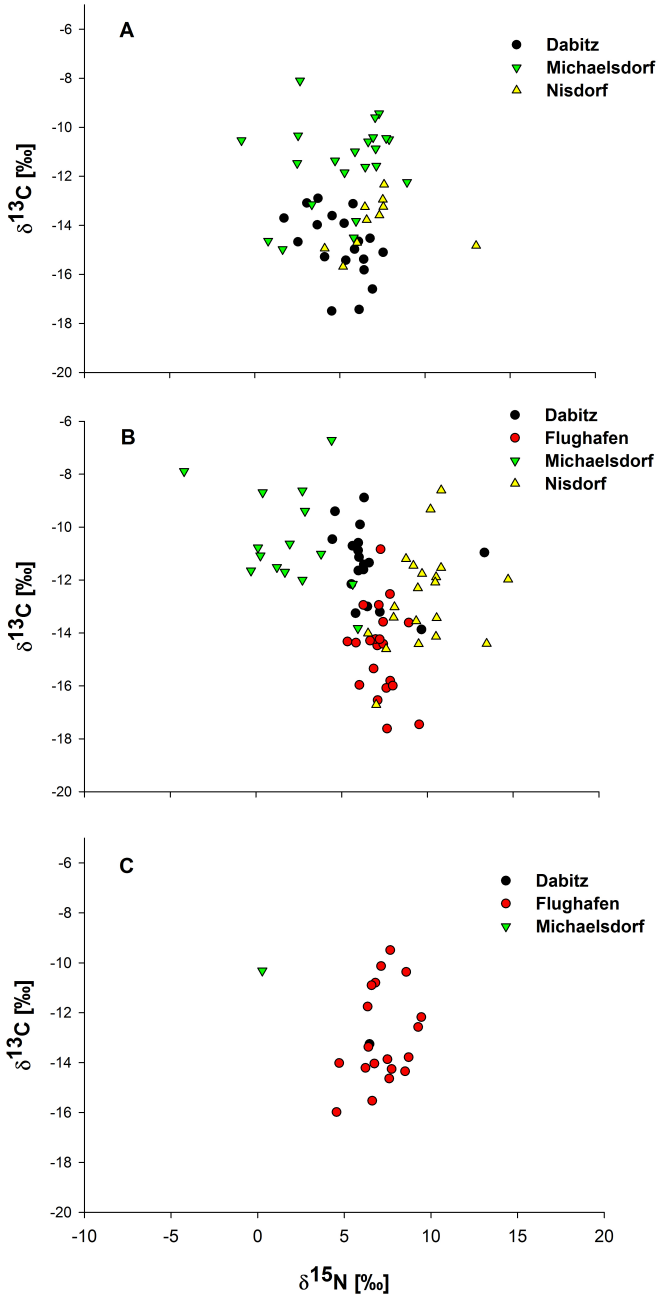

Figure 21: Plots of δ^{15}N and δ^{13}C measured in three genera collected from four stations of the DZBC over an annual cycle with A - *Chara sp.*, B - *Stuckenia pectinata* and C - *Myriophyllum spicatum*.

4.5 Conclusion

To our knowledge this is the first study describing the isotopic and elemental composition of macrophytes in the DZBC and with the aim to identify possible pattern. Macrophytes of the DZBC have a high variability in δ^{15}N, δ^{13}C and C:N ratios. The elemental composition is dependent on season and indicates the biomass development over an annual cycle. The isotopic compositions are mainly affected by the location. Single stations are influenced by physical and chemical factors, which obscure the interpretation of isotope signatures. Distinct difference of δ^{15}N in macrophytes between the stations could be found, but not the expected decrease with distance from the river Recknitz. Still, the monitoring technique "sewage plume mapping" (Costanzo *et al.*, 2005) could give additional information to describe the impact of N pollution inputs, where conventional measurements (nutrient concentrations) miss some processes. Neither differences in δ^{15}N ratios between segments nor intra-specific variability was found among the vascular and non-vascular species, indicating similar photosynthetic and uptake processes. Nitrogen and carbon stable isotopic composition of macrophytes showed up to be simple and useful indexes to describe the status of inner coastal waters and complemented existing monitoring programmes demanded by European Water Framework Directives.

5 DON in the Darss-Zingst Bodden Chain (DZBC) and its impact on macrophytes

5.1 Motivation

Anthropogenic activity has led to increased nutrient inputs to estuarine and coastal waters, which in turn has affected the nutritional status of aquatic systems and biochemical processes in there. Nitrogen can be the primary nutrient-limiting factor for plant, algal and microbial production and elevated N loading entering rivers, estuaries and coastal marine ecosystems can alter those aquatic environments. Changes in biomass, species composition and abundance of aquatic primary producers were often observed as consequences (Kovtun *et al.*, 2009; Lapointe *et al.*, 2005; Lotze *et al.*, 2000; Munkes, 2005). For example, a clear-water stage with macrophytes were superseded by a phytoplankton-dominated system in the Darss-Zingst Bodden Chain (DZBC), a shallow, inner coastal water of Germany (Schiewer, 1998; Schumann *et al.*, 2006). 30–40 years ago raised N inputs, due to agricultural and urban development, induced this shift. This effect is further supported by the morphology of the DZBC which is more influenced by the river run-off from the catchment area and less by marine inputs due to a narrow connection from the Baltic Sea.

The relationship between nitrogen and macrophytobenthos in such areas is of special interest since macrophytes like e. g. charophytes were used as indicators for eutrophication in accordance with the Water Framework Directive. The inter-annual distribution and abundances of macrophytes in the DZBC are heterogeneous (Selig *et al.*, 2009) and there is still a lack of knowledge how the growth and occurrence of macrophytes is regulated. Factors like light limitation (Angelstein *et al.*, 2009) and sedimentation (Kovtun-Kante *et al.*, 2014) could not explain those patchy seasonal pattern. Nutrient limitation or competition are more likely to be the important factors. But measurements of nutrient concentrations often did not show a relation between productivity or abundance of primary producers (Blindow and Meyer, 2015; Castro *et al.*, 2007; Fong *et al.*, 1993).

In contrary, the approach of stable isotope showed up to be a useful tool. In many studies, nitrogen stable isotope signatures of macrophytes have been shown to reflect N sources and can thus be used to monitor N contamination (Cole *et al.*, 2004; McClelland and Valiela, 1998; Riera *et al.*, 2000; Umezawa *et al.*, 2007; Viana *et al.*, 2011). Regarding the nutrient nitrogen most measurements and models just consider dissolved inorganic nitrogen (nitrate, nitrite and ammonium) as essential portion of N inputs to aquatic systems. Dissolved organic nitrogen (DON) as another portion of N was long neglected as possible nutrient source for macrophytes and other primary

producers due to its refractory nature. Investigations have falsified this erogenous consumption and proven that DON can be utilised by microbial organisms (McCallister *et al.*, 2006; Seitzinger and Sanders, 1997; Wiegner and Seitzinger, 2004) and phytoplankton (Bronk and Glibert, 1993; Maestrini *et al.*, 1999; Seitzinger, 2002). The uptake of DON by macrophytes and seagrasses was investigated in a limited number of studies (Tyler *et al.*, 2005; Van Engeland *et al.*, 2011; Vonk *et al.*, 2008). Those works have shown that the labile 'doughnut' fraction (Bronk *et al.*, 2007) of DON can be assimilated. Labile components like dissolved free amino acids (DFAA), dissolved combined amino acids (DCAA), DNA, RNA and urea represent the bioavailable and lower fraction of bulk DON. The utilization of humic and acid substances, the refractory and major components of the DON pool, is reserved to bacterial decomposition. The biological availability of DON and its ecosystem effects depends on its varying sources (Stepanauskas *et al.*, 2000; Wiegner *et al.*, 2006). Origins of DON can be river run off, terrestrial inputs, groundwater, atmospheric deposition, precipitation, release of bacteria, release of phytoplankton and photodegradation (Berman and Bronk, 2003; Hu and Smith Jr, 1998). Rivers, which are a major transport pathway for N from watersheds to coastal marine ecosystems, can have 10 % to over 80 % of their N in the form of dissolved organic N (Hedin *et al.*, 1995; Meybeck, 1982; Seitzinger and Sanders, 1997). Independent of spatial diverse sources of DON, information on the bioavailability of DON over seasonal cycles, is generally lacking.

A fundamental understanding of the effects of nutrient inputs from both inorganic and organic sources requires development of bioavailable nutrient loading budgets and knowledge of the response of coastal ecosystems to different forms of those nutrients. The current study addresses the dissolved component of organic N inputs to aquatic ecosystems. Even if only a minor fraction of DON is bioavailable, this part should be integrated in N loading budgets and considered as nutrient source. The major objective for this work was (1) to determine seasonal and spatial pattern of concentration and isotopic composition as well as proportion of DON to total N pool in a coastal inner water characterized by a salinity gradient. (2) The role of DON as nutrient on the development of macrophytes was investigated indirectly by using stable isotope data. (3) The link between DON, NO_3^- and PON were examined. Finally macrophyte stable isotopes were discussed as indicators for water quality management and identification of N contamination to such coastal systems.

5.2 Experimental set-up

Water samples and macrophytes were collected bi-weekly (June–September 2013 and March–June 2014) to monthly (November 2013–February 2014) over a whole year

from June 2013 to June 2014. Water samples were analysed for nutrient concentrations (NO_3^-, NO_2^-, NH_4^+), POM ($\delta^{15}N$ and $\delta^{13}C$ and C:N ratio), chlorophyll content and physical variables (salinity, temperature, oxygen saturation, pH). Isotope values of $\delta^{15}N$ and $\delta^{18}O$ in nitrate were analysed, when nutrient concentration (> 1 µmol L^{-1}) allowed measurements. Nitrogen isotope composition and concentrations of DON were analysed for only five time points, at least one per season. Six stations along the salinity gradient of the Darss-Zingst Bodden Chain were investigated: Recknitz, Pütnitz, Michaelsdorf, Meiningerbrücke, Dabitz and Nisdorf (Fig. 4 on page 17).
All water samples were taken near shore at the surface and frozen at -20 °C until further measurements. Nutrient concentrations were measured photometrically and isotope measurements were done with the IRMS. For measurement of concentration and nitrogen stable isotopes of DON, nitrate and ammonium (nitrite was negligible) were eliminated from samples with the diffusion-method after Sigman *et al.* (1997). Remaining samples with DON were oxidized to nitrate with the persulfate-oxidation. Finally the concentrations of the transformed samples (nitrate) were photometrically determined. δ^{15}DON (also samples which were oxidized to nitrate) and $\delta^{15}NO_3^-$ were analysed with the denitrifier-method after Sigman *et al.* (2001) and Casciotti *et al.* (2002). Dried macrophytes were analysed in the IRMS for nitrogen isotopes and N-contents. For further details in measurements see section 'Material and Methods'.

5.3 Results

5.3.1 Isotopic signature and concentration of DON

Concentration and nitrogen stable isotope values of DON were analysed over an annual cycle (June 2013–June 2014) at six stations of the DZBC. Macrophytes were dried, ground and analysed in the IRMS for stable and elemental composition. The concentrations of DON ranged from 3.7 µmol L^{-1} to 25.8 µmol L^{-1}. Minimum and maximum values were both found at station Meiningerbrücke in spring and in summer, respectively. The annual mean of all stations was 13.6 ± 5.3 µmol L^{-1}. δ^{15}N-DON had a mean of 6.5 ± 1.3 ‰. Lowest values with 4.8 ‰ were found at the station Meiningerbrücke in summer 2013 and highest with 10.3 ‰ in spring at the station Michaelsdorf. Significant differences in both, concentrations and δ^{15}N-DON, were found over an annual cycle in spring (ANOVA, Tukey-HSD, $p < 0.05$). With a mean of 8.4 ± 4.2 µmol L^{-1} the season spring had lowest DON concentration and differed significantly from all other seasons (Fig. 23). No significant differences were found for DON concentrations in summer, autumn and winter (16.1 ± 4.8 µmol L^{-1}, 13.9 ± 4.0 µmol L^{-1} and 13.6 ± 5.7 µmol L^{-1}). With 7.8 ± 1.4 ‰ highest δ^{15}N-DON values were found in spring (Fig. 22).

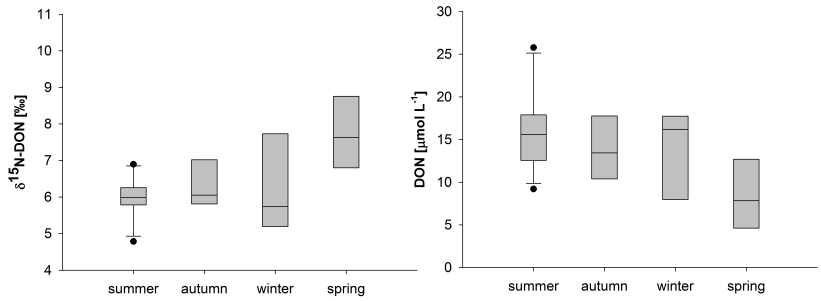

Figure 22: Box-Whisker-Plots showing the median (horizontal line inside boxes) and variability (box ends show interquartile range, vertical lines the full range, dots the outliers) of δ^{15}N-DON and DON concentrations measured at four seasons from 2013–2014. For autumn, winter and spring n = 6 (number stations) and for the season summer n = 12 because there were measurements for 2013 and 2014.

DON and concentration δ^{15}N-DON showed no distinct spatial pattern. With 12.9 ± 2.5 µmol L^{-1} and 15.2 ± 3.4 µmol L^{-1} the station Dabitz and Nisdorf were less variable in their DON concentrations than the other stations (Fig. 23). A significant difference of DON concentrations was only be found between the station Recknitz-Nisdorf and Recknitz-Meiningerbrücke (ANOVA, Tukey-HSD, $p < 0.05$).

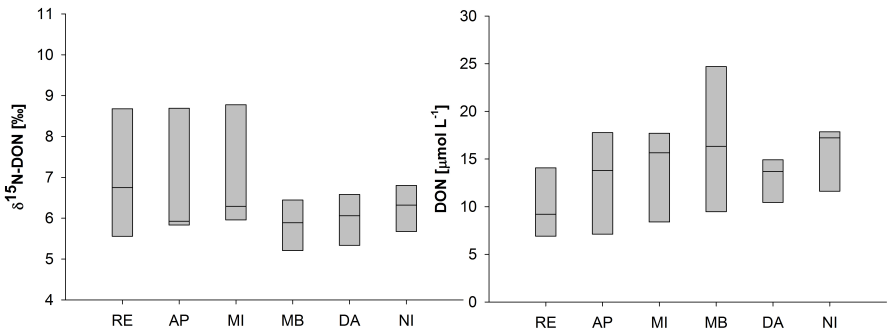

Figure 23: Box-Whisker-Plots showing the median (horizontal line inside boxes) and variability (box ends show interquartile range) of δ^{15}N-DON and DON concentrations measured at six stations in the Darss-Zingst Bodden Chain from June 2013–June 2014. Stations are arranged by salinity gradient (left to right increasing) with RE - Recknitz, P - Pütnitz, MI - Michaelsdorf, MB - Meiningerbrücke, DA - Dabitz and NI - Nisdorf.

The stations Recknitz, Pütnitz and Michaelsdorf (7.1 ± 1.6 ‰) had a higher variability in δ^{15}N-DON than the stations Meiningerbrücke, Dabitz and Nisdorf (6.0 ± 0.7 ‰). DON concentrations and δ^{15}DON ratios of the reference stations Zingst and Dierhagen from the Baltic Sea are similar to the values of the DZBC (Fig. 24). δ^{15}N-DON and ln DON concentration are only loosely correlated (r^2 = -0.43). The slope of the linear regression of δ^{15}N-DON and ln DON is the fractionation factor of -1.8 ‰ (Fig. 24). δ^{15}N-DON and DON concentration had a Spearman correlation coefficient of r_S = 0.672. The contribution of DON to total dissolved nitrogen (TDN) differed seasonally with an annual mean of 47.6 %. The lowest DON:TDN ratio with 3 % occurred at the Pütnitz in winter and highest with 89.4 % at station Nisdorf in summer 2013. At the station Recknitz the DON:TDN ratio was low (3.5–12 %) over the whole annual cycle.

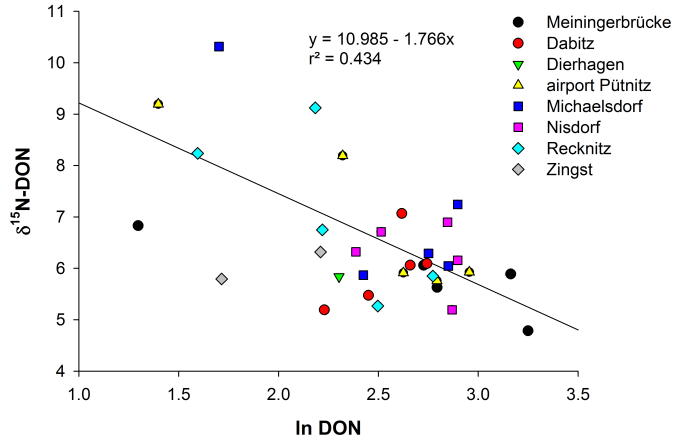

Figure 24: Plot of δ^{15}N-DON and ln DON concentrations from different stations of the DZBC from June 2013–June 2014. The slope of the regression line indicates the fraction factor ε.

5.3.2 Suspended PON concentrations and isotopes

The PON concentrations had an annual mean of all stations of 71.2 µmol L^{-1} with a minimum of 5.4 µmol L^{-1} at the station Recknitz in autumn and a maxima of 326.6 µmol L^{-1} at the Pütnitz also in autumn. The C-content had an annual mean of 844.8 µmol L^{-1} with lowest value of 67.6 µmol L^{-1} at station Recknitz in September and highest with 3040.5 µmol L^{-1} at station Pütnitz in August (Fig. 25). The C:N ratio had a range of 8.6–26.4 (both minimum and maximum were found in April) resulting in a mean of 12.0. The C:N ratio showed a distinct seasonality with highest values in May/June, which decreased until it reached the minimum from October–December.

The N- and C-content showed a spatial pattern: lowest values were found in the river Recknitz. Highest contents occurred at the Pütnitz and then decreasing with increasing salinity. Measurements of single stations are shown in Fig. 33 in the supplement. No seasonal or spatial trend could be found for δ^{13}POC (Fig. 25). δ^{15}N-PON ranged from -1.4 ‰ (Michaelsdorf in September) to 48.0 ‰ (Dabitz in October) and had a distinct peak in September over the annual mean of 8.8 ‰. The concentrations of PON and δ^{15}N-PN had a Spearman correlation factor of r_S = -0.18.

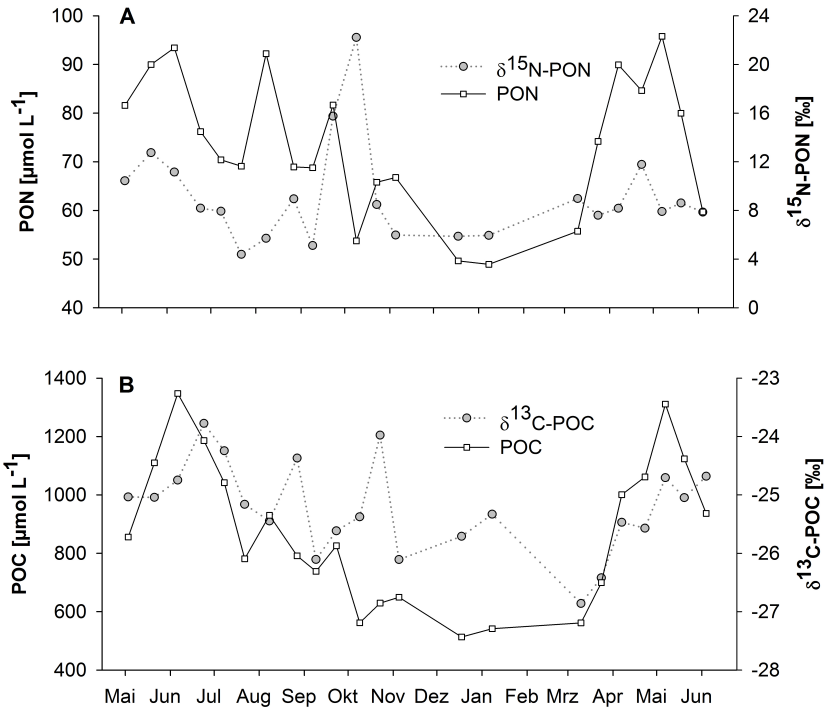

Figure 25: Mean of isotopic values and concentrations of suspended PON (A) and POC (B) from six stations of the DZBC. Error bars were renounced to keep an overview. For measurements at single stations see Fig. 33 in the supplement.

5.3.3 NO_3^- isotopic signature

Nitrate concentrations showed a clear seasonality, which were highest in winter and lowest in summer. In the river Recknitz the nitrate concentration was always higher compared to the other stations (see Fig. 35, supplemental material). δ^{15}N-NO_3^- range from 2.7–21.8 ‰ with a mean of 8.4 ‰ (Fig. 26). The variability of δ^{15}N-NO_3^- and δ^{18}O-NO_3^- was lowest in the river Recknitz and increased with increasing distance.

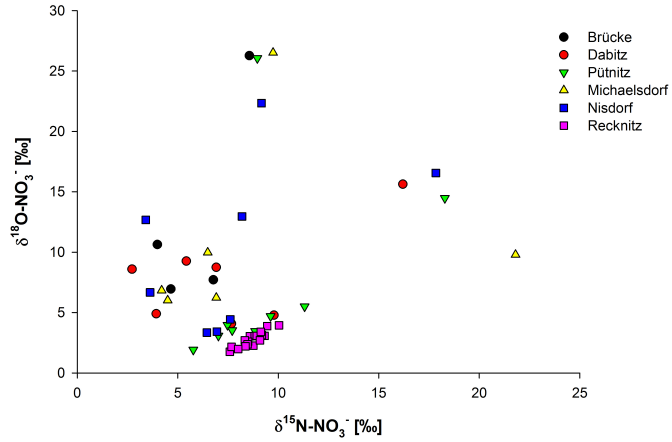

Figure 26: Plot of δ^{15}N and δ^{18}O of six stations from the DZBC over an annual cycle from June 2013–June 2014. Only when nitrate concentrations were high enough (> 1 µmol L^{-1}) measurement of isotopes were possible.

The slope of ln NO_3^- against δ^{15}N-NO_3^- is 3.6 (Fig. 27). With increasing NO_3^- concentrations δ^{15}N- values increased simultaneously (r_S = 0.648). Results are shown in Tab. 8 and Fig. 28. NO_3^- concentrations and $\delta^{15}NO_3^-$ ratios of the reference stations Zingst and Dierhagen from the Baltic Sea are similar to the values of the DZBC (Fig. 27).

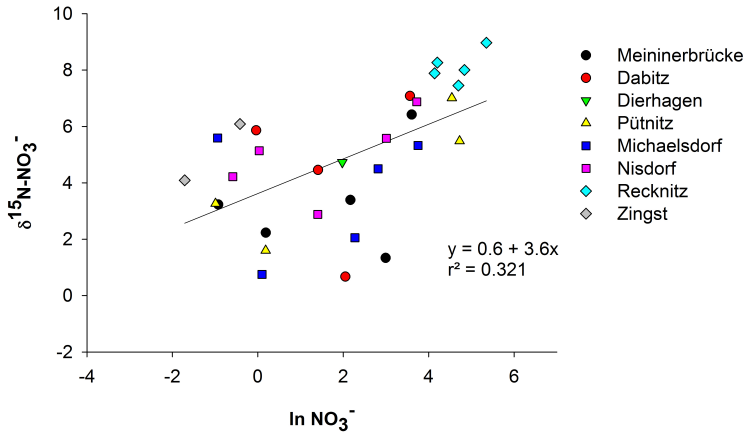

Figure 27: Plot of δ^{15}N-NO_3^- and ln NO_3^- concentrations from different stations of the DZBC from June 2013–June 2014. The slope of the regression line indicates the fraction factor ε.

5.3.4 Comparison of different nitrogen sources (POM, DON, NO_3^-)

Changes in concentration and stable isotopes of DON, nitrate and PON over time are shown in Fig. 28.

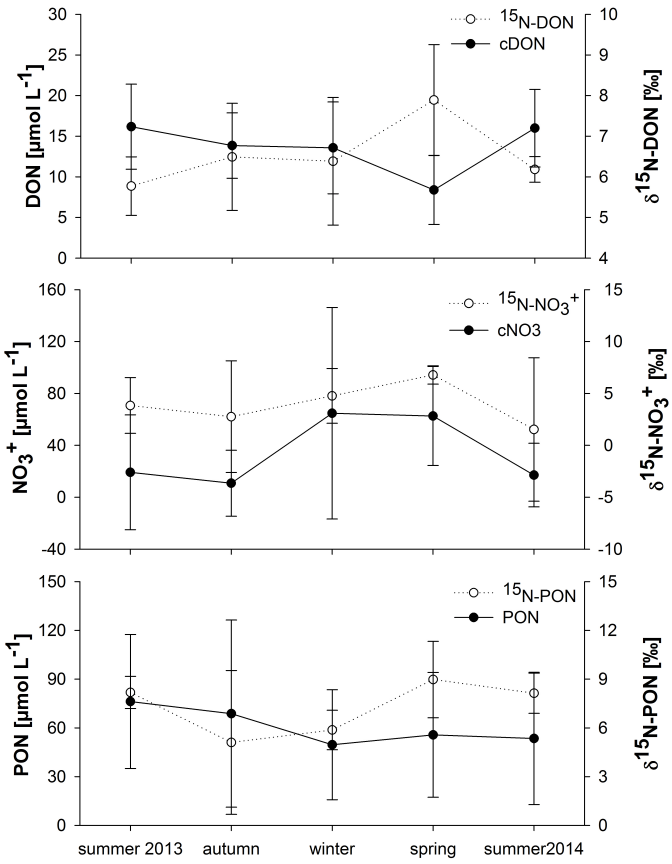

Figure 28: Concentration and nitrogen isotopic values in the Darss-Zingst Bodden Chain from June 2013—June 2014. The mean and deviation of six stations are shown with A - DON concentration and δ^{15}N-DON, B - Nitrate concentration and δ^{15}N-NO_3^- and C - PON concentration and δ^{15}PON.

Throughout the sampling period nitrate showed a clear seasonal trend in concentrations and isotopic composition. Both are elevated during winter and spring and decreased in summer and autumn. They had a Spearman correlation coefficient of $r^2 = 0.648$. DON concentrations and stable isotopes did not show a clear seasonality and changes over the year are not high. During spring DON concentrations are lower and δ^{15}DON values are elevated compared to other seasons. They are both negatively correlated

(r_S = -0.627). As DON, PON concentrations did not show a clear seasonality. Isotopic composition of PON are highest in spring and summer and lower in autumn and winter. They are loosely anti-correlated (r_S = -0.180). Results of Spearman correlations are shown in Tab. 8.

Table 8: Spearman correlation coefficients of measured nitrogen concentration and stable isotopic composition in the DZBC, June 2013–June 2014 for five sampling dates.

r_S	NO_3^-	DON	TDN	PON	δ^{15}N NO_3^-	δ^{15}N DON	δ^{15}N PON
NO_3^-		-0.406	0.991	-0.481	0.648	0.362	0.207
DON			-0.319	0.279	-0.378	-0.627	-0.362
TDN				-0.466	0.594	0.309	0.150
PON					-0.574	-0.052	-0.180
δ^{15}N-NO_3^-						0.337	0.16
δ^{15}N-DON							0.369
δ^{15}N-PON							

5.3.5 Nitrogen stable isotopic composition in macrophytes

Nitrogen isotopic composition of six species (*Chara aspera*, *Chara aspera*, *Chara baltica*, *Chara canescens*, *Chara tomentosa*, *Myriophyllum spicatum* and *Stuckenia pectinata*) from four stations (Pütnitz, Michaelsdorf, Dabitz and Nisdorf) range from 0.4–13.35 ‰. The overall mean independent of species, time and stations was 6.7 ‰. For further details of macrophytes isotopes see Chapter 2, where spatio-temporal pattern and differences in species and segments of single plants were investigated.

5.3.6 Sources of nitrogen for macrophytes

Plot of δ^{15}N-DON and δ^{15}N of macrophytes did not show a clear trend (Fig. 29). There is a weak correlation between δ^{15}N-DON and δ^{15}N-macrophytes of r^2 = 0.25. The correlation coefficient of δ^{15}N-NO_3^- and δ^{15}N-macrophytes is even lower with r^2 = 0.09.

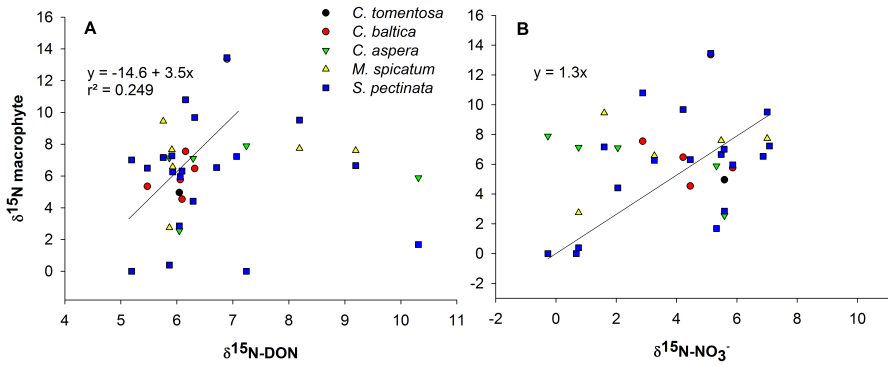

Figure 29: Plots of δ^{15}N in macrophytes from different species against A- δ^{15}N-DON and B - δ^{15}N-NO_3^-.

Independent of stations or seasons the median and ranges of macrophytes, DON, NO_3^- and PON are shown in Fig. 30. δ^{15}N of macrophytes (vascular and non-vascular) are not significantly different (ANOVA, Tukey-HSD, $p > 0.05$) and both groups have similar means of 6.8 ‰. Also the δ^{15}DON values with a mean of 6.6 ‰ did not show a significant difference to δ^{15}N in macrophytes (ANOVA, Tukey-HSD, $p > 0.05$). Nitrate in contrary, had a mean of 4.9 ‰ and is significantly lower than δ^{15}N of DON and macrophytes (ANOVA, Tukey-HSD, $p < 0.05$). PON has a mean of 8.8 ‰.

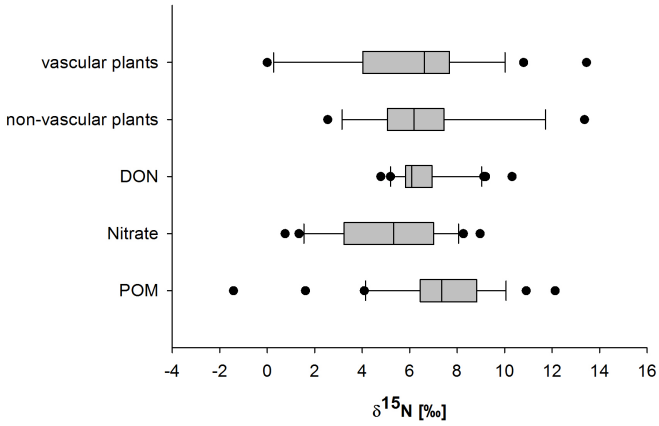

Figure 30: Box-Whisker-Plots showing the median (vertical line inside boxes) and variability (box ends show interquartile range, horizontal lines the full range, dots the outliers) of δ^{15}N for vascular plants (n = 26), non-vascular plants (n = 13), DON (n = 30), nitrate (n = 30) and PON (n = 30).

5.4 Discussion

In this chapter concentration and $\delta^{15}N$ values for DON, NO_3^-, PON and macrophytes collected from June 2013–June 2014 were presented and discussed with the specific focus on possible nitrogen sources for macrophytes in the DZBC. Goal of this study was to investigate (1) the seasonality (concentrations and stable isotope signatures) of DON and contribution to TDN and (2) whether DON is a potential nitrogen source for macrophytes. Furthermore, we investigated the correlation of nitrate, DON and PON to identify coupling between these nitrogen pools and discuss whether macrophytes stable isotopes can be used as markers of eutrophication and nitrogen contamination.

5.4.1 The DZBC - a highly variable inner coastal water

Since 1969 freshwater and marine inputs were collected during a monitoring program for the DZBC. To our knowledge the DON pool for this studying area was not discussed or integrated in models predicting eutrophication events and its consequences. DON measurements during the monitoring program were made only indirectly by the subtraction of TDN and DIN and differs from measurement made in this study, where inorganic components (mainly ammonia and nitrate, nitrite was negligible) were removed to get samples just remaining DON. In contrary, nutrient concentrations (ammonia, nitrate, nitrite), abiotic (pH, temperature, salinity, oxygen content, Secchi depth) and biotic variables (chlorophyll content) of the DZBC were well investigated in the last decades (Schiewer, 1998; Schumann *et al.*, 2006) until today. The next section will give only a short overview of those parameters. Figures of nutrient concentrations and abiotic variables over time are shown in the supplemental material. Limited water exchange between single lagoons and with the Baltic Sea via narrow channels lead to raising salinity gradient from west to east and range from 0–11 resulting from freshwater inputs (river Recknitz and Barthe) and salt water inputs from the Baltic Sea. During the sampling period the study area was always oxygen saturated (> 2 mg L^{-1}) in the water column (Fig. 34, supplemental material).

The river Recknitz, influenced by a catchment area eight times greater than the DZBC itself ($\backsim$1578 and 187 km^2, respectively), is the main source of high N loads. Nitrate concentrations showed a clear seasonality with highest values in winter of $\backsim$35 µmol L^{-1} and in summer during phytoplankton development nitrate and also ammonium fell drastically and often reached detection limit. In the river Recknitz the nitrate concentrations were always higher compared to the other stations (63–88 µmol L^{-1} in summer and 120–213 µmol L^{-1} in winter). Ammonium concentrations ranged between 0.5–19.5 µmol L^{-1} with an annual mean of 4.4 µmol L^{-1}. Based on quick bacterial turnover ammonium concentration did not only oscillate over year but also

daily. Nitrite concentrations are always near the detection limit (< 2.5 µmol L^{-1}) indicating denitrification processes (nitrite to N_2). Despite great efforts in nutrient reduction the total N loads are still high, even if they were reduced by 20–30 % in the last decades (Bachor, 2005). Total chlorophyll concentrations demonstrated the phytoplankton biomass development and had the highest annual mean with 93.7 mg m^{-3} at the station Pütnitz and then decreasing with increasing salinity until Nisdorf with an annual mean of 17.7 mg m^{-3}. The river Recknitz, where streaming inhibit phytoplankton blooms, had an annual mean in total chlorophyll of 6.0 mg m^{-3} (Fig. 36 , supplemental material).
Despite the clear spatial and temporal pattern in nutrient concentrations, chlorophyll content and salinity, the DZBC is a highly dynamic and complex system (hydrology, precipitation, wind). Although the lagoons got heavily polluted until the early 1990ies, they ecologically act efficiently as a filter and hence protect the open Baltic Sea from eutrophication (Schiewer, 1998).

5.4.2 Seasonality and spatial pattern of DON in the DZBC

Concentrations of DON are around 4–26 µmol L^{-1}. These concentrations ranges are comparable with other studies investigating estuarine or coastal environments (Berman and Bronk, 2003; Lara *et al.*, 1993; Schlarbaum *et al.*, 2011). Reference locations (Zingst and Dierhagen) from the outer coastal water measured in this study were 5.56 and 10.6 µmol L^{-1}, respectively. Görs *et al.* (2007) measured DON in the DZBC in 1996 and found depending on season 18–101 (summer) and 9–136 µmol L^{-1} (winter), respectively. In contrary, the outer coastal water of the Baltic Sea at the DZBC had much lower concentrations of 8–34 (summer) and 14–30 µmol L^{-1} (winter). Compared to the results of this study the concentration in the inner and outer coastal water of the DZBC are much higher which could be explained by (1) possible reduction of fertilizers, management of waste-water inputs and other actions to minimize anthropogenic loads in the last ten years or (2) by differences in DON measurements. Unfortunately DON can not be determined directly, because it concludes compartments with different molecular weights and charges. Every indirect method had disadvantages which lead to measuring inaccuracy (Graeber *et al.*, 2012a,b). Görs *et al.* (2007) calculated DON by subtraction DIN from TDN. TDN was measured by persulphate oxidation followed by nitrate determination. The persulphate oxidation was also used in this study, but before nitrate and ammonium were removed with the diffusion-method after Sigman *et al.* (1997). Such small varieties can lead to greater differences in results and direct comparison should be treated with caution.
A clear seasonality in DON (concentrations and isotopic values) could not be shown

which might be due to a high turnover of the labile fraction of DON of hours to days and, thus, more than five sampling points are necessary to identify pattern in time. However, in spring δ^{15}N-DON were significantly higher whereas concentrations of DON were lower. The bacterial degradation of labile as well as refractory DON compounds to NH_4^+ or the beginning (assimilation of nutrients) growing season of macrophytes and phytoplankton may explain this pattern. Persisting DON concentrations over the annual cycle are not surprising and these results, found in many other aquatic systems too, lead to the erogenous conclusion that DON is not utilised by primary producers (Knapp and Sigman, 2005). Though the labile fraction of DON was proven to be metabolised within hours to days by bacteria and phytoplankton and studies with high-resolution time scales should also be performed with macrophytes to understand the complex mechanism.

The fractionation factor of -1.8 support this hypothesis. In general a fractionation factor of ~ 5 ‰ indicates nitrate assimilation by phytoplankton (Sigman *et al.*, 2001), negative fractionation factors up to ~ -0.4 ‰ were only found for N_2-fixation processes (Sigman et al. 2009). In our study area many processes happen simultaneously (uptake of DON, release by bacteria, phytoplankton and macrophytes, photodegradation, remineralization by bacteria) in the field and led to this ambiguous signal. Sources and sinks of DON maybe in a kind of steady state, which would also explain relatively similar DON concentrations along the salinity gradient. However, concentration and δ^{15}N values of DON were negatively correlated (Spearman coefficient r_S = -0.627), which indicate a relation between those compartments even if the fractionation factor did not show this.

The DON:TDN ratio in contrary, showed a clear seasonality and was highest in summer (maximum of 89 %), when DIN is near the detection limit due to conversion into biomass and lowest in winter (minimum of 3 %) when the dying biomass is remineralised by bacteria and nutrients (DIN) are released. Which fractions of DON are bioavailable were not investigated in this study. Görs *et al.* (2007) identified the chemical composition of DON in the DZBC for the growing season 1996, which consist of 5 % DFAA, 36 % DCAA and 59 % remained unidentified. Thus ~ 40 % of bulk DON is comprised of bioavailable compartments. This was also shown by Wiegner *et al.* (2006) who found DON contribute 43 % to TDN in nine rivers of the USA. Seitzinger (2002) found 0–73 % of DON can be utilised depending on the origin (59 % from urban/suburban storm water run-off, > 30 % from agriculture and > 23 % from forest). The bioavailable fraction of DON during vegetation period (~ 36 % of TDN) seems not to be high, but still, can be a potential N source for macrophytes during DIN-limitation in summer, because N-demand of macrophytes especially of charophytes is low.

A gradual change of DON concentrations as with nitrate concentrations were not

found with increasing salinity, although gradually decreasing concentrations and also δ^{15}DON were expected towards the Baltic Sea due to the decreasing impact of the river Recknitz with distance from its mouth. Just the variability of DON concentrations were lowest at Dabitz and Nisdorf and can give a hint to raising influence of marine waters with relatively lower DON concentrations. The high variability of DON concentration for the other stations can be explained by the high influence of anthropogenic sources not only from the river Recknitz, Barthe and other small brooks but also from DON release of phytoplankton and sediments. Also no spatial differences were found for δ^{15}N-DON. But again the variability is much higher for the stations Recknitz, Pütnitz and Michaelsdorf, which are mainly influenced by riverine inputs. Since the range of δ^{15}N-DON is relatively high, conclusions to the origins of DON could not be made.

5.4.3 DON as N source for macrophytes

To our knowledge this is the first study investigating DON as N source for macrophytes in the field. There is evidence that with increasing δ^{15}DON also the δ^{15}N signature of macrophytes is raising (slope of 3.5, r^2 = 0.249, see Fig. 29). The relation for $\delta^{15}NO_3^-$ is much lower (slope of 0.4, r^2 = 0.086) and coincide with the results from Chapter 1, where uptake rates of DON for macrophytes where higher than nitrate. Comparing the ranges of the isotopic signatures there are distinct differences between DON (6.6 ‰) and NO_3^- (4.1 ‰) isotopic values in this study. Macrophytes (6.8 ‰) reflected the range of δ^{15}DON, while nitrate isotopic values were significantly lower. There is a lack of knowledge about fractionation during assimilation in macrophytes. With the assumption, that macrophytes do not fractionate in general or to be precise do not fractionate in N-limited systems, the signatures give an indirect hint that DON was utilised. Fractionation leads to preferred uptake of the lighter isotopes which result in lighter tissue of macrophytes and remaining heavier substrates. Brabandere *et al.* (2007) investigated nitrate assimilation in *Potamogeton perfoliatus* and identified a fractionation factor of 1.9–3.6 during nitrate assimilation. Other studies investigating fractionation in macrophytes (Carvalho and Eyre, 2011; Cohen and Fong, 2005; Teichberg *et al.*, 2006) and seagrasses (Martinetto *et al.*, 2006) found similarly low fractionation factors compared to fractionation during assimilation in phytoplankton (Cifuentes *et al.*, 1989; Needoba *et al.*, 2003; Waser *et al.*, 1998).

The variability of isotopic composition of macrophytes is highly variable and they reflect something more than only the nutrient source, in this case DON, where the variability is not comparatively wide. The range of δ^{15}N of DON is the result of microbially mediated diagenesis, photodegradation and other transformation processes. Unfortunately distinct sources of DON and their isotopic signature, could not be iden-

tified in this study. In contrary, the sources of nitrate could be identified. The isotope signatures δ^{15}N-NO_3^- and δ^{18}O-NO_3^- indicate manure/wastewater and nitrification as origin (Fig. 31).

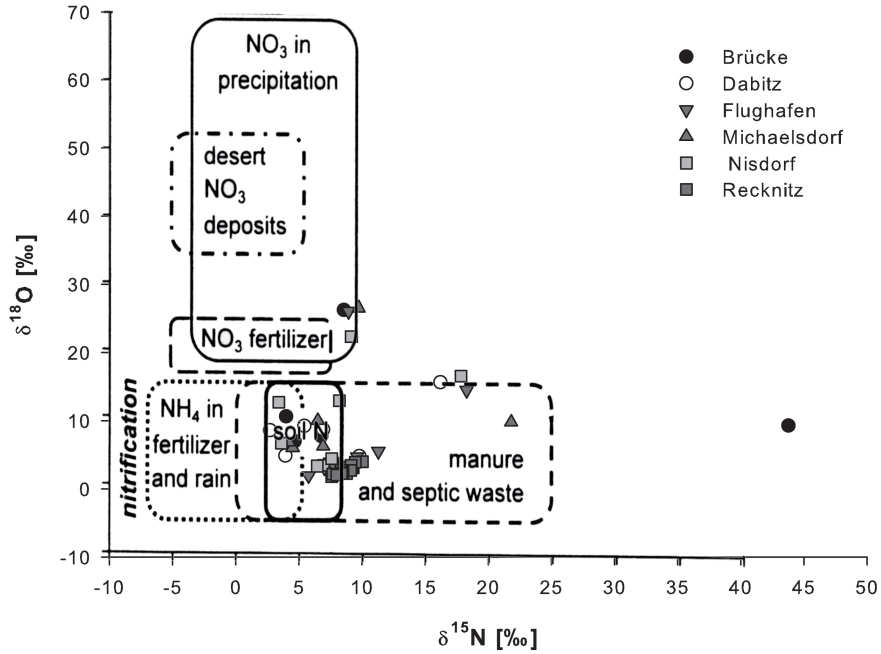

Figure 31: Plot of δ^{15}N and δ^{18}O from different sources (adapted from Kendall *et al.* (2007)). Only when nitrate concentrations were high enough (> 1 µmol L^{-1}) measurement of isotopes were possible.

The statistical analysis of EOF support these results (Fig. 32). The CCA correlations of macrophytes versus DON and nitrate are moderate with r^2 = 0.61 and r^2 = 0.68, respectively. Elevated δ^{15}N in macrophytes in summer and spring during growing season can be explained by assimilation of DON and also nitrate. As mentioned earlier fractionation was if any minor. δ^{15}N values of DON and nitrate were low in summer and autumn, and high in winter and spring. This rather unexpected values can be explained with overlapping processes. It has also to mentioned that result of EOF have to treat carefully since the data set with only five time point is extremely small. For example, the seasonal development of δ^{15}N in macrophytes of EOF are distinct from results of the complete annual data set shown in Chapter 2 (see Fig. 15 on 47, no seasonal pattern of δ^{15}N for macrophytes).

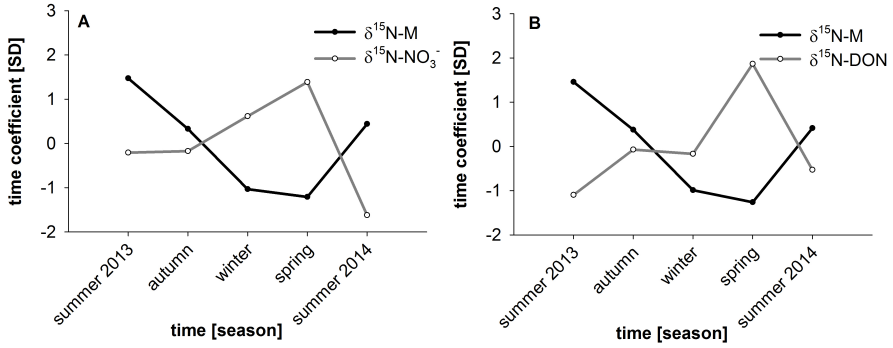

Figure 32: Time coefficients of the first empirical orthogonal function (EOF) pattern of four stations from Darss-Zingst Bodden Chain (DZBC) for A - ^{15}N of macrophytes and nitrate and B - ^{15}N of macrophytes and dissolved organic nitrogen (DON).

5.5 Conclusion

In this study addressing dissolved organic N in the DZBC, both concentrations and stable isotope signatures of DON were measured. Over an annual cycle the DON concentrations range from 4-26 µmol L^{-1} and δ^{15}N-DON had a mean of 6.5 ± 1.3 ‰. A clear seasonality was found in spring with elevated δ^{15}N-DON values and decreased DON concentrations indicating the degradation of DON to NH_4^+. DON contribute 89 % of TDN during the vegetation period and Görs *et al.* (2007) identified ~40 % of DON as labile fraction. With the assumption that the labile DON fraction can be used as N source by primary producers especially the macrophytes, which have relatively low N demand, N-limitation can be excluded. This hypothesis is supported by the comparison of isotope signature of DON and macrophytes which have similar ranges and means (6.6 ‰ and 6.8 ‰ respectively) contrary to nitrate (4.9 ‰). These results indirectly show the assimilation of DON by macrophytes and disprove the assumption of DON or more general N as influencing factor controlling the distribution and occurrence of macrophytes. In contrary, stable N isotopes of macrophytes should be considered as indicator for environmental changes. Other studies found direct relationships between wastewater N isotopes and macrophyte abundances where common methods like measurements of nutrient concentrations failed. A relation between DON and macrophytes could be found, but the expected decrease of ^{15}N in macrophytes and DON with increasing salinity could not be proven. The high variability of ^{15}N in macrophytes and DON can be attributed to possible sources and sinks and also the simultaneously processes. Not as with nitrate concentrations, which are mainly transported by the river

Recknitz the DON concentrations are spatially homogeneous. So the river Recknitz is not the main source of DON, where sources and sinks seem to be in a kind of steady state. Other possible source of DON are sediments and the bacterial degradation of PON. Further isotope investigations in the DZBC over a longer time period are necessary to make conclusion or predictions. The factors controlling the growth and distribution of macrophytes still remain a challenge for scientists in a complex and highly dynamic system like the DZBC. Finally DON seem to be a potential N source not only for macrophytes but also for bacteria and phytoplankton and should be integrated into N loading budgets on coastal ecosystems to avoid over- or underestimating.

6 Final conclusion and future outlook

Results of this dissertation could show that inorganic (NH_4^+ and NO_3^-) as well as dissolved organic nitrogen can be utilised as N source for macrophytes in the DZBC. An important finding is also the experimental set-up that has proven that AA as part of the labile DON can be taken up via roots and shoots and that they are preferred over NO_3^-. Data from field emphasise this observation in accordance with the N isotope signature from macrophytes and DON. Questions about the impact of epibionts, whether they inhibit (nutrient competition) or support macrophytes, while they provide nutrients via microbial degradation still remains open. Also the sediment in the field as additional pool for rooted macrophytes was not investigated and needs further observations.

For the first time these studies investigate isotopic signatures of macrophytes and N components in the DZBC. Unfortunately data from other studies about isotope signatures of DON in the DZBC do not exist. Viana *et al.* (2011) could demonstrate that N isotope signatures of macrophytes in an estuary could be used solely to comprehend the eutrophication process of the last decades. Since long-termed changes in nutrient concentration and light depth often could not predict abrupt changes in abundance or composition of primary producers in the DZBC (Blindow and Meyer, 2015), monitoring data about stable N isotopes should be considered as better indicator. Regardless of whether stable isotopes of macrophytes can indicate N sources and contamination of different N sources, there is still a lack of knowledge of fractionation in macrophytes. This gap should be closed, when using stable isotopes as tool.

With beginning of this study the N was assumed to be the limiting factor during the growing season for macrophytes. But during summer when DIN concentrations are low and the DON gets more important as N source the demand of N for macrophytes should be secured since DON concentrations stagnate on a constant level. N turned out not to be the limiting factor. Identification of N sources or contaminations and factors controlling macrophytes is important since they can buffer short nutrient contaminations and lead to an improvement of environmental conditions.

The seasonality and spatial pattern of isotopic values and concentrations of DON were investigated. The bioavailability of bulk DON in the DZBC was not considered in this study and also the quick turnover of the labile fraction of DON within hours to days could not be investigated during this sampling period. Other studies have shown that the bioavailablity can change depending on the origin. DON should be no longer neglected from N loading budgets at coastal waters and there is not only a lack of the whole N-budget (nitrate underestimate, TDN overestimate) but also all primary producers should be integrated in such aquatic systems.

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List of abbreviations

AA	amino acids mixture
ANOVA	analysis of variance
CAM	crassulacean acid metabolism
CN	carbon:nitrogen
CO	carbon oxide
CO_2	carbon dioxide
DA	Dervadas Alloy
DCAA	dissolved combined amino acids
DFAA	dissolved free amino acids
DIC	dissolved inorganic carbon
DIN	dissolved inorganic nitrogen
DON	dissolved organic nitrogen
DW	dry weight
DZBC	Darss-Zingst Bodden Chain
EA	elemental analyser
EDTA	Ethylenediaminetetraacetic acid
EOF	Empirical Orthogonal Functions
GC	gas chromatograph
HCl	hydrochloric acid
HCO_3	hydrogen carbonate
H_2SO_4	sulphuric acid
IRMS	isotope ratio mass spectrometer
MgO	magnesium oxide
N_2	elemental nitrogen
N	nitrogen
NaOH	sodium hydroxide solution
$NaNO_2$	sodium nitrite
$K_2S_2O_8$	potassium peroxodisulphate
KNO_3	potassium nitrate
NH_4^+	ammonium

NH_3	ammonia
NH_4Cl	ammonium chloride
N_2O	nitrite oxid
NO_2^-	nitrite
NO_3^-	nitrate
PEP	phosphoenolpyruvate
POC	particulate organic carbon
POM	particulate organic matter
PON	particulate organic nitrogen
RUBISCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SO_2	sulphur oxide
TDN	total dissolved nitrogen
TN	total nitrogen
TP	total phosphate
VCl_3	vanadium chloride

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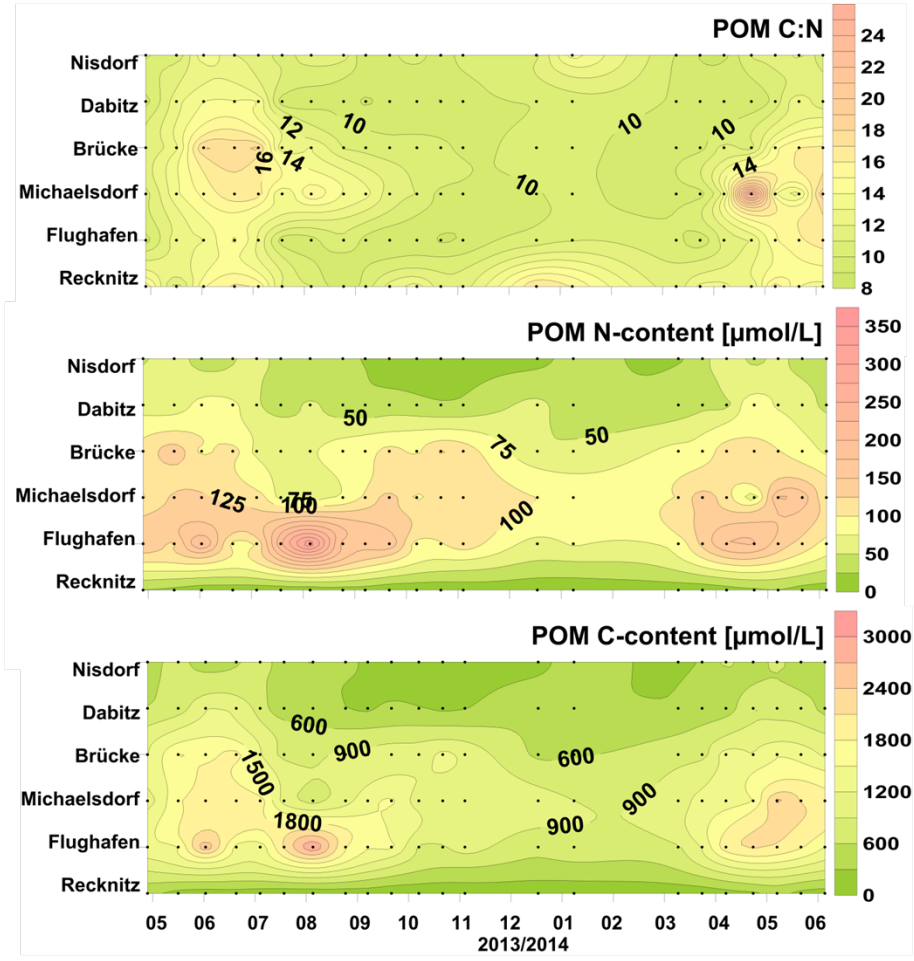
Supplement



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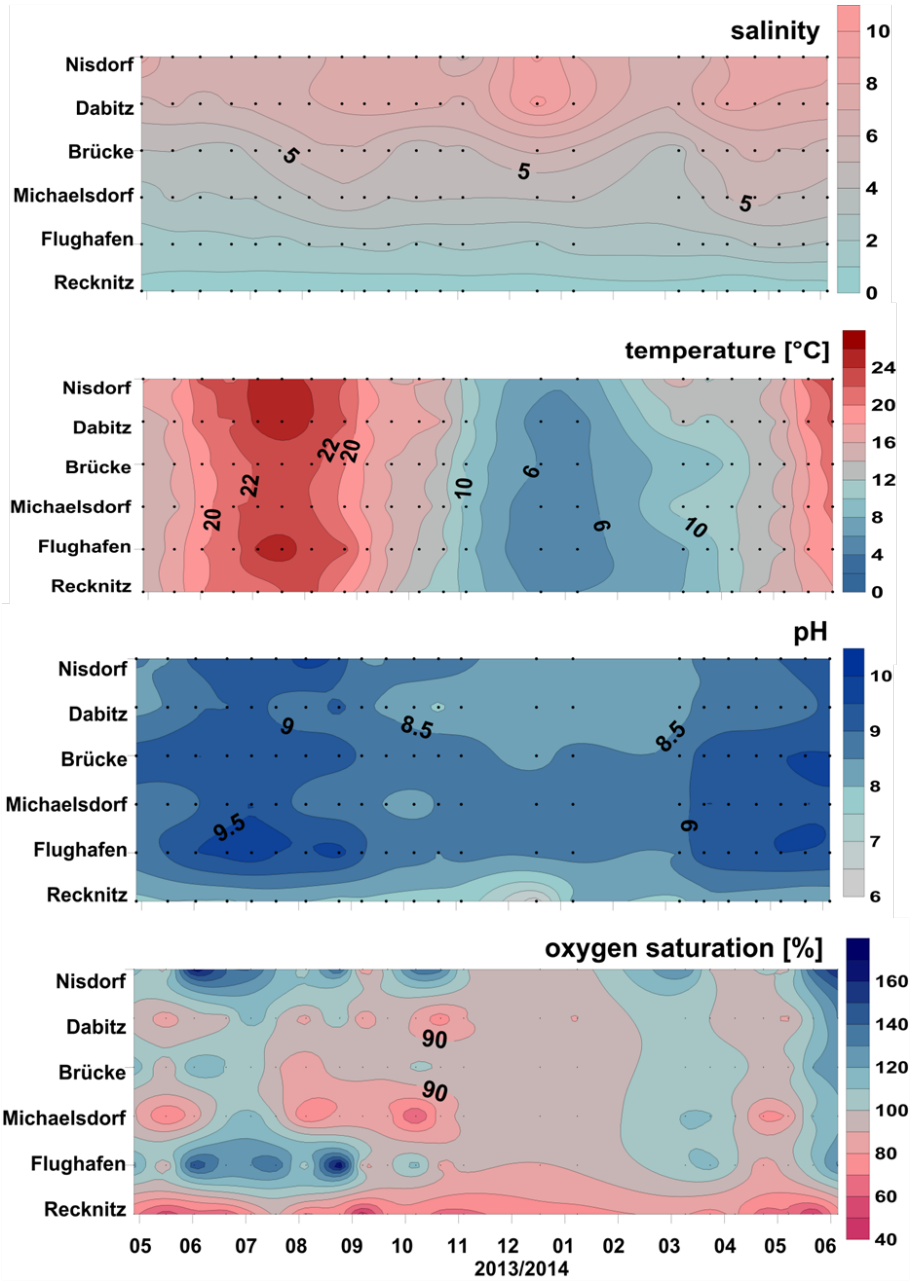

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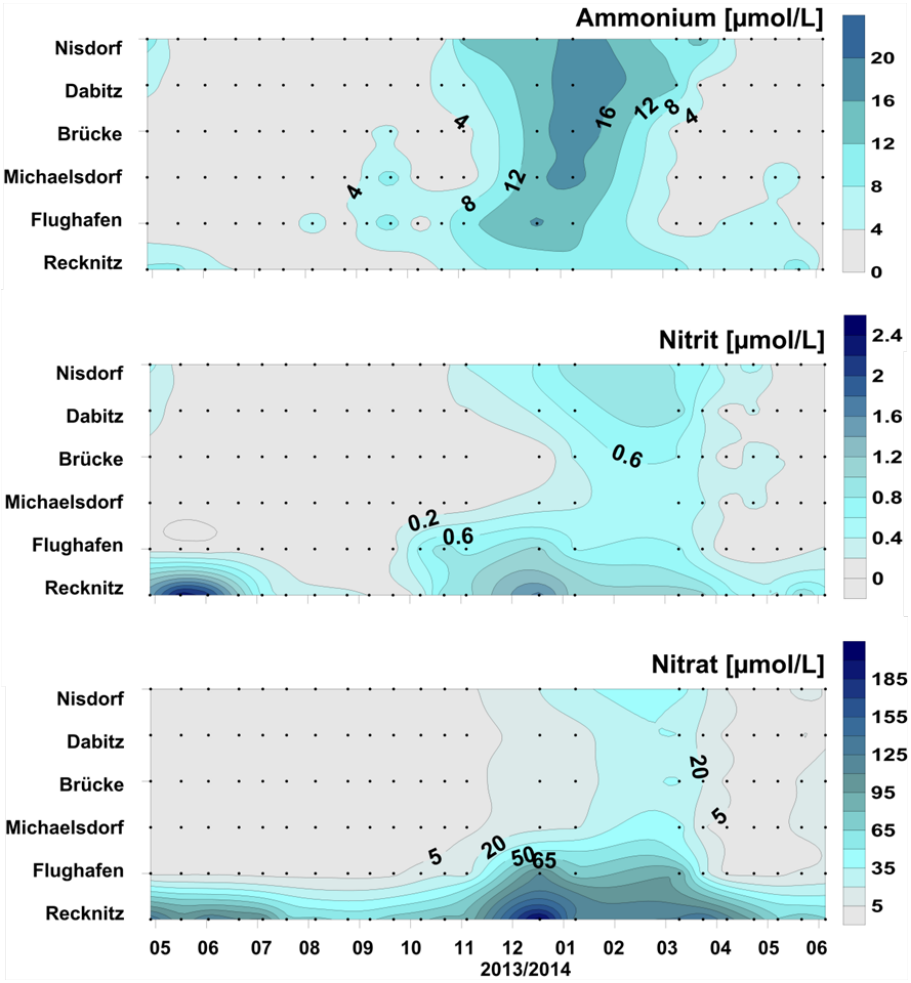

Figure 35: Filled contour plots of concentrations of NH_4^+, NO_3^- and NO_2^- from surface waters of six stations of the DZBC over an annual cycle (June 2013–June 2014). Plots were created with Surfer 10 and Grapher.

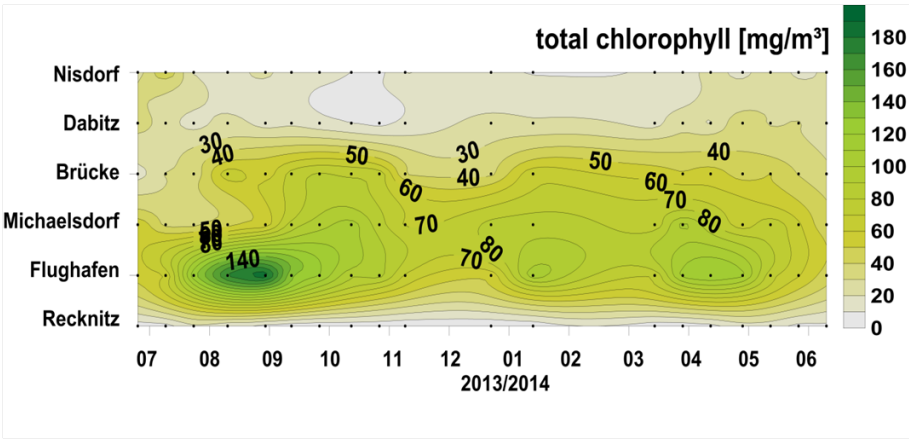

Figure 36: Filled contour plot of total chlorophyll from surface waters of six stations of the DZBC over an annual cycle (July 2013–June 2014). Plots were created with Surfer 10 and Grapher.

Acknowledgements

Der mögliche Beitrag von DON als Nährstoffquelle für Makropyhten der Darss-Zingster Boddenkette hat mich dreieinhalb Jahre beschäftigt und die Arbeit findet nun sein Ende. An dieser Stelle möchte ich den vielen Leuten, ohne die diese Arbeit nicht entstanden wäre, ganz herzlich bedanken. Noch vor Beginn der eigentlichen Arbeit haben mich meine Doktor-Mutter **Prof. Dr. Maren Voss** und mein Doktor-Vater **Prof. Dr. Hendrik Schubert** bei der Antragstellung und der eigentlichen Idee der Dissertation unterstützt und ich danke Ihnen für die Möglichkeit diese Doktorarbeit umzusetzen. Maren danke ich für die angenehme Betreuung, die immer sehr strukturiert und konstruktiv verlief und für mich nicht nur eine riesen Hilfe beim Schreiben der Arbeit und des Papers, sondern auch allgemein im Alltag als Wissenschaflter war. Hendrik danke ich ebenso für die unkomplizierte Betreuung und die vielen Freiräume die mir gelassen wurden, um meine eigenen Ideen und Experimente umzusetzen können. **Andreas Kleeberg** danke ich für die Zusage am Thesis-Komitee und die Diskussionen. Der **Fazit-Stiftung** danke ich für die zweieinhalbjährige Förderung der Arbeit mit einem Stipendium.

Ich hatte das Privileg, sowohl in die Arbeitsgruppe **'Aquatische Ökologie'** als auch **'Mariner Stickstoffkreislauf'** ganz herzlich aufgenommen zu werden und möchte mich für die entspannte Zusammenarbeit bedanken.

Im einzlenen möchte ich mich bei **Maike**, **Claudia**, **Anja** und **Petra** für die schönen, unterhalsamen Ausfahrten und erfolgreichen Probenahmen bedanken. Petra danke, dass du mir Fahrstunden mit Unibussen und Fahrsicherheit gegeben hast, das war doch recht nervenraubend zu Beginn. **Arne** danke für die Hiwis und allen weiteren Hilfestellungen jeglicher Art. **Frau Heinrich**, der guten Fee der Ökologie, danke ich für die unkomplizierte Hilfe bei nervigen Verwaltungskram. **Aisha** du warst der beste HIWI, den man sich nur wünschen kann und ich danke dir für die großartige Hilfe bei dem Tracerexperiment, für die endlosen Einwaagen und auch die lustigen Ausfahrten. **Iris** gilt ein besonders großes Dankeschön für die unglaublich vielen Messungen am Massenspektrometer, für die Einführung und Anleitung im Labor und den vielen wertvollen Tipps für technische/methodische Verbesserungen. Ohne dich wären die ganzen Ergebnisse nicht möglich gewesen. **Joachim** danke ich für die Einführungnen in EOF-Analysen

und deren Berechnungnen. Außerdem möchte ich mich bei **Henning**, **Anja**, **Anne** für die gemeinsame Zeit im Büro und die kleinen Sektfeiern (möge noch viele weitere Folgen) bedanken. **Anne** dir danke ich auch für das Beibringen von LaTex (so sieht die Arbeit doch schöner aus als mit Word) und den vielen Korrketuren. Ganz besonders möchte ich auch **Sandra** danken, die mein Interesse an der Wissenschaft überhapt erst geweckt und gefördert hat und mir jederezeit bei Problemen beistand und mich beratschlagt hat. Nebenbei bei danke ich ihr auch für das gemeinsame, erholsame Musizieren.

Ich möchte mich vor allem bei **Felix**, **Flo** und **Anne** für die geselligen Kneipenbesuche und dafür, dass ihr mich immer unterstütz, mir Mut zugesprochen und mich zum Lachen gebracht habt, bedanken. Ganz besonderen Dank an **Frau Dr. Anna-Marie Klamt** für die vielen aufbauenden Skypetelefonate und Besuche. **Frau Dr. Katrin Attermeyer** danke ich für die zahlreichen und sehr schnellen Korrekturen, die Besuche in Rostock und die Skyegespräche.

Vor allem möchte ich mich bei meiner lieben Familie bedanken, die immer an mich geglaubt und mich auf diesem Weg unterstütz hat wo sie nur konnte. Danke an **Mutti**, **Papa**, **Romy** und **Tony**. Danke auch an die Thums (**Veronika**, **Karsten**, **Benno** und **Jonas**). Zu guter Letzt möchte ich mich bei meinem liebevollen Partner und meiner besseren Hälfte, **Gregor**, für die unendliche Geduld vor allem in der Endphase, die Unterstützung und Aufmunterungen in schweren Zeiten, die aufbauenden Freizeitgestalungen und für die, wenn auch etwas seltenen und kurz geratenen, Nackenmassagen bedanken.

Declaration of Authorship

Hiermit erkläre ich, Christiane Volkmann, dass die vorliegende Arbeit mit dem Titel 'Zur Bedeutung organischen Materials für Makrophyten in Küstengewässern der Ostsee' selbstständig und ohne fremde Hilfe verfasst und keine anderen Hilfsmittel als angegeben, verwendet wurde. Insbesondere versichere ich, dass ich alle wörtlichen und sinngemäßen Übernahmen aus anderen Werken als solche kenntlich gemacht habe.

I hereby certify that the thesis 'The impact of organic material for macrophytes in coastal waters of the Baltic Sea' has been composed by me and is based on my own work, unless stated otherwise. I ensure that all references and verbatim extracts have been quoted, and all sources of information, including graphs and data sets, have been specifically acknowledged.

Rostock, 04.05.2016

Publications

Volkmann, C., Halbedel, S., Voss, M. and Schubert, H. (2016). The role of dissolved organic and inorganic nitrogen for growth of macrophytes in coastal waters of the Baltic Sea. *Journal of Experimental marine biology and ecology*, 477:22-30.