Cyanobacterial Nitrogen Fixation in the Baltic Sea

With focus on *Aphanizomenon* sp.

Jennie Barthel Svedén
Till mamma och pappa
ABSTRACT

Cyanobacteria are widely distributed in marine, freshwater and terrestrial habitats. Some cyanobacterial genera can convert di-nitrogen gas (N₂) to bioavailable ammonium, i.e. perform nitrogen (N) fixation, and are therefore of profound significance for N cycling. N fixation by summer blooms of cyanobacteria is one of the largest sources of new N for the Baltic Sea. This thesis investigated N fixation by cyanobacteria in the Baltic Sea and explored the fate of fixed N at different spatial and temporal scales. In Paper I, we measured cell-specific N fixation by Aphanizomenon sp. at 10 °C, early in the season. Fixation rates were high and comparable to those in late summer, indicating that Aphanizomenon sp. is an important contributor to N fixation already in its early growth season. In Paper II, we studied fixation and release of N by Aphanizomenon sp. and found that about half of the fixed N was rapidly released and transferred to other species, including autotrophic and heterotrophic bacteria, diatoms and copepods. In Paper III, we followed the development of a cyanobacterial bloom and related changes in dissolved and particulate N pools in the upper mixed surface layer. The bloom-associated total N (TN) increase was mainly due to higher particulate organic N (PON) concentrations, but also to increases in dissolved organic nitrogen (DON). About half the PON-increase could be explained by the sum of N-fixing cyanobacteria, other phytoplankton (>2µm) and zooplankton, indicating that production was stimulated by the N fixation. In Paper IV, we used a growth model based on measured photosynthesis–irradiance relationships to explore the production potential of Aphanizomenon sp. The model included data on irradiance, biomass, temperature and light attenuation (1999–2013). Until the bloom peak, the modelled production matched the measured biomass, indicating low production losses. Over the whole season, the modelled production could explain a substantial part of the summer TN increase, assuming that plausible losses (such as grazing or cell lysis) are retained within the upper mixed layer. Complementing the other data, we also investigated the nutrient content (Paper I) and varying cell width (Paper IV) of Aphanizomenon sp. By a combination of approaches, this thesis has contributed new information on cyanobacterial N fixation rates, the transfer of fixed N to other organisms in the food web and shown the potential for fixed N to stimulate summer primary and secondary production in the Baltic Sea.
LIST OF PAPERS


(IV) Svedén JB, Walve J. Production and nitrogen fixation by Baltic Sea *Aphanizomenon* sp. — estimates from a growth model. *Manuscript*

**Paper I**: Participation in experimental work, large part in microscopy and processing of nutrient content and mass spectrometry data, lead author responsible for writing, JBS and BA contributed equally to the study, **Paper II**: Participation in experimental work (2010 and 2011), smaller part in data processing and writing, **Paper III**: Main person responsible for field work, microscopy, data processing and writing, **Paper IV**: Main person responsible for microscopy, data processing and writing.

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**Related papers not included in thesis:**

*Scientific papers*


Popular science paper

ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µm</td>
<td>micrometer</td>
</tr>
<tr>
<td>(^{13})C</td>
<td>stable carbon isotope, used as label</td>
</tr>
<tr>
<td>(^{14})C</td>
<td>radioactive carbon isotope, used as label</td>
</tr>
<tr>
<td>(^{15})N</td>
<td>stable nitrogen isotope, used as label</td>
</tr>
<tr>
<td>Anammox</td>
<td>anaerobic ammonium oxidation</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>B.P.</td>
<td>before present</td>
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<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>CTD</td>
<td>instrument for conductivity, temperature and depth</td>
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<tr>
<td>DIN</td>
<td>dissolved inorganic nitrogen</td>
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<tr>
<td>DON</td>
<td>dissolved organic nitrogen</td>
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<tr>
<td>EA-IRMS</td>
<td>elemental analysis isotope ratio mass spectrometer</td>
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<tr>
<td>fmol</td>
<td>femtomole</td>
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<td>h</td>
<td>hour</td>
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<tr>
<td>HELCOM</td>
<td>Baltic Marine Environment Protection Commission - Helsinki Commission</td>
</tr>
<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>N</td>
<td>nitrogen</td>
</tr>
<tr>
<td>(N_2)</td>
<td>di-nitrogen gas</td>
</tr>
<tr>
<td>NH(_3)</td>
<td>ammonia</td>
</tr>
<tr>
<td>NH(_4^+)</td>
<td>ammonium</td>
</tr>
<tr>
<td>NO(_2^-)</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO(_3^-)</td>
<td>nitrate</td>
</tr>
<tr>
<td>N. spumigena</td>
<td>Nodularia spumigena</td>
</tr>
<tr>
<td>O(_2)</td>
<td>oxygen gas</td>
</tr>
<tr>
<td>P</td>
<td>phosphorus</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>PON</td>
<td>particulate organic nitrogen</td>
</tr>
<tr>
<td>POC</td>
<td>particulate organic carbon</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>SIMS</td>
<td>secondary ion mass spectrometry</td>
</tr>
<tr>
<td>SMHI</td>
<td>Swedish Meteorological and Hydrological Institute</td>
</tr>
<tr>
<td>SNMMP</td>
<td>Swedish National Marine Monitoring Program</td>
</tr>
<tr>
<td>sp./spp.</td>
<td>latin for species/species pluralis</td>
</tr>
<tr>
<td>TN</td>
<td>total nitrogen</td>
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</table>
INTRODUCTION

Cyanobacteria – a success story

Cyanobacteria are ancient organisms that evolved during the Archean or early Proterozoic eon (Schopf et al. 2007; Rasmussen et al. 2008). They constitute one of the most morphologically diverse prokaryotic phyla on Earth, with the ability to branch to filaments and differentiate specialized cells, and thereby attain diverse structures and functions (Adams and Duggan 1999; Kumar et al. 2010). The early-achieved morphological diversity (Schirrmeister et al. 2011) could explain their wide distribution, in marine, freshwater and terrestrial habitats, where they occur as unicellular or filamentous organisms, free-living or as endosymbionts. In aquatic environments they are often bloom-forming, at times dominating the phytoplankton community (Fig. 1).

![Sailing boat in a cyanobacteria bloom, between Oxelösund and Landsort, northern Baltic Proper, 2014-07-25. Photo: Swedish Coast Guard (Kustbevakningen)](image)

The ability of some cyanobacterial lineages to use atmospheric di-nitrogen ($N_2$) as nitrogen source, i.e. nitrogen (N) fixation, could have emerged early, as a response to depletion of bioavailable N (Navarro-González et al. 2001). In the Archean atmosphere, $N_2$ is believed to have been fixed abiotically to nitric oxide under the influence of lightning discharge, but the low yield of this process, combined with an increasing N demand, suggests a strong evolutionary selection for biological nitrogen fixation (Falkowski 1997; Navarro-González et al. 2001). An early origin of N fixation is also
implied by the highly conserved \textit{nif} genes, encoding for the enzyme nitrogenase (Post-gate 1982; Zehr et al. 1995; Böhme 1998). Nitrogenase catalyzes the energy-demanding reduction of \( \text{N}_2 \) to \( \text{NH}_3 \), which requires 16 ATP (Falkowski and Raven 2007).

A pronounced oxygenation of the Earth’s atmosphere occurred about 2.3 billion years ago (Bekker et al. 2004). This important event in the history of life has been ascribed to the production of elementary oxygen gas (\( \text{O}_2 \)) by phototrophic cyanobacteria (Canfield 2005). The increasing levels of atmospheric \( \text{O}_2 \) ultimately caused cyanobacteria to evolve different strategies to protect nitrogenase, which is deactivated by \( \text{O}_2 \). Among some filamentous cyanobacteria, these strategies included the formation of heterocysts, differentiated N-fixing cells (Adams and Duggan 1999). Cyanobacterial symbiosis later co-evolved to the chloroplasts of eukaryotic cells (e.g. Deusch et al. 2008) and the ancient history of cyanobacteria is thus fundamental for aerobic production on our planet.

**HETEROCYSTS**

Heterocysts are terminally differentiated cyanobacterial cells specialized in N fixation, but lacking oxygen production (Photosystem II activity). Their respiration rates are high and the glycolipid cell walls are thick in order to keep the enzyme nitrogenase in an anaerobic environment (Adams and Duggan 1999). The energy needed for N fixation is derived from photosynthesis or respiration of carbohydrates (Flores and Herrero 1994), which are imported from vegetative cells. In turn, the heterocyst exports glutamine, as a product of N fixation (Böhme 1998). The heterocyst frequency (number of heterocysts per total cell number) varies over the growth season and between species. For instance, Baltic Sea \textit{Aphanizomenon} sp. has a heterocyst frequency of 1–3% of the total cell number whereas Baltic Sea \textit{Nodularia spumigena} has a frequency of 5–10% (Walve and Larsson 2007; Ploug et al. 2010; Mohlin et al. 2012).

**The Baltic Sea case**

Cyanobacterial pigments (zeaxanthin) in Baltic Sea sediment cores indicate the presence of blooms already during the early Litorina Sea period, about 7000 years ago (Bianchi et al. 2000, see also section Study area). The prevailing N limitation of to-
day’s Baltic Proper may also date back to this period, when phosphorus (P) rich seawater is proposed to have entered the Litorina Sea (Bianchi et al. 2000). In marine environments, P is recycled more efficiently from sediments than in freshwater, due to chemical processes connected to the high concentrations of sulfate in sea salt (Blomqvist et al. 2004). A later emergence of blooms has been proposed to be coupled to increased populations and land-use with concomitant elevated nutrient loads during the Medieval Period (Zillén and Conley 2010). Both views are based on the assumption that an environment rich in P and with a low N:P ratio favors diazotrophic (N-fixing) cyanobacteria, which are able to circumvent the N limitation faced by the non-diazotrophic phytoplankton community. The riverine nutrient load to the Baltic Sea is generally supplied in a ratio that exceeds the Redfield N:P ratio of 16:1 (mol:mol) for optimum phytoplankton growth (HELCOM 2011; Redfield 1958), but internal processes favor a low N:P ratio of the Baltic Proper (Granéli et al. 1990). Transport of organic matter to bottom waters and sediments increases oxygen consumption and stimulates loss of N through denitrification and anammox (Vahtera et al. 2007; Bonaglia et al. 2014). At the same time, P (as phosphate) is released from the sediments during anoxic and low-oxygen conditions, and thus becomes available in the water column (Gunnars and Blomqvist 1997; Viktorsson et al. 2013). This results in a low inorganic N:P ratio in the winter water and a N-limited spring bloom, leaving a surplus of P, at least partly available for the summer bloom of N-fixing cyanobacteria.

**NUTRIENT LIMITATION**

The Redfield ratio expresses the main nutrient requirements for phytoplankton, being 106:16:1 (on molar basis) for C:N:P. If the dissolved nutrients available to phytoplankton deviate from this ratio, the nutrient in low concentration (in relation to the Redfield conditions) tends to become limiting for phytoplankton production. In the Baltic Sea the N:P ratio differs between the sub-basins, mainly due to biogeochemical processes coupled to salinity. The open Baltic Proper is generally N-limited, while there is a transition towards P-limitation in the Gulf of Bothnia (Granéli et al. 1990). However, a recent study has shown that the Bothnian Sea has transitioned to more N-limited conditions during the last decades, due to P-rich bottom waters entering from the northern Baltic Proper (Rolff and Elfwing 2015).
Heterocystous N-fixing cyanobacteria in the Baltic Sea

There are three known N-fixing genera of cyanobacteria in the Baltic Sea: *Aphanizomenon* sp., *Dolichospermum* spp. and *Nodularia spumigena* (Fig. 2). These are filamentous and heterocystous. In addition, they contain gas vesicles, enabling them to regulate their buoyancy in the water column (Walsby et al. 1995). The late summer surface blooms of *N. spumigena* are renowned for their toxicity and negative effects on amenity and recreational values (Sivonen et al. 2007). *N. spumigena* is the largest of the three and its comparably wide and long filaments often aggregate to surface scums at the end of the bloom (Fig. 1). *N. spumigena* mostly resides in the upper 5 m of the water column, and is found mainly in July–August and in the open sea. *Aphanizomenon* sp. usually blooms earlier than *N. spumigena* (June–August), does not form surface accumulations to the same extent and has a deeper depth distribution (Hajdu et al. 2007). In contrast to the other two diazotrophs, *Aphanizomenon* sp. can be found all year around, even at low abundances under the winter ice (Laamanen and Kuosa 2005). The filaments are mainly tightly packed together to mm-long needle-like colonies, that may contain several hundreds of filaments and thousands of cells (Fig. 2A). *Aphanizomenon* sp. is reportedly more common in the northern Baltic Proper, whereas *N. spumigena* is more abundant in the central and south part of the Baltic Sea (Stal et al. 2003; Wasmund et al. 2015). *Dolichospermum* spp., formerly known as *Anabaena* spp. (Wacklin et al. 2009), is the least abundant of the three but may occasionally reach high abundances (Wasmund et al. 2015). This genus is also the least studied, but has recently gained more interest and seems to have high N fixation rates (Klawonn 2015).

![Figure 2](image.png)

**Figure 2** (A) *Aphanizomenon* sp. colonies. (B) Single filament of *Aphanizomenon* sp. (4.5 µm wide). (C) Filament of *Dolichospermum* spp. (6 µm wide) (D) Filament of *Nodularia spumigena* (12 µm wide). Photos kindly provided by Helle Ploug (A) and Helena Höglander (B–D).
The two sides of Baltic Sea N-fixing cyanobacteria

During the last century, the Baltic Sea has been subject to several anthropogenic stressors, including e.g., contaminants, over-fishing and high nutrient loads (Elmgren 2001). The summer cyanobacterial blooms are perhaps the most conspicuous sign of eutrophication in the Baltic Sea. Cyanobacterial blooms have been frequently observed in the open waters of the Baltic Proper since the 1960s, whereas earlier they were mainly reported from coastal areas (Finni et al. 2001). There is a general perception that the blooms have increased in intensity and occurrence during the last decades, but their patchiness and inter-annual variability makes these assumptions challenging to confirm by regular monitoring. Data from the Swedish National Marine Monitoring Program (SNMMP) show a positive trend of cyanobacterial biomass at the coastal station B1 (northern Baltic Proper) since the early 1990s, but not during the last 15 years. The other SNMMP stations in the Baltic Proper show neither increasing or decreasing concentrations of cyanobacteria (Höglander et al. 2016), similar to other international monitoring stations in the Baltic Proper (Wasmund et al 2015). Using satellite derived time series data (1979–2013), Kahru and Elmgren (2014) found significantly more surface accumulations during the second half of the study period (1997–2013), but emphasized the inter-annual variability.

The toxicity of cyanobacterial blooms has been shown to have variable effects on other organisms in the food web (Karjalainen et al. 2007; Sukenik et al. 2015). The hepatotoxin nodularin (from *N. spumigena*) appears to be transferred and accumulated in the food web (Engström-Öst et al. 2002). Furthermore, the neurotoxin β-methylamino-L-alanine (BMAA), produced by a majority of the cyanobacterial species, has been found in Baltic Sea summer blooms, as well as bioaccumulated in zooplankton, fish and mussels (Jonasson et al. 2010), and recently also traced in humans (Berntzon et al. 2015).

Despite their negative effects, cyanobacteria are significant actors in the Baltic Sea nutrient cycling, as they provide N to the N-limited phytoplankton community (Vahtera et al. 2007). Several studies have shown a large leakage of newly fixed N from live cyanobacterial cells as ammonium (NH$_4^+$, Ploug et al. 2010; 2011) or dis-
solved organic nitrogen (DON, Glibert and Bronk 1994). This N has potential to alleviate the summer N-limitation, and hence increase the summer production of other phytoplankton. Released DON is likely available for growth of both phytoplankton and heterotrophic bacteria (Korth et al. 2012; Hoikkala et al. 2015), and can reach higher trophic levels via the microbial loop (Azam et al. 1983). Diazotrophically derived N has been shown to reach higher trophic levels, i.e. zooplankton, indirectly or by direct grazing (Wannicke et al. 2013; Karlson et al. 2015). Zooplankton is an important food source for fish larvae and fish species like herring (Clupea harengus) and sprat (Sprattus sprattus) (Arrhenius and Hansson 1993). The benthic community also utilizes cyanobacterial N (Karlson et al. 2014). However, direct sedimentation of cyanobacteria is generally considered a less important process compared to their remineralization in the upper mixed layer (Sellner 1997). In conclusion, cyanobacteria are both a cause and consequence of eutrophication, but also have the potential to increase overall productivity in the Baltic Sea, including beneficial ecosystem services such as fish production (Fig. 3).

Figure 3 During summer, cyanobacteria potentially stimulate the production of other phytoplankton by their release of N. The diazotrophically derived N is potentially also transferred to zooplankton by grazing on these phytoplankton, or directly on the cyanobacteria. Zooplankton are an important food source for juvenile fish. Fixed N might also reach the benthic community, through direct or indirect sedimentation. Elena Gorokhova and Per Holliland are acknowledged for pictures of plankton and fish.
SCOPE OF THE THESIS

Several previous studies have measured or estimated cyanobacterial N fixation in the Baltic Proper using different approaches (e.g., Rahm et al. 2000; Larsson et al. 2001; Wasmund et al. 2005; Ohlendieck et al. 2007; Rolff et al. 2007; Degerholm et al. 2008; Gustafsson et al. 2013), highlighting the significance of this source of new N. Yet, little is known about the pathways of fixed N in the Baltic Sea ecosystem. This thesis aims to contribute new knowledge about Baltic Sea cyanobacteria, their N fixation and the possible fate of the fixed N. The included papers present new data on cell-specific fixation rates of *Aphanizomenon* sp. at low temperatures (10 °C) and a fast transfer of fixed N to other species in the plankton community (*Paper I* and *Paper II*). The thesis further characterizes different N pools in the upper mixed layer during a cyanobacteria bloom (*Paper III*). The bloom-associated increase of total nitrogen (TN) in the upper mixed layer (Larsson et al. 2001; Rolff et al. 2007) has not been previously described in terms of different nitrogen fractions. Additionally, the potential of *Aphanizomenon* sp. to explain the TN increase is explored, using a model based on measured photosynthesis–irradiance relationships and *in situ* monitoring data (*Paper IV*). With special focus on *Aphanizomenon* sp. (Fig. 4) the thesis also presents new empirical data on nutrient content and cell size of this widespread species.

![Figure 4](image.png)

*Figure 4* Baltic Sea *Aphanizomenon* sp. colonies of different sizes and shapes. Photos: Helle Ploug and Jennie B. Svedén.
Nitrogen fixation and potential pathways for fixed N

Three methodological approaches have been used:

1. **Stable isotope tracer incubations**
   - Does *Aphanizomenon* sp. fix N in the early season at 10 °C? (*Paper I*)
   - Is fixed N released from *Aphanizomenon* sp. and transferred to other species within the food web? (*Paper II*)

2. **Field monitoring**
   - Which N pools constitute the bloom-associated increase in total N, and can the post-bloom N decrease be explained by sedimentation? (*Paper III*)

3. **Modelling**
   - Does modelled production of *Aphanizomenon* sp. have potential to explain total N increase? (*Paper IV*)

*Aphanizomenon* sp. close-up

- What is the volume-specific content of carbon (C), nitrogen (N) and phosphorus (P) in *Aphanizomenon* sp.? (*Paper I*)
- Does the cell width of *Aphanizomenon* sp. vary with water temperature during the growth season? (*Paper IV*)
STUDY AREA AND METHODS

Study area
The Baltic Sea is a geologically young, semi-enclosed sea characterized by estuarine-like circulation and hydrologically distinct sub-basins. The mean depth is only 54 m with the Landsort Deep (459 m) as the deepest part (Leppäranta and Myrberg 2009). Seawater enters from the North Sea through the narrow Danish straits in the south, while large rivers carry a substantial freshwater inflow. The salinity increases from 1–3 (PSU) in the north (Bothnian Bay) to ~10 in the south (Arkona Basin, Wulff et al. 1990). The narrow passages and shallow sills of the Baltic Sea cause a restricted water exchange with the ocean. The stratification of the water column, separating the less saline surface water from the denser bottom water below the halocline, further decreases water exchange in the deep areas, especially in the Baltic Proper. The occasional major inflows of water from the North Sea, required for ventilation of the bottom waters, have decreased in frequency and intensity since the mid-1970s (Schinke and Matthäus 1998). The long water retention time in the Baltic Sea, about 30 years (Döös et al. 2004), makes it vulnerable to, e.g., nutrient loads and contaminants.

Since the latest glaciation, the Baltic Sea has undergone several developmental stages, alternating between freshwater lake and inland sea. It was formed as a meltwater lake, the Baltic Ice Lake (~12 000 years Before Present, B.P.), when the Weichselian ice sheet retreated (Ignatius et al. 1981; Donner 1995). The opening of a strait in the lowland of central Sweden later drained the lake and led to saltwater influx from the ocean (in the west) and a brackish water stage, the Yoldia Sea (~10 600 years B.P., Donner 1995). The importance of the ocean connection during this stage has however been debated (Ignatius et al. 1981). Due to postglacial land rise (isostatic uplift), the opening towards the ocean narrowed (9 000–9 600 years B.P.) and marked the beginning of a new freshwater stage, the Ancylus Lake (Donner 1995). Decreased isostatic uplift and a rapid raise of the ocean level eventually allowed seawater to enter through the Danish Straits, establishing a new brackish stage, the Litorina Sea (~7 500 years B.P., consisting of several sub-stages), with higher salinities than in the present-day Baltic Sea (Ignatius et al. 1981; Donner 1995). As the Danish Straits narrowed, the salinity
decreased and about 300 years ago the Baltic Sea entered its recent stage (Donner 1995). This relatively short geological history of drastic changes, combined with low salinity, has resulted in a low species diversity (Elmgren and Hill 1997).

The specific study area of this thesis was the northern Baltic Proper (Fig. 5). Here, the salinity in the surface water is 6–7, but below the strong halocline at 60–80 m (Granéli et al. 1990; Carstensen et al. 2014) the salinity is higher (~10, Tuominen et al. 1998). A seasonal thermocline develops during summer at about 10–20 m depth (SNMMP-data, SHARK database, available at: smhi.se), having important implications for primary production. For the incubation experiments (Papers I and II), water was sampled at the SNMMP coastal station B1 (N 58°48', E17°37'). The field monitoring (Paper III) was performed at the SNMMP open sea stations BY31 (Landsort Deep, N 58°35', E 18°14') and BY29 (N 58°53', E 20°19') in the northwestern and northeastern Baltic Proper, respectively. Three additional stations in both areas were also sampled (C1–C3, C7–C9). Long-term SNMMP data from BY31 was used in the model (Paper IV).

**Figure 5** The sampling stations in the northern Baltic Proper.
Stable isotope tracer incubations

In nature, elements exist as different isotopes. These variations of an element have identical numbers of protons (and electrons), and usually exhibit equal chemical properties. Isotopes of the same element differ by the number of neutrons in the cell nucleus, resulting in different atomic masses. Many of the isotopes are radioactive, some with very short half-lives, while others are stable. Nitrogen has two stable isotopes, with atomic masses of 14 and 15, respectively. $^{14}$N is the naturally most abundant isotope (99.636%) while the heavier isotope $^{15}$N, with one additional neutron, is less common (0.3636%). This large difference in natural abundance, together with the nearly identical chemical behaviour, make $^{15}$N ideal as a tracer for N-cycling. We have used stable isotope techniques to study N fixation in Paper I and Paper II. In the same manner, the stable carbon isotopes $^{12}$C and $^{13}$C were used to study C-fixation, with $^{13}$C as tracer. In Paper I we also used the radioactive isotope (i.e. not stable) $^{14}$C as a tracer to study carbon uptake.

For both Paper I and Paper II, field-collected water samples containing Aphani-zomenon sp. were incubated in glass bottles closed with rubber stoppers, through which stable isotopes $^{13}$C-bicarbonate and $^{15}$N$_2$ were injected. Incubations were made under ambient temperature and light conditions, for different periods of the diel cycle. In Paper I, $^{15}$N$_2$ was injected as a bubble (Montoya et al. 1996), but in Paper II, we also used the method of injecting an aliquot of a pre-prepared $^{15}$N$_2$-enriched seawater (Klawonn et al. 2015). Because of the long equilibrium time of $^{15}$N$_2$-gas in water, the actual labeling percentage (%) is lower than the theoretical value when injecting a bubble, especially during short incubations (Mohr et al. 2010; Wilson et al. 2012; Klawonn et al. 2015). By injecting $^{15}$N$_2$ as a gas, we therefore faced an increased risk of underestimating the N fixation.

Different types of mass spectrometry were used to perform isotopic analyses. In all mass spectrometry, molecules are ionized and separated according to their different masses. Thereby, both different elements and their isotopes, all with unique atomic masses, can be identified and quantified. In Paper I and Paper II, samples were analyzed by elemental analysis isotope ratio mass spectrometry (EA-IRMS) and secondary ion mass spectrometry (SIMS). For EA-IRMS analyses of the $^{13}$C and $^{15}$N uptake
in biomass we used a Thermo Flash EA 1112 elemental analyzer coupled to an isotope ratio mass spectrometer (Thermo Delta Plus XP, Thermo Fisher Scientific). To study cell-specific uptake we used the SIMS type IMS 1280 (Cameca, Gennevilliers, France). The IMS 1280 uses a primary beam of cesium ions, with a spatial resolution of 1 µm, to ionize molecules from a thin layer of the sample. The simultaneous imaging of the sample allows for both cell identification and cell-specific measurements of the $^{13}$C- and $^{15}$N- uptake in mixed field populations (Wagner 2009, Musat et al. 2012).

In Paper II, samples were also analyzed by nanoSIMS (NanoSIMS 50L, Cameca). NanoSIMS 50L has a similar methodological principle as IMS 1280, but among other differences a higher spatial resolution (Hoppe et al. 2013), which is necessary for the identification of e.g., heterotrophic bacteria.

**Field monitoring**

In 2011, we studied the upper mixed layer N pools during the progress of the cyanobacterial bloom, through a biweekly sampling program (Paper III). From May to September, our field sampling campaign joined and extended the regularly performed SNMMP cruises. Water was sampled from the upper 20 m of the water column, in two areas (northwestern and northeastern Baltic Proper) with four stations each, using serial water bottles (0, 5, 10, 15 and 20 m). The samples from each depth were pooled in a water container. Subsamples were taken for cyanobacterial biomass, total N (TN) and different particulate and dissolved N fractions. Sediment traps (Larsson et al. 1986; Broman et al. 1990) were deployed at three stations in each area, below the thermocline at ~25 m depth (20–29 m) to collect particulate organic nitrogen (PON) from the upper mixed layer. Temperature, salinity and the resulting pycnocline was measured with a CTD instrument (Multi Parameter CTD 90M, Sea & Sun. Marine Tech).

After settling in Utermöhl chambers, filamentous cyanobacteria of the species/genera *Aphanizomenon* sp. (one filament width size class), *N. spumigena* (three size classes) and *Dolichospermum* spp. (two size classes) were counted under an inverted microscope (Leica DM IRB) at 100 times magnification. Standard analyses of inorganic N
(NH₄⁺ and NO₂⁻+NO₃⁻), TN, total dissolved N and PON (total and <10 µm) were performed at the SNMMP laboratory at the Department of Ecology, Environment and Plant Sciences (Stockholm University). Inorganic nutrient analyses used segmented flow analysis (SFA, modified ALPKEM O. I. Analytical Flow Solution IV methods # 319528, # 319526, # 319527). TN and total dissolved N was analyzed using oxidation of N with a modification of the Koroleff (1983) method. The resulting NO₃⁻ was measured with the method above. For analyses of PON in water and settled particles, filters and filter blanks were dried over night at 60°C, packed into tin-foils and analysed for PON on a Leco CHNS-932 Analyzer.

Modelling
In order to estimate total cyanobacterial production (including losses, e.g., by grazing and mortality) we developed a growth model (Paper IV). The model used a modified photosynthesis–irradiance equation from Platt et al. (1980) which was fitted to empirically determined photosynthesis–irradiance curves of *Aphanizomenon* sp. (Walve and Larsson 2007). The model analysis comprised 15 years of *in situ* data from the SNMMP, including water temperature, pycnocline depth, light attenuation and *Aphanizomenon* sp. biomass. Surface irradiance data were available from SMHI (STRÅNG data, available at: strang.smhi.se) and recalculated to depth-specific irradiances using light profile derived extinction coefficients (SNMMP). In the model, the hourly C-specific production at each depth was calculated according to;

\[ P^B(I) = P_s^B \left[ 1 - e^{(-\alpha I/P_s^B)} \right] e^{(-\beta I/P_s^B)} - R \]

where I is irradiance, \( P_s^B \) is the potential maximum photosynthetic rate (no photoinhibition), alpha (\( \alpha \)) defines the initial slope of the curve, beta, (\( \beta \)), defines the degree of photoinhibition and \( R \) defines respiration. \( P_s^B \), \( \alpha \) and \( \beta \), are temperature dependent and approximated from field data (Walve and Larsson 2007). We tested different respiration estimates found in the literature. The hourly C-specific production at each depth was multiplied by the biomass concentration at the corresponding depth, assuming
various depth distributions, and accumulated to daily and seasonal production estimates. The resulting seasonal production in C was converted to N using a C:N ratio of 6.3 (mol:mol, Walve and Larsson 2007).

Measurements of *Aphanizomenon* sp. volume-specific nutrient content and cell widths

In **Paper I**, we investigated the volume-specific nutrient content of *Aphanizomenon* sp. We picked colonies with a gold needle under the dissection microscope to obtain pure *Aphanizomenon* sp. samples. Lugol-preserved sub-samples were analysed by microscopy for both total cumulative filament length (×100 magnification, Leica DM IRB) and cellular dimensions (×1000 magnification, Olympus Vanox-T AH2). The volume was calculated assuming a cylindrical filament form. Corresponding sub-samples were analysed for total N and P according to the method described above. The carbon content was calculated with a C:N ratio from samples measured on a Leco CHNS-932 Analyzer (see above).

![Figure 6 Petri dish with *Aphanizomenon* sp. colonies, to be individually picked under the dissection microscope.](image)

**Paper IV** presents an empirical cell width–temperature relationship of *Aphanizomenon* sp. The cell width of 50–100 cells from 20 different sampling occasions from May to September (2011 and 2014) were measured under a microscope (Olympus Vanox-T AH2) at 1000-fold magnification. The temperature data (0–10 m) from the corresponding dates were from the SNMMP.
MAIN RESULTS AND DISCUSSION

High cell-specific N fixation rates by *Aphanizomenon* sp. in the early growth season (10 °C)

During summer, *Aphanizomenon* sp. is known to be an important N-fixer in the Baltic Proper (Degerholm et al. 2008; Ploug et al. 2010). With the background that *Aphanizomenon* sp. take up little or no combined N for most of the year (Zakrisson et al. 2014), we investigated early season N fixation, at 10 °C (*Paper I*). Stable isotope incubations at *in situ* light intensities during 24 h, showed an average net N fixation rate of 55 fmol N cell\(^{-1}\) d\(^{-1}\), with dark N fixation rates being 20% of those measured in light. Both C- and N fixation rates were high at this low temperature and even comparable to those reported for *Aphanizomenon* sp. in August at 19 °C, using the same methods (Ploug et al. 2010). The measured rates might also be underestimated, due to the method injecting \(^{15}\)N\(_2\) as a gas-bubble (see method section, Mohr et al. 2010; Klawonn et al. 2015).

The high respiration rates (23% of gross photosynthesis, measured by \(^{14}\)C-incubations and O\(_2\)-microsensors), likely reflected the energy needed for high N fixation rates. Nitrogen fixation continued during the afternoon when C-fixation appeared strongly photoinhibited.

High N fixation rates by *Aphanizomenon* sp. at early growth season temperatures (≤14 °C) have also been shown by Klawonn (2015). Long term data on *Aphanizomenon* sp. biomass and water temperature from national monitoring stations B1 and BY31 (SNMMP) show the initiation of the biomass increase at about 10–15 °C (see *Paper I*, Supplementary material), supporting the potential for a high cellular activity at these temperatures. Thus, *Aphanizomenon* sp. can take advantage of the usually high photosynthetically active radiation in June, when the temperature is still near 10 °C.
Fast transfer of fixed N from *Aphanizomenon* sp. to the microbial and classical food-web

In **Paper II**, we investigated the role of *Aphanizomenon* sp. in terms of N transfer to the plankton community. During four consecutive years of incubation studies, *Aphanizomenon* sp. showed high enrichment in $^{15}$N after incubations with $^{15}$N$_2$. However, no uptake of added $^{15}$NH$_4^+$ could be traced. These results are supported by the study by Zakrisson et al. (2014), who found no uptake of combined N by *Aphanizomenon* sp. in a Baltic Sea coastal area. In **Paper II**, we also showed that *Aphanizomenon* sp. released about 50% of its fixed N as $^{15}$NH$_4^+$, which was transferred in the food web within only a few hours, as shown by ammonium analysis of the sample water and the $^{15}$N enrichment in other planktonic organisms.

The organisms that showed enrichment from uptake of diazotrophically derived N included diatoms (*Chaetoceros* sp.), copepods, autotrophic and heterotrophic bacteria (both as free-living or attached to *Aphanizomenon* sp.). The results illustrate the fast transfer of fixed N in the food web (~5 hours). The transfer of fixed N to diatoms and copepods also indicate a plausible fate of the fixed N, since the heavy silica shells of diatoms and fecal pellets from copepods are an important part of the fast-sinking organic export from the euphotic layer (Ploug et al. 2008). The experiments further showed that picocyanobacteria were active C-fixers, but not able to fix N. Also Klawonn (2015) found no N fixation in the <5 µm fraction. Hence, Baltic Sea picocyanobacteria appear to use N released from heterocystous cyanobacteria, and are not themselves active fixers.

The bloom-associated TN-increase is mainly a result of increased PON — indicating stimulated production

The Baltic Sea summer blooms are associated with an increase in TN, which only to a minor extent can be explained by the biomass of filamentous cyanobacteria. In **Paper II**, we show that N fixed by *Aphanizomenon* sp. is released and transferred to other planktonic organisms. To investigate the large scale implications of this transfer, we followed a cyanobacterial bloom (2011) and characterized the TN increase in terms of different N fractions (**Paper III**). We found that the TN increase was mainly
due to an increase in PON, but also in dissolved organic nitrogen (DON). Filamentous cyanobacteria biomass contributed ~20% of the total PON increase and about 10% of the TN increase. About half of the PON increase and later decrease could be explained by the sum of cyanobacteria, other autotrophs (>2 µm) and zooplankton, indicating that cyanobacteria stimulate both primary and secondary production during the bloom.

The TN decrease after the bloom was mainly due to PON >10 µm, but did not result in higher sedimentation rates. Measured sedimentation could only explain a small part of the post-bloom N loss, and there was little settling of undecomposed cyanobacteria. In Paper II, the plausible indirect sedimentation through, e.g., diatoms, is recognized and the potential underestimation of sedimentation (Gustafsson et al. 2013) is discussed in Paper III. There is also a possibility that decaying cyanobacteria were present in our traps, but not recognizable by microscopy. Decaying cyanobacterial aggregates have been shown to sink out rapidly (Ploug 2008) and the presence of cyanobacterial akinetes (sinking resting cells) in sediments (Suikkanen et al. 2010) indicate direct sedimentation by cyanobacteria. Methodological challenges with sediment trap studies include contamination by vertically migrating organisms, as highlighted by Heiskanen (1995) and Heiskanen et al. (1998). We pre-screened the material with a 250 µm sieve to remove zooplankton, likely killed by the preservative. Some filamentous cyanobacteria were also caught in the sieve but adding these did not change their small contribution to the settled material. Even with screening, smaller organisms could potentially bias results when preservatives are used. On the other hand, not using preservatives in the traps poses the risk of in-trap mineralization of organic material and grazing by zooplankton. In Paper III, we also speculate that the uncertain post-bloom N-loss might be explained by water mixing or transfer to higher trophic levels, i.e. fish.

Cyanobacterial production has potential to explain TN-increase — indications from model results

In Paper III, we show that both primary and secondary production were stimulated during the cyanobacterial bloom. In Paper IV, we followed up on these results by exploring the production capacity of cyanobacteria with *Aphanizomenon* sp. as model
species. The seasonally accumulated modelled production estimated a total production, including what could potentially have been produced but lost by grazing, cell lysis, mixing or sedimentation. These losses, if retained within the upper mixed layer, have potential to contribute to a TN increase.

Until bloom peak, the modelled production was similar to the actual biomass, indicating low losses during the initial phase of the bloom. Also, these results illustrate that parameters included in the model (light, temperature, depth distribution) likely are the main controlling factors of biomass increase during the early season. After the bloom peak, the different model versions (varying depth distribution and respiration estimates) resulted in a broader range of production estimates. The bloom collapse, and hence the difference between the accumulated modelled production and the actual biomass, might be an effect of grazing (Wannicke et al. 2013) and viral or bacterial infections (Suttle 1994; Rashidan and Bird 2001). The model could also overestimate production due to phosphorus (P) limitation (Walve and Larsson 2007; Klawonn 2015). Additionally, sedimentation or mixing to below 20 m could decrease the biomass. The accumulated seasonal net N fixation estimated from the model was similar to the N fixation shown in empirical studies (Ohlendieck et al. 2000; Stal and Walsby 2000; Degerholm et al. 2008; Klawonn 2015). Depending on the respiration estimates and biomass depth distributions used, 11–95% of the measured TN increase could be explained by the modelled production of *Aphanizomenon* sp. during the time series (1999–2013) tested. Our results show that total cyanobacteria production, including plausible loss processes, has potential to explain the TN increase in summer, with the assumption that the production mainly resides in the upper 20 m.

Modified nutrient content and temperature-dependent cell width of *Aphanizomenon* sp.

Assumptions of cell sizes and their carbon content have important implications when biovolume and carbon biomass are calculated from abundance data. In **Paper I**, we present a measured volume-specific C-content for *Aphanizomenon* sp. (0.21 pg C µm$^{-3}$) that is about 24% higher than that of the HELCOM PEG biovolume estimation (0.16 pg C µm$^{-3}$; Olenina et al. 2006, version PEG_BVOL2013.xls), which is based
on diatoms and flagellates (Menden-Deuer and Lessard 2000). Previously, it has been shown that preservation with Lugol’s solution causes a 15 to 30% decrease in cell volume of other filamentous cyanobacteria, e.g., *Dolichospermum* spp. (Hawkins et al. 2005). Hence, for live cells, the nutrient content per cell volume may be similar to those of diatoms and flagellates. However, our measurements of the cellular nutrient content suggest that for samples preserved with Lugol’s solution, the use of the conventional conversion factors may considerably underestimate *Aphanizomenon* sp. carbon biomass. The effect of using different conversion factors was tested in *Paper III* and *Paper IV*.

The measured cell width of *Aphanizomenon* sp. varied between 4.2 and 5.0 µm (May–September) with a seasonal mean width of 4.5 µm in both 2011 and 2014. This mean width has also been presented in a study by Rolf et al. (2007) while Olenina et al. (2006) (version PEG_BVOL2013.xls) use the widths of 4.0 µm and 5.0 µm.

There was a negative linear relationship between cell diameter and temperature ($r^2=0.82$, $p<0.0001$, $n=19$), described by the equation;

$$w = -0.0593T + 5.3222$$

where $w$ is cell width (µm) and $T$ is temperature (°C). The effect of using different cell widths in calculations was tested in *Paper IV*.
SYNTHESIS AND OUTLOOK

Cyanobacteria are one of the main players in the eutrophication of the Baltic Sea, being both a cause and a consequence of the current situation. N fixation by cyanobacteria is one of the largest sources of combined N to the Baltic Proper (Larsson et al. 2001; Wasmund et al. 2005, Paper III). Knowledge about the fate of this fixed N is important for ecosystem management, since we want to reduce the negative effects of blooms without reducing beneficial production, i.e. of fish (Karlson et al. 2015). Here, we show that N fixation might be important earlier in the season than previously anticipated (Paper I) and that the transfer of fixed N in both the classical and microbial food web can be fast (Paper II). We could also demonstrate this transfer of N to other organisms on a large scale, as a clear increase in the PON fraction of the upper mixed layer, at the time of the bloom (Paper III). The biovolume of non-diazotrophic phytoplankton and zooplankton increased during the bloom, as did the PON <10 µm fraction, consisting, e.g., of pico- and nanoplanckton. The actual cyanobacteria biomass contributed only about 20% of the PON increase (Paper III), but exploring their production potential with *Aphanizomenon* sp. as a model organism, we found that the total N increase during the bloom might indeed be a result of cyanobacterial production (Paper IV). The thesis also present new empirical data on the nutrient content and temperature dependent cell width of *Aphanizomenon* sp. (Paper I and IV).

N-fixing cyanobacteria appear independent of bioavailable N, but rely on dissolved P, as phosphate or bioavailable dissolved organic P. In the Baltic Proper, the concentrations of phosphate are tightly coupled to the oxygen concentrations in the bottom waters. The physical properties of the Baltic Sea, e.g., the several shallow sills that restrict water exchange with the North Atlantic, make it susceptible to low-oxygen conditions. This natural vulnerability is enhanced by eutrophication and climate warming, factors that increase the areal extent of oxygen deficiency (Carstensen et al. 2014). Around the year 1999, there was an abrupt increase in the areal extent and volume of hypoxia (low oxygen) and anoxia (no oxygen, formation of toxic hydrogen sulphide) in the bottom sediments and bottom waters of the Baltic Sea. This areal extent of hypoxic and anoxic areas had not previously been documented (Hansson et al. 2011). The situation persists and around 20% of the present-day Baltic Proper is
considered anoxic. The large inflow of saline and oxygen rich water from the Kattegat in the end of 2014 appears to have had only short-term effects on the oxygen concentrations (Andersson 2016). The leakage of phosphate from these areas is the most important source of dissolved P in the Baltic Sea (Gunnars and Blomqvist 1997; Viktorsson et al. 2013). The large areas of hypoxia and anoxia are thus likely to continuously support the diazotrophic cyanobacteria with P (Carstensen et al. 2014).

Climate change is expected to increase the annual precipitation and surface water temperatures of the Baltic Sea. There are also projections of more extreme precipitation events (HELCOM 2013). These future climatic projections, in combination with eutrophication, have potential to promote the expansion of cyanobacteria blooms (O’Neil 2012; Pearl and Paul 2012; Hense et al. 2013). Changes in water temperature may have both a direct effect on growth rates and an indirect effect due to increased stratification of the water column (Jöhnk et al. 2008; Pearl and Huisman 2009). Experiments with Baltic Sea Aphanizomenon sp. and N. spumigena have shown a positive effect of increased temperature on biovolume and photosynthetic activity, but both species had lower biovolume when grown together, indicating the need for studies on the interactions between these and other species (Karlberg and Wulff 2013). 

N. spumigena and Aphanizomenon sp. have different temperature optima with the former growing better at higher temperatures (20–25 °C) (Lehtimäki et al. 1997). On the other hand, both cell division and N fixation rates in N. spumigena have been shown to decrease when exposed to elevated CO₂ concentrations (Czerny et al. 2009), highlighting the complexity of possible climate change effects. Aphanizomenon sp. has a lower temperature optimum (16–22 °C) (Lehtimäki et al. 1997) but as we show in Paper I, this species also has potential for high rates of N fixation already at 10 °C, which is advantageous in the competition for the P left in the upper mixed layer after the spring bloom. In Paper IV we propose that P is not limiting Aphanizomenon sp. during the initial biomass increase phase. A recent study has shown several changes in the phyloogy of the Baltic Sea, coupled to climate change (Kahru et al. 2016). The changes include earlier onsets of both the spring and summer bloom, and a shift of maximum chlorophyll-a concentrations from spring to summer. The observed change in light conditions, with cumulative irradiance maxima reached earlier in the season (Kahru et al. 2016), is likely favorable for Aphanizomenon sp. (Paper IV).
The SIMS technique (Paper I and II) allows us to study N fixation on a cellular level in mixed communities, thus greatly increasing the possibilities for future species-specific investigations of fixation rates, N transfer, and even the discovery of new, currently unknown, N-fixers. Different species may respond differently to environmental conditions and tools like SIMS will certainly be of use in current and future quests for information on N fixation in a changing environment. With SIMS, Klawonn (2015) could show high N fixation rates by *Dolichospermum* spp. The bloom dynamics of this short-blooming (Paper III) and relatively overlooked genera might be better captured, compared to in our study (Paper III), by more frequent, e.g. weekly, sampling. If the brief appearance of *Dolichospermum* spp. in our study was due to strong and selective grazing by zooplankton (Chan et al. 2006; Marino et al. 2006) this could be an important pathway for fixed N, especially if the fixation rates are as high as indicated. The formation and settling of akinetes might also be more important for this species (Suikkanen et al. 2010).

Additional studies on the changes in different PON fractions (e.g. <1µm and <3 µm) during the bloom would contribute more quantitative information concerning the pathways of fixed N. Whether cyanobacterial N is transferred within the classical food web or via smaller organisms in the microbial loop has implications for summer production efficiency. Novel tools, such as amino acid compound-specific stable isotope analysis (AA-CSIA), indicate both food source and trophic position of an organism (e.g., McClelland et al. 2003; Nielsen et al. 2015) and are thus of great interest for the elucidation of fixed N pathways in the food web. The post-bloom N decrease was to a large extent unresolved by our set-up (Paper III), indicating possible transfer to higher trophic levels (i.e., fish) or large underestimation of sedimentation (Gustafsson et al. 2004; 2013). The final fate of fixed N is still uncertain, e.g. to what extent the fixed N is lost through denitrification and anammox, or becomes available for the spring bloom (Vahtera et al. 2007; Bonaglia et al. 2014). The combination of small-scale tools, like SIMS, AA-CSIA and also genetic analysis (targeting the cellular and organism level), with larger scale monitoring of N pools and sedimentation patterns, might be a successful future direction for studying the pathways of fixed N in the Baltic Sea.
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SAMMANFATTNING

Cyanobakterier är vitt spridda i akvatiska och terrestra miljöer. En del släkten kan omvandla kvävgas (N₂) till biotillgängligt ammonium, dvs. utföra kvävefixering, och är därmed fundamentala för kvävets (N) kretslopp. Fixerat N från sommarblomningar av cyanobakterier är en av de största källorna till nytt N i Östersjön. Denna avhandling undersökte cyanobakteriers N-fixering i Östersjön och utforskade det fixerade kvävets potentiella vägar i olika rumsliga och tidsmässiga skalar. I Paper I mättes den cellspecifika N-fixeringen hos *Aphanizomenon* sp. vid 10 ºC, under tidig säsong. Fixeringshastigheten var hög, och jämförbar med den under sensommaren. Resultaten pekar på att *Aphanizomenon* sp. är en viktig kvävefixerare redan tidigt under säsongen.

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