



Sustainable Uses of
Baltic Marine Resources



SUBMARINER Report 1/2013:

Determining optimal growth conditions for the highest biomass microalgae species in Lithuanian part of the Curonian Lagoon

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Abstract

The depletion of the fossil fuels worldwide is creating a pressure for searching for renewable energy and fuel sources. Currently crops are already being used widely for the production of bioethanol via fermentation, which is being used as a fuel component or as a fuel itself. However, using crops creates a competition of energy industry with agriculture for arable land, potentially leading to an increase of price of the food products and dissatisfaction of advocates of Asian, Latin American and African starvation problem. Using arable lands is unacceptable since their quantity is limited, while the Earth population is increasing, and food production also has to be increased to meet the growing needs. Microalgae is an excellent alternative, which need less arable land or land at all for cultivation, since they can be grown in off-shore facilities. They are also rich in energy and fast-growing; the yield of oil from microalgae can be 10–20 higher than the yield from crops grown on a plot of land of same area. Although existing technologies of cultivating microalgae are expensive and the end-product cannot compete with fossil fuels by price, there is a lot of investment into the research on this subject and the improvement of the technologies. Currently, most of the development on cultivating microalgae is located in the U.S., however, research facilities are also emerging in Europe. The aim of this research was to identify microalgae species from Curonian Lagoon which could be potential candidates for cultivation in Lithuania, and to statistically determine how the accumulation of their biomass correlates with the changes in environmental conditions, using five years (2005-2009) monitoring data. Five species, frequently acquiring a much larger biomass (up to 68.8 mg L⁻¹) over other species, were identified. These species were: *Aphanizomenon flos-aquae* and *Planktothrix agardhii* of class *Cyanoophyceae*; *Actinocyclus normanii* f. *subsalsus*, *Diatoma tenuis* and *Stephanodiscus rotula* of class *Diatomophyceae*. Optimal cultivation conditions were suggested based on results of the analysis on the correlation of changes in environmental conditions with the changes in the biomass of these microalgae.

URL

The report is available for download at the SUBMARINER project's website at <http://publications.submariner-project.eu>

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Content

1. Introduction	4
2. Materials and Methods	7
3. Results and Discussion	9
4. Acknowledgements	12
References	14

1. Introduction

The depletion of fossil fuels (*i.e.* oil) together with the adverse environmental impacts of conventional energy sources is one of the most important emerging problems that humanity is facing today. For this reason, recently an active research is directed towards the development of renewable fuel sources in many countries. In the United States, the Energy Independence and Security Act (EISA) established new Renewable Fuel Standard of 36 billion gallons of renewable fuels (cellulose biofuels and biodiesel) to reach by 2022 (Ferrell and Sarisky-Reed, 2010). The cheapest and the most popular way to produce the fuel is by exploiting cultivated plants as a fuel source, since they are capable of generating complex molecules with little input (CO₂, inorganic fertilizers and water) and little maintenance (Lang et al., 2001). The fuel, obtained this way, is called *biofuel*. Renewable, non-toxic and biodegradable biofuel has high advantage over conventional sources as a future energy source (Amaro et al., 2011).

Microalgae are one of the most promising renewable sources of biofuel, because of accumulation of triacylglycerols (TAG) as a storage lipids up 50% of dry cell weight as well as higher photosynthetic efficiency and biomass production comparing with plants (Ferrell and Sarisky-Reed, 2010; Guan Hua et al., 2010; Abou-Shanab et al., 2011). Microalgae are microscopic single-cell, filaments, colonies or coenobia forming photosynthetic organisms which grow in different types of water bodies including thermal hot springs or extremely saline lakes. They include a large variety of evolutionally distinct species: from green algae, diatoms to e.g. cyanobacteria, which are biologically procaryotes, but traditionally classified as 'blue-green algae'. Microalgae can be cultivated in closed or semi-closed photobioreactors or in open shallow ponds to yield biomass, which can be then processed to extract a lipid part for producing fuels (Brennan and Owende, 2010). The algae biomass and its residues can also be used as a substrate for fermenting it into ethanol (also an important component of several species of fuel), for producing biogas, as a fertilizer, as a food supplement for animals used in aquaculture or stock-raising (Ferrell and Sarisky-Reed, 2010; Amaro et al., 2011). Or it can be simply burned to generate heat and electricity.

Accumulation of energy-rich reserve compounds like starch, oil occurs in many microalgae under nitrogen starvation conditions (Hu et al., 2008), however new technologies were applied to increase ethanol and oil accumulation in their biomass (Greenwell et al., 2010). Genetically engineered cyanobacteria from *Synechococcus* genus can directly produce ethanol and secrete it into the growth medium, where it can be easily extracted from (Deng and Coleman, 1999). This simplifies the bioethanol production, since it excludes the fermentation step from the process. According to Li et al. (2010) and Siaux et al. (2011), the amount of oil stored in microalgal cells could be increased by two fold after genetically shutting down starch biosynthesis in green algae *Chlamydomonas*. However, the number of species that have been successfully genetic transformed is still scarce. That emphasise the need to develop studies of gene expression regulation control applying new molecular biology tools to standardize genetic modifications of microalgae in future (Amaro et al., 2011). To avoid contamination of environment with modified organisms, the search and screen of the native species characteristics would be advantageous in the future as well.

The cultivation of microalgae similarly to macroalgae or macrophytes can also be combined with biological wastewater treatment (Brennan and Owende, 2010). NASA developed a novel advantageous technology of using microalgae grown in forward osmosis bags submerged into flowing wastewaters exists, which will be integrated into biorefineries to produce renewable energy products like diesel or jet fuel (<http://lunarscience.nasa.gov/articles/omega>).

Microalgae have been named a "third-generation" source of biofuel, with 1st being terrestrial crops

(corn, other agricultural products), and the 2nd being lignocellulosic agriculture and forest residue, non-food crops (Brennan and Owende, 2010). Microalgae as a fuel source are superior source compared to 1st and 2st generation sources because does not create a competition between the agricultural and fuel industries, since it does not need arable land and certain food products which are currently being used in the production of bioethanol (Amaro et al., 2011). They can be grown on wastewater or salt water and give a much higher yield of product per hectare of land used per year (Table 1) (<http://www.oilgae.com/algae/oil/yield/yield.html>)

Table 1: The theoretical yields of two types of biofuel (litres per hectare of land used per year) from different types of a starting substrate.

	Oil	Ethanol
Corn	170	3,100-3,900
Rapeseed	1200	
Sugar Cane		5,300-6,500
Oil Palm	6000	5,000-6,000
Microalgae	46,000-140,000	20,000

As of 2012, most of the development on cultivating microalgae is located in the U.S., although smaller markets are also expected to emerge in Europe and Asia (SBI Reports). The large scale industrial microalgae cultivation already exists, for such purposes as the use of cyanobacteria to produce ethanol (Algenol Biofuels, Florida, USA), or for the biological wastewater treatment using microalgae grown in bags made from forward osmosis membranes for (NASA, pilot studies). There is yet no established large-scale cultivation of microalgae for the production of biodiesel, primarily because of the high price of the final product, and, as a result, its inability to compete with cheaper fossil fuels (Amaro et al., 2011; Davis et al., 2011). Still, microalgae-derived biofuels might become an economically viable option with time as cultivation technologies develop, it scales up, and the investment grows: the proposed market value for algae fuels in 2015 is \$1.6 billion (SBI Reports).

Currently there is little research in Lithuania aimed on the cultivation of microalgae or algae overall, and no actual cultivation of algae for industrial purposes, although Lithuania has two natural conditions, which are very suitable for this: a large number of sunny days during the year and rich water resources available. This includes a highly eutrophied Curonian Lagoon, whose inorganic nutrients-rich waters can theoretically be used as a growth medium for microalgae. Recycling of the Lagoon waters would both provide a cheap source of nutrients for the microalgae cultivation and serve for ecology by removing the excess nutrients from water. However, if sun treated waters from a natural source are used, highly competitive species are required for cultivation, and otherwise they will be outgrown by other microalgae. This can be achieved by cultivating local isolated species that are already dominant in the local water reservoirs. They are also best adapted to the local conditions, and, therefore, they will theoretically grow to the optimal yield. Being native species they do not pose a danger for local ecosystem in case of any leak of cultivated microalgae into the natural water systems. Cultivation of indigenous algae species has a potential for regional biofuel production and biotechnological application (Chaichalerm et al., 2011; Odlare et al., 2011).

According to Energy policy (Reijnders L. 2006), introducing a new type for industry in the local market, such as algae cultivation, will not only serve as a basis for creating a renewable, local energy and fuel source, increasing the economic stability of the country in future, but will also



create new jobs and new infrastructure, which is very important for Lithuania with a high unemployment rate (13.7%, as of May 2012).

Current study focuses on the possibilities of application of native microalgae species for different combined uses in Lithuania. So the aim of this research was to identify the dominant microalgae species of the Curonian Lagoon, which would be the best possible candidates for the cultivation in the local area. The second aim was to identify what environmental conditions lead to the accumulation of biomass of these species, as this information would be useful in determining their optimal growth conditions in any future attempts of cultivation of these microalgae.

2. Materials and Methods

Monitoring data on the microalgae biomass variations and the changes of water physicochemical properties, obtained from the Marine Research Department (MRD) of the Ministry of Environment of the Republic of Lithuania, were used as a starting material for a statistical analysis used to determine the optimal growing conditions for microalgae.

The samples for the determination of microalgae species composition as well as water physicochemical conditions were collected during the course of 5 years, 2005-2009. The water samples were taken with a Ruttner water sampler at 14 monitoring stations of the Curonian Lagoon: monthly at stations 2, 5 and 12; 3-4 times per year – at the rest stations (Figure 1). The data (water temperature, current water level) from hydrometeorological posts located in the northern, central and southern parts of the lagoon were also analysed.

Multiple chemical, physical properties of water and other parameters were monitored. This includes:

Hydrochemical properties: pH, dissolved O₂ concentration, biological oxygen demand of water for 7 days (BOD₇), NO₂, NO₃, NH₄, PO₄ concentrations, overall nitrogen and phosphorus concentrations, SiO₂ concentration, salinity.

Physical properties: water temperature, water level.

Ecotoxicology: presence of oil products, detergents, Hg, Pb, Cu, Cd, Zn, Cr, Ni, and also 16 various organic pesticides and other pollutants (e.g. pentachlorophenol (PCP), benzo[k]fluoranthene, benzo[a]pyrene).

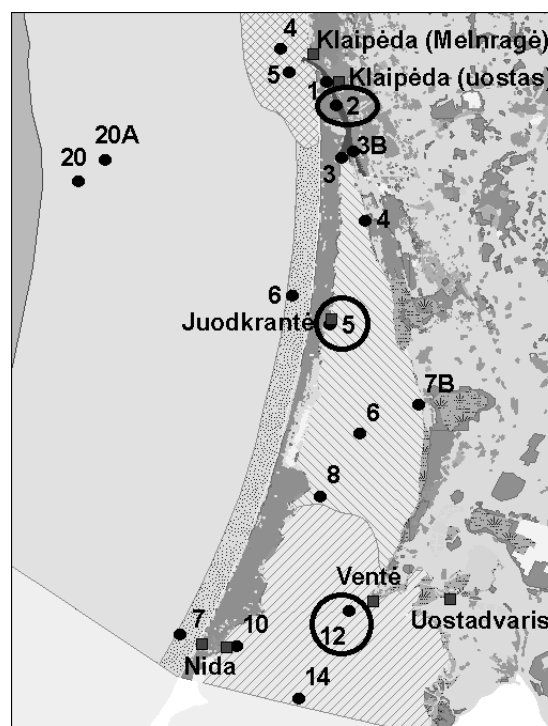
Other factors: wind direction, air temperature.

The following factors were taken into consideration when determining the optimal condition for microalgae biomass accumulation: total nitrogen and phosphorus concentrations, BOD₇, pH, water level, water temperature, water salinity.

The microalgae biomass in the samples was estimated by multiplying species abundance by the mean cell volume, according to stereometrical formulas (Oirik et al., 1988).

Curonian Lagoon is an estuary whose salinity changes from freshwater to brackish from south to north, and, therefore, the samples would cover both freshwater and saline water species.

The relationship between the microalgae biomass and the environmental conditions was evaluated using a statistical method known as the Canonical Correspondence Analysis to determine which environmental conditions have the strongest influence on the microalgae biomass. The data were



- Monitoring station
- Hydrometeorological post



log-transformed prior to analysis. In case of the biomass data, "1" was added to all the values to eliminate zeroes, and then the data were log-transformed. The biplots indicate whether there is a relationship between the independent variables (environmental conditions) and dependent variables (species biomass): the closer the lines are to each other, the stronger is the correlation between the two variables. Opposite directions of two indicate a negative correlation.

3. Results and Discussion

The amounts of inorganic and total nutrients (nitrogen and phosphorus) in water were mostly uniform across the monitoring stations, with slightly elevated levels observed at Station 12, the closest to the river Nemunas outflow. Other parameters were also similar, only the water salinity varied from 0.03-0.47 ‰ (average 0.08) on Station 12 to 2.92-6.77 ‰ (average 5.17) on Station 2 (Table 2). On the seasonal basis, the nutrient concentrations were decreased from early summer to mid-autumn, possibly due to the nutrient uptake from water by actively growing algae. The nutrients concentrations increase in spring is related with the river inflow from the catchment area and re-suspension processes from the sediments of the Curonian Lagoon. In all cases nutrient concentrations were below the Maximum Allowable Concentration (MAC).

Table 2: Average nutrients concentrations and other hydrochemical parameters in the Curonian Lagoon water across three monitoring stations in years 2005-2009. Standard deviations are shown in brackets (\pm). MAC - Maximum Allowable Concentration (according to Republic of Lithuania Environmental Protection Minister order No. D1-633).

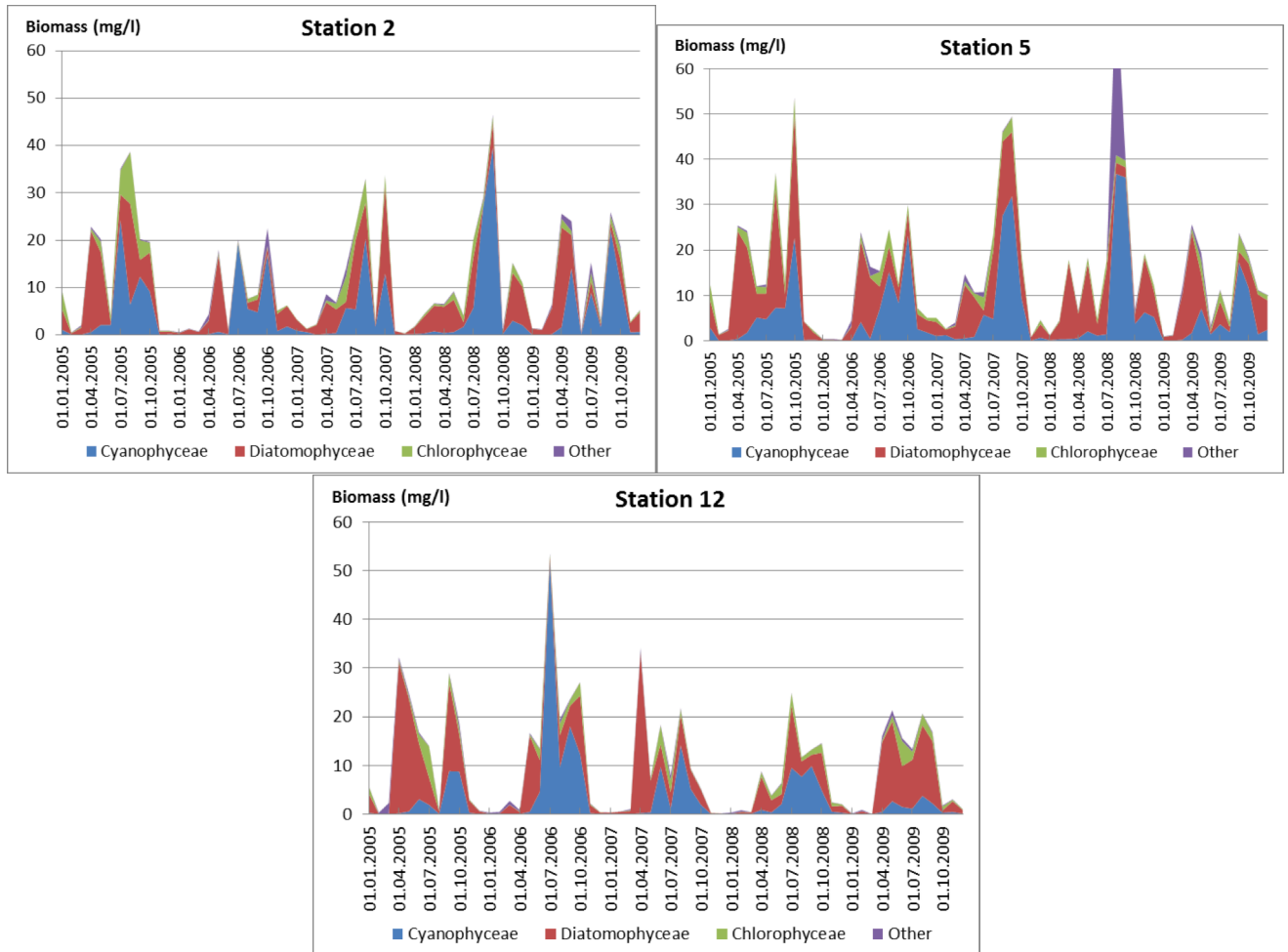
	BOD ₇ (mg/l)	pH	NO ₃ (mg/l)	NH ₄ (mg/l)	PO ₄ (mg/l)	N (mg/l)	P (mg/l)	Salinity
Station 2	4,35 ($\pm 1,74$)	8,75 ($\pm 0,49$)	0,54 ($\pm 0,63$)	0,08 ($\pm 0,07$)	0,02 ($\pm 0,02$)	1,35 ($\pm 0,61$)	0,07 ($\pm 0,02$)	5,17 ($\pm 0,92$)
Station 5	4,95 ($\pm 1,90$)	8,78 ($\pm 0,48$)	0,51 ($\pm 0,57$)	0,08 ($\pm 0,08$)	0,02 ($\pm 0,02$)	1,34 ($\pm 0,55$)	0,08 ($\pm 0,04$)	1,11 ($\pm 1,18$)
Station 12	5,14 ($\pm 2,09$)	8,58 ($\pm 0,41$)	0,90 ($\pm 0,75$)	0,09 ($\pm 0,07$)	0,03 ($\pm 0,02$)	1,78 ($\pm 0,67$)	0,09 ($\pm 0,03$)	0,08 ($\pm 0,08$)
MAC	6,00	6..9	2,30	1,00	0,40	2,50	0,10	

Figure 2 shows the regular annual variations of the microalgae biomass in three stations of the Curonian Lagoon. Total algae biomass increased between April and late September, reaching the maximum levels in August or September (up to 95.8 mg L⁻¹). In winter months, from December to early March, microalgae biomass was much lower (0.13-13.22 mg L⁻¹).

The dominant microalgae classes during annual cycle were *Cyanophyceae*, *Diatomophyceae* and *Chlorophyceae*. Diatoms was dominant during the cold month, whereas cyanobacteria and green algae during warmest months. During the microalgae biomass peak in water usually few or even single species dominate comprising as much as 40 to 78% of the total microalgae biomass in a water sample at a given time. From overall 526 algae species recorded in the Curonian Lagoon five species were selected based on high biomass and length period of their presence. The highest biomass of a single cyanobacteria species were higher if compare to the other microalgae species. The most frequently high biomass levels reaching species in the Curonian Lagoon of class *Cyanophyceae* were identified *Aphanizomenon flos-aquae* (comprising up to 68.8 mg L⁻¹ in July) and *Planktothrix agardhii* (up to 38.8 mg L⁻¹ in August), whereas of class *Diatomophyceae* – *Actinocyclus normanii* f. *subsalsus* (up to 50.5 mg L⁻¹ in October), *Diatoma tenuis* (up to 10.2 mg L⁻¹ in May) and *Stephanodiscus rotula* (up to 24.2 mg L⁻¹ in April). Diatoms *D. tenuis* and *S. rotula* were early spring, cold water blooming species, while all other species were accumulating largest biomass during the late summer and early autumn months.

The data on the variation of the biomass of these five microalgae species was compared to the data on the environmental conditions in the relevant monitoring years using the Canonical Correspondence Analysis (Figure 3).

Figure 2: Annual microalgae biomass variation at three monitoring stations (January 2005 – December 2009)



A. flos-aquae and *P. agardhii* biomass positively correlated with pH, salinity, BOD₇ levels and temperature, meaning they can be successfully cultivated in eutrophied water (e.g., in semi-permeable bags), such as water from the Curonian Lagoon or wastewater on wastewater treatment plants. However, since they are nitrogen-fixing species, it negatively correlated or was indifferent to dissolved nitrogen compounds. Therefore, if these cyanobacteria might be cultivated in water taken from natural sources with other microalgae present, nitrogen concentration has to be lowered to make them competitive over other species. Since they prefer higher temperatures (blooming occurred at water temperatures starting at 15°C and higher), it is better to cultivate them in shallower ponds, reservoirs or photobioreactors.

Photosynthetic capability and capacity for genetic engineering of cyanobacteria together with high species diversity and proliferation under on-going eutrophication and climate warming worldwide make them attractive candidates for use in bio-industrial applications and remediation (Pulz and Gross, 2004; Ducat et al., 2011). Cyanobacteria might be important for nutrients removal, especially

phosphorus, from highly eutrophicated water bodies or wastewaters. Due to optimal growth temperatures, the period of *Aphanizomenon* and *Planktothrix* species growth will be short and restricted to summer month in our country that diminishes their commercial applicability. There are still considerable obstacles to overcome to make biofuel production from cyanobacteria economically competitive with traditional fossil fuel sources, however industry for cyanobacterial products is developing towards higher added value products (isoprene, sugars, bioactive compounds, antioxidative substances, phycocyanin) that may be applied or already introduced in medicine or cosmetic, food industries (Eriksen, 2008; Ducat et al., 2011). Bloom forming *Aphanizomenon flos-aquae* are one of the most important cyanobacteria applied in biotechnology. *A. flos-aquae* are commonly used as human nutrition supplements (Pulz and Gross, 2004), however, they have to be cultivated in closed bioreactors under strictly controlled conditions to achieve high culture purity and species toxicity should be studied first.

S. rotula, on the contrary to cyanobacteria, is positively influenced by the concentration of nitrogen in water in case of all three stations. The biomass of this species is also negatively influenced by salinity level, i.e. it prefers less saline environments. It also has a strong (station 2) or very strong (stations 5 and 12) negative correlation with water temperature (the species has higher biomass at lower temperatures) and elevated phosphorus concentrations. This means *S. rotula* is a low temperature-tolerant organism which is possibly outgrown at higher temperatures by faster-growing species. *D. tenuis* is also cold-tolerant species, however, it is dominant at slightly higher temperatures than *S. rotula*, typically during mid to late spring. *A. normanii* f. *subsalsus*, unlike *D. tenuis* and *S. rotula*, is a warm water salinity tolerant diatom species, also positively influenced by phosphorus concentration. Since both cold water and warm water species are present amongst bloom forming microalgae species in the Curonian Lagoon, it is theoretically possible to cultivate microalgae under local conditions throughout the year, which is useful for Lithuania with its late spring and summer. Although the biomass yields during the colder months will be much smaller. Other data obtained are potentially useful in determining the optimal conditions for the cultivation of these microalgae species.

Diatoms can be used for the nitrogen compounds removal when the runoff from agricultural land dominates, but their success will be decided by the SiO_2 concentrations in the water (substances important for their skeleton construction). *Stephanodiscus* and *Diatoma* species showed negative whereas *Actinocyclus* slight positive correlation with BOD_7 , thus probably only latter diatom species may be successfully introduced in to wastewater treatment system.

Diatoms main reserve products are chrysolaminarin and oil which can reach up to 60% dry weight. So they are especially interesting for biotechnology because their physiological potential to accumulate high proportions of oils that may represent a future source for fuel, long chain polyunsaturated fatty acids or eicosapentaenoic acid (Pulz and Gross, 2004; Khozin-Goldberg et al., 2011). However, being the large size they are slow growing species, thus the positive balance between oil accumulation and biomass accumulation should be studied first. Diatoms are also widely used in aquaculture as a food for molluscs (Borowitzka 1997).



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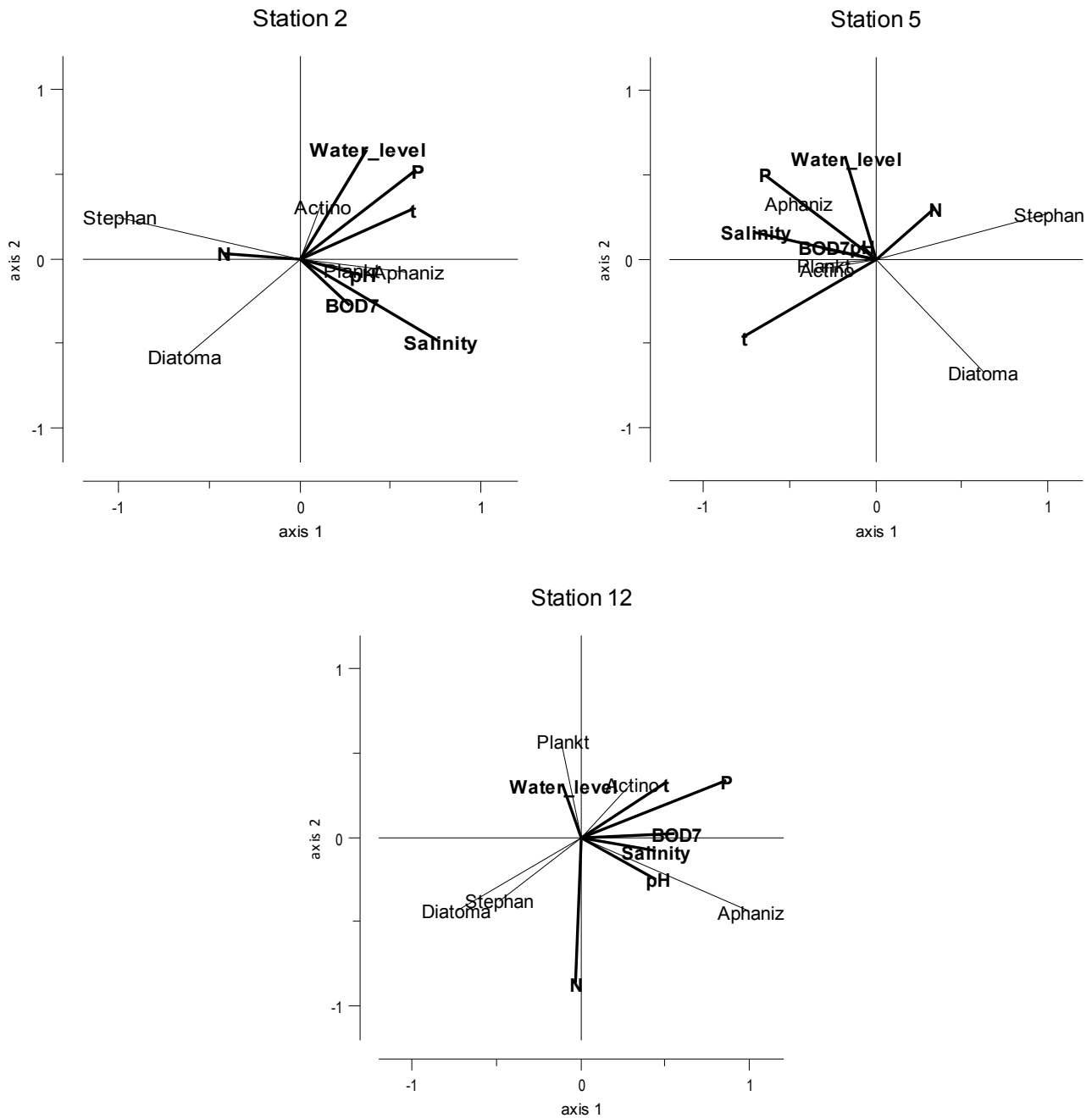


Figure 3: Ordination biplots based on the microalgae biomass-environmental conditions relationship analysis

Thin lines indicate the species, thick lines indicate the environmental variables.

Actino – *Actinocyclus normanii* f. *subsalsus*, Aphaniz – *Aphanizomenon flos-aquae*, Diatoma – *Diatoma tenuis*, Plankt – *Planktothrix agardhii*, Stephan – *Stephanodiscus rotula*.

BOD₇ – biological oxygen demand for 7 days; N – total nitrogen concentration, P– total phosphorus concentration.

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Improving the Baltic Sea environment and economies: Innovative approaches to the sustainable use of marine resources

The Baltic Sea Region faces enormous challenges including new installations, fishery declines, excessive nutrient input, the effects of climate change as well as demographic change. But novel technologies and growing knowledge also provide opportunities for new uses of marine ecosystems, which can be both commercially appealing and environmentally friendly. Through increased understanding and promotion of innovative and sustainable new uses of the Baltic Sea, SUBMARINER provides the necessary basis for the region to take a proactive approach towards improving the future condition of its marine resources and the economies that depend on them.

Activities

Compendium

Describing current and potential future marine uses

- Comprehensive inventory of current and new uses
- Strengths, weaknesses, opportunities and threats to the BSR
- Environmental and socioeconomic impacts
- State and availability of technologies
- Market potential
- Gaps and obstacles in the legal framework

Regional Strategies

Testing new uses in real conditions

- Feasibility studies for new uses
- Technological and financial needs
- Impacts on environmental and socioeconomic conditions within the area
- Specific legal constraints

BSR Roadmap

Recommending necessary steps across all disciplines to promote beneficial uses and mitigate against negative impacts

- Research topics
- Institutional and network initiatives
- Legal changes (e.g. spatial plans)
- Environmental regulations
- Economic incentives

BSR Network

Bringing relevant players together

- Business cooperation events
- Network structure (incl. membership, mission, independent finances, business plan, etc.)
- Virtual information and exchange platform
- Regional, national and BSR-wide roundtables and seminars on new marine uses

Partners

Poland

- **Lead Partner:** The Maritime Institute in Gdańsk
- Gdańsk Science and Technology Park

Germany

- Federal Ministry for the Environment, Nature Conservation and Nuclear Safety
- Norgenta North German Life Science Agency
- Kieler Wirkstoff-Zentrum am GEOMAR | Helmholtz Centre for Ocean Research Kiel
- University of Rostock
- BioCon Valley Mecklenburg-Vorpommern e.V.

Denmark

- ScanBalt
- Lolland Energy Holding

Sweden

- Royal Institute of Technology (KTH)
- The Royal Swedish Academy of Sciences
- Trelleborg Municipality

Estonia

- Tallinn University of Technology
- Entrepreneurship Development Centre for Biotechnology & Medicine

Lithuania

- Klaipeda University Coastal Research and Planning Institute
- Klaipeda Science and Technology Park

Latvia

- Ministry of Environmental Protection and Regional Development of the Republic of Latvia
- Environmental Development Association

Finland

- Finnish Environment Institute – SYKE

Project Duration

October 2010 – December 2013

Project Budget

- ERDF Co-Finance: € 2.8 million
- Partners' contribution: € 0.8 million
- Total Project Budget: € 3.6 million



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