Promjena sastava fitoplanktonskih lipida kao odgovor na okolišni stres

Novak, Tihana

Sveučilište u Zagrebu

PRIRODOSLOVNO-MATEMATIČKI FAKULTET

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Mentor: Dr. sc. Blaženka Gašparović

Zagreb, 2021

University of Zagreb

FACULTY OF SCIENCE

Tihana Novak

CHANGES IN PHYTOPLANKTON LIPID COMPOSITION AS A RESPONSE TO ENVIRONMENTAL STRESS

DOCTORAL THESIS

Supervisor: Blaženka Gašparović, PhD

Zagreb, 2021

Ovaj doktorski rad izrađen je u Laboratoriju za biogeokemiju mora i atmosfere, Zavoda za istraživanje mora i okoliša Instituta Ruđer Bošković u Zagrebu, pod mentorstvom dr.sc. Blaženke Gašparović, u sklopu Interdisciplinarnog doktorskog studija Oceanologije na Prirodoslovno-matematičkom fakultetu Sveučilišta u Zagrebu. Doktorski rad je izrađen kao dio aktivnosti u sklopu projekta Hrvatske zaklade za znanost: „Utjecaj okolišnog stresa na pojavnost i međudjelovanje biološki važnih organskih molekula i mikronutrijenata u morskom ekosustavu“ (AMBIOMERES, IP-11-2013-8607; voditelj projekta: dr. sc. Blaženka Gašparović).

Prvenstveno bih se zahvalila svojoj mentorici, doktorici Blaženki Gašparović na pruženoj prilici za rad u znanosti te godinama zajedničkog rada. Hvala Vam što ste svojom voljom i trudom omogućili da što bolje odradim svoj posao i što više naučim. Bez Vas ovo ništa ne bi bilo moguće.

Dr. sc. Jeleni Godrijan srdačno zahvaljujem na puno toga, prvim koracima u laboratoriju, upoznavanju s uzgojem do završnih koraka pisanja, ispravljanja, članaka i doktorskog rada. I naravno na jednom novom prijateljstvu.

Zahvaljujem svim članovima komisije, dr. sc. Zrinki Ljubešić, dr. sc. Tei Mišić Radić, dr. sc. Petru Žutiniću za pružene komentare i pomoć ne samo na doktoratu već i u višegodišnjoj suradnji.

Ovaj doktorski rad napravljen je kao sklop tri znanstvene publikacije te ne bi bio moguć bez doprinosa ovih brojnih koautora. Stoga zahvaljujem dr. sc. Ivni Vrana Špoljarić, dr. sc. Jeleni Godrijan, dr. sc. Danieli Pfannkuchen Marić, dr. sc. Snježani Kazazić, dr. sc. Milanu Čankoviću, dr. sc. Zrinki Ljubešić, dr. sc. Enisu Hrustiću, dr. sc. Marini Mlakar, dr. sc. Tamari Đakovac, dr. sc. Ingrid Ivančić, Nikoli Mediću, dr. sc. Ani Baričević, dr. sc. Mirti Smodlaka Tanković, dr. sc. Zhuoyi Zhu, dr. sc. Ruifeng, Zhang te prof. Jinzhou Du.

Članicama Laboratorija za biogeokemiju mora i atmosfere zahvaljujem na pomoći, savjetima te znanju koje ste mi pružile ovih godina. Velika mi je čast i izazov bio raditi doktorat u okruženju s toliko uspješnih znanstvenica.

Zahvaljujem Zdeslavu Zovku i dr. sc. Jeleni Dautović na pomoći pri laboratorijskom i terenskom radu.

Zahvaljujem svojim kolegicama za svu pomoć u pisanju i izradi ovog doktorata te posebice Ani za sve dane ludila u 10. krilu, Abri za sve službene i neslužbene savjete, Ivni za sve kave, Slađani za duge razgovore i Jeleni za srdačnost.

Hvala Matei za bezvremensku potporu.

Posebno zahvaljujem svojoj obitelji koja se već preko 33 godine aktivno bavi mojim problemima. Mama i tata hvala vam za sve što ste mi omogućili da dobijem i postignem sve što poželim. Hvala mojoj najboljoj sestri na potpori i razumijevanju koje samo sestra zna dati. Hvala mojem najboljem bratu na cjeloživotnom usmjeravanju i pretjeranoj brizi. I njihovim supružnicima na tehničkoj i moralnoj potpori u pisanju doktorata.

I mojem najboljem prijatelju Franku, hvala što si uvijek tu za mene.

Mojim dečkima

TEMELJNA DOKUMENTACIJSKA KARTICA

Sveučilište u Zagrebu

Prirodoslovno-matematički fakultet
Geološki odsjek Doktorski rad

PROMJENA SASTAVA FITOPLANKTONSKIH LIPIDA KAO ODGOVOR NA OKOLIŠNI STRES

Tihana Novak

Laboratorij za biogeokemiju mora i atmosfere,

Zavod za istraživanje mora i okoliša,

Institut Ruđer Bošković, Bijenička cesta 54, 10000 Zagreb

Sažetak: Cilj ovog doktorskog rada je ispitati kako promjene u okolišu utječu na sastav i kvantitetu proizvedene organske tvari fitoplanktona. Po prvi puta su sistematski ispitane lipidne klase kao markeri stresa u laboratorijski uzgojenim kulturama dijatomeje *Chaetoceros pseudocurvisetus* te okolišnim uzorcima vode različitih trofičkih statusa, Jadranskog mora i estuarija rijeka Wengchang, Kina, i Krke, Hrvatska. Od okolišnih čimbenika stresa istraženi su utjecaji promjene temperature i saliniteta te nedostatak hranjivih soli. Za analizu lipida korištene su metode tankoslojne kromatografije i masene spektrometrije. Dobiveni rezultati laboratorijskog uzgoja i terenskih uzoraka uspoređeni su te su pokazali da se u stresnim uvjetima stanice fitoplanktona sporije dijele, nakupljaju više lipida i izlučuju više otopljene organske tvari uključujući lipide. Zamijećena je promjena omjera fosfolipida i glikolipida u nedostatku fosfata, te nakupljanje triglicerida u nedostatku nitrata. Promjena saliniteta dovela je do promjena u sastavu masnih kiselina fosfolipida. Rod dijatomeja *Chaetoceros* pokazao se kao dobar modelni organizam za razumijevanje prilagodbe fitoplanktona na globalne promjene.

(145 stranica, 5 slika, 128 literaturna navoda, jezik izvornika: hrvatski)

Ključne riječi: *Chaetoceros pseudocurvisetus,* estuarij, hranjive soli, metabolizam lipida, salinitet, sjeverni Jadran, temperatura

Mentor: Dr.sc. Blaženka Gašparović, znanstveni savjetnik

Ocjenjivači: Dr. sc. Zrinka Ljubešić, izvanredni profesor

Dr. sc. Tea Mišić Radić, znanstveni suradnik

Dr. sc. Petar Žutinić, docent

Dr. sc. Jelena Godrijan, znanstveni suradnik (zamjena)

Rad prihvaćen: 8. siječnja 2021.

BASIC DOCUMENTATION CARD

University of Zagreb
Faculty of Science
Department of Geology

Doctoral Thesis

CHANGES IN PHYTOPLANKTON LIPID COMPOSITION AS A RESPONSE TO ENVIRONMENTAL STRESS

Tihana Novak
Laboratory for Marine and Atmospheric Biogeochemistry,
Division for Marine and Environmental Research,
Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb

Abstract: The aim of this dissertation is to examine how changes in the environment affect phytoplankton in terms of the quality and quantity of produced organic matter. For the first time, lipid classes were systematically examined as stress markers in cultures of diatoms *Chaetoceros pseudocurvisetus* and environmental water samples of different trophic statuses, the Adriatic Sea, the Wengchang River, China, and the Krka River, Croatia, estuaries. Among the environmental stress parameters, the effects of temperature and salinity changes and lack of nutrients were investigated. Thin layer chromatography and mass spectrometry methods were used for lipid analysis. The results obtained from cultivation experiments and field samples were compared and showed that under stressful conditions there is reduced phytoplankton growth, phytoplankton accumulate more lipids and excrete more dissolved organic matter including lipids. Remodeling of lipids occurs as a change in the ratio of phospholipids and glycolipids as a response to phosphate deficiency and the accumulation of triglycerides in the absence of nitrates in the growth medium. The change in salinity leads to changes in the fatty acid composition of phospholipids. The diatoms genus *Chaetoceros* has proven to be a good model organism for understanding the adaptation of phytoplankton to global changes.

(145 pages, 5 figures, 128 references, original in Croatian)

Keywords: *Chaetoceros pseudocurvisetus*, estuaries, lipid metabolism, Northern Adriatic Sea, nutrients, temperature, salinity

Supervisor: Dr.sc. Blaženka Gašparović, Senior Scientist

Reviewers: Dr. sc. Zrinka Ljubešić, Associate Professor
Dr. sc. Tea Mišić Radić, Research Associate
Dr. sc. Petar Žutinić, Assistant Professor
Dr. sc. Jelena Godrijan, Research Associate (substitute)

Theses accepted: 08. 01. 2021.

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II. Novak, T., Godrijan, J., Pfannkuchen Marić, D., Djakovac, T., Medić, N., Ivančić, I., Mlakar, M., Gašparović, B., 2019. **Global warming and oligotrophication lead to increased lipid production in marine phytoplankton**. Science of the Total Environment 668, 171-183 https://doi.org/10.1016/j.scitotenv.2019.02.372

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PROŠIRENI SAŽETAK

Fitoplankton doprinosi gotovo polovici globalne primarne proizvodnje te pokreće biogeokemijske procese u moru (Field i sur., 1998). Rast fitoplanktona uvjetovan je dostupnošću hranjivih soli, svjetlom, temperaturom, salinitetom te brojnim drugim čimbenicima čije promjene mogu predstavljati stres tim jednostaničnim organizmima (Falkowski i Raven, 2007; Guschina i Harwood, 2006, Falkowski 1998). Svi ti čimbenici mogu doprinijeti promjenama na molekularnoj razini stanica fitoplanktona, uključujući promjene u koncentraciji i sastavu lipida.

Ovaj doktorski rad predstavlja fundamentalna istraživanja biokemijskih procesa u moru posebice prilagodbama fitoplanktona na promjene u okolišu. Važan doprinos ovog istraživanja je u razumijevanju odgovora stanica fitoplanktona, posebice kroz promjenu sastava lipida, na različite nepovoljne okolišne pritiske. U ovom radu istraženi su utjecaj promjene temperature, nedostatak hranjivih soli fosfata i nitrata te promjene saliniteta na sastav i strukturu lipida dijatomeja. Ovo istraživanje ima originalni znanstveni doprinos u spoznajama o značajkama organske tvari u moru te kruženju ugljika. Po prvi puta su sustavno ispitane lipidne klase kao markeri stresa u laboratorijski uzgojenim kulturama dijatomeja te okolišnim uzorcima vode različitih trofičkih statusa. Za analizu uzoraka lipida primijenjena je metoda tankoslojne kromatografije unaprijeđena u Laboratoriju za biogeokemiju mora i atmosfere, te metoda masene spektrometrije unaprijeđena u Laboratorij za spektrometriju masa i funkcionalnu proteomiku, Instituta Ruđer Bošković.

Ova disertacija se temelji na tri objavljene publikacije čiji su rezultati raspravljeni te su doneseni zajednički zaključci. Uz pretpostavku da se fitoplankton prilagođava stresnim uvjetima kako bi opstao, cilj istraživanja, bio je odrediti promjene na molekularnoj razini, a prvenstveno količine, sastava i struktura lipida određenih fitoplanktonskih vrsta i fitoplanktonskih zajednica, te su postavljene četiri hipoteze koje su potvrđene u objavljenim publikacijama. U sve tri publikacije rezultati su pokazali da prilikom promjena u okolišu te u laboratorijskim uvjetima dolazi do promjene u sintezi staničnih lipida (hipoteza 1). U publikaciji III korištena je metoda tekućinske kromatografije visoke djelotvornosti (HPLC) gdje su se odredile promjene na razini masnih kiselina fosfolipida te je uočeno da promjene u okolišu posljedično uzrokuju promjene kod ispitivanih mikroorganizama i na molekularnom nivou (hipoteza 2). Rezultati dobiveni u publikacijama I i II, u kojima su provedeni eksperimenti uzgoja dijatomeja *Chaetoceros pseudocurvisetus* pri različitim uvjetima, potvrdili su da neki okolišni čimbenici imaju veći utjecaj na rast i razmnožavanje

fitoplanktona (hipoteza 3) te da dijatomeje mogu biti dobri modelni organizmi za ispitivanje utjecaja okolišnog stresa na fitoplankton (hipoteza 4).

U prvoj publikaciji (I) istražena je proizvodnja otopljenih lipida, lipidnih klasa i otopljenog organskog ugljika *in situ* u sjevernom Jadranu, te u *in vivo* istraživanjima dijatomeje *Chaetoceros pseudocurvisetus*. Dijatomeje su izolirane iz sjevernog Jadrana, te uzgajane u temperaturnom rasponu 10 – 30 °C te pri različitim koncentracijama fosfata. Dobiveni rezultati uspoređeni su s rezultatima uzorkovanja dvije trofički različite postaje sjevernog Jadrana, u razdoblju od veljače do kolovoza 2010. godine s temperaturnim rasponom 9,3 – 31,1 °C. S povišenjem temperature u kulturama je došlo do povećanog nakupljanja lipida posebice na temperaturama 25 i 30 °C, kada je puno značajniji dio proizvodnje lipida bio usmjeren u stvaranje otopljene lipidne frakcije. Utjecaj temperature na pojačano otpuštanje otopljenih lipida veći je u uvjetima nedostatka fosfata, što dovodi do zaključka da bi oligotrofna područja mogla biti osjetljivija na promjene temperature. Za razliku od monokultura, rezultati dobiveni na uzorcima iz sjevernog Jadrana ukazuju da uz temperaturu i mnogi okolišni čimbenici utječu na distribuciju, sastav i kruženje primarno proizvedenih lipida. U drugoj publikaciji (II) istražena je proizvodnja lipida i lipidnih klasa na uzorcima sjevernog Jadrana i dijatomeje *Chaetoceros pseudocurvisetus*. Dijatomeje su uzgajanje u temperaturnom rasponu 10 – 30 °C i pri koncentracijama hranjivih soli koje su oponašale oligotrofne i eutrofne uvjete. Dobiveni rezultati uspoređeni su s rezultatima uzorkovanja dvije trofički različite postaje sjevernog Jadrana, u razdoblju od ožujka 2013. do ožujka 2014. godine s temperaturnim rasponom 10,6 – 24,7 °C. Uočeno je pojačano nakupljanje lipida po stanici pri povišenim temperaturama i u oligotrofnim uvjetima. Dostupnost hranjivih soli ima jači utjecaj na remodeliranje lipida od povišenja temperature. U uvjetima nedostatka fosfata zamijećena je promjena omjera fosfolipida i glikolipida, dok je u uvjetima nedostatka nitrata prisutno nakupljanje triglicerida. U trećoj publikaciji (III) istražen je okolišni utjecaj promjene saliniteta, temperature i različite koncentracije hranjivih soli na proizvodnju i molekularni sastav fosfolipida dvaju različitih estuarija. Istraživanja su provedena u tropskom, eutrofnom estuariju rijeke Wenchang u Kini u rujnu 2014. godine te umjerenom, mezotrofnom estuariju rijeke Krke u Hrvatskoj u svibnju 2015. godine. U skladu s većom brojnošću fitoplanktona, koncentracije fosfolipida bile su više u estuariju rijeke Wenchang nego u estuariju rijeke Krke. Strukture masnih kiselina fosfatidilglicerola, fosfatidilkolina i fosfatidilinozitola bile su slične u svim uzorcima, iz čega se može zaključiti da su to molekule od vitalne važnosti te ih stanica održava srazmjerno nepromijenjenima. Promjene su uočene kod masnih kiselina fosfatidiletanolamina, fosfatidne kiseline i

fosfatidilserina, ovisno o različitim uvjetima dostupnosti hranjivih soli i temperature u estuarijima, ali i prema gradijentu saliniteta. Masne kiseline s dužim lancima nađene su u slatkoj vodi i morskim postajama najvećeg saliniteta, dok su u pravilu masne kiseline kraćih lanaca nađene u estuarijskoj vodi. To ukazuje na sposobnost fitoplanktona da remodelira ove fosfolipide ovisno o okolišnim uvjetima i strukturi fitoplanktonske zajednice.

Ovo istraživanje otvorilo je brojna pitanja i ideje za daljnji rad i projekte u području istraživanja lipida fitoplanktona. Važno je naglasiti kako bitan doprinos ovoga, a i istraživanja kao ovo je u razumijevanju trenutnih, ali i budućih biokemijskih procesa u moru. Korištenje lipida kao markera stresa omogućuje nam predviđanje odgovora fitoplanktona na promjene u okolišu.

THESIS SUMMARY

Phytoplankton contributes to almost half of the world's primary production and is driving biogeochemical processes in the sea (Field et al., 1998). Phytoplankton growth is influenced by the availability of nutrients, light, temperature, salinity, and numerous other factors whose changes can induce stress in these unicellular organisms (Falkowski and Raven, 2007; Guschina and Harwood, 2006; Falkowski 1998). All of these factors can contribute to the molecular changes in phytoplankton cells, including changes in lipids.

This doctoral thesis presents fundamental research on biochemical processes in the sea, particulary on adaptation of phytoplanton to environmental changes. An important contribution of this research is in understanding the response of phytoplankton cells, especially in terms of lipid composition, to various unfavorable environmental pressures. The effects of temperature change, phosphate and nitrate deficiency, and salinity changes were examined on lipid composition and structure in diatoms. This research also contributes to the understanding of the characteristics of the organic matter in the sea and the carbon cycle. For the first time, lipid classes as stress markers were systematically examined in both laboratory-grown diatom cultures and environmental water samples of different trophic statuses. For the analysis of lipid samples, thin layer chromatography improved in the Laboratory for Marine and Atmospheric Biogeochemistry, and mass spectrometry improved in the Laboratory for Mass Spectrometry and Functional Proteomics, Ruđer Bošković Institute, was applied.

The doctoral thesis is based on three published publications, which were discussed, and joint conclusions were reached. With the assumption that phytoplankton adapts to stress conditions in order to survive, the aim of the study was to determine changes at the molecular level, and in particular the amount, composition and structure of lipids of lipids of specific phytoplankton as well as the whole community. Four hypotheses have been set and confirmed in published publications. In all three publications, results showed that changes in cellular lipid synthesis occur during changes in conditions, be it in the environment or in laboratory (hypothesis 1). In the publication III, the HPLC method was used to determine changes in the fatty acid composition and it was observed that environmental changes consequently cause changes at the molecular level of tested microorganisms (hypothesis 2). The results obtained in publications I and II, where experiments were conducted on the cultivated diatom *Chaetoceros pseudocurvisetus,* confirmed that some environmental parameters have a greater impact on the growth and reproduction of phytoplankton (hypothesis 3) and that diatoms represent a good model organism for giving insight into the effects of environmental stress on phytoplankton (hypothesis 4).

The first publication (I) investigated the production of dissolved lipids, lipid classes and dissolved organic carbon in the *in situ* samples of the northern Adriatic and *in vivo* experiments of diatom *Chaetoceros pseudocurvisetus*. Diatoms were isolated from northern Adriatic and grown at a temperature range of 10 – 30 °C and with contrasting phosphate concentration. The obtained results were compared with the results obtained at two stations of the northern Adriatic characterized with different P availability, occupied the period from February to August 2010 with a temperature range of 9.3 − 31.1°C. In cultures, an increased accumulation of lipids was observed with increasing temperature, especially at temperatures of 25°C and 30°C, at which a significant part of lipid production was directed toward the dissolved phase. The effect of temperature on the enhanced release of dissolved lipids is greater under phosphorus-limiting conditions indicating that oligotrophic regions might be more vulnerable to temperature rise. If compared to monocultures, results from the northern Adriatic revealed that in addition to temperature, there are many other environmental factors that affect the distribution, composition and circulation of primarily produced lipids. The second publication (II) investigated the production of lipids and lipid classes on samples of the northern Adriatic and diatom *Chaetoceros pseudocurvisetus*. Diatoms were grown at a temperature range of 10 − 30 °C and at nutrient concentrations that mimicked oligotrophic and eutrophic conditions. The obtained results were compared with the samplings of two trophically different stations in the northern Adriatic, in the period from March 2013 to March 2014 with a temperature range of 10.6 − 24.7°C. Increased lipid accumulation per cell was observed at elevated temperatures and under oligotrophic conditions. Lipid remodeling was more affected by nutrient availability than by temperature increase. Remodeling of lipids occured as a change in the ratio of phospholipids and glycolipids in response to phosphate deficiency and the accumulation of triglycerides in response to nitrogen deficiency. The third publication (III) investigated the environmental impact on the production and molecular composition of phospholipids in two different estuaries. The research was conducted in the subtropical, eutrophic Whengchang River estuary in China in September 2014., and the moderate pristine, mesotrophic Krka River estuary in Croatia in May 2015. Consistent with the higher abundance of phytoplankton, phospholipid concentrations were higher in the Wengchang River estuary than in the Krka River estuary. In all samples, the fatty acid structures of phosphatidylglycerol, phosphatidylcholine and phosphatidylinositol were similar, indicating that these are molecules of vital importance, therefore cells kept them relatively unchanged. Changes were observed in the fatty acids phosphatidylethanolamine, phosphatidic acid and phosphatidylserine, depending on different conditions, but also on the

salinity gradient. Long-chain fatty acids were found in freshwater and marine stations of the highest salinity, while short-chain fatty acids were found in estuarine waters. This indicated the ability of phytoplankton to remodel these phospholipids depending on environmental conditions and the structure of the phytoplankton community.

This research opened a number of questions and ideas for further work and projects in the field of phytoplankton lipid research. It is necessary to emphasize an important contribution of this, and research like this, in understanding current and future biochemical processes in the sea. Using lipids as a stress marker allows us to predict phytoplankton responses to environmental changes.

1 UVOD

1.1 Organska tvar u vodenom okolišu

Organska tvar u vodenom okolišu smjesa je različitih spojeva koji se međusobno razlikuju po podrijetlu, sastavu te fizičko-kemijskim svojstvima. Organske molekule su sve molekule koje sadrže ugljik, isključivši okside (CO, CO_2, HCO_3^-, H_2CO_3, CO_3^{2-}), elementarne oblike ugljika te minerale. Prema podrijetlu organsku tvar u vodenim sustavima dijelimo na autohtonu, nastala unutar samog sustava, te alohtonu, u sustav unesenu vanjskim izvorima. Najveći dio organske tvari u moru (40 – 50 Pg C) godišnje nastao je autohtono i to primarnom i sekundarnom proizvodnjom dok je manji dio bakterijskom razgradnjom, autolizom mrtvih organizama, alohtonim unosom rijekama (godišnje oko 0,4 Pg C), otpadnim vodama ili iz atmosfere (Lee i sur., 1988; Libes, 2009). Organska tvar u moru predstavlja kontinuum u veličinskoj raspodjeli od malih molekula do 30 m velikih kitova. Kako bi ih metodološki lakše odredili organsku tvar u moru dijelimo na otopljenu (DOM, engl. *dissolved organic matter*) i čestičnu (POM, engl. *particulate organic matter)* organsku tvar. Sve što zaostaje na filtrima 0,22 – 1 μm definira se kao čestična organska tvar, a najčešće korišteni su filtri sa staklenim vlaknima (GF/F) veličine pora 0,7 μm (Hansell i Carlson, 2002). Funkcionalno je ova podjela bitna s obzirom da su čestice manje od 1 μm uglavnom lebdeće. Treba uzeti u obzir da najveći dio organske tvari u moru pripada otopljenoj frakciji (~ 89 %), dok ostatak dijelimo na detritus kao neživu (~ 9 %) te organizme kao živu (~ 2 %) partikularnu frakciju (Duursma i Dawson, 1981).

Otopljena organska tvar (DOM) sadrži najveće zalihe reaktivnog ugljika na Zemlji, kao i velike količine dušika i fosfora. Ona utječe na koncentraciju atmosferskog CO_2, hrana je heterotrofnim bakterijama, potiče fotokemijske reakcije, doprinosi uklanjanju hranjivih soli iz površine prianjanjem na veće čestice i kompleksira s metalima. Koncentracije otopljenog organskog ugljika (DOC, engl. *dissolved organic carbon*) u moru su reda 10 μmol, nekoliko μmol za otopljeni organski dušik (DON, engl. *dissolved organic nitrogen*) i reda desetine μmol za otopljeni organski fosfat (DOP, engl. *dissolved organic phosphate*). S obzirom da je najveći udio otopljenog ugljika, često se DOC koristi kao mjera za DOM. Najveće koncentracije DOM-a su u površinskom sloju mora s obzirom da se tamo odvija najveća sinteza organskih molekula. Niz termoklinu koncentracije DOM-a eksponencijalno opadaju. U pravilu, koncentracije DOC-a su puno veće od POC-a, posebice u dubokom moru. Manje od 10 % DOM-a je strukturno definirano, i to su uglavnom ugljikohidrati, a neki od sastojaka su izrazito hlapljivi kao npr. dimetil-sulfid i imaju važnu ulogu u kontroli klime (Libes, 2009).

Udio lipida u DOC-u u morskim i estuarijskim uzorcima uglavnom su male (0,8 – 4,5 %) (Mannino i Harvey, 1999; Marić i sur., 2013; Myklestad, 2000). Otopljeni lipidi predstavljaju svježi materijal nastao primarnom proizvodnjom i/ili odmah nakon stanične smrti (Hansell i Carlson, 2002).

Od tri kategorije molekula koje čine glavninu organske tvari: lipidi, proteini i ugljikohidrati, lipidi su najmanje istraženi, iako imaju važnu ulogu u brojnim biološkim procesima (Arts i sur., 2009). Dosta dugo se smatralo da imaju ulogu samo kao energetske rezerve i u strukturi stanica, no njihova uloga je višestruka. Kao prvo, bitni su za funkciju membrana i komunikaciju stanice s okolinom. Uz direktnu biološku važnost u samim stanicama imaju važnu ulogu u kruženju elemenata. Tako u ukupnom kruženju ugljika u moru sudjeluju s 15 – 25 %, a kao konzumenti PO_4 s 18 – 28 % (Hunter, 2015). Uočeno je da mogu imati i direktan utjecaj na Redfieldov omjer jer u uvjetima nedostatka fosfata u okolišu stanice fitoplanktona zamjenjuju fosfolipide sa sulfolipidima (Van Mooy i sur., 2009).

1.2 Biogeokemija lipida

Lipidi su biološki vrlo važne molekule bogate ugljikom koje sudjeluju u izgradnji i funkcioniranju stanica, a unutar molekule mogu sadržavati bitne elemente uključujući fosfor, sumpor i dušik. Različite molekule lipida nemaju jednaka strukturna i kemijska svojstva, ali se svrstavaju u istu skupinu na temelju jednake metode izolacije. Karakterizira ih fizičko svojstvo veće topljivosti u nepolarnim i slabo polarnim otapalima kao što su heksan, diklormetan te dietileter (Carey, 2003; Libes, 2009). Njihova karakteristika je da nisu direktno kodirani u genomu. Karakteristike kao lateralna organizacija, zakrivljenost, struktura membrana posljedica su prirode samoformirajuće membrane i ovise o zakonima fizike, termodinamike i termomehanike. Lipidi ne stvaraju polimere s kovalentnim vezama nego makroagregate kao npr. dvostruku membranu te se kao takvi mogu opisati kao savršeni balans reda i nereda (Hunter, 2015).

S obzirom na polarnost dijelimo ih u dvije skupine: (i) nepolarni lipidi - ugljikovodici, digliceridi, trigliceridi, steroli, slobodne masne kiseline, voskovi i masni alkoholi te (ii) polarni lipidi- fosfolipidi, pigmenti, monoacilgliceridi, glikolipidi i betain lipidi (Arts i sur., 2009) (Slika 1). Nepolarni lipidi uglavnom imaju ulogu u skladištenju energije (trigliceridi i voskovi), ali su i prekursori drugim molekulama (esteri) ili jednostavno raspadni produkti ostalih lipida (slobodne masne kiseline, masni alkoholi). Polarni lipidi imaju važnu ulogu u izgradnji staničnih membrana, posebice fosfolipidi koji grade fosfolipidni dvosloj koji čini

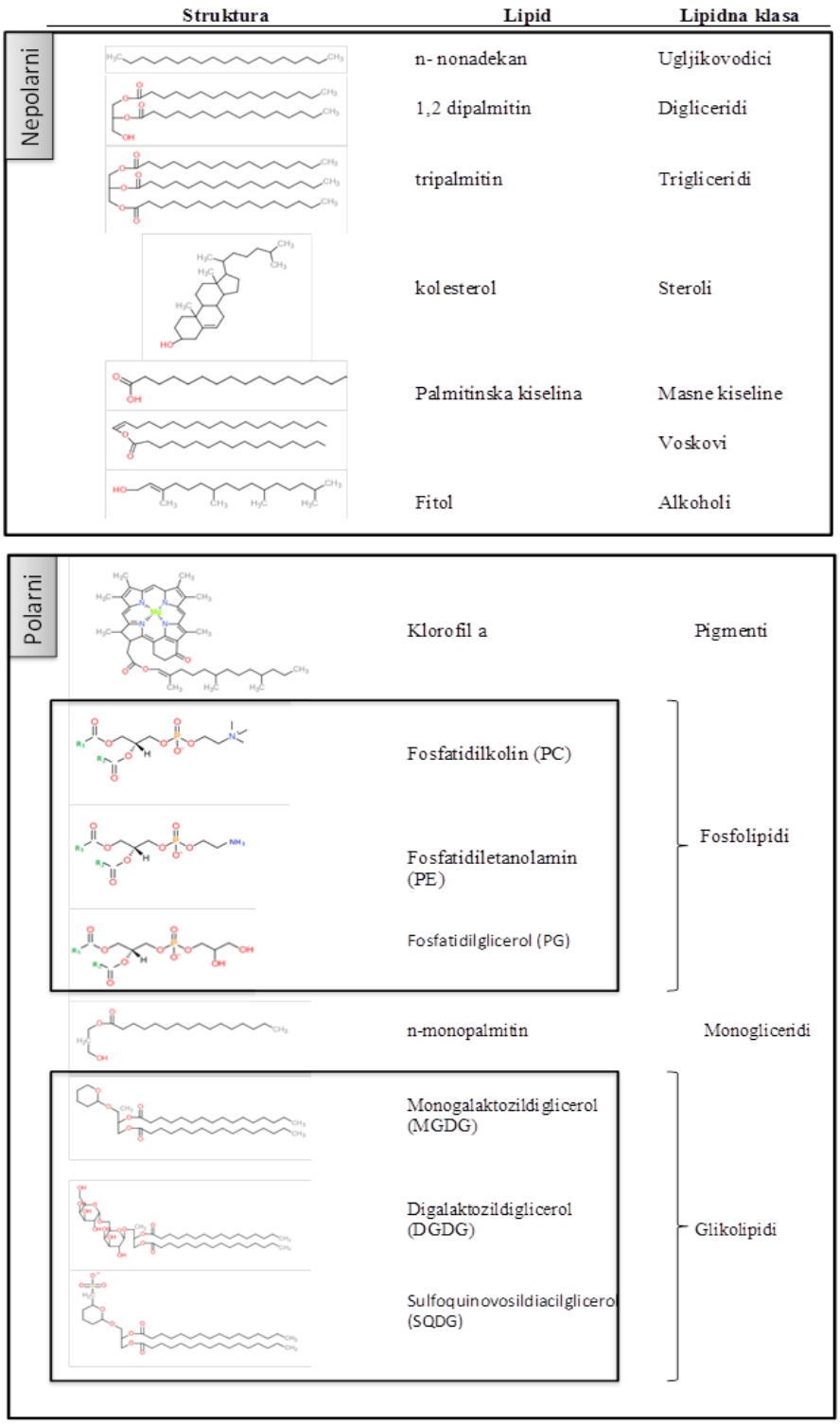
staničnu membranu. Neki polarni lipidi uz to imaju senzorsku ulogu (npr. inozitol lipidi i sfingolipidi) te su glavni intermedijeri u staničnom signalizacijskom putu, a time imaju važnu ulogu u reakciji stanica na okolišni stres (Arts i sur., 2009).

Biogeni ugljikovodici pojavljuju se u svim morskim organizmima i zauzimaju udio od oko 1 % ukupnih lipida organizama. Uglavnom se spominju kao indikatori zagađenja (Parrish, 1988). Masne kiseline glavni su konstituent većine morskih lipida i važan izvor energije za morske organizme. U sastavu morskih lipida dominiraju palmitinska kiselina (n-C16:0) te višestruko nezasićene masne kiseline, poglavito ω-3 masne kiseline n-C20:5 i n-C22:6 (Parrish, 1988). Trigliceridi, poznatiji kao masti i ulja su triesteri glicerola i masnih kiselina (Pine, 1994). Masne kiseline od kojih se sastoje trigliceridi fitoplanktona gotovo uvijek imaju paran broj ugljikovih atoma (n-C6:22), što je povezano s njihovom sintezom (Pine, 1994). U mnogim algama nepolarni trigliceridi čine značajan udio lipida te su veoma važan izvor energije za stanice (Parrish i Wangersky, 1991). Neki detektirani lipidi se pojavljuju u moru kao razgradni produkti drugih lipida. Tako digliceridi nastaju razgradnjom glicerida. Do stvaranja alkohola dolazi biološkom razgradnjom, hidrolizom voskova te klorofila *a* i *b*.

Glikolipidi zajedno s fosfolipidima glavni su konstituenti bioloških membrana. Glikolipidi najčešće sadrže jednu ili dvije molekule galaktoze povezane na sn-3 poziciju glicerola (Guschinia i Harwood, 2009). Glavni glikolipidi algi su monogalaktozildiglicerol (MGDG) i digalaktozildiglicerol (DGDG) te zauzimaju postotak od 40−55% i 15−35% ukupnih tilakoidnih lipida (Guschinia i Harwood, 2009). Također u značajnim količinama u tilakoidnim membranama eukariotskih algi zabilježen je lipid sulfokvinovosildiacilglicerol (SQDG). (Harwood, 1998).

Fosfolipidi su lipidi koji sadrže fosfatnu estersku skupinu. Građu fosfolipida čine alkohol glicerol ili sfingozin na koje se vežu masne kiseline i fosforilirani alkohol. Najjednostavniji fosfolipid naziva se fosfatidatom (diacilglicerol 3-fosfat) i nastaje esterifikacijom hidroksilnih skupina C1 i C2 masnim kiselinama, te atoma C3 fosfornom kiselinom. Do stvaranja fosfolipida dolazi stvaranjem esterske veze između hidroksilne skupine alkohola i fosfatne skupine fosfatidata (Pine, 1994). Glavni fosfolipidi u većini eukariotskih algi su fosfatidilkolin (PC), fosfatidiletanolamin (PE) te fosfatidilglicerol (PG). PC i PE su najzastupljeniji membranski lipidi i čine ~ 68 − 80% ukupnih fosfolipida (van Meer i sur., 2008). Osim njih u dostatnim količinama nalaze se i fosfatidilserin (PS), fosfatidilinozitol (PI) te difosfatidilglicerol (DPG) (Guschina i Harwood, 2006). Međusobno

se razlikuju po alkoholu vezanom za fosfatidat. Fosfolipide nalazimo u vanjskoj membrani kloroplasta, osim PG koji se u značajnim količinama nalazi u tilakoidnim membranama (Guschina i Harwood, 2006), gdje sudjeluju u fotosintetskom transportu elektrona (Wada i Murata, 2007).



Slika 1. Polarne i nepolarne klase lipida detektirane tankoslojnom kromatografijom.

U vrlo niskim koncentracijama lipidi su rasprostranjeni u svim morima i oceanima (Arts i sur., 2009), gdje se pojavljuju u otopljenoj i partikularnoj fazi (Parrish, 1988). Dobri su pokazatelji promjena organske tvari u vodenom okolišu, izvora, proizvodnje, karakterizacije, kruženja i raznolikosti. Karakterizacija lipida na molekularnoj razini koristi se kao pokazatelj izvora i prilagodbe fitoplanktona na okolišni stres (Arts i sur., 2009), podrijetla i procesa organske tvari (Mansour i sur., 1999), virusne infekcije fitoplanktona (Fulton i sur., 2014) i dr.

1.3 Biologija fitoplanktona

Fitoplankton je heterogena skupina jednostaničnih autotrofnih (miksotrofnih) organizama koji lebde u vodenom stupcu. S obzirom da govorimo o planktonu radi se o organizmima koji se kreću vodenim strujanjima pasivno i tonjenjem. Neki predstavnici imaju bičeve koji im omogućuju pasivno pokretanje unutar vodenog stupca. Dostupnost svjetla ograničavajući je faktor za život fitoplanktona te je njihov rast isključivo vezan za fotičku zonu. Većina fitplanktonskih stanica je gušća od vode te time imaju tendenciju tonjenja. Neke stanice imaju prilagodbe za usporavanje tonjenja kako bi povećale količinu vremena u fotičkoj zoni i omogućile daljnji rast i razmnožavanje.

Determinacijska svojstva pojedinih vrsta temeljena su na veličini, pigmentima i morfologiji. Najčešća podjela je u tri frakcije temeljene na veličini stanica: 1) mikroplankton (20 – 200 μm), 2) nanoplankton (2 – 20 μm) i 3) pikoplankton (0,2 – 2 μm) (Sieburth i sur., 1978). Novija podjela bazirana na analizi tibosomske DNA (rDNA) dijeli ih u četiri frakcije: pikonanoplankton (0,8 −5 μm), nanoplankton (5 − 20 μm), mikroplankton (20 − 180 μm) i mezoplankton (180−2000 μm) (de Vargas i sur., 2015). Ti sitni mikroorganizmi imaju veoma važnu ulogu u biosferi s obzirom da su glavni oceanski primarni proizvođači koji procesom fotosinteze uklanjaju CO_2 iz okoliša i proizvode kisik. Ukupna globalna neto primarna proizvodnja procijenjena je na 104,9 Pg ugljika godišnje, sa sličnim udjelom kopnene od 56,4 Pg ugljika (53,8 %) i oceanske od 48,5 Pg ugljika (46,2 %) komponente (Field i sur., 1998). Iako su odgovorni za gotovo pola svjetske primarne proizvodnje, oni čine tek 0,2 % biomase svih primarnih proizvođača (Field i sur., 1998). Taj nerazmjer između neto primarne proizvodnje i biomase posljedica je višestruko brže pretvorbe organske tvari u oceanima (u prosjeku 2 – 6 dana) nego na kopnu (prosjek 19 godina) (Falkowski i Raven, 2007; Field i sur., 1998). Sve više istraživanja se provodi koristeći fitoplankton kao modelni organizam, a uz to sve je više istraživanja novih vrsta fitoplanktona i njihovih zanimljivih značajki.

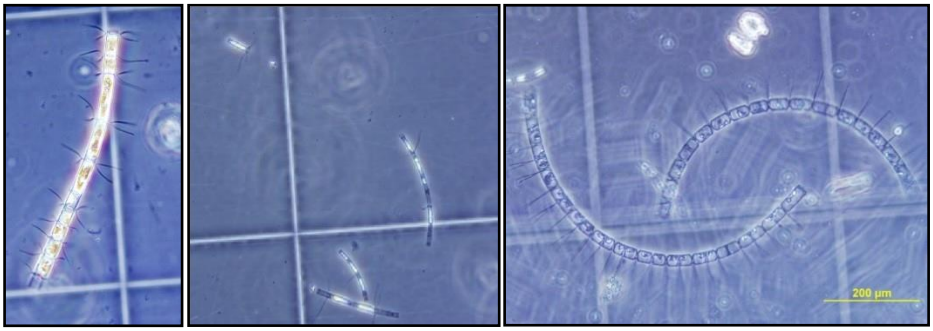
Zbog primarne proizvodnje fitoplankton ima značajnu ulogu u biološkoj pumpi. Biološka pumpa je kolektivni naziv za proces kruženja ugljika, iz atmosfere preko površine mora do konačnog tonjenja organske tvari. Proces započinje pretvaranjem anorganskog ugljika u organsku tvar fotosintezom, zatim djelomičnu razgradnju mikroorganizmima gdje ponovno postaje dostupan otopljeni ugljik. Organske čestice tonu, kada potonu ispod termokline u oceanima, ugljik iz te organske tvari nedostupan je za interakciju s atmosferom na nekoliko tisuća godina. Organske čestice koje potonu u sediment uklonjene su od interakcije s atmosferom na puno dulji period. U biološkoj pumpi vrlo su uspješne dijatomeje i kokolitoforidi s obzirom da stvaraju krutu čestičnu organsku tvar koja lakše tone te se uspješnije uklanja iz površine mora (Libes, 2009).

Dijatomeje su najzastupljenija i ekološki najuspješnija mikrofitoplanktonska skupina (Malviya i sur., 2016). Ti organizmi imaju veoma važnu ulogu u proizvodnji organske tvari i kruženju ugljika i silicija u morskim ekosustavima (Obata i sur., 2013), procjenjuje se da su odgovorne za 27 % globalne primarne proizvodnje (Malviya i sur., 2016). Do sada je zabilježeno preko 8000 vrsta u slatkim i morskim vodama, a procjena je da ima još 20 – 200 tisuća neistraženih vrsta (Appeltans i sur., 2012; Guiry, 2012).

Dijatomeje se pojavljuju u kolonijama ili kao zasebne stanice. Glavno obilježje im je kućica sagrađena od silicija u obliku petrijeve posudice, gdje gornja valva (epiteka) prelazi preko donje valve (hipoteke). Stanice se razmnožavaju spolno i nespolno. Pri nespolnom razmnožavanju dolazi do mitoze prilikom koje od epiteke i hipoteke nastaju stanice kćeri. Stanica kćer nastala iz epiteke jednake je veličine kao stanica majka, no stanica nastala iz hipoteke ima manji volumen. Time se progresivno smanjuje veličina stanica populacije te u trenutku kad populacija dosegne kritičnu veličinu, stanice prelaze u sporu iz koje se obnavljaja početna veličina stanica.

Konzumacijom dostupnih hranjivih soli u vodenom okolišu te fiksacijom CO_2 dijatomeje sintetiziraju ugljikohidrate, proteine i lipide (Zulu i sur., 2018). Lipidi su glavni konstituenti stanica dijatomeja, a prosječni udio lipida u stanici može doseći i 25% suhe tvari (Levitan i sur., 2014), a njihova proizvodnja varira ovisno o uvjetima rasta. Glavne komponente rezervnih lipida su trigliceridi (TG) i slobodne masne kiseline (FFA, engl. *free fatty acids*), koji uobičajeno čine 15 – 25% biomase stanica (Mangas-Sánchez i Adlercreutz, 2015). Rezultati ukazuju da dijatomeje imaju najveću sposobnost nakupljanja lipida u usporedbi s drugim klasama fitoplanktona (Hildebrand i sur., 2012). Za potrebe eksperimenata ovog doktorskog rada, izolirana je i uzgajana morska dijatomeja *Chaetoceros pseudocurvisetus* Mangin 1910**.** Genetski materijal kulture pohranjen je u GenBank pod

brojem MG385841 (18S DNA) i MG385842 (28S DNA). U morima i oceanima jedna od najbrojnijih porodica dijatomeja je *Chaetoceros* (Malviya i sur., 2016), a u sjevernom Jadranu česti predstavnik porodice je vrsta *Chaetoceros pseudocurvisetus* (slika 2) te je kao takva izabrana kao dobra predstavnica sustava.



Slika 2 Prikaz različitih veličina stanica dijatomeje *Chaetoceros pseudocurvisetus*.

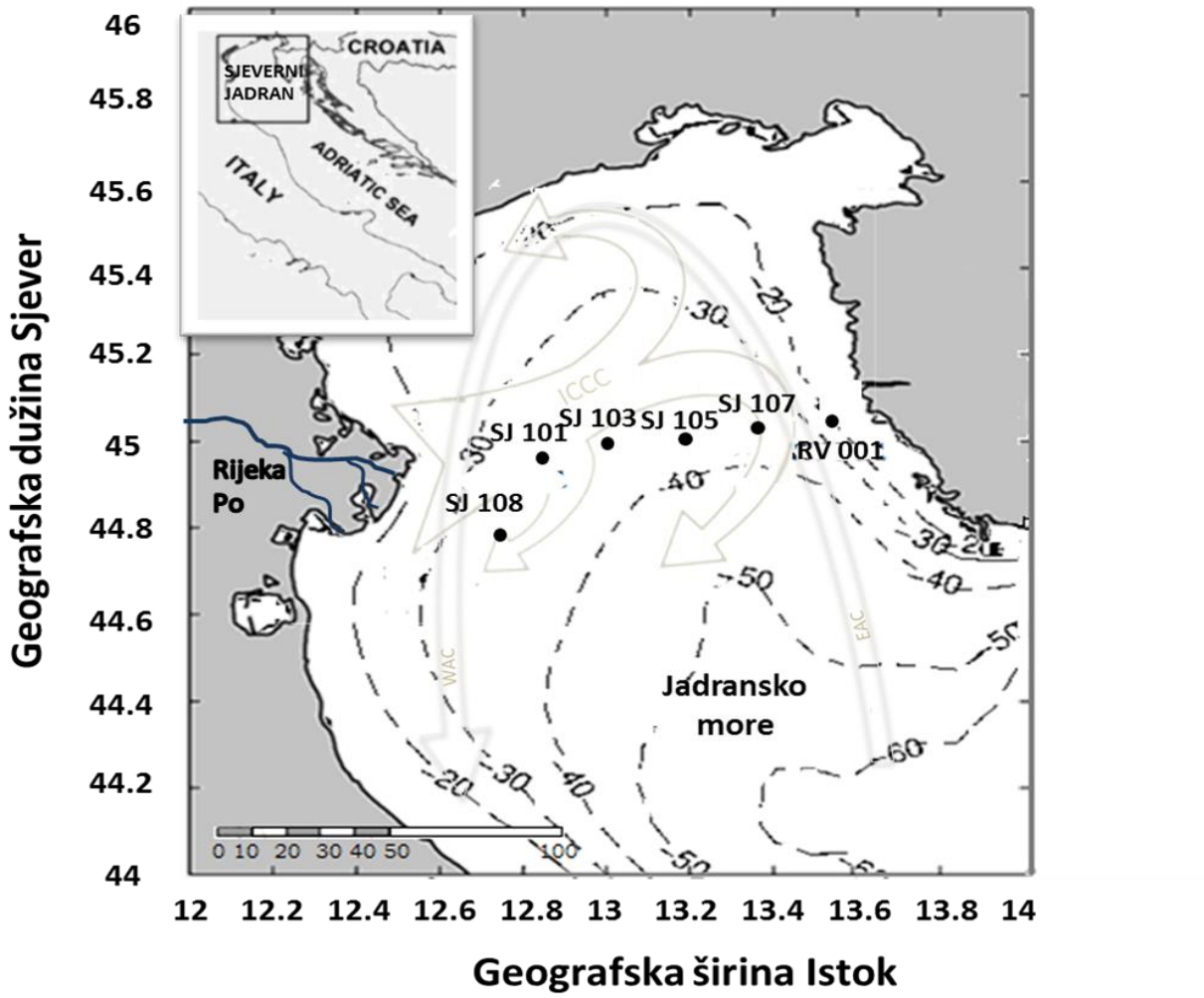
1.4 Područja istraživanja

Sjeverni Jadran najplići je dio Jadranskog mora s prosjekom dubine oko 30 m. Na temelju batimetrije i oceanografskih karakteristika Jadran se uz sjeverni dijeli još i na srednji i najdublji južni Jadran (Gačić i sur., 1999; Orlić i sur., 1992) Jadransko more dio je Mediterana te se proteže između Apeninskog i Balkanskog poluotoka. Duljine je otprilike 800 km, a širine u prosjeku 180 km (Russo i Artegiani, 1996). Klima je umjerenog tipa s temperaturom gotovo uvijek iznad 10 °C.

Trofički sastav sjevernog Jadrana pod utjecajem je atmosferskih čimbenika vjetra i temperature, te ponajviše slatkovodnim priljevom hranjivih soli iz bogate rijeke Po i obližnjih rijeka. To je jedno od najproduktivnijih područja Mediterana, na svim trofičkim razinama, od fitoplanktona do riba (Fonda i sur., 2005). Hranjivim tvarima bogate vode rijeka uvelike utječu na njegovu primarnu proizvodnju te stvaraju gradijent hranjivih soli koji se proteže od zapada prema istoku (Djakovac i sur., 2012). Sve fitoplanktonske vrste pokazuju jaki pad u biomasi niz gradijent zapad - istok, ali obrnuti gradijent uočen je za bioraznolikost jer manje hranjivih soli dovodi do veće bioraznolikosti (Fonda i sur., 2005). Cijelim Jadranom nanoplankton dominira nad mikroplanktonom i u gustoći i u brojnosti vrsta s većom zamjećenom abundancijom u sjeverenom djelu te uz obalu (Revelante i Gilmartin, 1976; Fonda Umani i sur., 2005). Viličić i sur. (2009) su u sjevernom Jadranu zabilježili 215 vrsta uključujući 16 kokolitoforida, 102 dijatomeje, 89 dinoflagelata, 2 silikoflagelata, 1 euglenoficeju i 1 krizoficeju. Dominantni zabilježeni fitoplankton bili su *Cerataulina pelagica, Chaetoceros socialis, Chaetoceros vixvisibilis* te *Pseudo-nitzschia* s gustoćom do 10^6 stanica po dm^3. U istraživanju sjevernog Jadrana uz obalu Istre zabilježena su 202 taksona

fitoplanktona, od kojih 107 do razine vrste. Najzastupljeniji zabilježeni rod dijatomeja bio je *Chaetoceros* (27 vrsta), te među dinoflagelatima rod *Ceratium* (18 vrsta) (Godrijan i sur., 2013.).

Značajno obilježje Jadrana su dvije površinske morske struje, istočna EAC (engl. *Eastern Adriatic Current*) koja donosi hladnu i vodu većeg saliniteta iz Jonskog mora u srednji i sjeverni Jadran, te zapadna WAC (engl. *Western Adriatic Current*) koja slatku vodu nosi uz zapadnu obalu u južni dio Jadrana (Orlić i sur., 1992b; Poulain, 2001; Zore-Armada, 1969). U centralnom i istočnom dijelu sjevernog Jadrana se u toplijim mjesecima stvaraju anticiklonalni vrtlozi koji u ljeto stvaraju jaku jugo-istočnu struju nazvanu Istarska obalna protustruja (ICCC, engl. *Istrian Coastal Countercurrent*,) (Supic i sur., 2003). Za posljedicu ova struja ima smanjenje saliniteta te povećanje koncentracija hranjivih soli i primarne proizvodnje (Djakovac i sur., 2012). Sjeverni Jadran trenutno je pod značajnim antropogenim utjecajem, uključujući unos hranjivih soli, urbanizacija obale, ribarstvo, turizam, itd. Do sada je u sjevernom Jadranu zamijećen pretjerani izlov (Fortibuoni i sur., 2010), eutrofija 80-ih godina prošlog stoljeća (Lotze i sur., 2011) te u zadnje vrijeme oligotrofija (Mozetič i sur., 2010) Zbog svojih je karakteristika vrlo istraživano područje te su kroz dosadašnja istraživanja i uspostavljene stalne istraživačke postaje (Slika 3). U razdoblju od 1972.do 2009. godine došlo je do značajnih promjena u stupnju trofičnosti sjevernog Jadrana što je utjecalo na sezonalnost i brojnost fitoplanktona. U usporedbi s prvim periodom (1972.− 2000.) u drugom periodu (2000.− 2009.) zamijećena je značajno manja brojnost fitoplanktona. U drugom periodu, na postaji SJ107 zamijećen je i pomak cvata na veljaču (najveći zabilježeni cvat bio je u veljači 2004. godine), a na postaji RV001 došlo je do općenitog smanjenja gustoće fitoplanktona tako da nije ni zamijećen proljetni cvat, jedino je povećana gustoća u srpnju i listopadu (Marić i sur., 2012.).

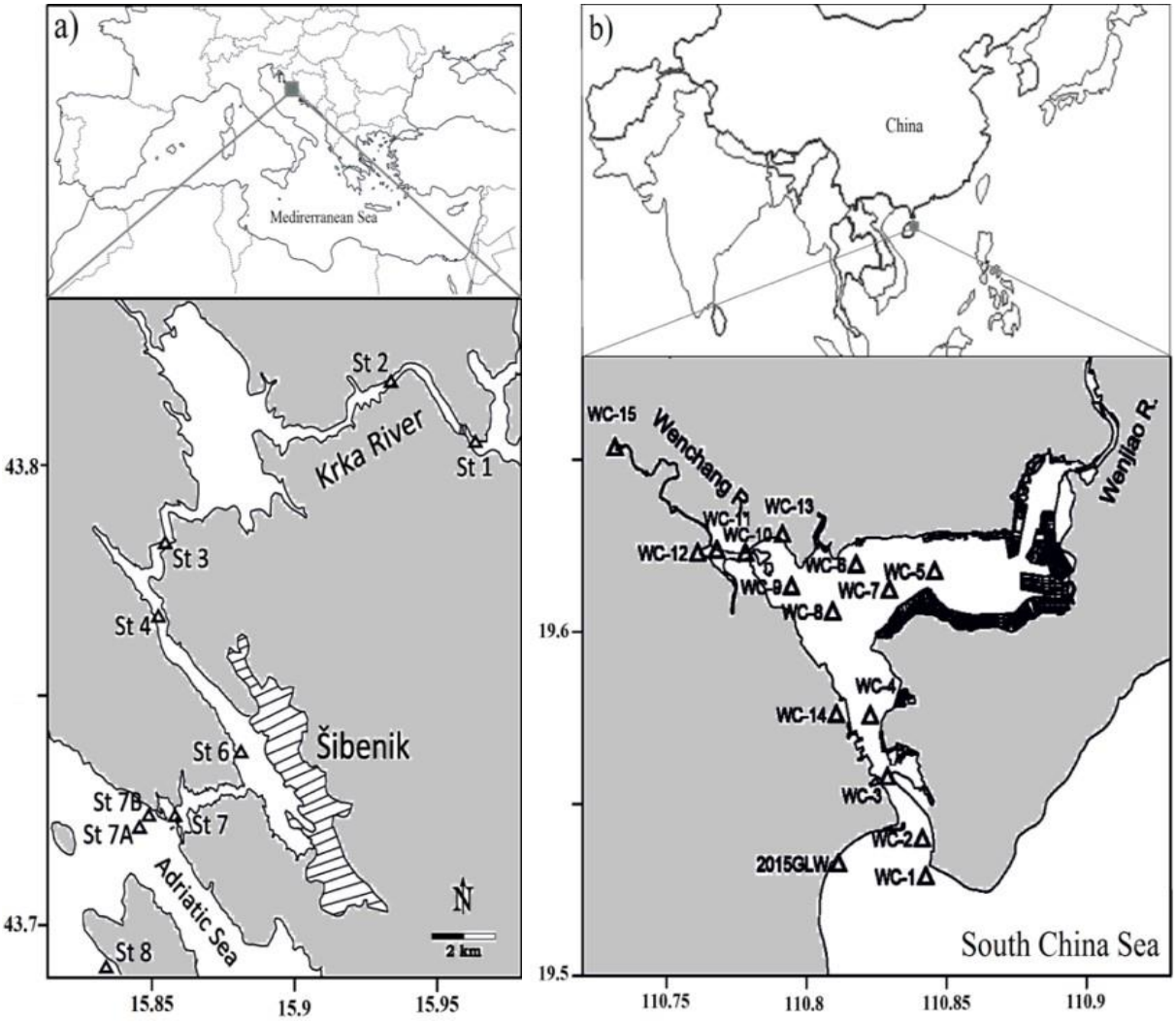


Slika 3. Prikaz sjevernog Jadrana s označenim morskim strujama i stalnim postajama. Prema Novak i sur., 2018.

Estuarij rijeke Krke (KRE) formiran je 49 km dugačkom rijekom Krkom u Jadransko more (Slika 4a). Dugačak je otprilike 25 km, uglavnom uzak s dva proširenja: Prokljanskim jezerom i Šibenskim zaljevom. Pod slabim je utjecajem plime i oseke, ali uz značajan donos slatke vode rijekom Krkom te je zbog toga visoko stratificiran. Sloj slatke i morske vode dijeli oštra haloklina (Žutić i Legović, 1987). Zbog veće koncentracije slatkovodnog fitoplanktona pristiglog iz jezera Visovac koncentracije klorofila *a* su znatno veće iznad nego ispod halokline (Viličić, i sur., 1989). Najveće koncentracije nanofitoplanktona su izmjerene u haloklini, gdje velike koncentracije hranjivih soli omogućavaju akumulaciju fitoplanktona (Cetinić i sur., 2006). Haloklina predstavlja barijeru za slatkovodni fitoplankton te tu često dolazi do njihovog raspada. Na području halokline izmjerena je povećana koncentracija feofitina, raspadnog produkta klorofila *a* (Žutić i Legović, 1987) te partikularne organske tvari (Cauwet, 1991). Donos materijala je vrlo slab te je estuarij vrlo bistar s eufotičkom

zonom 6 – 20 m. U usporedbi s drugim stratificiranim estuarijima koncentracija hranjivih soli je mala, no ipak puno veća nego u obalnim djelovima istočnog Jadranskog mora te se može smatrati eutrofnim područjem (Viličić, i sur., 1989). Koncentracije ortosilikata i nitrata smanjuju se nizvodno, dok je veća koncentracija ortofosfata i ukupnog fosfora u Šibenskom zaljevu zbog većeg antropogenog utjecaja (Legovic i sur., 1994). Od izmjerenih suspendiranih čestica 80 % zauzima fitoplankton s najvećim udjelom pikoplanktona manjeg od 1 μm koji uključuje cijanobakterije (0.6–0.8 μm) te male zelene alge (Moreira-Turcq i sur., 1993).

Estuarij rijeke Wenchang čini završetak rijeke Wenchang zajedno s lagunom. Veličine je približno 40 km^2 smještenim na koordinatama 19°36′ N, 110°49′ E na otoku Hainanu (Slika 4b). Hainan je najveći otok u Južnom kineskom moru. Rijeka Wenchang locirana je na istočnoj obali otoka te prolazi kroz Wenchang, glavni grad otoka prije nego što ulazi u lagunu sa sjeverno zapadne strane. Rijeka je glavni donositelj slatke vode te sedimenta i netretirane urbane otpadne vode u lagunu. Prosječna dubina estuarija je 3 m te završava uskim kanalom prosječne dubine 10 m. Estuarij je pod utjecajem poludnevnih izmjena plime i oseke koji dovode do dvosmjernog strujanja vode: uzvodno za vrijeme plime i nizvodno za vrijeme oseke. Do 1960 godine većina estuarija bila je prekrivena šumom mangrova koji su imali važnu ekološku funkciju u sedimentaciji i filtraciji donesene vode. Od tada dolazi do intenziviranja akvakulture koja sada zauzima otprilike 35% estuarija. Sada je to izrazito eutrofno područje zagađeno pretjeranom akvakulturom iz koje ulazi netretirani otpad koji uključuje ostatke hranjenja i feces uzgajanih životinja. U vanjskoj zoni estuarija, morskom djelu zabilježeni su obalni koraljni grebeni i polja morskih cvjetnica. (Herbeck i sur., 2011; Krumme i sur., 2012)

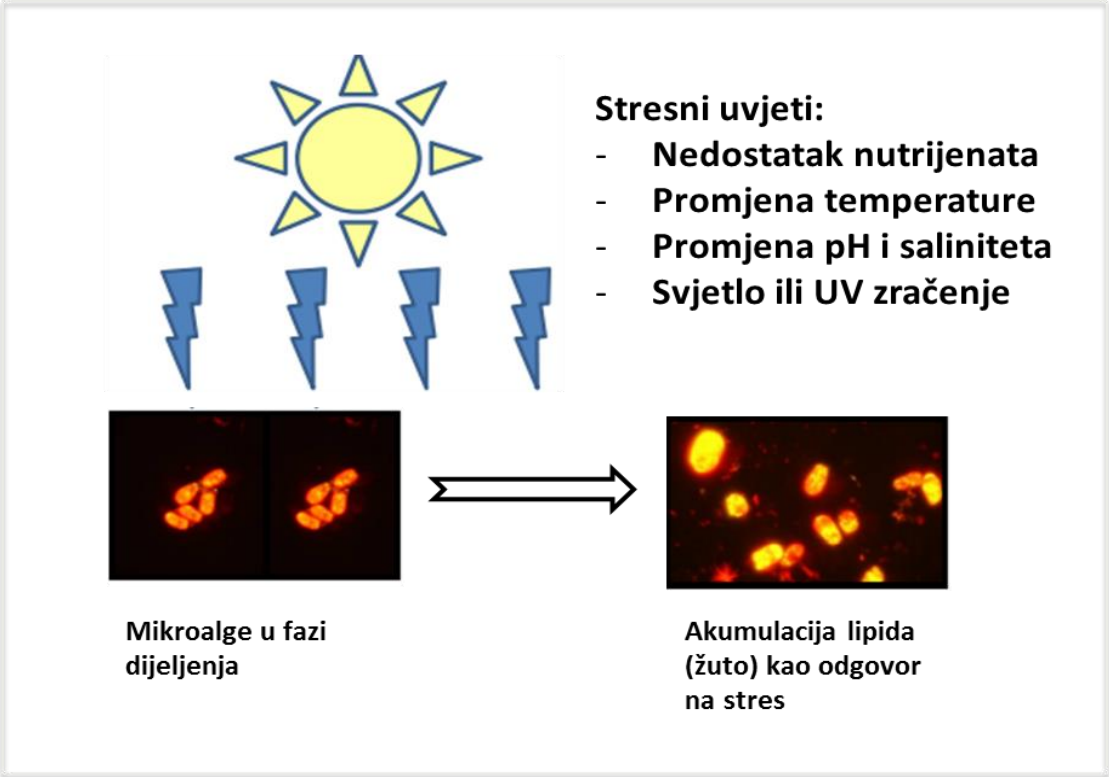


Slika 4. Estuarij rijeke Krke (a) i rijeke Wenchang (b) s istraživanim postajama. Prema Novak i sur., članak u procesu objave

1.5 Okolišni utjecaji na biogeokemiju lipida

Preživljavanje fitoplanktona uvelike ovisi o okolišu u kojem se nalazi. Njihov rast i razmnožavanje pod utjecajem je raznih okolišnih faktora kao što su temperatura, svjetlost, salinitet, količina i sastav hranjivih soli te koncentracija CO_2 (Guschina i Harwood, 2006; Palenik, 2015; Thompson, 1996; Yu i sur., 2014). Takve promjene posljedično utječu na molekularni sastav stanica fitoplanktona, te također i na kvalitetu i sastav lipida. Općenito je prihvaćeno da promjene najčešće dolaze na lipidnom dvosloju membrane kako bi se unutarstanične funkcije mogle neometano odvijati (Arts i sur., 2009). Posljednjih godina razvoj kromatografskih tehnologija u kombinaciji s masenom spektrometrijom dovelo je do otkrića novih 'neobičnih' klasa lipida i masnih kiselina i novih istraživanja o njihovim ulogama u funkcioniranju stanice fitoplanktona (Guschina i Harwood, 2006; Khotimchenko i

Vas'kovsky, 2004; Murakami i sur., 2018; Rontani i sur., 2004). U optimalnim uvjetima rasta fitoplankton proizvodi veliku količinu biomase, s malim udjelom lipida (Sharma i sur., 2012) (Slika 7).



Slika 5. Proizvodnja lipida u stresnim uvjetima. Preuzeto i prerađeno od Sharma i sur. 2012.

Temperatura je jedan od glavnih čimbenika koji utječe na rast fitoplanktona i proizvodnju lipida. Rast u nepovoljnim uvjetima utječe na kvalitetu fitoplanktonskih lipida te dolazi do nakupljanja neutralnih lipida (Sharma et al., 2012; Guschina i Harwood, 2006; Thompson, 1996). Uz nakupljanje najčešće zamijećena promjena u strukturi samih lipida je veća zastupljenost nezasićenih masnih kiselina prilikom smanjenja temperature (Sushchik i sur., 2003; Thompson, 1996). S obzirom na svoju stereokemiju, nezasićene masne kiseline s dvostrukim vezama ne mogu biti gusto pakirane kao zasićene čime se povećava fluidnost membrane. U kulturama uzgajanim u laboratoriju temperatura ima značajnu ulogu na rast stanica, a indirektno i na topljivost CO_2 i O_2 u uzgojnom mediju (Yu i sur., 2014). Ovisno o vrsti dijatomeje zamijećene su različite koncentracije lipida pri različitim temperaturama Primjeri različitih istraživanja na dijatomejama prikazani su u Tablici 1.

Tablica 1. Promjena u koncentraciji lipida kod različitih dijatomeja uzgajanih pri različitim temperaturama.

T uzgoja (°C)	Dijatomeja	Koncentracija lipida	Literaturni navod
[25] [27] [30] [33] [35]	***Chaetoceros sp.***	Najveći udio lipida pri 25°C	(Renaud i sur., 2002)
[20] [25] [30]	***Chaetoceros cf. wighamii***	Veća pri većim temperaturama (ovisi i o CO_2)	(Araújo i Garcia, 2005)
[10] [15] [20] [25]	***Chaetoceros calcitrans***	Varira, najveća pri 20 °C	(Thompson i sur., 1992)
	Chaetoceros gracilis ***Chaetoceros simplex***	Najveća pri 10°C te opada s porastom temperature	
[10] [15] [20] [25] [30] [35]	***Nitzchia closterum***	Najveća pri 20 °C	(Renaud i sur., 1995)
	Nitzchia palacea	Najveća pri 10°C	
[10] [15] [20] [25] [30]	***Phaeodactilum tricornatum***	U oblik, najveća na 10 i 30 °C	(Thompson i sur., 1992)
	Thalassiosira pseudonana	Varira, najveća na 25°C	

Dostupnost hranjivih soli određuje rast fitoplanktona no njihove koncentracije uvelike variraju u različitim vodenim masama. Izvori hranjivih soli u moru su raznoliki te uključuju: donos s kopna rijekama, potocima i nanosima, vertikalnim miješanjem vodenog stupca, recikliranjem u površinskim slojevima hranjenjem većih organizama (″sloppy feeding″), remineralizacijom bakterijama, otpadnim materijalima životinja te taloženjem iz atmosfere. Hranjive soli su anorganski spojevi makro- i mikroelemenata. Makroelementi su potrebni fitoplanktonu u velikim količinama, to su ugljik, vodik, kisik, dušik, fosfor te silicij bitan za stvaranje ljušturica dijatomeja. Mikroelementi uključuju željezo, mangan, bakar, cink, molibden, sumpor, kalij, kalcij, magnezij i klor. Nedostatak bilo kojeg od tih elemenata predstavlja stres za fitoplankton, koji se očituje različitim prilagodbama na molekularnoj razini. S obzirom da je ograničenje hranjivim solima bitan stresni faktor, puno znanstvenih radova objavljeno je na temu utjecaja hranjivih soli na sastav lipida različitih vrsta (Tablica 2). Dostupnost hranjivih soli je ograničavajući faktor u većini morskih sustava. Primjer je Artički ocean gdje tijekom zimskih mjeseci gotovo ne dolazi do fotosinteze iako zbog polarnog svjetla ima obilje svjetla. U tom je slučaju limitirajući faktor primarne produkcije nedostatak hranjivih soli u eufotičkoj zoni, kao rezultat intenzivne stratifikacije (Dunbar, 1975). U pravilu je dotok hranjivih soli u potpunosti ovisan o nestabilnosti vodenog stupca,

osim u dijelovima s jakim donosom hranjivih soli s kopna, npr. utjecaj rijeke Po na sjeverni Jadran (Duursma i Dawson, 1981)

Hranjive soli su bitan faktor za rast i razmnožavanje fitoplanktona te njihov nedostatak dovodi do stresnih uvjeta prilikom čega se smanjuje rast i stvaranje novih stanica. Kada se smanji proizvodnja biomase stanica više nema potrebe za stvaranjem novih membrana te se okreće nakupljanju proizvedenih masnih kiselina u trigliceride. Pri normalnim uvjetima ATP (adenozin trifosfat) i NADPH proizvedeni fotosintezom se troše prilikom stvaranja biomase oslobađajući ADP (adenozin difodfat) i $NADP^+$ koji se ponovno koriste kao receptori za fotosintezu. Kada je smanjena koncentracija hranjivih soli i proizvodnja biomase može doći do nedostatka $NADP^+$ što predstavlja problem stanici s obzirom da je fotosinteza ovisna o svjetlu i ne može se isključiti. Prilikom sinteze masnih kiselina, koje se akumuliraju u već spomenute trigliceride, koristi se NADPH te se time obnavlja koncentracija $NADP^+$ (Hu i sur., 2008; Sharma i sur., 2012).

Tablica 2. Proizvodnja lipida kod dijatomeja pri različitim limitacijama hranjivim solima

Limitirajuća hranjiva sol	Dijatomeja	Promjena lipida	Literaturni navod
Dušik (N)	***Chaetoceros muellerei***	Pet puta veća koncentracija lipida	(McGinnis i sur., 1997)
	Phaeodactilum tricornutum	Povećana koncentracija lipida (uglavnom TG)	(Burrows i sur., 2012; Yodsuwan i sur., 2017)
		Smanjena koncentracija nezasićenih masnih kiselina	(Breuer i sur., 2012)
		Akumulacija skladišnih lipida	(Larson i Rees, 1996)
	Thalasiosira pseudonana	Porast u ukupnoj koncentraciji masnih kiselina	(Jiang i sur., 2014)
Silicij (Si)	***Chaetoceros gracilis***	50% porast ukupnih lipida	(Adams i Bugbee, 2014)
Fosfor (P)	***Chaetoceros affinis***	Zamjena fosfolipida (PG) sulfolipidima (SQDG)	(Van Mooy i sur., 2009)
	Chaetoceros sp.	Povećana koncentracija lipida s većim udjelom C16:0 i C:18:1	Reitan i sur., 1994; Valenzuela i sur., 2013)
	Phaeodactilum tricornutum	Dvostruko povećanje koncentracije masnih kiselina	(Wurch i sur., 2011)
	Thalasiosira pseudonana	Zamjena fosfolipida drugim lipidima	(Hunter i sur., 2018; Martin i sur., 2011; Van Mooy i sur., 2009)

Dostupnost nitrata utječe na metabolizam fitoplanktona. Općenito se pokazao trend nakupljan ja lipida posebice triglicerida prilikom limitacije nitratima u različitim vrstama fitoplanktona (Sharma i sur., 2012; Thompson, 1996; Yeh i Chang, 2011). Limitaciju nitrata dijatomeje prevladavaju različitim prilagodbama; fiksacijom dušika pomoću simbioze s diazotrofnim cijanobakterijama (Poulton i sur., 2009), akumulacijom nitrata tijekom povoljnih uvjeta (Lomas i Glibert, 2000) te korištenjem organskog dušika (Morando i Capone, 2018).

Fosfat je ključna hranjiva sol iako većinom nije limitirajući u oceanima, no sve je više zamijećena njegova limitacija u obalnom morskom pojasu (Hoppe, 2003). U uvjetima nedostatka fosfata stanice imaju razvijene različite mehanizme preživljavanja. Prilikom starvacije fosfatom dolazi do zamjene fosfolipida glikolipidima: SQDG (Martin i sur., 2011; Mooy i sur., 2006; Van Mooy i sur., 2009), betaine lipidima (Van Mooy i sur., 2009; Martin i sur., 2011) te DGDG (Hartel i sur., 2000). Važnu ulogu za uspješno korištenje fosfata iz otopljene organske tvari ima ekstracelularni enzim alkalna fosfataza (AP) (Hoppe, 2003), čija aktivnost je također abilježena u zajednici sjeverno jadranskog fitoplanktona (Ivančić i sur., 2016, 2012).

Promjene **saliniteta** okoliša utječu na fitoplankton na tri načina: i) osmotski stres s direktnim utjecajem na stanični vodeni potencijal, ii) ionski stres pod utjecajem unosa ili gubitka iona, te iii) promjene u staničnoj ionskoj ravnoteži zbog selektivne propusnosti membrane (Kirst, 1990).

Promjene saliniteta u estuarijima su ogromne, od saliniteta 0 u riječnoj vodi do preko 33 u morskoj vodi. Prilikom takve nagle i velike promjene stanica nastoji očuvati nepromijenjeni turgor. Takav stres ponajviše djeluje na propusnost i fluidnost membrane stvarajući osmotski stres, toksičnost pretjeranim unosom Na i Cl iona te ionski disbalans unutar stanice (Elkahoui i sur., 2004; Kirst, 1989). U regulaciji membranskih funkcija najznačajniju ulogu imaju lipidi promjenom sastava sterola i polarnih lipida uključujući fosfolipide i glikolipide (Chen i sur., 2008; Parida i Das, 2005). Kod dijatomeje *Nitzschia laevis* povećanje saliniteta izazvalo je smanjenje neutralnih lipida, uz povećanu proizvodnju fosfolipida, glikolipida i ukupnih sterola. Nezasićenje masnih kiselina svih lipida poraslo je pri povećanju koncentracije NaCl s 10 na 20 gdm^{-3}, ali se smanjilo daljnjim povećanjem koncentracije NaCl na 30 gdm^{-3} (Chen i sur., 2008). Povećanje saliniteta od 0,4 mol dm-3 do 4 mol dm-3 NaCl kod kultura *Dunaiella salina* dovelo je do porasta ukupnih zasićenih masnih kiselina, a smanjenja nezasićenih masnih kiselina (Xu i Beardall, 1997). Sve te promjene ukazuju na smanjenje permeabilnosti i fluidnosti membrane pri povećanju saliniteta. Kod

dijatomeje *Chaetoceros cf. wighamii* zabilježeno je smanjenje ukupne proizvodnje lipida pri povećanju saliniteta s 25 na 35 (Araújo i Garcia, 2005).

2 CILJ I HIPOTEZE RADA

Uz pretpostavku da se fitoplankton prilagođava stresnim uvjetima kako bi opstao, cilj istraživanja je odrediti promjene na molekularnoj razini, a posebice u količini, sastavu i strukturi lipida određenih vrsta fitoplanktona i fitoplanktonskih zajednica.

Postavljene hipoteze odgovorene su u priloženim publikacijama:

1. Prilikom promjena u okolišu te u laboratorijskim uvjetima dolazi do promjene u sintezi staničnih lipida. (Publikacije I, II i III)
2. Promjene u okolišu posljedično uzrokuju promjene kod ispitivanih mikroorganizama i na molekularnom nivou. (Publikacija III)
3. Neki okolišni čimbenici imaju veći utjecaj na rast i razmnožavanje fitoplanktona. (Publikacije I i II)
4. Dijatomeje mogu biti dobri modelni organizmi za ispitivanje utjecaja okolišnog stresa na fitoplankton. (Publikacije I i II)

3 PUBLIKACIJE NA KOJIMA SE TEMELJI DOKTORSKI RAD

3.1 Publikacija I

Naslov: Enhanced dissolved lipid production as a response to the sea surface warming

Autori: Novak Tihana, Godrijan Jelena, Pfannkuchen Marić Daniela, Djakovac Tamara, Mlakar Marina, Baričević Ana, Tanković Smodlaka Mirta, Gašparović Blaženka

Časopis: Journal of Marine Systems

Godina objave: 2018.

DOI: https://doi.org/10.1016/j.jmarsys.2018.01.006

Journal of Marine Systems 180 (2018) 289–298

Contents lists available at ScienceDirect

Journal of Marine Systems

journal homepage: www.elsevier.com/locate/jmarsys

Enhanced dissolved lipid production as a response to the sea surface warming

Tihana Novak[a], Jelena Godrijan[a], Daniela Marić Pfannkuchen[b], Tamara Djakovac[b], Marina Mlakar[a], Ana Baricevic[b], Mirta Smodlaka Tanković[b], Blaženka Gašparović[a,*]

[a] Division for Marine and Environmental Research, Ruđer Bošković Institute, POB 180, HR–10002 Zagreb, Croatia
[b] Center for Marine Research, Ruđer Bošković Institute, G. Paliaga 5, HR-52210 Rovinj, Croatia

ARTICLE INFO

Keywords:
Dissolved lipids
DOC
Diatom *Chaetoceros pseudocurvisetus*
Northern Adriatic Sea
Global warming

ABSTRACT

The temperature increase in oceans reflects on marine ecosystem functioning and surely has consequences on the marine carbon cycle and carbon sequestration. In this study, we examined dissolved lipid, lipid classes and dissolved organic carbon (DOC) production in the northern Adriatic Sea, isolated diatom *Chaetoceros pseudocurvisetus* batch cultures grown in a wide temperature range (10–30 °C) and in contrasting nutrient regimes, phosphorus (P)-depleted and P-replete conditions. Additionally, lipids and DOC were analyzed in the northern Adriatic (NA) in two stations characterized with different P availability, occupied from February to August 2010 that covered a temperature range from 9.3 to 31.1 °C. To gain insight into factors governing lipid and lipid classes' production in the NA, apart from temperature (T), Chlorophyll *a*, phytoplankton community abundance and structure, nutrient concentrations were measured together with hydrographic parameters. We found enhanced accumulation of dissolved lipids, particulary glycolipids, with increasing T, especially during the highest in situ temperature. The effect of T on enhanced dissolved lipid release is much more pronounced under P-deplete conditions indicating that oligotrophic regions might be more vulnerable to T rise. Temperature between 25 and 30 °C is a threshold T range for *C. pseudocurvisetus*, at which a significant part of lipid production is directed toward the dissolved phase. Unlike monocultures, there are multiple factors influencing produced lipid composition, distribution and cycling in the NA that may counteract the T influence. The possible role of enhanced dissolved lipid concentration for carbon sequestration at elevated T is discussed. On the one hand, lipids are buoyant and do not sink, which enhances their retention at the surface layer. In addition, they are surface active, and therefore prone to adsorb on sinking particles, contributing to the C sequestration.

1. Introduction

Marine organic matter (OM) plays a key role in CO_2 sequestration capacity of the oceans. Operationally defined, marine OM is in dissolved and particulate form. Marine dissolved organic matter (DOM) represents one of the largest active pools of organic carbon in the global carbon cycle, constituting > 90% of total marine organic carbon inventories (Hedges, 1992; Kaiser and Benner, 2009). The phytoplankton community and heterotrophic organisms are the main source of OM in the sea (Libes, 2009). The photosynthetic production of DOM by phytoplankton can represent a substantial fraction of total primary production (Nagata, 2000; Pugnetti et al., 2006). There is a broad range of organic compounds freshly released by phytoplankton including carbohydrates, proteins, amino acids, lipids, nucleic acids, and to a lesser extent, other organic molecules involved in numerous metabolic processes (Thornton, 2014). Lipids are an important component of productivity in coastal areas. Lipids are carbon rich, of very high energetic value, thus representing important metabolic fuels. Different lipid molecular structures influence their reactivity. However, the molecular structure is not the only factor relevant for OM reactivity. The fate of OM also depends on environmental conditions (Wakeham and Canuel, 2006). Marine lipid characterization on a molecular level enables their use as good geochemical markers for the identification of OM processes in the sea, sources, and plankton adaptation to different stressors (Bourguet et al., 2009; Christodoulou et al., 2009; Gašparović et al., 2013; Van Mooy et al., 2006).

Carbon uptake and sequestration by the ocean (i.e. the biological pump) is mainly enabled by the export of sinking biogenic particles. OM partition between dissolved and particulate phases is an important factor in determining fate of organic carbon in the ocean (Thornton,

* Corresponding author.
E-mail address: gaspar@irb.hr (B. Gašparović).

https://doi.org/10.1016/j.jmarsys.2018.01.006
Received 31 May 2017; Received in revised form 15 January 2018; Accepted 17 January 2018
Available online 01 February 2018

2014), as it has implications to the organic matter export from the photic zone. Nowadays it is well known that DOC can contribute to the biological pump (Hansell and Carlson, 2001). As DOC does not sink, its export to the deep ocean/sea occurs through the water column overturn, and its incorporation on sinking marine particulate organic matter, POM (Hwang et al., 2006) or onto mineral particles (Wang and Lee, 1993). Marine DOM exhibits a spectrum of reactivity, from very fast turnover of the most bioavailable forms in the surface ocean to long-lived materials circulating within the ocean abyss (Hansell, 2013).

The most abundant components of the deep ocean DOM are carboxyl-rich alicyclic molecules that have structural similarities to lipid classes sterols and hopanoids (Hertkorn et al., 2006). Hwang and Druffel (2003) found that lipid-like material is a significant source of the uncharacterized organic carbon in the ocean. Although lipids in DOM may have an important role, there are few studies on dissolved lipids in the ocean in last 50 years (e.g. Parrish et al., 1988; Gerin and Goutx, 1994; Mannino and Harvey, 1999; Goutx et al., 2009; Marić et al., 2013). Dissolution from the particulate fraction is the main source of dissolved lipids in the marine environment is (Yoshimura et al., 2009). Parrish et al. (1988) measured profiles of dissolved marine lipid classes over the Scotian Slope and the Bedford basin. They found high concentrations of dissolved lipids (29–190 μg/L), with the highest dissolved lipid levels measured in the vicinity of pycnocline and composed primarily of acetone-mobile polar lipids (pigments, glycolipids). Gerin and Goutx (1994) investigated dissolved lipids in the Almeria-Oran frontal system. They found highly variable concentration (9–113 μg/L) and depth distribution. Dissolved lipid peaks were closely related to Chl *a*. Most dissolved lipid peaks were found to include alcohols and/or acetone mobile polar lipids as principal constituents. Mannino and Harvey (1999) suggested that, although lipids comprised a small portion of DOM, the composition of dissolved lipids has the potential to provide information on the source and diagenetic processing. Goutx et al. (2009) examined changes in concentration and composition of Iatroscan-measured dissolved lipids in the Ligurian Sea, NW Mediterranean. Dissolved lipid concentrations in 0–1000 m water column, varied from 5.3 to 48.5 μg/L, with highest concentration found in 0–50 m surface layer that coincides with phytoplankton biomass. Significant correlations between glycolipids and various phytoplankton pigments suggested that picoeucaryote phytoplankton were a major source of dissolved lipids. Marić et al. (2013) analyzed dissolved lipids in the northern Adriatic, and found that their concentration ranged from 10.3 to 70.6 mg/L, comprising 0.8–4.5% of the DOC. The investigated period was characterized by the dominance of glycolipids, phospholipids and free fatty acids in the dissolved fraction.

As marine DOM is a major reservoir of carbon, characterizing factors affecting the production is essential to understand the dynamics of the global carbon cycle. Surface temperatures are predicted to warm by 2–3 °C over the next 100 years (IPCC, 2001). Sea surface temperature data collected in the northern Adriatic Sea, evidenced a general warming through all seasons in the period 1988–1999, with respect to the period 1911–1987 (Russo et al., 2002). Temperature effect on DOM release has generally been overlooked (Thornton, 2014). In this study we performed microcosm incubations, covering the present temperature range of northern Adriatic (NA) (10–30 °C) with different nutrient amendments. This was done to test how temperature rise influences DOM, particularly dissolved lipid and lipid classes production, and how it is superposed on the effect of nutrients' availability. We selected to work with extracellular OM produced during diatom *Chaetoceros pseudocurvisetus* cultures growth, according to criteria that genera *Chaetoceros* are an important phytoplankton component in the NA and are frequently bloom-forming taxa (Bosak et al., 2016). In addition, we set out to investigate how annual temperature variations affect dissolved lipid production in the complex system, as the northern Adriatic area.

2. Materials and methods

2.1. Site description, sampling and basic environmental determinations

The northern Adriatic Sea is biologically the most productive region in the Mediterranean Sea (Harding et al., 1999). The NA is a highly variable, dynamic environment, with close coupling between river-borne nutrients, net productivity and vertical carbon fluxes. The most important source of the nutrients in the region is the Po River and the winter overturn of regenerated nutrients from the bottom layer, which does not exceed 50 m in the entire basin (Degobbis et al., 2000). It is a complex basin, the western part is greatly influenced by the Po River freshwater input, while its eastern part receives highly saline oligotrophic waters from the southern Adriatic. Chemical and biological processes are influenced by the hydrodynamic regime of the system, which changes strongly due to short-term meteorological phenomena that influence the circulation and vertical structure of the water column (Supić and Vilibić, 2006).

We sampled the NA monthly from the research vessel "Vila Velebita", at two stations that are considered hydrodynamically and trophically different: oligotrophic eastern station 107 (mostly depleted in PO_4) and mesotrophic/eutrophic western station 101 (Fig. 1). Seven cruises were made from February to August 2010 covering a temperature range from 10 to 30 °C. Samples were collected at the surface with 5 L Niskin bottles.

Temperature and salinity were measured using a CTD probe (Seabird SBE25, Sea–Bird Electronics Inc., Bellevue, Washington, USA).

Dissolved inorganic nitrogen (DIN) (calculated as sum of nitrates (NO_3), nitrites (NO_2), ammonium (NH_4)) and orthophosphates (PO_4) were determined aboard by spectrophotometric methods (Parsons et al., 1984), immediately after sample collection. The absorbance readings for all nutrients were made on Shimadzu UV-Mini 1240 spectrophotometer with 10 cm quartz cuvettes. Method accuracies for NO_3, NO_2, NH_4, and PO_4 were ± 3%, ± 3%, ± 5%, and ± 3%, respectively, and detection limits 0.05 μmol/L, 0.01 μmol/L, 0.1 μmol/L, and 0.02 μmol/L, respectively.

Subsamples for the determination of Chlorophyll *a* (Chl *a*) were filtered on Whatman GF/C filters. Following a 3 h extraction in 90% acetone (in the dark, with grinding after addition of acetone), Chl *a* concentrations were determined by a Turner TD–700 fluorometer (Parsons et al., 1984).

2.2. Phytoplankton analysis

Phytoplankton samples were collected using Niskin bottles, 200 mL were preserved in 2% (final concentration) formaldehyde neutralized with disodium tetraborate decahydrate and analyzed within one month from sampling. 50 mL sub-samples were settled for 40 h and analyzed by Zeiss Axiovert 200 microscope following Utermöhl method (1958). Total phytoplankton abundances include all species counted in the microphytoplankton (20–200 μm) and nanophytoplankton (2–20 μm) groups (Sieburth et al., 1978).

2.3. Phytoplankton cultures

We set out to investigate temperature dependent variability in the quantity and composition of organic matter released during growth in P-replete (F2 medium, 36 μmol/L PO_4) and P-depleted (F2 medium with PO_4 reduced to 1 μmol/L) conditions. Marine diatom *Chaetoceros pseudocurvisetus* monoclonal culture was selected for the microcosms experiments at 10, 15, 20, 25 and 30 °C. *C. pseudocurvisetus* colony was manually isolated using a micropipette from a net sample collected at the station SJ101 on 30th October 2014. The culture's genetic material is deposited in GenBank under Accession numbers MG385841 (18S DNA) and MG385842 (28S DNA). Batch culture of *C. pseudocurvisetus* was maintained in F2 medium (Guillard, 1975) in sterile VWR® Tissue

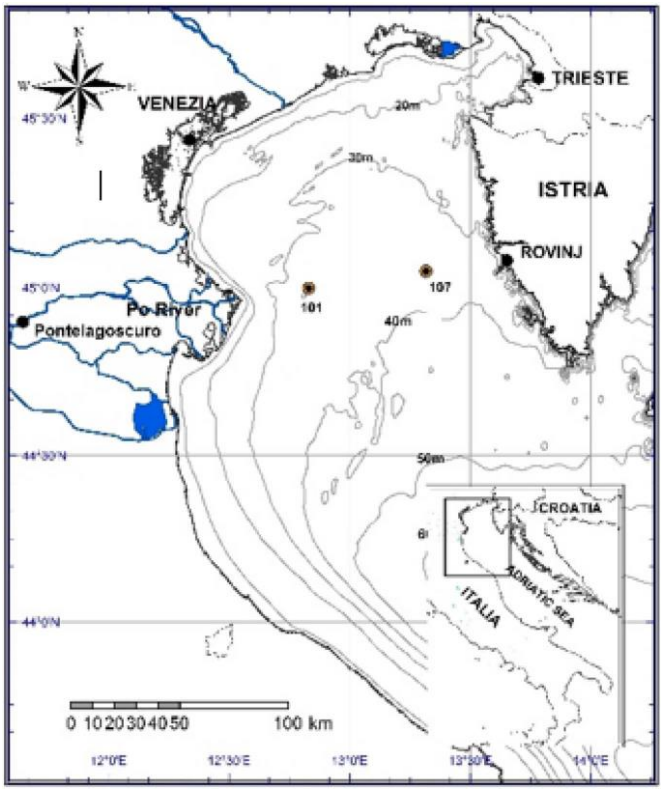


Fig. 1. Study area in the northern Adriatic Sea showing position of stations 107 and 101.

Culture Flasks (VWR, Radnor, Pennsylvania). The media was prepared in NA seawater rested for 2 months in the dark, filtered on sterile 0.22 µm white plain filters (Merck Milipore Ltd.) and boiled in microwave (Keller et al., 1988). Media amendments were added aseptically after sterilization. All experiments were done in duplicate. Cells were pre-conditioned in 50 mL, at experimental temperatures of growth prior to inoculation in 800 mL of medium.

We inoculated 10^5 cells of *C. pseudocurvisetus* in 800 mL of each batch culture medium at the beginning of the experiment. All cultures were grown on a 12/12 light/dark cycle under illumination of 4500 lx. Growth was terminated at onset (third day) of stationary growth phase. The growth phases were determined every second day by cell counting with Fuchs-Rosenthal Chamber hemocytometer under Olympus BX51-P polarizing microscope. Batch cultures were not axenic, however, we took all precautions to avoid further contamination with bacteria. Bacteria were not detected by microscopy, and cultures were not blurry, which would indicate bacterial contamination. During cell counting, we observed a negligible number of dead cells, lacking pigments and cell content. Therefore, analyzed OM is discussed as mainly produced by *C. pseudocurvisetus*. Samples for DOC and lipid analyses were performed from the culturing medium before inoculation and at the onset of stationary phase growth (third day). DOC and dissolved lipid data were obtained by deducing their values measured at the end of cultivation from those measured in the culturing medium.

2.4. Lipid analysis

In order to determine NA seawater dissolved lipid classes, 3 L of seawater was collected in glass containers and passed through a 200 µm stainless steel screen to remove zooplankton and larger particles. Immediately after sampling, seawater was filtered through 0.7 µm Whatman GF/F filters pre-burned at 450 °C for 5 h. Duplicates of 100 mL culture were filtered on precombusted 0.7 µm Whatman GF/F filters to determine the lipid composition of diatom *C. pseudocurvisetus*. Filtrates containing dissolved lipids were stored in dark bottles until liquid-liquid extraction with dichloromethane (twice at pH 8 and twice at pH 2), which was performed within 24 h for NA samples, and immediately for *C. pseudocurvisetus* samples. Ketone 2–nonadecanone was added as internal standard to each sample to estimate the recoveries in the subsequent steps of sample analysis. The extracts were evaporated to dryness under nitrogen atmosphere and redissolved in 20 to 50 µL dichloromethane, depending on sample concentration.

Lipid classes were determined by thin–layer chromatography–flame ionization detection (TLC–FID; Iatroscan MK–VI, Iatron, Japan). Eighteen lipid classes, which constitute total lipids, may be detected by this technique including hydrocarbons, wax esters and sterol esters (WE/SE herein after termed WE), fatty acid methyl esters (ME), fatty ketones (KET), fatty acid methyl esters, ketones, triacylglycerols (TG), free fatty acids, fatty alcohols, 1,3– and 1,2–diacylglycerols, sterols (ST), pigments (PIG), monoacylglycerols, three glycolipids (GL) (monogalactosyldiacylglycerols, digalactosyldiacylglycerols and

sulfoquinovosyldiacylglycerols), and three phospholipids (PL) (phosphatidylglycerols, phosphatidylethanolamines and phosphatidylcholines). Free fatty acids, fatty alcohols, 1,3– and 1,2–diacylglycerols, monoacylglycerols and fatty acid methyl esters are products of early lipid degradation representing degradation indices (DI). Lipid classes were separated on Chromarods SIII and quantified by an external calibration with standard lipid mixture, with a hydrogen flow of 160 mL/min and air flow of 2000 mL/min. Total lipid concentrations were obtained by summing all lipid classes quantified by TLC-FID. The standard deviation determined from duplicate runs accounted for 0–14% of the relative abundance of lipid classes. A detailed description of the procedure is described in Gašparović et al. (2014, 2015).

2.5. Dissolved organic carbon analysis

One liter of seawater and 50 mL of culture medium were filtered through 0.7 µm Whatman GF/F filters combusted at 450 °C/5 h for the DOC determination. Filtered samples for DOC analysis were collected in duplicates in the 22 mL glass vials combusted 450 °C/4 h. Samples were preserved with mercury chloride (10 mg/L) and stored at + 4 °C in the dark until analysis.

A model TOC–VCPH (Shimadzu) carbon analyzer with a platinum silica catalyst and a non–dispersive infrared (NDIR) detector for CO_2 measurements was used for DOC measurements and calibrated with potassium hydrogen phthalate. Concentration was calculated as an average of three replicates. The average instrument and Milli–Q blank correspond to 30 µg C/L with high reproducibility (1.5%).

2.6. Data analysis

Principal component analyses (PCA) was used to elucidate the relationships between temperature, DOM producers (phytoplankton) and DOM including DOC, dissolved lipid and lipid classes for *C. pseudocurvisetus* cultures and NA seawater samples.

3. Results

3.1. Chaetoceros pseudocurvisetus cultures

We determined how and to what extent phytoplankton production of dissolved organic matter (DOM) depends on temperature, and investigated DOC and total dissolved lipids (Lip_diss) including lipid classes. DOM production was examined for early stationary growth phases of cultures of diatom *C. pseudocurvisetus* that were grown under phosphorus deplete (P-depleted) and replete (P-replete) conditions.

Both DOC and Lip_diss concentrations increased in the temperature range from 10 to 30 °C together with their contribution to total organic carbon and total lipids, respectively (Table 1). DOC concentration increased 1.6 times between 10 and 30 °C in both P-depleted and P-replete cultures. The content of DOC to total organic carbon (TOC) (DOC(%)) increased with increasing T 3.2- and 2.6-fold for the P-depleted and P-replete culturing conditions, respectively. Lower Lip_diss concentrations in P-replete cultures, for which concentration of Lip_diss increased 1.9-fold between 10 and 30 °C, were measured. In P-depleted cultures Lip_diss concentrations increased 2.2-fold in the temperature range from 10 to 30 °C. Lipids made higher contribution to DOC at P-depleted than at P-replete conditions, with increasing tendency at higher temperatures (Supplementary materials, Fig. S1).

Concentrations and contributions of particular lipid class to total dissolved lipid concentration are presented in Table 1. Majority of lipid classes concentration, including GL, TG, ST and DI, increased with increasing temperature, apart from PL concentration that did not show temperature dependence for both P-depleted or P-replete cultures. For both growth regimes, the contribution of GL, TG, ST and particularly DI to Lip_diss increased with increasing T, while for PL it was the opposite.

Final *C. pseudocurvisetus* cell number decreased with temperature for both culturing conditions, with highest abundance recorded for 15 °C (Table 1). The concentration of released DOC and dissolved lipids per cell (data are not shown but can be calculated from the Table 1) was the lowest for 15 °C, and substantially highest at 30 °C, for both culturing conditions. In P-depleted conditions 16.6 pg DOC and 12.3 pg lipids (what would roughly be 8.6 pg lipid carbon assuming 70% carbon in lipids) were released per *C. pseudocurvisetus* cell at 15 °C, while in P-replete conditions 17.4 pg DOC and 5.9 pg lipids per cell (what would roughly be 4.1 pg lipid carbon per cell) were calculated. Much more DOC and Lip_diss were released in the cultures grown at 30 °C. In P-depleted conditions 13.9 and 14.6 times more DOC and Lip_diss, respectively, were released per cell at 30 °C (229.6 pg DOC and 125.8 pg lipid C) in comparison to 15 °C. For P-replete conditions the increase in DOM release was lower, 9.6 and 14.2 times more DOC and Lip_diss, respectively, were released per cell at 30 °C (166.1 pg DOC and 84.1 pg lipid C per cell) regarding 15 °C. Generally, more DOC and Lip_diss were excreted per cell in the less favorable growing conditions when less *C. pseudocurvisetus* cells developed (Fig. 2).

Principal component analyses of T and DOM variables of P-depleted and-P replete cultures are presented in Fig. 3. The first two principal components of P-depleted cultures (Fig. 3a) explained 85.1% of the total variability among the 14 variables. For P-replete cultures (Fig. 3b), the first two principal components explained 77.7% of the total variability among the 14 variables. For both culturing conditions T, DOC, DOC(%), three Lip_diss variables (concentration, contribution to total lipids (Lip_diss(%)), as well to DOC (Lip_diss in DOC)), the contribution of GL to the dissolved lipid pool (GL(%)), and the contribution of DI to the dissolved lipid pool (DI(%)) variables predominated in the high negative values of PC1. This indicates their positive correlation with temperature. These variables were inversely related to increase in cell abundances and the contribution of PL to dissolved lipid pool (PL(%)), indicating that increased T leads to lower cell abundances and lower PL content in dissolved OM pool. The GL contribution is mainly on the expense of PL.

3.2. Northern Adriatic

3.2.1. Environmental conditions

We followed DOM dynamics of surface waters at the P richer and P poor stations in the temperate northern Adriatic Sea. During the investigated period, temperature distribution (Fig. 4a) showed a regular sinusoidal annual cycle with minimum in February (9.26 and 9.55 °C at stations 101 and 107, respectively) and maximum in July (31.06 and 28.58 °C at stations 101 and 107, respectively). During the study period, salinity varied within 29.3–37.6 range, and 21.3 and 37.9 at stations 107 and 101, respectively (Fig. 4b). Lower salinities at station 101 coincide with increased Po River flows (data not shown). Spreading of low-salinity surface waters extended to station 107, corresponding to the surface water warming (Lyons et al., 2007) in the period from April to August.

Distribution of nutrients followed the Po River fresh water inflow. DIN concentration (Fig. 4c) was markedly higher at station 101 (1.23–73.69 µmol/L) than at station 107 (0.62–9.00 µmol/L). It peaked in June at station 101 and in July at station 107. Phosphate concentration (Fig. 4d) at station 101 ranged from 0.02 µmol/L to 0.37 µmol/L. The surface water at station 107 was entirely oligotrophic, and phosphate concentration was consistently < 0.02 µmol/L with exception in July when 0.09 µmol/L PO_4 was measured.

Concentrations of Chl *a* (Fig. 5a), used herein as an indicator of phytoplankton biomass, followed that of DIN and PO_4, and peaked in June at station 101 (8.9 µg/L) and in July at station 107 (1.7 µg/L). Phytoplankton abundance (Fig. 5b) was the highest in May (reaching 3.47×10^6 cells/L) at station 101, while the highest phytoplankton abundance at station 107 was recorded in June (0.30×10^6 cells/L). *Chaetoceros* taxa considerably contributed to phytoplankton community (Fig. 5c) with 91.6 and 79.8% in May and June, respectively, at station

Table 1
Temperature (T), average values of DOC and the contribution of DOC to TOC (in parentheses), total dissolved lipids and the contribution of Lip_diss to total lipid (dissolved and particulate) (in parentheses), and concentration and the contribution (in parentheses) of major lipid classes (phospholipids (PL), glycolipids (GL), triacylglycerols (TG), sterols (ST) and degradation indices (DI)) to total dissolved lipids, and cell abundances of *C. pseudocurvisetus* cultures grown in increasing temperatures and phosphorus depleted and replete conditions. The number of cells corresponds to 17, 11, 9, 11 and 10 days of stationary phase onset (growth termination) for temperatures 10, 15, 20, 25 and 30 °C, respectively.

T	DOC	Lip_diss	PL	GL	TG	ST	DI	Cell
°C	μg C/L (%)	μg/L (%)	μg/L (%)					cells/L
P-depleted cultures								
10	440 (12.9)	231.8 (54.5)	106.7 (46.1)	64.8 (28.0)	9.9 (4.8)	4.9 (2.1)	23.2 (11.7)	2.0E + 07 ± 8.1E + 05
15	458 (18.5)	312.2 (58.7)	136.2 (43.6)	83.7 (29.4)	9.0 (3.3)	5.7 (1.9)	43.2 (13.0)	2.8E + 07 ± 5.3E + 06
20	666 (23.2)	373.3 (76.8)	198.4 (53.2)	101.4 (27.0)	8.7 (2.6)	10.4 (1.5)	26.2 (7.0)	1.8E + 07 ± 1.6E + 06
25	626 (20.1)	306.2 (76.2)	132.8 (43.4)	86.5 (28.2)	11.6 (4.3)	7.0 (2.3)	42.6 (13.9)	1.1E + 07 ± 9.8E + 05
30	714 (40.9)	516.7 (76.6)	152.4 (29.5)	154.7 (29.9)	23.4 (5.0)	11.3 (3.8)	149.6 (29.5)	2.9E + 06 ± 3.1E + 05
P-replete cultures								
10	463 (14.2)	197.9 (57.7)	102.8 (51.9)	56.1 (28.3)	6.6 (3.3)	5.2 (2.6)	13.6 (7.3)	1.5E + 07 ± 9.0E + 05
15	649 (15.6)	258.2 (58.7)	93.2 (36.1)	74.7 (28.9)	3.0 (1.2)	7.0 (2.7)	24.6 (13.0)	3.7E + 07 ± 2.6E + 06
20	723 (15.4)	260.1 (71.2)	130.5 (50.2)	82.5 (31.7)	1.6 (0.6)	5.4 (2.1)	16.0 (6.2)	1.6E + 07 ± 2.2E + 06
25	650 (15.6)	364.5 (75.6)	160.4 (44.0)	123.1 (33.8)	12.9 (3.5)	7.7 (2.1)	40.3 (11.0)	1.7E + 07 ± 1.0E + 06
30	758 (37.0)	383.8 (73.8)	111.7 (29.1)	134.6 (35.1)	16.4 (4.3)	14.3 (3.7)	64.6 (16.8)	4.6E + 06 ± 3.4E + 05

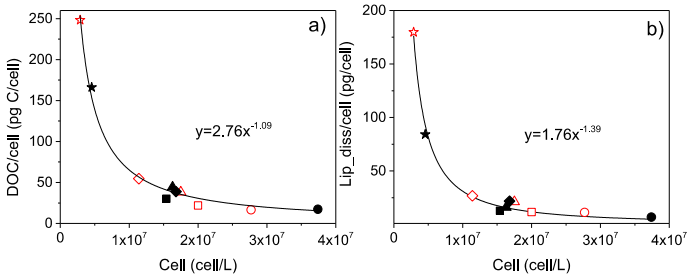


Fig. 2. Relationship between (a) average DOC excreted by *C. pseudocurvisetus* single cell and (b) average total dissolved lipid excreted by *C. pseudocurvisetus* single cell and *C. pseudocurvisetus* concentration for temperatures 10 (squares), 15 (circles), 20 (triangles), 25 (diamonds) and 30 °C (stars) for the P-depleted (open symbols) and P-replete (full symbols) growth conditions.

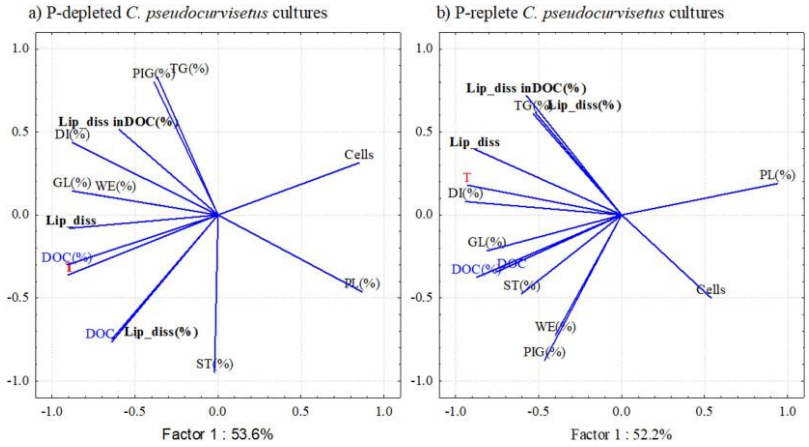


Fig. 3. Principal components analysis (PCA) of the variables: temperature, cell abundances (Cells), DOC, the content of DOC in TOC (DOC(%)), total dissolved lipids (Lip_diss), the content of Lip_diss in total lipids (Lip_diss(%)) and in DOC (Lip_diss in DOC(%)), the content of phospholipids (PL(%PL)), glycolipids (GL(%)), triacylglycerols (TG(%)), sterols (ST(%)), wax/steryl esters (WE(%)), pigments (PIG(%)) and degradation indices (%DI) in Lip_diss for the *C. pseudocurvisetus* cultures grown in P-depleted (a) and P-replete (b) conditions.

101. Meanwhile at station 107 maximum *Chaetoceros* taxa were detected in March and July when it contributed to phytoplankton community with 10.5 and 28.6%, respectively.

3.2.2. Dissolved organic matter production

Temporal distribution of DOC concentration exhibited February minimum (980 and 895 μg C/L at stations 101 and 107, respectively) and maximum in the warmest month of July (2275 and 1903 μg C/L at stations 101 and 107, respectively) (Table 2). DOC accounted for

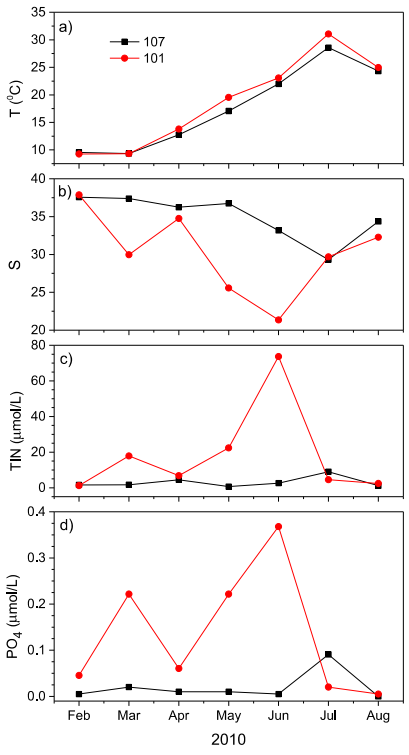


Fig. 4. Temporal distribution of (a) temperature, (b) salinity, (c) total inorganic nitrogen and (d) orthophosphate at stations 101 (circles) and 107 (squares) during the investigation period in 2010.

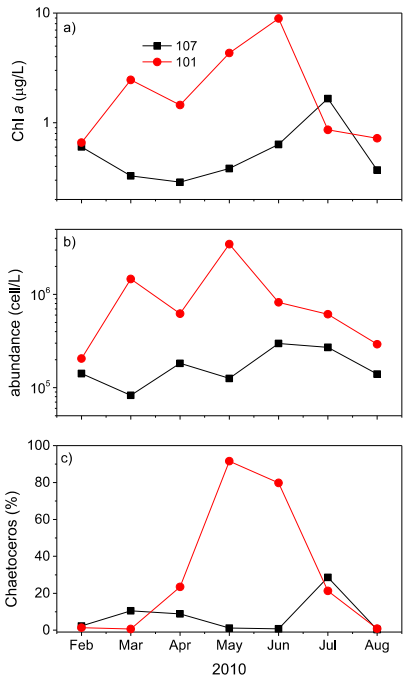


Fig. 5. Temporal distribution of (a) Chlorophyll *a*, (b) total phytoplankton abundance and (c) *Chaetoceros* taxa abundance contribution to total phytoplankton abundance at the mesotrophic station 101 (circles) and oligotrophic station 107 (squares) during the investigation period in 2010.

65–93% of the total organic carbon (TOC) at station 101 (data for particulate organic carbon of both stations are not shown) and 83–94% of the total organic carbon at the station 107 (Table 2). February was an exception, the contribution of DOC to TOC increased toward summer at station 101, while at station 107 trend was opposite, with exception in August (Table 2). Higher Lip_diss concentrations were measured for the oligotrophic, P-depleted station 107, than at mesotrophic station 101. Similarly to DOC, temporal distribution concentrations of Lip_diss increased from winter to summer and peaked in July to values of 45.6 and 50.7 µg/L at stations 101 and 107, respectively (Table 2). General trend of the contribution of Lip_diss to total lipid (dissolved and particulate; data for particulate lipids of both stations are not shown) is not clear. However, if February data were excluded, higher contributions were estimated for summer months (Table 2). Lipids made higher contribution to DOC at station 107 (average 2.0%) than station 101 (average 1.8%) (Supplementary materials, Fig. S2). Generally, more DOC and Lip_diss per Chl *a* was detected for the less favorable plankton growing conditions when lower phytoplankton biomass (determined by Chl *a*) was noted (Fig. 6).

The concentration of the majority of lipid classes, including GL, TG, ST and DI did not show clear relationship with temperature (Table 2). The same was assessed for their contribution to total dissolved lipids (Table 2). These results indicate that other parameters, not only T, define the concentration of DOM in the NA waters.

Evaluating the relationships between temperature and DOM production at NA stations 107 and 101 we performed PCA (Fig. 7). In order to be consistent with *C. pseudocurvisetus* culturing conditions, namely P depleted and P replete conditions, PCA for station 107 was performed only for months when Chl *a* was low (< 1 µg/L), assuming that phytoplankton as DOM producer, grew in P depleted conditions (bolded months in Table 2). Station 101 PCA analysis was performed only for months when Chl *a* was higher than 1 µg/L, assuming that phytoplankton grew in P favorable conditions (bolded months in Table 2).

The first two principal components of stations 107 and 101 (Fig. 7) explained 73.8% and 86.4%, respectively, of the total variability among the 14 variables. T, DOC, PL(%) and GL(%) variables, Lip_diss, and dissolved lipid content in DOC (Lip_diss in DOC(%)) predominated in the positive values of PC1 for station 107. This indicates their positive relationship. Inversely related to these variables were the increase in the content of DOC (DOC(%)), ST (ST(%)), WE (WE(%)), PIG (PIG(%)) and DI (DI(%)) in the lipid pool. All variables regarding Lip_diss (their concentration, contribution to total lipids as well to DOC) had the greatest negative effect on PC2, and were significantly negative correlated to Chl *a* and TG(%). This indicates that at P-depleted conditions more dissolved lipids may be expected for lower phytoplankton biomass as indicated by Chl *a*, and that in such conditions contribution of TG to Lip_diss is decreasing with increased dissolved lipid concentration. The parallel position of variables Chl *a* and TG(%) indicate that in P depleted conditions at station 107, there was higher TG share in total dissolved lipids with higher (but still low) phytoplankton abundance (Chl *a*).

T, DOC, three Lip_diss variables (concentration, contribution to total

Table 2
Temperature (T), DOC and the contribution of DOC to TOC (in parentheses), total dissolved lipids and the contribution of Lip_diss to total lipid (dissolved and particulate) (in parentheses), and concentration and the contribution (in parentheses) of major lipid classes (phospholipids (PL), glycolipids (GL), triacilglycerols (TG), sterols (ST) and degradation indices (DI)) to total dissolved lipids for the northern Adriatic oligotrophic station 107 and the mesotrophic station 101.

Month, 2010	T	DOC	Lip_diss	PL	GL	TG	ST	DI
	°C	μg C/L (%)	μg/L (%)	μg/L (%)				
Station 107								
Feb	9.55	895 (92.0)	16.3 (50.7)	3.4 (20.7)	3.5 (21.6)	1.1 (7.0)	0.5 (3.1)	6.5 (40.0)
Mar	9.34	1011 (94.0)	15.5 (48.3)	1.8 (11.4)	6.5 (42.2)	0.5 (3.2)	0.6 (4.1)	4.5 (29.2)
Apr	12.76	1109 (91.9)	46.9 (69.5)	10.4 (22.2)	7.2 (15.4)	0.6 (1.3)	3.1 (6.7)	19.9 (42.5)
May	17.06	1119 (89.2)	49.4 (57.5)	7.9 (16.0)	7.4 (15.0)	1.6 (3.2)	2.8 (5.6)	27.5 (55.6)
Jun	21.99	1592 (88.6)	42.5 (58.3)	13.3 (31.3)	18.0 (42.4)	2.8 (6.6)	0.8 (1.9)	6.6 (15.6)
Jul	28.58	1903 (82.7)	50.7 (58.1)	11.7 (23.1)	18.5 (36.4)	3.0 (5.9)	2.5 (5.0)	13.2 (26.0)
Aug	24.32	1630 (92.5)	47.5 (65.7)	14.1 (29.7)	23.0 (48.4)	0.7 (1.4)	0.7 (1.5)	7.5 (15.8)
Station 101								
Feb	9.26	980 (92.7)	21.3 (54.1)	5.3 (24.8)	8.3 (39.1)	0.4 (1.8)	0.7 (3.1)	5.6 (26.2)
Mar	9.32	1299 (65.1)	33.6 (33.6)	4.8 (14.4)	14.6 (43.6)	1.5 (4.5)	0.7 (2.2)	9.8 (29.2)
Apr	13.79	1323 (82.4)	27.2 (31.8)	6.0 (21.9)	9.9 (36.5)	1.5 (5.6)	0.8 (3.1)	8.7 (32.1)
May	19.55	1659 (73.2)	40.1 (43.9)	8.1 (20.3)	16.4 (40.9)	0.4 (1.1)	0.7 (1.8)	12.7 (31.7)
Jun	23.07	1853 (67.6)	91.2 (57.1)	19.7 (21.6)	38.4 (42.1)	3.6 (3.9)	1.5 (1.6)	9.0 (21.9)
Jul	31.06	2275 (88.3)	45.6 (43.0)	13.0 (28.6)	19.5 (42.8)	0.4 (0.8)	1.4 (3.0)	14.8 (32.5)
Aug	24.98	1852 (89.2)	34.5 (48.4)	11.9 (34.6)	15.0 (43.4)	0.3 (0.8)	0.8 (2.2)	4.4 (12.7)

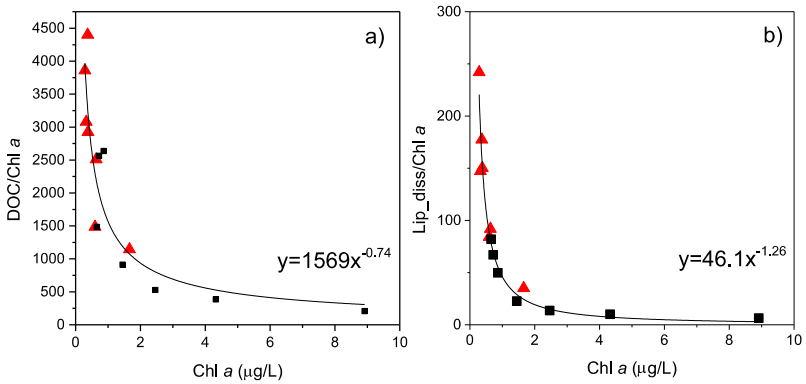


Fig. 6. Relationship between (a) DOC normalized to Chl *a* and (b) total dissolved lipids normalized to Chl *a* vs *Chl a* as an indicator of the phytoplankton biomass for the oligotrophic station 107 (triangles) and the mesotrophic station 101 (squares) in the northern Adriatic Sea.

lipids (Lip_diss(%)) as well to DOC (Lip_diss in DOC)), Chl *a*, PIG(%), and GL(%) variables predominated in the high positive values of PC1 for station 101. This indicates their positive correlation with temperature. Correlation between T and Chl *a* reflects not to their interconnection, but rather to coincidental increase of nutrient concentration at warmer months during 2010 at station 101, and the consequent increase in phytoplankton biomass (c.f. Figs. 4b and c, and 5a). Those variables were inversely related to DOC(%), ST(%) and DI(%), the same as observed for station 107. The increase in the contribution of glycolipids (GL(%)) to the lipid pool at station 101 was inversely related to phospholipid contribution (PL(%)).

4. Discussion

To understand the influence of T on DOM production in seas and oceans, a simple approach is required as a starting point, due to numerous influential parameters and the interplay of their influence on DOM production, its quality, and cycling. Therefore, we started with a simple system by analysing lipid classes and DOC production by diatom *C. pseudocurvisetus* batch cultures grown under different T and nutrient conditions. Furthermore, we investigated lipid classes and DOC from the northern Adriatic covering wide environmental conditions regarding T and nutrient availability. Our data should give insight for the development of models that predict T rise consequences on the biological pump.

Freshly produced DOM is composed of three major biochemical substances, proteins, carbohydrates, and lipids. Phytoplankton is the main lipid source in the oceans as well as in the NA, with heterotrophic bacteria contributing to much lower extent (Gašparović et al., 2013; Frka et al., 2011). Investigations of DOM production by diatoms is important, having in mind that diatoms constitute one of the ecologically most important groups of phytoplankton worldwide, among which *Chaetoceros* is the most abundant and diverse genus (Malviya et al., 2016).

4.1. Chaetoceros pseudocurvisetus cultures

DOM (DOC and dissolved lipids) production by *C. pseudocurvisetus* in T range from 10 to 30 °C is nonlinear (Fig. 2). The lowest amount of DOM per cell is released by P-depleted and P-replete cultures grown at 15 °C. This indicates that at 15 °C, energy, carbon and other essential elements are more engaged in the cell reproduction and growth, and

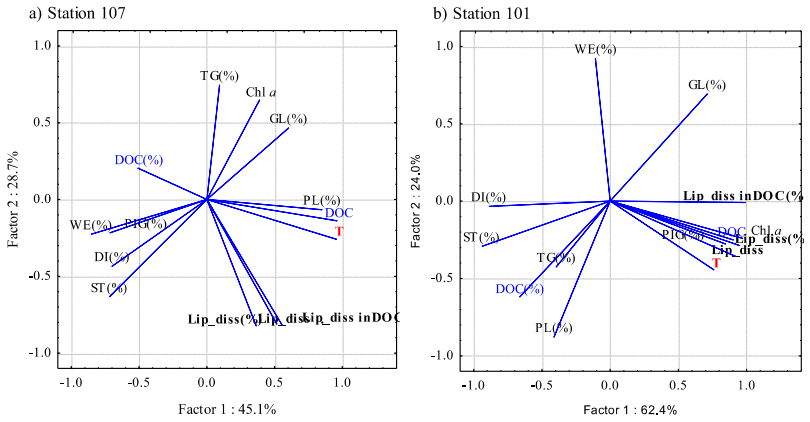


Fig. 7. Principal components analysis (PCA) of: temperature, Chl *a*, DOC, total dissolved lipids (Lip_diss), contribution of dissolved to total lipids (Lip_diss(%)) as well as contribution of dissolved lipids to DOC (Lip_diss in DOC), the contribution of phospholipids (%PL), glycolipids (%GL), triacylglycerols (%TG), sterols (%ST) and degradation indices (%DI) to Lip_diss for the northern Adriatic stations a) 107 and b) 101.

not in DOM release. Temperature rise reflects on *C. pseudocurvisetus* increased DOM release (Fig. 2), together with increased proportion of primary production directed toward DOM, including lipids, production with respect to POM production. An increase in total lipid content generally occurs at higher temperatures for microalgae cultures (Sharma et al., 2012). Increase above the optimal T value probably affects the function of enzymes involved in numerous cellular processes. Consequently, carbon, nitrogen and phosphorus cannot be optimally utilized by cell, but rather are released in the cell surrounding. This is especially pronounced for 30 °C, which is probably above the threshold temperature value, when cellular processes change for *C. pseudocurvisetus* isolated from the NA. Kim et al. (2011) also observed that elevated temperature disproportionately enhances the ratio of DOC to POC production in mesocosm experiment.

Comparing P-depleted and P-replete cultures, we have shown that increased DOC and Lip_diss release per cell is much more pronounced for the cultures growing in P scarcity. Plot of DOC or Lip_diss released per *C. pseudocurvisetus* cell against cell number (as a measure of growth success indicating poor to good growth conditions) (Fig. 2) shows that when *C. pseudocurvisetus* lives in a poor growth conditions (whether elevated temperature or P scarcity) there is generally lower cell abundance and those cells excrete much more DOC and lipids than those living in optimal growth conditions when cell abundance is higher. It is illustrated that there is a combined effect of temperature and nutrient scarcity on the pronounced DOM release. The data show that the temperature above threshold value affects *C. pseudocurvisetus* DOM production more significantly than nutrient limitation. DOC and Lip_diss release can be described by power functions: $DOC/cell = 2.76 \times cell^{-1.09}$ and $Lip\_diss/cell = 1.76 \times cell^{-1.39}$, respectively.

Not only DOC and dissolved lipid concentrations rise with increasing T, but also their content in TOC and total lipid pools, respectively. We have observed a very high content of dissolved lipids in DOC, up to 51% for P-depleted cultures grown at 30 °C (Supplementary materials, Fig. S1). Such high content of lipids in DOM might be explained by the fact that DOC, in our dataset, is composed only of fresh DOC. In contrast, most of DOM in the ocean and seas is refractory (Hansell, 2002), and content of dissolved lipids in DOM is much lower (Marić et al., 2013).

For both culturing conditions, content of glycolipids increases proportionally to T increase, while the opposite is observed for phospholipids. Obviously, these two groups of main membrane lipids (Lodish et al., 2004) play significant role in phytoplankton T accommodation. GL are predominantly located in photosynthetic membranes. They not only establish the lipid bilayer into which the photosynthetic complexes are embedded, but GL are also found within photosystems I and II structures (Jordan et al., 2001; Loll et al., 2005). Photosynthesis, which occurs in thylakoids, is the most heat-sensitive cellular function in photosynthetic organisms (Berry and Björkman, 1980). Yang et al. (2006) emphasized that in liposomes, containing only DGDG and/or MGDG, increase the photosystem II thermal stability, whereas phospholipids significantly decrease it. Further on, phosphorus scarcity has an impact on enhanced sulfo-glycolipid accumulation (Van Mooy et al., 2006).

Both, increasing T and P deficiency, cause substantial lipid degradation as concluded by both higher DI concentration and DI content in the dissolved lipid pool for those conditions. Other lipid classes showed T dependence in the whole T range investigated, with much higher proportion at 30 °C (Table 1), which was on the expense of PL. We assume P from PL is reused by alkaline phosphatase activity (Ivančić et al., 2016) for new cells, and that cells probably save P while releasing lipid classes mainly composed of C, H and O. It is to be concluded that the quality of dissolved lipids depends on the lipid producer physiological adaptation to the environmental conditions.

4.2. Northern Adriatic

DOC and Lip_diss concentrations in the NA were highest at the warmest month of July, when content of Lip_diss in total lipids increased. Generally, DOC is accumulating during summer in surface waters of seas and oceans (Børsheim and Myklestad, 1997; Giani et al., 2005; Shen et al., 2016). This DOM accumulation is explained by nutrient limitation of heterotrophic bacteria and subsequent microbial alteration of marine DOC (Shen et al., 2016; Fonda Umani et al., 2012). However, during the warm period in NA bacterial alkaline phosphatase, lipase and protease reach the highest activities (Celussi and Del Negro, 2012). NA is characterized by close bacteria-phytoplankton coupling (e.g. Puddu et al., 1998; Gašparović et al., 2013), and therefore bacteria should have a strong impact on fresh OM reworking. Bacteria modify the carbon cycle in different ways. As bacteria use behavioural and biochemical strategies to acquire organic matter, whether by the expression of enzymes to solubilize particulate organic matter, they

contribute to increased DOM pool, or by direct use of primary produced DOM, which is almost exclusively accessible to heterotrophic bacteria, they contribute to DOM removal from the sea (Azam and Malfatti, 2007).

Here we are pointing that increased temperatures also, usually in summer, likely have consequences on enhanced DOM (DOC and dissolved lipids) production by phytoplankton population. Furthermore, this effect is more pronounced for phosphorus-limited conditions when compared to the P-depleted station 107 with the mesotrophic occasionally P-replete station 101 (Fig. 6). The same we noticed for the batch culture experiments (Fig. 2).

Unlike monocultures, in the northern Adriatic the contribution of lipids to DOC is low (1.1–3.4%) (Supplementary materials, Fig. S2). This is explained by the fact that 90% of marine DOM is refractory and chemically stable (Hansell, 2002), while dissolved lipids represent fresh DOM formed from carbon fixed during primary production and released during life cycle and after cell death (programmed, viral lysis, and sloppy feeding on phytoplankton). Calculation of fresh DOC revealed it as a minor part (0–2%) of the DOC pool in the autumn to winter period in the northern Adriatic (Marić et al., 2013). Higher content of fresh DOC might be expected for the productive period that is investigated in this study.

The difference in the composition of lipid classes in the dissolved fraction is detected between monoculture experiment and the northern Adriatic. Unlike monocultures, there are multiple factors influencing fresh DOM composition, distribution and cycling in the seas that may counteract the T influence. This includes mixed phytoplankton population, phytoplankton species that are adapted to summer temperatures, nutrient input in colder season, changes in light availability, photochemistry and heterotrophy. Increased respiration rates at higher temperature (Vázquez-Domínguez et al., 2007) influence quantity and quality of phytoplankton produced DOM, which are labile compounds with a very short lifetime (Hopkinson et al., 2002). The dissolved lipid composition in the NA is modified by (i) strong bacteria-phytoplankton coupling (Puddu et al., 1998), and (ii) increased heterotrophic bacteria lipase activity during summer (Celussi and Del Negro, 2012). Although *C. pseudocurvisetus* batch cultures were not axenic, we took all precautions to avoid contamination with bacteria and therefore, analyzed lipids are discussed as produced by *C. pseudocurvisetus*. Furthermore, photochemical degradation was omitted by culturing conditions. Therefore, differences between laboratory and real system are expected.

The common feature for the monoculture experiments and NA samples is increased GL content for higher T. This feature is already noticed and explained with the role of GL in achieving thermal stability and prevention/mitigation of photooxidation (Gašparović et al., 2013). It seems that dissolved GL are less prone to degradation than TG, WE, ST and DI whose content in dissolved lipids does not correlated to T or is negatively correlated. Likewise, Tegelaar et al. (1989) reported on resistance of GL in marine sediments to degradation. GL do not possess biologically important phosphorus and nitrogen, and as such are probably less preferred substrate for plankton community. To explain lower susceptibility of GL to photochemical degradation in summer, we may also assume that GL fatty acids are more saturated. This makes sense in context that membrane fluidity is reduced for saturated/less unsaturated compounds (Los and Murata, 2004) which is needed at elevated temperatures.

Rising T would cause sinking of OM to a lesser extent (as DOM does not sink) and less carbon would be sequestered from the atmosphere to ocean/sea sediments (Thornton, 2014). The OM “quality” influences its cycling and capability to sequester carbon. The role of lipids in that process is questionable. On the one hand, lipids are buoyant and do not sink. This might lead to lipid surface remineralization due to prolonged period in surface waters, where they are produced. On the other hand, lipids are surface active, and therefore prone to adsorb on sinking particles, and as such may efficiently contribute to C sequestration. Indeed, Hwang and Druffel (2003) found selective lipid accumulation in the water column. Lipid saturation affects the export of carbon to the deep ocean (Gašparović et al., 2016).

5. Conclusions

From our model experiments on diatom batch cultures *C. pseudocurvisetus*, that were grown in a wide temperature range (10–30 °C) and in P-depleted and P-replete conditions that mimicked conditions in the northern Adriatic, and from the analyses of northern Adriatic samples, we can highlight the following conclusions:

- Temperature rise influences increased DOC and dissolved lipid production in the non-linear manner
- The temperature threshold range above which exponential release of lipids as well as DOC is between 25 and 30 °C for *C. pseudocurvisetus*
- The effect of T on DOC and dissolved lipid release is much more pronounced in phosphorus poor conditions, indicating that oligotrophic regions are more vulnerable to T rise.
- In complex systems, like seas and oceans, unlike the simple and controlled conditions in batch cultures, lipid quantity and quality differ and show different T dependence. Nutrient input in colder season, changes of light availability, mixed phytoplankton population, phytoplankton species that are adapted to summer temperatures, lipid sorption on inorganic and organic particles, photooxidation, and bacterial activity, all influence the dissolved lipid quantity and quality. All these parameters contribute hiding T effect on lipid release.
- Glycolipids are the only lipid class whose content in total lipids increased with rising T for both batch cultures and northern Adriatic samples, indicating their role in phytoplankton T acclimation and longer resistance to degradation in comparison to other classes.
- Rising T would cause lower OM sink (as DOM does not sink), and as such less carbon can be sequestered from the atmosphere to ocean/sea sediments. The role of lipids in that process is questionable: lipids are buoyant, but as very surface active they adsorb on sinking particles and consequently may contribute to the C sequestration.

Acknowledgments

Jasna Jakovčević and Margareta Buterer (Laboratory for processes in the marine ecosystem, IRB) are greatly acknowledged for sample collection and analyses of nutrients and Chl *a*. We thank Dr. Robert Precali for Chl *a* data and Zdeslav Zovko for DOC analysis. We thank the crew of the R/V Vila Velebita for their professional help and support. This work was funded by the grants from the Croatian Science Foundation under the projects IP-11-2013-8607 and UIP-2014-09-6563.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmarsys.2018.01.006.

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3.2 Publikacija II

Naslov: Global warming and oligotrophication lead to increased lipid production in marine phytoplankton

Autori: Novak Tihana, Godrijan Jelena, Pfannkuchen Marić Daniela, Djakovac Tamara, Medić Nikola, Ivančić Ingrid, Mlakar Marina, Gašparović Blaženka

Časopis: Science of the Total Environment

Godina objave: 2019.

DOI: https://doi.org/10.1016/j.scitotenv.2019.02.372

Science of the Total Environment 668 (2019) 171–183

Contents lists available at ScienceDirect

Science of the Total Environment

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Global warming and oligotrophication lead to increased lipid production in marine phytoplankton

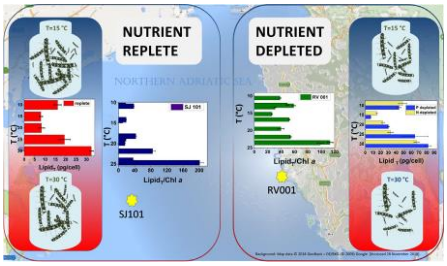
Tihana Novak [a,*], Jelena Godrijan [a,1], Daniela Marić Pfannkuchen [b], Tamara Djakovac [b], Nikola Medić [c], Ingrid Ivančić [b], Marina Mlakar [a], Blaženka Gašparović [a]

[a] Division of Marine and Environmental Research, Ruđer Bošković Institute, POB 108, HR-10002 Zagreb, Croatia
[b] Center for Marine Research, Ruđer Bošković Institute, G. Paliaga 5, HR-52210 Rovinj, Croatia
[c] Marine Biological Section, Department of Biology, University of Copenhagen, DK-3000 Helsingør, Denmark

HIGHLIGHTS

- Sea surface warming in the northern Adriatic leads to enhanced lipid production.
- Nutrient scarcity has influence alongside temperature on lipid accumulation.
- Nutrient status is more important for lipid remodeling than temperature.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 7 December 2018
Received in revised form 19 February 2019
Accepted 24 February 2019
Available online 27 February 2019

Editor: Sergi Sabater

Keywords:
Marine lipids
Temperature
Nutrients
Northern Adriatic Sea
Chaetoceros pseudocurvisetus
Diatoms

ABSTRACT

Earth temperature is rising and oligotrophication is becoming apparent even in coastal seas. In this changing environment, phytoplankton use carbon and nutrients to form important biomolecules, including lipids. However, the link between lipid production and changing environment is still unexplored. Therefore, we investigated the phytoplankton lipid production in the diatom *Chaetoceros pseudocurvisetus* cultures under controlled temperatures ranging from 10 to 30 °C and nutrient regimes mimicking oligotrophic and eutrophic conditions. Results were compared to plankton community's lipid production in the northern Adriatic at two stations considered as oligotrophic and mesotrophic during an annual monthly sampling. In order to gain detailed information on the investigated system, we supplemented lipid data with chlorophyll *a* concentrations, phytoplankton taxonomy, cell abundances and nutrient concentration along with hydrographic parameters. We found enhanced particulate lipid production at higher temperatures, and substantially higher lipid production in oligotrophic conditions. Enhanced lipid production has two opposing roles in carbon sequestration; it can act as a retainer or a sinker. Lipid remodeling, including change in ratio of phospholipids and glycolipids, is more affected by the nutrient status, than the temperature increase. Triacylglycerol accumulation was observed under the nitrogen starvation.

* Corresponding author.
E-mail address: tihana.novak@irb.hr (T. Novak).
[1] Current address: Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive East Boothbay, ME 04544, USA.

https://doi.org/10.1016/j.scitotenv.2019.02.372
0048-9697/

1. Introduction

Global warming is defined as an increase in combined air and sea surface temperatures (T) averaged over the globe and over a 30-year period (IPCC, 2018). If the warming continues at this rate, it is likely to reach an increase above 1.5 °C between 2030 and 2052, in respect to pre-industrial levels (IPCC, 2018). Increase in T leads to the variable acceleration of both, chemical as well as biological reactions. For example, biologically important processes such as photosynthesis and respiration react differently to ambient T changes. The T rise strongly favors respiration leading to conversion of organic carbon back to CO_2 (Allen et al., 2005). Global warming might also induce a series of cascading feedbacks in both terrestrial and marine carbon cycling processes (Boscolo-Galazzo et al., 2018). Many uncertainties exist in understanding (i) the quality and quantity of produced organic matter (OM) in surface productive layer, (ii) factors influencing the increased/decreased OM production, and (iii) the OM transport into deep ocean layers. These are important factors influencing the vertical redistribution of bioactive elements within the ocean, also impacting carbon sequestration and consequently climate change.

Oligotrophication across the world oceans is evidenced by measured decrease in chlorophyll *a* concentrations (Agusti et al., 2017) and reduced primary production (Behrenfeld et al., 2006). In Europe, the air T increased for 0.9 °C from 1901 to 2005, and consequently decreased precipitation (35–40%) was noted. This resulted in decreased river nutrient inputs, which were identified as one of the main causes of oligotrophication. Oligotrophicaton was particularly noted in the southern Europe, including the northern Adriatic watershed (Cozzi and Giani, 2011). Increased T also leads to greater stratification of the water column and reduced diffusion of the vertical nutrient supply (Agusti et al., 2017).

Phytoplankton are responsible for almost half of the total global primary production (Field et al., 1998). Within phytoplankton, diatoms are the most abundant and ecologically most successful group (Malviya et al., 2016), playing a significant role in OM production and carbon cycling in the marine ecosystem (Obata et al., 2013). By fixing CO_2 and consuming nutrients they synthesize carbohydrates, proteins, and lipids (Zulu et al., 2018). Lipids are an important component of marine productivity and an integral part of particulate organic matter (Parrish, 1988). As high-energy components rich in carbon, they are metabolic fuel as well as membrane and signal molecules for all organisms (Arts et al., 2009). Phytoplankton's ability to adapt to the changing environment is reflected in the production of variety of lipids (Thompson, 1996). Characterization of marine lipids on the molecular level enables their use as biogeochemical markers. They are useful in identification of sources and cycling of OM, as well as phytoplankton adaptation to the environmental stress (Guschina and Harwood, 2009). The distribution between different lipid classes and quantity of lipids depend on environmental factors and the stage of the cell cycle of primary producers (Zhukova and Aizdaicher, 2001).

Lipids are hydrophobic molecules structured mostly of hydrophobic fatty acid chains. In addition, many lipids have a hydrophilic head (phospholipids, glicolipids, betaine lipids). The hydrophobic character of lipids influences their accumulation on hydrophobic phase boundaries, such as the sea surface where they can be readily transformed by rich microbial community and ultraviolet radiation (UV) (Cunliffe et al., 2013). Hydrophilic parts of the molecule enable their adsorption onto mineral particles and larger high molecular weight organic particles (Morris and Eglinton, 1977). Futhermore, they interact with bulk carbohydrates via hydrogen bonding, nonpolar and electrostatic interactions (Kozarac et al., 2000). These processes might be responsible for fast sedimentation of particle associated lipids to sea or ocean floor. Therefore, lipids may have potential to sequester carbon from the upper ocean when associated with sinking particles. It was found that lipid-like material is a significant source of the uncharacterized organic carbon in the ocean (Hwang and Druffel, 2003). Recent studies highlighted the importance of lipid saturation for the carbon export from the surface to the deep ocean (Gašparović et al., 2016, 2017b, 2018a). Moreover, highly unsaturated novel phospholipids, possibly formed by cross-linking of unsaturated compounds in oceanic depths, are selectively preserved in the ocean. That made them both phosphorus and carbon carriers to the ocean depths (Gašparović et al., 2018b).

In this study, we aimed to investigate an impact of two environmental factors, T and nutrient availability, on lipid production by phytoplankton. We intended to determine which of these two factors could have more impact and if there is synergetic effect of the high T and nutrient scarcity. We first preformed experiments on phytoplankton monocultures in the laboratory and then proceeded to the in situ survey of the northern Adriatic ecosystem. As a model phytoplankton, we have chosen a diatom from the *Chaetoceros* family, common in the northern Adriatic (Bosak et al., 2016). We performed microcosm experiments in nutrient replete and depleted conditions, covering the recently registered data of the northern Adriatic annual T and nutrient variations. The northern Adriatic (Fig. 1) is characterized by a gradient of trophic conditions, from meso- and eutrophic (western) to oligotrophic (eastern) parts (Justić et al., 1995). Additionally, the northern Adriatic has large annual T variations (8–30 °C), especially in the upper water column (Gašparović, 2012). Our study provides an important contribution to projecting phytoplankton responses to future anthropogenic drivers.

2. Methods

2.1. Laboratory experiments

2.1.1. Monoclonal culture establishment

For the study of T influence on the cell growth and lipid production, we selected a representative *Chaetoceros* taxon. *Chaetoceros* occurs in the northern Adriatic all year round indicating its possibility to accommodate to wide T range (Bosak et al., 2016). We manually isolated one chain of *Chaetoceros* from a mesh sample collected at the SJ101 station in October 2014 (Fig. 1), and identified it as *Chaetoceros pseudocurvisetus*. The successfully grown monoclonal culture was maintained in f/2 medium (Guillard, 1975) at 15 °C, 4500 lx, on 12:12 h light/dark photoperiod, and was sub-cultured every 2–3 weeks.

2.1.2. DNA analysis

We performed a DNA analysis to confirm the microscopically preformed identification. We confirmed that the cultivated species was indeed *Chaetoceros pseudocurvisetus*. For the DNA extraction, 30 ml of the monoculture in exponential growth phase, was filtered through the 1.2 μm cellulose filter (Merck Millipore) and frozen at −80 °C. Genomic DNA was extracted and amplified following methodology described in Smodlaka Tanković et al. (2018). The obtained sequences were first aligned and then analyzed using software Geneious 11.1.5 (Kearse et al., 2012). For search and comparison of data with NCBI GenBank database BLAST algorithm was used (Altschul et al., 1997; Benson et al., 2010). We used the large subunit 28S as DNA barcode. The Nucleotide BLAST similarity search found the 28S sequence in the Gene Bank database (Theriot et al., 2010), and it showed 100% pairwise identity (on 100/query cover) with the sequences. The small and large subunit ribosomal RNA gene partial sequence was deposited in the GeneBank under accession numbers MG385841 FOR 18S DNA and MG385842 for 28S DNA.

2.1.3. Chaetoceros pseudocurvisetus cultures

In the experiment, *C. pseudocurvisetus* was cultured in 850 ml sterile VWR® Tissue Culture Flasks (VWR, Radnor, Pennsylvania), under five T (10, 15, 20, 25 and 30 °C) representing the T range in the northern Adriatic (Gašparović, 2012). Light intensity was 4500 LUX, and the cultures were maintained in the 12:12 h light/dark photoperiod. We adjusted the optimum concentrations of nutrients according to nutrient replete f/2 medium (Guillard, 1975). In the replete medium, average

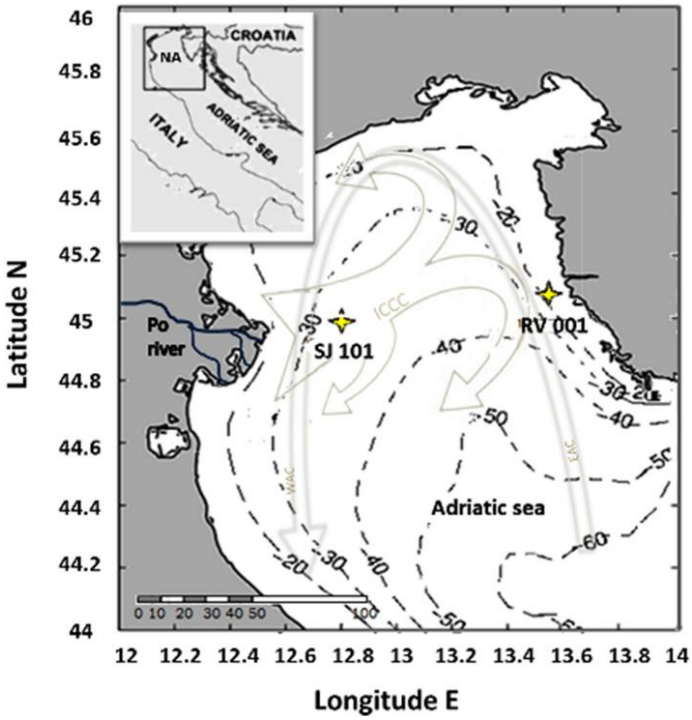


Fig. 1. Map of sampling stations SJ101 and RV001 in the northern Adriatic Sea with the scheme of winter prevailing Eastern Adriatic Current (EAC), Western Adriatic Current (WAC) and summer prevailing Istrian Coastal Countercurrent (ICCC) (after Supić et al. (2003)).

of orthophosphate (PO_4) was 36.33 ± 6.56 μmol/l and dissolved inorganic nitrogen (DIN=NO_3 + NO_2 + NH_4) of 1059 ± 181.1 μmol/l. In phosphorous depleted (P-depleted) medium PO_4 concentrations were 0.6 ± 0.1 μmol/l. Concentrations of DIN in nitrogen depleted medium (N-depleted) were 7.6 ± 1 μmol/l. Concentration of nutrients was measured before inoculation of cells. Corresponding N/P ratios were 29.4, 1765, and 0.2 for replete P-depleted and N-depleted media, respectively. Aseptic techniques in all culture manipulations were used, even though the cultures were monoclonal rather than axenic. To ensure a low organic content, seawater was taken during the winter on the oligotrophic side of the northern Adriatic, when phytoplankton activity was low. Collected water was pre-filtered through 0.7 μm Whatman GF/F filters and rested for 2 months in the dark. Rested water was filtered again through sterile 0.22 μm white plain filters (Merck Milipore ltd.), and boiled in microwave (Keller et al., 1988) before adding sterile media amendments.

We started the experiment by inoculating 10^5 cells into each 800 ml batch culture medium, in duplicates. Before inoculation, cells were kept in f/2 replete media in 250 ml VWR® Tissue Culture Flasks (VWR, Radnor, Pennsylvania) under T of experiment. To establish the grow rate of *C. pseudocurvisetus*, we preformed cell counts every two days with Fuchs-Rosenthal Chamber hemocytometer under an Olympus BX51-P polarizing microscope. We terminated the growth by filtering samples through 0.7 μm Whatman GF/F filters, at the beginning of the stationary phase. Filters cleaned of organic matter by pre-burning at 450 °C for 4 h were used. After filtration, filters were frozen at −80 °C and stored until the lipid extraction.

In order to calculate DIN and PO_4 uptake ratios, their concentrations were determined at the beginning and at the end of 15 °C batch culture experiments, using standard spectrophotometric methods (Parsons et al., 1984). DIN and PO_4 uptake ratios were calculated as the difference between their initial and final concentrations.

Total particulate organic matter (POC) was measured using the high-temperature catalytic oxidation method. POC was analyzed by solid sample module SSM-5000A connected to Shimadzu TOC-VCPH carbon analyzer, calibrated with glucose (Sugimura and Suzuki, 1988). POC concentrations were corrected based on blank filter measurements. The average filter blank with the instrument blank corresponds to 0.005 mg/1. The reproducibility for the glucose standard was 3%.

2.1.4. Alkaline phosphatase activity

To determine the *C. pseudocurvisetus* alkaline phosphatase activity (APA), an additional experiment was performed. *C. pseudocurvisetus* (initial concentrations 1.65×10^5 cell/l) was inoculated in triplicate in the replete and P-depleted medium. Culture batches were incubated at 15 °C, with a light dark cycle of 12:12 h, in sterile 250 ml vented culture flasks (easy flasks, Nuclon, Denmark). The experiment lasted for 10 days. The APA was measured on days 3, 6, 8, and 10. The APA was measured by florogenic substrate, methylumbeliferyl phosphate (MUF-P), according to the procedure described in Hoppe (1983). The MUF-P was dissolved in 2-methoxyethanol and diluted in filtered (0.22 μm pore size) and autoclaved sea water prior to use. Fifty μl of substrate (concentration 250 μM) was added in 200 μl of live monoclonal cultures (final concentration 50 μM). The MUF-P hydrolysis product methylumbelliferone (MUF)

fluorescence was measured by Tecan M200 Pro spectrofluorimeter (excitation at 365 nm and emission at 460 nm). The reaction was incubated at 16 °C in the dark and fluorescence was measured at intervals of 0, 10, 30 and 60 min. The fluorescence increased linearly over the incubation time. The APA (nmol/lxs) was calculated as the difference between two measurements divided by the incubation time after calibration of instrument with the MUF. To produce a standard curve, a range of the MUF concentrations (0–1500 μM) was used. Cell-specific activity was calculated by dividing APA by the cell abundance.

2.2. In situ measurements

2.2.1. Site description, environmental measurements and sample collection

The northern Adriatic is a shallow basin, with an average depth of 30 m (Orlić et al., 1992). It is under eutrophic pressure by the Po River at its western part and oligotrophic influence from the middle Adriatic Sea at the eastern part (Djakovac et al., 2012). In warmer months (from April to October) anticyclonic circulation in the central and eastern part of the northern Adriatic induces a strong southeast current known as Istrian Coastal Countercurrent (ICCC) (Supić et al., 2003) (Fig. 1). ICCC transports nutrient replete water toward eastern, oligotrophic part of the northern Adriatic, where it increases primary production (Giani et al., 2012).

We performed the sampling at two northern Adriatic stations with opposing trophic conditions. Station SJ101 is situated close to the Po River inflow and is usually considered eu-mesotrophic. Station RV001 is situated 1 nautical mile from the city of Rovinj, and is prevalently oligotrophic (Fig. 1). Surface water samples (0 m) were collected monthly, during research vessel Vila Velebita cruises from March 2013 to March 2014. In June, December, and February, sampling was not possible due to the bad weather conditions. Seawater T and salinity (S) were determined by CTD probe (SBE 25 Sea logger CTD, Sea-Bird Electronics, Inc., Bellevue, Washington, USA). Water samples for the analysis of nutrients, chlorophyll *a* (Chl *a*), lipids, and phytoplankton were collected with 5 l Niskin bottles.

For the lipid analysis, 3 l of water was pre-filtered through a 200 μm stainless steel screen and collected in glass bottles. Pre-filtration was preformed to remove larger particles including zooplankton. Immediately after, seawater sample was filtered on 0.7 μm pore size grade GF/F Whatman® glass microfiber filters, cleaned of organic matter by pre-burning at 450 °C for 4 h. Filters were stored in cryotubes, first in liquid nitrogen, and then at −80 °C until further analysis.

Subsamples for nitrate (NO_3), nitrite (NO_2) and orthophosphate (PO_4) determination were measured on board by standard spectrophotometric methods (Parsons et al., 1984). Ammonium (NH_4) was analyzed using modified indo-phenol method (Ivančić and Degobbis, 1987).

For chlorophyll *a* (Chl *a*) determination, subsamples were filtered on GF/C Whatman® glass microfiber filters. Filters were grinded and Chl *a* was extracted in 90% acetone for 3 h to 24 h (Arar and Collins, 1997) in the dark. Final Chl *a* concentrations were determined by turner TD-700 fluorimeter (Parsons et al., 1984).

2.2.2. Quantitative and qualitative phytoplankton analysis

Samples (200 ml) for the identification and enumeration of phytoplankton cells were preserved in neutralized formaldehyde (2% final concentration) solution (Kemika d.d. Zagreb, Croatia) (Throndsen, 1978). Phytoplankton cells were enumerated (Utermöhl method) using an inverted microscope (Zeiss Axiovert 200; Zeiss GmbH, Oberkochen, Germany) equipped with phase contrast and differential interference contrast optics (Lund et al., 1958; Utermöhl, 1958). Phytoplankton were identified to the lowest possible taxonomic rank using determination keys listed in Godrijan et al. (2012).

2.3. Lipid extraction and analysis

We have extracted particulate lipids by one–phase solvent mixture of dichloromethane–methanol–water (Blight and Dyer, 1959). As an internal standard, we added 10 μg n-hexadecanone into each sample. After extraction, samples were evaporated under nitrogen atmosphere to dryness and stored at −20 °C. Right before lipid separation, samples were re-dissolved in 20–40 μl dichloromethane. Eighteen lipid classes were separated on Chromarods SIII and quantified by an external calibration with standard lipid mixture and by thin–layer chromatography–flame ionization detection (TLC–FID) (Iatroscan MK–VI, Iatron, Japan), with a hydrogen flow of 160 ml/min and air flow of 2000 ml/min (Gašparović et al., 2015, 2017a). Each sample was analyzed in duplicate. Total lipid concentration is a sum of all lipid classes quantified by the flame ionization. Determined lipids include following classes: wax esters and sterol esters (WE/SE); fatty acid methyl esters (ME); fatty ketone hexadecanone (KET, internal standard); triacylglycerols (TG); free fatty acids (FFA); fatty alcohols (ALC); 1,3-diacylglycerols (1,3 DG); sterols (ST); 1,2-diacylglycerols (1,2 DG); pigments (PIG); monoacylglycerols (MG); mono- and di-galactosyldiacylglycerols (MGDG and DGDG); sulfoquinovosyldiacylglycerols (SQDG), phosphatidylglycerols (PG); phosphatidylethanolamines (PE); and phosphatidylcholine (PC). Concentration of glycolipids (GL) is expressed as a sum of MGDG, DGDG, and SQDG; and phospholipids (PL) as a sum of PG, PE, and PC. Analysis procedure was described by Gašparović et al. (2015, 2017a) in detail. We would like to point out that in the monoculture experiment we discussed WE/SE TLC band as SE, while in the northern Adriatic samples the band was discussed as WE. Namely, WE are zooplankton storage lipids (Lee et al., 2006) and cannot be found in phytoplankton monocultures. Since the WE/SE TLC band contains only small amounts of sterol esters (Hudson et al., 2001) it is referred to as zooplankton wax esters for the northern Adriatic samples.

2.4. Data analysis

We calculated the growth rate with the equation:

$$\mu = \frac{1}{t}\ \ln\left(\frac{N_m}{N_0}\right)$$

N_o and N_m are algal concentrations at the beginning and at the end of batch culture experiment, and t is time in days (Thompson et al., 1992).

To evaluate the relationship between different measured parameters Principal Component Analysis (PCA) in Statistica Release 7 software was used. The strength of a linear association between two variables was evaluated by Pearson correlation coefficientsusing Excel 2016 software.

3. Results

3.1. Chaetoceros pseudocurvisetus cultures

3.1.1. Chaetoceros pseudocurvisetus physiology

We have studied the effect of T changes and nutrient availability on the growth rate and lipid production of diatom *C. pseudocurvisetus*. The cells were grown at 10, 15, 20, 25 and 30 °C, and in three opposing media: replete, P-depleted, and N-depleted. *C. pseudocurvisetus* cell counts, growth rates, and average carbon content are presented in Table 1. As a measure of the cell carbon content we used POC and normalized it to cell numbers. This was done because all POC in the experiment was produced during *C. pseudocurvisetus* growth, as it was zero at the beginning of the experiment. These data were used to calculate percentage of intracellular lipid carbon (carbon allocated to lipids).

Maximum cell abundance was detected at 15 °C for all batches, and minimum was detected at 10 °C for replete, at 20 °C for P-depleted and at 30 °C for N-depleted. There is a significant difference in growth rates

Table 1
Cell number counted at stationary growth phase, growth rate and average carbon content for *Chaetoceros pseudocurvisetus* grown at five different temperatures and different media (replete, P- and N-depleted).

Batch medium	Temperature	Cell number	Growth rate	Average carbon content
	(°C)	(cell × 10^6/l)	(cell/day)	(pgC/cell)
replete	10	9.16 ± 3.2	0.26 ± 0.03	333.4 ± 104.5
	15	25.8 ± 11.7	0.48 ± 0.04	63.1 ± 12.3
	20	13.4 ± 0.8	0.67 ± 0.01	243 ± 118.3
	25	19 ± 4.5	0.72 ± 0.03	169.1 ± 56.3
	30	2.8 ± 0.6	0.35 ± 0.03	201.9 ± 6.5
P-depleted	10	6.2 ± 1.8	0.29 ± 0.01	551.6 ± 13.8
	15	21 ± 7.7	0.51 ± 0.04	74.5 ± 19
	20	1.1 ± 0.6	0.29 ± 0.1	341.8 ± 30.9
	25	10.3 ± 6.3	0.43 ± 0.07	241.3 ± 38.5
	30	9.6 ± 0.8	0.43 ± 0.01	294.9 ± 20
N-depleted	10	4.3 ± 1	0.28 ± 0.00	872.2 ± 7.8
	15	20.7 ± 2.6	0.47 ± 0.07	164.3 ± 26
	20	13.7 ± 1.2	0.67 ± 0.01	149.1 ± 24.3
	25	6.5 ± 0.7	0.66 ± 0.02	648 ± 76.7
	30	4.2 ± 1	0.50 ± 0.04	324.2 ± 74.4

depending on T and medium. The highest growth rate in replete and N-depleted growth was detected between 20 °C and 25 °C, and lowest at 10 °C. In P-depleted medium, the highest growth rate was detected at 15 °C, and lowest at 10 °C and 20 °C. The number of days for the cultures to reach the stationary phase were 14.2 ± 1.6, 10.7 ± 0.5, 7.2 ± 0.2, 7.7 ± 0.0, 8.7 ± 0.5 for 10, 15, 20, 25 and 30 °C, respectively.

To investigate if *C. pseudocurvisetus* overcomes the PO_4 deficiency by alkaline phosphatase (AP) activation, we measured APA of batch cultures grown in replete and P-depleted media at 15 °C (Fig. 2). *C. pseudocurvisetus* activate AP at both growing conditions. Cell-specific APA was much higher in cultures grown under P-limited conditions.

We tested the DIN/PO_4 uptake for *C. pseudocurvisetus* grown at 15 °C, what is considered as optimal T for their growth (Novak et al., 2018). N/P uptake ratio was 16.4 ± 0.2 in replete medium which corresponds to the Redfield ratio (Redfield, 1934). More phosphorous was consumed (N/P = 2.6 ± 0.3) in N-depleted and more nitrogen (N/P = 72.9 ± 4.2) in P-depleted medium.

3.1.2. Chaetoceros pseudocurvisetus lipid production

The T and nutrient availability (Fig. 3 and supplementary Table S1) affected lipid production. To get an insight into lipid production we normalized lipids to number of cells. Lipid classes are divided in two groups. First, the cell lipids with two subgroups: (i) lipids that are located predominantly in membranes (membrane lipids) (PL, GL, ST, and PIG) and (ii) intracellular reserve lipids (SE, inert storage forms of sterols, and TG being phytoplankton metabolic energy reserves). Second group are free fatty acids, alcohols, diacylglycerols, and monoacylglycerols that represent cell lipid degradation indices (DI). Membrane lipids have the highest contribution in cell lipids. *C. pseudocurvisetus* synthesized the lowest cell lipids quantity at 15 °C in replete < P-depleted < N-depleted order. The highest lipid content was detected at 30 °C in replete < N-depleted < P-depleted order. In the T range 20 to 30 °C, the cell lipid content increased more than twice under nutrient depleted conditions, compared to replete conditions. Phospholipid cell content increased in N-limited, as well as in P-limited conditions. TG cell content was the highest for N-depleted cultures. The highest contribution of lipid carbon to the total carbon was at 30 °C under all described growth conditions. The lowest contribution of lipid carbon to the total carbon was at 10 °C under N-depleted, 15 °C under P-depleted and 20 °C under the replete conditions. The highest content of cell membrane lipids was detected at 30 °C. Reserve lipids also have the highest content in total lipids at 30 °C, with an exception of P-depleted medium where the highest content was measured at 15 °C. DI content per cell was the highest at 30 °C, following the cell lipids trend.

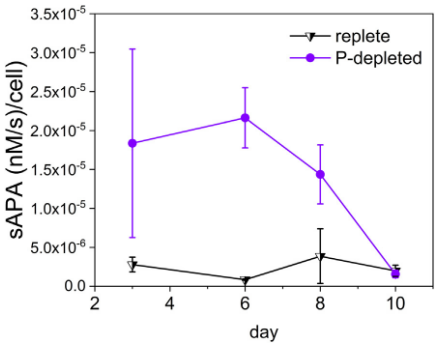


Fig. 2. Cell-specific alkaline phosphatase activity (sAPA) of *C. pseudocurvisetus* for growth in replete (triangles) and P-depleted (circles) medium.

The contribution of membrane lipid classes to cell lipids varies depending on T and growth medium. As calculated (Supplementary Table S1) GL contribution (%GL) was the highest at 15 °C in all batches (max. in replete 56.4%). The highest PL contribution (%PL) was detected at 30 °C in replete medium (45.3%), at 20 °C in P-depleted medium (47.7%), and at 25 °C in N-depleted medium (26.2%). PIG contributions (%PIG) peaked at 30 °C in replete medium (11.1%), at 25 °C in both N-depleted (25.6%) and P-depleted medium (7.7%). In general, ST contributions (%ST) exhibited a decreasing trend with T. TG contribution (%TG) tended to increase at high T for replete and P-depleted growth conditions. At N-depleted conditions %TG was high at all T and much higher in comparison to replete and P-depleted media (an average of 15.7 ± 2.1%).

To evaluate the relationship between T and produced cell lipids distribution, which might suggest cell lipid remodeling, the principal component analysis (PCA) for each culture medium was preformed (replete, P-depleted, and N-depleted) (Fig. 4). PCA of nutrient replete conditions explained 82.8% total variability between seven variables. The first principal component (PC1) had the highest negative loadings for temperature T, %PL, and %PIG, whereas, positive loadings were observed for %GL and %ST. Such distribution of variables indicates increased PL and PIG contribution, and decreased GL and ST contribution with T rise. Reserve lipids TG and SE contributions were not correlated with the T rise.

PCAs of P-depleted conditions explained 77.8% of the total variability between seven variables. The T dominated at positive value of PC2 and correlated only with %TG. Other variables did not correlate to T. Such distribution indicates that P depletion is more important for the lipid classes' distribution than T rise. PCAs of the N-depleted conditions explained 87.1% of total variability between seven variables. The T dominated at the positive value of PC2 and correlated only with %SE. Other variables did not correlate to T. Therefore, we assume that nitrogen depletion is more important for the lipid classes' distribution than T rise.

The strength of a linear association between two variables from the PCA analysis for all growth conditions are evaluated by Pearson correlation coefficients, *r* (Supplementary Table S2). Although there are multiple influences on a particular lipid parameter, Pearson correlation coefficients are significant for those parameters that appeared significantly correlated in the PCA analysis.

3.2. Northern Adriatic

3.2.1. Environmental conditions

We investigated the influence of T and nutrient availability on lipid production at two northern Adriatic stations, SJ101 and RV001, with contrasting trophic status. T (Fig. 5a) at both stations revealed sinusoidal annual curves with maxima in July and August, and a minimum in

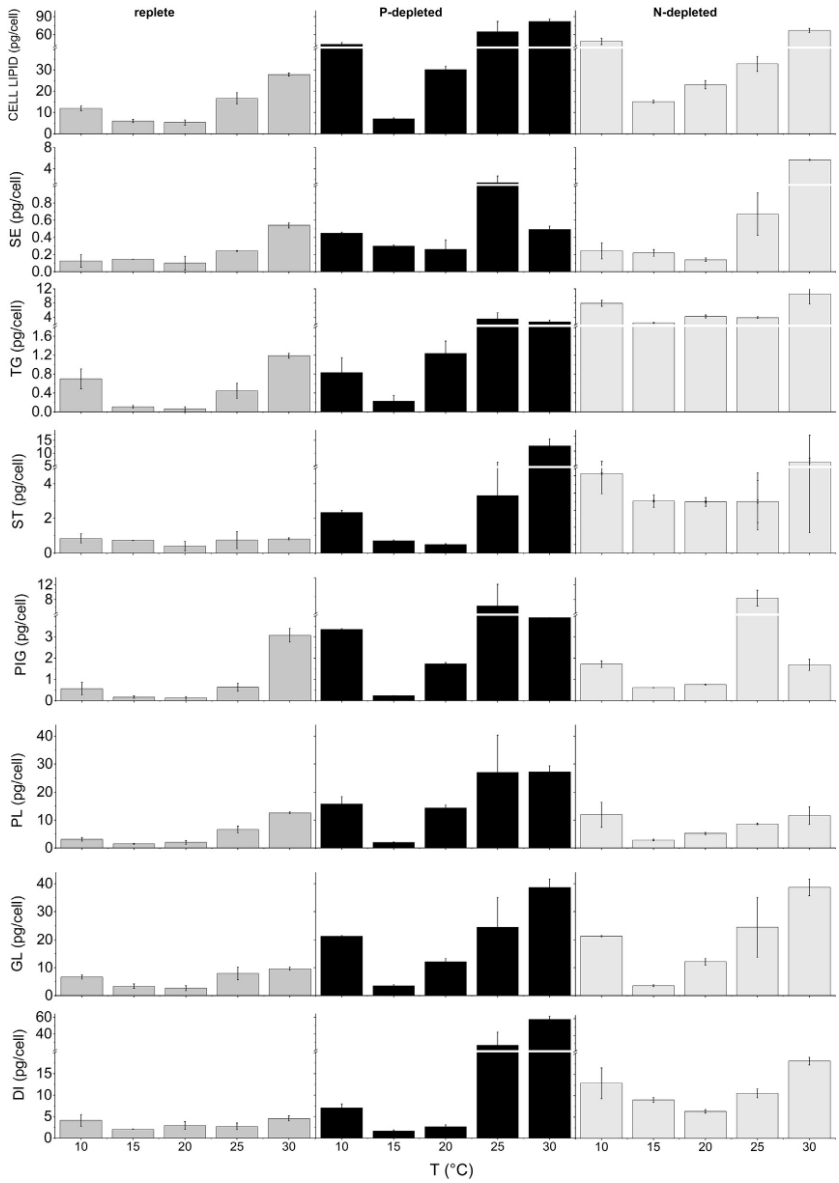
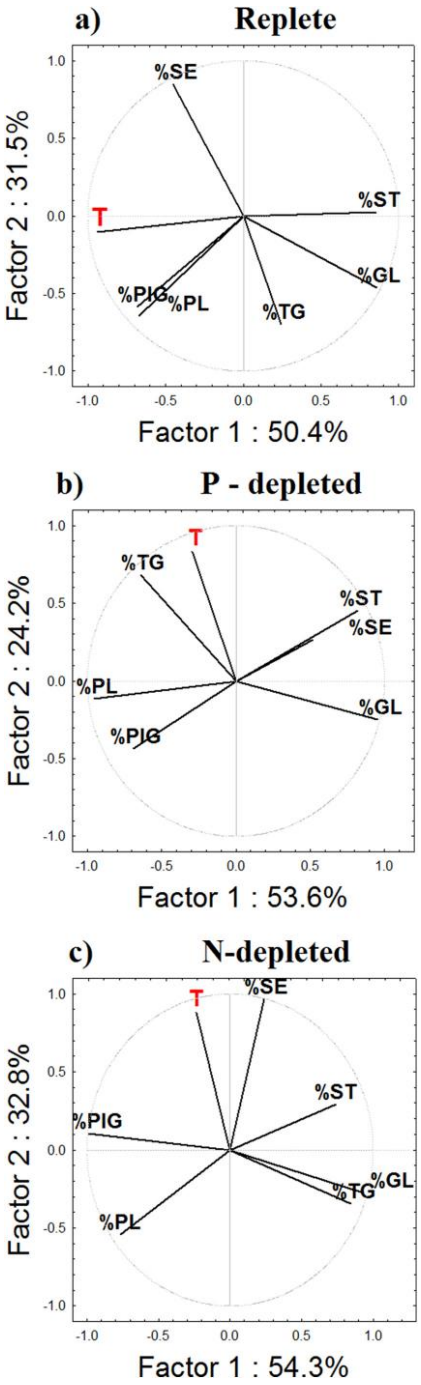


Fig. 3. Main parameters of lipid production normalized to *C. pseudocurvisetus* cell abundance in three different batch cultures, replete (left pannel), P- (middle pannel) and N-depleted (right pannel) at five temperatures (10, 15, 20, 25 and 30 °C) at the end of the experiments. Abbreviations: sterol esters (SE), triacylglycerols (TG), sterols (ST), pigments (PIG), phospholipids (PL), glycolipids (GL), and degradation indices (DI).

March 2013. Station SJ101 had higher nutrient concentrations (Fig. 5c and d) than station RV001 that correlated with S variations (Fig. 5b). Average surface PO_4 and DIN concentrations were 0.12 μmol/l and 15.33 μmol/l, respectively, for station SJ101, and 0.05 μmol/l and 2.22 μmol/l, respectively, for station RV001. Exact values for described parameters are given in the Supplementary Table S3.

Chl *a* distribution (Fig. 6a) followed the distribution of nutrients, with higher concentrations observed at station SJ101 (0.09–10.02 μg/



l). The highest peak was detected in March at station SJ101 (10.02 μg/l). Concentrations of Chl *a* were much lower at RV001 (0.16–0.76 μg/l) with maximum in November, in accordance to regular autumn water column mixing.

Phytoplankton abundances and taxonomy (nano and micro fraction) were determined for both stations. The contribution of *Chaetoceros* taxa in the phytoplankton community is given in Fig. 6b. Higher dominance of *Chaetoceros taxa* was determined in March 2013 at station SJ101, with the highest contribution in total phytoplankton community (50%). All sets of values for Chl *a* and the contributions of *Chaetoceros* taxa, from Fig. 6, are given in the Supplementary Table S3.

3.2.2. Lipid production and composition

Starting from the fact that phytoplankton is the main lipid producer in seas and oceans (Gašparović et al., 2014), we normalized lipid concentration to Chl *a* (Fig. 7, Supplementary Table S3). The highest cell lipid/Chl *a* values were calculated for July at both stations, when T was 24.67 °C for station SJ101 and 23.41 °C for station RV001. The cell lipid/Chl *a* ratio was more or less uniform within T range from 10 to 20 °C, while there was a rise from 20 °C upward at both stations. Although, lipid production at station RV001 was much lower than at SJ101, there was also lower phytoplankton biomass (measured as Chl *a*, Fig. 6a). Consequently, there was a higher average ratio of cell lipid/Chl *a* for station RV001. The average values were 48.4 and 56.1 for stations SJ101 and RV001, respectively. The highest PL cellular content was measured in July at station SJ101, and the lowest was in March with measured temperatures: 24.67 °C and 14.33 °C, respectively. In general, higher content of PL/Chl *a* was detected at the nutrient poorer station RV001 than at the nutrient richer station SJ101. The highest and lowest GL cellular contents were measured at station SJ101 in September (T = 21.8 °C) and March (T = 14.33 °C), respectively. The trend of lipid content degradation indices (DI) per Chl *a* followed the trend of total cell lipids content, with the highest values observed during the warmer season.

As can be calculated from the lipid data and the measured temperature (Supplementary Table S3), the lipid classes contribution to the cell lipids decrease in the following order: membrane lipids > degradation indices > reserve lipids. The highest contribution of membrane lipids to the total lipids was measured in September at station SJ101 (91.64%) at T 21.82 °C, and the lowest contribution in August at station SJ101 (51.4%) at low T 24.07 °C. The membrane lipids contribution was on average 66.0 ± 10.1% at SJ101, and 65.5 ± 8.4% at RV001. GL at station SJ101 (average 42.9 ± 9.2%) and PL at station RV001 (average 46.8 ± 6.5%) were among the highest contributors to the membrane lipids. ST and PIG had a low contribution to membrane lipids: on average ST and PIG contributed 15.4 ± 6.9%, and 3.5 ± 2.8%, respectively to the total membrane lipids at station SJ101; and at station RV001 9.7 ± 3.5%, and 5.0 ± 2.7%, respectively. Reserve lipids (TG and WE) had a small contribution to the total lipids. Based on the results presented in the Supplementary Table S3, average contribution of TG to the total lipids was calculated to be 4.0 ± 3.5% at station SJ101, and 4.3 ± 3.1% at station RV001. Months with higher dissolved inorganic nitrogen (DIN) inputs (>10 μmol/l) had lower TG contribution (2.7 ± 1%). The average contributions of WE to the total lipids were 2.5 ± 1.2%, and 4.8 ± 2.5% at stations SJ101 and RV001, respectively.

We preformed PCA to evaluate the relationship between T, contribution of lipid classes to the total lipids (indicating lipid remodeling), and available nutrients for both northern Adriatic stations. Two PCAs of northern Adriatic stations SJ101 and RV001 (Fig. 8a and b), explained 56.5% and 62.7% of the total variability among 9 variables. Temperature, %TG, and %PIG were not correlated in the plane defined by PC1 and

Fig. 4. Principal component analysis (PCA) for the variables; temperature (T), contribution of different lipid classes to total cell lipids: triacylglycerol (%TG), sterol ester (%SE), sterol (%ST), pigment (%PIG), phospholipid (%PL), and glycolipid (%GL) at replete (a), P-depleted (b) and N-depleted (c) growth conditions.

PC2 at station SJ101 (Fig. 8a). PC1 had the highest positive value for DIN, PO_4, %ST, and %GL and the highest negative value for %PL and %WE. The strength of a linear association between two variables from PCA were evaluated by Pearson correlation coefficients, *r* (Supplementary Table S5). Although, there are multiple influences on a particular lipid parameter, Pearson correlation coefficients were mainly significant for those parameters that appeared significantly correlated in the PCA. Results indicate that at the nutrient richer station, nutrient availability is more important for the lipid classes' distribution than T. The PCA at station RV001 (Fig. 8b) shows that T had the greatest negative loading of PC2 together with %GL, while the highest positive loadings of PC2 were for %TG and %WE. This suggests that the GL contribution increases with T rise. The greatest positive PC1 loadings were evident for DIN, %PL, and %PIG. The greatest negative PC1 loadings were for %ST. The PO_4 variable was not correlated in the plane defined by PC1 and PC2. The %PL and %GL were inversely correlated for both stations, indicating their interchange depending on the environmental conditions. Pearson correlation coefficients were predominantly significant for those parameters that appeared significantly correlated in the PCA analysis (Supplementary Table S5).

We aimed to evaluate the influence of nutrient availability on cell lipid distribution under both the optimal and high T scenarios. We performed a PCA for the T range 15–20 °C, which is considered an optimal T range for phytoplankton growth. This was compared to PCA for the T range from 20 to 25 °C considered as the high T range for phytoplankton growth (Fig. 9a and b). For the optimal T range the PC1 had the greatest positive loadings for DIN and %ST (Fig. 9a), while the greatest negative PC1 loadings were noted for %TG, %WE, and %PIG. The greatest positive PC2 loadings were for PO_4 and %PL, while the greatest negative loading on PC2 was for %GL. This indicates that at the optimal T range northern Adriatic lipid class distribution is „by the book". This includes more PL and less GL at higher PO_4 concentrations (Van Mooy et al., 2006), as well as an increase in TG contribution which coincide with a decrease in DIN availability (Bourguet et al., 2009; Parrish and Wangersky, 1987). Pearson correlation coefficients were mostly significant for those parameters that appeared significantly correlated in the PCA analysis (Supplementary Table S6).

For the higher T range the greatest positive PC1 loadings were for % PL, %PIG, %TG, and %WE (Fig. 9b), while %GL and DIN had the greatest negative effect on PC1. Variables PO_4 and %ST were explained by PC2 with negative loading values. The PO_4 increase does not result in greater PL contribution to total lipids. PCA shows that the relationship between nutrients and lipid classes is more complex at higher T than at the optimal T range, indicating the important role of higher T for the lipid biochemistry. Pearson correlation coefficients were mostly significant for those parameters that appeared significantly correlated in the PCA analysis (Supplementary Table S6).

4. Discussion

Phytoplankton response to T increase and nutrient availability reduction was the focus of our study. We investigated the possible cascade effect on lipid production in response to T rise as the primary, and nutrient depletion as the secondary, ecosystem influence. We compared the experimental results of the model diatom *Chaetoceros* with a one-year monthly sampling of the complex northern Adriatic system, a characteristic coastal sea ecosystem highly influenced by the global change processes. Prior studies noted the importance of T influence on phytoplankton metabolism referring to lipid production (e.g. Opute, 1974; Toseland et al., 2013), but they rarely took into account both laboratory and in situ experiments. During our one-year sampling period (2013–2014) T higher than 25 °C were not observed at RV001 and SJ101, even though there were events of surface water T up to 30 °C in northern Adriatic (e.g. Novak et al., 2018).

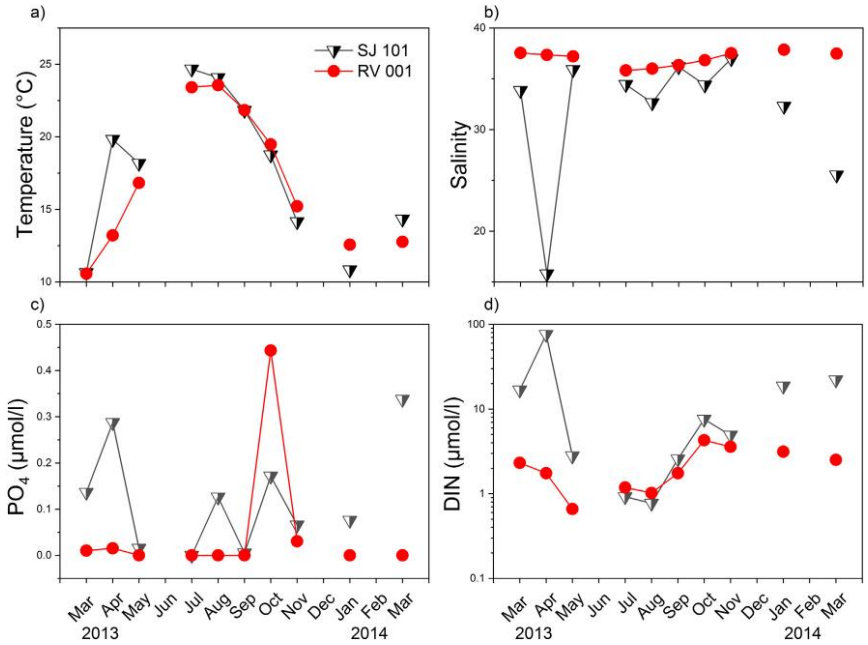


Fig. 5. The northern Adriatic environmental parameters: temperature (a), salinity (b), orthophosphate (c) and dissolved inorganic nitrogen (d) at the mesotrophic station SJ101 (triangles) and oligotrophic station RV001 (circles) during the investigation period in 2013–2014.

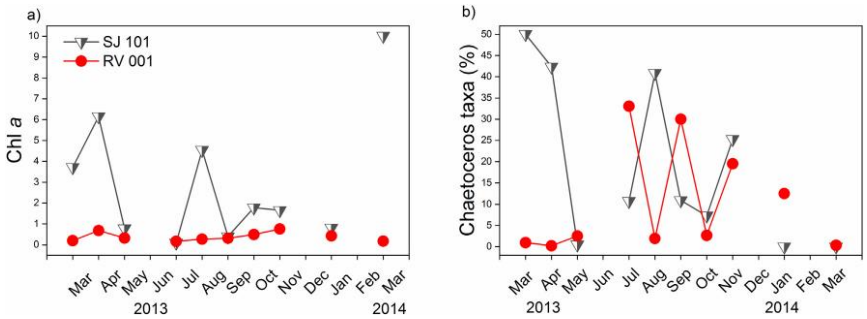


Fig. 6. The northern Adriatic phytoplankton status. Chlorophyll *a* (a) and contribution of *Chaetoceros* taxa abundance to phytoplankton community abundance (b) at the mesotrophic station SJ101 (triangles) and the oligotrophic station RV001 (circles) during the investigation period in 2013–2014.

Each phytoplankton species has an optimal T range for growth. An optimum T for *C. pseudocurvisetus* growth is 15 °C. At this T, the highest cell abundance and the lowest lipid cell content were measured, together with the lowest primary production directed to dissolved fraction (Novak et al., 2018). The highest T used in the *C. pseudocurvisetus* cultivation (30 °C) lead to the cell abundance stagnation and significant cell lipid accumulation. Increased cellular content of lipids at the highest growth T parallels *C. pseudocurvisetus* reduced reproduction. We assume that reproduction slowed down due to thermo-sensitivity of enzymes involved while accumulation of lipids continued. However, at the lowest T growth (10 °C) *C. pseudocurvisetus* showed high lipid cellular content as well. Sharma et al. (2012) have shown that under optimal growth conditions, also regarding T range, large amount of phytoplankton biomass is produced, with relatively low lipid content. Growing in environmentally unfavorable conditions, many phytoplankton species alter their lipid biosynthetic pathways toward the formation and accumulation of neutral ones (Sharma et al., 2012). This is observed for *Ochromonas danica* in T range 15 to 30 °C (Aaronson, 1973), three diatoms *N. paleacea* in T range 15–25 °C *N. closterium* and *Isochrysis* sp. (PS 11) in T range 20–30 °C (Renaud et al., 1995). However, opposing to our and referenced findings two *Chaetoceros* species, *C. cymplex* and *C. gracilis*, showed a decline in lipid per cell in T range from 10 to 25 °C (Thompson et al., 1992). Other six investigated microalgae species showed inconsistent relationship between T and lipid content (Thompson et al., 1992). Nonetheless, at the extreme high or low growth T a decrease has been observed in the microalgae lipid production (Aaronson, 1973; Opute, 1974). Opute (1974) suggested that this effect was caused by discontinuation of growth due to irreversible damage on enzymes. Our experiment was based on T range typically occurring in the NA, and major extremes (below 10 and above 30 °C), were not taken into account. Within this T range *C. pseudocurvisetus* was able to grow and reproduce, and no discontinuation of growth was observed. However, we unsuccessfully tried to grow *C. pseudocurvisetus* at 7 °C since cells did not divide.

In addition to T, increased lipid production in *C. pseudocurvisetus* is also affected by lack of nutrients. Phosphorus scarcity appeared to have a greater role in lipid accumulation at higher T. Oppositely; nitrogen deprivation has a higher influence on enhanced lipid cell accumulation at lower T. As an example, N starvation resulted in increased total lipid content of cell for some species up to 50% (Schuhmann et al., 2012).

The northern Adriatic phytoplankton community followed the tendency of *C. pseudocurvisetus* monoculture experiment. The highest lipid content per Chl *a* was observed in winter and particularly in warmest summer months. Together with significant increase in cell lipids, lipid degradation indices content increased with rising T, both, for *C. pseudocurvisetus* cultures as well as the northern Adriatic phytoplankton community. This suggests that the enhanced lipid production and lipid degradation processes take place at high T. P-depleted conditions influence even greater lipid accumulation as observed at P-depleted station RV001. This indicates that nutrient scarcity has an additional effect on phytoplankton lipid accumulation alongside the high T, as shown in the example of the northern Adriatic.

The T rise and nutrient shortage influence higher production of all lipid classes, as observed in monoculture experiments and for the northern Adriatic. There are two major membrane lipids PL and GL. PL mainly reside in the plasma membrane and many endoplasmic membrane systems, while GL are enriched in the chloroplast (Guschina and Harwood, 2009). We detected higher PL content per Chl *a* for the nutrient poorer station RV001. The same increased PL content per cell was observed for *C. pseudocurvisetus* grown in both P and N scarcity. We propose that PL quota for cells living in replete conditions is lower in comparison to depleted conditions. This could be due to PL dilution during higher rate of cell divisions in replete condition. Also, cell that are in nutrient stress, and cannot successfully divide, might store P in phospholipids (Abida et al., 2015). In case of favorable environmental conditions, P from PL could be re-allocated to any P containing molecule important for vital function(s) (e.g. DNA and RNA). Additional forms of P reserves for PL synthesis in cells are polyphosphates (Martin et al., 2014). Polyphosphates might be formed in cells that were pre-grown in nutrient replete conditions, and used during cell growth under investigated nutrient depleted conditions. GL accumulation was observed at temperatures higher than 19 °C in the northern Adriatic. This accumulation was explained as a mechanism to achieve thermal stability (Gašparović et al., 2013).

Scarcity of N is reflected in the increased content of TG per cell or per Chl *a*. *C. pseudocurvisetus* accumulates lipids in N-depleted medium, mostly due to significant increase of TG, approximately two times more than in other conditions. In situ samples respond in the same pattern, the oligotrophic northern Adriatic station RV001 showed higher TG production when compared to mesotrophic station SJ101. Increased TG/Chl *a* was observed at both stations for months when the lowest DIN concentrations were measured. Nonetheless, N-limitation can be overcome by nitrogen fixation by marine diazotrophs in oceans and seas (Capone, 2001). Diatoms are found to have N fixation (Poulton et al., 2009), N storage (Lomas and Glibert, 2000) and direct utilization of organic N (Morando and Capone, 2018) capabilities. A general trend of TG accumulation as a response to N-depletion has been observed in numerous taxa (Sharma et al., 2012; Thompson, 1996).

There is a synergetic impact of T rise and nutrient scarcity on lipid remodeling. The nutrient status appeared to be more important for lipid composition remodeling than T, at least within T range covered by our investigation. Lipid remodeling occurs in higher T range, in particular phospholipid remodeling, indicating that increased T interferes with PL biosynthesis. The contribution of PL and GL to the cell lipids

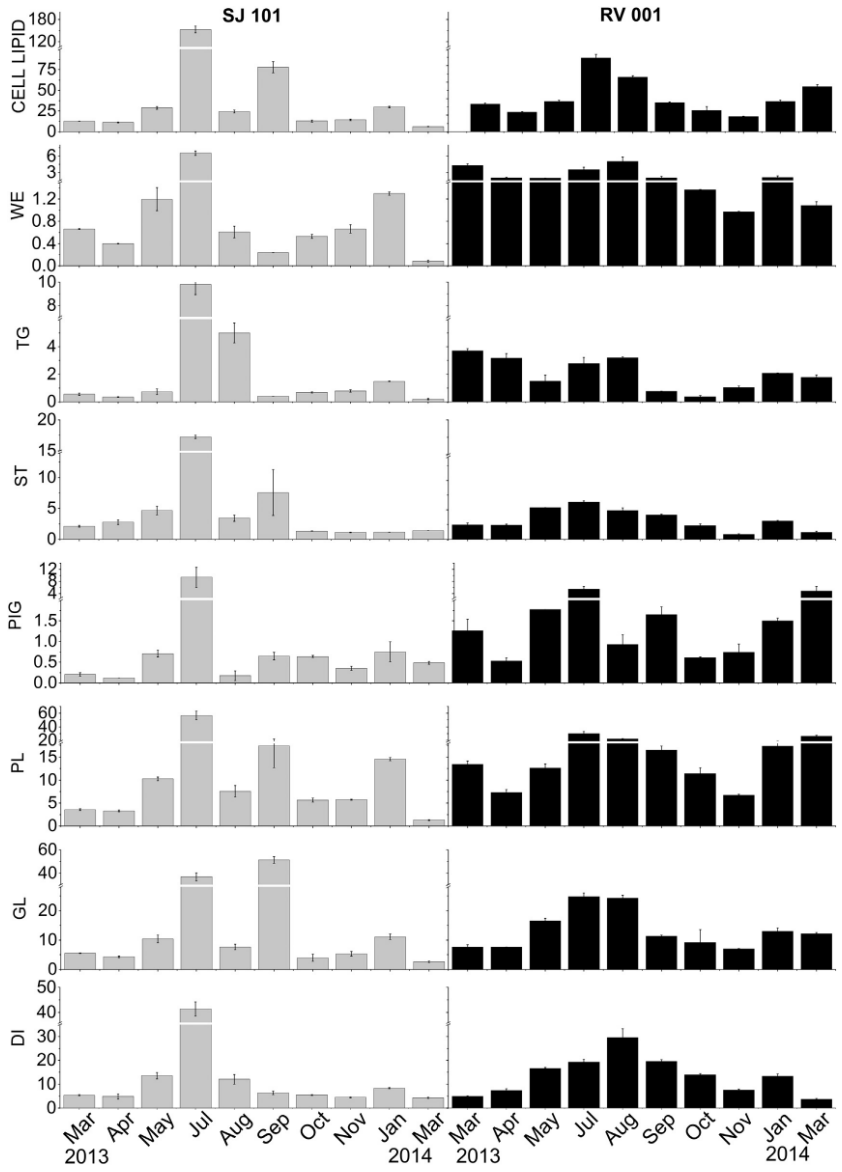


Fig. 7. Lipid production measured at stations SJ101 (left pannel) and RV001 (right pannel) of the northern Adriatic in the period from March 2013 to March 2014. All lipid values are given based on Chl *a*. Abbreviations: wax esters (WE), triacylglycerols (TG), sterols (ST), pigments (PIG), phospholipids (PL), glycolipids (GL), and degradation indices (DI).

interchange depends on T and nutrient availability. Decreased PL contribution to cell lipids, at the expense of GL, was promoted by P scarcity, this was observed for both the P depleted station RV001 and in P-depleted *C. pseudocurvisetus* cultures. At optimal T range (15–20 °C) lipid classes' distribution followed published trends. This entailed enhanced PL contribution in P favorable conditions in parallel with decline in GL (Van Mooy et al., 2006), and accumulation of TG that followed DIN depletion (Bourguet et al., 2009; Parrish and Wangersky, 1987).

C. pseudocurvisetus metabolism changed in dependence to nutrient availability. *C. pseudocurvisetus* used N and P in Redfield ratio 16.4 ± 0.2 indicating a balanced metabolism in the nutrient favorable medium. The metabolism was directed toward the synthesis of N-rich molecules

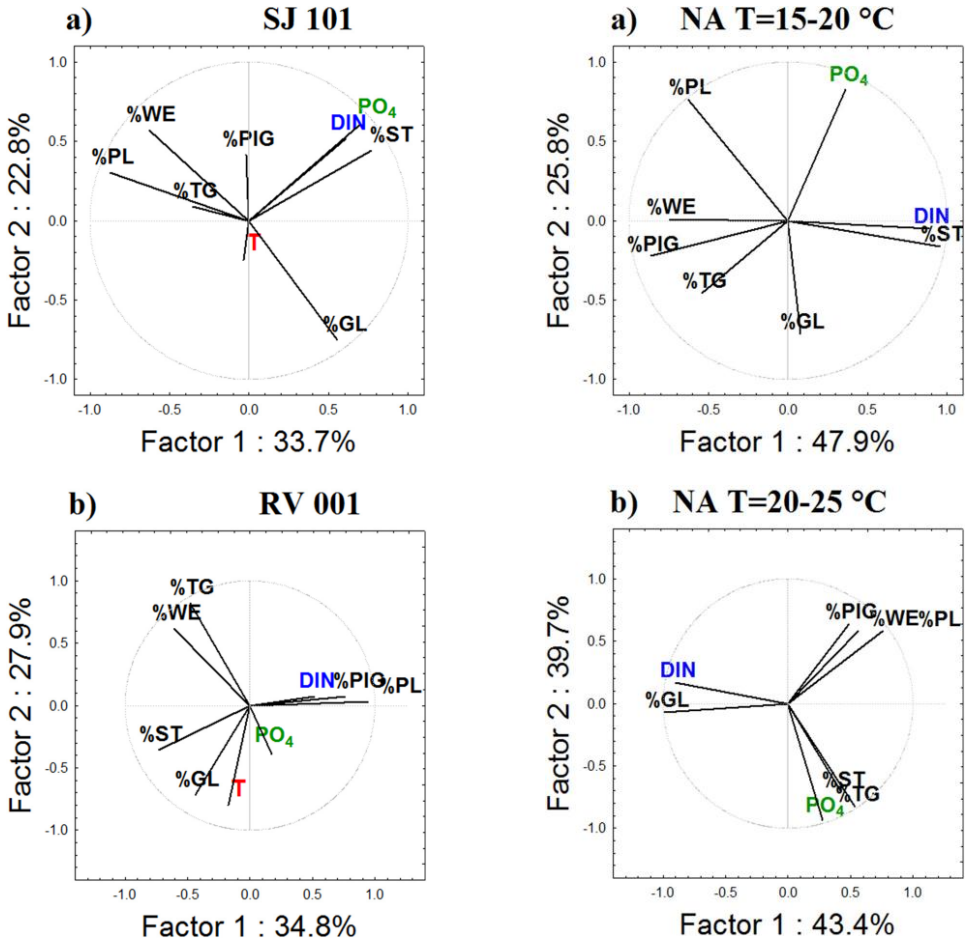


Fig. 8. Principal component Analysis (PCA) for variables; temperature (T), dissolved inorganic nitrogen (DIN), orthophosphate (PO4), contribution of cell lipid classes: triacylglycerol (%TG), wax esters (%WE), sterols (%ST), pigments (%PIG), phospholipids (%PL), glycolipids (%GL) measured at two northern Adriatic stations SJ101 (a) and RV001 (b).

Fig. 9. Principal component Analysis for variables; temperature (T), dissolved inorganic nitrogen (DIN), orthophosphate (PO4), contribution of cell lipid classes: triacylglycerol (%TG), wax esters (%WE), sterols (%ST), pigments (%PIG), phospholipids (%PL), glycolipids (%GL) measured for the both northern Adriatic stations for two temperature ranges, 15–20 °C (a) and 20–25 °C (b).

in the P-depleted medium (Grosse et al., 2017), indicated by the high ratio of N/P uptake (72.9 ± 4.2). Under these conditions cells have several mechanisms to overcome P-deficiency by itself, including alkaline phosphatase (AP) activation (Hoppe, 2003) (also employed by *C. pseudocurvisetus*) and PL substitution with GL: SQDG (Van Mooy et al., 2006, 2009; Martin et al., 2011), betaine lipid (Van Mooy et al., 2009; Martin et al., 2011), and DGDG (Hartel et al., 2000). While N-limitation with DIN concentration < 1 μmol/l (Justić et al., 1995) was occasionally noticed in the northern Adriatic (Gašparović et al., 2013), the northern Adriatic is a P-limited sea (Ivančić et al., 2016). Phytoplankton can form P reserves in the form of polyphosphates when P favorable conditions occur (Martin et al., 2014; Romans et al., 1994). The northern Adriatic phytoplankton community, especially diatoms, under P deficiency, induces great APA in order to obtain P from dissolved organic matter (Ivančić et al., 2012, 2016). *C. pseudocurvisetus* N/P uptake (2.6 ± 0.3) was low in N-depleted medium, indicating lower N-containing molecules synthesis (enzymes, proteins). This leads to TG accumulation, molecules containing only carbon, hydrogen and oxygen.

High T and oligotrophication influence cell carbon allocation toward the synthesis of carbon rich molecules, including lipids. This will be reflected in the modification of the carbon pump, as different biomolecules have different decay constants. The highest decay constant have carbohydrates and it further decreases from proteins to lipids (Benner and Amon, 2015). The efficiency of lipids in carbon sequestration might be antagonistic. Lipid buoyancy very likely enhances their retention in the surface layer of the ocean, where remineralization processes take place. However, their adsorption to particles (Morris and Eglinton, 1977) influences lipid removal to deeper ocean/sea layers via the sedimentation process. Accumulation of GL, as observed in nutrient poorer station RV001, may assist more successful lipid sedimentation. Namely,

GL are less attractive substrates for microbial degradation, due to their molecular composition (carbon, hydrogen, sulphur, and oxygen), without the essential P and N.

5. Conclusions

With this study, we can conclude that rising sea T caused enhanced lipid accumulation for the *Chaetoceros pseudocurvisetus* and northern Adriatic phytoplankton population. This effect was more pronounced in conditions of nutrient scarcity. Taking into account monoculture experiments, P scarcity enhanced lipid accumulation at higher T more than N depletion, while it was opposite for lower T. Between the two influential parameters on lipid synthesis, lack of nutrients had a greater role. We assume that oligotrophic seas and oceans, like tropical oceans and the eastern Mediterranean Sea, would be areas of higher lipid accumulation. Consequently, this change in carbon allocation will be reflected in the carbon pump alteration as different biomolecules have different biogeochemistry in water column.

CRediT authorship contribution statement

Tihana Novak: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Jelena Godrijan:** Data curation, Formal analysis, Investigation, Methodology, Writing - review & editing. **Daniela Marić Pfannkuchen:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing - review & editing. **Tamara Djakovac:** Data curation, Formal analysis, Investigation, Writing - review & editing. **Nikola Medić:** Data curation, Formal analysis, Investigation, Writing - review & editing. **Ingrid Ivančić:** Data curation, Formal analysis, Investigation, Methodology, Resources, Writing - review & editing. **Marina Mlakar:** Conceptualization, Funding acquisition, Resources, Supervision, Validation, Writing - review & editing. **Blaženka Gašparović:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing - original draft, Writing - review & editing.

Acknowledgements

We would like to thank Dr. Robert Precali for Chl *a* data. Jasna Jakovčević and Margareta Buterer are greatly acknowledged for sample collection and analyses of nutrients and Chl *a*. We thank Ivan Grabar for *C. pseudocurvisetus* cultures' nutrient analysis. We thank the crew of the R/V Vila Velebita for their professional help and support. We also thank Brittney Honisch and Dr. Catherine Mitchell for their help with English language edits. This work was funded by the grants from the Croatian Science Foundation under the projects IP-11-2013-8607 Appearance and interaction of biologically important organic molecules and micronutrient metals in marine ecosystem under environmental stress and Life strategies of phytoplankton in the northern Adriatic UIP-2014-09-6563.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.02.372.

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3.3 Publikacija III

Naslov: Impact of environmental conditions on phospholipid fatty acid composition

Autori: Vrana Špoljarić Ivna, Novak Tihana, Gašparović Blaženka, Kazazić Snježana, Čanković Milan, Ljubešić Zrinka, Hrustić Enis, Mlakar Marina, Du Jinzhou, Zhang Ruifeng, Zhu Zhuoyi

Časopis: Aquatic ecology

Godina objave: 2020.

DOI: https://doi.org/10.1007/s10452-020-09805-6

Aquat Ecol
https://doi.org/10.1007/s10452-020-09805-6

Impact of environmental conditions on phospholipid fatty acid composition: implications from two contrasting estuaries

Ivna Vrana Špoljarić · Tihana Novak · Blaženka Gašparović · Snježana P. Kazazić · Milan Čanković · Zrinka Ljubešić · Enis Hrustić · Marina Mlakar · Jinzhou Du · Ruifeng Zhang · Zhuoyi Zhu

Received: 3 May 2020 / Accepted: 20 October 2020

Abstract Phospholipid (PL) composition has a tremendous influence on the cell integrity and physiological competency. At the same time, plankton PL make important metabolic fuels for higher trophic levels. The goal of this study was to identify environmental control on PL production and their molecular identity of the suspended particles in two different estuaries. We conducted research in subtropical, eutrophic Wenchang River Estuary in China and temperate pristine, mesotrophic Krka River Estuary in Croatia. In agreement with the more abundant phytoplankton, PL concentrations were much higher in the Wenchang River Estuary (30.3–178.2 μg L^{-1}) than in the Krka River Estuary (8.4–18.8 μg L^{-1}). Given that six PL classes investigated (phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylserine (PS)) have different roles in the cell, we expected their different fatty acid composition in different environments. We found small differences in the fatty acid composition of PC, PG, and PI between two estuaries. These results suggest that the essential

Handling Editor: Télesphore Sime-Ngando.

I. Vrana Špoljarić · T. Novak · B. Gašparović (✉) · M. Čanković · M. Mlakar
Laboratory for Marine and Atmospheric Biogeochemistry, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia
e-mail: gaspar@irb.hr

S. P. Kazazić
Laboratory for Mass Spectrometry and Functional Proteomics, Division of Physical Chemistry, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia

Z. Ljubešić
Department of Biology, Faculty of Science, University of Zagreb, Rooseveltov trg 6, 10000 Zagreb, Croatia

E. Hrustić
Institute for Marine and Coastal Research, University of Dubrovnik, Kneza Damjana Jude 12, 20000 Dubrovnik, Croatia

M. Mlakar
Laboratory for physical chemistry of traces, Division for Marine and Environmental Research, Ruđer Bošković Institute, Bijenička cesta 54, 10000 Zagreb, Croatia

J. Du · Z. Zhu
State Key Laboratory of Estuarine and Coastal Research, East China Normal University, No. 500 Dongchuan Road, Shanghai 200241, People's Republic of China

R. Zhang
School of Oceanography, Shanghai Jiao Tong University, Shanghai 200030, People's Republic of China

Published online: 03 November 2020

fatty acid compositions of these PL in estuarine plankton are relatively constant in order to preserve membrane functions and/or cell processes in which they are involved regardless of environmental conditions. In contrast, PE, PA and PS fatty acid composition substantially differed between two estuaries as well as throughout the salinity gradient in each estuary. This suggests the adaptability of plankton to remodel these PL depending on the environmental conditions and the plankton community structure. Good environmental conditions (favorable N/P ratio, temperature) are important for increased PL content (% in POC and total lipids) in estuarine plankton and increased essential polyunsaturated fatty acid content in PL, which is beneficial to higher trophic levels.

Keywords Phospholipids · Fatty acids · Estuaries · Temperate · Subtropical · Phytoplankton pigments

Introduction

Coastal regions are considered key climate change hot spots worldwide (IPCC 2014). Estuaries are among the most productive environments on Earth. They receive substantial inputs of nutrients and organic matter from the mainland that support high rates of plankton metabolism and primary production (Cloern et al. 2014). This is reflected in higher trophic levels making estuaries a favorable environment for commercially important fish and shellfish farming. In general, estuaries are highly heterogeneous and complex ecosystems characterized by high biodiversity (Cloern et al. 2014; Muylaert et al. 2009).

Among the three major biochemical compounds, lipids, proteins, and carbohydrates, lipids are present in the lowest concentrations, but play disproportional roles in numerous essential biological processes (Arts et al. 2001). They are carbon-rich, with very high energetic value, thus representing important metabolic fuels for higher trophic levels (Lee et al. 1971; Parrish 1998). In addition, the molecular structures of lipids contain important heteroatoms, including phosphorus, nitrogen, sulfur and oxygen. For all organisms' life and growth, energy, space and nutrients are required. One of the key nutrients is phosphorus. It is assimilated into essential molecules, such as nucleic acids, ATP, and phospholipids (PL). Phospholipids are engaged in (i) establishing the permeability barrier for cells and cell organelles, (ii) providing the matrix for the assembly and function of a wide variety of catalytic processes, (iii) acting as donors in the synthesis of macromolecules, and (iv) actively influencing the functional properties of membrane-associated processes (Dowhan 1997; Dowhan et al. 2008).

Phospholipids, including phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylserines (PS), phosphatidylinositols (PI) and phosphatidic acids (PA), are predominantly located in extra-chloroplast membranes of the cell. Phosphatidylglycerols (PG) are the exception as they are the only PL located in thylakoid membranes, involved in photosynthetic transport of electrons (Wada and Murata 2007). Phosphatidylcholines and PE are the most abundant structural lipids in membranes, consisting of $\sim$ 68–80% of the total PL (van Meer et al. 2008), while PI, PA and PS are usually minor components of the total PL. They are important signal and regulatory molecules in phytoplankton cells. Phosphatidic acids and PS are also precursors for biosynthesis of other PL (Khozin-Goldberg 2016).

The content of PL in total lipids of selected algal species that belong to Haptophyta, Rodophyta, Chlorophyta and Bacillariophyta range from 1 to 52% (Guschina and Harwood 2009). Investigations of impact of temperature and nutrient availability on growth of diatom *Chaetoceros pseudocurvisetus* revealed that PL share in total lipids increases with temperature both in replete and phosphorus (P)-depleted conditions (Novak et al. 2019). Lipid content of marine bacteria is low, ranging from 1.7 to 7.3% of organic carbon, with PL as main lipids, ranging from 51 to 96% (Goutx et al. 1990a; 1990b). Phospholipids contain fatty acid residues of variable chain lengths and degrees of unsaturation. Lipid composition depends on the species and environmental conditions (Li et al. 2005; Guiheneuf et al. 2010; Schwenk et al. 2013; Hixson and Arts 2016; Hernando et al. 2018). Changes in the primary producers' essential polyunsaturated fatty acid content in aquatic environment may be an ecological risk for the higher trophic levels (Müller-Navarra et al. 2000). Omega-3 polyunsaturated fatty acids are essential nutrients with a wide range of health benefits. The most common marine omega-3 polyunsaturated fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), whose primary sources are marine algae and phytoplankton. Their absorption into tissues of organisms of

higher trophic levels is more efficient from PL than from triacylglycerols (Schunck et al. 2012).

In this study, we conducted an in-depth investigation of the PL in two contrasted estuaries. We combined those results with diverse environmental data. The Krka River Estuary (KRE) in Croatia and the Wenchang River Estuary (KRE) in China substantially differed in temperature and nutrient status, and consequently phytoplankton community structure. Using high-performance liquid chromatography (HPLC)/electrospray ionization (ESI) tandem mass spectrometry (MS/MS), we performed phospholipid fatty acid (PLFA) profiling of six most abundant phospholipids: PC, PG, PE, PA, PI and PS. This study aimed to address forcing variables responsible for PL abundance and to define the influence of different environmental conditions on PL composition. To the best of our knowledge, this is the first study that provides complete fatty acid profiling of the main six phospholipid classes in estuaries.

Methods

Study sites and sample collection

The Wenchang and Wenijao Rivers enter the WRE (Fig. 1a). It is a shallow system with a water depth of max 3 m (Liu et al. 2011). It is characterized by a tropical monsoonal climate. Its temperature is lower in the dry season (November–April) (23.3–28.7 °C) than in the wet season (May–October) (27.0–33.6 °C) (Li et al. 2014). Riverine input, groundwater discharge and aquaculture effluents are the major source of nutrients entering into the WRE (Liu et al. 2011). The concentrations of inorganic nutrients vary along salinity gradient and seasonally, being ~ 0–100 µmol L^{-1} NO_3^-, ~ 0–5.5 µmol L^{-1} NO_2^-, ~ 0–70 µmol L^{-1} NH_4^+, ~ 0–1.3 µmol L^{-1} PO_4^{3-}, and ~ 5–150 µmol L^{-1} SiO_4^{4-} (Liu et al. 2011). Chlorophyll *a* (Chl *a*) concentrations in WRE are tide and season (dry/wet) dependant, ranging from 0 to 27 µg L^{-1} (Herbect et al. 2011). Dissolved organic carbon (DOC) content in the WRE reaches the values up to 20 mg L^{-1} (Herbeck et al. 2013), indicating its highly eutrophic character.

The Krka River Estuary is a 25 km long estuary that spreads from the Skradinski Buk waterfalls to the Šibenik Channel (Fig. 1b). The water depth gradually increases from 5 m below the waterfalls to 43 m at the mouth. It is temperate estuary with annual temperature variations between 4.5 °C and 28.8 °C (Gržetić et al. 1991; Cetinić et al. 2006). The main sources of nutrients in this estuary are the Krka River, city of Šibenik (Gržetić et al. 1991), and numerous submarine groundwater discharges connected to the karst aquifer

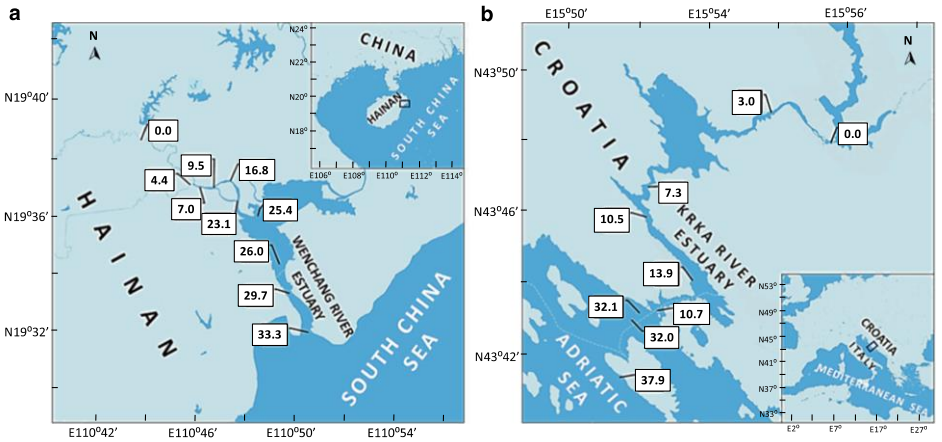

Fig. 1 Sampling stations named after corresponding salinities; **a** the Wenchang River Estuary (WRE) and **b** the Krka River Estuary (KRE)

(Liu et al. 2019). Measured nutrient concentrations vary within ranges of 0–59.2 µmol L^{-1} NO_3^-, 0–1.1 µmol L^{-1} NO_2^-, 0–13.2 µmol L^{-1} NH_4^+, 0–1.73 µmol L^{-1} PO_4^{3-}, and 0–65.8 µmol L^{-1} SiO_4^{4-} (Gržetić et al., 1991; Svensen et al. 2007). The KRE is highly stratified as a result of its sheltered position and low tidal movements (0.2–0.5 m) (Gržetić et al., 1991). The boundary layer between fresh and salty waters is characterized by a steep halocline that varies in thickness and depth, dependent on freshwater inflow and wind. Most of the primary production takes place in the 0.2–4 m brackish layer above the sharp halocline (Svensen et al. 2007). In situ primary production (measured in winter, autumn and late summer) varies mostly from 1 to 30 mg C m^{-3} h^{-1} (Gržetić et al. 1991). Seasonal distribution of phytoplankton biomass is characterized with highest biomass in spring and autumn–winter period and lowest during summer stratification, with Chl *a* ranging from 0.07 to 4.73 µg L^{-1} (Bužančić et al. 2012). Krka is a pristine river with DOC of only 0.5 mg L^{-1} (Louis et al. 2009), while DOC concentration in KRE is on average 1 mg L^{-1} (Lechtenfeld et al. 2013).

Samples were collected using 5-L Niskin bottles from the surface water (depth of 0.5 m) following the salinity (S) gradient from riverine end-member (S = 0) to marine end-member (S = 37.9 in the KRE and S = 33.3 in the WRE) (Fig. 1). Water sampling was performed from September 4th to 9th 2014 in the KRE and from May 8th to 10th 2015 in the WRE.

In this paper, our main focus was on estuaries, i.e., brackish waters with variable salinities (S) and inhabited by freshwater, estuarine, and marine water phytoplankton. Therefore, data obtained from the estuaries were exclusively discussed. Data obtained from freshwater and marine water end-member are also shown in the figures for comparisons, but are omitted from the discussion.

Basic environmental analysis

Temperature (T), salinity and pH of the KRE and WRE water samples were measured in situ by multiparameter probes HQ40D (Hach Lange, Germany) and Multi 350i (WTW, Geotech Environmental Equipment, Denver, USA), respectively.

Samples (50 mL) for the analysis of ammonium (NH_4^+) were stabilized by addition of 2 mL of phenol solution (1 mol L^{-1}; 95% ethanol) (Ivančić and Degobbis 1984) and stored in the dark at 4 °C. Samples (500 mL) for all other nutrients were stored at − 20 °C. The concentrations of total inorganic nitrogen (TIN) (TIN = nitrate (NO_3^-), nitrite (NO_2^-), and NH_4^+), and orthophosphate (PO_4^{3-}) were determined by spectrophotometric methods following Strickland and Parsons (1972).

Pigment analysis

While generally Chl *a* is used as a convenient proxy of phytoplankton biomass, many other phytoplankton pigments exhibit chemotaxonomic associations that might be used in the characterization of phytoplankton assemblages (Gibb et al. 2000). We have detected fucoxanthin (*fuco*, diatoms) and peridinin (*perid*, dinophytes), two marker pigments mostly associated with microphytoplankton. We found also marker pigments more typical of nanophytoplankton: chlorophyll c3 (*chl c3*, prymnesiophytes and chrysophytes), butanoyloxyfucoxanthin (*but*, chrysophytes), 19′–hexanoyloxyfucoxanthin (*hex*, prymnesiophytes), chlorophyll *b* (*chl b*, chlorophytes and prasinophytes), violaxanthin (*viola*, chlorophytes and prasinophytes), alloxanthin (*allo*, chrysophytes), and lutein (*lut*, chlorophytes and prasinophytes). Finally, we found also a typical picophytoplankton marker pigment zeaxanthin (*zea*, cyanobacteria) (Jeffrey and Vesk 1997).

For the pigment determination, 1 L of seawater was filtered through 0.7 µm Whatman GF/F filters preburned at 450 °C for 5 h and preserved in − 80 °C liquid nitrogen until the analysis. The extraction in 4 ml of cold 90% acetone was performed by sonication, and the extracts were collected by centrifugation. The composition of phytoplankton pigments, soluble in organic solvents, was analyzed by HPLC following the method by Barlow et al. (1997). Acetone extracts were mixed 1:1 (v/v) with 1 M ammonium acetate and injected into the HPLC system with 3-mm Thermo Hypersil-Keystone column MOS2, C-8, 120 A pore size, 150 × 4.6 mm (Thermo Hypersil-Keystone, Bellefonte, PA, USA). Pigments were separated at the flow rate of 1 mL min^{-1} using a linear gradient program with duration of 40 min by using solvent A and B. Solvent A consisted of 70:30 (v/v) methanol: 1 M ammonium acetate, while solvent B was 100% methanol. Chlorophylls and carotenoids were detected by the absorbance at 440 nm (SpectraSYSTEM,

Model UV 2000, Thermo Fischer Scientific, USA). Qualitative and quantitative analyses of individual pigments were performed by the external standard calibration using authentic pigment standards (VKI, Denmark).

Particulate organic carbon (POC)

For the POC determination, 0.12–1 L of estuarine water was filtered through 0.7 µm Whatman GF/F filters pre-burned at 450 °C for 5 h. A solid sample module SSM-5000A connected to a Shimadzu TOC-VCPH carbon analyzer calibrated with glucose was used for POC analysis. Concentrations of POC were corrected based on blank filter measurements. The average filter blank, including the instrument blank, corresponded to 5 µg C L^{-1}. The reproducibility for the glucose standard was 3%.

Lipid analysis

For the lipid class determination, 0.5–3 L of riverine/estuarine/seawater was collected in glass containers and passed through the 200-µm stainless steel screen to remove zooplankton and larger particles. It was followed by filtration through 0.7 µm Whatman GF/F filters pre-burned at 450 °C for 5 h. Particulate lipids were extracted by a modified one-phase solvent mixture of dichloromethane-methanol-water (Bligh and Dyer 1959). N-hexadecanone was added as internal standard to each sample to estimate the lipid recoveries in the subsequent steps of the sample analysis. The extracts were evaporated to dryness under nitrogen gas, stored at − 20 °C for 1 day and dissolved in 20 µL dichloromethane immediately before analysis.

Lipid classes were determined by thin-layer chromatography with flame ionization detection (TLC-FID) (Iatroscan MK-VI, Iatron, Japan). The classes were separated on Chromarods SIII and quantified by external calibration with standard lipid mixture, with a hydrogen flow of 160 mL min^{-1} and air flow of 2000 mL min^{-1}. The standard deviation determined from duplicate runs accounted for 1–14% of the lipid classes′ relative abundance. Eighteen lipid classes were detected by this technique (including hydrocarbons, wax and steryl esters, fatty acid methyl esters, ketone (standard hexadecanone), triacylglycerols, free fatty acids, alcohols, 1,3-and 1,2-diacylglycerols, sterols, pigments, monoacylglycerols, monogalactosyldiacylglycerols, digalactosyldiacylglycerols, sulfoquinovosyldiacylglycerols, mono- and di-phosphatidylglycerols, phosphatidylethanolamines and phosphatidylcholines). The separation scheme involved subsequent elution steps in solvent systems of increasing polarity followed by a subsequent partial burn of Chromarods. Total lipid concentrations were obtained by summing all lipid classes quantified by TLC-FID. Detailed procedures are described in Gašparović et al. (2015; 2017).

Separation of PL present in sample mixture was carried out using the UltiMate 3000 Rapid Separation HPLC (RSLC) (Dionex, Germany) system. Acquity UPLC BEH C18 (2.1 × 100 mm with 1.7 µm particles) (Waters, Milford, Massachusetts, USA) column was maintained at 50 °C, while gradient elution was employed. The solvent system included solution A: LC–MS grade methanol/ultrapure water (1:1, v:v; 10 mM NH4-acetate, 0.1% formic acid) and solution B: LC–MS-grade isopropanol (10 mM ammonium acetate, 0.1% formic acid). The gradient started from 55% A/45% B, reached 90% B in 40 min, 99% B in 2 min and remained there for 10 min, then to 45% B in 1 min, followed by equilibration for 22 min. The flow rate was 0.15 mL min^{-1}, and injected volume of sample mixture was 10 µL. Immediately before analysis, dichloromethane was evaporated and sample was redissolved in a solution of methanol: chloroform (1:2, v:v). HPLC system was online with amaZon ETD ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) for fatty acid composition analysis. The mass spectrometer was equipped with the standard ESI ion source (nebulizer pressure: 8 psi; drying gas flow rate 5 L min^{-1}; drying gas temperature 250 °C; the potential on the capillary ± 4500 V). The lipid profiling was performed in both positive and negative ion modes. The data were collected at a mass range of m/z = 100–1200. The ESI MS/MS was performed using collision energy of 1 eV. For PC species, the positive ionization mode was used ($[M + H]^+$), while for PE, PS, PI, PG and PA species negative ionization mode was used ($[M\text{-}H]^-$). For the discovery, annotation, and putative identification of phospholipids, we used an in-house assembled lipid library derived from LIPID MAPS (https://www.lipidmaps.org/). Here, composition of PL classes is discussed in terms of variety of PL molecular species.

We present phospholipid fatty acid profiling, ranging from 14 to 22 carbon atoms (C14–C22).

Data analysis

The principal component analysis (PCA) was performed in order to determine influence of environmental parameters on fatty acid composition of six targeted PL and to find out PL classes′ markers for phytoplankton groups revealed by pigment analysis. The PCA was performed using Statistica Release 7 software. PCA was carried out after log-transformation of the data to reduce the influence of extreme values or outliers. Due to the large number of PLFA variables (> 100), after preliminary PCAs that included all PLFA, the significantly correlated variables (factor loadings ≥ 0.5) were selected for further PCA. Variables that were discarded are listed in the Supplementary Tables S1a and b.

For the calculation of lipid contribution to POC, we assumed that carbon content of lipids was 70%.

Results

Environmental conditions

Temperature variations in the WRE were within a 3–4 °C range (28.1 to 31.5 °C) with a decreasing trend toward marine end-member (Fig. 2a). The KRE was characterized by temperature ranging from 21.9 to 26.2 °C, with a temperature increase toward marine end-member (Fig. 2b). Concentrations of TIN were significantly higher in the WRE (4.0–154.9 µmol L^{-1}) than in the KRE (1.9–5.8 µmol L^{-1}). The concentrations of PO_4^{3-} varied within 0.48–1.98 µmol L^{-1} and 0.21–0.69 µmol L^{-1} in the WRE and KRE, respectively (Fig. 2a and b). As a result, ratio of N/P was much higher, with wider range as well, in the WRE (2.2–232.5, average 77.6) than in the KRE (7.6–24.4, average 12.0).

Autotrophic plankton community was much more abundant in the WRE in comparison to the KRE (Fig. 3a and b). Concentrations of Chl *a* ranged from 2.45 to 87.71 µg L^{-1} (average 30.10 µg L^{-1}), and from 0.28 to 1.31 µg L^{-1} (average 0.85 µg L^{-1}) in the WRE and KRE, respectively.

The relative abundance of autotrophic plankton biomarker pigments differed for the two estuaries (Fig. 3a and b). Diatoms (pigment *fuco*) were the dominant phytoplankton group in both estuaries. In the WRE, besides diatoms, cyanobacteria (*zea*) dominated the community. The phytoplankton community substantially differed in the KRE. A significant contribution of the pigment *chl c3* suggested an important abundance of prymnesiophytes and chrysophytes. Among these two groups, chrysophytes were more abundant in less saline water according to pigment *lut*, while the substantial contribution of the pigment *hex* indicated important involvement of prymnesiophytes in the more saline waters.

POC and lipids

The concentrations of POC in the WRE and KRE varied between 1015 and 5363 µg L^{-1} (average 2869 µg L^{-1}) and 140 and 441 µg L^{-1} (average 274 µg L^{-1}), respectively (Fig. 4a and b). The total lipid (TL) concentrations varied between 128.6 and 661.0 µg L^{-1} (average 328.1 µg L^{-1}) and 27.7 and 49.3 µg L^{-1} (average 39.4 µg L^{-1}) in the WRE and KRE, respectively (Fig. 3a and b). Within the TL, the concentrations of PL ranged from 30.3 to 178.2 µg L^{-1} (average 89.4 µg L^{-1}) and from 8.4 to 18.8 µg L^{-1} (average 11.9 µg L^{-1}) in the WRE and KRE, respectively (Fig. 4a and b). Total lipid and PL carbon contributions to the POC content (TL_C and PL_C) in the WRE ranged from 5.4 to 12.1% (average 8.8%) and from 1.3 to 3.7% (average 2.4%), respectively. In the KRE, TL_C and PL_C ranged from 6.9 to 16.2% (average 11.1%) and from 1.7to 6.5% (average 3.5%), respectively (Fig. 4a and b). Phospholipids contributed more to TL in the KRE than in the WRE. Phospholipids constituted 22.4–31.0% (average 27.5%) and 17.0–39.9% (average 30.8%) of TL in the WRE and KRE, respectively.

Phospholipidomics

Phospholipid molecular diversity

Greater molecular diversity (Fig. 5a and d) was found in the WRE. For both estuaries molecular diversity decreased in the order of PC > PG > PA > PE > PI > PS, being on average 107 (110) PC species, 50 (31) PG species, 39 (16) PA species, 30 (15) PE species, 9 (11) PI species and 1 (2) PS species in the WRE (in parentheses for the KRE). Molecular

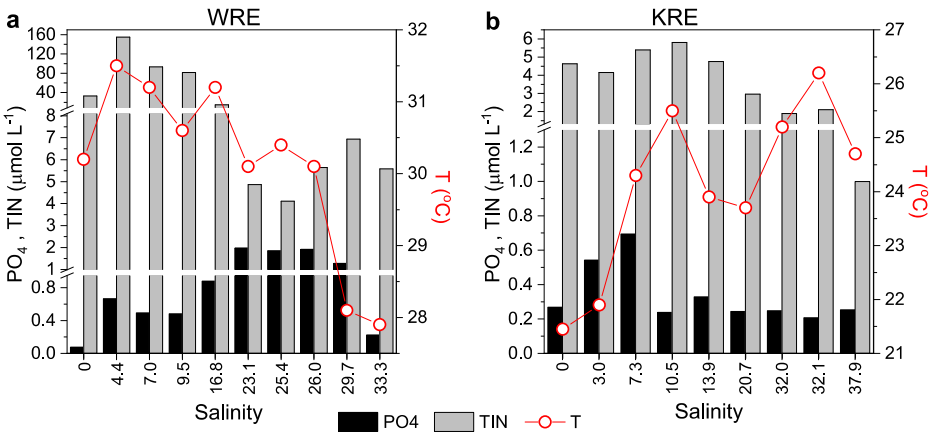

Fig. 2 Environmental properties in the **a** WRE and **b** KRE and their freshwater and marine water end-members: PO_4^{3-} (black column), TIN (grey column) and temperature (circles). (Color figure online)

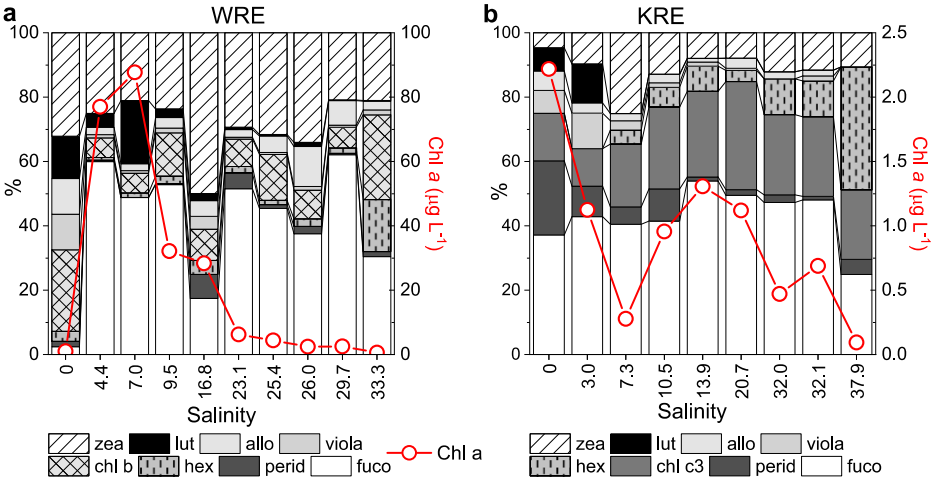

Fig. 3 Chlorophyll *a* content and the relative abundance of pigments of the major autotrophic plankton groups (%) in the **a** WRE and **b** KRE and their freshwater and marine water end-members. Note different scales for Chl *a* in two estuaries

diversity generally decreased toward the marine end-member, with the exception of PA and PE in the WRE.

Phospholipid saturation/unsaturation

The highest degree of unsaturation was observed for PG in both estuaries: double bonds 1.50–2.57 (average 2.09) and 1.57–2.35 (average 2.07) for the WRE and KRE, respectively (Fig. 5b and e). The greatest double bond variability was observed for PS (0–1.25 in the WRE and 0.50–2.50 in the KRE) and for PI (0.79–1.88 in the WRE and 0.79–3.00 in the KRE). The lowest double bond variability in both estuaries was observed within PC, 1.53–2.05 (average 1.70) in the WRE and

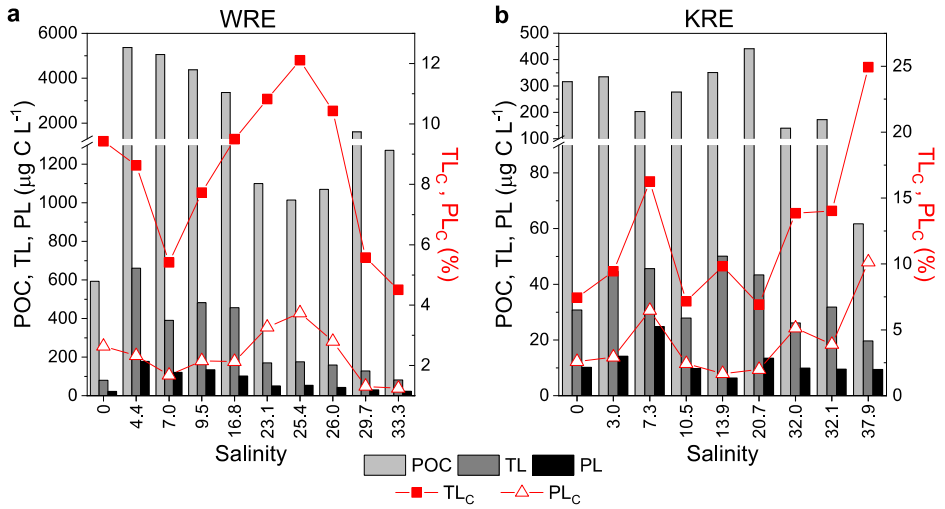

Fig. 4 Organic matter in the **a** WRE and **b** KRE and their freshwater and marine water end-members: particulate organic carbon (POC), total lipids (TL), phospholipids (PL), the contribution of TL carbon to POC (TL_C) and the contribution of PL carbon to POC (PL_C)

1.56–1.79 (average 1.66) in the KRE. Unsaturation of PE and PA in the WRE was characterized with double bonds 0.74–1.45 (average 1.10), and 1.35–2.26 (average 1.66), respectively. In the KRE, unsaturation of PE and PA included double bonds 1.00–2.00 (average 1.49), and 1.15–1.85 (average 1.59), respectively. On average, PL species unsaturation in the WRE (average double bonds 1.2–1.9) was lower than that in the KRE (average double bonds 1.4–2.4). The average polyunsaturated fatty acid relative content (%) (Supplementary Fig. S1) was highest for PG and PC in both estuaries, being 44.1 and 44.7% in the WRE, and 47.0 and 43.5% in the KRE, respectively. In comparison, the relative content of polyunsaturated fatty acids in PE was 19.1% in the WRE, and was much higher, 32.3%, in the KRE. In addition, average polyunsaturated fatty acid content in PA was 34.3 and 30.6% in the WRE and KRE, respectively. Their average relative contents in PI were 40.8 and 30.0%, whereas in a few detected PS it was 4.2 and 40.5% in the WRE and KRE, respectively.

The average relative distributions of double bonds in PL are shown in Fig. 6a and b. Detailed double bond relative distributions (all samples) are shown in Supplementary Fig. S2 for the WRE and KRE. Due to the few PS found in both estuaries, PS is omitted from Figs. 6, 7, 8, 9, and Supplementary Figs. S5 and S6. Common features for all samples of both estuaries are observed. Most common fatty acids in all PL were saturated and those with one double bond. Phosphatidylcholines contained more fatty acid acyl chains with three and four double bonds with respect to other PL, whereas PG contained predominantly fatty acyl chains with one double bond.

Phospholipid acyl chain length

Phospholipid fatty acyl chain lengths (acyl carbon number) varied among stations and different PL in the WRE with no apparent patterns (Fig. 5c and f). Phospholipid PI were characterized by the average longest fatty acyl chain lengths in both estuaries (18.8 and 19.0 in the WRE and KRE, respectively). In comparison, average fatty acyl chain lengths of PC, PG and PA were 18.0, 17.9, and 17.8, respectively, in the WRE, and 17.7, 18.2 and 17.6, respectively, in the KRE. The average fatty acyl chain lengths of a few PS detected were 16.7 and 17.0 in the WRE and KRE, respectively. The regularity and general trend were observed for the KRE: the longest fatty acyl chain

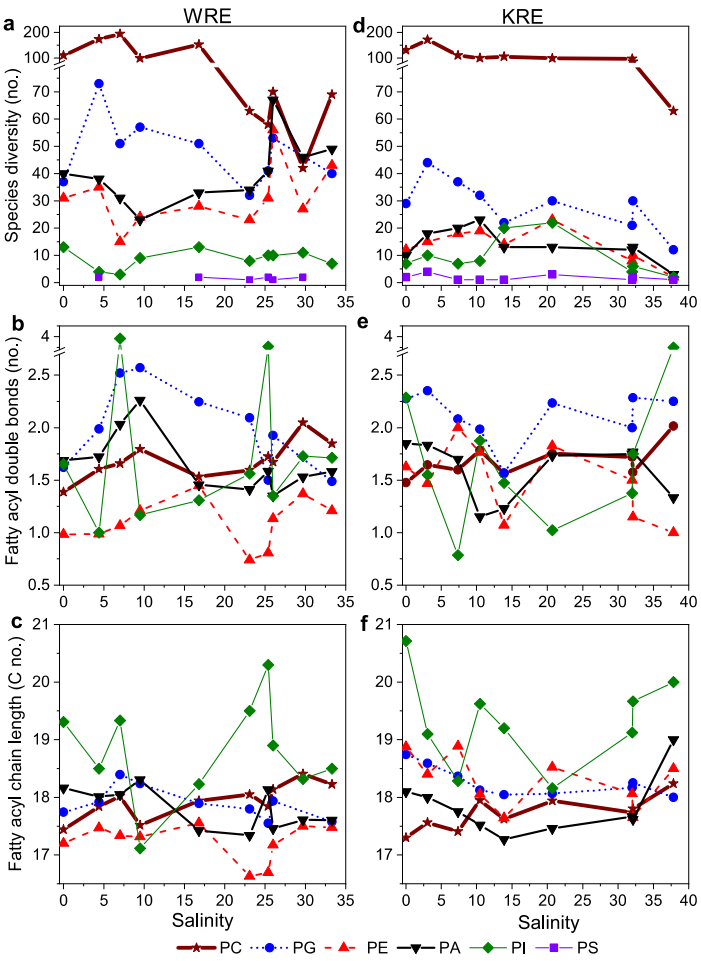

Fig. 5 The average phospholipid characteristics in **a–c** the WRE and **d–f** the KRE and their freshwater and marine water end-members. **a** and **d** the number of molecular species (diversity), **b** and **e** fatty acyl double bond (number of DB) and **c** and **f** fatty acyl chain length (number of carbon atoms) of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI) and phosphatidylserines (PS). Due to very few PS species, it is omitted from Fig. 5b,c,e and f

lengths were detected in freshwater and marine end-members, while fatty acyl chain lengths were on average shorter in estuarine waters.

The relative distributions of PL average number of fatty acyl chain lengths are shown in Fig. 6c and d. Detailed PL fatty acyl chain lengths and relative distributions (all samples) are shown in Supplementary Fig. S3 for the WRE and KRE. Generally, fatty acids with 18 carbon atoms (C18) were most common in PG and C22 in PI.

Phospholipid fatty acid composition

The relative distribution of identified fatty acids (%) within each PL class is presented in Fig. 7 and in Supplementary Tables S2-S7 for the WRE and KRE. The average relative distributions are shown in Supplementary Fig. S4 and Supplementary Table S8. The composition of fatty acids in PC (Fig. 7a, Supplementary Table S2) was similar for the two estuaries and deviation in the content of individual

fatty acid did not affect the uniformity of the general pattern. The most common fatty acids in PC were 16:0, 16:1, 18:1, 18:2, 18:3 and 18:4, while PG (Fig. 7b, Supplementary Table S3) were characterized by fatty acid 18:1 with the substantial contribution of 16:1 and 20:5. A pattern appeared also for the PE, PA and PI (Fig. 7c, d and e, respectively, and Supplementary Tables S4–S6). Fatty acids 16:0, 16:1, 18:0 and 18:1 were most common in PE, while 16:0, 16:1 and 18:1 were most common in PA. Phosphatidylinositol was found to be enriched with fatty acids 22:0 and 22:6 with respect to other PL. The fatty acid relative distributions were mainly retained from the river across the estuaries to the nearby sea, particularly for PC and PG.

The average contribution of odd-chain fatty acids, which are bacterial biomarkers (Dalsgaard et al. 2003), was 8.7% in both estuaries (PS is omitted from the calculation) (Supplementary Fig. S4 and Supplementary Table S8). The content of odd-chain fatty acid varied in individual PL; it was the lowest in PG (average 8.3 and 1.7%), average 15.2 and 7.4% in PE, and average 10.4 and 11.3% in PA for the WRE and KRE, respectively. Their content was on average 8.3% in PC for both estuaries. The contribution of odd-chain fatty acids to total fatty acids fluctuated considerably in PI and PS, from 0% to 29.5% in PI and from 0 to 50% in PS for both estuaries.

We were interested whether any PL contained higher percentages of eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) (Fig. 7

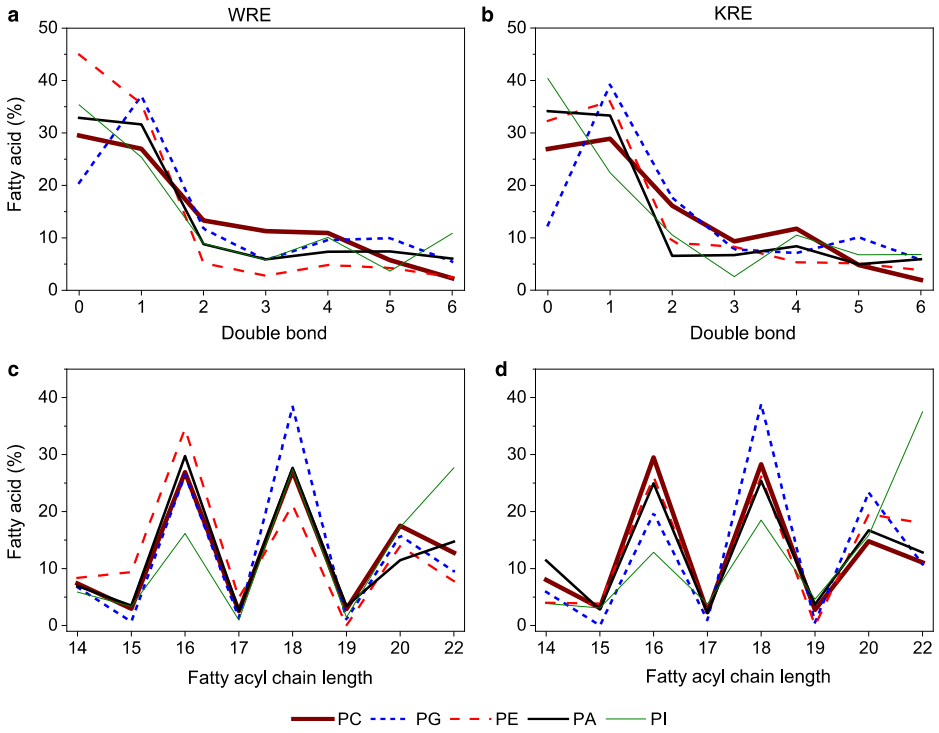

Fig. 6 **a** and **b** The average relative distribution of double bonds (%) and **c** and **d** fatty acyl chain length (number of carbon atoms) (%) of phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidic acid (PA) in **a** and **c** the WRE and **b** and **d** the KRE

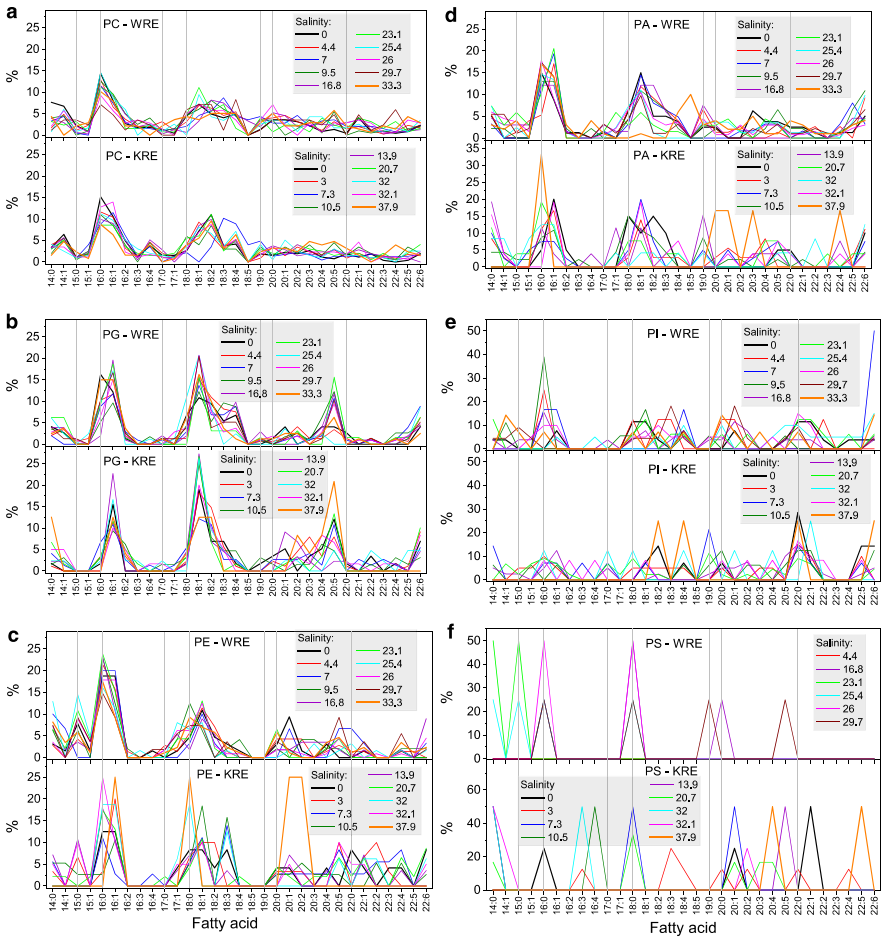

Fig. 7 Phospholipid fatty acid relative distribution (%) in the Wenchang River Estuary (WRE) and the Krka River Estuary (KRE), and their freshwater and marine water end-members. Data are presented for **a** phosphatidylcholine (PC), **b** phosphatidylglycerol (PG), **c** phosphatidylethanolamine (PE), **d** phosphatidic acid (PA), **e** phosphatidylinositol (PI), and **f** phosphatidylserine (PS)

and Supplementary Fig. S5). EPA contributed to fatty acid composition on average 3.3, 9.2, 4.1 and 3.0% in PC, PG, PE and PA in the WRE and on average 2.4, 7.8, 5.5 and 4.8% in PC, PG, PE and PA in the KRE, respectively. In comparison, the contribution of DHA was 2.2, 6.0, 2.8 and 6.4% in PC, PG, PE and PA, respectively, in the WRE, and 2.0, 6.5, 4.3 and 6.6% in PC, PG, PE and PA, respectively, in the KRE. EPA was the dominant polyunsaturated fatty acid in PG with respect to other PL in the eutrophic WRE samples and was often the dominant polyunsaturated fatty acid in PG in the mesotrophic KRE samples. Occasionally, PI could be a source of DHA.

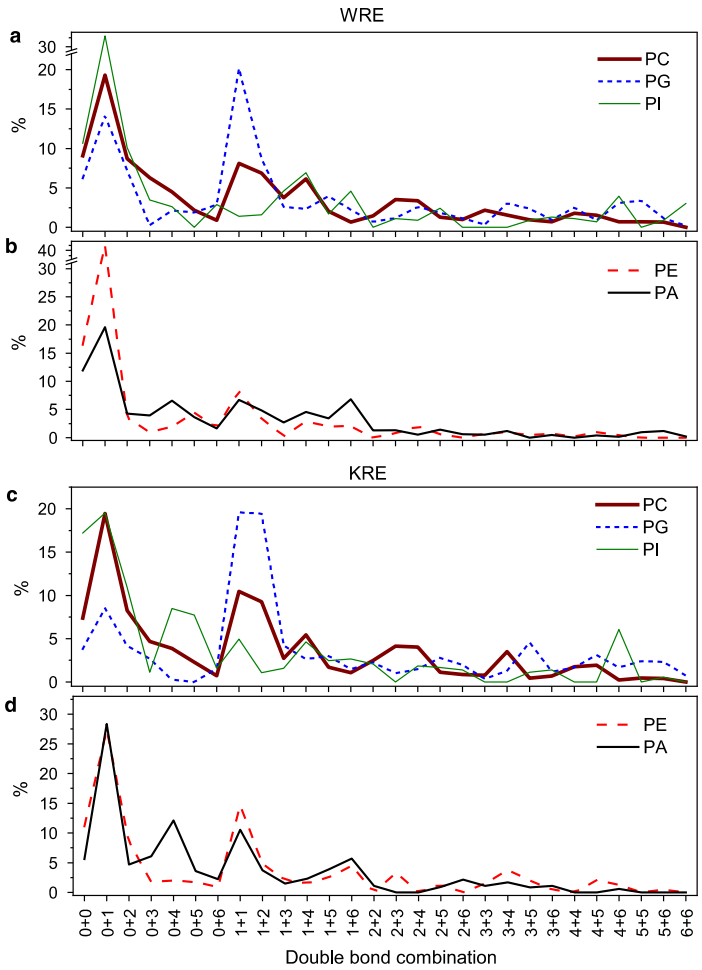

Fig. 8 The average relative distribution of fatty acids' double bond combinations (%) of **a** and **c** phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) and **b** and **d** phosphatidylethanolamine (PE) and phosphatidic acid (PA) in **a** and **b** the WRE and **c** and **d** the KRE

The combinations of fatty acyl double bonds and chain lengths

The average relative distribution combination of double bonds in two fatty acyl chains and the combination of two fatty acyl chain lengths are presented in Figs. 8 and 9 and Supplementary Tables S9 and S10 for the WRE and KRE. Details for all samples are given in Supplementary Figs. S8 and S9 and Supplementary Tables S11–S22. Again, common features were noticed. The dominant double bond combination of two fatty acid chains in PC, PE, PA and PI was one saturated and one unsaturated fatty acid with one double bond (0 + 1), while PG was

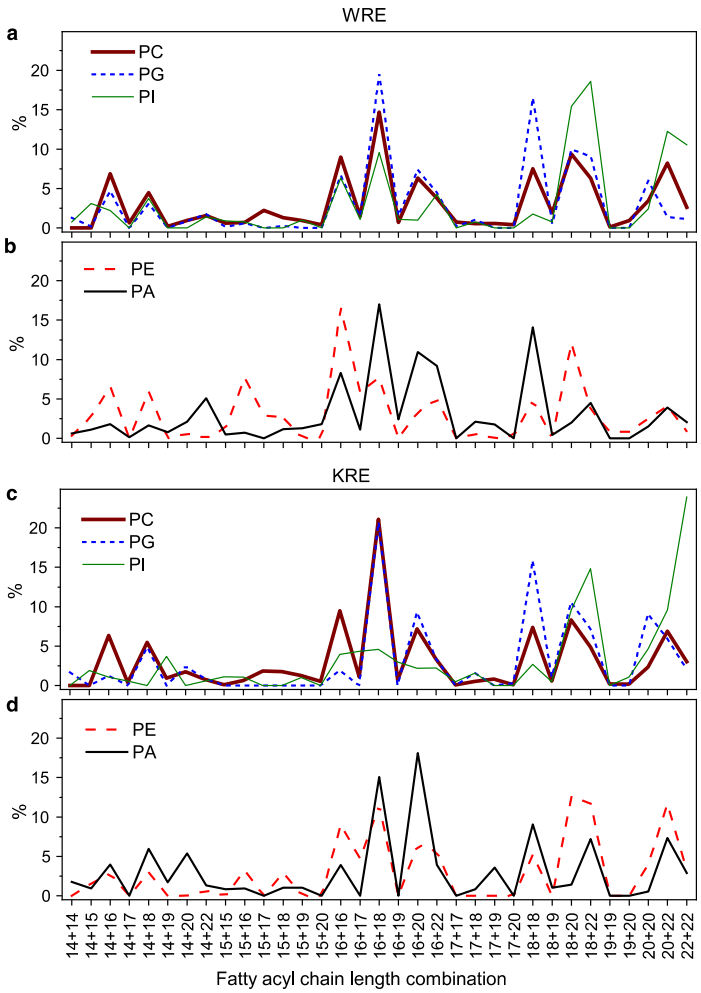

Fig. 9 The average relative distribution of fatty acyl chain length (number of C atoms) combination (%) of **a** and **c** phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylinositol (PI) and **b** and **d** phosphatidylethanolamine (PE) and phosphatidic acid (PA) in **a** and **b** the WRE and **c** and **d** the KRE

characterized by double bond combination 1 + 1 being the most common species (Fig. 8 and Supplementary Tables S11-S16).

The relative distribution of the fatty acyl chain length combination (%) exhibited similarities between two estuarine systems (Fig. 9, Supplementary Fig. S9 and Supplementary Tables S17–S22). This is especially evident in PC for which the combination of fatty acyl chain lengths C16 and C18 (16 + 18) was the most common. PG was characterized by the predominance of fatty acid combination 16 + 18 and 18 + 18. Combinations of longer fatty acid chains

dominated within PI, whereas fewer similarities were evident for PE and PA.

Influence of environmental conditions on phospholipid fatty acid composition

The PCA incorporating all PLFA data and variables including temperature, salinity, pH, oxygen, Chl *a*, TIN and PO_4^{3-} concentrations were performed to define possible influence of environmental conditions on fatty acid composition of six targeted PL. The analyses showed the different effects of environmental parameters on the fatty acid composition of PL for the two estuaries (Supplementary Fig. S6). The greatest positive PC1 loadings for the WRE samples had temperature, TIN, Chl *a* and O_2 with fatty acid 18:3 in PC, fatty acids 18:3, 18:4, 20:3 and 22:2 in PG, fatty acids 16:0 and 20:2 in PE and 18:2, 18:3, and 22:4 in PA. For the KRE samples, the greatest positive PC1 loadings had TIN, Chl *a* and O_2 with fatty acids 14:0, 15:1, 16:2, 17:0 and 22:2 in PC, fatty acids 18:3 and 22:6 in PG, fatty acid 16:0, 18:1 and 20:4 in PE, fatty acids 16:1 and 18:1 in PA. The greatest negative PC1 loadings for the WRE samples had salinity and fatty acids 15:0, 22:3, 22:4 and 22:6 in PC, fatty acid 18:1 in PG, fatty acid 15:0 in PE, fatty acid 20:0 in PA. The greatest negative PC1 loadings for the KRE samples had salinity and fatty acids 15:0, 20:3, 20:4, 20:5, 22:0 and 22:4 in PC, fatty acids 14:0, 20:2, 20:4 and 20:5 in PG, fatty acids 16:1, 18:0, 20:3, 20:4, 22:0 and 22:4 in PE and fatty acids 16:0 and 20:1 in PA. The greatest positive PC2 loadings for the WRE samples had PO_4^{3-}, fatty acid 22:2 in PC, fatty acids 14:0 and 16:1 in PG, fatty acids 15:1 and 17:1 in PE, fatty acids 14:0, 15:0 and 20:1 in PA. For the KRE samples, the greatest negative PC2 loadings had fatty acids 14:1, 15:1, 16:4 and 18:3 in PC, fatty acid 18:0 in PG, fatty acid 22:4 in PE. These analyses suggest that only common feature for the two estuaries was increased contribution of longer chain fatty acids (C20 and C22) at higher salinities.

Analysis of possible phospholipid fatty acids as specific phytoplankton group(s) markers

Intending to define possible PC, PG, PE and/or PA fatty acids as specific markers for phytoplankton group(s), we performed PCA considering estuarine phytoplankton marker pigments and PLFA variables. After preliminary PCA, the significantly correlated variables (factor loadings ≥ 0.5) were selected for further PCA (Supplementary Fig. S7). For both estuaries, correlations of PLFA with some pigment were observed. At the same time, many pigments did not show any significant correlation with PLFA, including *fuco*, *allo*, *hex* and *viola* in the WRE and *allo* and *zea* in the KRE. Different PLFA correlated with the particular pigment for the two estuaries. For example, *perid* was grouped with PC22:2 in the WRE, while *perid* was grouped with PC14:1, PA18:0 and PA18:2 in the KRE.

Discussion

Phytoplankton play central roles in food webs and global cycling of elements (C, P, N). Changes in their cellular compositions affect organic matter flux both to higher trophic levels and to deep waters (Falkowski et al. 2004). In this paper, we focused on the plankton PLFA composition of two very different estuaries with regard to temperature, nutrient loads, and consequently different phytoplankton communities. Data on the total lipid-derived fatty acid compositions in the estuaries, seas/oceans and phytoplankton monocultures are relatively abundant (e.g., Scribe et al. 1991; Galois et al. 1996; Derieux et al. 1998; Canuel 2001; Pedrosa-Pamies et al. 2018). However, data on total PLFA are scarce (Table 1a). Here, we took the step forward of carrying out a more detailed PLFA characterization in two estuaries by analyzing the fatty acid composition of individual PL including PC, PG, PE, PA, PI and PS.

Consistent with significantly higher nutrient concentrations, autotrophic plankton abundance, according to the Chl *a* content, was much higher in the eutrophic WRE than in the KRE. In general, eutrophic coastal regions in the tropics are sites of high phytoplankton biomass (Cotovicz et al. 2018). In line with very different environmental conditions, the phytoplankton community differed between the estuaries, but also across salinity gradient in each estuary. However, it is not surprising that we found dominance of diatoms in both estuaries knowing that coastal river plumes are in general the places of diatom growth owing to the continuous nutrient inputs (Wawrik and Paul 2004). The abundance of cyanobacteria in the

Table 1 Literature review on phospholipids

(a) Total phospholipid fatty acid investigations

PLFA use	Location	References
Determination of the major biogenic contributors to the surface and underlying waters	The Mediterranean Sea	Brinis et al. (2004)
Identification of the sources of organic matter	The York River Estuary, USA	Palomo and Canuel (2010)
Estimation of the plankton community structure	The Scheldt Estuary, border region between Belgium and the Netherlands	Boschker et al. (2005)
Determining microbial community structures	The upwelling area, northern Chile	Espinosa et al. (2009)
Identification of the sources of carbon	The Gulf of Carpentaria, Australia	Rothlisberg et al. (1994)
Identification of the biomarkers to quantify bacterial biomass and to characterize microbial community structure	A wide variety of sediments, ranging from intertidal to coastal, shelf and deep-sea sediments, waters, and soils	Kunihiro et al. (2014)
Identification of the chemotaxonomic markers for phytoplankton species	The Scheldt Estuary, border region between Belgium and the Netherlands	Dijkman and Kromkamp (2006)

(b) Total phospholipid concentrations

Concentrations ($\mu g\ L^{-1}$)	Trophic status	Location	References
0.87–1.23	Oligotrophic	The Mediterranean Sea	Brinis et al. (2004)
~ 20–800	Highly eutrophic	The Scheldt Estuary, border region between Belgium and the Netherlands	Dijkman and Kromkamp (2006)
0.6–5.4	–	The upwelling area, northern Chile	Espinosa et al. (2009)
0.88–16.65	–	The southern North Sea	Le Guitton et al. (2017)
30.3–178.2	Eutrophic	The Wenchang River Estuary, China	This study
8.4–18.8	Mesotrophic	The Krka River Estuary, Croatia	This study

(c) Phospholipid content in total lipids

Content (%)	Trophic status	Location	References
6–20	–	The surface mixed layer near the edge ofthe Scotian Shelf and fromBedford Basin	Parrish and Wangersky (1988)
8–39	Oligotrophic	The west Mediterranean Sea	Gérin and Goutx (1994)
20–50	Eutrophic	The marine Rogoznica Lake	Penezić et al. (2010)
11–55	A wide range of trophic states	The East Atlantic Ocean	Gašparović et al. (2014)
29–39	–	The nearshore and offshore waters of a Great Lake	Smith et al. (2007)
22–31	Eutrophic	The Wenchang River Estuary, China	This study
17–46	Mesotrophic	The Krka River Estuary, Croatia	This study

WRE reflects influence of high temperature on their dominance (Mesquita et al. 2020).

Here we assumed that PLFA were mainly of autotrophic plankton origin (Gašparović et al. 2014). However, the contribution of heterotrophic bacteria cannot be neglected. The average contribution of bacterial fatty acid markers (odd chain fatty acids) to total fatty acids was 8.7% in both estuaries, which was considerable. Total PL content is dependent on plankton biomass; consequently, higher

concentrations of PL were detected in the eutrophic WRE (30.3–178.2 μg L^{-1}) than in the mesotrophic KRE (8.4–18.8 μg L^{-1}). Literature and our data on PL concentrations (Table 1b) expectedly show that total PL content increases from the oligotrophic to eutrophic aquatic environment.

The PL relative content (%) in plankton is influenced by environmental conditions and is species specific (Guschina and Harwood 2009). The PL content in TL in different aquatic environments spans across a wide range, 6–55% (Table 1c). Here, PL relative content (averages 27.5 and 30.1% in TL in the WRE and KRE, respectively) is in accordance with earlier findings. Higher PL relative content (%) in both POC and TL was obtained in the KRE than in the WRE (i.e., more PL synthesized per Chl *a* in the KRE than in the WRE). These results indicate importance of more favorable N/P ratio in the KRE than in the WRE. Unfavorable environmental conditions lead to phytoplankton lipid remodeling. Phytoplankton development under oligotrophic conditions, especially during nitrogen deficiency, leads to the triacylglycerol accumulation at the expense of other lipid classes, including PL (Parrish and Wangersky 1987; Bourguet et al. 2009; Novak et al. 2019). Phosphorus limitation and high seawater temperatures lead to enhanced glycolipid instead of phospholipid accumulation (Gašparović et al. 2013).

Here found large PL molecular diversity, especially within PC, possibly reflects a complex community (freshwater, estuarine and marine), as well as their responses to fluctuations in environmental conditions, e.g., salinity, nutrient concentrations, light intensity, temperature, ...

Although we investigated two notably different estuaries, similarities in the PLFA composition, number of double bonds and fatty acyl chain lengths were observed, particularly for PC, PG and PI. We assume that plankton maintains basic PC, PG and PI fatty acid composition to preserve the roles they play in the cell. At the same time, the composition of other, less common, fatty acids in PC, PG and PI differed between stations and the estuaries. The main PC features that were essential for optimal function in estuarine plankton membranes were: (1) fatty acids 16:0, 16:1, 18:1, 18:2, 18:3 and 18:4, (2) higher content of unsaturated fatty acids with three and four double bonds with respect to other PL, (3) dominance of double bond combination in two fatty acid chains $0 + 1$, and (4) relative invariability of total unsaturation with respect to other PL. These could indicate the conservatism of cellular PC synthesis in terms of preserving the integrity of the cell itself and maintaining the physicochemical properties of membranes, at least for the environmental conditions in estuaries covered by this study and phytoplankton groups detected.

The only PL present at measurable level in thylakoid membranes are PG (Wada and Murata 2007). Higher fatty acid unsaturation in PG, with respect to other investigated PL, can be expected given the role of PG in photosynthetic electron transport in thylakoids (Wada and Murata 2007), knowing that fatty acid unsaturation improves photosynthetic electron flux across more liquid thylakoid membrane (Siegenthaler and Murata 2004). The most important PG fatty acids 18:1, 16:1 and to a lesser extent 20:5 were probably favorable for maintaining thylakoid membrane function. It seems that double bond combination $1 + 1$ and fatty acyl chain length combinations $16 + 18$ and $18 + 18$ were basic in PG and important for PG proper functioning.

Long-chain fatty acids C20 and C22, were probably important for PI to successfully conduct particular cellular function(s), including the role in cell growth, signal transduction processes and membrane anchoring of proteins in plants (Riekhof and Benning 2009).

The great variability in fatty acid saturation/unsaturation and chain lengths of PE, PA, and particularly PS indicate that fatty acid composition of those PL is species-specific, dependent on the plankton growth phase and/or a response to diverse environmental conditions.

Two investigated estuaries differed, among others, in water temperature. Since unsaturated fatty acids are important in adjusting membrane fluidity, it is suggested that the degree of unsaturation of the membrane lipid fatty acids increases with temperature decrease (Murata and Los 1997). Our data on fatty acid unsaturation in the two estuaries, i.e., higher polyunsaturated fatty acid contribution to total fatty acids detected in the colder KRE, are in the agreement with previous reports. However, the fatty acid composition is influenced by season, as well. Connelly et al. (2016) found more saturated fatty acids during winter in the Beaufort Sea shelf and explained by fatty acid cycling and/or fatty acids from heterotrophs.

Statistical PCA indicated that the increase in salinity possibly influenced the increased proportion of long chain fatty acids in PL. Fatty acid remodeling is continuous process that is triggered by changing environmental conditions (e.g., Urzica et al. 2013), and depends on the phytoplankton growth phase (e.g., Boelen et al. 2017). At the same time, fatty acid remodeling can be accomplished very fast, within an hour or two (Urzica et al. 2013; Rai and Gaur 2001). Therefore, we concluded that due to the complex and different environmental conditions and plankton community structures in the WRE and KRE we did not identify particular environmental parameters responsible for the synthesis of specific fatty acid(s) of six PL classes investigated. Also, as indicated by the PCA analysis (Fig. S7), we could not define particular PLFA marker(s) for phytoplankton group(s), most likely due to complex phytoplankton composition and different environmental conditions in the WRE and KRE.

Autotrophic plankton is the origin of the long-chain omega-3 polyunsaturated fatty acids EPA and DHA to higher trophic levels. They have key roles in the marine species growth and are critical to their survival (Jónasdóttir 2019). We found that there was no significant difference between two estuaries regarding EPA and DHA proportion in PL. Eventually, PG and PI might be an important source of EPA and DHA, respectively.

Conclusions

Herein, we present the comprehensive analysis of six main phospholipids (PC, PG, PE, PA, PI and PS) at the level of individual lipid species, for the two notably different estuaries, the pristine and temperate Krka River Estuary and the eutrophic, subtropical Wenchang River Estuary. Favorable nutrient conditions, found in the KRE, lead to enhanced PL content of plankton, which are at the same time richer in polyunsaturated fatty acids that consequently benefit higher trophic levels.

Estuarine plankton maintains favorable PC, PG and PI fatty acid composition for optimal membrane function(s) in which these PL are involved, irrelevant of the environmental conditions and plankton community structure. This suggests that mechanisms of preserving essential fatty acid composition is universal, despite probable energy investment by the cell due to the advantages of maintaining fine control of cell functioning. It is indicated that several features are preferable for the role of PG, including higher acyl chain unsaturation with respect to other PL. Regarding PC and their functions, important are C16 and C18 fatty acids. PC are characterized by lower variability of total unsaturation with respect to other PL. In comparison, long-chain fatty acids (C22 and C20) seem to be important for the roles of PI in the estuarine plankton cells. The fatty acid composition of PE, PA, and PS differed between the estuaries as well as throughout the salinity gradient in each estuary. This suggests the adaptability of plankton to remodel these PL depending on the environmental conditions as well as difference in the plankton community structure.

Acknowledgements This work was funded by the grant from the Croatian Science Foundation under the project IP-11-2013-8607, by a grant of the Bilateral collaboration between Croatia and China (No. 6–8 project in 2013), and by Ministry of Science and Technology in China (No. 2014CB441503). The authors thank Dr. Jelena Dautović for carrying out POC analyses of samples from the Krka River Estuary.

Author contributions IVŠ and SK did HPLC/MS/MS lipid and data analysis, TN analyzed and processed TLC-FID lipid data, MČ did environmental data analysis, ZLj analyzed pigments, EH and RZ performed nutrient analysis. BG, MM and ZZ conceived, planned and initiated the study; TN, MČ, JD, RZ, ZZ and BG performed field sampling, BG and IVŠ wrote the first manuscript draft. All authors discussed the results, edited the manuscript and approved the final submitted manuscript.

Funding This work was funded by the grant from the Croatian Science Foundation under the project IP-11-2013-8607, by a grant of the Bilateral collaboration between Croatia and China (No. 6–8 project in 2013), and by Ministry of Science and Technology in China (No. 2014CB441503).

Data availability Data are available from the corresponding author on reasonable request.

Code availability Not applicable.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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4 RASPRAVA

Obzirom da su lipidi, za razliku od ostalih organskih molekula, najstabilniji (Benner i Amon, 2015) promjena u sintezi organskih molekula stanica fitoplanktona utječe na biološku pumpu. Lipidi imaju moguću dvostruku ulogu u uklanjanju organske tvari u moru. Dosta su plovni te se mogu dulje zadržavati u površinskim dijelovima, gdje dolazi do remineralizacije. No oni se također uspješno vežu za čestice što pospješuje proces sedimentacije te njihovo uklanjanje iz površinskih slojeva (Morris i Eglinton, 1977).

Dosadašnje studije istraživale su utjecaj stresa na metabolizam lipida fitoplanktona, ali su rijetko uzimale u obzir i *in vivo* i *in situ* eksperimente (npr. Abida i sur., 2015; Opute, 1974; Toseland i sur., 2013; Van Mooy i sur., 2009). Kako bismo što detaljnije definirali utjecaje pojedinih zasebnih čimbenika na strukturu i sastav lipida eksperimenti su započeti s jednostavnijim laboratorijskim uzgojima dijatomeje *Chaetoceros pseudocurvisetus* izolirane iz Sjevernog Jadrana te uspoređeni s kompleksnim sustavima estuarija i Jadranskog mora.

U *in situ* i *in vivo* uzorcima određen je sastav i količina lipida. ukupni lipidi normalizirani su po broju stanica za *in vivo* uzorke te po količini klorofila a (Chl *a*) za *in situ* uzorke kako bi se mogla uspoređivati proizvodnju lipida u različitim sustavima. Uzeto je u obzir da su lipidi glavni proizvođači lipida u moru (Gašparović i sur., 2014.). Promjenom uvjeta u *in vivo* uzgoju došlo je do promjena u sintezi staničnih lipida. Pri optimalnim uvjetima uzgoja, koji su se odnosili na temperaturu od 15 °C, salinitet 30 i omjer hranjivih soli N/P=16, u *in vivo* uzorcima zamijećena je manja akumulacija lipida po stanici, što ukazuje da se energija, ugljik i osnovni elementi više koriste za dijeljenje stanica i rast populacije. Pri takvim uvjetima zamijećen je najveći prinos stanica (prosječna specifična brzina rasta od 0,5 d^{-1}) i smanjeni doprinos lipida u DOM-u. Dosadašnja istraživanja također navode da pri optimalnim uvjetima rasta fitoplankton proizvodi veliku količinu biomase, s malim udjelom lipida po stanici (Sharma i sur., 2012). Zamijećena je razlika u strukturi lipida (sastavu, omjerima) između *in vivo* i *in situ* uzetih uzoraka. U kompleksnim okolišnim sustavima više čimbenika djeluje uzajamno na strukturu i sastav lipida te je utjecaj pojedinih čimbenika definiran multivarijantnim statističkim metodama. U *in vivo* uzorcima hranjive soli su pokazale da imaju važniju ulogu u promjeni sastava staničnih lipida nego temperatura, barem za raspon temperatura pokriven ovim istraživanjem. Temperatura iznad kritične vrijednosti više utječe na proizvodnju DOM-a zbog pojačane primarne proizvodnje usmjerene na otopljenu frakciju kao i zbog raspada stanica. Stanice se sporije dijele što rezultira manjim brojem stanica, te se posljedično ugljik, dušik i fosfor ne mogu koristiti te njihova koncentracija raste u DOM-u. Rast u nepovoljnim uvjetima utječe na kvalitetu

fitoplanktonskih lipida te dolazi do akumulacije neutralnih lipida (Sharma i sur., 2012; Guschina i Harwood, 2006; Thompson, 1996), što je u zamijećeno i u *in situ* i *in vivo* uzorcima ovog rada nakupljanjem neutralnih lipida pri nedostatku hranjivih soli i povišenju temperature. Ponajviše je zabilježena akumulacije triglicerida po stanici prilikom limitacije nitratima.

Na *in situ* estuarijskim uzorcima uočili smo da dolazi do promjena u dvostrukim vezama fosfolipida pri promjeni temperature i saliniteta. Najčešća promjena u strukturi samih lipida je povećanje zastupljenosti nezasićenih masnih kiselina prilikom smanjenja temperature (Sushchik i sur., 2003; Thompson, 1996). Rezultati ovog doktorskog rada u skladu su s prethodnim istraživanjima. Veća je nezasićenost u ukupnom broju masnih kiselina u hladnijem estuariju rijeke Krke nego u toplijem estuariju rijeke Whengchang.

Povoljni uvjeti u *in situ* uzorcima estuarija rijeke Krke, doveli su do povećanja koncentracije fosfolipida, koji su bogatiji nezasićenim masnim kiselinama. Struktura masnih kiselina fosfolipida fosfatidilglicerola (PG), fosfatidilkolina (PC) i fosfatidilinozitola (PI) slična je u svim uzorcima uzetim u estuarijima s različitim trofičkim i temperaturnim uvjetima iz čega se može zaključiti da su navedeni lipidi od vitalne važnosti te ih stanica održava nepromijenjenima. Do promjena dolazi kod masnih kiselina fosfolipida fosfatidiletanolamina (PE), fosfatidne kiseline (PA) i fosfatidilserina (PS) i to ovisno o okolišnim uvjetima, uključujući gradijent saliniteta.

Dijatomeje su dominantno zastupljene u fitoplanktonu i doprinose uvelike primarnoj proizvodnji (Malviya i sur., 2016) te je veoma je bitno da se uključe u istraživanja. Prilikom uzgoja problem predstavlja životni ciklus dijatomeja. One s vremenom smanjuju svoje stanice te u trenutku kad je populacija dosegne kritičnu veličinu prelaze u sporu iz koje se obnavljaju velike stanice. Iako, uspoređujući uzgoje u kojima su se pojavile stanice različitih veličina primjećeno je da veličina stanice nije predstavljala problem s obzirom da nije došlo do promjene omjera lipida unutar same stanice i ostalih uvjeta uzgoja.

4.1 Promjena temperature

Utjecaj promjene temperature na sastav i strukturu partikularnih i otopljenih lipida prikazan je u publikacijama I, II i III. Globalno zatopljenje kombinacija je povećanja temperature zraka i površine mora u posljednjih 30 godina. Ukoliko se ovaj trend nastavi predviđanja su da će temperatura narasti 2 – 3 °C (IPCC, 2014), a nova upozorenja ukazuju da bi taj trend trebalo zaustaviti na maksimalnih 1.5 °C (IPCC, 2018). Mjerenja površinske

temperature sjevernog Jadrana ukazuju na povećanje u razdoblju od 1979. – 2017. godine (Vilibić i sur., 2019). U ovim istraživanjima proveli smo laboratorijske eksperimente na morskoj dijatomeji *Chaetoceros pseudocurvisetus* uzgajanoj na rasponu temperatura od 10 – 30 °C kako bi istražili njezin utjecaj na proizvodnju partikularnih i otopljenih lipida. Svaka vrsta ima optimalnu temperaturu za rast i razvoj. Najveća koncentracija lipida u stanicama *Chaetoceros sp.* (CS256) izmjerena je kada su stanice uzgajane na 25 °C (16,8 % suhe tvari), dok je optimalni raspon temperatura za uzgoj bio 27 – 30 °C (Renaud i sur., 2002). Pri temperaturi 25 °C i dodatku CO_2 najveću stopu rasta imala je dijatomeja *Chaetoceros cf. wighami* (Araújo i Garcia, 2005). U našim uzgojima s razmakom od 5 °C optimalnom se pokazala temperatura od 15 °C, pri kojoj je došlo do najvećeg eksponencijalnog rasta stanica *Chaetoceros pseudocurvisetus* s najmanjom koncentracijom lipida po stanici, zajedno s najmanjom primarnom proizvodnjom usmjerenom prema otopljenoj frakciji (najmanje stvaranje DOM-a). Najveća temperatura uzgoja od 30 °C dovela je do stagnacije razmnožavanja i značajne akumulacije lipida unutar stanica, a isti trend zabilježen je na nižoj temperaturi od 10 °C. Povećanje temperature utjecalo je na pojačano otpuštanje DOM-a, zajedno s povećanom primarnom proizvodnjom usmjerenom prema DOM-u u odnosu na POM. U eksperimentu izvedenom u zatvorenom i kontroliranom mezokozmos uzorku volumena 3 dm^3 također je uočeno povećanje DOC-a u odnosu na POC prilikom povećanja temperature (Kim i sur., 2011). Za zaključiti je da su pri temperaturi od 15 °C stanice u idealnom temperaturnom uvjetu i usmjeravaju stvaranje lipida za rast i razmnožavanja. Pri povišenim temperaturama, u našem slučaju temperatura od 30 °C, dolazi do raspada i izlijevanja staničnog materijala, usporava se razmnožavanje stanica koje u sebi akumuliraju lipide, a veliki dio primarne proizvodnje usmjeren je prema otopljenoj frakciji. Zbog svega toga dolazi do povećanja koncentracije otopljenog ugljika u okolini.

U okolišnim uvjetima mjeren je utjecaj temperature na cijelu populaciju fitoplanktona, tj. na ukupnu proizvodnju lipida u sustavu. Primijećeno je da najveća koncentracija lipida po stanici (mjereno na klorofil *a*) dobivena u ljetnim mjesecima kada su i izmjerene više temperature. Također, zabilježen je porast broja lipida indikatora degradacije (alkoholi, slobodne masne kiseline i dr.) pri povećanim temperaturama. Minimalna izmjerena koncentracija DOC-a i otopljenih lipida bila je najmanja u zimi, a maksimalna u ljeti. Uobičajeno je zamijećena veća akumulacija DOC-a u površinskim vodama tijekom ljetnih mjeseci (Giani i sur., 2005) što pokazuje da pri povišenju temperature dolazi do pojačane

proizvodnje lipida i degradacije samih lipida**.** Na drugim kulturama zabilježeno je također povećanje ukupne koncentracije lipida prilikom povećanja temperature (Sharma i sur. 2012).

Proizvodnja lipida je bila veća u estuariju rijeke Whengchang nego u estuariju rijeke Krke, no to je također ovisilo o trofičkom statusu estuarija. Udio ukupnih lipida u čestičnoj organskoj tvari, bio je veći u estuariju rijeke Krke, gdje su zabilježene niže temperature nego u estuariju rijeke Whengchang. Također je nađen i veći udio fosfolipida u ukupnim lipidima u estuariju rijeke Krke. Navedeni rezultati su u skladu s literaturnim podacima da se veća količina fosfolipida stvara u optimalnim uvjetima dostupnosti hranjivih soli i rasponu temperature bez ekstremnih vrijednosti, dok pri stresu dolazi do većeg nakupljanja neutralnih lipida (Sharma i sur., 2012).

Nezasićene masne kiseline važne su u održavanju fluidnosti membrane, za očekivati je da će nezasićenost masnih kiselina rasti sa smanjenjem temperature (Murata i Los, 1997). Naše istraživanje potkrepljuje tu tvrdnju, s obzirom da je zabilježen veći udio nezasićenih masnih kiselina u hladnijem estuariju rijeke Krke, nego u toplijem estuariju rijeke Whengchang.

Zajednička karakteristika monokultura i eksperimenata na sjevernom Jadranu je da je zabilježeno povećanje glikolipida pri višim temperaturama, kao što je već zabilježeno u Gašparović i sur., 2013. Očito su GL manje podložni degradaciji od ostalih membranskih lipida čija koncentracija u otopljenim lipidima ne korelira s temperaturom ili negativno korelira. Razlog tome bi mogao biti u svojstvu glikolipida ne posjeduju biološki važni fosfor i dušik te kao takvi nisu atraktivni za mikrobiološku razgradnju.

4.2 Promjena dostupnosti hranjivih soli

Utjecaj promjena dostupnosti hranjivih soli na sastav i strukturu partikularnih i otopljenih lipida prikazan je u publikacijama I, II i III. Oligotrofne sustave karakterizira nedostatak hranjivih soli i slaba primarna proizvodnja, a sam proces nastajanja manje produktivnog sustava naziva se oligotrofikacija, što je suprotno eutrofikaciji, tj. odgovoru sustava na prekomjeran unos hranjivih soli velikom primarnom proizvodnjom koja dovodi do prevelikog rasta fitoplanktona (Stockner i sur., 2000). I jedan i drugi slučaj predstavljaju disbalans povoljnih uvjeta.

Mjerenja klorofila *a* (Agusti i sur., 2017) i primarne proizvodnje (Behrenfeld i sur., 2006) u moru sve više ukazuju na trend oligotrofikacije na globalnoj razini. U sjevernom Jadranu globalni porast temperature doveo je do smanjenja količina oborina (35 – 40%) što je

dovelo do smanjenja pritoka hranjivih soli rijekama i zamijećene oligotrofikacije (Cozzi i Giani, 2011; Mozetič i sur., 2010). Justić i sur., 1995 su definirali da su za povećanje produktivnosti sustava važna dva paralelna procesa: povećanje dotoka nitrata i fosfata, te njihov balans. Sjeverni Jadran karakterizira disbalans N/P omjera te vrlo niske koncentracije PO_4 (Gašparović i sur., 2013). Za potrebe usporedbe odnosa utjecaja hranjivih soli uzete su dvije postaje, jedna definirana kao mezotrofna te jedna oligotrofna. Estuarij rijeke Krke definiran je kao oligotrofno područje, osim luke Šibenik (Svensen i sur., 2007), a estuarij rijeke Whengchag zbog pretjerane akvakulture i unosa hranjivih soli netretiranim vodama kućanstava i poljoprivrede izrazito je eutrofno područje.

Zbog trofičke različitosti istraživanih sustava dobiveni rezultati pokazali su utjecaj različite dostupnosti i omjera hranjivih soli na sastav lipida fitoplanktonske zajednice. Uvjeti uzgoja oponašali su uvjete suviška hranjivih soli (F2 medij) i disbalansa u odnosu na manjak nitrata ili manjak fosfata.

Kao i u dosadašnjim istraživanjima (Sharma i sur., 2012; Thompson, 1996; Yeh i Chang, 2011; Lynn i sur., 2000) zamijećeno je da prilikom limitacije nitratima dolazi do akumulacije triglicerida. Prilikom uzgoja *Chaetoceros pseudocurvisetusa* u nedostatku nitrata zabilježena je dvostruko veća akumulacija triglicerida po stanici nego u optimalnim uvjetima. Navedeni trend pratile su i fitoplanktonske stanice iz uzoraka Sjevernog Jadrana, gdje je oligotrofna postaja RV001 imala veću koncentraciju triglicerida po klorofilu *a* u usporedbi s mezotrofnom postajom SJ101. Također koncentracija triglicerida pokazala je negativnu korelaciju s koncentracijom otopljenog dušika (DIN) u sakupljenim uzorcima.

Rezultati su pokazali da se stanice koje žive u nedostatku esencijalnih hranjivih soli sporije dijele i dosežu manji broj stanica u stacionarnoj fazi uzgoja te izlučuju više DOC-a i otopljenih lipida nego stanice u uvjetima s dovoljno hranjivih soli. Uspoređujući uzgoje s nedostatkom nitrata i nedostatkom fosfata zaključeno je da se više DOC-a i otopljenih lipida oslobađa prilikom nedostatka fosfata.

U slučaju nedostataka fosfata uočen je trend povećanja glikolipida u odnosu na fosfolipide, što je uočeno i na oligotrofnijoj postaji sjevernog Jadrana RV001 i u kulturama koje su rasle u uvjetima limitacije fosfatom. U tim uvjetima stanice često mijenjaju lipidni sastav, primjerice zamjenjuju fosfolipide s glikolipidima: SQDQ (Van Mooy i sur., 2006, 2009; Martin i sur., 2011), DGDG (Hartel i sur., 2000) te betaine lipidima (Van Mooy i sur., 2009; Martin i sur., 2011). U rasponu temperatura (15 − 20 °C) distribucija lipida pokazivala je uobičajen trend, gdje je koncentracija fosfata pozitivno korelirala s udjelom fosfolipida i negativno s udjelom glikolipida. Pri većim temperaturama taj trend nije uočen.

Metabolizam uzgojene kulture *Chaetoceros pseudocurvisetus* također se mijenjao ovisno o dostupnosti hranjivih soli. U mediju bogatom hranjivim solima unos N/P bio je 16,4 ± 0,2, dok se veća količina N u odnosu na P unijela prilikom limitacije fosfatom, gdje je N/P omjer iznosio 72,9 ± 4,2. Tijekom N limitacije unos N/P bio je manji 2,6 ± 0,3, te je prilikom toga došlo do manjeg stvaranja dušikom bogatih molekula i veće akumulacije TG koji sadrže samo C, O i H u strukturi molekule. Dosadašnja istraživanja (Gašparović i sur., 2014; Gérin i Goutx, 1994; Penezić i sur., 2010), ali i rezultati dobiveni ovim istraživanjem pokazuju da ukupna koncentracija fosfolipida raste od oligotrofije prema eutrofiji, ali udio fosfolipida u ukupnim lipidima ima obrnuti trend. U prilog tome idu veći udio fosfolipida na oligotrofnoj postaji RV001, pojačana koncentracija fosfolipida po stanici u uzgojima *Chaetoceros pseudocurvisetus* s limitirajućim hranjivim solima, te u estuariju rijeke Krke. Za pretpostaviti je da je kvota fosfolipida u eutrofnim uvjetima manja nego u oligotrofnim. Vjerojatni uzrok tome je povećano dijeljenje stanica prilikom rasta u hranjivim solima bogatim uzgojima. Također, stanice koje su u stresu i ne mogu se uspješno dijeliti, akumuliraju fosfor u fosfolipidima (Abida i sur., 2015). U optimalnim uvjetima fosfor se premješta u vitalno bitnije molekule kao npr. DNA i RNA.

4.3 Promjena saliniteta

Utjecaj saliniteta na sastav i strukturu lipida prikazan je u publikaciji III. Rezultati ukazuju da stres uzrokovan promjenom saliniteta dovodi do skraćivanja duljine masnih kiselina fosfolipida slatkovodnog fitoplanktona, dok fitoplankton koji raste u povoljnim uvjetima saliniteta (slatkovodni pri S=0 i morski pri S=35−38, ovisno o moru ili oceanu) sintetizira fosfolipide koji sadrže masne kiseline s dužim lancima. U estuariju rijeke Krke uočen je trend produljivanja lanaca masnih kiselina niz estuarij, izuzevši uzorak saliniteta 0. Inače, skraćivanje i produljivanje lanaca masnih kiselina je proces koji je potaknut promjenama u okolišu, a može se odvijati vrlo brzo, unutar sat ili dva (Rai i Gaur, 2001; Urzica i sur., 2013).

U publikaciji III napravljena je analiza glavnih komponenti (PCA, engl. *principal component analysis*) uzoraka estuarija rijeke Krke i estuarija rijeke Whengchang koja je pokazala određene korelacije masnih kiselina i saliniteta. Negativnu korelaciju u uzorcima estuarija rijeke Whengchang imali su salinitet i masne kiseline n-C15:0, n-C22:3, n-C22:4 i n-C22:6 u PC, masne kiseline n-C18:1 u PG, masna kiselina n-C15:0 u PE i masna kiselina n-C20:0 u PA. Negativnu korelaciju u KRE uzorcima pokazali su salinitet i masne kiseline n-

C15:0, n-C20:3, n-C20:4, n-C20:5, n-C22:0 i n-C22:4 za PC, masne kiseline n-C14:0, n-C20:2, n-C20:4 i n-C20:5 u PG, masne kiseline n-C16:1, n-C18:0, n-C20:3, n-C20:4, n-C22:0 i n-C22:4 u PE te masnih kiselina n-C16:0 i n-C20:1 u PA. Prema dobivenim rezultatima nije bilo moguće odrediti specifičnu masnu kiselinu kao indikator promjene saliniteta. Rezultati istraživanja provedenih na masnim kiselinama mikroalge *Nanochloropsis oculata* uzgajanim na salinitetima 25, 35, i 45 ‰ nisu pokazivali značajnu razliku, kao ni razine zasićenosti u odnosu na ta tri paralelna uzgoja (Na i sur., 2012). I sastav masnih kiselina *Phaeodactylum tricornutum* ostaje jednaka na različitim salinitetima (Qiao i sur., 2016.) Međutim, u uzgoju halotolerantne alge *Dunaliella salina* zabilježena je pozitivna korelacija zasićenosti masnih kiselina i saliniteta (Xu i Beardall, 1997). Iako se dugo pretpostavljalo da je za izmjenu vode tijekom osmotskog šoka najzaslužnija propusnost membrane, u posljednjih nekoliko godina sve se više ističe i uloga vodenih kanala (akvaporina) i membranskih proteina (Rai i Gaur, 2001).

4.4 Zajednički utjecaj više čimbenika

U svom prirodnom okolišu fitoplankton je pod simultanim utjecajem više čimbenika stresa. Kao ekstremni primjer su morske alge u zoni plime i oseke, koje podnose mehanički stres, izmjenu vodenog i suhog okoliša, isušivanje, različite intenzitete svjetla, temperature i saliniteta unutar samo jednog ciklusa plime i oseke (Davison i Pearson, 1996). U ovim istraživanjima zamijećene su različite promjene u okolišu kao npr. promjene svjetla, izmjene dana i noći, promjene u dotoku hranjivih soli, dnevnu i sezonsku izmjenu temperature, promjene saliniteta. Neke promjene su sezonske, dok su neke kratkotrajne (kao npr. promjena saliniteta u estuarijima) te fitoplankton mora odraditi veoma brzu prilagodbu kako bi preživio. Uočeno je da se promjene u lipidnom dvosloju membrane, a i modeliranje masnih kiselina mogu odviti unutar par sati (Martin i sur., 2011; Urzica i sur., 2013).

Trendovi u istraživanjima, a i ova istraživanja ukazuju da okolišni faktori koji utječu na fotosintezu i proizvodnju lipida mijenjaju količinu lipidnih klasa te dovode do promjena sastava lipida i stanica fitoplanktona u sustavu (Guschina i Hardwood 2009). Uočeno je da povoljni uvjeti u okolišu dovode do stvaranja veće količine fosfolipida i glikolipida, dok se u nepovoljnijim uvjetima sinteza lipida više usmjeruje u stvaranje energetskih rezervi lipida kao što su trigliceridi (Sayanova i sur., 2017; Sharma i sur., 2012). Dobiveni rezultati na kulturama i *in situ* uzorcima Jadrana i estuarija pokazuju da stanice kada žive u lošijim uvjetima (povišena temperatura ili nedostatak hranjivih soli) slabije rastu, akumuliraju više

lipida po stanici i ispuštaju više DOC-a i otopljenih lipida nego one koje žive u idealnim uvjetima. Ukupna izmjerena koncentracija lipida u sustavu bila je veća u eutrofnijim područjima i uzgojima, ali samo radi povećane produktivnosti sustava.

Do određene temperature utjecaj temperature i hranjivih soli na otpuštanje DOM je simultani, no kada temperatura prijeđe prag tolerancije (što je temperatura od 30 °C za uzgajani *Chaetoceros pseudocurvisetus*) ona ipak više utječe na proizvodnju DOM-a od limitacije hranjivim solima. Rezultati su pokazali da je na postajama bogatijim hranjivim solima, dostupnost hranjivih soli važnija u raspodjeli lipida od temperature. Na akumulaciju lipida po stanici veći utjecaj na većim temperaturama ima limitacija fosfatom, a na manjim temperaturama limitacija nitratima.

Uočeno je da estuarijski fitoplankton, neovisno o uvjetima, održava poželjni sastav masnih kiselina fosfatidilglicerola, fosfatidilkolina i fosfatidilinozitola što ukazuje da su to fosfolipidi odgovorni za normalno funkcioniranje stanica. Velika varijabilnost fosfolipidnih masnih kiselina, zasićenosti i duljine lanaca masnih kiselina u uzorcima estuarija rijeke Whengchang i estuarija rijeke Krke indiciraju da je sastav masnih kiselina ovisan o vrsti fitoplanktona, te ovisi o fazi rasta i/ili odgovora na okolišne uvjete. Osnovni čimbenici odgovorni za uspješan rast fitoplanktona su temperatura i svjetlost, a kada su oni unutar specifičnih optimuma vrste tada ta vrsta tolerira veći raspon saliniteta (Kirst, 1990).

Promjene u višestruko nezasićenim masnim kiselinama (PUFA, engl. *poliunsaturated fatty acids*) mogu dovesti do značajnih ekoloških promjena (Müller-Navarra i sur., 2000), s obzirom da su ω-3 nezasićene masne kiseline esencijalni elementi s brojnim dobrobitima za više trofičke organizme (Jónasdóttir, 2019). Najčešće morske PUFA su eikosapentaenoična kiselina (EPA, n-C20:5) i dokosaheksaenoična kiselina (DHA, n-C22:6), čiji glavni izvor je fitoplankton. Njihova apsorpcija u tkiva viših organizama veća je iz fosfolipida nego iz triglicerida (Rossmeisl i sur., 2012). U uzorcima estuarija rijeke Krke i rijeke Whengchang nije pronađena značajnija razlika s obzirom na udio EPA i DHA u fosfolipidima. Međutim, kao značajni izvor EPA pokazao se PL fosfatidilglicerol, a DHA PL fosfatidilinozitol. Okolišni uvjeti imali su utjecaj na proizvodnju lipida i masnih kiselina kod uzgoja *Phaeodactylum tricornutum*. Smanjenje koncentracije EPA uočeno je prilikom limitacije dušikom (1,24 prema 12,35 – 49,40 mg dm^{-3}) i povećanjem temperature (25 °C prema 15 °C), a promjena saliniteta od 15 do 35 i različita osvijetljenost (50 – 150 μmol $m^{-2}s^{-1}$) nisu doveli do promjena u količini EPA u 7 dana uzgoja (Qiao i sur., 2016).

4.5 Dijatomeje kao modelni organizmi

Zbog velike zastupljenosti u fitoplanktonskoj zajednici, dijatomeje predstavljaju jednu od ekološki najvažnijih grupa, a među njima *Chaetoceros* je jedna od najbrojnijih. *Chaetoceros* je zabilježen u Jadranu tijekom cijele godine što ukazuje na njegovu prilagodbu na različite okolišne uvjete (Bosak i sur., 2016). Dominacija dijatomeja zamjećena je i u oba istraživana estuarija, što je uobičajeno s obzirom da je poznato da su riječna ušća bogata dijatomejama s obzirom na veliki pritok hranjivih soli rijekama (Wawrik i Paul, 2004). Dijatomeje imaju ključnu ulogu u kruženju ugljika i silicija u oceanima, a zaslužni su za gotovo pola oceanske primarne proizvodnje (Malviya i sur., 2016).

Posljednjih godina jako je porastao interes za istraživanje dijatomeja i njihovih molekularnih komponenta, s obzirom na njihov potencijal u proizvodnji različitih bioaktivnih komponenti i kemikalija za industrijske primjene (Vinayak i sur., 2015). Primjerice, dijatomeje su bogate pigmentima karotenoidima, koji se uvelike koriste kao dodatci prehrani, u farmaciji i kozmetici (Fu i sur., 2015; Vílchez i sur., 2011), a pokazuju najveću sposobnost akumulacije lipida u stresnim uvjetima među algama (Hildebrand i sur., 2012b). Dijatomeje su također perspektivni kandidat za razvoj različitih bioproizvoda s dodanom vrijednošću prema održivoj bio-ekonomiji (Yi i sur., 2017).

4.6 Doprinos istraživanja

U Laboratoriju za biogeokemiju mora i atmosfere tijekom proteklih godina unaprijeđeni su protokoli za uzgoj morskih i slatkovodnih dijatomeja u stresnim uvjetima. Protokol za ekstrakciju i tankoslojnu kromatografiju lipida unaprijeđen je kako bi se što bolje definirale klase lipida uzgojenih kultura i okolišnih uzoraka.

Ovo istraživanje otvorilo je brojna pitanja, ali i ideje za daljnji rad u tom području. Osim ovdje prikazanih stresora bilo bi zanimljivo uključiti i druge čimbenike kao npr. pH, promjenu intenziteta svjetla, ali i antropogene utjecaje zagađenjem. Zanimljivo bi bilo nastaviti eksperimente i na drugim skupinama fitoplanktona te vidjeti njihov odgovor u promjeni lipida. Osim isključivo lipida, bilo bi zanimljivo unaprijediti metode te usporediti kako se u određenim uvjerima mijenjaju omjeri sintetiziranih glavnih biomolekula, lipida, proteina i šećera.

Eksperimenti utjecaja saliniteta provedeni na slatkovodnim i morskim kulturama algi, te uspoređeni s estuarijskim uzorcima pokazuju zanimljive preliminarne rezultate

remodeliranja lipida u stanicama. Tu ipak najzanimljiviju ulogu ima halotermna alga *Dunaiellla salina* koja je također održavana u Laboratoriju za biogeokemiju mora i atmosfere.

Istraživanje lipida i drugih spojeva iz algi veoma je aktivno područje. Mnoge novije studije uključuju i manje poznate, a i novo otkrivene vrste (Guschina i Harwood, 2006; Mucko i sur., 2020; Qiao i sur., 2016; Tanković i sur., 2018; Wurch i sur., 2011). Također, sve je više naglasak na korištenje molekularnih tehnologija, ne samo kako bi se shvatili mehanizmi stvaranja lipida i njihovih funkcija, nego kako bi se stvorila kodirajuća DNA za specijalne enzime koji bi se kasnije koristili za proizvodnju genetički modificiranih organizama (Guschinia i Hardwood, 2006). Osim bitnog doprinosa razumijevanju ekologije mnogih različitih vrsta zajednica, primjena stresa često se koristi kao eksperimentalni pristup s praktičnom vrijednosti. Za primjer *Dunaliella salina,* u stresnim uvjetima producira količine karotenoida, glicerola, lipida, vitamina, minerala i proteina dovoljne za komercijalno dobivanje tih kemikalija (Hosseini i Shariati, 2009). Istraživanja kao naša pomoći će nam za predviđanje odgovora fitoplanktona na promjene u okolišu, upravo korištenjem lipida kao biomarkera stresnih uvjeta.

5 ZAKLJUČCI

1. Optimalna temperatura za uzgoj dijatomeje *Chaetoceros pseudocurvisetus* je 15 °C. Pri toj temperaturi stanice usmjeravaju asimilaciju ugljika u rast i razmnožavanje. Prilikom odstupanja od optimalne temperature u uzgoju stanice veliki dio primarne proizvodnje usmjeravaju u otopljenu frakciju i dijelom se raspadaju te dodatno povećavaju koncentraciju otopljene organske tvari u okolini.
2. Reakcija fitoplanktona na okolišni stres je sinteza molekula bogatih ugljikom, uključujući lipide. Iako dolazi do smanjenja dijeljenja stanica, aktivna proizvodnja masnih kiselina i lipida i dalje se nastavlja. S obzirom da stanice nemaju potrebu za stvaranjem novih membrana, stvoreni lipidi, najčešće neutralni, se akumuliraju u stanici i koriste u trenutku povoljnijih okolišnih uvjeta. Najčešće zabilježena promjena je nakupljanje triglicerida, staničnih rezervi masnih kiselina, prilikom rasta stanica u mediju i okolišu s nedostatkom dušikovih spojeva.
3. Posljedica rasta fitoplanktona u uvjetima manjka fosfata i u modelnom eksperimentu i u uzorcima prikupljenim na oligotrofnim postajama je povećanje udjela fosfolipida u ukupnim staničnim lipidima. Za zaključiti je da se stanice u stresu uzorkovanom nedostatkom hranjivih soli ne mogu uspješno dijeliti te nakupljaju fosfolipide. Prilikom rasta u povoljnim koncentracijama fosfora on se premješta u vitalno bitnije molekule kao što su npr. DNA i RNA.
4. Utjecaj temperature i dostupnosti hranjivih soli na otpuštanje DOM-a i modeliranje lipida simultan je u određenim temperaturnim rasponima. Kada temperatura prijeđe prag tolerancije (što je temperatura od 30 °C za uzgajani *Chaetoceros pseudocurvisetus*) ona više utječe na proizvodnju DOM-a od limitacije hranjivim solima. U optimalnom rasponu temperatura ($15 - 20$ °C) sastav lipida ovisi u dostupnosti hranjivih soli. To se očituje većim udjelom glikolipida u odnosu na fosfolipide pri nedostatku fosfata te većim udjelom triglicerida u ukupnim lipidima pri nedostatku dušika.
5. Metabolizam stanica u kulturi *Chaetoceros pseudocurvisetus* mijenja se ovisno o dostupnosti hranjivih soli. Omjer asimiliranog dušika i fosfora mijenjao se u odnosu na njihove koncentracije u mediju i odstupa od Redfieldovog 16:1. Stoga, dolazimo do zaključka da razlike u uvjetima uzgoja dovode do promjena u elementarnom sastavu stanica fioplanktona i organske tvari, što bi potencijalno moglo dovesti i do modifikacije zaliha hranjivih soli u moru.

6. Promjena saliniteta dovodi do promjena u sastavu masnih kiselina fosfolipida. Masne kiseline s dužim lancima uočene su u slatkoj vodi i morskim postajama najvećeg saliniteta, dok su u pravilu masne kiseline kraćih lanaca nađene u estuarijskoj vodi. To ukazuje da je promjena dužine lanca masnih kiselina fosfolipida jedan od mehanizama prilagodbe na stres u uvjetima varijabilnog saliniteta.
7. Struktura masnih kiselina fosfatidilglicerola, fosfatidilkolina i fosfatidilinozitola slična je u svim uzorcima uzetim u estuarijima s različitim trofičkim i temperaturnim uvjetima iz čega se može zaključiti da su to molekule od vitalne važnosti zbog čega ih stanica održava relativno nepromijenjenima. Do promjena dolazi kod masnih kiselina fosfatidiletanolamina, fosfatidne kiseline i fosfatidilserina, i to ovisno o različitim uvjetima, ali i po gradijentu saliniteta. To navodi na zaključak da prilagodljivost planktona za preoblikovanje ovih fosfolipida ovisno o uvjetima okoliša i strukturi zajednice planktona.
8. Promjene lipida u stanicama dijatomeja *Chaetoceros pseudocurvisetus* uzgajanim pod različitim stresorima u skladu su s rezultatima uzoraka uzetih u sjevernom Jadranu te se može zaključiti da je ta vrsta dobar modelni predstavnik tog sustava.

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7 ŽIVOTOPIS

Tihana Novak (rođ. Sesar) rođena je u Zagrebu 28.6.1987. Osnovnu i srednju školu završila je u Zagrebu. Dodiplomski studij Biologije završila je 2009. godine, a diplomski studij Ekologije i zaštite prirode – modul more 2012. godine na Prirodoslovno matematičkom fakultetu u Zagrebu. Od 2015. godine studentica je poslijediplomskog studija Oceanologije na Prirodoslovno matematičkom fakultetu u Zagrebu. Tihana je od 2015. godine doktorand na projektu Hrvatske zaklade za znanost: Utjecaj okolišnog stresa na pojavnost i međudjelovanje biološki važnih organskih molekula i mikronutrijenata u morskom ekosustavu (AMBIOMERES) pod voditeljstvom dr. sc. Blaženke Gašparović. Trenutno je zaposlena u Laboratoriju za biogeokemiju mora i atmosfere u Zavoda za istraživanje mora i okoliša, Instituta Ruđer Bošković. Koautorica je na 6 znanstvenih radova, od kojih je prvi autor na 2 znanstvena rada u časopisima koje citira Web of science (WOS) baza podataka. Koautorica je na 22 priopćenja u obliku postera ili predavanja. Sudjelovala je u aktivnostima dvije COST akcije ES1408 The European Network For Algal-Bioproducts (EUALGAE) te CA15121 Advancing marine conservation in the European and contiguous seas (MarCons).

8 PRILOZI

1. Popis znanstvenih aktivnosti

Publikacije u CC časopisima:

1. Novak, Tihana; Godrijan, Jelena; Marić Pfannkuchen, Daniela; Djakovac, Tamara; Mlakar, Marina; Baricevic, Ana; Smodlaka Tanković, Mirta; Gašparović, Blaženka (2018) Enhanced dissolved lipid production as a response to the sea surface warming, Journal of marine systems, 180 , 289-298 https://doi.org/10.1016/j.jmarsys.2018.01.006
2. Novak, Tihana; Godrijan, Jelena; Pfannkuchen, Daniela Marić; Đakovac, Tamara; Medić, Nikola; Ivančić, Ingrid; Mlakar, Marina; Gašparović, Blaženka (2019) Global warming and oligotrophication lead to increased lipid production in marine phytoplankton, Science of The Total Environment, 668, 171-183 https://doi.org/10.1016/j.scitotenv.2019.02.372
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2. Popis korištenih kratica

Chl *a*	klorofil a
DGDG	digalaktozildiglicerol
DHA	dokosaheksaenoična masna kiselina (C 22:6)
DIN	otopljeni anorganski dušik (engl. *dissolved inorganic nitrogen*)
DOC	otopljeni organski ugljik (engl. *dissolved organic carbon*)
DOM	otopljena organska tvar (engl. *dissolved organic matter*)
DON	otopljeni organski dušik (engl. *dissolved organic nitrogen*)
DOP	otopljeni organski fosfat **(**engl. *dissolved organic phosphorous*)
DNA	deoksiribonukleinska kiselina (engl. *deoxyribonucleic acid*)
EAC	Istočna jadranska struja (engl. *Eastern Adriatic Current*)
EPA	eikosapentaenoična masna kiselina (C 20:5)
GL	glikolipidi
ICCC	Istarska obalna protustruja (engl. *Istrian Coastal Countercurrent*)
MGDG	monogalaktozildiglicerol
PC	fosfatidilkolin
PE	fosfatidiletanolamin
PG	fosfatidilglicerol
PL	fosfolipidi
POC	partikularni organski ugljik (engl. *particulate organic carbon*)
POM	partikularna organska tvar (engl. *particulate organic matter*)
PUFA	višestruko nezasićene masne kiselinama (engl. *poliunsaturated fatty acids*)
rDNA	ribosomska deoksiribonukleinska kiselina
RV	Rovinj
SJ	sjeverni Jadran
SQDG	sulfokvinovosildiacilglicerol
TG	trigliceridi
WAC	zapadna jadranska struja (engl. *Western Adriatic Current*)

3. Dodatni materijali objavljenih članaka

Supporting material for **Enhanced dissolved lipid production as a response to the sea surface warming**

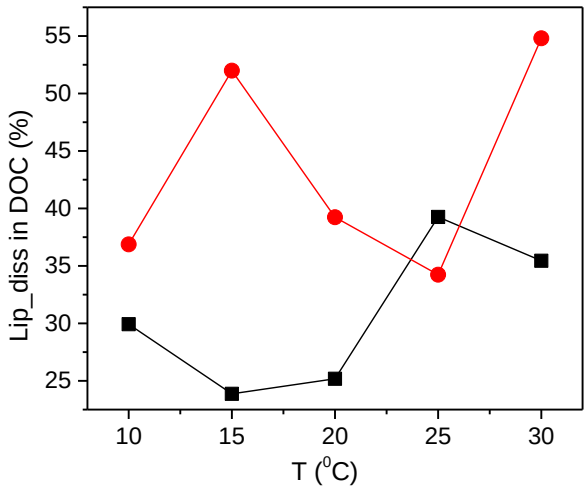

Fig. S1. Average contribution of dissolved lipids to DOC for the P-depleted (circles) and P-replete (squares) *C. pseudocurvisetus* growth conditions.

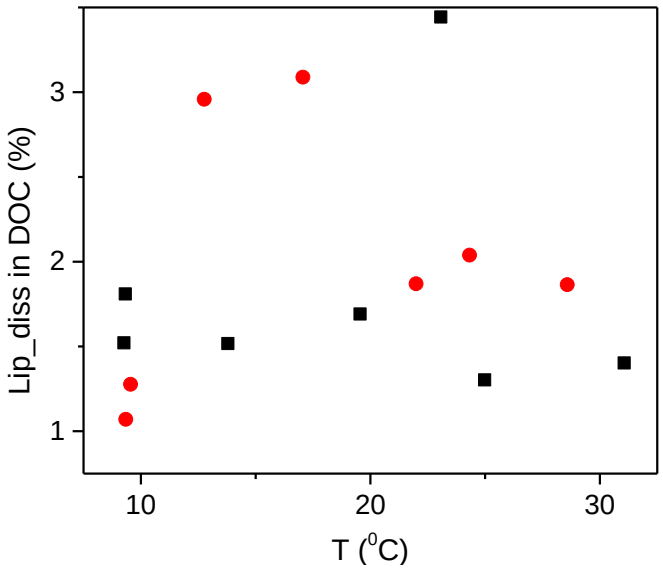

Fig. S2. Contribution of dissolved lipids to DOC for oligotrophic station 107 (circles) and mesotrophic station 101 (squares).

Supporting material for "**Global warming and oligotrophication lead to increased lipid production in marine phytoplankton**"

Tihana Novak[a] *, Jelena Godrijan[a,1], Daniela Marić Pfannkuchen[b], Tamara Djakovac[b], Nikola Medić[c], Ingrid Ivančić[b], Marina Mlakar[a], Blaženka Gašparović[a]

[a] *Division of Marine and Environmental Research, Ruđer Bošković Institute, POB 108, HR-10002 Zagreb, Croatia*

[b] *Center for Marine Research, Ruđer Bošković Institute, G. Paliaga 5, HR-52210 Rovinj, Croatia*

[c] *Marine Biological Section, Department of Biology, University of Copenhagen, DK-3000 Helsingør, Denmark*

[1] Current adress: *Bigelow Laboratory for Ocean Sciences, 60 Bigelow Drive East Boothbay, ME 04544, USA*

Table S1. Main parameters of lipid production in three different batch cultures (replete, P- and N-depleted) at five temperatures (10, 15, 20, 25 and 30 °C): total cell lipids, contribution of lipid carbon to total cell carbon in parenthesis, cell lipid classes and degradation indices (DI) at the end of the experiments normalized to cell.

Batch medium	T	Cell lipids (% lipid carbon)	Cell lipids						Degradation indices
			Membrane lipids				Reserve lipids		
			PL	GL	ST	PIG	TG	SE	DI
	(°C)		pg/cell						
replete	10	12.0±1.1 (3.6)	3.1±0.7	6.6±0.7	0.8±0.3	0.6±0.3	0.7±0.2	0.1±0.1	4.1±1.4
	15	6.0±0.8 (6.7)	1.5±0.2	3.4±0.8	0.7±0.0	0.2±0.1	0.1±0.0	0.1±0.0	2.1±0.1
	20	5.4±1.2 (2.7)	2.0±0.7	2.6±0.9	0.4±0.3	0.1±0.1	0.1±0.0	0.1±0.1	3.0±0.9
	25	16.7±2.6 (6.9)	6.6±1.2	7.9±2.3	0.8±0.5	0.6±0.6	0.4±0.2	0.2±0.0	2.7±0.8
	30	27.8±0.7 (11.3)	12.6±0.3	9.6±0.6	0.8±0.1	3.1±0.3	1.2±0.1	0.5±0.0	4.6±0.6
P-depleted	10	44.0±2.6 (5.7)	15.8±2.6	21.2±0.2	2.3±0.1	3.4±0.0	0.8±0.3	0.5±0.0	7.1±0.9
	15	7.1±0.4 (5.6)	2.0±0.1	3.6±0.3	0.7±0.1	0.2±0.0	0.2±0.1	0.3±0.0	1.7±0.2
	20	30.3±1.5 (6.4)	14.4±1.0	12.1±1.1	0.5±0.0	1.7±0.1	1.2±0.3	0.3±0.1	2.6±0.4
	25	64.7±17.5 (6.6)	27.0±13.3	24.4±10.6	3.3±2.4	5.0±2.3	3.5±1.7	1.4±1.3	27.0±15.3
	30	82.0±3.9 (34.0)	27.2±2.1	38.7±3.0	9.0±1.4	3.9±0.2	2.7±0.5	0.5±0.0	57.5±3.7
N-depleted	10	48.9±5.29 (3.9)	11.9±4.4	22.7±2.8	4.3±0.1	1.7±0.2	8.0±0.8	0.2±0.1	13.0±3.6
	15	15.1±0.7 (6.3)	2.9±0.3	7.9±0.6	1.1±0.1	0.6±0.0	2.4±0.1	0.2±0.0	9.0±0.6
	20	23.0±1.87 (9.6)	5.2±0.4	11.7±1.8	1.0±0.2	0.8±0.0	4.2±0.5	0.1±0.0	6.3±0.4
	25	32.8±3.46 (4.1)	8.6±0.3	10.3±1.8	1.0±0.2	8.4±2.1	3.9±0.2	0.7±0.2	10.6±1.1
	30	66.8±3.39(14.7)	11.6±3.2	30.9±1.0	6.3±0.5	1.7±0.3	10.6±2.9	5.7±0.1	18.2±0.9

Table S2. Pearson correlation coefficients, *r*, for variables used in PCA analysis (Figs. 4a, b and c) for replete, P-depleted and N-depleted growth conditions. Values in red are highly correlated, in blue are moderately correlated and in black are not correlated.

	T	%PL	%GL	%SE	%ST	%TG	%PIG
Replete							
T		0.9428	-0.9093	0.2486	-0.7084	-0.1863	0.6172
%PL			-0.9177	-0.0057	-0.8473	-0.0680	0.6073
%GL				-0.0980	0.8110	-0.1442	-0.8597
%SE					0.4807	-0.6826	-0.0186
%ST						-0.4309	-0.6976
%TG							0.5461
%PIG							
P-depleted							
T		0.1609	-0.4178	-0.3112	0.2608	0.6012	-0.1070
%PL			-0.8815	-0.5149	-0.9092	0.5242	0.5536
%GL				0.3046	0.7057	-0.8373	-0.5645
%SE					0.3576	0.1279	-0.4853
%ST						-0.2847	-0.6197
%TG							0.1762
%PIG							
N-depleted							
T		-0.2778	-0.4106	0.7836	-0.1406	-0.3483	0.3100
%PL			-0.6394	-0.6457	-0.6303	-0.4130	0.6581
%GL				-0.0753	0.4800	0.9063	-0.9307
%SE					0.5015	-0.1275	-0.1413
%ST						0.3491	-0.7021
%TG							-0.9044
%PIG							

Table S3. The values of northern Adriatic environmental parameters and lipid production. Temperature (T), salinity (S), orthophosphate (PO_4), dissolved inorganic nitrogen (DIN), Chlorophyll *a* (Chl *a*) and contribution of Chaetoceros taxa abundance to phytoplankton community abundance at stations SJ101 and RV001 during the investigation period in 2013-2014. Lipid results are given for cell lipids and lipid degradation indices (DI). All lipid values are given based on Chl *a* (Lipid/Chl *a*).

Station	Month	T	S	PO4	DIN	Chl a	Chaetoceros	Cell lipids total	Cell lipids						Degradation indicies
									Membrane lipids				Reserve lipids		
		(°C)		μmol/l		(μg/L)	%		PL	GL	ST	PIG	TG	WE	DI
SJ101	March	10.64	33.84	0.14	16.80	3.72	50	12.6±0.2	3.5±0.2	5.5±0.1	2.1±0.1	0.2±0.0	0.6±0.1	0.7±0.0	5.4±0.2
	April	19.82	15.79	0.29	76.15	6.15	42	11.1±0.5	3.3±0.2	4.3±0.3	2.7±0.3	0.1±0.0	0.3±0.0	0.4±0.0	4.8±1.0
	May	18.17	35.9	0.02	2.79	0.77	0	28.9±1.6	10.6±0.4	10.7±1.3	4.8±0.7	0.7±0.1	0.8±0.2	1.2±0.2	14.0±1.4
	July	24.67	34.43	0.00	0.92	0.09	11	152.5±8.9	63.4±7.0	41.2±3.8	19.3±0.3	10.5±3.7	10.9±1.0	7.3±0.4	48.5±3.3
	August	24.07	32.64	0.13	0.77	4.54	41	24.2±1.8	7.5±1.2	7.6±0.9	3.4±0.5	0.2±0.1	5.0±0.7	0.6±0.1	11.9±2.0
	September	21.82	36.17	0.01	2.57	0.40	11	78.1±6.7	17.5±4.7	51.7±2.9	7.6±3.7	0.7±0.1	0.4±0.0	0.2±0.0	6.4±0.7
	October	18.75	34.39	0.17	7.56	1.78	7	12.9±1.3	5.7±0.4	4.0±1.2	1.3±0.0	0.6±0.0	0.7±0.0	0.5±0.0	5.5±0.2
	November	14.15	36.96	0.07	4.92	1.66	25	14.4±0.8	5.9±0.1	5.5±0.8	1.2±0.1	0.4±0.1	0.8±0.1	0.7±0.1	4.6±0.2
	January	10.83	32.28	0.08	18.63	0.82	0	29.8±1.0	14.3±0.3	10.9±0.9	1.1±0.0	0.7±0.2	1.4±0.0	1.3±0.0	8.1±0.2
	March	14.33	25.53	0.34	22.14	10.02	0	6.1±0.3	1.3±0.1	2.6±0.2	1.4±0.0	0.5±0.0	0.2±0.0	0.1±0.0	4.3±0.2
RV001	March	10.57	37.54	0.01	2.31	0.20	1	33.4±1.2	13.7±0.7	7.7±0.8	2.5±0.3	1.3±0.3	3.8±0.2	4.4±0.3	5.0±0.2
	April	13.21	37.35	0.02	1.75	0.68	0	23.7±0.8	7.5±0.7	7.8±0.1	2.5±0.2	0.5±0.1	3.3±0.3	2.1±0.0	7.5±0.8
	May	16.82	37.21	0.00	0.66	0.33	3	36.7±1.3	11.6±0.8	15.2±0.8	4.9±0.0	1.6±0.3	1.4±0.4	1.8±0.0	15.3±0.4
	July	23.41	35.82	0.00	1.18	0.16	33	89.4±4.2	37.0±3.7	30.3±1.6	7.8±0.2	6.6±1.1	3.4±0.5	4.3±0.5	23.7±1.3
	August	23.56	35.99	0.00	1.02	0.27	2	66.1±1.6	24.2±0.5	26.5±1.1	5.4±0.5	1.0±0.3	3.5±0.1	5.5±0.9	32.5±4.1
	September	21.84	36.35	0.00	1.75	0.31	30	35.1±0.9	15.9±0.8	10.9±0.3	4.0±0.1	1.6±0.2	0.7±0.0	2.0±0.3	18.8±0.7
	October	19.48	36.83	0.44	4.29	0.49	3	25.7±4.6	11.6±1.3	9.3±4.4	2.3±0.3	0.6±0.0	0.4±0.1	1.4±0.0	14.1±0.5
	November	15.21	37.52	0.03	3.58	0.76	20	18.3±0.3	7.1±0.2	7.4±0.1	0.9±0.1	0.8±0.2	1.1±0.1	1.0±0.0	8.0±0.4
	January	12.57	37.85	0.00	3.14	0.42	13	36.8±1.6	16.4±1.1	12.2±1.1	2.9±0.1	1.4±0.1	2.0±0.0	2.0±0.3	12.1±0.8
	March	12.77	37.47	0.00	2.51	0.17	0	54.7±2.2	30.2±1.3	14.1±0.4	1.4±0.2	5.6±1.7	2.0±0.2	1.3±0.1	4.2±0.4

Table S4. List of phytoplankton species with frequency of appearance larger than 5% at stations SJ101 and RV001 for the investigated period. Number of samples with recorded presence (N), Frequency of appearance (FRE), maximum cell abundances (cells L-1) (MAX), averaged cell abundances (cells L-1) over the whole dataset (AVG) and standard deviation (SD).

Species	**N**	**FRE**	**MAX**	**AVG**	**STDEV**
Coccolithophyceae					
Coccolithophyceae miscellaneous <10 µm	66	69%	99400	16831	22624
Coccolithophyceae miscellaneous 10-20 µm	32	34%	17040	3040	4114
Acanthoica quattrospina Lohmann	9	9%	2840	1914	965
Calciosolenia brasiliensis (Lohmann) J.R.Young	22	23%	1420	410	396
Calciosolenia murrayi Gran, 1912	16	17%	5680	1043	1262
Calciopappus caudatus Gaarder & Ramsfjell	12	13%	7100	1511	1726
Emiliania huxleyi (Lohmann) W.W.Hay & H.P.Mohler	74	78%	369200	23806	56045
Ophiaster sp. Gran, 1912	18	19%	2840	1171	758
Rhabdosphaera clavigera var. *stylifera* (Lohmann) Kleijne and Jordan	13	14%	2840	1872	999
Syracosphaera pulchra Lohmann, 1902	37	39%	8520	1759	1583
Syracosphaera pulchra Lohmann, 1902 HOL *oblonga* type, sensu Young et al., 2003	8	8%	48280	16153	18132
Bacillariophyceae					
Bacillariophyceae miscellaneous MICRO	22	23%	1140	394	258
Bacillariophyceae miscellaneous NANO	48	51%	15620	3543	3330
Bacteriastrum furcatum Shadbolt	17	18%	18240	4368	5640
Bacteriastrum hyalinum Lauder	18	19%	23180	4911	5976
Bacteriastrum jadranum Godrijan, Maric & Pfannkuchen	6	6%	9940	4733	3058
Cerataulina pelagica (Cleve) Hendey	55	58%	21300	2940	4356
Chaetoceros affinis Lauder	17	18%	4560	1502	1333
Chaetoceros brevis F.Schütt	7	7%	1520	580	526
Chaetoceros circinalis (Meunier) K.G.Jensen & Moestrup	10	11%	39900	9671	13178
Chaetoceros curvisetus Cleve	15	16%	45440	9535	12487
Chaetoceros danicus Cleve	12	13%	4560	1373	1524
Chaetoceros diversus Cleve	7	7%	93720	30773	38009
Chaetoceros lauderi Ralfs ex Lauder	10	11%	7410	3102	2787
Chaetoceros lorenzianus Grunow	6	6%	2090	1008	568
Chaetoceros rostratus Ralfs	7	7%	2660	1054	922
Chaetoceros socialis H.S.Lauder	15	16%	924370	189660	299418
Chaetoceros sp. Ehrenberg	34	36%	92300	5671	15407
Chaetoceros tortissimus Gran	9	9%	6840	3030	2412
Chaetoceros vixvisibilis Schiller	9	9%	5700	3306	1496
Chaetoceros wighamii Brightwell	5	5%	3420	2078	1231
Chaetoceros contortus F.Schütt	6	6%	10640	3895	3552
Coscinodiscus sp. Ehrenberg	8	8%	380	101	116
Cyclotella sp. (Kützing) Brébisson	22	23%	44020	8407	11674
Cylindrotheca closterium (Ehrenberg) Reimann & J.C.Lewin	40	42%	20520	2429	3684
Dactyliosolen mediterraneus (H.Peragallo) H.Peragallo	8	8%	1140	551	366
Detonula pumila (Castracane) Gran	7	7%	1520	906	467
Diploneis bombus (Ehrenberg) Ehrenberg	39	41%	19880	2349	3536
Diploneis sp. Ehrenberg ex Cleve	9	9%	2840	917	783
Diplopsalis sp. complex R.S.Bergh, 1881	18	19%	380	78	83
Eucampia cornuta (Cleve) Grunow	9	9%	1520	689	566
Guinardia flaccida (Castracane) H.Peragallo	42	44%	2090	439	459
Guinardia striata (Stolterfoth) Hasle	33	35%	4560	1216	1200
Hemiaulus hauckii Grunow ex Van Heurck	34	36%	2840	348	527

Leptocylindrus danicus Cleve	12	13%	3800	1012	1196
Leptocylindrus minimus Gran	5	5%	3040	1406	904
Lioloma pacificum (Cupp) Hasle	25	26%	53200	7611	13540
Navicula sp. Bory	21	22%	2280	436	451
Nitzschia incerta (Grunow) M.Peragallo	10	11%	380	248	140
Paralia sulcata (Ehrenberg) Cleve	30	32%	19880	2281	3771
Pleurosigma angulatum (J.T.Quekett) W.Smith	8	8%	950	319	273
Pleurosigma sp. W.Smith	35	37%	1520	369	355
Pseudo-nitzschia spp. H.Peragallo	52	55%	346560	38486	66115
Rhizosolenia alata Brightwell	7	7%	380	229	143
Rhizosolenia alata f. *gracillima* (Cleve) Grunow	43	45%	5320	1155	1093
Rhizosolenia alata var. *indica* (Peragallo) Ostenfeld	6	6%	190	72	55
Rhizosolenia calcar-avis Schultze	26	27%	380	158	144
Rhizosolenia fragilissima Bergon	31	33%	38340	4907	7393
Rhizosolenia imbricata Brightwell	50	53%	5680	836	1143
Rhizosolenia robusta G.Norman ex Ralfs	10	11%	190	55	45
Skeletonema costatum (Greville) Cleve	12	13%	850420	231923	342153
Thalassionema nitzschioides (Grunow) Mereschkowsky	79	83%	14440	2055	2752
Thalassiosira angulata (W.Gregory) Hasle	7	7%	1520	881	467
Thalassiosira rotula Meunier	11	12%	2850	1286	945
Thalassiosira sp. Cleve	12	13%	5680	1242	1554
Dinophyceae					
Dinophyceae miscellaneous MICRO	14	15%	1420	708	478
Dinophyceae miscellaneous NANO	81	85%	42600	5150	7528
Alexandrium sp.	5	5%	1420	548	490
Ceratium candelabrum (Ehrenberg) F.Stein	5	5%	80	48	16
Ceratium extensum (Gourret) A.Cleve	6	6%	380	97	127
Ceratium falcatum (Kofoid) Jörgensen	6	6%	120	53	30
Ceratium furca (Ehrenberg) Claparède & Lachmann	36	38%	760	174	192
Ceratium furca var. *eugrammum* (Ehrenberg) Schiller	5	5%	190	70	60
Ceratium fusus (Ehrenberg) Dujardin	65	68%	2840	212	373
Ceratium trichoceros (Ehrenberg) Kofoid	13	14%	200	79	55
Ceratium tripos (O.F.Müller) Nitzsch	15	16%	200	61	48
Gonyaulax polygramma F.Stein	13	14%	950	258	282
Goniodoma acuminatum (Ehrenberg) F.Stein	7	7%	380	197	161
Gymnodinium sp. F.Stein	10	11%	9940	1302	2884
Gyrodinium fusiforme Kofoid & Swezy	10	11%	570	233	175
Gyrodinium sp. Kofoid & Swezy	9	9%	8520	2031	2654
Heterocapsa sp. F.Stein	29	31%	48280	7786	12469
Kofoidinium velelloides Pavillard	10	11%	190	87	51
Minuscula bipes (Paulsen) Lebour	9	9%	2840	1667	1135
Oxytoxum caudatum Schiller	7	7%	2130	623	636
Podolampas elegans F.Schütt	9	9%	760	280	289
Prorocentrum micans Ehrenberg	18	19%	570	191	158
Prorocentrum minimum (Pavillard) J.Schiller	28	29%	14200	2994	3713
Prorocentrum triestinum J.Schiller	15	16%	8520	2711	2172
Protoperidinium diabolus (Cleve) Balech	5	5%	1140	268	436
Protoperidinium steinii (Jørgensen) Balech	8	8%	190	103	56
Protoperidinium tuba (J.Schiller) Balech	8	8%	5680	1066	1750
*Pseliodinium vaubani*i Sournia	11	12%	1140	220	307
Scrippsiella sp. Balech ex A.R.Loeblich III	9	9%	11360	2372	3321
Torodinium sp. Kofoid & Swezy	24	25%	950	231	273
Dictyochophyceae					
Dictyocha fibula Ehrenberg	19	20%	2840	787	756
Ebriidea					
Hermesinium adriaticum Zach	9	9%	710	186	216
Nano Chlorophyceae miscellaneous	65	68%	65320	10716	14231
Nano Chrysophyceae miscellaneous	9	9%	8520	3787	3510

Nano Cryptophyceae miscellaneous	87	92%	193120	22883	35289
Nano Prasinophyceae miscellaneous	13	14%	8520	3168	2239

Table S5. Pearson correlation coefficients, *r*, for variables used in PCA analysis (Figs. 6a and b) for stations SJ101 and RV001. Values in red are highly correlated, in blue are moderately correlated and in black are not correlated.

	T	PO4	DIN	%PL	%GL	%WE	%ST	%TG	%PIG
SJ101									
T		-0.2369	-0.1437	-0.1164	-0.1188	-0.3235	0.1106	0.3934	-0.0027
PO4			0.6763	-0.4119	-0.0836	-0.2233	0.6970	-0.0283	0.2592
DIN				-0.2276	0.0085	0.0073	0.6056	-0.2796	-0.1652
%PL					-0.6487	0.7001	-0.6523	0.1181	0.1076
%GL						-0.6613	-0.0065	-0.5148	-0.3462
%WE							-0.1840	0.0378	0.0279
%ST								-0.1026	0.1602
%TG									-0.1637
%PIG									
RV001									
T		0.1578	0.6481	-0.1000	0.4988	-0.2552	0.3362	-0.5821	-0.1258
PO4			0.6481	0.1782	0.1622	-0.1039	0.0594	-0.3444	-0.2943
DIN				0.4650	-0.1175	-0.1275	-0.4763	-0.1329	-0.0723
%PL					-0.5706	-0.4637	-0.6466	-0.4786	0.6497
%GL						-0.2769	0.3685	-0.3205	-0.4346
%WE							0.1909	0.7455	-0.5922
%ST								-0.0084	-0.5057
%TG									-0.3063
%PIG									

Table S6. Pearson correlation coefficients, *r*, Pearson correlation coefficients for variables used in PCA analysis (Figs. 7a and b) for the both northern Adriatic stations for two temperature ranges, 15-20 °C and 20-25 °C (b). Values in red are highly correlated, in blue are moderately correlated and in black are not correlated.

	T	PO4	DIN	%PL	%GL	%WE	%ST	%TG	%PIG
15-20 °C									
T		0.7591	0.5268	0.0788	-0.5509	-0.6234	0.5984	-0.6733	-0.6291
PO4			0.3853	0.3637	-0.3695	-0.0755	0.1266	-0.5679	-0.5269
DIN				-0.5841	0.0463	-0.7007	0.7967	-0.1807	-0.7075
%PL					-0.6475	0.4292	-0.7216	0.0459	0.3633
%GL						0.3985	0.0614	-0.0407	-0.1333
%WE							-0.8438	0.1122	0.4201
%ST								-0.4438	-0.7149
%TG									0.7251
%PIG									
20-25 °C									
T		0.3313	-0.8872	0.2245	-0.6168	0.2796	0.3585	0.6271	0.2745
PO4			-0.4002	-0.3449	-0.2026	-0.3718	0.6882	0.9360	-0.4947
DIN				-0.4689	0.8257	-0.5317	-0.3169	-0.6798	-0.2610
%PL					-0.8200	0.7148	0.0086	-0.1348	0.7623
%GL						-0.5495	-0.4359	-0.4477	-0.5538
%WE							-0.3787	-0.1315	0.3058
%ST								0.7092	-0.0676
%TG									-0.3063
%PIG									