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BIOAKTIVNI POTENCIJAL I KEMIJSKI SASTAV HIDROALKOHOLNIH EKSTRAKATA DIJATOMEJA SJEVERNOG JADRANA

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BIOAKTIVNI POTENCIJAL I KEMIJSKI SASTAV HIDROALKOHOLNIH EKSTRAKATA DIJATOMEJA SJEVERNOG JADRANA

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Rad je izrađen u laboratorijima Centra za istraživanje mora-Institut Rođer Bošković u Rovinju, BIOcentra u Zagrebu, Zavoda za prehrambenu tehnologiju i biotehnologiju Kemijsko-tehnološkog fakulteta Sveučilišta u Splitu, Sveučilišnog odjela za studije mora u Splitu, Instituta za jadranske kulture i melioraciju krša u Splitu te Odjelu za kemiju Prirodoslovno-matematičkog fakulteta u Splitu.

Sažetak

Morske dijatomeje su široko rasprostranjene mikroalge čija komercijalna upotreba još uvijek nije prisutna zbog niza tehnoloških prepreka. Pronalazak tehnoloških riješenja za održivu proizvodnju i iskorištavanje dijatomejne biomase omogućilo bi bolje iskorištenje dijatomeja i njihovih spojeva u područjima poput nanotehnologije, prehrambene i farmaceutske industrije. U ovom doktorskom radu istraživan je bioaktivni potencijal i kemijski sastav dijatomeja *Chaetoceros costatus* (CIM935), *Chaetoceros socialis* (CIM929), *Thalassiosira rotula* (CIM861) i *Skeletonema grevillei* (CIM876) izoliranih iz sjevernog Jadrana. Cilj rada bio je odrediti utjecaj različitih ekstrakcijskih otapala, uzgojnih sustava i uzgojnih medija bez određenih makronutrijenata na bioaktivni potencijal i kemijski sastav dijatomejnih ekstrakata. Antioksidacijski potencijal dijatomejnih ekstrakata procijenjen je metodom snage redukcije feri-iona, 2,2-difenil-1-pikrilhidrazilni testom i metodom određivanja kapaciteta apsorpcije kisikovih radikala, a u kemijskoj analizi korištena je metoda Folin Ciocalteu za određivanje ukupnog sadržaja fenolnih spojeva, plinska kromatografija s masenom spektrometijom i tekućinska kromatografija ultra visoke učinkovitosti spregnutom sa spektrometrijom masa visoke rezolucije. U odnosu na aceton i heksan, veći ekstrakcijski prinos, antioksidacijski potencijal te prinos biološki aktivnih spojeva zabilježen je korištenjem etanola u ekstrakciji dijatomeje *C. costatus*. Ekstrakcija biomase *T. rotula* u otopini s većim volumnim udjelima etanola u hidroalkoholnoj smjesi omogućila je veći antioksidacijski potencijal i prinos komercijalno važnih spojeva poput eikozapentaenske kiseline i 24-metilenkolesterola. Bioreaktor je omogućio brži rast te veći prinos biomase *S. grevillei* u odnosu na termostatsku miješalicu (inkubator), a ekstrakti *S. grevillei* dobiveni iz biomase uzgojene u bioreaktoru bilježili su povećanje antioksidacijskog potencijala i sadržaja bioaktivnih spojeva poput masnih kiselina i sterola tijekom rasta. U odnosu na silicij i fosfor, najveći utjecaj na povećanje antioksidacijske aktivnosti i sadržaja derivata pigmenata u vrsti *C. costatus* zabilježen je pri nedostatku dušika u uzgojnom mediju, međutim isti utjecaj nije zabilježen u vrsti *C. socialis*. Navedeni rezultati ukazuju na izniman bioaktivni potencijal dijatomejnih ekstrakata te daju osnovne smjernice za daljnja biotehnološka istraživanja s ciljem omogućavanja daljnje komercijalizacije.

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BIOACTIVE POTENTIAL AND CHEMICAL COMPOSITION OF HYDROALCOHOLIC EXTRACTS OF DIATOMS FROM THE NORTH ADRIATIC

Roberta Frleta Matas

Thesis performed in the laboratories of the Center for Marine Research - Institute Ruđer Bošković in Rovinj, BIOcenter in Zagreb, Department of Food Technology and Biotechnology at the Faculty of Chemical Technology in Split, University Department of Marine Studies in Split, Institute for Adriatic Culture and Karst Reclamation in Split, and Department for Chemistry at the Faculty of Science in Split

Abstract

Marine diatoms are a diverse group of microalgae with widespread distribution, yet their commercial exploitation remains limited due to various technological constraints. The development of sustainable technological solutions for the cultivation and valorization of diatom biomass could significantly enhance the utilization of these organisms and their bioactive metabolites across fields such as nanotechnology, functional food production, and pharmaceutical applications. This doctoral thesis investigated the bioactive potential and chemical composition of four diatom species: *Chaetoceros costatus* (CIM935), *Chaetoceros socialis* (CIM929), *Thalassiosira rotula* (CIM861), and *Skeletonema grevillei* (CIM876), all isolated from the northern Adriatic Sea. The primary objective of the study was to evaluate the influence of different extraction solvents, cultivation systems, and macronutrient-deficient media on the antioxidant capacity and chemical profiles of diatom extracts. Antioxidant potential was assessed using the ferric reducing antioxidant power (FRAP) assay, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and the oxygen radical absorbance capacity (ORAC) method. Total phenolic content (TPC) was determined using the Folin–Ciocalteu method, and chemical profile using gas chromatography–mass spectrometry (GC-MS), and ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry (UHPLC-HRMS). Among the solvents tested, ethanol provided superior extraction yields, enhanced antioxidant activity, and a higher concentration of biologically active compounds in *C. costatus* compared to acetone and hexane. In the case of *T. rotula*, increasing the volumetric ratio of ethanol in hydroalcoholic mixtures significantly improved antioxidant potential and the recovery of commercially relevant compounds such as eicosapentaenoic acid (EPA) and 24-methylenecholesterol. Cultivation of *S. grevillei* in a bioreactor resulted in accelerated growth and greater biomass productivity compared to a conventional thermostatic shaker (incubator). Furthermore, extracts obtained from bioreactor-cultured biomass showed higher antioxidant activity and higher concentrations of bioactive constituents, particularly fatty acids and sterols, over the growth period. Among the tested macronutrients, nitrogen deprivation had the most pronounced effect on enhancing antioxidant activity and pigment derivative content in *C. costatus*. However, a similar response was not observed in *C. socialis*, indicating species-specific metabolic responses to nutrient stress. Collectively, these findings underscore the notable bioactive potential of diatom extracts and offer valuable insights for the optimization of cultivation and extraction strategies. They provide a foundation for further biotechnological research aimed at facilitating the commercial application of diatom-derived bioactive compounds.

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Sadržaj

1. PREGLED OBJAVLJENIH RADOVA

Preliminarno istraživanje. **Frleta Matas R**., Čagalj M., Jelusić, K., Radman, S., Šimat V. (2025). The effect of solvent choice on antioxidant potential and chemical composition of extracts from microalgae *Chaetocerus costatus. Phycology*, 5(1), 8. https://doi.org/10.3390/phycology5010008

Rad 1. **Frleta Matas R**., Popović M, Čagalj M., Šimat V. (2023). The marine diatom *Thalassiosira rotula*: chemical profile and antioxidant activity of hydroalcoholic extracts. *Frontiers in Marine Science*, 10, 1221417. https://doi.org/10.3389/fmars.2023.1221417

Rad 2. **Frleta, R.,** Popović, M., Smital, T., Šimat, V. (2022). Comparison of growth and chemical profile of diatom *Skeletonema grevillei* in bioreactor and incubation-shaking cabinet in two growth phases. *Marine Drugs*, 20, 697. https://doi.org/10.3390/md20110697

Rad 3. **Frleta Matas, R.**, Radman, S.; Čagalj, M.; Šimat, V. (2024). Influence of nutrient deprivation on the antioxidant capacity and chemical profile of two diatoms from genus *Chaetoceros*. *Marine Drugs*, 22, 96. https://doi.org/10.3390/ md22020096

2. UVOD

Morske mikroalge, čiji se broj vrsta u moru procjenjuje na čak 300 000, predstavljaju važan izvor hrane u morskim ekosustavima (Rumin i sur. 2020; Duran i sur. 2021). Najzastupljenija skupina morskih mikroalgi odgovorna za više od 40 % ukupne primarne proizvodnje oceana su dijatomeje (Field i sur., 1998). Dijatomeje su fotosintetičke mikroalge karakteristične po silikatnoj staničnoj stijenci frustuli, unutar koje se nalazi sadržaj bogat različitim skupinama spojeva poput pigmenata, masnih kiselina, sterola, proteina, peptida i polisaharida (Nieri i sur., 2023). Navedeni spojevi nositelji su temeljne biološke aktivnosti u dijatomejama, primarno antioksidacije. Antioksidansi se definiraju kao tvari koje imaju sposobnost zaštite stanica od nastanka i nakupljanja slobodnih radikala čime onemogućavaju neželjene procese koji dovode do oksidacijskog stresa i razvoja kroničnih bolesti poput kardiovaskularnih i neurodegenraditvnih bolesti (Mendonça i sur., 2022). Oksidacijski stres je patološko stanje koje nastaje u uslijed nagomilavanja slobodnih radikala poput kisikovih ili dušikovih, što dovodi do oštećenja stanica, tkiva i organa. Slobodni kisikovi radikali (ROS, engl. *reactive oxygen species*) i slobodni dušikovi radikali (RNS, engl. *reactive nitrogen species*) su visoko reaktivne molekule koje u organizmu nastaju kao nusprodukti normalnog metabolizma, posebno u mitohondrijima, ali i kao odgovor na vanjski stres (npr. UV zračenje, zagađenja, toksine). I ROS i RNS mogu imati korisnu ulogu u staničnom signaliziranju, ali njihova prekomjerna proizvodnja dovodi do oksidativnog stresa i oštećenja stanica (Mendonça i sur., 2022). Održavanje ravnoteže između njihove proizvodnje i eliminacije ključno je za zdravlje stanica i organizma. Proces antioksidacijskog djelovanja može biti: i) izravan (neutralizacijom slobodnih radikala kroz donaciju elektrona bez da sami postanu reaktivni); ii) neizravan (neutralizacijom slobodnih radikala kroz poticanje sinteze enzima i molekula koje štite stanice ili obnavljaju oštećenja). Jedna od važnih karakteristika gotovo svih antioksidansa je višestruka biološka aktivnost, poznatija i kao polifarmakološki učinak. Polifarmakologija je koncept u kojem molekula, kao djelatna tvar, ima višestruko djelovanje zbog čega je učinkovitija u djelovanju kod složenih bolesti poput raka, kod kojih je u nastanak i razvoj bolesti uključeno više proteina i metaboličkih puteva (Mendonça i sur., 2022). Antioksidacijski testovi predstavljaju brz i učinkovit način za preliminarni probir ekstrakata iz kojih se može procijeniti njihov bioaktivni potencijal.

Istraživanja na dijatomejama pokazala su da sadrže veliki broj različitih biološki aktivnih spojeva zbog čega se smatraju važnom skupinom mikroalgi s antioksidacijskim potencijalom i mogućnošću primjene njihove biomase i ekstrakata u industrijama poput farmaceutske,

kozmetičke i prehrambene, proizvodnji biodizela te u području nanotehnologije (Sathasivam i sur. 2019; Ummalyma i sur. 2022).

Jednu od glavnih komponenti unutarstaničnog sadržaja dijatomejnih stanica čine lipidi čiji udio iznosi i do 25 % lipida/suhu masu dijatomeja (Yi i sur. 2017). Uz neutralne lipide (triacilgliceroli), polarne lipide (glikolipidi i fosfolipidi) i oksilipine u najzastupljenije lipidne spojeve spadaju i masne kiseline i steroide (sterola) (Yi i sur., 2017). Dijatomeje sadrže široki raspon masnih kiselina te su izvrstan izvor višestrukonezasićenih masnih kiselina (VNMK) poput eikosapentaenske (EPK), dokozaheksaenske (DHK) i dokozapentaenske kiseline (DPK) koje imaju blagotvoran utjecaj na ljudsko zdravlje (sprječavaju i smanjuju rizike od razvoja bolesti krvožilnog sustava, upalnih i autoimunih bolesti, dijabetesa te neurodegenerativnih bolesti i dr.) (Calder, 2017; Li i sur. 2019; Bárcenas-Pérez i sur. 2021). Osim masnih kiselina, dijatomeje sadrže derivate masnih kiselina u obliku primarnih amida masnih kiselina (PAMK) koji imaju važnu ulogu u kontroli različitih fizioloških procesa poput kretanja, spavanja i angiogenze (Farrell i sur., 2012). Također, PAMK imaju dokazana bioaktivna svojstva poput antimikrobnih, antidijabetičkih, antitumorskih i antihelminitičkih (D'Oca i sur., 2010; Dembitsky, 2022; Kabara i sur., 1972; Tanvir i sur., 2018). Dijatomeje su poznate i kao dobar izvor esencijalnih triterpenoida sterola koji posjeduju brojne biološke aktivnosti, a klasificirani su kao spojevi sigurni za upotrebu u hrani (Aldini i sur. 2014; Ras i sur. 2014; Wang i Seibert 2017; Jaramillo-Madrid i sur. 2020; Cutignano i sur. 2022). Jedan od najzastupljenih sterola u dijatomeja je 24-metilenkolesterol, važan intermedijer u biosintezi fitosterola (Rampen i sur. 2010; Gallo i sur. 2020). Posljednjih nekoliko desetljeća popularnost fitosterola raste zbog pozitivnih zdravstvenih učinaka među kojima su dokazani snižavanje razine kolesterola, protuupalno, antioksidacijsko i antidijabetičko djelovanje te hepatoprotektivne i neuroprotektivne sposobnosti (Sañé i sur. 2021; Jie i sur. 2022). Druga važna skupina spojeva u stanicama dijatomeja su pigmenti, od kojih su najzastupljeniji klorofili (klorofil *a* i klorofil *c*), fukoksantin, *β*-karoten i ksantofili (diatoksantin, diadinoksantin, violaksantin, anteraksantin, zeaksantin). Uloga pigmenata u dijatomejnim stanicama je fotosintetska i fotoprotektivna, a zbog dokazanih bioloških aktivnosti (antioksidativne, antiupalne, antitumorske) imaju potencijal za komercijalnu primjenu u prehrambenoj, kozmetičkoj i farmaceutskoj industriji (Kuczynska i sur., 2015). Uzgoj dijatomeja u kontroliranim uvjetima još uvijek nije raširen ni komercijaliziran zbog tehnoloških prepreka koje se odnose na optimizaciju uzgoja, učinkovito prikupljanje (engl. *harvesting*) i ekstrakciju ciljanih spojeva (Gonzalez-Fernandez i Muñoz 2017).

Metoda ekstrakcije i odabir pogodnog otapala imaju ključnu ulogu u izdvajanju pojedinih skupina spojeva te izravno utječu na prinos i kemijski profil ekstrakata dijatomeja. Ekstrakcijski prinos predstavlja masu ekstrahiranog materijala iz početne tvari, a sustavna analiza kojom se identificira sastav, koncentracija te karakteristike spojeva u uzorku ekstrahiranog materijala predstavlja kemijski profil. U cilju postizanja maksimalnog prinosa željenih spojeva često se koristi metoda ekstrakcije potpomognuta ultrazvukom (EPU). Mehanizam rada EPU zasniva se na provedbi mehaničke vibracije (usljed djelovanja ultrazvučnih valova) koje dopiru u stanicu uzrokujući ekspanzijske i kompresijske cikluse unutarstaničnog sadržaja (lipidi, pigmenati i drugih bioaktivni spojevi), a potpomognuti termičkim efektom uzrokuju degradaciju stanične stijenke i oslobađanje unutarstaničnog sadržaja u otapalo (Mason i sur. 1996; Kong i sur., 2014; Staroveški, 2018; Vernès i sur. 2020; Mienis i sur., 2024). Ovaj oblik ekstrakcije je ekološki prihvatljiv, prikladan za rad s malom količinom uzorka te skraćuje vrijeme ekstrakcije.

S druge strane, odabir ekstrakcijskog otapala vrši se na temelju indeksa polariteta, primjerice heksan (0,0-0,1) < kloroform (4,1) < metanol (5,1) = aceton (5,1) < etanol (5,2) < voda (9,0), vodeći se pri tom da je polarnost ciljanih spojeva u ekstrakciji približno jednaka polarnosti otapala (Foo i sur., 2015). Etanol je često korišteno polarno otapalo koje zbog svoje amfipatske prirode ima mogućnost interakcije s nepolarnim i polarnim molekulama pa se zbog toga često koristi u ekstrakciji spojeva iz mikroalgi. Efikasnost ekstrakcije spojeva sa širokim rasponom polariteta dodatno se povećava korištenjem otapala u obliku hidroalkoholnih smjesa (Jacotet-Navarro i sur. 2018). Štoviše, veća biološka aktivnost zabilježena je u ekstraktima pripremljenim korištenjem hidroalkoholnih smjesa u odnosu na ekstrakte pripremljene isključivo u vodi ili etanolu, a najveći prinos bioaktivnih spojeva postiže se korištenjem hidroalkoholnih smjesa u rasponu od 50 – 80 % (Silva i sur. 2022; Jacotet-Navarro i sur. 2018; Monteiro i sur. 2020; Čagalj i sur. 2021). Korištenje "zelenih" otapala kao što su hidroalkoholne smjese smatra se ekološki prihvatljivim.

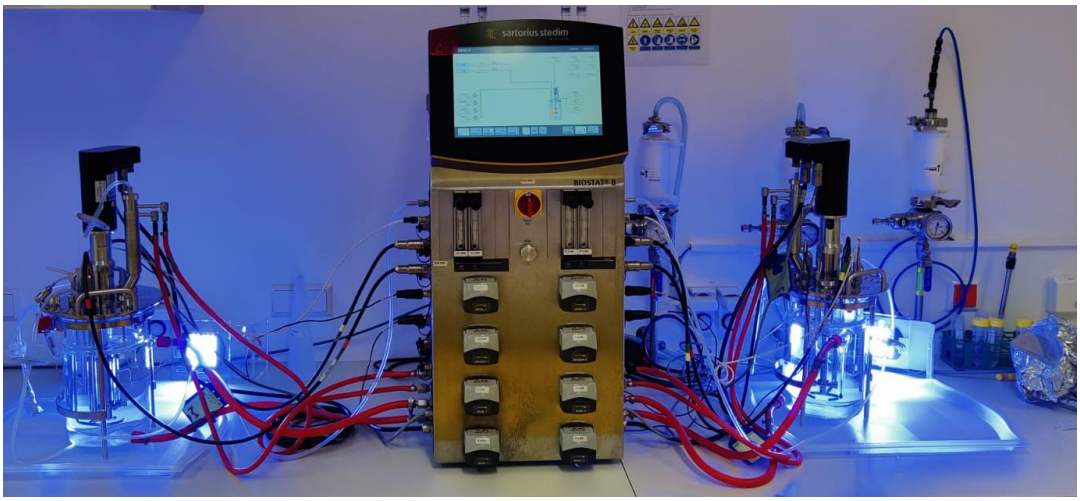
Optimizacija uzgojnog ciklusa dijatomeja jedan je od ključnih koraka kojim se osigurava maksimalan prinos njihove biomase što posljedično omogućava postizanje većeg ekstrakcijskog prinosa. Povećana proizvodnja metabolita kod dijatomeja uglavnom je uvjetovana promjenom niza abiotičkih parametara poput temperature, saliniteta, pH, intenziteta svjetlosti, udijela CO_2 i dostupnim hranjivim tvarima (Lin i sur. 2018; Gatamaneni i sur. 2018; Vello i sur. 2023). Laboratorijski uzgoj najčešće se provodi u inkubatorima te rezultira nižom brojnosti dijatomeja u odnosu na prirodno okruženje. Inkubatori za uzgoj mikroalgi su uređaji koji imaju mogućnost regulacije temperature, unutar kojih se nalazi izvor svjetlosti poput LED ili fluorescentnog svjetla

s mogućnošću postavljanja željenog fotoperioda, a u svrhu postizanja kontroliranih uvjeta uzgoja (Slika 1).

Slika 1. Laboratorijski uzgoj dijatomeje *Skeletonema grevillei* u inkubatorima s integriranim sustavom miješanja (vlastita fotografija)

Osnovni nedostatak laboratorijskog uzgoja je nedostatan prinos biomase i iskoristivost hranjivih tvari iz medija (Stonik 2015). Konvencionalni uzgoj u inkubatorima općenito daje mali prinos biomase, a trošak proizvodnog napora (kombinacija energije, rada, materijala i vremena potrebnih za održavanje proizvodnog procesa) ga premašuje čime je dodatno umanjena profitabilnost procesa. Prvi bioreaktor razvijen je 1997. godine u Japanu u cilju proizvodnje veće biomase dijatomeje *Nitzschia* sp. (Fukami i sur. 1997). U kontekstu uzgoja i biotehnologije, aspekt homogenosti prvenstveno se odnosi na jednoliku raspodjelu stanica mikroalgi u mediju čime se postiže jednolika dostupnost hranjivih tvari i svjetlosti ključnih za uspješan rast. Tijekom uzgoja nužno je stalno miješanje kojim se sprječava sedimentacija stanica (Slika 2).

Nedostatak hranjivih tvari, neprimjerena temperatura te različiti izvori svjetlosti predstavljaju stresne uzgojne uvjete koji dodatno potiču različite metaboličke procese, poput proizvodnje karotenoida i biosinteze lipida. Pri stresnim uvjetima uzgoja povećava se proizvodnja pojedinih primarnih i sekundarnih metabolita koji imaju ulogu u obrani stanice, a kemijska karakterizacija sintetiziranih spojeva u unutarstaničnom sadržaju (primarni i sekundarni metaboliti) prilično je složen postupak (Vuppaladadiyam i sur. 2018).



Slika 2. Laboratorijski uzgoj dijatomeje *Skeletonema grevillei* u bioreaktoru s LED svjetlom (vlastita fotografija)

Za pogodan rast i proizvodnju metabolita nužni su makronutrijenti, posebno silicij (Si), dušik (N) i fosfor (P). U dijatomeja Si predstavlja ključan nutrijent za izgradnju frustule, a indirektno je uključen i u procese replikacije deoksiribonukleinske kiseline (DNK) i diobe stanica (Orefice i sur. 2019; Shrestha i Hildebrand 2015). Većina asimiliranog N koristi se za sintezu proteina i nukleinskih kiselina, a također je esencijalan u biosintezi drugih molekula poput lipida i različitih šećera (Orefice i sur. 2019). Fosfor je važan dio membrana dijatomejnih stanica gdje je prisutan u fosfolipidima, a sastavni je dio nukleinskih kiselina te neizostavan za proizvodnju kemijske energije u obliku nikotinamid adenin dinukleotid-fosfata (NADPH) i adenozin trifosfata (ATP) (Lovio-Fragoso i sur. 2021). Nedostatak ili potpuni izostanak Si, N i P mogu biti okidači za povećanu metaboličku proizvodnju i nakupljanje različitih bioaktivnih spojeva u stanicama dijatomeja međutim metabolički odgovori često se razlikuju među vrstama (Lovio-Fragoso i sur. 2021; Curcuraci i sur. 2022; Smith i sur. 2016; Yu i sur. 2016). Poznato je da veća koncentracija Si, N i P pozitivno utječe na rast dijatomeja, dok manjak ili potpuni izostanak potiču sintezu spojeva kao što su pigmenti, masne kiseline ili fenoli (Orefice i sur. 2019; Curcuraci i sur. 2022). Kod vrste *Chaetoceros muelleri* najveći fiziološki stres izaziva nedostatak N što rezultira povećanjem udjela lipida unutar stanica (Lin i sur. 2018; Gao i sur. 2013). Isti metabolički odgovor zabilježen je i kod vrste *Phaeodactylum tricornutum* (Curcuraci i sur. 2022). Izazivanje fiziološkog stresa nedostatkom/manjkom hranjiva u uzgojnom mediju često je korištena strategija za poticanje stanica mikroalgi na povećanu sintezu bioaktivnih spojeva, a metabolički odgovori često se razlikuju među vrstama (Lovio-Fragoso i sur. 2021).

Pronalazak tehnoloških rješenja i uzgojnih parametara kojima bi se povećala biomasa dijatomeja, uvjeta uzgoja u okviru kojih bi se povećala proizvodnja ciljanih metabolita, postizanje većeg ekstrakcijskog prinosa i bolji bioaktivni potencijal ekstrakata od ključne su važnosti za njihovu komercijalizaciju i širu industrijsku primjenu. Također, vrlo je važno proizvodnju usmjeriti prema korištenju svih dijelova dijatomeja (npr. silikatne ljušturice koje zaostaju nakon ekstrakcije), čime bi se omogućila maksimalna ekonomska isplativost i ekološka održivost komercijalnog uzgoja dijatomeja.

2.1. Ciljevi preliminarnog istraživanja i objedinjenih radova

Preliminarno istraživanje

- Usporediti učinkovitost i prinos ekstrakcije, antioksidativni potencijal i kemijski sastav ekstrakata dijatomeje *Chaetoceros costatus* dobivenih koristeći ultrazvučnu ekstrakciju (EPU) različitim otapalima – acetonom, etanolom i heksanom,.

Rad 1

- Odrediti razlike u ekstrakcijskom prinosu, kemijskom profilu i antioksidacijskom potencijalu dijatomeje *Thalassiosira rotula* korištenjem različitih hidroalkoholnih mješavina (50 % i 70 % etanol).

Rad 2

- Odrediti krivulje rasta, kemijski profil i antioksidacijski potencijal dijatomeje *Skeletonema grevillei* u standardnom F/2 mediju tijekom dvije faze rasta (eksponencijalne i stacionarne faze) u dva uzgojna sustava, bioreaktoru i inkubatoru (termostatiranoj miješalici).

Rad 3

- Ispitati utjecaj ograničenja nutrijenata (dušika, fosfora i silicija) u F/2 uzgojnom mediju na antioksidativni kapacitet i kemijski profil (pigmenti, fenolni spojevi, masne kiseline, steroli) dviju vrsta dijatomeja iz roda *Chaetoceros – Chaetoceros costatus* i *Chaetoceros socialis.*

3. MATERIJALI I METODE

3.1. Odabir dijatomejnih kultura

Za potrebe istraživanja izolirano je devet vrsta dijatomeja (*C. costatus* (CIM935), *Chaetoceros curvisetus* (CIM951), *Chaetoceros dydimus* (CIM827), *C. socialis* (CIM929), *Leptocylindrus sp.* (CIM900), *Pseudonitzschia sp* (CIM927), *S. grevillei* (CIM876), *Skeletonema sp.* (CIM936), *T. rotula* (CIM861)) na području sjevernog Jadrana, u okviru sezonskog monitoringa Instituta Ruđer Bošković. Vrste su pročišćene, genetski identificirane i preuzete iz kolekcije kultura Centra za istraživanje mora Instituta Ruđer Bošković u Rovinju. Najbolju prilagodbu (u pogledu rasta) na laboratorijske uvjete uzgoja pokazale su vrste *C. costatus* (CIM935), *C. socialis* (CIM929), *S. grevillei* (CIM876) i *T. rotula* (CIM861) koji su odabrani za provedbu pojedinih istraživanja. Zbog smanjene stope rasta i progresivnog razvoja morfoloških deformacija pri dugotrajnom laboratorijskom uzgoju, u pojedinim istraživanjima ovog rada korištene su različite vrste dijatomeja.

3.2. Uzgoj i određivanje krivulje rasta dijatomeja

Rast dijatomeja (*C. costatus* (CIM935), *C. socialis* (CIM929), *S. grevillei* (CIM876) i *T. rotula* (CIM861)) praćen je prebrojavanjem dijatomejnih stanica u graduiranoj Sedgwick Rafter komorici na temelju koje je određena krivulja rasta prema standardnoj proceduri (Karlson i sur., 2010). Odabrane vrste (*C. costatus* (CIM935), *C. socialis* (CIM929), *S. grevillei* (CIM876) i *T. rotula* (CIM861)) inokulirane su u stacionarnoj fazi rasta u F/2 uzgojni medij. Medij za uzgoj dijatomeja pripremljen je korištenjem filtrirane sterilne morske vode uz dodatak makronutrijenata (Si, N i P) te mikronutrijena (metala u tragovima i vitamina) prema standardnim postupcima (Andersen, 2010).

3.2.1. Uzgojni parametri u preliminarnom istraživanju

Vrsta *C. costatus* uzgajana je u inkubatoru u šest Erlenmeyer tikvica, a svaka tikvica sadržavala je 1,5 L F/2 uzgojnog medija te 100 ml inokuluma dijatomeje *C. costatus* (10^5 stanica/mL). *Chaetoceros costatus* uzgajan je pri temperaturi od 18 °C, fotoperiodu (svjetlo:tama) 18:6 i intezitetu svjetlosti 2500 luxa (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italija). Prikupljanje kulture izvršeno je po ulasku kulture u stacionarnu fazu rasta filtracijom kroz filter od staklenih mikrovlakana (Grade GF/F Whatman). Dijatomejna biomasa prikupljena je falcon

epruvete te zamrznuta, a zatim liofilizirana (FreeZone 2.5, Labconco, Kansas City, MO, SAD) prije postupka ekstrakcije.

3.2.2. Uzgojni parametri u Radu 1.

Dijatomeja *T. rotula* uzgajana je u inkubatoru u šest Erlenmeyerovih tikvica volumena 5 L. Svaka tikvica je sadržavala 1,5 L F/2 medija u koji je inokulirano 150 mL inokuluma *T. rotula* brojnosti 10^5 stanica/mL. Do postizanja stacionarne faze rasta, dijatomeja *T. rotula* uzgajana je na 17 °C, pri fotoperiodu (svjetlo:tama) 16:8 pod LED svjetlom (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italija) intenziteta od 2500 luxa. Dijatomejna biomasa prikupljena je u stacionarnoj fazi rasta filtracijom kroz filter od staklenih mikrovlakana (Grade GF/F Whatman). Prikupljena biomasa prebačena je pomoću strugača stanica u falcon epruvete te zamrznuta. Prije postupka ekstrakcije, zamrznuta biomasa *T. rotula* podvrgnuta je procesu liofilizacije (FreeZone 2.5, Labconco, Kansas City, MO, SAD).

3.2.3. Uzgojni parametri u Radu 2.

Dijatomeja *S. grevillei* uzgajana je u F/2 mediju u dva uzgojna sustava: bioreaktoru (BS) (BiostatB Twin, Sartorius, Goettingen, Njemačka) i u Erlenmeyer tikvici volumena 5 L u termostatskoj mješalici tj. inkubatoru (IS) (Certomar T plus, Sartorius, Goettingen, Njemačka). Za svaki sustav korištene su dvije replike, od kojih je svaka sadržavala 2 L F/2 medija u kojem je inokulirano 50 mL *S. gravillei* (9×10^4 stanica/mL). U oba sustava dijatomeje su uzgajane na 18 °C s postavljenim fotoperiodom (svjetlo:tama) 12:12 h te intenzitetom svjetlosti od 2500 luxa. U BS-u korišteno je LED svjetlo (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italija) dok su u IS-u korištene fluorescentne lampe (Fluora T8, Osram, Garching, Njemačka). Tijekom uzgoja u BS miješanje se provodilo pomoću propelera pri 70 okretaja/min a brzina aeracije postavljena je na 1 L/min, dok je u IS brzina mješanja iznosila 50 okretaja/min kako bi se izbjeglo prolijevanje kultura. Prikupljanje biomase izvršeno je u eksponencijalnoj fazi uzgoja (nakon 192 h) i na početku stacionarne faze rasta (nakon 312 h) filtracijom kroz filter od staklenih mikrovlakana (Grade GF/F Whatman).

3.2.4. Uzgojni parametri u Radu 3.

Dijatomeje *C. costatus* i *C. socialis* uzgajane su u Erlenmeyer tikvici volumena 2 L koja je sadržavala 500 mL uzgojnog medija i 20 mL inokuluma dijatomeje *S. grevillei* (10^5 stanica/mL) u slijedećim uvjetima: u standardnom F/2 mediju (kontrolna skupina, K); u F/2 mediju bez dodatka

fosfora (P-), dušika (N-) i silicija (Si-). Sve skupine (K, P-, N-, Si-) uzgajane su u inkubatoru pri temperaturi od 18 °C, intezitetu svjetlosti od 2500 luxa (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italija) i fotoperiodu (svjetlo:tama) 16:8. Uzgoj je proveden u dva ponavljanja.

3.3. Metode ekstrakcije dijatomejne biomase

3.3.1. Ekstrakcijski parametri u prelimiranom istraživanju

U ekstrakciji liofilizirane biomase *C. costatus* za ekstrakcijska otapala odabrani su aceton, etanol i heksan. Ekstrakcijski proces podpomognut je korištenjem ultrazvuka u ultrazvučnoj kupelji (DU-100 Digital Ultrasonic Cleaner, Giorgio Bormac, Carpi, Italija) na frekvenciji od 40 kHz i 40 °C tijekom 1 sata. Svi uzorci su centrifugirani (Rotafix 32A, Hettich, Tuttlingen, Njemačka) na 3.220 *g* tijekom 5 minuta. Sakupljeni supernatanti (ekstrakti) filtrirani su kroz filter mješanog celuloznog estera promjera pore 0,45 µM (LGG, Meckenheim, Njemačka) te upareni do suhog pomoću centrifugalnog usparivača (RC10-22, Jouan, Herblain, Francuska).

3.3.2. Ekstrakcijski parametri u Radu 1.

Liofilizirana biomasa dijatomeje *T. rotula* ekstrahirana je u hidroalkoholnim mješavinama 50 % i 70 % etanola, EPU metodom u ultrazvučnoj kupelji (Transsonic Tp 310H, Elma Schmidbauer GmbH,Singen, Njemačka) pri frekvenciji od 40 kHz i temperaturi od 40 °C tijekom 1 sata. Nakon ekstrakcije, uzorci su centrifugirani (Centric 150, Tehtnica, Slovenija) 10 minuta na sobnoj temperaturi i 2.515 *g*. Dobiveni supernatant (ekstrakt) filtriran je korištenjem filter celuloznog estera promjera pora 0,45 µm (LGG, Meckenheim, Njemačka) te uparen do potpune suhoće pomoću centrifugalnog usparivača (RC10-22, Jouan, Herblain, Francuska).

3.3.3. Ekstrakcijski parametri u Radu 2.

Prikupljena biomasa dijatomeje *S. grevillei* prenesena je u falcon epruvete te resuspendirana u 10 mL 70 % etanola. Postupak ekstrakcije proveden je u duplikatu korištenjem EPU pomoću ultrazvučne sonde (Sonoplus HD 3200, Bandalin, Berlin, Njemačka) na 20 kHz ± 500 Hz tijekom 5 minuta, pri rasponu od 30 % od maksimalne amplitude 308 µm i tretmanima od 20 s. Pauza između tretmana bila je 40 s. Nakon ekstrakcije, uzorci su centrifugirani (Lynx 4000 centrifuga, Thermo Scientific, Waltham, Massachusetts, SAD) na 29.097 *g* na 4 °C tijekom 30 minuta. Sakupljeni supernatant (ekstrakt) filtriran je kroz sterilni acetat celulozni filter promjera

pora 0,2 μm (LGG, Meckenheim, Njemačka) i uparen do potpune suhoće pomoću centrifugalnog usparivača (RC10-22, Jouan, Herblain, Francuska).

3.3.4. Ekstrakcijski parametri u Radu 3.

Ekstrakcija liofilizirane biomase dijatomeja *C. costatus* i *C. socialis* provedena je korištenjem 70 % etanola te potpomognuta ultrazvukom u ultrazvučnoj kupelji (DU-100 Digital ultrasonic cleaner, Giorgio Bormac, Carpi, Giorgio Bormac, Carpi, Italija) frekvenciji 40 kHz pri temperaturi od 50 °C tijekom 1 sata. Uzorci su centrifugirani (Rotafix 32A, Hettich, Tuttlingen, Njemačka) 5 minuta pri sobnoj temperaturi i 3.220 *g* dobiveni supernatanti su filtrirani kroz filter mješanog celuloznog estera promjera pore 0,45 μM (LGG, Meckenheim, Njemačka) i osušen centrifugalnim isparivačem (RC10-22, Jouan, Herblain, Francuska).

3.4. Kemijska identifikacija spojeva

3.4.1. Spektrofotometrijska metoda određivanja ukupnog sadržaja fenolnih spojeva – Folin Ciocalteu

Određivanje ukupnog sadržaja fenola (USF) u ekstraktima dijatomeja provedeno je Folin–Ciocalteu metodom (Amerine i Ough, 1980). U volumen destilirane vode od 1,5 mL dodano je 25 μL ekstrakata *T. rotula* te 25 μL Folin-Ciocalteu reagensa. Nakon dodavanja reagensa smjese su promiješane i ostavljene 1 min. Zatim je dodano 475 μL destilirane vode i 375 μL 20 % otopine natrijeva karbonata. Uzorci su ostavljeni 2 h na sobnoj temperaturi u mraku, a apsorbancija je izmjerena na 765 nm pomoću spektrofotometra (SPECORD 200 Plus, Edition 2010, Analytik Jena AG, Jena, Njemačka). Analiza uzoraka provedena je u 4 ponavljanja, a rezultati izraženi u mg galne kiseline (mg GK)/g suhe dijatomeje (g SD) (u Radu 1) te mg galne kiseline (mg GK)/ litri ekstrakta (L EX) (u Radu 3).

3.4.2. Kromatografske metode

U kemijskoj analizi dijatomejnih ekstrakata korištene su kromatografske metode. Odvajajući kemijske spojeve iz smjese na temelju njihovih interakcija s pokretnom i nepokretnom fazom omogućava se precizna identifikacija i kvantifikacija spojeva (de Souza i sur. 2023).

3.4.2.1. Plinska kromatografija

U identifikaciji spojeva korištena je plinska kromatografija s masenom spektrometijom (GC – MS) prema metodi Torras-Claveria i sur. (2010) s manjim izmjenama. U suhe ekstrakte dijatomeja dodan je derivatizacijski agens NO-bis(trimetilsilil)trifluoracetamid (BSTFA) (50 µL), a identifikacija spojeva provedena je pomoću plinske kromatografije (engl. *gas chromatography* (GC), Nexis C-2030, Shimadzu, Kyoto, Japan), zajedno s detektorom masene spektrometrije (engl. *mass spectrometry* (MS)) (Shimadzu QP2020 NX, Kyoto, Japan), opremljenim otvorom za podjeljeno/nepodjeljeno (engl. *split*/*splitless*) ubrizgavanje. U analizi je korištena kapilarna kolona od fuzioniranog silicija (duljine 30 m × unutarnji promjer 0,25 mm i.d., debljina filma 0,25 m) (SH-5MS, Shimazu, Japan). Ultra čisti helij korišten je kao plin nosač s protokom od 1 mL/min. Analize su provedene s MS punim skeniranjem (35-750 *m*/*z*). Za kalibraciju masenog spektrometra korišten je kalibracijski standard perfluorotributilamin, pri energiji ionizacije udarom elektrona od 70 eV. U analizi je postavljen sljedeći program temperature kolone; ekvilibracija pećnice od 3 minute s početnom temperaturom od 120 °C tijekom 3 minute koja je povećana na 292 °C brzinom od 5 °C/min a zatim povećana na 320 °C brzinom od 30 °C/min te držana izotermno 17 minuta. Budući da komercijalne knjižnice nisu nudile odgovarajuće masene spektre za identifikaciju svih derivatiziranih oblika spojeva, isti uzorci analizirani su u nederivatiziranom obliku. Analize uzoraka u nederivatiziranom obliku provedena je prema istom protokolu, ali je umjesto derivatizirajućeg sredstva korišteno 50 µL diklorometana. Identifikacija spojeva u derivatiziranom i nerderivatiziranom obliku provedena je usporedbom masenih spektara i retencijskih vremena s bazama podataka Wiley 12 i NIST 2020 (NIST, 2020; Oberacher, 2012). Svi uzorci ubrizgani su i analizirani u duplikatu.

3.4.2.2. Tekućinska kromatografija

Druga kromatografska tehnika u identifikaciji spojeva dijatomejnih ekstrakata bila je tekućinska kromatografija ultra-visoke učinkovitosti spregnuta sa spektrometrijom masa visoke rezolucije (engl. *ultra-high-performance liquid chromatography-high-resolution mass Ssectrometry* (UHPLC-ESI-HRMS)). Kemijska analiza ekstrakata provedena je UHPLC-ESI-HRMS-om, a spojevi su identificirani na temelju navedenog elementarnog sastava u kombinaciji s MS/MS spektrima s razinama pouzdanosti 2 (vjerojatna struktura) i 3 (moguća struktura). Prilikom identifikacije spoja, program analizira spektre masa (MS i MS/MS) i predlaže molekularne formule na temelju omjera mase i naboja (*m*/*z*) detektiranih iona. Tandemska masena spektrometrija (MS/MS) fragmentira molekularni ion, čime se dobiva uvid u substrukture i

funkcionalne skupine u analiziranoj molekuli. Razina pouzdanosti 2 podrazumjeva da su dokazi o elementarnom sastavu i fragmetaciji značajni, iako mogu postojati alternativne strukture, predložena formula molekule može se smatrati vjerojatnom. S druge strane, pouzdanost na razini 3 ukazuje na to da iako se može predložiti struktura na temelju dostupnih podataka razina sigurnosti je manja u odnosu na razinu 2, čime se struktura tada smatra mogućom. U analizi je korišten ExionLC AD UHPLC sustav (AB Sciex, Concord, ON, Kanada) spojen na kvadrupolni vremenski-proletni (Q-TOF) maseni spektrometar TripleTOF 6600+ (AB Sciex, Concord, ON, Kanada) s *duospray* ionskim izvorom. Analitička kolona Acquity UPLC BEH Phenyl-Hexyl (Waters, Milford, MA, SAD) 2,1 mm × 100 mm s veličinom čestica od 1,7 µm korištena je za kromatografsko odvajanje spojeva. Za mobilnu fazu A korištena je voda a za mobilnu fazu B acetonitril, koje su sadržavale 0,1 % mravlje kiseline. Tijekom cijele analize, brzina protoka (0,4 mL/min) i temperatura (30 °C) bile su konstantne. Elucija je započela na 2 % B i držana je 0,6 min, nakon čega je uslijedio linearni B gradijent do 100 % do 18,5 min. Od 18,5-25 min, elucija je ponovno bila izokratska na 100 % B. Ionizacija elektrosprejom postavljena je u pozitivnom načinu rada (ESI+) s disocijacijom izazvanom sudarom (CID) u načinu prikupljanja ovisnom o informacijama (IDA) za dobivanje MS/MS spektra mase. Detaljan opis parametara opisan je radu Radman i sur. (2022.). Podaci masenog spektrometra obrađeni su pomoću programa ACD/Spectrus Processor 2021.1.0 (ACD/Labs, Toronto, ON, Kanada). Na temelju masenih spektara i prijavljenih elementarnih sastava spojeva u kombinaciji s rezultatima pretraživanja u bazama podataka MassBank, Lipid Maps, ChemSpider i ChEBI predložena je identifikacija spojeva.

3.5. Metode određivanja antioksidacijskog potencijala dijatomejnih ekstrakata

Za procjenu ukupne antioksidativne aktivnosti ekstrakata dijatomeja primijenjene su tri različite analitičke metode: FRAP (engl. *ferric reducing antioxidant power*), DPPH (engl. *2,2-diphenyl-1-picrylhydrazyl*) i ORAC (engl. *oxygen radical absorbance capacity*). Njihova kombinacija korištena je zbog različitih mehanizama djelovanja, budući da pojedinačan pristup ne pruža dovoljno pouzdanu procjenu sveukupnog antioksidacijskog potencijala. Temeljem mehanizma djelovanja metode se dijele na metode temeljene na prijenosu atoma vodika (engl. *hydrogen atom transfer*, HAT) i metode temeljene na prijenosu elektrona (engl. *electron transfer*, ET) (Li i sur. 2014). Jedna od metoda koja djeluje prema HAT mehanizmu je DPPH, a procjenjuje sposobnost određenog antioksidansa u neutralizaciji 2,2-difenil-1-pikrilhidrazil radikala što se očituje u mjerljivoj promjeni broje iz ljubičaste u žutu (Kedare i Singh 2011). Uz DPPH još jedna

metoda prema HAT mehanizmu je i ORAC kojom se mjeri sposobnost antioksidansa da inhibira oksidaciju uzrokovanu peroksil radikalima, što se očituje u gubitku fluorescencije (Chaves i sur. 2020). Metoda FRAP kvantificira reducirajuću snagu antioksidansa mjereći kapacitet redukcije feri iona (Fe^{3+}) u fero ione (Fe^{2+}), a prema mehanizmu djelovanja spada u ET metode. Ključne razlike među metodama odnose se na vrstu radikala koju detektiraju i kompleksnost izvođenja. Dok DPPH i FRAP omogućuju brzu i jednostavnu analizu sposobnosti uklanjanja slobodnih radikala ili redukcijske snage, ORAC zahtijeva složeniji i vremenski zahtjevniji postupak, ali se smatra relevantnijim za procese u biološkim sustavima.

Za mjerenje redukcijske aktivnosti (FRAP test), dijatomejnih ekstrakata u radovima korištena je spektrofotometrijska metoda (Benzie i Strain, 1996) modificirana za primjenu u mikrotitarskim pločicama (Čagalj i sur., 2022). Volumen otopine FRAP reagensa od 300 µL dodan je u jažice mikrotitarskih pločica te je apsorbancija izmjerena na valnoj duljini od 592 nm pomoću čitača (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, SAD). Nakon dodavanja 10 µL pripremljenih uzoraka dijatomejnih ekstrakata ponovno izmjerena promjena apsorbancije. Razlika u apsorbanciji između FRAP reagensa prije dodavanja uzorka i 4 minute nakon dodavanja uspoređena je s zabilježenom vrijednosti absorbancije za standardnu otopinu Troloxa. Uzorci su testirani u četiri ponavljanja, a rezultat je izražen u µM trolox ekvivalenta (µM TE).

Metodom 2,2-difenil-1-pikrilhidrazilnog testa procijenjena je sposobnost ekstrakata dijatomeja u hvatanju DPPH radikala (Čagalj i sur., 2022). Volumen otopine DPPH radikala od 290 µL apsorbancije od 1,2 nm pipetiran je u jažice mikrotitarske pločice, a mjerenja su provedena na valnoj duljini od 517 nm. U jažice je dodano 10 µL ekstrakata a smanjenje apsorbancije izmjereno je pomoću čitača (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, SAD) nakon jednog sata. Testiranje je provedeno u četiri ponavljanja a antioksidacijska aktivnost ekstrakata dijatomeje izražena je kao postotak inhibicije (% inhibicije) DPPH radikala.

Metoda određivanja kapaciteta apsorpcije kisikovih radikala određena je prema modificiranoj metodi opisanoj u radu Burčul i sur. (2018). Prije provedbe analize uzorci su razrijeđeni 1:10 u Radu 1 i 2, 1:1000 u preliminarnom istraživanju dok su u Radu 3 testirani nerazrijeđeni uzorci ekstrakata. Volumen razrijeđenih uzoraka od 25 µL dodan je u jažice mikrotitarske pločice u koje je prodhodno dodano 150 µL 4,2 mM fluoresceina (3',6'-dihidroksispiro[izobenzofuran-1(3H),9'-[9H] ksantan]-3-jedan). Pločice su termostatirane na 37 °C u trajanju od 30 minuta, a zatim je

dodano 25 µL 2,2'-azobis (2-amidinopropan) dihidroklorida (AAPH). Na valnim duljinama od 485 i 520 nm obavljena su mjerenja ekscitacije, odnosno emisije svake minute u periodu od 80 min. Rezultati su izraženi u µM trolox ekvivalenta po litri ekstrakta (TE/LE) u preliminarnom istraživanju, Radovima 1 i 3, dok su u Radu 2 rezultati izraženi u mM trolox ekvivalenta po gramu suhog ekstrakta (TE/mgE), a svi uzorci testirani su u tri ponavljanja.

3.6. Statistička analiza

Za izražavanje statističke razlike između gustoće stanica, ekstrakcijskog prinosa, USF te rezultata antioksidacijske aktivnosti korištena je analiza varijance (jednosmjerna ANOVA praćena Fisherovim testom najmanje značajne razlike) (Šimat i sur., 2020). Analize su provedene pomoću Statgraphics Centurion-Ver. 16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, SAD).

4. REZULTATI

4.1. Preliminarni rezultati

S ciljem odabira najpogodnijeg ekstrakcijskog otapala za postizanje maksimalnog bioaktivnog potencijala te prinosa bioaktivnih spojeva u daljnjem istraživanju, liofilizirana biomasa dijatomeje *C. costatus* (CIM 935) ekstrahirana je korištenjem acetona, etanola i heksana. Dobivenim ekstraktima određen ekstrakcijski prinos i antioksidacijska aktivnost (DPPH i ORAC), dok je kemijska identifikacija acetonskih, etanolnih i heksanskih ekstrakta dijatomeje *C. costatus* provedena tekućinskom kromatografijom vrlo visoke učinkovitosti spregnutom sa spektrometrijom masa visoke rezolucije (engl. *ultra-high-performance liquid chromatography-high-resolution mass spectrometry*, UHPLC-ESI-HRMS) (Slika 3) (detaljno opisano u poglavlu 3. Materijali i metode).

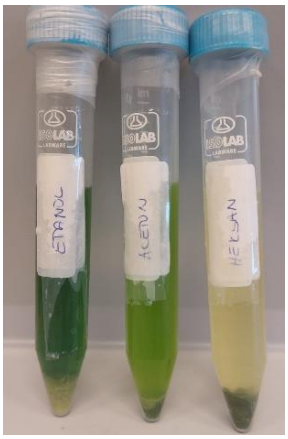
Ekstrakcija etanolom (390 mg EX/g LD) rezultirala je značajno višim prinosom ekstrakcije ($p < 0,05$) od acetona i heksana, čak 3 puta većim prinosom nego heksanom (120 mg EX/g LD) i 13 puta većim prinosom nego acetonom (30 mg EX/g LD) (Tablica 1).

Tablica 1. Masa suhih ekstrakta dijatomeje *Chaetoceros costatus* dobivena ekstrakcijom liofilizirane biomase acetonom, etanolom i heksanom.

Otapalo	**m** (mg EX/g LD)
Aceton	30[a]
Etanol	390[b]
Heksan	120[c]

[a-c] *Različita slova u istom stupcu označavaju značajnu razliku (p>0,05) između uzoraka.*

Za određivanje antioksidacijskog potencijala korišteni su DPPH i ORAC (Tablica 2). Najveći postotak inhibicije DPPH zabilježen je u etanolnom ekstraktu (9,17 ± 0,99 % inhibicije) dok je ekstrakcija acetonom rezultirala najvećom ORAC vrijednošću ekstrakta (50,66 ± 2,22 µM trolox ekvivalenta na litru ekstrakta (µM TE/LE). Korištenjem oba testa nije bilo moguće odrediti antioksidacijsku aktivnost ekstrakta u heksanu zbog kemijskih svojstava otapala (niske točke vrelišta).



Slika 3. Acetonski, etanolni i heksanski ekstrakti liofilizirane biomase dijatomeje *Chaetoceros costatus* (vlastita fotografija).

Tablica 2. Rezultati DPPH i ORAC testova za acetonske, etanolne i heksanske ekstrakte dijatomeje *Chaetoceros costatus*

	DPPH (% inhibicije)	**ORAC** (μM TE/LE)
Aceton	3,51 ± 0,47[a]	50,66 ± 2,22[a]
Etanol	9,17 ± 0,99[b]	37,10 ± 3,29[b]
Heksan	NO	NO

[a,b] *Različita slova u istom stupcu označavaju značajnu razliku (p>0,05) između uzoraka;* TE *– trolox ekvivalent;* NO *– vrijednost nije određena;*

U sva tri ekstrakta dijatomeja UHPLC-ESI-HRMS-om identificirani su pigmenti i derivati pigmenata, derivati masnih kiselina te steroidi i derivati steroida. U acetonskim ekstraktima identificirano je 37 spojeva, u etanolnim 34 dok je u ekstraktima u heksanu zabilježeno tek 20 spojeva.

U skupini pigmenata i njihovih derivata zabilježini su monoterpenski lakton (loliolid, br. 1), pet ksantofila i derivata (br. 2–3, 5–7), dva derivata klorofila *b* (feoforbid b (br. 4), jedan derivat feofitina (br. 9), feofitin *b* (br. 10), pet derivata klorofil *a* (feoforbid a (br. 8), divinilfeofitin *a* (br. 11), 151-hidroksi-lakton-feofitin *a* (br. 12), 132-hidroksi-feofitin *a* (br. 13) i feofitin *a* (br. 14)). Ekstrakcija acetonom rezultirala je najvećim prinosom pigmenata i njegovih derivata s najvećim sadržajem feofitina *a* (br. 14). Za razliku od acetona i heksana, u etanolnim ekstraktima *C. costatus* identificiran je feoforbid *b* (br. 4), a najveći prinos bilježio je spoj 13^2-hidroksi-feofitin *a* (br. 13.). Heksan je omogućio ekstrakciju tek tri spoja iz ove skupine.

U skupini derivata masnih kiselina identificirano je osam primarnih amida masnih kiselina (PAMK), a najzastupljeniji bio je oleamid (br. 22). Uz oleamid u ekstraktima dijatomeje *C. costatus* zabilježena je prisutnost sljedećih PAMK: linoleamid (br. 19), stearamid (br. 23), palmitoleamid (br. 18), palmitamid (br. 20), miristamid (br. 16), gondamid (br. 25), i erucamid (br. 27). Osim PAMK identificirana su četiri glicerofosfokolina (br. 28–31), tri estera masnih kiselina (br. 17, 21, 24) i dva diacilglicerola (br. 32-33), dugolančana masna kiselina C:20 (br. 26) i sfingolipid heksadekasfinganin (br. 15).

U skupini sterola i derivata identificirano je pet (br. 34–38) spojeva. U svim analiziranim uzorcima (3β)-3-hidroksistigmast-5-en-7-on (br. 37) bio je najzastupljeniji steroidni derivat. Identificirana su i dva biljna sterola, kampesterol (br. 34) i stigmastatrien (br. 36) te dva derivata sterola (br. 35, 38).

Tablica 3. Glavni nehlapivi spojevi u acetonskim, etanolnim i heksanskim ekstraktima u uzorku dijatomeje *Chaetoceros costatus* identificirani tekućinskom kromatografijom vrlo visoke učinkovitosti spektrometrije masa visoke rezolucije (UHPLC-ESI-HRMS).

Redni broj	Naziv spoja	Masa	[M+H]+	Molekulska formula	Površina vrha (proizvoljne jedinice)		
					Aceton	Etanol	Heksan
PIGMENTI I DERIVATI							
1	Loliolid	196,11	197,12	$C_{11}H_{16}O_3$	$4,63\times10^6$	-	-
2	Apo-10-fukoksantinal	424,26	425,27	$C_{27}H_{36}O_4$	$2,63\times10^4$	$8,26\times10^3$	-
3	Halocintiaksantin acetat	640,41	641,42	$C_{42}H_{56}O_5$	$2,47\times10^6$	$2,55\times10^5$	-
4	Feoforbid *b*	606,25	607,26	$C3_5H_{34}N_4O_6$		$1,37\times10^3$	-
5	Fukoksantin	658,42	659,43	$C_{42}H_{58}O_6$	$5,13\times10^6$	$4,55\times10^5$	-
6	Diatoksantin	566,41	567,42	$C_40H_{54}O_2$	$5,76\times10^5$	$1,67\times10^3$	-
7	Fukoksantinol	616,41	617,42	$C_40H_{56}O_5$	$1,30\times10^5$	$5,08\times10^3$	-
8	Feoforbide *a*	592,27	593,28	$C_{35}H_{36}N_4O_5$	$8,30\times10^6$	$7,98\times10^4$	$2,67\times10^3$
9	3-[21-Metoksikarbonil-4,8,13,18-tetrametil-20-okso-9,14-divinil-3,4-didehidro-3-24,25-dihidroforbinil] propanska kiselina	588,24	589,25	$C_{35}H_{32}N_4O_5$	$2,13\times10^5$	$1,37\times10^5$	-
10	Feofitin *b*	884,55	885,55	$C_{55}H_{72}N_4O_6$	$3,40\times10^5$	$4,45\times10^4$	-
11	Divinil feofitin *a*	868,55	869,56	$C_{55}H_{72}N_4O_5$	$4,58\times10^5$	$3,83\times10^4$	-
12	15^1-hidroksi-lakton-feofitin *a*	902,56	903,56	$C_{55}H_{74}N_4O_7$	$1,67\times10^6$	$3,06\times10^5$	$1,90\times10^3$
13	13^2-hidroksi-feofitin *a*	886,56	887,57	$C_{55}H_{74}N_4O_6$	$1,53\times10^6$	$7,00\times10^6$	-

14	Feofitin *a*	870,57	871,57	$C_{55}H_{74}N_4O_5$	$3,58\times10^7$	$7,06\times10^5$	$1,41\times10^4$
	DERIVATI MASNIH KISELINA						
15	Heksadekasfinganin	273,27	274,27	$C_{16}H_{35}NO_2$	$4,51\times10^6$	$2,08\times10^5$	$8,56\times10^5$
16	Miristamid	227,23	228,23	$C_{14}H_{29}NO$	$4,14\times10^4$	$8,02\times10^4$	$1,11\times10^5$
17	Monomiristin	302,25	303,25	$C_{17}H_{34}O_4$	$8,49\times10^4$	$1,67\times10^4$	$1,68\times10^4$
18	Palmitoleamid	253,24	254,25	$C_{16}H_{31}NO$	$1,06\times10^5$	$2,01\times10^5$	$2,25\times10^5$
19	Linoleamid	279,26	280,26	$C_{18}H_{33}NO$	$2,62\times10^5$	$3,73\times10^5$	$4,26\times10^5$
20	Palmitamid	255,26	256,26	$C_{16}H_{33}NO$	$5,47\times10^5$	$6,57\times10^5$	$9,82\times10^5$
21	Monopalmitin	330,28	331,28	$C_{19}H_{38}O_4$	$3,77\times10^6$	$6,49\times10^5$	$7,05\times10^5$
22	Oleamide	281,27	282,28	$C_{18}H_{35}NO$	$6,56\times10^6$	$7,00\times10^6$	$7,03\times10^6$
23	Stearamid	283,29	284,30	$C_{18}H_{37}NO$	$1,96\times10^5$	$2,58\times10^5$	$1,87\times10^5$
24	Monostearin	358,31	359,32	$C_{21}H_{42}O_4$	$3,70\times10^6$	$3,51\times10^6$	$3,98\times10^6$
25	Gondamid	309,30	310,31	$C_20H_{39}NO$	$1,06\times10^5$	$9,4\times10^4$	$3,4\times10^4$
26	Arahidonska kiselina	304,24	305,25	$C_20H_{32}O_2$	$6,34\times10^4$	-	-
27	Erucamid	337,33	338,34	$C_{22}H_{43}NO$	2.18×10^6	$2,00\times10^6$	$3,12\times10^5$
28	l-(9-oktadecenoil)-2-(9-pentadecenoil)-glicero-3-fosfokolin	743,55	744,55	$C_{41}H_{78}NO_8P$	$3,08\times10^4$	$5,46\times10^4$	-
29	1-(11,14-eikosadienoil)-2-heptadekanoil-glicero-3-fosfoserin	801,55	802,56	$C_{43}H_80NO_10P$	$5,82\times10^4$	$2,73\times10^4$	$2,45\times10^4$
30	1-oktadekanoil-2-(9,12-heptadekadienoil)-glicero-3-fosfokolin	771,58	772,59	$C_{43}H_{82}NO_8P$	$4,43\times10^4$	$7,14\times10^4$	-

31	l-(9-oktadecenoil)-2-(9-nonadecenoil)-glicero-3-fosfokolin	799,61	800,62	$C_{45}H_{86}NO_8P$	$2,85\times10^5$	$5,73\times10^4$	-
32	Dipalmitin	568,51	569,51	$C_{35}H_{68}O_5$	$2,66\times10^5$	-	-
33	1-oktadekanoil-2-heksadekanoil-*sn*-glicerol	596,54	597,55	$C_{37}H_{72}O_5$	$1,55\times10^5$	$5,11\times10^4$	$8,42\times10^3$
	STEROIDI I DERIVATI						
34	Chola-5,22-dien-3-ol	342,29	343,30	$C_{24}H_{38}O$	$9,00\times10^4$	$7,72\times10^4$	$7,76\times10^4$
35	β-Stigmasterol	394,36	395,37	$C_{29}H_{46}$	$1,02\times10^3$	$9,60\times10^3$	$8,80\times10^3$
36	Kampesterol	400,37	401,38	$C_{28}H_{48}O$	$1,96\times10^5$	-	-
37	(3β)-3-Hidroksistigmast-5-en-7-on	428,37	429,37	$C_{29}H_{48}O_2$	$7,60\times10^5$	$2,30\times10^5$	$7,77\times10^3$
38	24-Hidroperoksi-24-vinil-kolesterol	444,36	445,37	$C_{29}H_{48}O_3$	$5,13\times10^4$	$1,58\times10^4$	-

U acetonskim i etanolnim ekstraktima nisu zabilježena značajnija odstupanja u antioksidacijskoj aktivnosti i kvantiteti identificiranih spojeva. Međutim, zbog značajno većeg ekstrakcijskog prinosa od čak 13 puta etanol se pokazao pogodnijim otapalom i odabran je za ekstrakciju nehlapivih spojeva dijatomeja u daljnjem istraživanju.

4.2. Sažeti pregled rezultata objedinjenih radova

4.2.1. Rad 1. The marine diatom *Thalassiosira rotula*: chemical profile and antioxidant activity of hydroalcoholic extracts

U ovom radu istražen je utjecaj korištenja dvije vrste hidroalkoholnih smjesa, 50 %-tnim i 70 %-tnim etanolom u ekstrakciji dijatomeje *T. rotula* (CIM861). Određen je ekstrakcijski prinos i USF dok je bioaktivni potencijal ekstrakata procijenjen *in vitro* antioksidativnim testovima DPPH, FRAP i ORAC, a kemijski profil derivatiziranih ekstrakata analiziran je korištenjem GC-MS-om (detaljno opisano u poglavlu 3. Materijali i metode). Ekstrakcija liofilizirane biomase dijatomeje provedena je pomoću ultrazvuka. Veći ekstrakcijski prinos zabilježen je kod dijatomejnih ekstrakata dobivenih 70 %-tnim etanolom i prosječna masa prinosa bila je 0,21 ± 0,01 g EX/g SD. U istim ekstraktima zabilježen je veći sadržaj ukupnih fenola, 5,80 ± 0,32 mg GK/g SD, kao i snažnije antioksidacijsko djelovanje i to sa sve tri metode (DPPH inhibicija od 17,53 % ± 0,56 %, FRAP od 766,67 ± 34,69 μM TE i ORAC od 58,87 ± 2,03 μM TE/LE). Kemijska analiza GC-MS-om potvrdila je prisutnost 19 spojeva u etanolnim ekstraktima pripravljenim sa 70 %-tnim etanolom, dok je u ekstraktima dobivenim ekstrakcijom 50 %-tnim etanolom utvrđena prisutnost 16 spojeva. Dominantni spojevi zabilježeni u 50 %-tnim i 70 %-tnim etanolskim ekstraktima su masne kiseline: miristinska, palmitelaidna, palmitinska, zatim dvije višestruko nezasićene masne kiseline EPK i DPK te sterol, 24-metilenkolesterol. Nadalje, ekstrakcija s većim udjelom etanola (70 %) u vrste *T. rotula* rezultirala je većim sadržajem miristinske kiseline, EPK, DPK i 24-metilenkolesterola. Rezultati ovog istraživanja potvrdili su da je 70 %-tni etanol, ekstrakcijsko otapalo koje osim što osigurava veći prinos nezasićenih masnih kiselina i sterola pozitivno utječe i na povećanje antioksidacijske aktivnost dijatomejnih ekstrakata.

4.2.2. Rad 2. Comparison of growth and chemical profile of diatom *Skeletonema grevillei* in bioreactor and incubation-shaking cabinet in two growth phases

Cilj istraživanja bio je usporediti rast, kemijske profile i antioksidacijsku aktivnost dijatomeje *S. grevillei* uzgojene u BS i u IS u eksponencijalnoj (nakon 192 h) i na početku stacionarne faza rasta (nakon 312 h). Rast je praćen prebrojavanjem stanica u graduiranoj Sedgewick Rafter komorici, a prisutni spojevi identificirani su u derivatiziranom obliku korištenjem GC-MS-a dok je antioksidativni kapacitet procijenjen DPPH i ORAC metodom (detaljno opisano u poglavlu 3. Materijali i metode). Nakon 168 h uzgoja, zabilježen je značajan statistički rast (p<0.05) *S. grevillei* u BS u odnosu na rast u IS, a zabilježena razlika zadržala se do kraja uzgojnog perioda. Također, dvostruko veća masa suhog ekstrakta dobivena je za biomasu uzgojenu u BS i to u obje faze raste. Po završetku uzgojnog perioda, u biomasi iz BS identificirano je 42, a iz IS 34 spoja, dok je nakon 192 h bioreaktorska biomasa bilježila 44 spoja, a ona iz IS 33. U oba uzgojna sustava i u obje faze rasta, oleamid je bio najdominantniji spoj, međutim zabilježen je njegov značajni pad s produljenjem uzgojnog ciklusa. Uz oleamid, u oba sustava zabilježeni su slijedeći dominantni spojevi: palmitelaidna kiselina, glicerol monostearat, miristinska kiselina, kolesterol, eikozapentaenska kiselina, 1-monopalmitin i 24-metilenkolesterol. Ekstrakt biomase *S. grevillei* dobivene nakon 312 h uzgoja u BS pokazao je najbolji antioksidacijski potencijal s DPPH inhibicijom od 11,4 ± 1 % te ORAC vrijednosti 93,3 ± 8,4 mM TE/mgE. Rezultati ove studije potvrdili su da je BS pogodniji sustav za uzgoj u odnosu na IS jer omogućava brži rast i veći prinos biomase bogate širokim spektrom visokovrijednih bioaktivnih spojeva.

4.2.3. Rad 3. Influence of nutrient deprivation on the antioxidant capacity and chemical profile of two diatoms from genus *Chaetoceros*

U ovom radu uspoređen je utjecaj potpunog nedostatka makronutrijenata fosfora, dušika ili silicija u uzgojnom medijom, na antioksidacijski potencijal i kemijski profil dijatomeja *C. socialis* i *C. costatus*. Identifikacija spojeva provedena je UHPLC-ESI-HRMS-om, a antioksidacijski potencijal određen je metodama DPPH, FRAP te ORAC (detaljno opisano u poglavlu 3. Materijali i metode). U ekstraktima vrsta *C. costatus* i *C. socialis* uzgojenim u različitim uzgojnim uvjetima identificirani su pigmenti, masne kiseline, steroli te njihovi derivati. Najzastupljeniji derivat pigmenta u svim ekstraktima bio je feofitin *a* , osim u ekstraktima obje vrste dobivenih iz biomase uzgojem bez prisustva silicija. Loliolid, feoforbid *a*, feofitin *b* i tri derivata klorofila *a* najzastupljeniji su derivati pigmenata u vrsti *C. costatus* uzgojenoj bez dušika. U skupini derivata masnih kiselina, među detektiranim primarnim amidima masnih kiselina najzastupljeniji spoj bio je oleamid. Najveći sadržaj PAMK zabilježen je u kontrolnom uzorku, dok je opadanje njihovog sadržaja zabilježeno u obje vrste pri izostanku ključnih nutrijenata u uzgojnom mediju, osobito dušika. U skupini sterola i derivata detektirano je pet spojeva, a njihov sadržaj varirao je od uzorka do uzorka i nije bio usporediv među vrstama. Sadržaj ukupnih fenola u ekstraktima vrste *C. costatus* zabilježen je u rasponu od 46,25 ± 1,08 do 89,38 ± 6,21 mg GK/L, a u *C. socialis* od 29,58 ± 1,08 do 54,17 ± 1,18 mg GK/L. U odnosu na *C. socialis*, rezultati svih korištenih antioksidacijskih testova ukazuju na veći antioksidativni potencijal dijatomeje *C. costatus*, osobito pri uzgoju u mediju bez dušika. Rezultati ovog istraživanja ukazuju na veći antioksidacijski potencijal dijatomeje *C. costatus* te veći sadržaj biološki aktivnih spojeva, osobito pri izostanku dušika u uzgojnom mediju zbog čega utjecaj nutrijenata na kemijski sastav i biološku aktivnost treba razmatrati na razini vrste.

5. RASPRAVA

Postizanje maksimalnog ekstrakcijskog prinosa jedan je od glavnih izazova u proizvodnji mikroalgalne biomase (Svenning i sur. 2020). Ultrazvuk se pokazao uspješnom metodom koja u procesu ekstrakcije omogućava pucanje dijatomejnih frustula čime se pospješuje izlazak unutarstaničnog sadržaja, dok topivost spojeva prisutnih unutar stanica ovisi o odabiru otapala pogodne polarnosti (Suroy i sur. 2014). Za razliku od acetona i heksana, zbog amfipatske prirode etanola moguća je istovremena ekstrakcija polarnih i nepolarnih spojeva poput lipida i karotenoida iz vrste *Chaetoceros calcitrans* (Purkan i sur., 2022). U nedavnoj studiji Ferdousa i sur. (2025), usporedili su prinos ekstrakcije, antioksidativna aktivnost, ukupni sadržaj fenola i flavonoida te citotoksičnost vrsta morskih zelenih mikroalgi *Tetraselmis* sp. i *Nannochloropsis* sp. te dijatomeja *Chaetoceros* sp. i *Thalassiosira* sp. ekstrahiranih metanolom, etanolom, acetonom, heksanom, diklorometanom, kloroformom i etil acetatom. U prelimiranom istraživanju ovog doktorskog rada postignut je prinos ekstrakcije od 39 %, što je 18 % više nego u predhodno navedenoj studiji. Poznato je da korištenje alkohola u ekstrakciji dijatomeja pozitivno utječe na povećanje bioaktivnog potencijala omogućavajući veću antioksidacijsku aktivnost ekstrakata (Manivannan i sur., 2012). Hemalatha i sur. (2015) procijenili su antioksidacijski potencijal i ukupni sadržaj fenola metanolnih, acetonskih i heksanskih ekstrakata tri morske dijatomeje, *Chaetoceros curvisetus*, *Thalassiosira subtilis* i *Odontella aurita*. Zabilježene vrijednosti udjela (%) inhibicije DPPH testom za acetonski ekstrakt *C. curvisetus* nisu se razlikovale od vrijednosti procijenjenih u prelimiranom istraživanju ovog doktorskog rada pri istraživanju ekstrakta *C. costatus*. U studiji Hemalathae i sur (2015.) zabilježena je statistički značajna (p>0,05) razlika u vrijednostima DPPH inhibicije korištenjem različitih otapala (metanol>aceton>heksan), kao i u preliminarnom istraživanju ovog rada. U studiji Ferdous i sur. (2025) liofilizirana biomasa dijatomeje *Chaetoceros* sp. ekstrahirana je različitim otapalima (metanol, etanol, aceton, heksan, diklorometan, kloroform i etil acetat) u EPU. U ekstrakatima dijatomeje određen je USF i ukupan sadržaj flavonoida a bioaktivni potencijal procijenjen je antioksidacijskim i citotoksičnim testovima. Jednako rezultatima prelimiranog istraživanja ovog istraživanja, vrijednosti DPPH inhibicije etanolnih ekstrakata *Chaetoceros* sp. bile su značajno veće od acetonskih, dok je za heksanske ekstrakte zabilježena najniži udio (%) inhibicije. Izbor prikladnog otapala utječe na ekstrakcijski prinos kao i na učinkovitost u ekstrakciji željenih spojeva. Aceton se pokazao najučinkovitijim otapalom u ekstrakciji pigmenata iz biomase dijatomeja (Pasquet i sur., 2011). Osim što omogućava ekstrakciju različitih klasa pigmenata, dodatna prednost korištenja acetona je u onemogućavanju hidrolize klorofila *a* te postizanju najvećeg prinosa pigmenata u kratkom vremenu, čime

nadmašuje etanol i heksan (Warkoyo i Saati, 2011). Aceton se pokazao prikladnim otapalom za ekstrakciju pigmenata iz dijatomeja zbog sposobnosti ekstrakcije pigmenata poput fukoksantina, klorofila *c* i diadinoksantina uz minimalnu razgradnju (Pasquet i sur., 2011). Rezultati prelimiranog istraživanja ovog rada potvrđuju visoku učinkovitost acetona u ekstrakciji pigmenata iz biomase dijatomeje *C. costatus,* a osim najvećeg prinosa pigmenta, aceton je omogućio i ekstrakciju najvećeg broja različitih klasa pigmenata. U ekstraktima *C. costatus* u prelimiranom istraživanju, najdominantniji pigment bilo je feofitin *a* i njegovi derivati koji su predhodno zabilježeni u vrstama roda *Chaetoceros* (Saide i sur., 2020). Zbog lipofilne prirode feofitina u biološkim uzorcima topivost je bolja pri korištenju dietiletera, acetona i kloroforma no zbog negativnog utjecaja na okoliš, dugoročno korištenje ovih otapala smatra se neodrživim (Kim i sur., 2020; Nakazato, 1995). Feofitini se mogu ekstrahirati i etanolom, međutim etanolna ekstrakcija pokazala je nižu učinkovitost u usporedbi s korištenjem prethodno navedenih organskih otapala (FAO, 1987). Dijatomeje su mikroalge s izrazito raznolikim profilom pigmenata. Zbog velikog prinos pigmenata u procesu ekstrakcije, dijatomeje imaju potencijal za industrijsku upotrebu u prehrambenoj (bojila u hrani, razvoj funkcionalnih napitaka) i kozmetičkoj industriji (bojila u kozmetici). Poznato je da su feofitini biološki vrlo aktivne molekule koje imaju potencijal za primjenu u medicini zbog širokog spektra bioloških aktivnosti poput antitumorskog, antimikrobnog i antioksidativnog djelovanja (Gomes i sur., 2020; Higashi-Okai i sur., 1998; Hsu i sur., 2013; Kusmita i sur., 2015). S druge strane, heksan je često korišteno otapalo u ekstrakciji masnih kiselina no njegova učinkovitost u ekstrakciji masnih kiselina značajno se razlikuje ovisno o vrsti dijatomeja i metodi korištenoj za razbijanja stanica (Svenning i sur., 2020). Zbog nepolarnih svojstava, heksan omogućava i učinkovitu ekstrakciju lipofilnih sterolnih spojeva kao što su kampesterol i stigmasterol (Jaramillo-Madrid i sur., 2020a; Jaramillo-Madrid i sur., 2020b). U prelimiranom istraživanju najmanji prinos masnih kiselina i sterola zabilježen je u ekstrakciji heksanom. Etanol je posebno učinkovit u ekstrakciji spojeva s hidrofilnim i lipofilnim svojstvima, a izrazito učinkovitim pokazao se u ekstrakciji nekoliko klasa spojeva iz mikroalgi, poput polarnih lipida i različitih nepolarnih spojeva kao što su masne kiseline (Georgiopoulou i sur., 2023). Ekstrakcija acetonom i etanolom u prelimiranom istraživanju ovog doktorskog rada omogućila je relativno jednak prinos masnih kiselina i njihovih derivata, a ekstrakcija acetonom dodatno je rezultirala najvećim prinosom sterola i derivata. U istom istraživanju u vrsti *C. costatus,* identificirano je 19 spojeva u skupini derivata masnih kiselina od kojih je osam PAMK, a najbolja topivost PAMK zabilježena je u etanolu. Najzastupljeniji PAMK u svim ekstraktima *C. costatus* bio je oleamid (br. 22) koji je prethodno zabilježen u dijatomeji *Skeletonema marinoi* (Zulfiqar i sur., 2023). Poznato je da oleamid pruža mnoge zdravstvene prednosti osobito u

liječenju/prevenciji artritisa, ateroskleroze, tromboze i raka koje se temelje na predhodno zabilježenim biološkim aktivnostima (Ameamsri i sur., 2020; Hameed i sur., 2016; Moon i sur., 2018; Shao i sur., 2016). Antimikrobno djelovanje oleamida protiv bakterije *Staphylococcus aureus* (zona inhibicija = 25 mm) potvrđeno je u istraživanju ekstrakta gljive *Colletotrichum gloeosporioides* (Premjanu i Jaynthy, 2015). Jednako djelovanje zabilježeno je i protiv *S. aureus* (MIC = 125 uL/mL) korištenjem ekstrakta *Diaporthe schini*, gdje je oleamid zabilježen kao dominantniji metabolit (dos Reis i sur., 2019). Nadalje, antifungalna i antimikrobna aktivnost protiv *Aspergillus flavus* i *Klebsiella pneumonia* zabilježena su korištenjem oleamida iz ekstrakata kore cimeta (Hameed i sur., 2016). Između ostalog, oleamid je prepoznat i kao potencijalan algicid u kontroli cvjetanja cijanobakterija, posebno protiv *Microcystis aeruginosa* NIES -843 (Shao i sur., 2016). Uz PAMK, dijatomeje su važan izvor sterola. Steroli u ekstraktima dijatomeje *C. costatus* poput kampesterola, 24-hidroperoksi-24-vinil-kolesterola i kola-5,22-dien-3-ola identificirani su u preliminarnom istraživanju, a poznato je da imaju izvanredan potencijal za primjenu u raznim industrijama kao što su farmaceutska, kozmetička i prehrambena (Ameamsri i sur., 2020). Taj potencijal temelji se na biološkim aktivnostima poput citotoksičnog, antitipanosomalnog, protuupalnog i antimikobakterijskog djelovanja. Primjerice, (3β)-3-hidroksistigmast-5-en-7-on i stigmastatrien imaju potencijal za upotrebu u farmaceutskoj industriji za razvoj inovativnih formulacija zbog svoje jedinstvene strukture (Ameamsri i sur., 2020).

U ekstrakciji predhodno navedenih skupina spojeva, često je korištenje hidroalkoholnih mješavina. Korištenjem smjesa etanola i vode koje kombiniraju polarnost vode i lipofilnost etanola omogućena je ekstrakcija spojeva širokog spektra polaranosti. Ekstrakcija mikroalgi *Nannochloropsis gaditana* i *Chlorella* sp. mješavinom etanola i vode rezultirala je većim ekstrakcijskim prinosom u usporedbi sa smjesama metanola i vode (Monteiro i sur., 2020). Osim toga, veći ekstrakcijski prinos u obje vrste uočen je u EPU korištenjem 80 %-tnog etanola u usporedbi s 50 %-tnim etanolom. Rezultati Rada 1 u ovom istraživanju potvrđuju veći ekstrakcijski prinos korištenjem većeg volumnog udjela etanola kod ekstrakcije dijatomeje *T. rotula*. Osim povećanja ekstrakcijskog prinosa dijatomeje *T. rotula*, ekstrakcija većim udjelom etanola (70 % etanol) značajno je utjecala na bioaktivni potencijal ekstrakta, povećanjem antioksidativnog potencijala. Ekstrakti *T. rotula* pripremljeni hidroalkoholnom smjesom od 70 % etanola u Radu 1 bilježili su 46 % inhibiciju DPPH testom u odnosu na metanolne ekstrakte *T. subtilis* u istraživanju Hemalathae i sur. (2015.). Na povećanje antioksidacijske aktivnosti utjecaj imaju i različiti lipidni spojevi. Najčešće dominantne masne kiseline u dijatomejama su miristinska, palmitinska, palmitelaidinska i EPK (Yi i sur., 2017; Zulu i sur., 2018), što je potvrđeno i u ekstraktima vrste *T.*

rotula istraživane u Radu 1. Sabia i sur. (2018) zabilježili su prisutnost miristinske kiseline, palmitinske kiseline i palmitoleinske kiseline u dijatomeji *Thalassiosira pseudonana*. Miristinska kiselina, poznata kao C14:0, prisutana je u svim živim organizmima, a može se koristiti i kao potencijalno sredstvo protiv gljivice *Candida sp.* otporne na lijekove, dok Javid i sur. (2020) i Prasath i sur., (2019) među glavnim biološkim aktivnostima miristinske kiseline ističu antiparazitsko, antivirusno, antifungalno i antitumorsko djelovanje. S druge strane, palmitinska kiselina (C16:0) je važna u sintezi dviju masnih kiselina, *n*-3 (kao što je DPK) i *n*-6 (kao što je EPK) (Nieri i sur., 2023). Zhu i sur. (2021) su zabilježili antiproliferativno i antimetastatsko djelovanje palmitinske kiseline protiv raka prostate. Palmitelaidinska kiselina je trans izomer palmitoleinske kiseline. U istraživanju kontrole stvaranja biofilma gram-negativnih i gram-pozitivnih bakterija palmitelaidnom kiselinom, zabilježena je inhibicija od 25 % kod bakterije *Escherichie coli* pri koncentraciji od 256 μg mL^{-1} i inhibicija od 21 % kod bakterije *S. aureus* pri koncentraciji od 16 μg mL^{-1} (Yuyama i sur., 2020). Generalno, dijatomeje predstavljaju dobar izvor VNMK, što potvrđuju i rezultati Rada 1 ovog doktorskog rada, gdje je zabilježen udio EPK i DPK od gotovo 20 % kod *T. rotula* ekstrahirane 70 % etanolom (Maadane i sur., 2015). Osim što je u sintezi masnih kiselina posredni spoj između EPK i DHK, DPK pruža mnoge zdravstvene prednosti, poput smanjenja rizika od razvoja koronarnih bolesti i razine kolesterola u plazmi, dok je EPK važna za neurološki razvoj te ima protuupalni učinak i smanjuje rizik od hipertenzije, Crohnove bolesti i koronarne bolesti bolesti srca (Calder, 2017; Lozano-Muñoz i sur., 2020). Bie i sur. (2020) objavili su antitumorsko djelovanje EPK protiv raka jajnika povećanjem imunomodulatorne aktivnosti dok je u oralnim infekcijama, potvrđeno antibakterijsko djelovanje protiv parodontnih bolesti (Sun i sur., 2016). Rod *Thalassiosira* pokazao se dobrim izvorom fitosterola 24-metilenkolesterol s udjelom >60 % u ukupnom sastavu sterola, također poznatog i kao 24-metikolesta5,24(28)-dien-3β-ol (Gallo i sur., 2020; Nieri i sur., 2023; Rampen i sur., 2010). Jaramillo-Madrid i sur. (2020b) identificirali su 24-metilenkolesterol u tri vrste *Thalassiosira*, uključujući *T. rotula* dok su Cutignano i sur. (2022) prvi put izvijestili o citotoksičnom i antiproliferativnom djelovanju 24-metilenkolesterola ekstrahiranog iz *T. rotula* protiv raka grudi za stanične linije raka MCF7 i pluća A549.

Jedan od ključnih koraka u osiguravanju dostatnih količina dijatomeja za daljnju primjenu je proizvodnja većih količina biomase. U Radu 2, brži rast i veći prinos ekstrakta *S. grevillei* zabilježen je tijekom rasta u bioreaktorskom sustavu u obje faze rasta (stacionarnoj i eksponencijalnoj). U istraživanju Raniello i sur. (2007) dijatomeje *Cocconeis neothumensis* veći ekstrakcijski prinos dobiven je ekstrakcijom biomase uzgojene u bioreaktoru a isto je zabilježeno

i u Radu 2. U odnosu na ekstrakte dobivene iz biomase *S. grevillei* uzgojene u IS u Radu 2, veći ekstrakcijski prinos te veći udio masnih kiselina i sterola dobiven je ekstraktcijom biomase *S. grevillei* uzgojene u BS. Najdominantniji spoj u oba uzgojna sustava (BS i IS) bio je oleamid dok su spojevi s udjelom većim od 5 % u BS-u bili palmitelaidna kiselina, glicerol monostearat, kolesterol, 24-metilenkolesterol, miristinska kiselina i EPK kiselina, a u IS-u glicerol monostearat, palmitelaidna kiselina, miristinska kiselina i 1-monopalmitin. U istraživanju Rada 2 veća akumulacija EPK određena je u BS-u u stacionarnoj fazi rasta u odnosu na eksponencijalnu. Mogući uzrok je smanjena dostupnost nutrijenata nakon dužeg uzgojnog perioda koja je potaknula akumulaciju EPK. Ovu činjenicu dodatno potvrđuje analiza antioksidacijske aktivnosti prikazane u Radu 2 kojom je potvrđena veća antioksidacijska aktivnost korištenjem oba testa (DPPH i ORAC) pri prelasku iz eksponencijalne u stacionarnu fazu rasta u BS-u i IS-u. Tijekom stacionarne faze rasta ograničena količina hranjivih tvari u uzgojnom mediju potiče proizvodnju EPK, a poznato je da spojevi poput masnih kiselina (EPK) i sterola (kolesterola) doprinose povećanju antioksidacijskog potencijala kao i cijelokupnog bioaktivnog potencijala (Ferreira i sur., 2021; Goiris i sur., 2015; Zhang i sur., 2021).

Ograničenje i/ili nedostatak nutrijenata ključni su u metaboličkoj proizvodnji brojnih spojeva u dijatomejama. Među različitim vrstama dijatomeja razlikuju se mehanizami prilagodbe na nutritivni stres (Goiris i sur., 2015). Jedan od mehanizama zaštite protiv oksidativnog stresa nedostatkom nutrijenata je povećana proizvodnja fenolnih spojeva. U istraživanju dijatomeje *Phaeodactylum tricornutum* pri nedostatku dušika (N−) tijekom 15 dana, zabilježene su značajne razlike u ukupnom sadržaju polifenola u usporedbi s kulturom uzajanom u standardnim uvjetima uzgoja (N+) (Curcuraci i sur., 2022). Tijekom rasta u mediju obogaćenom dušikom (3,07 ± 0,17 mg GK/g SD) dijatomeja *P. tricornutum* proizvela je veći sadržaj fenolnih spojeva u usporedbi s *P. tricornutum* uzgojenoj pri ograničenju dušikom, u čijim je ekstraktima određen znatno manji sadržaj fenolnih spojeva (1,12 ± 0,00 mg GK/g SD). Isti trend kao i u predhodno navedenim istraživanjima zabilježen je i u Radu 3 gdje je uz značajno veći sadržaj ukupnih fenola određeni u vrsti *C. costatus* u odnosu na *C. socialis* zabilježen i manji sadržaj fenolnih spojeva u obije vrsta pri potpunom izostanku nutrijenata (P, N i Si) u uzgojnom mediju. U prethodnom istraživanju mikroalge *Tetraselmis marina* izolirane iz sjevernog Jadrana, pronađena je pozitivna korelacija između antioksidativne aktivnosti i USF-a (Trentin i sur., 2022). Najveća antioksidacijska aktivnost u Radu 3 zabilježena je pri nedostatku dušika u uzgoju vrste *C. costatus*, međutim isti trend nije zabilježen i za *C. socialis.* U radu Curcuraci i sur. (2022), antioksidativno djelovanje dijatomeje *P. tricornutum* uzgojene bez N procijenjeno je DPPH i FRAP testovima. Oba

antioksidacijska testa potvrdila su statistički značajno niži antioksidativni kapacitet za *P. tricornutum* uzgojenom pri nedostatku dušika u mediju. Isti trend smanjenja antioksidacijskog potencijala opažen je i u istraživanju vrste zelene mikroalge *Dunaliela salina* u uzgoju bez N (Singh i sur., 2016). S druge strane, Jeyakumar i sur. (2020) odredili su najveću aktivnost DPPH testom u vrste *Isochrysis* sp. u uzgoju pri nedostatku N s inhibicijom od 85 %, dok je biomasa uzgojena u mediju bogatim dušikom (75 %) te kontrolnom mediju (64 %) pokazala nižu inhibiciju. Uzgoj bez ključnih nutrijenata najčešće pridonosi povećanju USF i pigmenata što značajno utječe na povećanje antioksidativnog kapaciteta (Kuczynska i sur., 2015). U Radu 3, ekstraktima *C. costatus* u svim tretmanskim skupinama identificiran je fukoksantin, spoj s izrazitim potencijalnom za primjenu u prehrambenoj i farmaceutskoj industriji te feoforbid derivat klorofila poznat po antitumorskoom, antioksidativnom, imunostimulirajućem, neuroprotektivnom i protuupalnom djelovanju (Saide i sur., 2020). Collier i Grossman (1992) otkrili su da nedostatak dušika u mediju tijekom uzgoja cijanobakterije *Synechococcus* sp. rezultira degradacijom klorofila. Moguće je da su upravo ovi derivati pridonijeli povećanom antioksidativnom djelovanju u istraživanju u Radu 3, osobito u vrste *C. costatus*. Monoterpenski hidroksilakton loliolid, produkt je fotooksidativne razgradnje karotenoida fukoksantina, poznatog antioksidansa široko rasprostranjenog u makroalgama (El Hattab i sur., 2008; Park i sur., 2019; Percot i sur., 2009; Radman i sur., 2021; Radman, Čižmek, i sur., 2022; Repeta, 1989; Yang i sur., 2011). U istraživanju taloženje sa propela Menzel i sur. (2003) koristili su ga kao biomarker u haptofitima, dijatomejama, dinoflagelatima i eustigmatofitima. Važni pigmenti u dijatomejama su i feofitini, najjednostavniji derivati klorofila kod kojih je atom Mg odstranjen iz porfirinskog prstena. Feofitini kao i feoforbidi pokazali su antioksidativno djelovanje (Hsu i sur., 2013; Lanfer-Marquez i sur., 2005; Saide i sur., 2020). Najzastupljeniji PAMK u obije vrste roda *Chaetoceros* u svim testiranim uzgojnim medijima u istraživanju Rada 3 bio je oleamid, najviše proučavan PAMK s potencijalom u lječenju Alzheimerove bolesti (Ano i sur., 2015; D'Oca i sur., 2010; Farrell i sur., 2012; Tanvir i sur., 2018). Za razliku od pigmenta, nedostatak N, P i Si utjecao je na smanjenje sadržaja PAMK u vrsta *C. costatus* i *C. socialis* osobito u uzgoju bez N. Uz PAMK-a u Radu 3 identificirano je 5 sterola (kampasterol (br. 35), deoksidirani stigmasteol (br.38) i tri derivata sterola (br. 34, 36, 37) za koje je predhodno zabilježeno antioksidativno, antikarcinogeno i protuupalno djelovanje (Borowitzka i sur., 2016). U *C. costatus* uzgojenom bez N identificiran je (3β)-3-hidroksistigmast-5-en-7-on, sterol poznat po antimalarijskoj aktivnost (Indriani i sur., 2020).

6. ZAKLJUČCI

- Usporedbom ekstrakcijskog prinosa korištenjem različitih otapala (aceton, etanol i heksan) u ekstrakciji dijatomeje *C. costatus*, najveći prinos zabilježen je korištenjem etanola. Bioaktivni potencijal potvrđen je analizom antioksidacijskog potencijala te je u etanolnim ekstraktima zabilježena tri puta veća sposobnost hvatanja slobodnog DPPH radikala, dok su ORAC vrijednosti acetonskih ekstrakta bile veće za 27 %. Zbog najnižeg ekstrakcijskog prinosa te najmanjeg broja identificiranih spojeva u ekstraktima, heksan se nije pokazao učinkovitim otapalom. Kemijski profil *C. costatus* ukazuje na sadržaj različitih pigmenata i primarnih amida masnih kiselina za koje je zabilježen širok spektar biološke ativnosti. Aceton je omogućio najučinkovitiju ekstrakciju pigmenta i derivata pigmenata s feofitinom *a* kao najzastupljenijim spojem. Ekstrakcija etanolom omogućila je najveći prinos oleamida, najzastupljenijeg spoja u skupini derivata masnih kiselina, dok je (3β)-3-hidroksistigmast-5-en-7-on bio najzastupljeniji steroidni derivat u etanolnim i heksanskim ekstraktima. S obzirom da nije utvrđena značajna razlika u antioksidacijskom potencijalu te kemijskom profilu acetonskih i etanolnih ekstrakata dijatomeje *C. costatus*, zbog tri puta većeg ekstrakcijskog prinosa etanolna ekstrakcija pokazala se učinkovitijom za ekstrakciju dijatomejne biomase (Preliminarno istraživanje).
- U ekstrakciji dijatomeje *T. rotula* hidroalkoholna smjesa 70 %-tnog etanola omogućila je veći ekstrakcijski prinos u odnosu na 50 %-tni etanol. Zabilježen je veći sadržaj ukupnih fenola, a za iste ekstrakte zabilježena je i veća antioksidacijska aktivnost korištenjem sva tri testa (FRAP, DPPH i ORAC). Miristinska kiselina, palmitelaidna kiselina, palmitinska kiselina, eikozapentaenske i dokozapentaenske kiseline identificirane su kao dominantni spojevi u oba ekstrakta. U ekstrakciji 70 % etanolom zabilježeni su veći prinosi komercijalno važne eikozapentaenske kiseline kao i 24-metilenkolesterola, potencijalno važnog spoja za farmaceutsku industriju zbog potvrđenog antitumorskog djelovanja. Štoviše, 24-metilenkolesterol identificiran je u udjelu većem od 10 % u 70 %-tnom etanolnom ekstraktu *T. rotula*. Hidroalkoholna ekstrakcija 70 %-tnim etanolom osim što se pokazala učinkovitom u ekstrakciji *T. rotula*, omogućila je veći bioaktivni potencijal ekstrakata *T. rotula,* koja se pokazala važnim izvorom brojnih masnih kiselina i sterola (Rad 1.).
- Usporedbom uzgoja *S. grevillei* u bioreaktorskom i inkubatorskom sustavu, brži prirast i ulazak u eksponencijalnu i stacionarnu fazu rasta zabilježen je u bioreaktoru. Uzgoj u bioreaktorskom sustavu rezultirao je većim ekstrakcijskim prinosom u obje faza rasta u odnosu na inkubatorski sustav. U procjeni bioaktivnog potencijala c određena je veća antioksidacijska aktivnost DPPH i ORAC testom u obje faze rasta (eksponencijalnoj i stacionarnoj). U oba uzgojna sustava

identificirane su palmitelaidna kiselina, glicerol monostearat, miristinska kiselina, kolesterol, eikozapentaenska kiselina, 1-monopalmitin i 24-metilenkolesterol, a za sve navedene spojeve prethodne studije zabilježile su različite biološke aktivnosti. Bioreaktorski sustav pokazao se povoljnim za postizanje bržeg rasta dijatomeja *S. grevillei* koji omogućava veći ekstrakcijski prinos te antioksidacijski potencijal ekstrakata dijatomeja, a značajne razlike u kemijskom profilu između ekstrakta dobivenih iz biomase uzgojem u bioreaktorskom i inkubatorskom sustavu nisu zabilježene (Rad 2.).

- Rast u uzgojnom mediju bez fosfora, dušika ili silicija u vrsta *C. costatus* i *C. socialis* rezultirao je nižim ukupnim sadržajem fenola. S druge strane, u procjeni bioaktivnog potencijala značajno povećanje antioksidativnog kapaciteta zabilježeno je u ekstraktima *C. costatus* uzgojenog u mediju bez dušika, dok isti trend nije uočen kod *C. socialis*. Uzgoj bez dušika potaknuo je proizvodnju pigmentnih derivata, loliolida, feoforbida *a*, feofitina *b* i tri derivata klorofila *a* u dijatomeje *C. costatus*. Za razliku od pigmenata, uzgoj bez dušika doveo je do značajnog smanjenja sadržaja primarnih amida masnih kiselina u obje vrste. Utjecaj nedostatka hranjivih tvari u uzgojnom mediju na biološku aktivnost ekstrakata dijatomeja treba promatrati na razini vrste, s obzirom da rezultati ovog istraživanja kao i predhodne studije ukazuju na značajne razlike na razini roda (Rad 3.).

7. LITERATURA

Aldini, R., Micucci, M., Cevenini, M., Fato, R., Bergamini, C., Nanni, C., Cont, M., Camborata, C., Spinozzi, S., Montagnani, M., Roda, G., D'Errico-Grigioni, A., Rosini, F., Roda, A., Mazzella, G., Chiarini, A., Budriesi, R. (2014). Antiinflammatory effect of phytosterols in experimental *Murine colitis* model: prevention, induction, remission study. *PLoS ONE*, *9*(9), e108112. https://doi.org/10.1371/journal.pone.0108112

Ameamsri, U., Chaveerach, A., Sudmoon, R., Tanee, T., Peigneur, S., Tytgat, J. (2020). Oleamide in *Ipomoea* and *Dillenia* species and inflammatory activity investigated through ion channel inhibition. *Current Pharmaceutical Biotechnology*, *22*(2), 254–261. https://doi.org/10.2174/1389201021666200607185250

Amerine, M. A. Ough, C. S. (1980). *Methods for Analysis of Musts and Wines*. Wiley. New York, SAD.

Andersen, R.A. (2004) *Algal Culturing Techniques, 1st ed.*. Academic Press: Cambridge, MA, SAD.

Ano, Y., Ozawa, M., Kutsukake, T., Sugiyama, S., Uchida, K., Yoshida, A., Nakayama, H. (2015). Preventive effects of a fermented dairy product against Alzheimer's disease and identification of a novel oleamide with enhanced microglial phagocytosis and anti-inflammatory activity. *PLOS ONE, 10*(3), https://doi.org/10.1371/journal.pone.0118512

Benzie, I. F. F., Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the frap assay. *Analytical Biochemistry*, *239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292

Bie, N., Han, L., Meng, M., Zhang, Y., Guo, M., Wang, C. (2020). Anti-tumor mechanism of eicosapentaenoic acid (EPA) on ovarian tumor model by improving the immunomodulatory activity in F344 rats. *Journal of Functional Foods*, *65*, 103739. https://doi.org/10.1016/j.jff.2019.103739

Borowitzka, M. A., Beardall, J., Raven, J. A. (2016). *The Physiology of Microalgae*. Springer International Publishing, Cham, Švicarska. https://doi.org/10.1007/978-3-319-24945-2

Burčul, F., Generalić Mekinić, I., Radan, M., Rollin, P., Blažević, I. (2018). Isothiocyanates: cholinesterase inhibiting, antioxidant, and anti-inflammatory activity. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *33*(1), 577–582. https://doi.org/10.1080/14756366.2018.1442832

Calder, P. C. (2017). Omega-3 fatty acids and inflammatory processes: From molecules to man. *Biochemical Society Transactions*, *45*(5), 1105–1115. https://doi.org/10.1042/BST20160474

Chaves, N., Santiago, A., & Alías, J. C. (2020). Quantification of the Antioxidant Activity of Plant Extracts: Analysis of Sensitivity and Hierarchization Based on the Method Used. *Antioxidants, 9*(1), 76. https://doi.org/10.3390/antiox9010076

Collier, J. L., Grossman, A. R. (1992). Chlorosis induced by nutrient deprivation in Synechococcus sp. strain PCC 7942: not all bleaching is the same. *Journal of Bacteriology, 174*(14), 4718–4726. https://doi.org/10.1128/jb.174.14.4718-4726.1992

Curcuraci, E., Manuguerra, S., Messina, C. M., Arena, R., Renda, G., Ioannou, T., Amato, V., Hellio, C., Barba, F. J., Santulli, A. (2022). Culture conditions affect antioxidant production, metabolism and related biomarkers of the microalgae *Phaeodactylum tricornutum*. *Antioxidants, 11*(2), 1–17. https://doi.org/10.3390/antiox11020411

Cutignano, A., Conte, M., Tirino, V., Del Vecchio, V., De Angelis, R., Nebbioso, A., Altucci, L., Romano, G. (2022). Cytotoxic Potential of the marine diatom *Thalassiosira rotula*: insights into bioactivity of 24-methylene cholesterol. *Marine Drugs, 20*(10), 595. https://doi.org/10.3390/md20100595

Čagalj, M., Skroza, D., Tabanelli, G., Özogul, F., Šimat, V. (2021). Maximizing the antioxidant capacity of *Padina pavonica* by choosing the right drying and extraction methods. *Processes, 9*(4), 587. https://doi.org/10.3390/pr9040587

Čagalj, M., Skroza, D., Razola-Díaz, M. D. C., Verardo, V., Bassi, D., Frleta, R., Mekinić, I. G., Tabanelli, G., Šimat, V. (2022). Cariations in the composition, antioxidant and antimicrobial activities of *Cystoseira compressa* during seasonal growth. *Marine Drugs, 20*(64), 1–14. https://doi.org/10.3390/md20010064

D'Oca, C. D. R. M., Coelho, T., Marinho, T. G., Hack, C. R. L., da Costa Duarte, R., da Silva, P. A., D'Oca, M. G. M. (2010). Synthesis and antituberculosis activity of new fatty acid amides. *Bioorganic & Medicinal Chemistry Letters, 20*(17), 5255–5257. https://doi.org/10.1016/j.bmcl.2010.06.149

de Souza, A. L. C., do Rego Pires, A., Moraes, C. A. F., de Matos, C. H. C., dos Santos, K. I. P., Campos e Silva, R., Acuña, S. P. C., dos Santos Araújo, S. (2023). Chromatographic methods for separation and identification of bioactive compounds. *Drug Discovery and Design Using Natural Products* (pp. 153–176). Springer Nature Švicarska. https://doi.org/10.1007/978-3-031-35205-8_6

Dembitsky, V. M. (2022). Microbiological aspects of unique, rare, and unusual fatty acids derived from natural amides and their pharmacological profile. *Microbiology Research, 13*(3), 377–417. https://doi.org/10.3390/microbiolres13030030

dos Reis, C. M., da Rosa, B. V., da Rosa, G. P., do Carmo, G., Morandini, L. M. B., Ugalde, G.

A., Kuhn, K. R., Morel, A. F., Jahn, S. L., Kuhn, R. C. (2019). Antifungal and antibacterial activity of extracts produced from *Diaporthe schini*. *Journal of Biotechnology*, *294*, 30–37. https://doi.org/10.1016/j.jbiotec.2019.01.022

Duran, S. K., Kumar, P., Sandhu, S. S. (2021). A review on microalgae strains, cultivation, harvesting, biodiesel conversion and engine implementation. *Biofuels*, *12*(1), 91–102. https://doi.org/10.1080/17597269.2018.1457314

El Hattab, M., Culioli, G., Valls, R., Richou, M., Piovetti, L. (2008). Apo-fucoxanthinoids and loliolide from the brown alga *Cladostephus spongiosus f. verticillatus* (Heterokonta, Sphacelariales). *Biochemical Systematics and Ecology*, *36*(5–6), 447–451. https://doi.org/10.1016/j.bse.2007.08.016

FAO, "Chlorophylls," Rim, Italija, 1987. Pristupljeno: 17. sječnja, 2024. Dostupno: https://www.fao.org/fileadmin/user_upload/jecfa_additives/docs/Monograph1/Additive-128.pdf

Farrell, E. K., Chen, Y., Barazanji, M., Jeffries, K. A., Cameroamortegui, F., i Merkler, D. J. (2012). Primary fatty acid amide metabolism: conversion of fatty acids and an ethanolamine in N18TG2 and SCP cells. *Journal of Lipid Research*, *53*(2), 247–256. https://doi.org/10.1194/jlr.M018606

Ferdous, U. T., Nurdin, A., Ismail, S., Shaari, K., Norhana Balia Yusof, Z. (2025). A comparative study on antioxidant properties, total phenolics, total flavonoid contents, and cytotoxic properties of marine green microalgae and diatoms. *Journal of Genetic Engineering and Biotechnology*, *23*(1), 100456. https://doi.org/10.1016/j.jgeb.2024.100456

Ferreira, M., Teixeira, C., Abreu, H., Silva, J., Costas, B., Kiron, V., Valente, L. M. P. (2021). Nutritional value, antimicrobial and antioxidant activities of micro- and macroalgae, single or blended, unravel their potential use for aquafeeds. *Journal of Applied Phycology*, *33*(6), 3507–3518. https://doi.org/10.1007/s10811-021-02549-2

Field C.B., Behrenfeld M.J., Randerson J.T., Falkowski P. (1998). Primary Production of the Biosphere. Biochemical Society Transactions, 281(5374), 237–240. https://doi.org/10.1042/bst0040954

Foo, S. C., Yusoff, F. M., Ismail, M., Basri, M., Khong, N. M. H., Chan, K. W., Yau, S. K. (2015). Efficient solvent extraction of antioxidant-rich extract from a tropical diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968. *Asian Pacific Journal of Tropical Biomedicine*, *5*(10), 834–840. https://doi.org/10.1016/j.apjtb.2015.06.003

Fukami, K., Nishimura, S., Ogusa, M., Asada, M., Nishijima, T. (1997). Continuous culture with deep seawater of a benthic food diatom *Nitzschia* sp. *Hydrobiologia*, *358*, 245–249.

https://doi.org/10.1023/A:1003141104693

Gallo, C., Landi, S., d'Ippolito, G., Nuzzo, G., Manzo, E., Sardo, A., Fontana, A. (2020). Diatoms synthesize sterols by inclusion of animal and fungal genes in the plant pathway. *Scientific Reports, 10*(1), 1–13. https://doi.org/10.1038/s41598-020-60993-5

Gao, Y., Yang, M., & Wang, C. (2013). Nutrient deprivation enhances lipid content in marine microalgae. *Bioresource Technology*, *147*, 484–491. https://doi.org/10.1016/j.biortech.2013.08.066

Gatamaneni, B. L., Orsat, V., Lefsrud, M. (2018). Factors Affecting Growth of Various Microalgal Species. *Environmental Engineering Science*, *35*(10), 1037–1048. https://doi.org/10.1089/ees.2017.0521

Georgiopoulou, I., Tzima, S., Louli, V., Magoulas, K. (2023). Process Optimization of Microwave-Assisted Extraction of Chlorophyll, Carotenoid and Phenolic Compounds from *Chlorella vulgaris* and Comparison with Conventional and Supercritical Fluid Extraction. *Applied Sciences, 13*(4), 2740. https://doi.org/10.3390/app13042740

Goiris, K., Van Colen, W., Wilches, I., León-Tamariz, F., De Cooman, L., Muylaert, K. (2015). Impact of nutrient stress on antioxidant production in three species of microalgae. *Algal Research, 7*, 51–57. https://doi.org/10.1016/j.algal.2014.12.002

Gomes, T. A., Zanette, C. M., Spier, M. R. (2020). An overview of cell disruption methods for intracellular biomolecules recovery. *Preparative Biochemistry & Biotechnology*, 1–20. https://doi.org/10.1080/10826068.2020.1728696

Gonzalez-Fernandez, C., Muñoz, R. (2017). *Microalgae-Based Biofuels and Bioproducts. From Feedstock Cultivation to End-products.* Elsevier, Amsterdam, The Netherlands https://doi.org/10.1016/C2015-0-05935-4

Hameed, I. H., Altameme, H. J., Mohammed, G. J. (2016). Evaluation of antifungal and antibacterial activity and analysis of bioactive phytochemical compounds of *Cinnamomum zeylanicum* (Cinnamon bark) using gas chromatography-mass spectrometry. *Oriental Journal of Chemistry*, *32*(4), 1769–1788. https://doi.org/10.13005/ojc/320406

Hemalatha, A., Parthiban, C., Saranya, C., Girija, K., Anantharaman, P. (2015). Evaluation of antioxidant activities and total phenolic contents of different solvent extracts of selected marine diatoms. *Indian Journal of Geo-Marine Sciences, 44*(10), 1630–1636.

Higashi-Okai, K., Otani, S., & Okai, Y. (1998). Potent suppressive activity of pheophytin *a* and *b* from the non-polyphenolic fraction of green tea (*Camellia sinensis*) against tumor promotion in mouse skin. *Cancer Letters, 129*(2), 223–228. https://doi.org/10.1016/S0304-3835(98)00113-X

Hsu, C.-Y., Chao, P.-Y., Hu, S.-P., Yang, C.-M. (2013). The antioxidant and free radical scavenging activities of chlorophylls and pheophytins. *Food and Nutrition Sciences*, *04*(08), 1–8. https://doi.org/10.4236/fns.2013.48A001

Indriani, I., Aminah, N. S., Puspaningsih, N. N. T. (2020). Antiplasmodial activity of stigmastane steroids from *Dryobalanops oblongifolia* stem bark. *Open Chemistry*, *18*(1), 259–264. https://doi.org/10.1515/chem-2020-0027

Jacotet-Navarro, M., Laguerre, M., Fabiano-Tixier, A. S., Tenon, M., Feuillère, N., Bily, A., Chemat, F. (2018). What is the best ethanol-water ratio for the extraction of antioxidants from rosemary? Impact of the solvent on yield, composition, and activity of the extracts. *Electrophoresis*, *39*(15), 1946–1956. https://doi.org/10.1002/elps.201700397

Jaramillo-Madrid, A. C., Abbriano, R., Ashworth, J., Fabris, M., Pernice, M., Ralph, P. J. (2020a). Overexpression of key sterol pathway enzymes in two model marine diatoms alters sterol profiles in *Phaeodactylum tricornutum*. *Pharmaceuticals*, *13*(12), 481. https://doi.org/10.3390/ph13120481

Jaramillo-Madrid, A. C., Ashworth, J., Ralph, P. J. (2020b). Levels of diatom minor sterols respond to changes in temperature and salinity. *Journal of Marine Science and Engineering*, *8*(2). https://doi.org/10.3390/JMSE8020085

Javid, S., Purohit, M. N., Yogish Kumar, H., Ramya, K., Mithuna, N. F. A., Salahuddin, M. D., Prashantha Kumar, B. R. (2020). Semisynthesis of myristic acid derivatives and their biological activities: a critical insight. *Journal of Biologically Active Products from Nature*, *10*(6), 455–472. https://doi.org/10.1080/22311866.2020.1865836

Jeyakumar, B., Asha, D., Varalakshmi, P., Kathiresan, S. (2020). Nitrogen repletion favors cellular metabolism and improves eicosapentaenoic acid production in the marine microalga *Isochrysis* sp. CASA CC 101. *Algal Research*, *47*, 101877. https://doi.org/10.1016/j.algal.2020.101877

Jie, F., Yang, X., Wu, L., Wang, M., Lu, B. (2022). Linking phytosterols and oxyphytosterols from food to brain health: origins, effects, and underlying mechanisms. *Critical Reviews in Food Science and Nutrition*, *62*(13), 3613–3630. https://doi.org/10.1080/10408398.2020.1867819

Kabara, J. J., Swieczkowski, D. M., Conley, A. J., Truant, J. P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrobial Agents and Chemotherapy*, *2*(1), 23–28. https://doi.org/10.1128/AAC.2.1.23

Karlson, B., Cusack, C. and Bresnan, E. 2010. Microscopic and molecular methods for quantitative phytoplankton analysis. *Intergovernmental Oceanographic Commission of UNESCO*. IOC Manuals and Guides, (55), pp-110.

Kedare, S. B., Singh, R. P. (2011). Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology*, *48*(4), 412–422. https://doi.org/10.1007/s13197-011-0251-1

Kim, S. B., Bisson, J., Friesen, J. B., Pauli, G. F., Simmler, C. (2020). Selective chlorophyll removal method to "degreen" botanical extracts. *Journal of Natural Products*, *83*(6), 1846–1858. https://doi.org/10.1021/acs.jnatprod.0c00005

Kong, W., Liu, N., Zhang, J., Yang, Q., Hua, S., Song, H., Xia, C. (2014). Optimization of ultrasound-assisted extraction parameters of chlorophyll from *Chlorella vulgaris* residue after lipid separation using response surface methodology. *Journal of Food Science and Technology*, *51*(9), 2006–2013. https://doi.org/10.1007/s13197-012-0706-z

Kuczynska, P., Jemiola-Rzeminska, M., Strzalka, K. (2015). Photosynthetic pigments in diatoms. *Marine Drugs*, *13*(9), 5847–5881. https://doi.org/10.3390/md13095847

Kusmita, L., Puspitaningrum, I., Limantara, L. (2015). Identification, isolation and antioxidant activity of pheophytin from green tea (*Camellia sinensis* (L.) Kuntze). *Procedia Chemistry*, *14*, 232–238. https://doi.org/10.1016/j.proche.2015.03.033

Lanfer-Marquez, U. M., Barros, R. M. C., Sinnecker, P. (2005). Antioxidant activity of chlorophylls and their derivatives. *Food Research International*, *38*(8–9), 885–891. https://doi.org/10.1016/j.foodres.2005.02.012

Li, Y., Ghasemi Naghdi, F., Garg, S., Adarme-Vega, T., Thurecht, K. J., Ghafor, W., Tannock, S., Schenk, P. M. (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. *Microbial Cell Factories*, *13*(1), 14. https://doi.org/10.1186/1475-2859-13-14

Lin, Q., Zhuo, W. H., Wang, X. W., Chen, C. P., Gao, Y. H., & Liang, J. R. (2018). Effects of fundamental nutrient stresses on the lipid accumulation profiles in two diatom species *Thalassiosira weissflogii* and *Chaetoceros muelleri*. *Bioprocess and Biosystems Engineering*, *41*(8), 1213–1224. https://doi.org/10.1007/s00449-018-1950-z

Lovio-Fragoso, J. P., de Jesús-Campos, D., López-Elías, J. A., Medina-Juárez, L. Á., Fimbres-Olivarría, D., Hayano-Kanashiro, C. (2021). Biochemical and molecular aspects of phosphorus limitation in diatoms and their relationship with biomolecule accumulation. *Biology*, *10*(7). https://doi.org/10.3390/biology10070565

Lozano-Muñoz, I., Muñoz, S., Díaz, N. F., Medina, A., Bazaes, J., Riquelme, C. (2020). Nutritional enhancement of farmed salmon meat via non-GMO *Nannochloropsis gaditana*: eicosapentaenoic acid (EPA, 20:5 *n*-3), docosapentaenoic acid (DPA, 22:5 *n*-3) and vitamin D3 for human health. *Molecules*, *25*(20). https://doi.org/10.3390/molecules25204615

Maadane, A., Merghoub, N., Ainane, T., El Arroussi, H., Benhima, R., Amzazi, S., Bakri, Y., Wahby, I. (2015). Antioxidant activity of some Moroccan marine microalgae: PUFA profiles, carotenoids and phenolic content. *Journal of Biotechnology*, *215*, 13–19. https://doi.org/10.1016/j.jbiotec.2015.06.400

Manivannan, K., Anantharaman, P., Balasubramanian, T. (2012). Evaluation of antioxidant properties of marine microalga *Chlorella marina* (Butcher, 1952). *Asian Pacific Journal of Tropical Biomedicine*, *2*(1), S342–S346. https://doi.org/10.1016/S2221-1691(12)60185-3

Mendonça, J. d. S., Guimarães, R. d. C. A., Zorgetto-Pinheiro, V. A., Fernandes, C. D. P., Marcelino, G., Bogo, D., Freitas, K. d. C., Hiane, P. A., de Pádua Melo, E. S., Vilela, M. L. B., Nascimento, V. A. d. (2022). Natural Antioxidant Evaluation: A Review of Detection Methods. *Molecules*, *27*(11), 3563. https://doi.org/10.3390/molecules27113563

Menzel, D., van Bergen, P. F., Schouten, S., Sinninghe Damsté, J. S. (2003). Reconstruction of changes in export productivity during Pliocene sapropel deposition: a biomarker approach. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *190*, 273–287. https://doi.org/10.1016/S0031-0182(02)00610-7

Mienis, E., Vandamme, D., Foubert, I. (2024). Resuspended freeze-dried *Nannochloropsis* as a model laboratory system for concentrated fresh *Nannochloropsis* in ultrasound cell disruption experiments. *Frontiers in Marine Science*, *11*. https://doi.org/10.3389/fmars.2024.1359090

Monteiro, M., Santos, R. A., Iglesias, P., Couto, A., Serra, C. R., Gouvinhas, I., Barros, A., Oliva-Teles, A., Enes, P., Díaz-Rosales, P. (2020). Effect of extraction method and solvent system on the phenolic content and antioxidant activity of selected macro- and microalgae extracts. *Journal of Applied Phycology*, *32*(1), 349–362. https://doi.org/10.1007/s10811-019-01927-1

Moon, S.-M., Lee, S. A., Hong, J. H., Kim, J.-S., Kim, D. K., Kim, C. S. (2018). Oleamide suppresses inflammatory responses in LPS-induced RAW264.7 murine macrophages and alleviates paw edema in a carrageenan-induced inflammatory rat model. *International Immunopharmacology*, *56*, 179–185. https://doi.org/10.1016/j.intimp.2018.01.032

Nakazato, M. (1995). *Method of Producing Water-Soluble Sodum Pheophorbdea*. *19*, 5. US5378835A

Nieri, P., Carpi, S., Esposito, R., Costantini, M., Zupo, V. (2023). Bioactive molecules from marine diatoms and their value for the nutraceutical industry. *Nutrients*, *15*(2), 1–23. https://doi.org/10.3390/nu15020464

NIST, N. I. of S. and T. (2022). NIST Chemistry WebBook. NIST Standard Reference Database. Pristupljeno: 17, siječanj 2025. Dostupno: https://webbook.nist.gov/chemistry/

Oberacher, H. (2012). *Wiley Registry of Tandem Mass Spectral Data, MS for ID*. Wiley, Hoboken,

NJ, SAD.

Orefice, I., Musella, M., Smerilli, A., Sansone, C., Chandrasekaran, R., Corato, F., Brunet, C. (2019). Role of nutrient concentrations and water movement on diatom's productivity in culture. *Scientific Reports*, *9*(1), 1–10. https://doi.org/10.1038/s41598-018-37611-6

Purkan, P.; Nurlaila, H.; Baktir, A.; Hadi, S.; Soemarjati, W. Microalgal lipid from *Chaetoceros calcitrans* and its conversion to biodiesel through ex and in-situ transesterification. *Rasayan Journal of Chemistry*, 43–52. https://doi.org/10.31788/RJC.2022.1558063

Park, S. H., Kim, D. S., Kim, S., Lorz, L. R., Choi, E., Lim, H. Y., Hossain, M. A., Jang, S., Choi, Y. I., Park, K. J., Yoon, K., Kim, J.-H., Cho, J. Y. (2019). Loliolide presents antiapoptosis and antiscratching effects in human keratinocytes. *International Journal of Molecular Sciences*, *20*(3), 651. https://doi.org/10.3390/ijms20030651

Pasquet, V., Chérouvrier, J.-R., Farhat, F., Thiéry, V., Piot, J.-M., Bérard, J.-B., Kaas, R., Serive, B., Patrice, T., Cadoret, J.-P., Picot, L. (2011). Study on the microalgal pigments extraction process: Performance of microwave assisted extraction. *Process Biochemistry*, *46*(1), 59–67. https://doi.org/10.1016/j.procbio.2010.07.009

Percot, A., Yalçın, A., Aysel, V., Erduğan, H., Dural, B., Güven, K. C. (2009). Loliolide in marine algae. *Natural Product Research*, *23*(5), 460–465. https://doi.org/10.1080/14786410802076069

Prasath, K. G., Sethupathy, S., Pandian, S. K. (2019). Proteomic analysis uncovers the modulation of ergosterol, sphingolipid and oxidative stress pathway by myristic acid impeding biofilm and virulence in *Candida albicans*. *Journal of Proteomics*, *208*, 103503. https://doi.org/10.1016/j.jprot.2019.103503

Premjanu, N., Jaynthy, C. (2015). Identification and characterization of antimicrobial metabolite from an endophytic fungus, *Colletotrichum gloeosporioides* isolated from *Lannea corammendalica*. *International Journal of ChemTech Research*, *7*(1), 369–374.

Radman, S., Cikoš, A.-M., Babić, S., Čižmek, L., Čož-Rakovac, R., Jokić, S., Jerković, I. (2022). *In vivo* and *in vitro* antioxidant activity of less polar fractions of *Dasycladus vermicularis* (Scopoli) Krasser 1898 and the chemical composition of fractions and macroalga volatilome. *Pharmaceuticals*, *15*(6), 743. https://doi.org/10.3390/ph15060743

Radman, S., Cikoš, A.-M., Flanjak, I., Babić, S., Čižmek, L., Šubarić, D., Čož-Rakovac, R., Jokić, S., Jerković, I. (2021). Less Polar compounds and targeted antioxidant potential (*in vitro* and *in vivo*) of *Codium adhaerens* C. Agardh 1822. *Pharmaceuticals*, *14*(9), 944. https://doi.org/10.3390/ph14090944

Radman, S., Čižmek, L., Babić, S., Cikoš, A.-M., Čož-Rakovac, R., Jokić, S., Jerković, I. (2022).

Bioprospecting of less-polar fractions of *Ericaria crinita* and *Ericaria amentacea*: developmental toxicity and antioxidant activity. *Marine Drugs*, *20*(1), 57. https://doi.org/10.3390/md20010057

Rampen, S. W., Abbas, B. A., Schouten, S., Damsté, J. S. S. (2010). A comprehensive study of sterols in marine diatoms (Bacillariophyta): implications for their use as tracers for diatom productivity. *Limnology and Oceanography*, *55*(1), 91–105. https://doi.org/10.4319/lo.2010.55.1.0091

Raniello, R., Iannicelli, M. M., Nappo, M., Avila, C., Zupo, V. (2007). Production of *Cocconeis neothumensis* (Bacillariophyceae) biomass in batch cultures and bioreactors for biotechnological applications: Light and nutrient requirements. *Journal of Applied Phycology*, *19*(4), 383–391. https://doi.org/10.1007/s10811-006-9145-4

Ras, R. T., Geleijnse, J. M., Trautwein, E. A. (2014). LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: A meta-analysis of randomised controlled studies. *British Journal of Nutrition*, *112*(2), 214–219. https://doi.org/10.1017/S0007114514000750

Repeta, D. J. (1989). Carotenoid diagenesis in recent marine sediments: II. Degradation of fucoxanthin to loliolide. *Geochimica et Cosmochimica Acta*, *53*(3), 699–707. https://doi.org/10.1016/0016-7037(89)90012-4

Rumin, J., Nicolau, E., de Oliveira, R. G., Fuentes-Grünewald, C., Picot, L. (2020). Analysis of scientific research driving microalgae market opportunities in Europe. *Marine Drugs*, *18*(5), https://doi.org/10.3390/md18050264

Sabia, A., Clavero, E., Pancaldi, S., Salvadó Rovira, J. (2018). Effect of different CO_2 concentrations on biomass, pigment content, and lipid production of the marine diatom *Thalassiosira pseudonana*. *Applied Microbiology and Biotechnology*, *102*(4), 1945–1954. https://doi.org/10.1007/s00253-017-8728-0

Saide, A., Lauritano, C., Ianora, A. (2020). Pheophorbide *a*: State of the Art. *Marine Drugs*, *18*(5), 257. https://doi.org/10.3390/md18050257

Sañé, E., Del Mondo, A., Ambrosino, L., Smerilli, A., Sansone, C., Brunet, C. (2021). The recent advanced in microalgal phytosterols: bioactive ingredients along with human-health driven potential applications. *Food Reviews International*, 1–20. https://doi.org/10.1080/87559129.2021.1938115

Sathasivam, R., Radhakrishnan, R., Hashem, A., AbdAllah, E. F. (2019). Microalgae metabolites: A rich source for food and medicine. *Saudi Journal of Biological Sciences*, *26*(4), 709–722. https://doi.org/10.1016/j.sjbs.2017.11.003

Shao, J., He, Y., Li, F., Zhang, H., Chen, A., Luo, S., Gu, J. D. (2016). Growth inhibition and possible mechanism of oleamide against the toxin-producing cyanobacterium *Microcystis aeruginosa* NIES-843. *Ecotoxicology*, *25*(1), 225–233. https://doi.org/10.1007/s10646-015-1582-x

Shrestha, R. P., Hildebrand, M. (2015). Evidence for a regulatory role of diatom silicon transporters in cellular silicon responses. *Eukaryotic Cell*, *14*(1), 29. https://doi.org/10.1128/EC.00209-14

Silva, M., Kamberovic, F., Uota, S. T., Kovan, I. M., Viegas, C. S. B., Simes, D. C., Gangadhar, K. N., Varela, J., Barreira, L. (2022). Microalgae as potential sources of bioactive compounds for functional foods and pharmaceuticals. *Applied Sciences (Switzerland)*, *12*(12). https://doi.org/10.3390/app12125877

Šimat, V., Vlahović, J., Soldo, B., Generalić Mekinić, I., Čagalj, M., Hamed, I., Skroza, D. (2020). Production and characterization of crude oils from seafood processing by-products. *Food Bioscience*, *33*, 100484. https://doi.org/10.1016/j.fbio.2019.100484

Singh, P., Baranwal, M., & Reddy, S. M. (2016). Antioxidant and cytotoxic activity of carotenes produced by *Dunaliella salina* under stress. *Pharmaceutical Biology*, *54*(10), 2269–2275. https://doi.org/10.3109/13880209.2016.1153660

Smith, S. R., Glé, C., Abbriano, R. M., Traller, J. C., Davis, A., Trentacoste, E., Vernet, M., Allen, A. E., Hildebrand, M. (2016). Transcript level coordination of carbon pathways during silicon starvation-induced lipid accumulation in the diatom *Thalassiosira pseudonana*. *New Phytologist*, *210*(3), 890–904. https://doi.org/10.1111/nph.13843

Stonik, V. S., Stonik, I. (2015). Low-molecular-weight metabolites from diatoms: Structures, biological roles and biosynthesis. *Marine Drugs*, *13*(6), 3672–3709. https://doi.org/10.3390/md13063672

Sun, M., Zhou, Z., Dong, J., Zhang, J., Xia, Y., Shu, R. (2016). Antibacterial and antibiofilm activities of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) against periodontopathic bacteria. *Microbial Pathogenesis*, *99*, 196–203. https://doi.org/10.1016/j.micpath.2016.08.025

Suroy, M., Moriceau, B., Boutorh, J., Goutx, M. (2014). Fatty acids associated with the frustules of diatoms and their fate during degradation-A case study in *Thalassiosira weissflogii*. *Deep-Sea Research Part I: Oceanographic Research Papers*, *86*, 21–31. https://doi.org/10.1016/j.dsr.2014.01.001

Svenning, J. B., Dalheim, L., Vasskog, T., Matricon, L., Vang, B., Olsen, R. L. (2020). Lipid yield from the diatom *Porosira glacialis* is determined by solvent choice and number of

extractions, independent of cell disruption. *Scientific Reports*, *10*(1), 1–10. https://doi.org/10.1038/s41598-020-79269-z

Tanvir, R., Javeed, A., Rehman, Y. (2018). Fatty acids and their amide derivatives from endophytes: new therapeutic possibilities from a hidden source. *FEMS Microbiology Letters*, *365*(12). https://doi.org/10.1093/femsle/fny114

Torras-Claveria, L., Berkov, S., Jáuregui, O., Caujapé, J., Viladomat, F., Codina, C., Bastida, J. (2010). Metabolic profiling of bioactive *Pancratium canariense* extracts by GC-MS. *Phytochemical Analysis*, *21*(1), 80–88. https://doi.org/10.1002/pca.1158

Trentin, R., Custódio, L., Rodrigues, M. J., Moschin, E., Sciuto, K., da Silva, J. P., Moro, I. (2022). Total Phenolic levels, in vitro antioxidant properties, and fatty acid profile of two microalgae, *Tetraselmis marina* strain IMA043 and naviculoid diatom strain IMA053, Isolated from the North Adriatic Sea. *Marine Drugs*, *20*(3), 207. https://doi.org/10.3390/md20030207

Ummalyma, S. B., Sirohi, R., Udayan, A., Yadav, P., Raj, A., Sim, S. J., Pandey, A. (2022). Sustainable microalgal biomass production in food industry wastewater for low-cost biorefinery products: a review. *Phytochemistry Reviews*, *3*. https://doi.org/10.1007/s11101-022-09814-3

Vello, V., Phang, S.-M., Poong, S.-W., Lim, Y.-K., Ng, F.-L., Shanmugam, J., Gopal, M. (2023). New report of *Halamphora subtropica* (Bacillariophyta) from the Strait of Malacca and its growth and biochemical characterisation under nutrient deprivation. *Regional Studies in Marine Science*, *62*, 102947. https://doi.org/10.1016/j.rsma.2023.102947

Vuppaladadiyam, A. K., Prinsen, P., Raheem, A., Luque, R., Ming Zhao. (2018). Microalgae cultivation and metabolites production: a comprehensive review. *Biofuels, Bioproducts and Biorefining*, *12*, 304–324. https://doi.org/10.1002/bbb

Wang, J. K., Seibert, M. (2017). Prospects for commercial production of diatoms. *Biotechnology for Biofuels*, *10*(1), 1–13. https://doi.org/10.1186/s13068-017-0699-y

Warkoyo Warkoyo, Saati, E. A. (2011). The solvent effectiveness on extraction process of seaweed pigment. *Makara of Technology Series*, *15*(1). https://doi.org/10.7454/mst.v15i1.850

Yang, X., Kang, M. C., Lee, K. W., Kang, S. M., Lee, W. W., Jeon, Y. J. (2011). Antioxidant activity and cell protective effect of loliolide isolated from *Sargassum ringgoldianum* subsp. *coreanum*. *ALGAE*, *26*(2), 201–208. https://doi.org/10.4490/algae.2011.26.2.201

Yi, Z., Xu, M., Di, X., Brynjolfsson, S., Fu, W. (2017). Exploring valuable lipids in diatoms. *Frontiers in Marine Science*, *4*(1), 1–10. https://doi.org/10.3389/fmars.2017.00017

Yu, S. J., Shen, X. F., Ge, H. Q., Zheng, H., Chu, F. F., Hu, H., Zeng, R. J. (2016). Role of sufficient

phosphorus in biodiesel production from diatom *Phaeodactylum tricornutum*. *Applied Microbiology and Biotechnology*, *100*(15), 6927–6934. https://doi.org/10.1007/s00253-016-7641-2

Yuyama, K. T., Rohde, M., Molinari, G., Stadler, M., Abraham, W. R. (2020). Unsaturated fatty acids control biofilm formation of *Staphylococcus aureus* and other gram-positive bacteria. *Antibiotics*, *9*(11), 1–11. https://doi.org/10.3390/antibiotics9110788

Zhang, K., Li, T., Shan, X., Lu, R., Zhang, S., Xu, H. (2021). Cholesterol: Bioactivities, Structural Modification, Mechanisms of Action, and Structure-Activity Relationships. *Mini-Reviews in Medicinal Chemistry*, *21*(14), 1830–1848. https://doi.org/10.2174/1389557521666210105123320

Zhu, S., Jiao, W., Xu, Y., Hou, L., Li, H., Shao, J., Zhang, X., Wang, R., Kong, D. (2021). Palmitic acid inhibits prostate cancer cell proliferation and metastasis by suppressing the PI3K/Akt pathway. *Life Sciences*, *286*, 120046. https://doi.org/10.1016/j.lfs.2021.120046

Zulfiqar, M., Stettin, D., Schmidt, S., Nikitashina, V., Pohnert, G., Steinbeck, C., Peters, K., Sorokina, M. (2023). Untargeted metabolomics to expand the chemical space of the marine diatom *Skeletonema marinoi*. *Frontiers in Microbiology*, *14*. https://doi.org/10.3389/fmicb.2023.1295994

Zulu, N. N., Zienkiewicz, K., Vollheyde, K., Feussner, I. (2018). Current trends to comprehend lipid metabolism in diatoms. *Progress in Lipid Research*, *70*, 1–16. https://doi.org/10.1016/j.plipres.2018.03.001

8. RADOVI OBJEDINJENI U DOKTORSKOJ DISERTACIJI

8.1. Preliminarno istraživanje. The effect of solvent choice on antioxidant potential and chemical composition of extracts from microalgae *Chaetocerus costatus*

phycology

MDPI

Communication

The Effect of Solvent Choice on Antioxidant Potential and Chemical Composition of Extracts from Microalgae *Chaetocerus costatus*

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Abstract: This study aim to compare the extraction yield, antioxidant potential, and chemical composition of *Chaetoceros costatus* extracted with acetone, ethanol, and hexane. The freeze-dried diatom biomass was extracted by ultrasonication for 1 h at 40 °C. The antioxidant capacity was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and oxygen radical absorbance capacity (ORAC), while the chemical profiles of the extracts were analyzed using high-performance liquid chromatography with high-resolution mass spectrometry with electrospray ionization (UHPLC-ESI-HRMS). The ORAC assay showed a 27% higher activity of the acetone extract, while the DPPH assay showed almost 3-fold higher DPPH inhibition. Pigments, fatty acids, sterols, and their derivatives were identified in all extracts. The chemical composition of ethanolic and acetonic extracts did not differ significantly, and hexane yielded the fewest compounds. The results of this study will contribute to extraction challenges that limit biotechnological application and exploitation of diatoms.

Keywords: diatoms; extraction solvent; antioxidant potential; chemical profile

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1. Introduction

The production of microalgae holds significant potential for many different sectors, including the production of biofuels, environmental protection, pharmaceuticals, and food supplements [1–5]. Diatoms are a group of microalgae that have exceptional potential to produce numerous high valuable compounds for commercial use [6]. The genus *Chaetoceros* can synthesize various amino acids, lipids, pigments, terpenoids, and polysaccharides, which are promising therapeutic and nutraceutical agents [7,8]. In addition, *Chaetoceros* sp. extracts also showed antioxidant and cytotoxic activities [9]. A wider application of microalgae, as well as diatoms, is primarily hindered by numerous biotechnological barriers. Besides the challenges of cultivation, contamination risks, and harvesting, one of the biggest challenges in microalgae production is the efficient processing of the microalgae biomass, i.e., extraction [10–12].

Extraction efficacy is improved by microwaves, ultrasounds, pressure and/or enzyme uses [13–15]. Microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) are the most used and proven effective for the extraction of microalgae biomass [13]. In general, although MAE outperforms UAE in terms of overall extraction efficiency,

UAE is easier to use when extracting smaller samples such as diatom biomass [16]. By combining the techniques, extraction yields could be significantly improved, with the best approach being UAE of previously lyophilized biomass [17]. However, the selection of the appropriate extraction techniques should be based on the selected species and the properties of the cell wall.

An efficient extraction process should maximize the yield of high-quality bioactive compounds from microalgae biomass at the same as time minimizing processing costs and environmental impact. High energy costs, the use of toxic solvents, and the generation of large quantities of waste are the main reasons why traditional techniques are not economically and ecologically sustainable for the long term [18,19]. Compared to other extraction techniques, UAE is very efficient, and requires less time and solvent [15,20], thus is considered environmentally friendly (reduces energy consumption and waste generation). The introduction of UAE in the food industry would ensure economic sustainability and contribute to a circular economy with minimal environmental impact [21]. The study of the species *Chaetoceros calcitrans* emphasizes the importance of using innovative methods such as UAE for efficient lipid extraction, but the effectiveness of the different techniques in the extraction of species of the genus *Chaetoceros* has not really been investigated so far.

Choosing a suitable solvent and/or solvent ratio is also a crucial step in optimizing extraction to achieve maximum extraction efficiency [22]. Solvent selection is based on polarity, i.e., hexane (0.0–0.1) < acetone (5.1) < ethanol (5.2) < water (9.0), where the polarity index of the solvent and the target compound should be approximately the same to ensure maximum yield [23–25]. According to ref. [9], acetone, hexane and ethanol are commonly used solvents in the extraction of microalgae. Acetone is an effective solvent for carotenoids such as violaxanthin, lutein, zeaxanthin, and carotene, as well as for polar lipids, but is also used for the extraction of other pigments [26]. In chlorophyll extraction, ethanol is particularly effective as it also extracts other bioactive compounds such as *n*-3 fatty acids, while hexane is primarily used to extract neutral lipids [26,27].

In order to determine the most suitable solvent for the extraction of diatoms, the aim of this study was to compare the differences in extraction yield, antioxidant activity, and chemical profile of the diatom *Chaetoceros costatus* extracted with acetone, ethanol, and hexane in combination with UAE.

2. Materials and Methods

2.1. Cultivation Conditions

A diatom strain of *C. costatus* (CIM935) was isolated from the northern Adriatic Sea and donated from the culture collection of the Center for Marine Research of the Ruđer Bošković Institute (Rovinj, Croatia). Cultivation was performed by adding 100 mL of diatom inoculum in the stationary growth phase (10^5 cells/mL) in an Erlenmeyer flask with 1.5 L of F/2 medium prepared according to the recipe [28]. During a cultivation period of 15 days, the diatoms were exposed to a light intensity of 2500 lux (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italy) and a 16:8 light:dark cycle, while the temperature was maintained at 18 °C. Cultivation was performed in six replicates.

2.2. Extraction Protocol

The diatom biomass was harvested during the stationary growth phase using glass microfiber filters (class GF/F Whatman). The collected diatom biomass was freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO, USA) and extracted with acetone, ethanol, and hexane. Extraction of 100 mg of lyophilized diatom biomass was performed in 8 mL of each solvent, while the extraction process was assisted by ultrasonication in an ultrasonic bath (DU-100 Digital Ultrasonic Cleaner, Giorgio Bormac, Carpi, Italy) at a frequency of 40 kHz

and 40 °C for 1 h. All samples were centrifuged (Rotafix 32A, Hettich, Tuttlingen, Germany) at 3220× *g* (4000 rpm) for 5 min. The collected supernatants were filtered through a 0.45 µM mixed cellulose ester filter (LGG, Meckenheim, Germany) and evaporated to dryness using a centrifugal evaporator (RC10-22, Jouan, Herblain, France).

2.3. Antioxidation Assays

Prior to the analyses, dried acetone, ethanol, and hexane extracts of *C. costatus* were redissolved at a concentration of 10 mg/mL in the solvents previously used for extraction.

The diatom extract's ability to scavenge DPPH radicals was evaluated [29]. After adding 290 µL of DPPH radical solution prepared in 96% ethanol with an initial absorbance of 1.2 to the wells of the microtiter plate, measurements were performed at 517 nm (SynergyHTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). One hour after adding 10 µL of the extracts to the wells, the decrease in absorbance was measured using a plate reader. The antioxidant activity of the diatom extracts was measured by calculating the percentage of inhibition of DPPH radicals that they could prevent (% inhibition).

The ORAC assay was performed in accordance with previously published procedures [30,31]. To the wells of a microtiter plate containing 150 µL of 4.2 mM fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1(3H),9′-[9H] xanthan]-3-one), 25 µL of the diatom extracts were added. A total of 25 µL of AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride) was added to the wells after they were thermostated at 37 °C for 30 min. Measurements of excitation and emission wavelengths at 485 and 520 nm were taken every minute for eighty minutes. Results were expressed in Trolox equivalents/L (µM TE/L). Both assays were performed in triplicate.

2.4. Chemical Analysis by Ultra-High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UHPLC-ESI-HRMS)

UHPLC-ESI-HRMS analyses were performed using an ExionLC AD UHPLC system (AB Sciex, Concord, ON, Canada) coupled to a quadrupole time-of-flight (Q-TOF) mass spectrometer TripleTOF 6600+ (AB Sciex, Concord, ON, Canada) with a duospray ion source. The compounds were separated chromatographically using the Acquity UPLC BEH Phenyl-Hexyl analytical column (Waters, Milford, MA, USA) 2.1 mm 100 mm with a particle size of 1.7 µM. 0.1% formic acid was present in both acetonitrile (mobile phase B) and water (mobile phase A). Throughout the analysis, the oven temperature of 30 °C and the flow rate of 0.4 mL/min remained constant. A linear B gradient up to 100% was maintained for 18.5 min after elution started at 2% B and was maintained for 0.6 min. Elution was again isocratic at 100% B between 18.5 and 25 min. Electrospray ionization in positive mode (ESI+) with collision-induced dissociation (CID) in information-dependent acquisition (IDA) mode was configured for the acquisition of MS/MS mass spectra. Our previous article [32] contains a detailed explanation of the parameters. ACD/Spectrus Processor 2021.1.0 software (ACD/Labs, Toronto, ON, Canada) was used to process the mass spectrometer data. It was proposed to identify the compounds based on their mass spectra, the reported elemental compositions and the results of the searches performed in the MassBank, Lipid Maps, ChemSpider, and ChEBI databases.

2.5. Statistical Analysis

The statistical difference between the DPPH and ORAC assay results obtained for various culture media of each species was expressed using analyses of variance (one-way ANOVA followed by Fisher's least significant difference test) [33]. Statgraphics Centurion-Ver. 16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, USA) was used to conduct the analyses.

3. Results and Discussion

3.1. Extraction Yield and Antioxidant Potential

The ethanolic extraction (390 mg/g DW diatom) resulted in a significantly higher extraction yield ($p < 0.05$) than acetone and hexane (Figure 1). Extraction with ethanol resulted in a 3-fold higher yield than with hexane (120 mg/g DW diatom) and a 13-fold higher yield than with acetone (30 mg/g DW diatom).

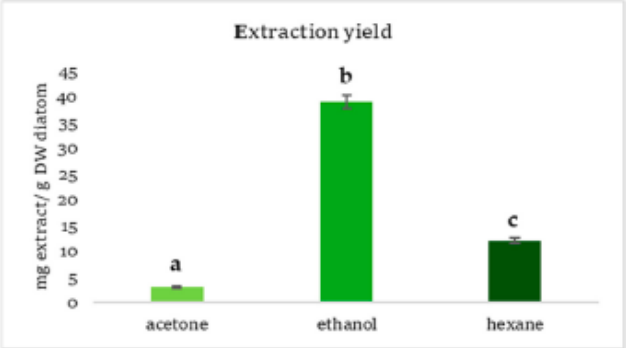

Figure 1. Extraction yield of the diatom *Chaetoceros costatus* extracted with acetone, ethanol and hexane. Different letters indicate significant difference ($p < 0.05$) between the samples ($n = 6$).

The medium polarity and better solubility properties enable higher extraction efficiency compared to acetone and hexane. Due to its amphiphilic nature, ethanol enables the extraction of polar and non-polar compounds such as lipids and carotenoids from *C. calcitrans* species [34]. In a recent comparative study, the extraction yield, antioxidant activity, total phenolic and flavonoid content and cytotoxicity of the marine green microalgae species *Tetraselmis* sp. and *Nannochloropsis* sp. and the diatoms *Chaetoceros* sp. and *Thalassiosira* sp. extracted with different solvents were investigated [9]. In our study, an extraction yield of 39% was achieved, which is 18% higher than in the above-mentioned study.

DPPH and ORAC were used to determine the antioxidant potential (Table 1). The highest percentage of DPPH inhibition was found in the ethanol extract (9.17 ± 0.99% inhibition), while the extract obtained by extraction in acetone gave the highest ORAC value (50.66 ± 2.22 µM TE/L). Due to the chemical properties of hexane (low boiling point), it was not possible to determine the antioxidant potential of the diatom extracts of *C. costatus* using DPPH and ORAC assays.

Table 1. DPPH (% inhibition) and ORAC (µM TE/L) results of acetone, ethanol and hexane extract of *Chaetoceros costatus*.

Solvent	DPPH (% Inhibition)	ORAC (µM TE/L)
Acetone	3.51 ± 0.47^{a}	50.66 ± 2.22^{a}
Ethanol	9.17 ± 0.99^{b}	37.10 ± 3.29^{b}
Hexane	ND	ND

ND—non detected; different letters in the same column indicate significant difference ($p < 0.05$) between the samples. (error type—standard error of the variance; $n = 4$).

Earlier studies reported a higher antioxidant activity of diatoms extracted with alcohols such as methanol [35]. Hemalatha et al. [36] evaluated antioxidant properties and total phenolic content of metanolic, acetone and hexane extracts of three selected marine diatoms, *Chaetoceros curvisetus*, *Thalassiosira subtilis* and *Odontella aurita*. The recorded DPPH inhibition values for acetone extract of *Chaetoceros* sp. did not differ from the values estimated in

this study, although the extraction was not assisted by ultrasound. Hemalatha et al. [36] also reported a statistically significant ($p > 0.05$) difference in DPPH values when different solvents (methanol > acetone > hexane) were used, which was also observed in this study. In the study by Ferdous et al. [9] on *Chaetoceros* sp. species, freeze-dried biomass was extracted with different solvents using ultrasound and the antioxidant properties, total phenolic and flavonoid content and cytotoxic properties of the crude extracts were evaluated. Similarly to the results of our study, Ferdous et al. reported that the DPPH scavenging capacity values of ethanolic extracts of *Chaetoceros* sp. were significantly higher than those of acetone extracts, while hexane extracts gave the lowest value.

3.2. Non-Target Screening of Non-Volatile Compounds in Acetone, Ethanol and Hexane Extract

Acetone, ethanol, and hexane extracts of *C. costatus* were analyzed using ultra-high-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-ESI-HRMS). The extraction efficiency of the target compounds and the overall extraction yield are primarily influenced by the choice of a suitable solvent.

Acetone has proven to be the most effective solvent for the extraction of pigments from diatoms [37]. Besides being able to extract a wide range of pigments, an additional advantage of this solvent is that it limits the hydrolysis of chlorophyll and maximizes yield in a short time, outperforming ethanol and hexane [38]. Due to its ability to extract pigments such as fucoxanthin, chlorophyll C, and diadinoxanthin with minimal degradation, acetone is the preferred extraction solvent for pigments in diatoms [37]. Hexane has proven to be an excellent solvent for fatty acid extraction, especially for polar fractions. However, its efficiency in fatty acid extraction varies greatly depending on the diatom species and the cell disruption method used [29,39]. Due to its non-polar properties, it also enables efficient extraction of lipophilic sterol compounds such as campasterol and stigmasterol [40,41]. Ethanol is particularly effective in extracting bioactive compounds with hydrophilic and lipophilic properties, making it a suitable solvent for microalgae extractions. It has also been shown to be highly effective in the extraction of several classes of compounds from microalgae, such polar lipids and some non-polar compounds such as fatty acids [22]. In this study, acetone extraction also gave the highest pigment yield with a wide range of different pigment classes, while hexane extraction did not give the highest fatty acid and sterol yield. In addition, extraction with acetone and ethanol gave similar yields of fatty acids and their derivatives, while acetone resulted in the highest yield of sterols and derivatives.

All present compounds were identified based on the given elemental composition and MS/MS spectra with confidence level 2 (probable structure) and 3 (possible structure) [42]. In acetone extract, 37 compounds was identified, in ethanolic extract 34, and only 20 compounds in hexane extract. Identified compounds were classified in three groups: pigments and derivatives, fatty acid derivatives, and steroids and derivatives (Table 2). While certain components are more soluble in specific solvents, overall, acetone has the great extraction power among the groups of compounds (Figure 2).

In *C. costatus* extracts were detected a monoterpene lactone (loliolide, no. 1), five xanthophylls and derivatives (no. 2–3, 5–7), two derivatives of chlorophyll *b* (pheophorbide *b* (no. 4), one pheophytine derivative (no. 9), a pheophytin *b* (no. 10), and five derivatives of chlorophyll *a* (pheophorbide *a* (no. 8), divinylpheophytin *a* (no. 11), 151-hydroxy-lactone-pheophytin *a* (no. 12), 13^2-hydroxy-pheophytin *a* (no. 13), and pheophytin a (no. 14)).

Table 2. Major non-volatile compounds in acetone, ethanol and hexane extracts of *Chaetoceros costatus* identified using high-performance liquid chromatography–high-resolution mass spectrometry with electrospray ionization (UHPLC-ESI–HRMS).

No.	Compound	Mass	[M+H]+	Molecular Formula	Peak Area (Arbitrary Unit)		
					Acetone	Ethanol	Hexane
	PIGMENTS AND DERIVATIVES						
1	Loliolide	196.110	197.11722	$C_{11}H_{16}O_3$	4.63×10^6	-	-
2	Apo-10-fucoxanthinal	424.261	425.26864	$C_{27}H_{36}O_4$	2.63×10^4	8.26×10^3	-
3	Halocynthiaxanthin acetate	640.413	641.42005	$C_{42}H_{56}O_5$	2.47×10^6	2.55×10^6	-
4	Pheophorbide *b*	606.248	607.25511	$C_{35}H_{34}N_4O_6$		1.37×10^3	-
5	Fucoxanthin	658.423	659.43062	$C_{42}H_{58}O_6$	5.13×10^6	4.55×10^6	-
6	Diatoxanthin	566.412	567.41966	$C_{40}H_{54}O_2$	5.76×10^5	1.67×10^3	-
7	Fucoxanthinol	616.413	617.42005	$C_{40}H_{56}O_5$	1.30×10^5	5.08×10^3	-
8	Pheophorbide *a*	592.269	593.27585	$C_{35}H_{36}N_4O_5$	8.30×10^6	7.98×10^4	2.67×10^3
9	3-[21-methoxycarbonyl4,8,13,18-tetramethyl-20-oxo-9,14-divinyl-3,4-didehydro-3-24,25-Dihydrophorbinyl]propanoic Acid	588.237	589.24455	$C_{35}H_{32}N_4O_5$	2.13×10^5	1.37×10^5	-
10	Pheophytin *b*	884.545	885.55246	$C_{55}H_{72}N_4O_6$	3.40×10^5	4.45×10^4	-
11	Divinyl pheophytin *a*	868.550	869.55755	$C_{55}H_{72}N_4O_5$	4.58×10^5	3.83×10^4	-
12	15^1-hydroxy-lactonepheophytin *a*	902.556	903.56303	$C_{55}H_{74}N_4O_7$	1.67×10^6	3.06×10^5	1.90×10^3
13	13^2-hydroxy-pheophytin *a*	886.561	887.56811	$C_{55}H_{74}N_4O_6$	1.53×10^6	7.00×10^6	-
14	Pheophytin *a*	870.566	871.5732	$C_{55}H_{74}N_4O_5$	3.58×10^7	7.06×10^5	1.41×10^4
	FATTY ACID DERIVATIVES						
15	Hexadecasphinganine	273.267	274.27406	$C_{16}H_{35}NO_2$	4.51×10^6	2.08×10^5	8.56×10^5
16	Myristamide (tetradecanamide)	227.225	228.23219	$C_{14}H_{29}NO$	4.14×10^4	8.02×10^4	1.11×10^5
17	Monomyristin (2,3-dihydroxypropyl tetradecanoate)	302.246	303.25299	$C_{17}H_{34}O_4$	8.49×10^4	1.67×10^4	1.68×10^4
18	Palmitoleamide (hexadec-9-enamide)	253.241	254.24784	$C_{16}H_{31}NO$	1.06×10^5	2.01×10^5	2.25×10^5
19	Linoleamide (Octadeca-9,12-dienamide)	279.256	280.26349	$C_{18}H_{33}NO$	2.62×10^5	3.73×10^5	4.26×10^5
20	Palmitamide (hexadecanamide)	255.256	256.26349	$C_{16}H_{33}NO$	5.47×10^5	6.57×10^5	9.82×10^5
21	Monopalmitin (2,3-dihydroxypropyl hexadecanoate)	330.277	331.28429	$C_{19}H_{38}O_4$	3.77×10^6	6.49×10^5	7.05×10^5
22	Oleamide (Octadec-9-enamide)	281.272	282.27914	$C_{18}H_{35}NO$	6.56×10^6	7.00×10^6	7.03×10^6
23	Stearamide (octadecanamide)	283.288	284.29479	$C_{18}H_{37}NO$	1.96×10^5	2.58×10^5	1.87×10^5
24	Monostearin (2,3-dihydroxypropyl octadecano-ate	358.308	359.31559	$C_{21}H_{42}O_4$	3.70×10^6	3.51×10^6	3.98×10^6
25	Gondamide (Icos-11-enamide)	309.303	310.31044	$C_{20}H_{39}NO$	1.06×10^5	9.4×10^4	3.4×10^4
26	Arachidonic acid (Icosa-5,8,11,14-tetraenoic acid)	304.240	305.24751	$C_{20}H_{32}O_2$	6.34×10^4	-	-
27	Erucamide (Docos-13-enamide)	337.334	338.34174	$C_{22}H_{43}NO$	2.18×10^6	2.00×10^6	3.12×10^5
28	1-(9-Octadecenoyl)-2-(9-pentadecenoyl)-glycero-3-phosphocholine	743.547	744.55378	$C_{41}H_{78}NO_8P$	3.08×10^4	5.46×10^4	-
29	1-(11,14-Eicosadienoyl)-2-heptadecanoyl-glycero-3-phosphoserine	801.552	802.55926	$C_{43}H_{80}NO_{10}P$	5.82×10^4	2.73×10^4	2.45×10^4
30	1-Octadecanoyl-2-(9,12-heptadecadienoyl)-glycero-3-phosphocholine	771.578	772.58508	$C_{43}H_{82}NO_8P$	4.43×10^4	7.14×10^4	-
31	1-(9-Octadecenoyl)-2-(9-nonadecenoyl)-glycero-3-phosphocholine	799.609	800.61638	$C_{45}H_{86}NO_8P$	2.85×10^5	5.73×10^4	-
32	Dipalmitin	568.507	569.51395	$C_{35}H_{68}O_5$	2.66×10^5	-	-
33	1-Octadecanoyl-2-hexadecanoyl-sn-glycerol	596.538	597.54525	$C_{37}H_{72}O_5$	1.55×10^5	5.11×10^4	8.42×10^3

Table 2. *Cont.*

No.	Compound	Mass	[M+H]+	Molecular Formula	Peak Area (Arbitrary Unit)		
					Acetone	Ethanol	Hexane
			STEROIDS AND DERIVATIVES				
34	Chola-5,22-dien-3-ol	342.292	343.29954	$C_{24}H_{38}O$	9.00×10^4	7.72×10^4	7.76×10^4
35	Campesterol	394.360	395.36723	$C_{29}H_{46}$	1.02×10^3	9.60×10^3	8.80×10^3
36	24-Hydroperoxy-24-vinyl-cholesterol	400.371	401.37779	$C_{28}H_{48}O$	1.96×10^5	-	-
37	(3β)-3-Hydroxystigmast-5-en-7-one	428.365	429.37271	$C_{29}H_{48}O_2$	7.60×10^5	2.30×10^5	7.77×10^3
38	Stigmastatriene	444.360	445.36762	$C_{29}H_{48}O_3$	5.13×10^4	1.58×10^4	-

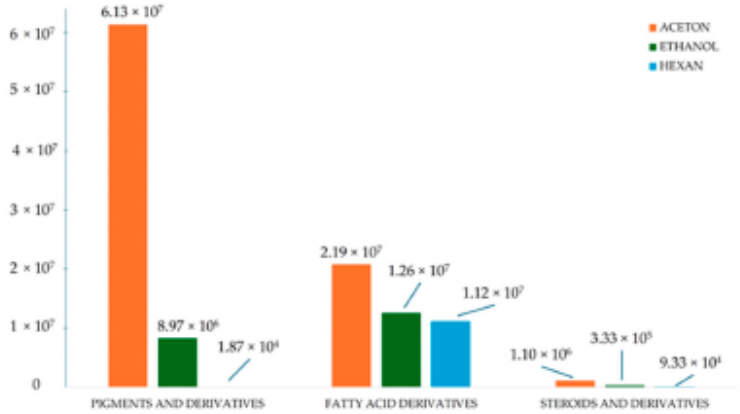

Figure 2. Comparison of the extraction efficiency of various solvents across different groups of components.

In general, extraction with acetone resulted in the highest yield of pigments and derivatives, and the highest content of pheophytin a (no. 14) was found. In contrast to acetone, a pheophorbide *b* (no. 4) was identified in ethanolic extracts of *C. costatus*, while the highest yield was recorded for the compound 13^2-hydroxy-pheophytin *a* (no 13). Hexane enabled the extraction of only three compounds from the group of pigments and derivatives.

Pheophytins and pheophorbides are biologically highly active molecules that have already been reported in *Chaetoceros* species [43,44]. Due to their various biological activities such as anticancer, antimicrobial, and antioxidant effects, they can be used as therapeutic agents in various medical applications [45–48]. They are soluble in various organic solvents, but the solubility of pheophytins from biological samples is better in dietileter, acetone, and chloroform than in ethanol, due to their lipophilic nature [49,50]. Although pheophytins can also be extracted with ethanol, this solvent showed a lower efficiency compared to the previously mentioned organic solvents [51]. The high yield and diverse profiles of diatom pigments offer a wide range of possibilities for industrial use. In addition to their role as colorants, they also have photoprotective and antioxidant properties. For this reason, they are attractive for various purposes, e.g., for the development of functional food supplements, natural colorants in cosmetics, and potential pharmaceutical ingredients.

In the group of fatty acid derivatives, 19 compounds were identified, eight of which were primary fatty amides (PFAAs). Compared to acetone, solubility of PFAAs is better in ethanol. That is because of ability to form hydrogen bonds which enhances solubility of fatty acids [52]. The most abundant compound was oleamide (no. 22) in ethanolic extracts, and PFAA was previously reported in *Skeletonema grevillei* and *Skeletonema marinoi* species [29,53]. Oleamide is known to provide many health benefits, but it is also known

for its biological activity, such as anti-inflammatory, antimicrobial and antialgal [54–56]. In the comparison between the analyzed samples, a C:16 sphingoid bases sphingolipid hexadecasphinganine (no. 15) in acetone extracts was the second compound by abundance. Hexadecasphingosine is a secondary metabolite about which only a few articles have been published to date. The key role of sphingolipids in diatom cells is to modify the properties of the cell membrane, helping to maintain membrane fluidity in response to osmotic stress and temperature [57]. While sphingolipids in higher plants play a crucial role in developmental processes such as pollen development, in diatoms, they are more focused on cell adaptation, while structural diversity is enabled by modifications such as long-chain base hydroxylation and fatty acid variations [58,59]. Sphingolipid structures can generate enzymes such as sphingoid base hydroxylase and ceramide synthase, which enable diatoms to respond rapidly to changing environmental conditions in the marine environment [58,60]. In addition to their role in organizing the cell membrane, they therefore also play a role as signaling molecules, which is why they are extremely important for marine microalgae such as diatoms [58,59].

In the group of sterols and derivatives were identified five compounds (no. 34–38). In all analyzed samples, (3*β*)-3-Hydroxystigmast-5-en-7-one (no. 37) was the most abundant steroid derivative. Two plant sterols, campesterol (no. 34) and stigmastatriene (No. 36), and two sterol derivatives (no. 35, 38) were also identified. In addition to algal and plant sterols, diatoms can also synthesize animal sterols [6,13,61]. Most sterols and their derivatives have antioxidant effects in addition to anticancer and anti-inflammatory properties, which contributes to the greater antioxidant potential of microalgae extracts. The sterols found in diatoms such as campesterol, 24-hyroperoxy-24-vinyl-cholesterol and chola-5,22-dien-3-ol have extraordinary potential for application in a variety of industries such as pharmaceuticals, cosmetics, and food [29]. This potential is based on the previously reported biological activities of sterols such as cytotoxic, antitypanosomal, anti-inflammatory and antimycobacterial effects. For example, (3β)-3-hydroxystigmast-5-en-7-one and stigmastatriene have the potential to be used in the pharmaceutical industry for the development of innovative formulations due to their unique structure [29].

Of the solvents used in this study, hexane has the most negative impact on the environment regarding atmospheric emissions and aquatic toxicity [62]. In contrast to hexane, acetone has a lower level of toxicity and a relatively favorable safety profile. In this study, ethanol is the most suitable option with a minimal environmental footprint, although it has a mixed ecological performance. Despite minimal limitations, ethanol is a suitable solvent, as it is produced renewably and has lower health risks [63]. Another advantage of using ethanol is that it supports the circular economy, which makes it particularly attractive in terms of a sustainable extraction process [63,64]. After the extraction process, the remaining diatom biomass can be used effectively (biofertilizer, nanotechnology, animal feed additive, etc.), which increases the economic and environmental sustainability of the production process.

4. Conclusions

Extraction of diatom *C. costatus* biomass with ethanol gave a significantly higher yield in comparison to acetone and hexane extract. The DPPH assay showed almost three times higher DPPH inhibition, while the ORAC assay showed 27% higher activity of the acetone extract. Compounds from the groups pigments and derivatives, fatty acid derivatives, as well as sterols and derivatives were detected in all analyzed extracts. There was no significant difference in number of detected compounds between the ethanolic and acetonic extracts, while hexane had significantly lower number of compounds. Pheophytin *a* was the most abundant pigment detected in the acetonic extract, while oleamide from the ethanolic

extract showed the highest abundance in the fatty acid derivatives group. In all samples, (3β)-3-hydroxystigmast-5-en-7-one was the most abundant compound in group of steroid derivatives. Due to a significantly higher extraction yield of up to 13 times, ethanol proved to be a much more suitable solvent for diatom extraction. This study contributes to the knowledge on the choice of solvent for efficient extraction of the diatom *C. costatus* in order to maximize the yield of bioactive compounds. Future research should investigate the bioactive potential in more detail with regard to application in the pharmaceutical and food industries.

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References

1. Wang, X.; Ma, S.; Kong, F. Microalgae Biotechnology: Methods and Applications. *Bioengineering* **2024**, *11*, 965. [CrossRef]
2. Vingiani, G.M.; De Luca, P.; Ianora, A.; Dobson, A.D.W.; Lauritano, C. Microalgal Enzymes with Biotechnological Applications. *Mar. Drugs* **2019**, *17*, 459. [CrossRef] [PubMed]
3. Mutanda, T.; Naidoo, D.; Bwapwa, J.K.; Anandraj, A. Biotechnological Applications of Microalgal Oleaginous Compounds: Current Trends on Microalgal Bioprocessing of Products. *Front. Energy Res.* **2020**, *8*, 598803. [CrossRef]
4. Mulluye, K.; Bogale, Y.; Bayle, D.; Atnafu, Y. Review on Microalgae Potential Innovative Biotechnological Applications. *Biosci. Biotechnol. Res. Asia* **2023**, *20*, 35–43. [CrossRef]
5. Bashir, M.; Farooq, M.; Khalid, S.; Ali, Q. The role of microalgae in different biotechnology applications. *Bull. Biol. Allied Sci. Res.* **2022**, *2022*, 25. [CrossRef]
6. Frleta, R.; Popović, M.; Smital, T.; Šimat, V. Comparison of Growth and Chemical Profile of Diatom *Skeletonema grevillei* in Bioreactor and Incubation-Shaking Cabinet in Two Growth Phases. *Mar. Drugs* **2022**, *20*, 697. [CrossRef]
7. Bhattacharjya, R.; Bansal, H.; Santoshi, S.; Rastogi, S.; Tiwari, A. Characterization of natural compounds derived from diatom *Chaetoceros gracilis* as potential therapeutic agents: An in-silico networking and docking study. *Algal Res.* **2024**, *83*, 103712. [CrossRef]
8. Tachihana, S.; Nagao, N.; Katayama, T.; Hirahara, M.; Yusoff, F.M.; Banerjee, S.; Shariff, M.; Kurosawa, N.; Toda, T.; Furuya, K. High Productivity of Eicosapentaenoic Acid and Fucoxanthin by a Marine Diatom *Chaetoceros gracilis* in a Semi-Continuous Culture. *Front. Bioeng. Biotechnol.* **2020**, *8*, 602721. [CrossRef] [PubMed]
9. Ferdous, U.T.; Nurdin, A.; Ismail, S.; Shaari, K.; Norhana Balia Yusof, Z. A comparative study on antioxidant properties, total phenolics, total flavonoid contents, and cytotoxic properties of marine green microalgae and diatoms. *J. Genet. Eng. Biotechnol.* **2025**, *23*, 100456. [CrossRef]

10. Novoveská, L.; Nielsen, S.L.; Eroldoğan, O.T.; Haznedaroglu, B.Z.; Rinkevich, B.; Fazi, S.; Robbens, J.; Vasquez, M.; Einarsson, H. Overview and Challenges of Large-Scale Cultivation of Photosynthetic Microalgae and Cyanobacteria. *Mar. Drugs* **2023**, *21*, 445. [CrossRef] [PubMed]
11. Borowitzka, M.A.; Vonshak, A. Scaling up microalgal cultures to commercial scale. *Eur. J. Phycol.* **2017**, *52*, 407–418. [CrossRef]
12. Rumin, J.; Junior, R.G.d.O.; Bérard, J.-B.; Picot, L. Improving Microalgae Research and Marketing in the European Atlantic Area: Analysis of Major Gaps and Barriers Limiting Sector Development. *Mar. Drugs* **2021**, *19*, 319. [CrossRef] [PubMed]
13. Quitério, E.; Grosso, C.; Ferraz, R.; Delerue-Matos, C.; Soares, C. A Critical Comparison of the Advanced Extraction Techniques Applied to Obtain Health-Promoting Compounds from Seaweeds. *Mar. Drugs* **2022**, *20*, 677. [CrossRef] [PubMed]
14. Ventura, S.P.M.; Nobre, B.P.; Ertekin, F.; Hayes, M.; Garciá-Vaquero, M.; Vieira, F.; Koc, M.; Gouveia, L.; Aires-Barros, M.R.; Palavra, A.M.F. Extraction of value-added compounds from microalgae. In *Microalgae-Based Biofuels and Bioproducts*; Elsevier: Amsterdam, The Netherlands, 2017; pp. 461–483. [CrossRef]
15. Grosso, C.; Valentão, P.; Ferreres, F.; Andrade, P. Alternative and Efficient Extraction Methods for Marine-Derived Compounds. *Mar. Drugs* **2015**, *13*, 3182–3230. [CrossRef] [PubMed]
16. Shahi, T.; Zonouzi, A.; Beheshti, B.; Almasi, M. Comparison of Four Lipid Extraction Methods from *Microalgae dunaliella* sp. for Biodiesel Production. *Iran. J. Chem. Chem. Eng.* **2020**, *39*, 371–378.
17. Onay, M.; Sonmez, C.; Oktem, H.A.; Yucel, M. Evaluation of Various Extraction Techniques for Efficient Lipid Recovery from Thermo-Resistant Microalgae, Hindakia, Scenedesmus and Micractinium Species—Comparison of Lipid Extraction Methods from Microalgae. *Am. J. Anal. Chem.* **2016**, *07*, 141–150. [CrossRef]
18. Chemat, F.; Vian, M.A.; Cravotto, G. Green Extraction of Natural Products: Concept and Principles. *Int. J. Mol. Sci.* **2012**, *13*, 8615–8627. [CrossRef] [PubMed]
19. Chemat, F.; Vian, M.A.; Fabiano-Tixier, A.-S.; Nutrizio, M.; Jambrak, A.R.; Munekata, P.E.S.; Lorenzo, J.M.; Barba, F.J.; Binello, A.; Cravotto, G. A review of sustainable and intensified techniques for extraction of food and natural products. *Green Chem.* **2020**, *22*, 2325–2353. [CrossRef]
20. Louie, K.B.; Kosina, S.M.; Hu, Y.; Otani, H.; de Raad, M.; Kuftin, A.N.; Mouncey, N.J.; Bowen, B.P.; Northen, T.R. Mass Spectrometry for Natural Product Discovery. In *Comprehensive Natural Products III*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 263–306. [CrossRef]
21. Şahin, S.; Kurtulbaş, E. Green Extraction and Valorization of By-Products from Food Processing. *Foods* **2024**, *13*, 1589. [CrossRef] [PubMed]
22. Georgiopoulou, I.; Tzima, S.; Louli, V.; Magoulas, K. Process Optimization of Microwave-Assisted Extraction of Chlorophyll, Carotenoid and Phenolic Compounds from *Chlorella vulgaris* and Comparison with Conventional and Supercritical Fluid Extraction. *Appl. Sci.* **2023**, *13*, 2740. [CrossRef]
23. Foo, S.C.; Yusoff, F.M.; Ismail, M.; Basri, M.; Khong, N.M.H.; Chan, K.W.; Yau, S.K. Efficient solvent extraction of antioxidant-rich extract from a tropical diatom, *Chaetoceros calcitrans* (Paulsen) Takano 1968. *Asian Pac. J. Trop. Biomed.* **2015**, *5*, 834–840. [CrossRef]
24. Reichardt, C.; Welton, T. *; Solvents and Solvent Effects in Organic Chemistry*; Wiley: Hoboken, NJ, USA, 2010. [CrossRef]
25. Resonac Corporation. Polarities of Solvents. Available online: https://www.shodex.com/en/dc/06/0117.html (accessed on 30 December 2024).
26. Morcelli, A.V.; da Silva Andrade, W.; Frankenberg, C.L.C.; Rech, R.; Marcílio, N.R. Extraction of Chlorophylls and Carotenoids from Microalgae: COSMO-SAC-Assisted Solvent Screening. *Chem. Eng. Technol.* **2021**, *44*, 12271232. [CrossRef]
27. Pila, A.N.; Cuello, M.C.; Schmitd, R.M.; Chamorro, E. Microalgae lipid extraction: A novel lab-scale method within a biorefinery approach (fractioning). *Rev. Tecnol. Cienc.* **2022**, *45*, 31–45. [CrossRef]
28. Andersen, R.A. *Algal Culturing Techniques*, 1st ed.; Academic Press: Cambridge, MA, USA, 2004.
29. Matas, R.F.; Popović, M.; Čagalj, M.; Šimat, V. The marine diatom *Thalassiosira rotula*: Chemical profile and antioxidant activity of hydroalcoholic extracts. *Front. Mar. Sci.* **2023**, *10*, 1221417. [CrossRef]
30. Burčul, F.; Mekinić, I.G.; Radan, M.; Rollin, P. Isothiocyanates: Cholinesterase inhibiting, antioxidant, and anti-inflammatory activity. *J. Enzym. Inhib. Med. Chem.* **2018**, *33*, 577–582. [CrossRef] [PubMed]
31. Prior, R.L.; Wu, X.; Schaich, K. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302. [CrossRef] [PubMed]
32. Radman, S.; Cikoš, A.-M.; Babić, S.; Čižmek, L.; Čož-Rakovac, R.; Jokić, S.; Jerković, I. In Vivo and In Vitro Antioxidant Activity of Less Polar Fractions of *Dasycladus vermicularis* (Scopoli) Krasser 1898 and the Chemical Composition of Fractions and Macroalga Volatilome. *Pharmaceuticals* **2022**, *15*, 743. [CrossRef]
33. Šimat, V.; Vlahović, J.; Soldo, B.; Mekinić, I.G.; Čagalj, M.; Hamed, I.; Skroza, D. Production and characterization of crude oils from seafood processing by-products. *Food Biosci.* **2020**, *33*, 100484. [CrossRef]
34. Purkan, P.; Nurlaila, H.; Baktir, A.; Hadi, S.; Soemarjati, W. Microalgal lipid from *Chaetoceros calcitrans* and its conversion to biodiesel through ex and in-situ transesterification. *Rasayan J. Chem.* **2022**, 43–52. [CrossRef]

35. Manivannan, K.; Anantharaman, P.; Balasubramanian, T. Evaluation of antioxidant properties of marine microalga *Chlorella marina* (Butcher, 1952). *Asian Pac. J. Trop. Biomed.* **2012**, *2*, S342–S346. [CrossRef]
36. Hemalatha, A.; Parthiban, C.; Saranya, C.; Girija, K.; Anantharaman, P. Evaluation of antioxidant activities and total phenolic contents of different solvent extracts of selected marine diatoms. *Indian J. Geo-Mar. Sci.* **2015**, *44*, 1630–1636.
37. Pasquet, V.; Chérouvrier, J.-R.; Farhat, F.; Thiéry, V.; Piot, J.-M.; Bérard, J.-B.; Kaas, R.; Serive, B.; Patrice, T.; Cadoret, J.-P.; et al. Study on the microalgal pigments extraction process: Performance of microwave assisted extraction. *Process Biochem.* **2011**, *46*, 59–67. [CrossRef]
38. Warkoyo, W.; Saati, E.A. The solvent effectiveness on extraction process of seaweed pigment. *Makara J. Technol.* **2011**, *15*, 2. [CrossRef]
39. Svenning, J.B.; Dalheim, L.; Vasskog, T.; Matricon, L.; Vang, B.; Olsen, R.L. Lipid yield from the diatom *Porosira glacialis* is determined by solvent choice and number of extractions, independent of cell disruption. *Sci. Rep.* **2020**, *10*, 22229. [CrossRef] [PubMed]
40. Jaramillo-Madrid, A.C.; Ashworth, J.; Ralph, P.J. Levels of Diatom Minor Sterols Respond to Changes in Temperature and Salinity. *J. Mar. Sci. Eng.* **2020**, *8*, 85. [CrossRef]
41. Jaramillo-Madrid, A.C.; Abbriano, R.; Ashworth, J.; Fabris, M.; Pernice, M.; Ralph, P.J. Overexpression of Key Sterol Pathway Enzymes in Two Model Marine Diatoms Alters Sterol Profiles in *Phaeodactylum tricornutum*. *Pharmaceuticals* **2020**, *13*, 481. [CrossRef]
42. Blaženović, I.; Kind, T.; Ji, J.; Fiehn, O. Software Tools and Approaches for Compound Identification of LC-MS/MS Data in Metabolomics. *Metabolites* **2018**, *8*, 31. [CrossRef] [PubMed]
43. Matas, R.F.; Radman, S.; Čagalj, M.; Šimat, V. Influence of Nutrient Deprivation on the Antioxidant Capacity and Chemical Profile of Two Diatoms from Genus *Chaetoceros*. *Mar. Drugs* **2024**, *22*, 96. [CrossRef] [PubMed]
44. Saide, A.; Lauritano, C.; Ianora, A. Pheophorbide a: State of the Art. *Mar. Drugs* **2020**, *18*, 257. [CrossRef] [PubMed]
45. Higashi-Okai, K.; Otani, S.; Okai, Y. Potent suppressive activity of pheophytin a and b from the non-polyphenolic fraction of green tea (*Camellia sinensis*) against tumor promotion in mouse skin. *Cancer Lett.* **1998**, *129*, 223–228. [CrossRef] [PubMed]
46. Gomes, R.A.; Teles, Y.C.F.; Pereira, E.d.O.; Rodrigues, L.A.d.S.; Lima, E.d.O.; Agra, M.d.F.; Souza, M.d.F.V.d. Phytoconstituents from *Sidastrum micranthum* (A. St.-Hil.) Fryxell (*Malvaceae*) and antimicrobial activity of pheophytin a. *Braz. J. Pharm. Sci.* **2015**, *51*, 861–867. [CrossRef]
47. Hsu, C.-Y.; Chao, P.-Y.; Hu, S.-P.; Yang, C.-M. The Antioxidant and Free Radical Scavenging Activities of Chlorophylls and Pheophytins. *Food Nutr. Sci.* **2013**, *04*, 1–8. [CrossRef]
48. Kusmita, L.; Puspitaningrum, I.; Identification, L.L. Isolation and Antioxidant Activity of Pheophytin from Green Tea (*Camellia sinensis* (L.) Kuntze). *Procedia Chem.* **2015**, *14*, 232–238. [CrossRef]
49. Nakazato, M. Method of Producing Water-Soluble Sodium Pheophorbide A. US5378835A, 20 September 1993.
50. Kim, S.B.; Bisson, J.; Friesen, J.B.; Pauli, G.F.; Simmler, C. Selective Chlorophyll Removal Method to 'Degreen' Botanical Extracts. *J. Nat. Prod.* **2020**, *83*, 1846–1858. [CrossRef] [PubMed]
51. Chlorophylls. FAO: Rome, Italy, 1987. Available online: https://www.fao.org/fileadmin/user_upload/jecfa_additives/docs/Monograph1/Additive-128.pdf (accessed on 24 December 2024).
52. Heryanto, R.; Hasan, M.; Abdullah, E.C.; Kumoro, A.C. Solubility of Stearic Acid in Various Organic Solvents and Its Prediction using Non-ideal Solution Models. *Sci. Asia* **2007**, *33*, 469. [CrossRef]
53. Zulfiqar, M.; Stettin, D.; Schmidt, S.; Nikitashina, V.; Pohnert, G.; Steinbeck, C.; Peters, K.; Sorokina, M. Untargeted metabolomics to expand the chemical space of the marine diatom *Skeletonema marinoi*. *Front. Microbiol.* **2023**, *14*, 1295994. [CrossRef]
54. Shao, J.; He, Y.; Li, F.; Zhang, H.; Chen, A.; Luo, S.; Gu, J.-D. Growth inhibition and possible mechanism of oleamide against the toxin-producing cyanobacterium *Microcystis aeruginosa* NIES-843. *Ecotoxicology* **2016**, *25*, 225–233. [CrossRef] [PubMed]
55. Hameed, I.; Altameme, H.; Mohammed, G. Evaluation of Antifungal and Antibacterial Activity and Analysis of Bioactive Phytochemical Compounds of *Cinnamomum zeylanicum* (Cinnamon Bark) using Gas Chromatography-Mass Spectrometry. *Orient. J. Chem.* **2016**, *32*, 1769–1788. [CrossRef]
56. Ameamsri, U.; Chaveerach, A.; Sudmoon, R.; Tanee, T.; Peigneur, S.; Tytgat, J. Oleamide in *Ipomoea* and *Dillenia* Species and Inflammatory Activity Investigated through Ion Channel Inhibition. *Curr. Pharm. Biotechnol.* **2021**, *22*, 254–261. [CrossRef] [PubMed]
57. Li, Y.; Lou, Y.; Mu, T.; Ke, A.; Ran, Z.; Xu, J.; Chen, J.; Zhou, C.; Yan, X.; Xu, Q.; et al. Sphingolipids in marine microalgae: Development and application of a mass spectrometric method for global structural characterization of ceramides and glycosphingolipids in three major phyla. *Anal. Chim. Acta* **2017**, *986*, 82–94. [CrossRef]
58. Liu, N.-J.; Hou, L.-P.; Bao, J.-J.; Wang, L.-J.; Chen, X.-Y. Sphingolipid metabolism, transport, and functions in plants: Recent progress and future perspectives. *Plant Commun.* **2021**, *2*, 100214. [CrossRef] [PubMed]
59. Michaelson, L.V.; Napier, J.A.; Molino, D.; Faure, J.-D. Plant sphingolipids: Their importance in cellular organization and adaption. *Biochim. Biophys. Acta (BBA)—Mol. Cell Biol. Lipids* **2016**, *1861*, 1329–1335. [CrossRef] [PubMed]

60. Haslam, T.M.; Feussner, I. Diversity in sphingolipid metabolism across land plants. *J. Exp. Bot.* **2022**, *73*, 2785–2798. [CrossRef] [PubMed]
61. Fagundes, M.B.; Vendruscolo, R.G.; Wagner, R. Sterols from microalgae. In *Handbook of Microalgae-Based Processes and Products*; Elsevier: Amsterdam, The Netherlands, 2020; pp. 573–596. [CrossRef]
62. Mikkelsen, S.H.; Warming, M.; Cowi; Denmark, A.; Syska, J.; Voskian, A. Survey of n-hexane. Copenhagen. 2014. Available online: www.mst.dk/english (accessed on 30 December 2024).
63. Connelly, A. Going Green—Solvents. Available online: https://andyjconnelly.wordpress.com/2016/11/22/going-green-solvents/ (accessed on 30 December 2024).
64. Carré, P.; Borah, C.D.; Piofczyk, T.; Gärtner, M.; Bothe, S.; Hadjiali, S. Solvent solutions: Comparing extraction methods for edible oils and proteins in a changing regulatory landscape. Part 2: Hazards control. *OCL* **2024**, *31*, 33. [CrossRef]

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The marine diatom *Thalassiosira rotula*: chemical profile and antioxidant activity of hydroalcoholic extracts

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The cosmopolitan centric diatom *Thalassiosira rotula* produces compounds in its natural habitat that can inhibit copepod reproduction. Moreover, it has been reported to possess compounds with therapeutic effects beneficial for health care. In this experiment, the extraction yield, total phenolic content (TPC), antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging ability, ferric reducing/antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC), and chemical profile by gas chromatography-mass spectrometry (GC-MS) analyses of *T. rotula* were investigated. Extractions were performed with 50% and 70% ethanol. A higher extraction yield [0.21 ± 0.01 g extract/g dry weight (DW) diatom] was observed for 70% ethanol. In addition, higher TPC (5.80 ± 0.32 mg gallic acid equivalents (GAE)/g DW diatom) and antioxidant activity [DPPH inhibition of 17.53% ± 0.56%, FRAP of 766.67 ± 34.69 µM Trolox equivalents (TE), and ORAC of 58.87 ± 2.03 µM TE] were observed for this extract. Myristic acid, palmitelaidic acid, palmitic acid, eicosapentaenoic acid, 24-methylenecholesterol, and docosapentaenoic acid were identified as dominant compounds in both extracts, while extraction in 70% ethanol yielded a higher content of fatty acids such as myristic acid, eicosapentaenoic acid, docosapentaenoic acid, and sterol 24-methylenecholesterol. Thus, it can be concluded that extraction of *T. rotula* with 70% ethanol improves antioxidant activity and provides a higher yield of compounds such as polyunsaturated fatty acids and sterols. Therefore, the species *T. rotula* could be considered a sustainable source of essential fatty acids and other bioactive compounds for further applications.

KEYWORDS

diatom, *Thalassiosira rotula*, antioxidant activity, chemical profile, myristic acid, EPA, DPA, 24-methylenecholesterol

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1 Introduction

The number of microalgae species is estimated to be about 300,000, making them a remarkable natural resource for investigations with possible use of the results in various biotechnological applications (Rumin et al., 2020; Duran et al., 2021). The use of marine microalgae to produce valuable compounds is recognized as a renewable and environmentally friendly production system. In general, microalgae are capable of producing nutraceuticals such as pigments (carotenoids), polyunsaturated fatty acids (PUFAs), sterols, phenolic compounds, terpenes, and sulfated polysaccharides (Menaa et al., 2021; Silva et al., 2022). Microalgae and their metabolites have been studied for applications in agriculture, food, pharmaceutical, and cosmetic industries (Borowitzka, 2018; Michalak et al., 2020; Alvarez et al., 2021; Lafarga & Acién, 2022). In the marine environment, diatoms are a predominant group of microalgae responsible for over 40% of the total primary production of the oceans (Field et al., 1998).

With the improvement of microscopy, metabarcoding, analytical analysis, and genetic analysis, research related to the use of diatoms for industrial applications has expanded considerably (Sharma et al., 2021). One of the genera that has been studied for the production of bioactive compounds is *Thalassiosira* (Sabia et al., 2018; Bhattacharjya et al., 2020; Marella & Tiwari, 2020; Baldisserotto et al., 2021). *Thalassiosira* sp. are known as marine centric diatoms with lipid content greater than 50% (Bhattacharjya et al., 2020). Lipids are among the major components of the intracellular content of diatom cells and account for nearly 25% of the dry weight (DW) (Yi et al., 2017). Diatoms contain a wide range of fatty acids, from C14:0 to C22:6, and many strains are considered excellent sources of *n*-3 fatty acids. Eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and docosapentaenoic acid (DPA) are important *n*-3 fatty acids found in microalgae (Li et al., 2019; Bárcenas-Pérez et al., 2021). The use of microalgal oil could reduce the pressure of intensive fishing on stocks and the risks associated with contamination with heavy metals, organic pollutants, and seasonal variations present in fish oil (Alves Martins et al., 2013; Remize et al., 2021). In contrast to the extraction of microalgae oil, the extraction and purification of fish oil (EPA and DHA) are difficult due to the removal of fishy odor and instability caused by the high content of PUFA (Li et al., 2019). However, consumption of these fatty acids provides health benefits in preventing and reducing the risk of various diseases such as coronary heart disease, diabetes, hypertension, inflammatory and autoimmune diseases, and neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and depression (Calder, 2017). In addition to PUFA, diatoms are also known to be a good source of sterols, essential triterpenoids found in all eukaryotes (Jaramillo-Madrid et al., 2020). Sterols are compounds with numerous biological activities and classified as safe compounds for food use by the consensus panel of the European Atherosclerosis Society (Aldini et al., 2014; Ras et al., 2014; Wang & Seibert, 2017; Cutignano et al., 2022). Diatoms are capable of synthesizing animal, plant, and algal sterols (Gallo et al., 2020). One of the most abundant sterols in centric diatoms is 24-methylenecholesterol, an important intermediate in the biosynthesis of phytosterols (Rampen et al., 2010; Gallo et al., 2020; Frleta et al., 2022). Phytosterols have gained popularity in recent decades due to their health-promoting properties (Luo et al., 2015). They have shown antioxidant, anti-inflammatory, and antidiabetic activity, as well as cholesterol-lowering, hepatoprotective, and neuroprotective ability (Sañé et al., 2021; Jie et al., 2022). The extraction protocol for all mentioned compounds and the choice of solvent play a crucial role and directly affect the extract yield as well as the biological activity and chemical profile of the extracts. Hydroalcoholic mixtures are considered a good choice for extraction because they allow the extraction of compounds with a wide range of polarities (Jacotet-Navarro et al., 2018). In addition, the use of "green" solvents such as ethanol for extraction is known to be environmentally friendly, and such extracts are acceptable for use in the food industry. Currently, research efforts are focused on maximizing the extraction of the desired compounds using ultrasound, the less time-consuming, environmentally friendly, and convenient method. A previous study reported higher biological activity of extracts prepared with a hydroalcoholic mixture compared to extracts prepared with water or ethanol alone (Silva et al., 2022). In the literature, the highest yield of bioactive compounds for the hydroalcoholic mixtures is reported in the range of 50%–80% (Jacotet-Navarro et al., 2018; Monteiro et al., 2020; Čagalj et al., 2021). The aim of this study was to 1) compare the differences in extraction yield and antioxidant capacity of the extracts when different hydroalcoholic mixtures were used for the extractions, 2) identify and compare the dominant compounds in *Thalassiosira rotula* extracts with potential for biotechnological applications.

2 Materials and methods

2.1 Cultivation and harvesting

The diatom T. rotula (CIM861) shown in Figure 1 was isolated from fresh seawater sample from the northern Adriatic Sea by the Center for Marine Research of the Ruđer Bošković Institute (Rovinj, Croatia). A standardized purification protocol was used to establish a culture, and the strain was additionally confirmed by molecular identification. The diatom was cultured in six Erlenmeyer flasks in a volume of 5 L. Each flask contained 1.5 L of F/2 medium and was inoculated with 150 mL of the same inoculum (10^5 cells/mL). The F/2 culture medium was prepared according to the previously described recipe (Andersen, 2010). T. rotula was cultured at 17°C and exposed to a 16:8 light:dark cycle with LED light (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italy) of 2,500 lux intensity. Diatom biomass was harvested at the stationary phase by filtration with glass microfiber filters (Grade GF/F Whatman). Biomass collected on the filter was transferred to falcon tubes using a cell scraper. Prior to extraction, samples were freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO, USA).

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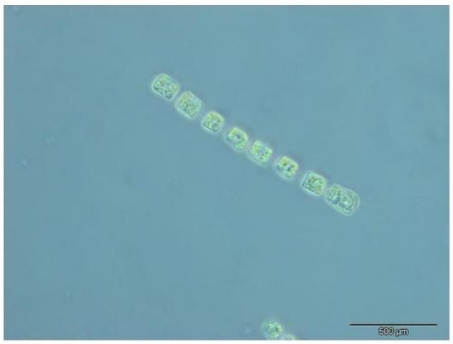

FIGURE 1
Diatom *Thalassiosira rotula* under the light microscope (BX51, Olympus, Japan) at 400× magnification.

2.2 Extraction of diatom biomass

Freeze-dried diatom biomass was extracted in 50% and 70% ethanol by ultrasound-assisted extraction (UAE) using an ultrasonic bath (Transsonic Tp 310H, Elma Schmidbauer GmbH, Singen, Germany) at a frequency of 40 kHz and 40°C for 1 *h*. After extraction, the samples were centrifuged (Centric 150, Tehtnica, Slovenia) for 10 *min* at room temperature and 2,515 × g. The supernatants obtained were filtered through a 0.45-µm mixed cellulose ester filter (LGG, Meckenheim, Germany) and concentrated using a centrifugal evaporator (RC10-22, Jouan, Herblain, France).

2.3 Determination of total phenolic content and antioxidation activity assays

The determination of total phenolic content (TPC) in diatom extracts was performed by the Folin–Ciocalteu method (Amerine and Ough, 1980). Briefly, 1.5 mL of distilled water and 25 µL of *T. rotula* extracts were mixed with 25 µL of Folin–Ciocalteu reagent. After adding the reagent, the mixtures were stirred and left for 1 min. Then, 475 µL of distilled water and 375 µL of 20% sodium carbonate solution were added. Samples were left for 2 h at room temperature in the dark, and absorbance was measured at 765 nm using a spectrophotometer (SPECORD 200 Plus, Edition 2010, Analytik Jena AG, Jena, Germany). Results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g DW diatom).

The antioxidant potential of *T. rotula* extracts was evaluated using ferric reducing/antioxidant power (FRAP), ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, and oxygen radical absorbance capacity (ORAC).

Samples were diluted 1:10 for the FRAP assay. FRAP was used to measure reducing activity (Benzie & Strain, 1996). Briefly, 300 µL of the FRAP reagent solution was added to the microplate wells, and absorbance was measured at 592 nm using a plate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). The change in absorbance was recorded after 4 min following the addition of 10 µL of sample in FRAP reagent. The difference in absorbance between the FRAP reagent before addition of the sample and 4 min after addition was compared to a value determined for the Trolox standard solution.

The ability of diatom extracts to scavenge DPPH radicals was also evaluated in 96-well microplates (Čagalj et al., 2022). A volume of 290 µL of DPPH radical solution with an initial absorbance of 1.2 nm was pipetted into the microplate wells, and measurements were performed at 517 nm. One hour after addition of 10 µL of the *T. rotula* extracts to the wells, the decrease in absorbance was measured using a plate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). The antioxidant activity of diatom extracts was expressed as the percentage of inhibition of DPPH radicals (% inhibition).

The ORAC technique was performed according to previously described procedures (Prior et al., 2003; Burčul et al., 2018). After a 1:10 dilution, 25 µL of the samples were added to wells of a microtiter plate that contained 150 µL of 4.2 mM fluorescein (3',6'-dihydroxyspiro[isobenzofuran-1(3H),9'-[9H] xanthan]-3-one). The plates were thermostatted at 37°C for 30 min, and then 25 µL of 2,2'-Azobis (2-amidinopropane) dihydrochloride, AAPH was added. Measurements were performed at excitation and emission wavelengths of 485 and 520 nm every minute for 80 min. Results were expressed in µM Trolox equivalents (µM TE). All assays were performed in triplicate.

2.4 Chemical analyses

Compounds were identified according to Frleta et al. (2022). Briefly, 50 µL of derivatizing agent (NO-Bis (trimethylsilyl) trifluoroacetamide-BSFA) was added to dry T. rotula extracts. Gas chromatography (GC; Nexis GC-2030, Shimadzu, Kyoto, Japan) equipped with mass spectrometry (MS) detector (Shimadzu QP2020 NX) and split/splitless injection port was used to identify the compounds. Analyses were performed using ultrapure helium as the carrier gas (flow rate = 1 mL/min) and a fused silica capillary column (length 30 *m* × inside diameter 0.25 *mm* i.d., film thickness 0.25 *m*) (SH-5MS, Shimazu, Japan). The mass spectrometer was calibrated using perfluorotributylamine at an electron impact ionization energy of 70 eV. Analyses were performed using a full MS scan (35–750 m/z). The column temperature program included an equilibration time of 3 *min*, the initial temperature of 120°C was held for 3 *min* and then increased to 292°C at a rate of 5°C/min, followed by a final increase to 320°C at a rate of 30°C/min and isothermal holding for 17 *min*. The derivatized compounds were identified by comparing their mass spectra and retention times of trimethylsilyl (TMS) derivatives with commercial databases NIST 2020 and Wiley (Oberacher, 2012; NIST, 2022). All samples were injected and analyzed in duplicate.

10.3389/fmars.2023.1221417

2.5 Statistical analyses

Analyses of variance (one-way ANOVA, followed by Fisher's least significant difference test) were used to express the statistical difference between samples in terms of extract yield, TPC, FRAP, DPPH, and ORAC assays. Analyses were performed using Statgraphics Centurion-Ver.16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, USA).

3 Results and discussion

3.1 Extraction yield

The extraction was performed with hydroalcoholic mixtures to extract compounds with a wide range of polarities. Moreover, these solvents were chosen to obtain higher yields of bioactive compounds and to preserve their activity. The extract yields obtained from *T. rotula* are shown in Figure 2. Extraction with 70% ethanol resulted in a higher yield (0.21 ± 0.01 g extract/g DW diatom) compared to 50% ethanol (0.17 ± 0.01 g extract/g DW diatom).

One of the biggest challenges in reducing production costs is maximizing extraction yields from microalgal biomass (Svenning et al., 2020). In diatom extraction, the choice of solvent and extraction method plays a critical role in solubilizing compounds from diatom frustules (Suroy et al., 2014). Svenning et al. (2020) reported sonication as an effective method for the disruption of diatom cells, while the extraction yield of lipids was highly dependent on the polarity of the solvent. Mixtures of ethanol and water have a synergistic effect in which the water acts as a swelling agent while the ethanol breaks the bonds between the matrix and solutes, which is also evident in this study where a higher extraction yield is reported for the 70% ethanol extraction (Şahin & Şamlı, 2013). In the extraction of two microalgal species, *Nannochloropsis gaditana* and *Chlorella* sp., ethanol-water mixtures resulted in higher extraction yields compared to methanol-water mixtures (Monteiro et al., 2020). In addition, higher extraction yields were observed for both species in UAE with 80% ethanol compared to 50% ethanol.

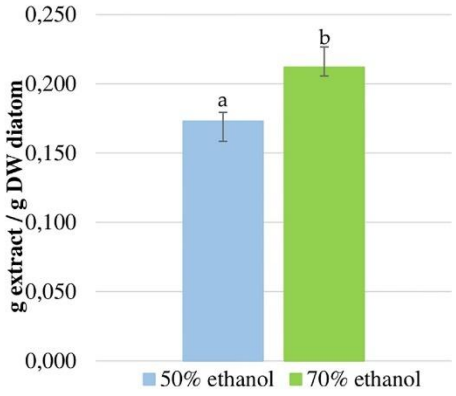

FIGURE 2
The average mass of extract obtained by extraction with 50% and 70% ethanol/g of dry weight of diatom *Thalassiosira rotula*. Different letters indicate statistically significant differences ($p < 0.05$).

3.2 GC-MS compound identification

The identified compounds in derivatized form in ethanolic extracts of the diatom T. rotula are listed in Table 1 with their retention index (RI), similarity, proportion, molecular weight, and mass spectra [relative intensity (m/z)]. The amount of compound in the sample was calculated based on the areas obtained and refers only to the proportion in the injected sample, since the method is not quantitative. All compounds were identified based on matched RI and similarity spectra ≥90% and confirmed according to previous studies (Zulu et al., 2018; Bhattacharjya et al., 2020; Gallo et al., 2020; Frleta et al., 2022).

A total of 16 compounds were identified by GC-MS in the 50% ethanolic extracts, while 19 compounds were identified in the 70% ethanolic extracts. In the 50% ethanolic extract, dominant compounds with a proportion greater than 10% were fatty acids, namely, myristic acid (30.6%), palmitelaidic acid (15.52%), and palmitic acid (10.05%). Compared to the 50% ethanolic extract, the content of myristic acid and palmitelaidic acid in the 70% ethanolic extract was lower by 34% and 8%, respectively, while the content of palmitic acid was higher by 15%. The predominant compounds were the same in both extracts, except for 24-methylenecholesterol, the proportion of which exceeded 10% only in the 70% ethanolic extract. Its amount was almost 20% higher in the 70% ethanolic extract. High levels of two PUFAs, DPA and EPA, were found in both extracts. However, higher efficiency in the extraction of PUFA is obtained by extraction with the higher ethanol content in the solvent. Nearly 10% of DPA and EPA were detected in a 70% ethanol extract, while a 50% ethanol extract contained nearly 30% and 18% less of these PUFAs, respectively. In addition to neophytadiene, which constituted 4.02% in the 70% ethanol extract, linoleic and stearic acids, glycerol, pyroglutamic acid, and uracil were identified, but in lower amounts, while they were not detected in the 50% ethanol extract. On the other hand, no heptadecanoic acid was identified in the 70% extract, which was present in the 50% ethanolic extract in an amount of 0.35%.

In conclusion, six compounds were found to be dominant in both extracts. These are listed in Table 2 along with their chemical formula, subclass in chemical classification, and reported biological activities.

The predominant fatty acids in diatoms are myristic, palmitic, palmitelaidic, and EPA, which is also confirmed in this study (Yi et al., 2017; Zulu et al., 2018). Sabia et al. (2018) reported the predominance of myristic acid, palmitic acid, and palmitoleic acid in *Thalassiosira pseudonana*. Myristic acid, also known as C14:0, is present in all living organisms. This natural bioactive compound could be used as a potential agent against drug-resistant *Candida*

TABLE 1 List of compounds identified in diatom *Thalassiosira rotula* extracted by 50% and 70% ethanol.

No.	Retention index	Similarity (%)		Proportion (%)		Common name of identified compound	Molar weight	Relative intensity (*m/z*)
		50% ethanol	70% ethanol	50% ethanol	70% ethanol			
1	1,267	n.d.	93	n.d.	0.71	Glycerol, 3TMS derivative	308	147(100), 73(98), 205(81), 103(41), 117(36), 218(30), 206(15), 148(15), 133(13), 204(11)
2	1,331	n.d.	93	n.d.	0.14	Uracil, 2TMS derivative	256	241(100), 99(66), 255(56), 256(54), 147(48), 73(46), 113(28), 242(20), 126(16), 44(14)
3	1,526	n.d.	95	n.d.	0.23	Pyroglutamic acid, 2TMS	273	156(100), 73(47), 147(15), 157(13), 258(11), 230(8),75(5),45(5), 158(4), 74(4)
4	1,653	96	96	0.45	0.35	Dodecanoic acid, TMS derivative	272	257(100), 117(92), 73(79), 75(57), 132(43), 129(30), 258(20), 145(19), 131(17), 55(16)
5	1,753	92	92	2.92	3.27	Tridecanoic acid, TMS derivative	286	117(100), 271(89), 73(78), 75(53), 132(47), 129(33), 145(22), 272(18), 131(16), 55(16)
6	1,840	n.d.	94	n.d.	4.02	Neophytadiene	278	95(100), 68(95), 82(83), 57(75), 69(65), 123 (60), 55(56), 43(54), 83(49), 67(47),
7	1,853	95	95	30.60	20.32	Myristic acid, TMS derivative	300	117(100), 285(99), 73(76), 132(52), 75(48), 129(33), 145(25), 286(23), 131(14), 55(14)
8	1,950	90	90	1.25	1.41	Pentadecanoic acid, TMS derivative	214	299(100), 117(97), 73(73), 132(49), 75(45), 129(34), 145(26), 300(23), 55(15), 43(14)
9	2,030	94	94	15.52	12.42	Palmitelaidic acid, TMS derivative	326	311(100), 117(96), 73(88), 75(70), 129(60), 55(40), 145(35), 96(30), 41(24), 98(23)
10	2,054	93	93	10.05	11.57	Palmitic Acid, TMS derivative	328	117(100), 313(77), 73(75), 132(56), 75(44), 129(33), 145(26), 314(18), 43(18), 55(16)
11	2,093	90	n.d.	0.35	n.d.	Heptadecanoic acid, TMS derivative	342	117(100), 327(88), 73(74), 132(55), 75(50), 145(47), 129(44), 201(24), 43(24), 55(22)
12	2,204	91	91	7.15	9.99	Docosapentaenoic acid, TMS derivative	402	79(100), 73(82), 75(72), 93(61), 108(56), 91 (53), 67(48), 105(41), 80(37), 117(35)
13	2,214	n.d.	93	n.d.	1.69	Linoleic acid, TMS derivative	352	73(100), 75(81), 337(74), 67(72), 81(62), 95 (52), 262(48), 55(41), 117(38), 96(36)
14	2,221	92	93	2.58	1.23	Oleic acid, TMS ester	354	73(100), 117(91), 339(84), 75(70), 129(61), 55(42), 145(35), 96(32), 69(26), 67(26),
15	2,248	n.d.	93	n.d.	1.52	Stearic acid, TMS derivative	356	341(100), 117(94), 73(70), 132(59), 75(41), 129(34), 145(32), 342(28), 43(18), 55(16),
16	2,380	94	93	7.68	9.35	Eicosapentaenoic Acid, TMS derivative	374	79(100), 73(87), 91(77), 75(69), 117(67), 93 (58), 67(58),119(53), 106(53), 105(50)
17	2,561	92	95	0.57	1.34	Doconexent, TMS derivative	374	73(100), 79(89), 91(87), 117(65), 67(64), 93 (57), 108(49), 75(49), 105(48), 119(43)
18	2,575	93	94	0.86	0.58	2-Palmitoylglycerol, 2TMS derivative	474	218(100), 129(94), 73(57), 147(46), 103(41), 313(36), 203(32), 191(25), 219(22), 57(21)
19	2,607	94	94	5.86	5.08	1-Monopalmitin, 2TMS derivative	474	371(100), 372(31), 73(23), 239(19), 147(19), 203(15), 129(13), 57(13), 459(10), 43(10)
20	2,767	92	94	1.25	0.95	2-Monostearin, 2TMS derivative	502	129(100), 218(88), 103(65), 73(62), 131(43), 147(40), 341(33), 57(33), 43(30), 203(29)
21	2,800	92	92	4.73	3.80	Glycerol monostearate, 2TMS derivative	502	399(100), 400(28), 73(20), 147(17), 203(13), 129(13), 57(12), 43(10), 487(10), 267(9)
22	3,257	–	–	8.19	10.03	24-methylenecholesterol derivate*	484	129(100), 386(48), 73(44), 296(41), 95(38), 119(37), 69(34), 341(32), 107(32), 81(32)

*The compound was identified using mass spectra from a previous study (Yang et al., 2021; Frleta et al., 2022). The values of the proportions are averages of two replicates with SD values of less than 0.01%. n.d. - not detected.

TABLE 2 The main compounds identified in 50% and 70% ethanol extracts of the diatom *Thalassiosira rotula*.

Common name	Molecular formula	Subclass of compounds	Biological activities	References
Myristic acid	$C_{14}H_{28}O_2$	Straight chain fatty acids	antioxidant, antibacterial, antifungal, immune-modulating, antiviral, antiparasitic	(Liu et al., 2019; Prasath et al., 2019; Hoang et al., 2020; Javid et al., 2020)
Palmitelaidic acid	$C_{16}H_{30}O_2$	Unsaturated fatty acids	antibiofilm, antimicrobial	(Yuyama et al., 2020)
Palmitic acid	$C_{16}H_{32}O_2$	Straight chain fatty acids	anti-inflamatory, antiproliferative, antimetastatic	(Zhu et al., 2021)
Eicosapentaenoic acid	$C_{20}H_{30}O_2$	Unsaturated fatty acids	antioxidant, anticancer, antibacterial, anti-inflamatory	(Sun et al., 2016; Calder, 2017; Bie et al., 2020; Xiao et al., 2022)
24-methylenecholesterol	$C_{29}H_{46}O$	Ergosterols and C24-methyl derivatives	anticancer	(Cutignano et al., 2022)
Docosapentaenoic acid	$C_{22}H_{34}O_2$	Unsaturated fatty acids	anticholesterol, anticardiac	(Calder, 2017; Lozano-Muñoz et al., 2020)

species, while Javid et al. (2020) highlighted the antiparasitic, antiviral, antifungal, and anticancer properties as the main activities of myristic acid (Prasath et al., 2019; Javid et al., 2020). On the other hand, palmitic acid (C16:0) is an important compound from which the biosynthetic pathway of two classical fatty acid families starts: *n*-3 (such as DPA) and *n*-6 (such as EPA) (Nieri et al., 2023). Zhu et al. (2021) reported antiproliferative and antimetastatic activities of palmitic acid against prostate cancer. Palmitelaidic acid is the trans isomer of palmitoleic acid. In a study on the control of biofilm formation of Gram-negative and Gram-positive bacteria by palmitelaidic acid, 25% inhibition of *Escherichia coli* at a concentration of 256 μg mL^{-1} and 21% inhibition of *Staphylococcus aureus* at a concentration of 16 μg mL^{-1} were observed by this unsaturated fatty acid (Yuyama et al., 2020).

Diatoms also contain large amounts of PUFA *n*-3 fatty acids (Maadane et al., 2015). In this study, nearly 20% of EPA and DPA were extracted from *T. rotula* with 70% ethanol. DPA is an intermediate compound between EPA and DHA and has many health benefits, such as lowering the risk of coronary heart disease and plasma cholesterol levels, while EPA is important for neurological development, has an anti-inflammatory effect, and lowers the risk of hypertension, Crohn's disease, and coronary heart disease (Calder, 2017; Lozano-Muñoz et al., 2020). Bie et al. (2020) reported the antitumor activity of EPA on ovarian cells by enhancing the immunomodulatory activity. In oral infections, it showed antibacterial activities against periodontal diseases (Sun et al., 2016).

As a good source of sterols, centric diatoms of the genus *Thalassiosira* are characterized by a dominance of the phytosterol 24-methylenecholesterol (>75%), also known as 24-methycholesta-5,24(28)-dien-3β-ol (Rampen et al., 2010; Gallo et al., 2020; Nieri et al., 2023). In accordance with our results, Jaramillo-Madrid et al. (2020) reported the presence of 24-methylenecholesterol in three *Thalassiosira* species, including *T. rotula*. Cutignano et al. (2022) were the first to report the cytotoxic and antiproliferative activity of 24-methylenecholesterol extracted from *T. rotula* on MCF7 breast cancer and A549 lung cancer cell lines in their study. These results suggest that extraction with 70% ethanol may be suitable for the preparation of an extract with a higher content of this sterol for further biomedical applications.

3.3 Total phenolic content and antioxidation capacity

The TPC of diatom extracts is shown in Figure 3. Due to their different polarities, hydroalcoholic mixtures should be the best choice for the extraction of phenolic compounds (Medina-Torres et al., 2017). *T. rotula* extracted with 70% ethanol had an almost 2-fold higher phenolic content (5.80 ± 0.32 mg GAE/g DW biomass) compared to the extract obtained with 50% ethanol (3.17 ± 0.29 mg GAE/g DW biomass).

When comparing our results with other *Thalassiosira* strains, Syed Ali et al. (Asha Shalini et al., 2019) found a higher amount of TPC in the methanolic extract of *T. weissflogii* (11.34 mg GAE g^{-1} DW). On the other hand, Hemalatha et al. (2015) found more than 10-fold lower TPC for *T. subtilis* methanolic extract compared to our strain. Moreover, authors tested different extracts, methanol, acetone, and hexane, from which methanolic extract showed the

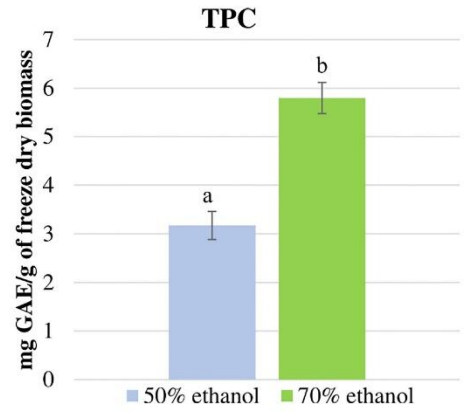

FIGURE 3
Total phenolic content (TPC) in the extracts obtained by extraction with 50% and 70% ethanol/g of freeze-dried diatom *Thalassiosira rotula*. Different letters indicate statistically significant differences ($p < 0.05$).

highest phenolic content. It is evident that the phenolic content varies greatly between species and is also influenced by the choice of solvent.

The full antioxidant potential of *T. rotula* extracts was measured using FRAP, DPPH, and ORAC assays, which are based on different mechanisms of action. DPPH and ORAC are based on hydrogen atom transfer, while FRAP is based on electron transfer (Granato et al., 2018). The results are shown in Figure 4. *T. rotula* extracted at 70% showed higher antioxidant activity in this study, which was measured by three different methods. Using the FRAP assay, almost 3-fold higher reducing activity was observed for the 70% ethanolic extract (766.67 ± 34.69 μM TE). The percentage of inhibition of DPPH radicals was 12-fold lower for the 50% ethanol extract, while the ability of the 70% ethanol extract to scavenge peroxyl radicals was more than 47% higher than that of the 50% ethanol extract.

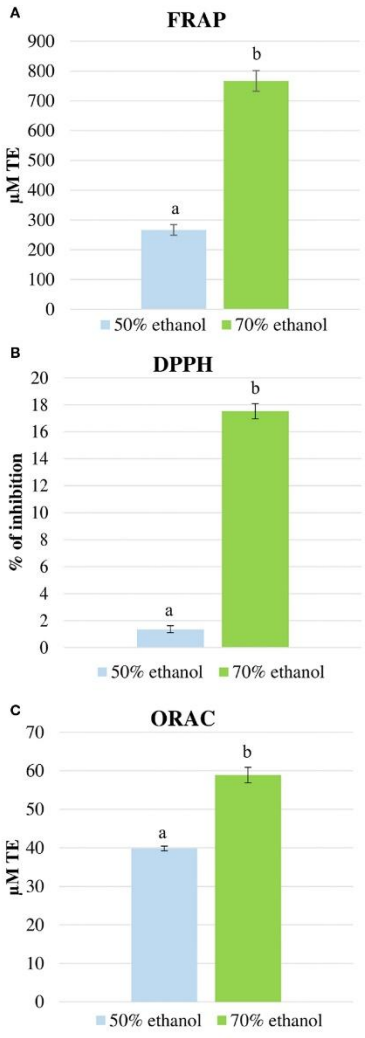

FIGURE 4
Ferric reducing/antioxidant power (FRAP) **(A)**, 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH) **(B)**, and the oxygen radical absorbance capacity (ORAC) **(C)** results for the 50% and 70% ethanolic extract of *Thalassiosira rotula*. Different letters denote statistically significant differences ($p < 0.05$).

The choice of extraction solvent had a significant effect on the variation of DPPH inhibition (Henriques et al., 2007; Hemalatha et al., 2015). Compared to the acetone and hexane extract, Hemalatha et al. (2015) showed the highest DPPH inhibition with the methanol extract of *T. subtilis* (12.51%). The methanol extract showed 46% lower inhibition in their study compared to our results for the 70% ethanol extract of *T. rotula*. Silva et al. (2022) reported almost three times higher percent DPPH inhibition by the ethanolic extract of the diatom *Skeletonema marinoi* compared to the water extract. This is similar to our study, where an increase in antioxidant activity was observed for the extract with a higher percentage of ethanol in the solvent mixture in all assays used.

4 Conclusions

The results confirmed that extraction in 70% ethanol provided a higher extraction yield of diatom *T. rotula*. In addition, the 70% ethanol extract showed higher TPC and antioxidant capacity by three methods. Myristic acid, palmitelaidic acid, palmitic acid, EPA, and DPA were identified as dominant compounds in both extracts. In addition, 24-methylenecholesterol was identified in 70% ethanolic extract in amounts greater than 10%. All of the above compounds have been shown to be biologically active in previous studies, and some compounds such as EPA are already being used commercially as therapeutics and food supplements. Higher yields of commercially important compounds (e.g., EPA), as well as compounds that could be used in the pharmaceutical industry in the future (e.g., 24-methylenecholesterol), were extracted in 70% ethanol. The results confirm that *T. rotula* is a diatom with a wide range of compounds that have exceptional potential. Therefore, future work should focus on studying the biological activities of the identified compounds *in vivo*, clinical trials, and methods to increase diatom production for commercial use.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors without undue reservation.

Author contributions

RF: conceptualization, methodology, formal analysis, investigation, data curation, writing—original draft preparation, and visualization. MP: methodology, validation, formal analysis, data curation, and writing—review and editing. MČ: methodology, formal analysis, data curation, and writing—review and editing. VŠ: conceptualization, methodology, validation, investigation, writing

—review and editing, visualization, and supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1221417/full#supplementary-material

References

Aldini, R., Micucci, M., Cevenini, M., Fato, R., Bergamini, C., Nanni, C., et al. (2014). Antiinflammatory effect of phytosterols in experimental murine colitis model: prevention, induction, remission study. *PLoS One* 9 (9), e108112. doi: 10.1371/journal.pone.0108112

Alvarez, A. L., Weyers, S. L., Goemann, H. M., Peyton, B. M., and Gardner, R. D. (2021). Microalgae, soil and plants: A critical review of microalgae as renewable resources for agriculture. *Algal Res.* 54, 102200. doi: 10.1016/j.algal.2021.102200

Alves Martins, D., Rocha, F., Castanheira, F., Mendes, A., Pousão-Ferreira, P., Bandarra, N., et al. (2013). Effects of dietary arachidonic acid on cortisol production and gene expression in stress response in Senegalese sole (*Solea senegalensis*) post-larvae. *Fish Physiol. Biochem.* 39 (5), 1223–1238. doi: 10.1007/s10695-013-9778-6

Amerine, M. A., and Ough, C. S. (1980). *Methods for Analysis of Musts and Wines* (New York: Wiley).

Andersen, R. A. (2010). *Algal culturing techniques* (Amsterdam: Elsevier, Academic Press).

Asha Shalini, A., Syed Ali, M., Anuradha, V., Yogananth, N., and Bhuvana, P. (2019). GCMS analysis and invitro antibacterial and anti-inflammatory study on methanolic extract of *Thalassiosira weissflogii*. *Biocatal. Agric. Biotechnol.* 8 (7), 1463–1481. doi: 10.1016/j.bcab.2019.101148

Baldisserotto, C., Sabia, A., Guerrini, A., Demaria, S., Maglie, M., Ferroni, L., et al. (2021). Mixotrophic cultivation of *Thalassiosira pseudonana* with pure and crude glycerol: Impact on lipid profile. *Algal Res.* 54, 102194. doi: 10.1016/j.algal.2021.102194

Bárcenas-Pérez, D., Lukeš, M., Hrouzek, P., Kubáč, D., Kopecký, J., Kaštánek, P., et al. (2021). A biorefinery approach to obtain docosahexaenoic acid and docosapentaenoic acid *n*-6 from *Schizochytrium* using high performance countercurrent chromatography. *Algal Res.* 55, 102241. doi: 10.1016/j.algal.2021.102241

Benzie, I. F. F., and Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a measure of "Antioxidant power": the FRAP assay. *Anal. Biochem.* 239 (1), 70–76. doi: 10.1006/abio.1996.0292

Bhattacharjya, R., Marella, T. K., Tiwari, A., Saxena, A., Singh, P. K., and Mishra, B. (2020). Bioprospecting of marine diatoms *Thalassiosira*, *Skeletonema* and *Chaetoceros* for lipids and other value-added products. *Mar. Drugs* 10 (5), 1–10. doi: 10.1016/j.biortech.2020.124073

Bie, N., Han, L., Meng, M., Zhang, Y., Guo, M., and Wang, C. (2020). Anti-tumor mechanism of eicosapentaenoic acid (EPA) on ovarian tumor model by improving the immunomodulatory activity in F344 rats. *J. Funct. Foods* 65, 103739. doi: 10.1016/j.jff.2019.103739

Borowitzka, M. A. (2018). "Microalgae in Medicine and Human Health: A Historical Perspective." in *Microalgae in Health and Disease Prevention* I. A. Levine and J. Fleurence ed. (Kidlington, United Kingdom: Elsevier), 195–210.

Burčul, F., Generalić Mekinić, I., Radan, M., Rollin, P., and Blažević, I. (2018). Isothiocyanates: cholinesterase inhibiting, antioxidant, and anti-inflammatory activity. *J. Enzyme Inhib. Med. Chem.* 33 (1), 577–582. doi: 10.1080/14756366.2018.1442832

Čagalj, M., Skroza, D., Razola-Díaz, M. D. C., Verardo, V., Bassi, D., Frleta, R., et al. (2022). Variations in the Composition, Antioxidant and Antimicrobial Activities of *Cystoseira compressa* during Seasonal Growth. *Mar. Drugs* 20 (64), 1–14. doi: 10.3390/md20010064

Čagalj, M., Skroza, D., Tabanelli, G., Özogul, F., and Šimat, V. (2021). Maximizing the antioxidant capacity of *Padina pavonica* by choosing the right drying and extraction methods. *Processes* 9 (4), 587. doi: 10.3390/pr9040587

Calder, P. C. (2017). Omega-3 fatty acids and inflammatory processes: From molecules to man. *Biochem. Soc Trans.* 45 (5), 1105–1115. doi: 10.1042/BST20160474

Cutignano, A., Conte, M., Tirino, V., Del Vecchio, V., De Angelis, R., Nebbioso, A., et al. (2022). Cytotoxic Potential of the Marine Diatom *Thalassiosira rotula*: Insights into Bioactivity of 24-Methylene Cholesterol. *Mar. Drugs* 20 (10), 595. doi: 10.3390/md20100595

Duran, S. K., Kumar, P., and Sandhu, S. S. (2021). A review on microalgae strains, cultivation, harvesting, biodiesel conversion and engine implementation. *Biofuels* 12 (1), 91–102. doi: 10.1080/17597269.2018.1457314

Field, B.C., Behremdeld, M. J., Randerson, J. T., and Falkowski, P. (1998). Primary production of the biosphere: integrating terrestrial and oceanic components. *Science* 281, 237–240: 5374. doi: 10.1126/science.281.5374.237

Frleta, R., Popović, M., Smital, T., and Šimat, V. (2022). Comparison of growth and chemical profile of diatom *Skeletonema grevillei* in bioreactor and incubation-shaking cabinet in two growth phases. *Mar. Drugs* 20 (11), 697. doi: 10.3390/md20110697

Gallo, C., Landi, S., d'Ippolito, G., Nuzzo, G., Manzo, E., Sardo, A., et al. (2020). Diatoms synthesize sterols by inclusion of animal and fungal genes in the plant pathway. *Sci.Rep* 10 (1), 1–13. doi: 10.1038/s41598-020-60993-5

Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L. D., Hidalgo, F. J., et al. (2018). Antioxidant activity, total phenolics and flavonoids contents: Should we ban *in vitro* screening methods? *Food Chem.* 264, 471–475. doi: 10.1016/j.foodchem.2018.04.012

Hemalatha, A., Parthiban, C., Saranya, C., Girija, K., and Anantharaman, P. (2015). Evaluation of antioxidant activities and total phenolic contents of different solvent extracts of selected marine diatoms. *Indian J. Mar. Sci.* 44 (10), 1630–1636.

Henriques, M., Silva, A., and Rocha, J. (2007). "Extraction and quantification of pigments from a marine microalga: a simple and reproducible method," in *Communicating current research and educational topics and trends in applied microbiology* (FORMATEX), 586–593.

Hoang, L., Beneš, F., Fenclová, M., Kronusová, O., Švarcová, V., Řehořová, K., et al. (2020). Phytochemical composition and *in vitro* biological activity of Iris spp. (iridaceae): A new source of bioactive constituents for the inhibition of oral bacterial biofilms. *Antibiotics* 9 (7), 1–24. doi: 10.3390/antibiotics9070403

Jacotet-Navarro, M., Laguerre, M., Fabiano-Tixier, A. S., Tenon, M., Feuillère, N., Bily, A., et al. (2018). What is the best ethanol-water ratio for the extraction of antioxidants from rosemary? Impact of the solvent on yield, composition, and activity of the extracts. *Electrophoresis* 39 (15), 1946–1956. doi: 10.1002/elps.201700397

Jaramillo-Madrid, A. C., Ashworth, J., and Ralph, P. J. (2020). Levels of diatom minor sterols respond to changes in temperature and salinity. *J. Mar. Sci. Eng.* 8 (2), 85. doi: 10.3390/JMSE8020085

Javid, S., Purohit, M. N., Yogish Kumar, H., Ramya, K., Mithuna, N. F. A., Salahuddin, M. D., et al. (2020). Semisynthesis of myristic acid derivatives and their biological activities: A critical insight. *J. Biol. Act. Prod. Nat.* 10 (6), 455–472. doi: 10.1080/22311866.2020.1865836

Jie, F., Yang, X., Wu, L., Wang, M., and Lu, B. (2022). Linking phytosterols and oxyphytosterols from food to brain health: origins, effects, and underlying mechanisms. *Food Sci. Nutr.* 62 (13), 3613–3630. doi: 10.1080/10408398.2020.1867819

Lafarga, T., and Acién, G. (2022). Microalgae for the food industry: from biomass production to the development of functional foods. *Foods* 11 (5), 765. doi: 10.3390/foods11050765

Levine, I. A., and Fleurence, J. (2018). "Microalgae in medicine and human health: A historical perspective," in *Microalgae in Health and Disease Prevention* (Academic Press), 195–210. doi: 10.1016/B978-0-12-811405-6.00009-8

Li, X., Liu, J., Chen, G., Zhang, J., Wang, C., and Liu, B. (2019). Extraction and purification of eicosapentaenoic acid and docosahexaenoic acid from microalgae: A critical review. *Algal Res.* 43, 101619. doi: 10.1016/j.algal.2019.101619

Liu, C., Yuan, C., Ramaswamy, H. S., Ren, Y., and Ren, X. (2019). Antioxidant capacity and hepatoprotective activity of myristic acid acylated derivative of phloridzin. *Heliyon* 5 (5), e01761. doi: 10.1016/j.heliyon.2019.e01761

Lozano-Muñoz, I., Muñoz, S., Díaz, N. F., Medina, A., Bazaes, J., and Riquelme, C. (2020). Nutritional enhancement of farmed salmon meat via non-GMO *Nannochloropsis gaditana*: Eicosapentaenoic acid (EPA, 20:5 *n*-3), docosapentaenoic acid (DPA, 22:5 *n*-3) and vitamin D3 for human health. *Molecules* 25 (20), 4615. doi: 10.3390/molecules25204615

Luo, X., Su, P., and Zhang, W. (2015). Advances in microalgae-derived phytosterols for functional food and pharmaceutical applications. *Mar. Drugs* 13 (7), 4231–4254. doi: 10.3390/md13074231

Maadane, A., Merghoub, N., Ainane, T., El Arroussi, H., Benhima, R., Amzazi, S., et al. (2015). Antioxidant activity of some Moroccan marine microalgae: Pufa profiles, carotenoids and phenolic content. *J. Biotechnol.* 215, 13–19. doi: 10.1016/j.jbiotec.2015.06.400

Marella, T. K., and Tiwari, A. (2020). Marine diatom *Thalassiosira weissflogii* based biorefinery for co-production of eicosapentaenoic acid and fucoxanthin. *Bioresour. Technol.* 307, 123245. doi: 10.1016/j.biortech.2020.123245

Medina-Torres, N., Ayora-Talavera, T., Espinosa-Andrews, H., Sánchez-Contreras, A., and Pacheco, N. (2017). Ultrasound assisted extraction for the recovery of phenolic compounds from vegetable sources. *Agronomy* 7 (3), 47. doi: 10.3390/agronomy7030047

Menaa, F., Wijesinghe, U., Thiripuranathar, G., Althobaiti, N. A., Albalawi, A. E., Khan, B. A., et al. (2021). Marine algae-derived bioactive compounds: A new wave of nanodrugs? *Mar. Drugs* 19 (9), 1–36. doi: 10.3390/md19090484

Michalak, I., Dmytryk, A., and Chojnacka, K. (2020). "Algae Cosmetics." in *Encyclopedia of Marine Biotechnology* K. Se-Kwon ed. (Pondicherry, India: Wiley), 65–85.

Monteiro, M., Santos, R. A., Iglesias, P., Couto, A., Serra, C. R., Gouvinhas, I., et al. (2020). Effect of extraction method and solvent system on the phenolic content and antioxidant activity of selected macro- and microalgae extracts. *J. Appl. Phycol.* 32 (1), 349–362. doi: 10.1007/s10811-019-01927-1

Nieri, P., Carpi, S., Esposito, R., Costantini, M., and Zupo, V. (2023). Bioactive molecules from marine diatoms and their value for the nutraceutical industry. *Nutrients* 15 (2), 1–23. doi: 10.3390/nu15020464

NIST (2022)NIST chemistry webBook. In: *NIST Standard Reference Database Number 69*. Available at: https://www.nist.gov/ (Accessed 1 May 2023).

Oberacher, H. (2012). *Wiley Registry of Tandem Mass Spectral Data, MS for ID* (New York: Wiley).

Prasath, K. G., Sethupathy, S., and Pandian, S. K. (2019). Proteomic analysis uncovers the modulation of ergosterol, sphingolipid and oxidative stress pathway by myristic acid impeding biofilm and virulence in *Candida albicans*. *J. Proteomics.* 208, 103503. doi: 10.1016/j.jprot.2019.103503

Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., et al. (2003). Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. *J. Agric. Food Chem.* 51 (11), 3273–3279. doi: 10.1021/jf0262256

Rampen, S. W., Abbas, B. A., Schouten, S., and Damsté, J. S. S. (2010). A comprehensive study of sterols in marine diatoms (Bacillariophyta): Implications for their use as tracers for diatom productivity. *Limnol. Oceanogr.* 55 (1), 91–105. doi: 10.4319/lo.2010.55.1.0091

Ras, R. T., Geleijnse, J. M., and Trautwein, E. A. (2014). LDL-cholesterol-lowering effect of plant sterols and stanols across different dose ranges: A meta-analysis of randomised controlled studies. *Br. J. Nutr.* 112 (2), 214–219. doi: 10.1017/S0007114514000750

Remize, M., Brunel, Y., Silva, J. L., Berthon, J. Y., and Filaire, E. (2021). Microalgae *n*-3 PUFAs production and use in food and feed industries. *Mar. Drugs* 19 (2), 1–29. doi: 10.3390/MD19020113

Rumin, J., Nicolau, E., de Oliveira, R. G., Fuentes-Grünewald, C., and Picot, L. (2020). Analysis of scientific research driving microalgae market opportunities in Europe. *Mar. Drugs* 18 (5), 264. doi: 10.3390/md18050264

Şahin, S., and Şamlı, R. (2013). Optimization of olive leaf extract obtained by ultrasound-assisted extraction with response surface methodology. *Ultrason. Sonochem.* 20 (1), 595–602. doi: 10.1016/j.ultsonch.2012.07.029

Sabia, A., Clavero, E., Pancaldi, S., and Salvadó Rovira, J. (2018). Effect of different CO2 concentrations on biomass, pigment content, and lipid production of the marine diatom *Thalassiosira pseudonana*. *Appl. Microbiol. Biotechnol.* 102 (4), 1945–1954. doi: 10.1007/s00253-017-8728-0

Sañé, E., Del Mondo, A., Ambrosino, L., Smerilli, A., Sansone, C., and Brunet, C. (2021). The recent advanced in microalgal phytosterols: bioactive ingredients along with human-health driven potential applications. *Food Rev. Int.* 39 (4), 1859–1878. doi: 10.1080/87559129.2021.1938115

Sharma, N., Simon, D. P., Diaz-Garza, A. M., Fantino, E., Messaabi, A., Meddeb-Mouelhi, F., et al. (2021). Diatoms biotechnology: various industrial applications for a greener tomorrow. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.636613

Silva, M., Kamberovic, F., Uota, S. T., Kovan, I. M., Viegas, C. S. B., Simes, D. C., et al. (2022). Microalgae as potential sources of bioactive compounds for functional foods and pharmaceuticals. *Appl. Sci.* 12 (12), 5877. doi: 10.3390/app12125877

Sun, M., Zhou, Z., Dong, J., Zhang, J., Xia, Y., and Shu, R. (2016). Antibacterial and antibiofilm activities of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) against periodontopathic bacteria. *Microb. Pathog.* 99, 196–203. doi: 10.1016/j.micpath.2016.08.025

Suroy, M., Moriceau, B., Boutorh, J., and Goutx, M. (2014). Fatty acids associated with the frustules of diatoms and their fate during degradation-A case study in *Thalassiosira weissflogii*. *Deep Sea Res.* 86, 21–31. doi: 10.1016/j.dsr.2014.01.001

Svenning, J. B., Dalheim, L., Vasskog, T., Matricon, L., Vang, B., and Olsen, R. L. (2020). Lipid yield from the diatom *Porosira glacialis* is determined by solvent choice and number of extractions, independent of cell disruption. *Sci. Rep.* 10 (1), 1–10. doi: 10.1038/s41598-020-79269-z

Wang, J. K., and Seibert, M. (2017). Prospects for commercial production of diatoms. *Biotechnol. Biofuels* 10 (1), 1–13. doi: 10.1186/s13068-017-0699-y

Xiao, B., Li, Y., Lin, Y., Lin, J., Zhang, L., Wu, D., et al. (2022). Eicosapentaenoic acid (EPA) exhibits antioxidant activity via mitochondrial modulation. *Food Chem.* 373, 131389. doi: 10.1016/j.foodchem.2021.131389

Yang, J., Li, C., and Zhang, Y. (2021). Engineering of *Saccharomyces cerevisiae* for 24-methylene-cholesterol production. *Biomolecules* 11 (11), 1–13. doi: 10.3390/biom11111710

Yi, Z., Xu, M., Di, X., Brynjolfsson, S., and Fu, W. (2017). Exploring valuable lipids in diatoms. *Front. Mar. Sci.* 4. doi: 10.3389/fmars.2017.00017

Yuyama, K. T., Rohde, M., Molinari, G., Stadler, M., and Abraham, W. R. (2020). Unsaturated fatty acids control biofilm formation of *Staphylococcus aureus* and other gram-positive bacteria. *Antibiotics* 9 (11), 1–11. doi: 10.3390/antibiotics9110788

Zhu, S., Jiao, W., Xu, Y., Hou, L., Li, H., Shao, J., et al. (2021). Palmitic acid inhibits prostate cancer cell proliferation and metastasis by suppressing the PI3K/Akt pathway. *Life Sci.* 286, 120046. doi: 10.1016/j.lfs.2021.120046

Zulu, N. N., Zienkiewicz, K., Vollheyde, K., and Feussner, I. (2018). Current trends to comprehend lipid metabolism in diatoms. *Prog. Lipid Res.* 70, 1–16. doi: 10.1016/j.plipres.2018.03.001

8.3. Rad 2. Comparison of Growth and Chemical Profile of Diatom *Skeletonema grevillei* in Bioreactor and Incubation-Shaking Cabinet in Two Growth Phases

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Comparison of Growth and Chemical Profile of Diatom *Skeletonema grevillei* in Bioreactor and Incubation-Shaking Cabinet in Two Growth Phases

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Abstract: Marine microalgae, diatoms, are considered a source of a wide range of high-value compounds, and numerous studies indicate their biotechnological potential in the food and feed industry, cosmetic industry, nanotechnology, pharmaceutical industry, biodiesel production, fertilizers, and wastewater treatment. The aim of this study was to compare the growth, chemical profiles, and antioxidant activity of the diatom *Skeletonema grevillei* cultivated in a bioreactor and an incubation-shaking cabinet at different growth phases (after 192 and 312 h). Growth was monitored by evaluating cell density with the Sedgewick Rafter chamber, and the collected biomass was extracted with 70% ethanol assisted by ultrasound. Extracts were evaporated to dryness and compounds were identified in derivatized form by gas chromatography and mass spectrometry (GC-MS) analysis, while antioxidant capacity was evaluated by DPPH and ORAC. Significantly faster growth was observed in the bioreactor than in the incubation-shaking cabinet. Oleamide, palmitelaidic acid, glycerol monostearate, myristic acid, cholesterol, eicosapentaenoic acid, 1-monopalmitin, and 24-methylene cholesterol were identified as the major compounds in both systems. Among them, oleamide was the dominant compound in both systems. It is also shown that prolonging the cultivation period had a direct effect on increasing the extract yield. The highest DPPH inhibition (11.4 ± 1%) and ORAC values (93.3 ± 8.4 mM TE) were obtained for the *S. grevillei* extract recovered from the bioreactor after 312 h. The obtained results contribute to the possibility of using *S. grevillei* for various biotechnological applications in the future.

Keywords: diatom; bioreactor; incubation-shaking cabinet; derivatized compounds; antioxidant activity

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1. Introduction

Market demand is changing rapidly, directing research toward finding new ways to use marine resources as a renewable and sustainable source of compounds that can be used in a variety of industries. Among the great biodiversity found in the oceans and seas, marine microalgae are recognized as microorganisms with exceptional biotechnological potential, especially in industries directed to production of pharmaceuticals, functional food ingredients, and food products [1]. Although the application of microalgae is mainly related to the food industry, many species have potential for wastewater treatment, biodiesel production, and nutraceuticals [2]. Despite their great potential, the wider application of a larger number of species is still not economically viable due to

numerous challenges, mainly related to technological barriers.

Diatoms are photosynthesizing microalgae with cell walls of transparent opaline silica containing compounds such as pigments, sterols, and fatty acids [3]. Currently, the most commercially important diatom species is *Dunaliella salina*. As the biotechnological potential of diatoms is constantly being discovered, the number of studies on different species is increasing. Diatoms have shown promising features that make them ideal candidates for this form of production, as their intracellular content can be used for the production of biodiesel and valuable food components, and the silicate shell can be used as a material for nanotechnology [4]. In terms of commercial use, the genus *Skeletonema* is an unconventional group of microalgae, but since it is non-toxic, it is suitable for use in the food industry [5]. In fact, the Food and Drug Administration considers *Skeletonema* sp. as generally recognized as safe (GRAS) [6]. Some species such as S. *marinoii* have been described in the literature [7,8]; however, there are no reports on *S. grevillei*. Nevertheless, the cultivation of microalgae for wider use slowed down due to technological obstacles, mainly related to the optimization of growth, harvesting, and extraction efficiency of targeted compounds [9]. In addition to the removing of technological barriers, cultivation should be directed toward the utilization of all production components (bio-based refinery), which would increase the economic profitability and environmental sustainability of the commercial use of diatoms.

Diatoms are among the most flexible and productive eukaryotic microalgae characterized by exceptional adaptability to changing environmental conditions (e.g., temperature and light). Mechanisms such as extracellular polysaccharide layers, cell wall thickness, and morphologically distinct resting stages minimize the potential damage caused by stress conditions [10–12]. On the other hand, their abundance in cultivation under laboratory conditions is much lower than in the natural environment. Therefore, optimization of cultivation and selection of suitable initiators for higher production of metabolites is of utmost importance. Increased production of primary and secondary metabolites is associated with stressful cultivation conditions (e.g., nutrient deficiency, non-optimal temperature, different light sources), while chemical characterization of the intracellular content (primary and secondary metabolites) is quite complex. Promoting metabolite production through nutrient deprivation and introducing other stress growth conditions often result in lower biomass yield, which is a common problem in microalgae production. The main challenges are obtaining a sufficient amount of biomass for biotechnological tests, problems related to the effects of nutrients and environmental changes on biosynthesis, and impurities [4]. Conventional cultivation in incubators typically produces little biomass, and the cost of the production effort exceeds the biomass yield and profitability. In 1997, Fukami et al. [13] developed the first bioreactor to promote biomass production of the diatom *Nitzschia* sp. In general, cultivation in bioreactors achieves greater homogeneity so that all cells have the same availability of key factors for successful growth, such as nutrients and light. While the production of higher amounts of primary metabolites is associated with optimal growth conditions, the accumulation of secondary metabolites is usually associated with stressful conditions [14]. Microalgae are known to produce high-value bioactive compounds such as polyunsaturated fatty acids (PUFA), carotenoids, phenolic compounds, sterols, and sulfated polysaccharides with many health and therapeutic benefits [6,15]. Nevertheless, there is little information on the cultivation potential and chemical profile of the genus *Skeletonema*. Some species such as *S. marinoii*, *S. dohrnii* and *S. costatum* have been previously studied [7,16–18]; however, there are no reports on *S. gravileii*. In a nutrient-deprived environment, *Skeletonema* species are able to increase lipid production [10], and even produce higher amounts of omega-3 eicosapentaenoic acid (EPA) [19]. Under light and temperature stress, these microorganisms produce components that exhibit antioxidant activity, while the selection of a suitable cultivation system can significantly increase biomass yields, i.e., extract yields [20,21]. In addition, metabolic processes in diatoms are extremely fast, and the production of various molecules may also depend on the growth phases [8].

Biotechnological improvements are possible by selecting suitable microalgal strains capable of producing large amounts of various bioactive compounds under optimal conditions. Such a form of microalgae cultivation would make commercialization faster and more profitable. Therefore, the aim of this study was to determine and compare the growth of *S. grevillei* in a standard F/2 medium using two cultivation systems (bioreactor and incubation-shaking cabinet). In addition, ethanolic extracts were prepared for each system at two growth stages and their chemical profiles (dominant compounds) and antioxidant potential were determined.

2. Results and Discussion

2.1. Comparison of Growth Curves in BRC and EIS

Considering that extending the duration of the cultivation period beyond the stationary phase would result in the decay of diatom cells, the end of cultivation in this study was defined as the time at which one of the systems reached maximum growth. This was considered the beginning of the stationary phase and in the bioreactor system (BRC) it was reached after 312 h.

The results of growth in a BRC and the incubation-shaking cabinet (EIS) system during the cultivation period are shown in Figure 1. First, the growth rate in each system over time was examined. After 144 h of cultivation, a statistically significant ($p < 0.05$) change (onset of exponential growth) was observed in the EIS system, while in the BRC system this was observed one day earlier. During the first 144 h, there was no statistically significant difference ($p > 0.05$) in cell number between the EIS and BRC systems. A statistically significant ($p < 0.05$) difference between the systems was first observed after 168 h and persisted until the end of cultivation. The total mass of dry extracts collected in the exponential and at the beginning of the stationary growth phase is shown in Figure 2. The weight of the dry extract was higher in the BRC system at both collection times. The results indicate that the higher homogeneity in the BRC system provided by the impeller and airflow was crucial for the uniform availability of light and nutrients, which consequently led to faster growth of diatoms in this system.

Since the early days of diatom cultivation in bioreactors, it has been known that cell homogeneity allows maximum utilization of limiting nutrients [13]. Although the lack of nutrients often stimulates the metabolism to produce secondary metabolites, these conditions cause disturbances in the growth of diatom cultures. Deficiencies in nutrients such as N and Si negatively affect growth, motility, and often cell morphology, but they are found to be important in stimulating diatom metabolism to produce more valuable metabolites [22–25]. Ramirez et al. [26] compared the growth of two diatoms, *N. epithemioides* and *Nitzschia* sp. at different temperatures, light intensities and silicate levels. The addition of silicate was found to be more suitable to stimulate growth than modification of light and temperature. Raniello et al. [20] recorded greater biomass production of the diatom *Cocconeis neothumensis* under different light and nutrient conditions in a bioreactor than in a batch culture in Petri dishes. Significantly higher extract yield (24 ± 5 mg of dry extract) was obtained in the bioreactor compared to batch cultures (17 ± 3 mg of dry extract), which is comparable to the results of extract yield between BRC and EIS systems in this study [20].

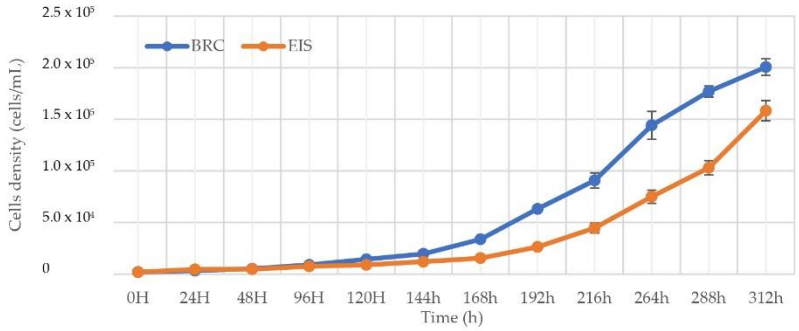


Figure 1. Growth curves for *Skeletonema grevillei* in a bioreactor and incubation-shaking cabinet during cultivation period of 312 h.

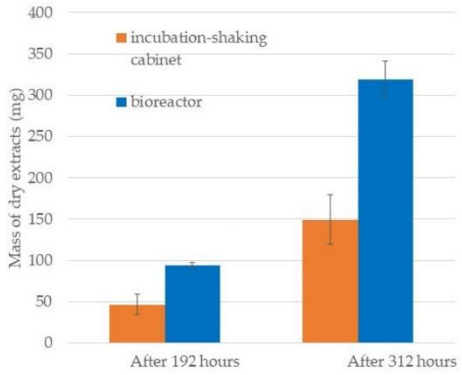


Figure 2. The average mass of dry *Skeletonema grevillei* extracts from bioreactor and incubation-shaking cabinet obtained from total diatom biomass after cultivation periods of 192 and 312 h.

2.2. Identification of Compounds by GC-MS

The compounds identified in a BRC, and EIS are listed in Tables 1 and 2 along with their retention index, percentage of similarity, proportion, and molecular weight. The retention times and MS *m*/*z* of the compounds is given in the Supplementary Material (Table S1). The chromatograms obtained are shown in Figure 3. Since the method is not quantitative, the proportions of the compounds refer only to the injected sample. The amount of each compound in the injected sample volume is expressed as a percentage calculated based on the areas obtained. In Tables 1 and 2, for the compounds identified in derivatized or non-derivatized form, only those with ≥85% similarity spectra and matching RI were considered. In addition, all compounds with similarity less than 90% were confirmed in non-derivatized form. All listed compounds were confirmed according to previous studies on the chemical profiling of disatoms [7,27,28].

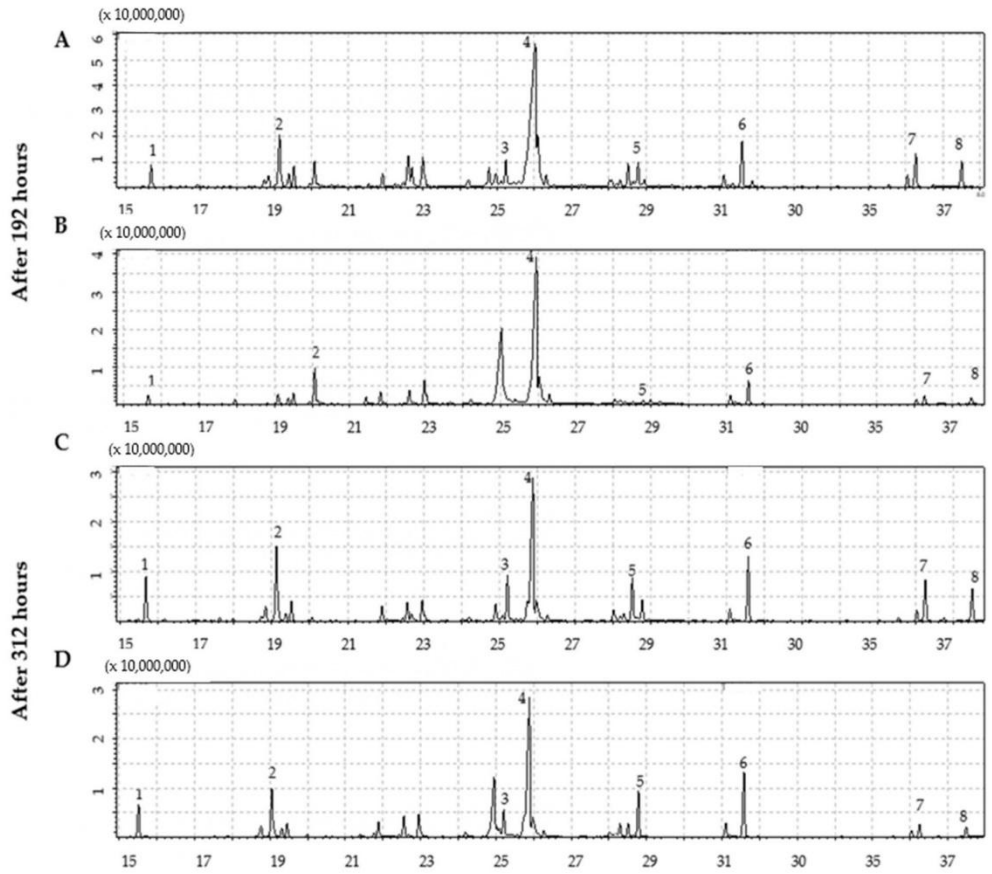


Figure 3. The chromatograms of derivatized dominant compounds in *Skeletonema grevillei* extracts tentatively identified by GC-MS in: (**A**,**C**) bioreactor, (**B**,**D**) incubation-shaking cabinet (**1**—myristic acid, **2**—palmitelaidic acid, **3**—eicosapentaeonic acid, **4**—oleamide or 9- octadecenamide, **5**—1-monopalmitin, **6**—glycerol monostearate, **7**—cholesterol, **8**—24-methylene cholesterol).

Table 1. List of tentatively identified compounds from *Skeletonema grevillei* extracts by GC-MS in bioreactor after 192 and 312 h.

No.	Retention Index		Similarty (%)		Proportion (%)		Identified Compound	Molar Weight
	BRC192	BRC312	BRC192	BRC312	BRC192	BRC312		
1	1242	n.d.	96	n.d.	0.05	n.d.	Benzoic acid, TMS derivative	194
2	1253	n.d.	86	n.d.	0.02	n.d.	Octanoic acid, TMS derivative	216
3	1269	1265	94	92	0.13	0.21	Glycerol, 3TMS derivative	308
4	1348	1345	95	92	0.08	0.05	Nonanoic acid, TMS derivative	230
5	1451	1450	95	91	0.02	0.02	Decanoic acid, TMS derivative	244
6	n.d.	1499	n.d.	94	n.d.	0.13	Malic acid, 3TMS derivative	350
7	1534	n.d.	91	n.d.	0.01	n.d.	1-Dodecanethiol	202
8	1653	1499	97	93	0.05	0.03	Dodecanoic acid, TMS derivative	272
9	1691	n.d.	85	n.d.	0.03	n.d.	Tetradecanenitrile	326
10	1753	1752	94	94	0.10	0.14	Tridecanoic acid, TMS derivative	286

11	1767	n.d.	91	n.d.	0.01	n.d.	1-Tetradecanol, TMS derivative	286
12	1788	1787	93	93	0.06	0.17	Loliolide, TMS	268
13	1853	1852	95	96	2.55	6.50	Myristic acid, TMS derivative	300
14	n.d.	1877	n.d.	90	n.d.	0.28	(*Z*)-3-Hexenyl β -glucopyranoside, 4TMS derivative	550
15	1879	n.d.	94	n.d.	0.11	n.d.	Palmitoleonitrile	235
16	1930	n.d.	86	n.d.	0.05	n.d.	(*E*)-13-Methyltetradec-9-enoic acid, TMS derivative	312
17	1952	1951	94	85	0.17	0.49	Pentadecanoic acid, TMS derivative	314
18	2009	2008	89	88	0.97	0.63	Eicosapentaenoic acid, TMS derivative	374
19	2031	2030	96	97	7.37	12.15	Palmitelaidic acid, TMS	326
20	2053	2052	96	95	2.31	2.73	Palmitic acid, TMS derivative	328
21	2076	n.d.	89	n.d.	0.43	n.d.	9-Octadecynenitrile	261
22	2083	2082	97	97	2.96	0.38	(*Z*)-9-Octadecenenitrile	263
23	2151	2094	92	83	0.04	0.03	Heptadecanoic acid, TMS derivative	342
24	n.d.	2157	n.d.	93	n.d.	0.04	Palmitoleamide	253
25	n.d.	2163	n.d.	87	n.d.	0.01	1-Octadecanol, TMS derivative	342
26	n.d.	2178	n.d.	85	n.d.	0.03	Octadecanamide	319
27	2184	2184	92	97	1.48	2.09	Phytol, TMS derivative	368
28	2216	2215	88	92	0.47	0.44	Linoleic acid, TMS derivate	352
29	2230	2229	94	94	2.36	0.90	(*Z*)-Oleic acid, TMS derivative	354
30	2251	2250	97	95	0.88	0.65	Stearic acid, TMS derivative	356
31	n.d.	2256	n.d.	86	n.d.	0.05	11-Methyloctadec-12-enoic acid, TMS derivative	368
32	n.d.	2331	n.d.	90	n.d.	0.16	(all-*Z*)-5,8,11,14,17-Eicosapentaenoic acid, methyl ester	330
33	2337	n.d.	92	n.d.	0.10	n.d.	(*Z*)-10-Nonadecenoic acid,TMS	368
34	2365	2362	95	96	2.30	2.50	9-Octadecenamide	281
35	n.d.	2373	n.d.	90	n.d.	0.68	(*Z*)-5,8,11-Eicosatrienoic acid, TMS derivative	378
36	2381	2380	94	96	3.29	6.00	Eicosapentaenoic acid, TMS derivative	374
37	2430	2422	83	82	49.84	33.65	Oleamide, TMS derivative	353
38	2450	2448	95	95	1.24	0.70	Steramide, TMS derivative	355
39	2564	2564	97	89	0.54	0.19	Doconexent, TMS derivative	400
40	2578	2578	93	87	1.39	1.09	2-Palmitoylglycerol, 2TMS derivative	474
41	2610	2610	96	96	2.91	2.89	1-Monopalmitin, 2TMS derivative	474
42	2771	2770	90	90	1.46	1.59	2-Monostearin, 2TMS derivative	502
43	2787	2787	93	90	0.34	0.10	1-Monooleoylglycerol, 2TMS derivative	500
44	2805	2804	97	96	5.92	9.30	Glycerol monostearate, 2TMS derivative	502
45	2826	2825	88	81	0.57	0.10	(*Z*)-Docos-13-enamide, TMS	409
46	n.d.	2997	n.d.	89	n.d.	0.15	Eicosanoic acid, 2,3-bis-(OTMS) propyl ester	530
47	2998	n.d.	93	n.d.	0.09	n.d.	2,3-Dihydroxypropyl icosanoate, 2TMS derivative	530
48	3066	3066	89	88	0.09	0.10	2-Arachidonoylglycerol, 2TMS	522

							derivative	
49	3163	3162	96	96	3.97	6.21	Cholesterol, TMS derivative	458
50	n.d.	3201	n.d.	90	n.d.	0.40	Desmosterol, TMS derivative	456
51	3261	3260	90 *	88 *	3.03	5.09	24-Methylene cholesterol *	398 *
52	3380	3380	88	81	0.15	0.20	Isofucosterol, TMS	484
53	3672	3672	90	92	0.06	0.80	Oleanolic acid 2TMS	600

* A compound identified in non-derivatized form and its molecular weight (other data for this compound, RI, and proportion, were obtained for the derivatized form) as well as with literature data comparison for its derivatized form.; n.d.—not detected. BRC192—after 192 h in a bioreactor; BRC312—after 312 h in a bioreactor.

Table 2. List of tentatively identified compounds by GC-MS in incubation-shaking cabinet after 192 and 312 h.

No.	Retention Index		Similarity (%)		Proportion(%)		Identified Compound	Molar Weight
	EIS192	EIS312	EIS192	EIS312	EIS192	EIS312		
1	1335	n.d.	95	n.d.	0.10	n.d.	Nonanoic acid, TMS derivative	230
2	1444	1379	94	92	0.03	0.03	Decanoic acid, TMS derivative	244
3	n.d.	1448	n.d.	75	n.d.	0.01	Butanedioic acid, TMS derivate	350
4	1650	1630	93	95	0.04	0.05	Dodecanoic acid, TMS derivative	272
5	1691	n.d.	93	n.d.	0.02	n.d.	Tetradecanenitrile	209
6	1751	1739	88	93	0.03	0.13	Tridecanoic acid, TMS derivative	286
7	1787	1773	86	92	0.01	0.22	Loliolide, TMS	268
8	1805	1796	88	80	0.05	0.02	Azelaic acid, 2TMS derivative	332
9	n.d.	1831	n.d.	86	n.d.	0.06	Myristoleic acid, trimethylsilyl ester	298
10	1852	1845	95	96	1.59	5.12	Myristic acid, TMS derivative	300
11	1877	n.d.	94	n.d.	0.08	n.d.	Palmitoleonitrile	235
12	1898	n.d.	96	n.d.	0.19	n.d.	Heptadecanenitrile	251
13	n.d.	1956	n.d.	82	n.d.	0.03	Hexadecanenitrile	251
14	1951	1947	93	94	0.10	0.23	Pentadecanoic acid, TMS derivative	314
15	n.d.	1960	n.d.	93	n.d.	0.05	1-Hexadecanol, TMS derivative	314
16	1968	1964	96	95	0.66	0.25	Tetradecanamide	227
17	2031	2026	88	96	1.63	7.89	Palmitelaidic acid, TMS	326
18	2052	2049	96	96	1.65	2.07	Palmitic acid, TMS derivative	328
19	2076	n.d.	89	n.d.	0.43	n.d.	9-Octadecynenitrile	261
20	2082	2078	97	97	6.10	0.37	(Z)-9-Octadecenenitrile	263
21	2151	2149	91	80	0.04	0.02	Heptadecanoic acid, trimethylsilyl ester	342
22	2158	2155	97	96	0.97	0.36	Palmitoleamide	253
23	n.d.	2161	n.d.	86	n.d.	0.05	1-Octadecanol, TMS derivative	342
24	2178	2176	98	97	1.93	0.52	Hexadecanamide	255
25	2184	2183	93	97	0.20	2.06	Phytol, TMS derivative	368
26	n.d.	2213	n.d.	88	n.d.	0.24	Linoleic, TMS derivative	352
27	2251	2250	95	95	0.75	0.53	Stearic acid, TMS derivative	356
28	n.d.	2364	n.d.	96	n.d.	13,97	9-Octadecenamide	281
29	n.d.	2380	n.d.	96	n.d.	3.78	Eicosapentaenoic acid, TMS derivative	374
30	2427	2422	84	82	73.31	35,53	Oleamide, TMS derivative	353
31	2449	2419	95	95	1.37	0.75	Octadecanamide, N-TMS derivate	355
32	2571	n.d.	93	n.d.	0.81	n.d.	13-Docosenamide, (Z)-	337
33	2578	2577	93	94	0.32	1.88	2-Palmitoylglycerol, 2TMS derivative	474
34	2610	2610	94	96	0.47	6.92	1-Monopalmitin, 2TMS derivative	474
35	2771	2771	89	91	1.28	2.20	2-Monostearin, 2TMS derivative	502
36	2804	2804	95	96	3.52	10,64	Glycerol monostearate, 2TMS derivative	502

37	2998	2997	87	80	0.04	0.10	2,3-Dihydroxypropyl icosanoate, 2TMS derivative	530
38	3162	3160	96	96	1.21	1.97	Cholesterol, TMS derivative	458
39	3260	3529	88 *	89 *	1.05	1.59	24-Methylene cholesterol *	398 *
40	n.d.	3379	n.d.	88	n.d.	0.13	Isofucosterol, O-TMS	484
41	n.d.	3672	n.d.	90	n.d.	0.22	Oleanolic acid 2TMS	600

* A compound identified in non-derivatized form and its molecular weight (other data for this compound, RI, and proportion, were obtained for the derivatized form) as well as with literature data comparison for its derivatized form.; n.d.—not detected. EIS192—after 192 h in an incubation-shaking cabinet; EIS312—after 312 h in an incubation-shaking cabinet.

The difference in the number of compounds identified in the BRC system after 192 and 312 h of cultivation was 44 and 42 compounds, respectively. Compared to BRC, a lower number of compounds were identified in both phases of the EIS system, 33 after 192 h and 34 after 312 h of cultivation. Oleamide (also known as 9-octadecenamide or (*Z*)-9-octadecenamide) was dominant in both cultivation systems and at both phases. After 192 h, the percentage of oleamide in BRC and EIS was 49.84% and 73.31%, respectively. In both systems, there was a significant decrease with the extension of the cultivation period, by 16.19% in BRC and 37.78% in EIS.

Compounds with a proportion higher than 5% were palmitelaidic acid, glycerol monostearate, cholesterol, 24-methylenecholesterol, myristic acid, and EPA acid in BRC and glycerol monostearate, palmitelaidic acid, myristic acid, and 1-monopalmitin in EIS. The second most abundant compound in BRC was palmitelaidic acid, while in EIS it was glycerol monostearate. Between the two samplings, the proportion of palmitelaidic acid increased from 7.37% to 12.15% in BRC and from 1.63% to 7.89% in EIS. On the other hand, the proportion of glycerol monostearate increased 3-fold in EIS (from 3.52% to 10.64%) while the increase was lower in BRC at the end of the cultivation period (from 5.92% to 9.30%). To our knowledge, this is the first time it has been identified in marine diatoms. In both systems, myristic acid increased by 3.95% in EIS and 3.53% in BRC, with an additional culture period of 120 h. After 312 h of cultivation, EPA was identified in both systems but the proportion in BRC was higher than in EIS, 6.40% and 3.78%, respectively. In addition, after 192 h, EPA was not detected in the EIS, while in BRC, it was recorded in proportion of 3.29%. The proportion of 1-monopalmitin in BRC remained almost unchanged (from 2.91% to 2.89%), and the same compound showed a sharp increase in EIS (from 0.47% to 6.92%) with the extension of cultivation.

In addition, an increase in tridecanoic acid, loliolide, pentadecanoic acid, palmitic acid, phytol, 2-monostearin, and loliolide was observed in both systems over cultivation time, while the proportion of decanoic acid remained unchanged. At the end of the cultivation period, some newly identified compounds in a proportion of less than 1%, were malic acid, (Z)-3-hexenyl-β-glucopyranoside, palmitoleamide, 1-octadecanol, desmosterol in BRC and butanedioic acid, hexadecanenitrile, 1-hexadecanol in EIS.

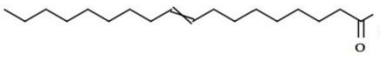
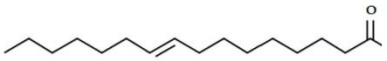
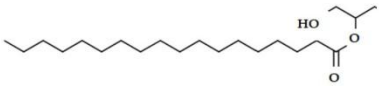
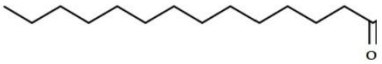
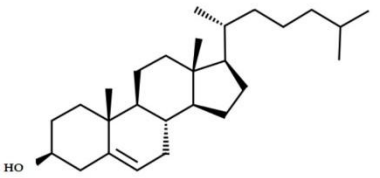
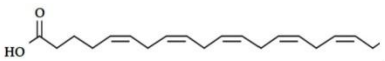
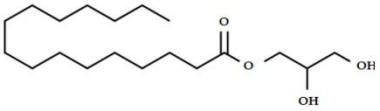
There were eight compounds that were predominant in both systems. These are listed in Table 3, with chemical formula and structure and biological activity reported in recent studies.

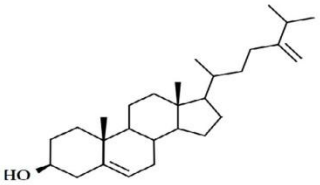
Oleamide is an amide derived from oleic acid. It is found in green algae such as *Chromochloris zofingiensis* and in few terrestrial plants. There are numerous biological activities related to this compound [29–31]. In general, oleamide from green algae is used in the treatment/prevention of arthritis, atherosclerosis, thrombosis, and cancer [32]. It was reported that oleamide from terrestrial plants (*Ipomoea* and *Dillenia* species) excretes anti-inflammatory properties [29]. Furthermore, oleamide from the fungus *Colletotrichum gloeosporioides* has confirmed antimicrobial activity against *Staphylococcus aureus* (zone of inhibition = 25 mm) [33]. The extract of *Diaporthe schini*, in which oleamide was a dominant metabolite, also showed antimicrobial activity against *S. aureus* (MIC = 125 uL/mL) [34]. Furthermore, antifungal, and antimicrobial activity of oleamide from cinnamon bark

was recorded against *Aspergillus flavus* and *Klebsiella pneumonia* [35]. It is also recognized as a potential algicide in the control of cyanobacterial blooms, especially against *Microcystis aeruginosa* NIES -843, a toxin-producing cyanobacterium [30]. Oleamide was found to be the main metabolite among the various bioactive compounds found in the green microalgae *Tetraselmis tetrathela* when cultivated at a salinity of 30 ppm [36].

Fatty acids are known for their antimicrobial and antibiofilm activity. Monounsaturated palmitelaidic acid showed antibiofilm activity against Escherichia coli, inhibiting 25% of biofilm formation at 256 µg/mL, while inhibition of S. aureus was 21% at a concentration of 16 µg/mL [37]. In general, diatoms are known producers of long-chain fatty acids such as omega-3 EPA [38]. EPA is known for its health-beneficial effects, so it is used in dietary supplements and nutraceuticals. During the stationary growth phase of diatoms, the limited amount of nutrients from the culturing media is promoting the production of EPA in the cells [39]. In our study, we reported a higher accumulation of EPA in a BRC during the stationary growth phase, which was likely caused by nutrient deficiency. Moreover, myristic acid was identified as the most abundant fatty acid in *S. grevillei* for both cultivation systems. Marzec et al. [27] also found the myristic acid to be the most abundant fatty acid in the diatom *Halamphora* cf. *salinicola* (strain SZCZM1454A). Myristic acid has been described for a variety of biological activities, including antifungal, antiviral, anticancer, and antiparasitic activities [40].

Table 3. The most dominant compounds identified from bioreactor and incubation-shaking cabinet in *Skeletonema grevillei* extracts.

No.	Compounds	Molecular formula	Structure	Properties
1.	**Oleamide**	$C_{18}H_{35}NO$		Anti-inflammatory, antialgal, antimicrobial and antifungal [30,31,34–36]
2.	**Palmitelaidic acid**	$C_{16}H_{30}O_2$		Antibiofilm activity [37]
3.	**Glycerol monostearate**	$C_{21}H_{42}O_4$		Anthelmintic [41]
4.	**Myristic acid**	$C_{14}H_{28}O_2$		Antifungal, antiviral, anticancer and antiparasitic [40]
5.	**Cholesterol**	$C_{27}H_{46}O$		Anticancer, anticardiac, anti-inflammatory, antimicrobial, anti-psychotic, antioxidative [42]
6.	**Eicosapentaenoic acid**	$C_{20}H_{30}O_2$		Cardioprotective, neuroprotective, anti-inflammatory, anticancer, antimicrobial and antioxidative [43–46]
7.	**1-monopalmitin**	$C_{19}H_{38}O_4$		Antiviral [47]

8.	**24-methylene cholesterol**	$C_{28}H_{46}O$		Anticancer [48]

An important cellular metabolite in triacylglycerol (TAG) biosynthesis is 1-monopalmitin. When TAGs are synthesized more intensively, a decrease in the content of 1-monopalmitin can be observed [49]. In *S. grevillei* we observed the decrease of 1-monopalmitin, probably due to the synthesis of fatty acids such as eicosaonic, EPA, and 5,8,11-eicosatrienoic acids.

Glycerol monostearate is known as the glycerol ester of stearic acid. There is no available literature on this compound from diatoms. So far it has been extracted from plant *Dichapetalum filicaule* and reported for antihelminthic activity against hookworms [41]. In general, glycerol monostearate from terrestrial plants is widely used to extend shelf-life and as an emulsifier in the food industry.

One of the most interesting pieces of information about the metabolic system of diatoms is their ability to synthesize animal and plant sterols [15]. In this study, the results showed a three-fold higher production of sterols in the BRC (12.70%) system compared to the EIS (3.69%) at the end of the cultivation period. The same ratios in sterol production between BRC and EIS can also be seen after 192 h of cultivation. The most abundant animal sterol in diatoms is cholesterol, which has a wide range of biological activities [42,50]. We identified three sterol compounds in derivatized extracts of *S. grevillea*, cholesterol, desmosterol, and isofucosterol. In addition to cholesterol, 24-methylene cholesterol was found among the dominant compounds. The proportion of 24-methylene cholesterol in BRC was 3.44% higher than in EIS at the end of the cultivation period. The mass spectra of the fourth sterol compound, 24-methylenecholesterol in derivatized form (Figure 4) was identified at a retention index of 3259. Since it was not available from the commercial libraries, the compound was confirmed in its non-derivatized form and in alignment with the literature data. Yang et al. [28] have provided the GC-MS spectrum for BSTFA derivatized 24-methylene cholesterol, which is in accordance with our results, while Brooks et al. [51] provided RI for the BSTFA derivatized 24-methylene cholesterol, which is also in alignment with our data.

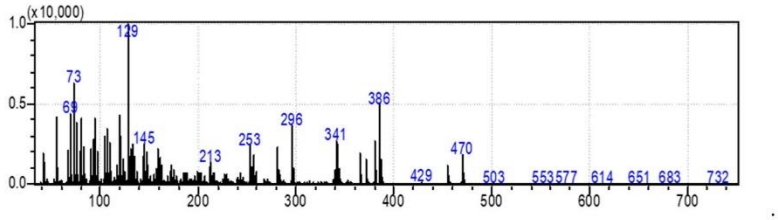


Figure 4. Mass spectra of derivatized sterol compound 24-methylene cholesterol in *Skeletonema grevillei* extracts.

A wide range of biological activities such as anticancer, anticardiac, anti-inflammatory, antimicrobial, anti-psychotic, and antioxidative have been reported for cholesterol [42]. In addition, Cutignano et al. [48] reported the anticancer activity of 24-methylene cholesterol against breast and lung cancer.

These results are consistent with previous studies that characterized the genus *Skeletonema* by a high content of sterols. Sterols with chemotypes of animals (cholesterol and desmosterol), plants (24-methylene cholesterol) and alga (fucosterol) were previously identified in the diatom species *S. marinoi* [7]. Cultivation conditions, especially a decrease in the amount of available nutrients, cause stress to microalgal cells that stimulates the metabolic production of sterols [52]. The modification of the cultivation conditions significantly affects the sterol profile [15]. A similar impact was observed in this study, at the beginning of the stationary phase in BRC when the amount of nutrients was limited higher sterol content was observed. Based on the sterol profile, it was originally hypothesized that diatoms could synthesize sterols by cyclization of lanosterol and cycloartneoids [50,7,53]. However, Gallo et al. [7] confirmed that the *S. marinoi* strain does not possess genes for lanosterol synthetase, but that desmosterol and cholesterol are produced via cycloartenoid pathways [15]. Since *S. marinoi* belongs to the same order as *S. grevillei*, it can be hypothesized that the biosynthesis of sterols occurs in a similar manner.

2.3. Antioxidation Activity Assays

The results of antioxidant activity (DPPH and ORAC) of diatom extracts are shown in Figure 5. At the tested concentration (10 mg dry extract/mL), the DPPH radical inhibition of the extracts from both systems was low. The highest DPPH inhibition (11.4 ± 1%) was observed for the extract from BRC after 312 h. Extracts from the stationary phase showed higher inhibition results for both systems.

In addition to the DPPH method, the ORAC method was chosen because it best simulates in vivo conditions and provides results for antioxidant levels equivalent to those in biological systems [54]. For the ORAC assay, the diatom extracts were diluted 10-fold. The highest ORAC value of 93.3 ± 8.4 mM TE was obtained for the extract from BRC after 312 h. Similar to DPPH inhibition results, ORAC values increased over cultivation period.

Fatty acids and sterols are recognized as compounds with strong antioxidant activity [42,55]. The increase in EPA and cholesterol over the cultivation period might be the reason why the antioxidant activity of *S. grevillei* extracts also increased. A statistically significant difference ($p < 0.05$) in antioxidant activity was detected between cultivation systems at the same growth stage for ORAC, but not for DPPH.

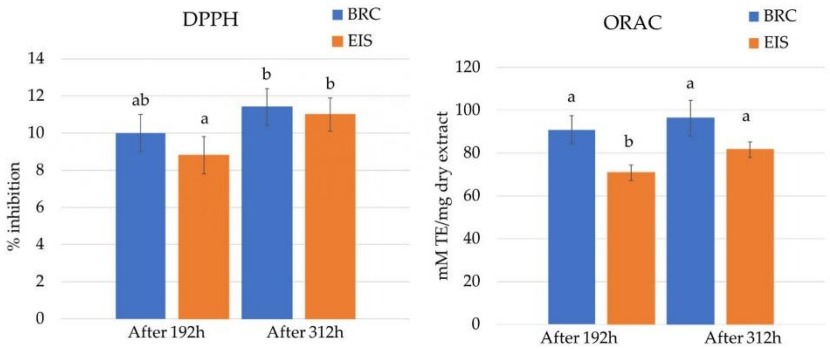


Figure 5. DPPH inhibition (**left**) and ORAC (**right**) results of *Skeletonema grevillei* extracts from bioreactor and incubation-shaking cabinet after 192 and 312h. Different letters denote statistical difference ($p < 0.05$).

Antioxidant activity of aqueous and ethanolic extracts of marine microalgae *Tetraselmis* sp. IMP3, *Tetraselmis* sp. CTP4, and *Skeletonema* sp. was evaluated using the methods ferric ion reducing antioxidant power (FRAP), DPPH, and 2,2′-azino-bis

(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) [56]. The highest antioxidant activity was found with the ABTS method, reaching a reduction of more than 80% in aqueous extracts of *Skeletonema* sp. while the results of ethanolic extracts obtained with the DPPH method were comparable to those obtained in the present study.

3. Materials and Methods

3.1. Chemicals

LiCrosolv Ethanol (gradient grade), Sigma Aldrich (St. Louis, USA) was used for extraction. In GC/MS analysis NO-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma-Aldrich (St. Louis, USA) was used as a derivatization reagent while dichlormethane (Gram-mol, Croatia) was used as a solvent for the non-derivatized sample.

3.2. Experimental Design

The strain of *S. grevillei* (CIM876), isolated from the northern Adriatic Sea, was donated from the culture collection of the Center for Marine Research of the Ruđer Bošković Institute (Rovinj, Croatia). The diatom was cultured in F/2 medium in two parallel systems: (i) in a bioreactor (BRC) (BiostatB Twin, Sartorius, Goettingen, Germany), (ii) in a 5-L Erlenmeyer flask set in an incubation-shaking cabinet (EIS) (Certomar T plus, Sartorius, Goettingen, Germany). Two replicas were used for each system, each containing 2 L of F/2 medium inoculated with 50 mL of *S. gravillei* (9×10^4 cells/mL). The medium was prepared according to the previously described recipe [57].

In both systems, diatoms were cultured at 18 °C with a light–dark photoperiod set to 12 h. The light intensity was 7500 lux in both cultivation systems. In BRC, a LED light (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italy) was used (a combination of cool white and blue), and fluorescent lamps (Fluora T8, Osram, Garching, Germany) were used in EIS. In BRC, the diatoms were stirred with an impeller at 70 rpm and the air flow rate set at 1 L/min, while in EIS they were shaken at a low speed (50 rpm) to avoid spillage.

3.3. The Growth Curve Determination

To compare the growth rate in BRC and EIS systems, 5 mL of the cultures were collected every 24 h of the cultivation period under sterile conditions. The growth curve was determined by counting the cells in a Sedgewick Rafter chamber using a light microscope (Sundew MCXI600, Micros, St. Veit/Glan, Austria) at 200xX magnification [58].

3.4. Collection and Extraction of Diatom Biomass

The biomass was collected at the exponential and at the beginning of stationary growth phases. These were determined at 192 and 312 h of culturing. Using the filtration glass microfiber filters (Grade GF/F Whatman) at 3.12 psi pressure the biomass was collected and transferred to falcon tubes and re-suspended in 10 mL of 70% ethanol. Extraction was performed in duplicate by ultrasound-assisted extraction (UAE) using an ultrasound probe (Sonoplus HD 3200, Bandalin, Berlin, Germany) set at 20 kHz ± 500 Hz. The extraction was performed for 5 min, at an amplitude of 30% and treatments of 20 s. The break between treatments was 40 s. The samples were centrifuged (Lynx 4000 centrifuge, Thermo Scientific, Waltham, Massachusetts, USA) at 29,097× *g*, at 4 °C for 30 min to separate the silicate frustule. The collected supernatant was filtered through a sterile CA filter with a pore diameter of 0.2 μm (LGG, Meckenheim, Germany) and evaporated in a centrifugal evaporator (RC10-22, Jouan, Herblain, France) until completely dry. The mass of the dry extract was recorded.

3.5. Identification of Compounds by GC/MS

The identification of compounds was done according to Torras-Claveria et al. with minor modifications [59]. Briefly, derivatizing agent (BSTFA) (50 μL) was added to the

dry extracts. The identification of the compounds was determined using gas chromatography (GC, Nexis GC-2030, Shimadzu, Kyoto, Japan), coupled with a mass spectrometry (MS) detector (Shimadzu QP2020 NX), equipped with a split/splitless injection port. The analyses were performed using a fused silica capillary column (length 30 m × inside diameter 0.25 mm i.d., film thickness 0.25 m) (SH-5MS, Shimazu, Japan). Ultra-pure helium was used as carrier gas with a 1 mL/min flow rate. Analyses were performed with MS full scan (35–750 m/z). A perfluorotributylamine was used for calibration of the mass spectrometer, at an electron impact ionization energy of 70 eV. The column temperature program was oven equilibration time of 3 min; initial temperature 120 °C for 3 min, increased to 292 °C at a rate of 5 °C /min, then increased to 320 °C at a rate of 30 °C/min and held isothermal for 17 min. Identification of the compounds in derivatized extracts was performed by comparing their trimethylsilyl (TMS) derivative mass spectra and GC retention times with Wiley 12 & NIST 2020 databases [60,61]. All samples were injected and analyzed in duplicate.

Since commercial libraries did not offer adequate mass spectra for the identification of compounds in derivatized forms, the same samples were analyzed in non-derivatized form as well. The analyses were performed according to the same protocol, but 50 μL dichloromethane was used instead of the derivatizing agent. The derivatized and non-derivatized compounds were identified by their retention index and mass spectra using the Wiley 12 & NIST 2020 database.

3.6. Antioxidant Activity of Diatom Extracts

Prior to analyses, dried extracts were dissolved in 70% ethanol at a concentration of 10 mg/mL. Two assays, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and oxygen radical absorbance capacity (ORAC) were used to determine the antioxidant potential of the extracts.

DPPH radical scavenging ability of diatom extracts was measured in 96-well microplates, following the method previously reported by Čagalj et al. [62]. Briefly, DPPH radical solution (290 μL) and prepared sample (10 μL) were pipetted in microplate wells. The absorbance was measured at 517 nm and the initial absorbance of the DPPH solution was set at 1.2. After 1 h, the decrease in the absorbance was measured using the plate reader (Synergy HTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). The antioxidant activity of extracts was expressed as DPPH radical inhibition percentages (% inhibition). ORAC method was performed using previously published protocols [63,64]. The samples were diluted in a 1:10 ratio and 25 μL of the prepared sample and 150 μL of fluorescein were added to each well of the microtiter plate. For 30 min, the plates were thermostated at 37 °C followed by the addition of 25 μL of AAPH. The measurements were recorded every minute over 80 min, at excitation and emission wavelengths of 485 and 520 nm. Results are expressed as μM Trolox equivalents (mM TE). Both assays were done in triplicate.

3.7. Statistical Analyses

Cell density data, collected and recorded using the Sedgewick Rafter method, were used for the analysis. The analyses of variance (one-way ANOVA followed by Fisher's least significant difference test) was used to determine the significant changes ($p < 0.05$) in the growth rate in each system over time and the difference between the EIS and BRC systems, as well as antioxidant activities [65]. Analyses were performed using Statgraphics Centurion-Ver.16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, USA).

4. Conclusions

This study confirms that bioreactor cultivation is a successful system for the cultivation of diatoms. Optimal growth conditions in bioreactors allowed faster entry into the exponential and stationary growth phases. It could be hypothesized that prolongation of

cultivation would result in slightly higher biomass in the bioreactor; however, the prolongation would also result in the death of diatom cells. The results of the chemical profiles revealed that oleamide was most abundant compound in the BRC and EIS systems, and that palmitelaidic acid, glycerol monostearate, myristic acid, cholesterol, eicosapentaenoic acid, 1-monopalmitin and 24-methylene cholesterol were also abundant. All of the mentioned compounds have been previously characterized for their biological activities, which is why some of them are already used in industry, while the other compounds have the potential for biotechnological applications. Moreover, in this study, tentative identification of derivatized 24-methylene cholesterol was obtained, which was not previously found in its derivatized form in the commercial libraries but is known from the literature. For precise identification techniques such HR-MS or NMR should be used. In addition, ethanolic extracts of *S. grevillei* showed low antioxidant activity at the tested concentration, so further studies should focus on the biological activity at higher concentrations and quantitative determination of the biologically active compounds. It should also be emphasized that, in addition to the extracts, silicate diatom frustules are also used in nanotechnology, which will certainly affect the profitability of the production of these microalgae in the future. Future research should focus on finding conditions under which it is possible to maximize the production of metabolites that have the potential for biotechnological applications.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md20110697/s1. Additional information of identified compound such as, retention time and m/z, available in Supplementary Table S1 List of identified compounds from *Skeletonema grevillei* extracts by GC-MS in incubation-shaking cabinet after 312 hours. .

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References

1. Sathasivam, R.; Radhakrishnan, R.; Hashem, A.; Abd_Allah, E.F. Microalgae Metabolites: A Rich Source for Food and Medicine. *Saudi J. Biol. Sci.* **2019**, *26*, 709–722. https://doi.org/10.1016/j.sjbs.2017.11.003.
2. Ummalyma, S.B.; Sirohi, R.; Udayan, A.; Yadav, P.; Raj, A.; Sim, S.J.; Pandey, A. Sustainable Microalgal Biomass Production in Food Industry Wastewater for Low-Cost Biorefinery Products: A Review. *Phytochem. Rev.* **2022**, *3*, 1–23. https://doi.org/10.1007/s11101-022-09814-3.
3. Spaulding, S.A.; Potapova, M.G.; Bishop, I.W.; Lee, S.S.; Gasperak, T.S.; Jovanoska, E.; Furey, P.C.; Edlund, M.B. Diatoms.Org: Supporting Taxonomists, Connecting Communities. *Diatom Res.* **2021**, *36*, 291–304. https://doi.org/10.1080/0269249X.2021.2006790.
4. Stonik, V.S.; Stonik, I. Low-Molecular-Weight Metabolites from Diatoms: Structures, Biological Roles and Biosynthesis. *Mar. Drugs* **2015**, *13*, 3672–3709. https://doi.org/10.3390/md13063672.

5. Benavente-Valdés, J.R.; Méndez-Zavala, A.; Hernández-López, I.; Carreón-González, B.A.; Velázquez-Arellano, M.E.; Morales-Oyervides, L.; Montañez-Saénz, J.C. Unconventional Microalgae Species and Potential for Their Use in the Food Industry. *Cultured Microalgae for the Food Industry*; Current and Potential Applications; Academic Press:Cambridge, MA, USA, 2021; pp. 49–71. https://doi.org/10.1016/B978-0-12-821080-2.00010-1.
6. Silva, M.; Kamberovic, F.; Uota, S.T.; Kovan, I.M.; Viegas, C.S.B.; Simes, D.C.; Gangadhar, K.N.; Varela, J.; Barreira, L. Microalgae as Potential Sources of Bioactive Compounds for Functional Foods and Pharmaceuticals. *Appl. Sci.* **2022**, *12*, 5877. https://doi.org/10.3390/app12125877.
7. Gallo, C.; Landi, S.; D'Ippolito, G.; Nuzzo, G.; Manzo, E.; Sardo, A.; Fontana, A. Diatoms Synthesize Sterols by Inclusion of Animal and Fungal Genes in the Plant Pathway. *Sci. Rep.* **2020**, *10*, 4204. https://doi.org/10.1038/s41598-020-60993-5.
8. Vidoudez, C.; Pohnert, G. Comparative Metabolomics of the Diatom *Skeletonema Marinoi* in Different Growth Phases. *Metabolomics* **2012**, *8*, 654–669. https://doi.org/10.1007/s11306-011-0356-6.
9. Gonzalez-Fernandez, C.; Muñoz, R. *Microalgae-Based Biofuels and Bioproducts: From Feedstock Cultivation to End-Products*; Woodhead Publishing Series in Energy; Woodhead Publishing: Sawston, UK, 2017.
10. Gao, G.; Wu, M.; Fu, Q.; Li, X.; Xu, J. A Two-Stage Model with Nitrogen and Silicon Limitation Enhances Lipid Productivity and Biodiesel Features of the Marine Bloom-Forming Diatom *Skeletonema Costatum*. *Bioresour. Technol.* **2019**, *289*, 121717. https://doi.org/10.1016/j.biortech.2019.121717.
11. Yuan, W.; Gao, G.; Shi, Q.; Xu, Z.; Wu, H. Combined Effects of Ocean Acidification and Warming on Physiological Response of the Diatom *Thalassiosira Pseudonana* to Light Challenges. *Mar. Environ. Res.* **2018**, *135*, 63–69. https://doi.org/10.1016/j.marenvres.2018.01.016.
12. Souffreau, C.; Vanormelingen, P.; Verleyen, E.; Sabbe, K.; Vyverman, W. Tolerance of Benthic Diatoms from Temperate Aquatic and Terrestrial Habitats to Experimental Desiccation and Temperature Stress. *Phycologia* **2010**, *49*, 309–324. https://doi.org/10.2216/09-30.1.
13. Fukami, K.; Nishimura, S.; Ogusa, M.; Asada, M.; Nishijima, T. Continuous Culture with Deep Seawater of a Benthic Food Diatom *Nitzschia* Sp. *Hydrobiologia* **1997**, *358*, 245–249. https://doi.org/10.1023/A:1003141104693.
14. Vuppaladadiyam, A.K.; Prinsen, P.; Raheem, A.; Luque, R.; Zhao, M. Microalgae Cultivation and Metabolites Production: A Comprehensive Review. *Biofuels Bioprod. Biorefining* **2018**, *12*, 304–324. https://doi.org/10.1002/bbb.1864.
15. Fagundes, M.B.; Vendruscolo, R.G.; Wagner, R. *Sterols from Microalgae*; Elsevier Inc.: Amsterdam, The Netherlands, 2020. https://doi.org/10.1016/B978-0-12-818536-0.00021-X.
16. Iglesias, S.; Míguez, C.; Sánchez, A.; Cancela, A.; Álvarez, X. *Thalassiosira Pseudonana* and *Skeletonema Costatum* Biomass Optimization: Cultivation, Harvesting, Extraction of Oils and Biodiesel and Pelletization of the Residue. *J. Sea Res.* **2022**, *187*, 102243. https://doi.org/10.1016/j.seares.2022.102243.
17. Sharmin, T.; Monirul Hasan, C.M.; Aftabuddin, S.; Rahman, M.A.; Khan, M. Growth, Fatty Acid, and Lipid Composition of Marine Microalgae *Skeletonema Costatum* Available in Bangladesh Coast: Consideration as Biodiesel Feedstock. *J. Mar. Biol.* **2016**, *2016*, 6832847. https://doi.org/10.1155/2016/6832847.
18. Thangaraj, S.; Sun, J. The Biotechnological Potential of the Marine Diatom *Skeletonema Dohrnii* to the Elevated Temperature and PCO2. *Mar. Drugs* **2020**, *18*, 259. https://doi.org/10.3390/md18050259.
19. Jiang, X.; Han, Q.; Gao, X.; Gao, G. Conditions Optimising on the Yield of Biomass, Total Lipid, and Valuable Fatty Acids in Two Strains of *Skeletonema Menzelii*. *Food Chem.* **2016**, *194*, 723–732. https://doi.org/10.1016/j.foodchem.2015.08.073.
20. Raniello, R.; Iannicelli, M.M.; Nappo, M.; Avila, C.; Zupo, V. Production of *Cocconeis Neothumensis* (Bacillariophyceae) Biomass in Batch Cultures and Bioreactors for Biotechnological Applications: Light and Nutrient Requirements. *J. Appl. Phycol.* **2007**, *19*, 383–391. https://doi.org/10.1007/s10811-006-9145-4.
21. Ingebrigtsen, R.A.; Hansen, E.; Andersen, J.H.; Eilertsen, H.C. Light and Temperature Effects on Bioactivity in Diatoms. *J. Appl. Phycol.* **2016**, *28*, 939–950. https://doi.org/10.1007/s10811-015-0631-4.
22. Bondoc, K.G.V.; Heuschele, J.; Gillard, J.; Vyverman, W.; Pohnert, G. Selective Silicate-Directed Motility in Diatoms. *Nat. Commun.* **2016**, *7*, 10540. https://doi.org/10.1038/ncomms10540.
23. Leynaert, A.; Fardel, C.; Beker, B.; Soler, C.; Delebecq, G.; Lemercier, A.; Pondaven, P.; Durand, P.E.; Heggarty, K. Diatom Frustules Nanostructure in Pelagic and Benthic Environments. *Silicon* **2018**, *10*, 2701–2709. https://doi.org/10.1007/s12633-018-9809-0.
24. Liu, Y.; Song, X.; Cao, X.; Yu, Z. Responses of Photosynthetic Characters of *Skeletonema Costatum* to Different Nutrient Conditions. *J. Plankton Res.* **2013**, *35*, 165–176. https://doi.org/10.1093/plankt/fbs080.
25. Gao, G.; Shi, Q.; Xu, Z.; Xu, J.; Campbell, D.A.; Wu, H. Global Warming Interacts with Ocean Acidification to Alter PSII Function and Protection in the Diatom *Thalassiosira Weissflogii*. *Environ. Exp. Bot.* **2018**, *147*, 95–103. https://doi.org/10.1016/j.envexpbot.2017.11.014.
26. Ramirez, E.E.; Gonzales, M.A.; Cifuentes, A.S.; Inostroza, I.; Urrutia, R.E. Culture and Growth of Two Benthic Diatoms Species Isolated from the Salar Del Huasco (North of Chile, 20° S) at Different Conditions of Temperature, Light and Nutrient. *Gayana Bot.* **2015**, *72*, 165–176. https://doi.org/10.4067/S0717-66432015000200001.
27. Marzec, M.; Dąbek, P.; Witkowski, A.; Monedeiro, F.; Pomastowski, P.; Buszewski, B.; Nowak, I. Lipid Constituents of Diatoms (*Halamphora*) as Components for Production of Lipid Nanoparticles. *Pharmaceutics* **2022**, *14*, 1171. https://doi.org/10.3390/pharmaceutics14061171.
28. Yang, J.; Li, C.; Zhang, Y. Engineering of *Saccharomyces Cerevisiae* for 24-Methylene-Cholesterol Production. *Biomolecules* **2021**, *11*, 1710. https://doi.org/10.3390/biom11111710.

29. Ameamsri, U.; Chaveerach, A.; Sudmoon, R.; Tanee, T.; Peigneur, S.; Tytgat, J. Oleamide in *Ipomoea* and *Dillenia* Species and Inflammatory Activity Investigated through Ion Channel Inhibition. *Curr. Pharm. Biotechnol.* **2020**, *22*, 254–261. https://doi.org/10.2174/1389201021666200607185250.
30. Shao, J.; He, Y.; Li, F.; Zhang, H.; Chen, A.; Luo, S.; Gu, J.D. Growth Inhibition and Possible Mechanism of Oleamide against the Toxin-Producing Cyanobacterium *Microcystis Aeruginosa* NIES-843. *Ecotoxicology* **2016**, *25*, 225–233. https://doi.org/10.1007/s10646-015-1582-x.
31. Leitner, P.D.; Jakschitz, T.; Gstir, R.; Stuppner, S.; Perkams, S.; Kruus, M.; Trockenbacher, A.; Griesbeck, C.; Bonn, G.K.; Huber, L.A.; et al. Anti-Inflammatory Extract from Soil Algae *Chromochloris Zofingiensis* Targeting TNFR/NF-KB Signaling at Different Levels. *Cells* **2022**, *11*, 1407. https://doi.org/10.3390/cells11091407.
32. Moon, S.M.; Lee, S.A.; Hong, J.H.; Kim, J.S.; Kim, D.K.; Kim, C.S. Oleamide Suppresses Inflammatory Responses in LPS-Induced RAW264.7 Murine Macrophages and Alleviates Paw Edema in a Carrageenan-Induced Inflammatory Rat Model. *Int. Immunopharmacol.* **2018**, *56*, 179–185. https://doi.org/10.1016/j.intimp.2018.01.032.
33. Premjanu, N.; Jaynthy, C. Identification and Characterization of Antimicrobial Metabolite from an Endophytic Fungus, Colletotrichum Gloeosporioides Isolated from *Lannea Corammendalica*. *Int. J. ChemTech Res.* **2015**, *7*, 369–374.
34. Dos Reis, C.M.; da Rosa, B.V.; da Rosa, G.P.; do Carmo, G.; Morandini, L.M.B.; Ugalde, G.A.; Kuhn, K.R.; Morel, A.F.; Jahn, S.L.; Kuhn, R.C. Antifungal and Antibacterial Activity of Extracts Produced from *Diaporthe Schini*. *J. Biotechnol.* **2019**, *294*, 30–37. https://doi.org/10.1016/j.jbiotec.2019.01.022.
35. Hameed, I.H.; Altameme, H.J.; Mohammed, G.J. Evaluation of Antifungal and Antibacterial Activity and Analysis of Bioactive Phytochemical Compounds of *Cinnamomum Zeylanicum* (Cinnamon Bark) Using Gas Chromatography-Mass Spectrometry. *Orient. J. Chem.* **2016**, *32*, 1769–1788. https://doi.org/10.13005/ojc/320406.
36. El-Kassas, H.Y.; El-Sheekh, M.M. Induction of the Synthesis of Bioactive Compounds of the Marine Alga *Tetraselmis Tetrathele* (West) Butcher Grown under Salinity Stress. *Egypt. J. Aquat. Res.* **2016**, *42*, 385–391. https://doi.org/10.1016/j.ejar.2016.10.006.
37. Yuyama, K.T.; Rohde, M.; Molinari, G.; Stadler, M.; Abraham, W.R. Unsaturated Fatty Acids Control Biofilm Formation of *Staphylococcus Aureus* and Other Gram-Positive Bacteria. *Antibiotics* **2020**, *9*, 788. https://doi.org/10.3390/antibiotics9110788.
38. Yang, R.; Wei, D.; Xie, J. Diatoms as Cell Factories for High-Value Products: Chrysolaminarin, Eicosapentaenoic Acid, and Fucoxanthin. *Crit. Rev. Biotechnol.* **2020**, *40*, 993–1009. https://doi.org/10.1080/07388551.2020.1805402.
39. Zulu, N.N.; Zienkiewicz, K.; Vollheyde, K.; Feussner, I. Current Trends to Comprehend Lipid Metabolism in Diatoms. *Prog. Lipid Res.* **2018**, *70*, 1–16. https://doi.org/10.1016/j.plipres.2018.03.001.
40. Javid, S.; Purohit, M.N.; Yogish Kumar, H.; Ramya, K.; Mithuna, N.F.A.; Salahuddin, M.D.; Prashantha Kumar, B.R. Semisynthesis of Myristic Acid Derivatives and Their Biological Activities: A Critical Insight. *J. Biol. Act. Prod. Nat.* **2020**, *10*, 455–472. https://doi.org/10.1080/22311866.2020.1865836.
41. Chama, M.A.; Dziwornu, G.A.; Waibel, R.; Osei-Safo, D.; Addae-Mensah, I.; Otchere, J.; Wilson, M. Isolation, Characterization, and Anthelminthic Activities of a Novel Dichapetalin and Other Constituents of *Dichapetalum Filicaule*. *Pharm. Biol.* **2016**, *54*, 1179–1188. https://doi.org/10.3109/13880209.2015.1059861.
42. Zhang, K.; Li, T.; Shan, X.; Lu, R.; Zhang, S.; Xu, H. Cholesterol: Bioactivities, Structural Modification, Mechanisms of Action, and Structure-Activity Relationships. *Mini-Rev. Med. Chem.* **2021**, *21*, 1830–1848. https://doi.org/10.2174/1389557521666210105123320.
43. Ceccarelli, V.; Ronchetti, S.; Marchetti, M.C.; Calvitti, M.; Riccardi, C.; Grignani, F.; Vecchini, A. Molecular Mechanisms Underlying Eicosapentaenoic Acid Inhibition of HDAC1 and DNMT Expression and Activity in Carcinoma Cells. *Biochim. Biophys. Acta—Gene Regul. Mech.* **2020**, *1863*, 194481. https://doi.org/10.1016/j.bbagrm.2020.194481.
44. Bie, N.; Han, L.; Meng, M.; Zhang, Y.; Guo, M.; Wang, C. Anti-Tumor Mechanism of Eicosapentaenoic Acid (EPA) on Ovarian Tumor Model by Improving the Immunomodulatory Activity in F344 Rats. *J. Funct. Foods* **2020**, *65*, 103739. https://doi.org/10.1016/j.jff.2019.103739.
45. Ferreira, M.; Teixeira, C.; Abreu, H.; Silva, J.; Costas, B.; Kiron, V.; Valente, L.M.P. Nutritional Value, Antimicrobial and Antioxidant Activities of Micro- and Macroalgae, Single or Blended, Unravel Their Potential Use for Aquafeeds. *J. Appl. Phycol.* **2021**, *33*, 3507–3518. https://doi.org/10.1007/s10811-021-02549-2.
46. Ribeiro-Vidal, H.; Sánchez, M.C.; Alonso-Español, A.; Figuero, E.; Ciudad, M.J.; Collado, L.; Herrera, D.; Sanz, M. Antimicrobial Activity of Epa and Dha against Oral Pathogenic Bacteria Using an in Vitro Multi-Species Subgingival Biofilm Model. *Nutrients*, **2020**, *12*, 2812. https://doi.org/10.3390/nu12092812.
47. Cheng, J.-C.; Liaw, C.-C.; Lin, M.-K.; Chen, C.-J.; Chao, C.-L.; Chao, C.-H.; Kuo, Y.-H.; Chiu, Y.-P.; Peng, Y.-S.; Hui-Chi, H. Anti-Influenza Virus Activity and Chemical. *Molecules* **2020**, *25*, 4427.
48. Cutignano, A.; Conte, M.; Tirino, V.; del Vecchio, V.; de Angelis, R.; Nebbioso, A.; Altucci, L.; Romano, G. Cytotoxic Potential of the Marine Diatom Thalassiosira Rotula: Insights into Bioactivity of 24-Methylene Cholesterol. *Mar. Drugs* **2022**, *20*, 595. https://doi.org/10.3390/md20100595.
49. Shi, Y.; Chen, Z.; Li, Y.; Cao, X.; Yang, L.; Xu, Y.; Li, Z.; He, N. Function of ORFC of the Polyketide Synthase Gene Cluster on Fatty Acid Accumulation in *Schizochytrium Limacinum* SR21. *Biotechnol. Biofuels*, **2021**, *14*, 163. https://doi.org/10.1186/s13068-021-02014-9.
50. Rampen, S.W.; Abbas, B.A.; Schouten, S.; Damsté, J.S.S. A Comprehensive Study of Sterols in Marine Diatoms (Bacillariophyta): Implications for Their Use as Tracers for Diatom Productivity. *Limnol. Oceanogr.* **2010**, *55*, 91–105. https://doi.org/10.4319/lo.2010.55.1.0091.
51. Brooks, C.J.W.; Cole, W.J.; McIntyre, H.B.; Smith, A.G. Selective Reactions in the Analysis and Characterization of Steroids by Gas Chromatography-Mass Spectrometry. *Lipids* **1980**, *15*, 745–755. https://doi.org/10.1007/BF02534028.

52. Kumari, P.; Kumar, M.; Reddy, C.R.K.; Jha, B. *Algal Lipids, Fatty Acids and Sterols*; Woodhead Publishing Ltd.: Sawston, UK, 2013. https://doi.org/10.1533/9780857098689.1.87.
53. Barrett, S.M.; Volkman, J.K.; Dunstan, G.A.; LeRoi, J.-M. Sterols of 14 Species of Marine Diatoms (Bacillariophyta). *J. Phycol.* **1995**, *31*, 360–369. https://doi.org/10.1111/j.0022-3646.1995.00360.x.
54. Bank, B.G.; Schauss, A. Antioxidant Testing : An ORAC Update. *Nutraceuticals World* **2004**, *7*, 68–71.
55. Xiao, B.; Li, Y.; Lin, Y.; Lin, J.; Zhang, L.; Wu, D.; Zeng, J.; Li, J.; Liu, J.w.; Li, G. Eicosapentaenoic Acid (EPA) Exhibits Antioxidant Activity via Mitochondrial Modulation. *Food Chem.* **2022**, *373*, 131389. https://doi.org/10.1016/j.foodchem.2021.131389.
56. Cardoso, C.; Pereira, H.; Franca, J.; Matos, J.; Monteiro, I.; Pousão-Ferreira, P.; Gomes, A.; Barreira, L.; Varela, J.; Neng, N.; et al. Lipid Composition and Some Bioactivities of 3 Newly Isolated Microalgae (*Tetraselmis* Sp. IMP3, *Tetraselmis* Sp. CTP4, and *Skeletonema* Sp.). *Aquac. Int.* **2020**, *28*, 711–727. https://doi.org/10.1007/s10499-019-00489-w.
57. Andersen, R.A. *Algal Culturing Techniques*; Elsevier Inc.: Amsterdam, The Netherlands, 2010.
58. Karlson, B.; Caroline, C.; Bresnan, E. *Microscopic and Molecular Methods for Quantitative Phytoplankton Analysis*; United Nations Educational, Scientific and Cultural Organization (UNESCO): Paris, France, 2010.
59. Torras-Claveria, L.; Berkov, S.; Jáuregui, O.; Caujapé, J.; Viladomat, F.; Codina, C.; Bastida, J. Metabolic Profiling of Bioactive Pancratium Canariense Extracts by GC-MS. *Phytochem. Anal.* **2010**, *21*, 80–88. https://doi.org/10.1002/pca.1158.
60. NIST. NIST Chemistry WebBook. Available online: https://webbook.nist.gov/chemistry/ (accessed on 11 October 2022).
61. Oberacher, H. *Wiley Registry of Tandem Mass Spectral Data, MS for ID*; Wiley: Hoboken, NJ, USA, 2012.
62. Čagalj, M.; Skroza, D.; del Carmen Razola-Díaz, M.; Verardo, V.; Bassi, D.; Frleta, R.; Generalić Mekinić, I.; Tabanelli, G.; Šimat, V. Variations in the Composition, Antioxidant and Antimicrobial Activities of *Cystoseira Compressa* during Seasonal Growth. *Mar. Drugs* **2022**, *20*, 64. https://doi.org/10.3390/md20010064.
63. Burčul, F.; Generalić Mekinić, I.; Radan, M.; Rollin, P.; Blažević, I. Isothiocyanates: Cholinesterase Inhibiting, Antioxidant, and Anti-Inflammatory Activity. *J. Enzym. Inhib. Med. Chem.* **2018**, *33*, 577–582. https://doi.org/10.1080/14756366.2018.1442832.
64. Prior, R.L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for Hydrophilic and Lipophilic Antioxidant Capacity (Oxygen Radical Absorbance Capacity (ORACFL)) of Plasma and Other Biological and Food Samples. *J. Agric. Food Chem.* **2003**, *51*, 3273–3279. https://doi.org/10.1021/jf0262256.
65. Šimat, V.; Vlahović, J.; Soldo, B.; Generalić Mekinić, I.; Čagalj, M.; Hamed, I.; Skroza, D. Production and Characterization of Crude Oils from Seafood Processing By-Products. *Food Biosci.* **2020**, *33*, 100484. https://doi.org/10.1016/j.fbio.2019.100484.

8.4. Rad 3. Influence of Nutrient Deprivation on the Antioxidant Capacity and Chemical Profile of Two Diatoms from Genus *Chaetoceros*.

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Influence of Nutrient Deprivation on the Antioxidant Capacity and Chemical Profile of Two Diatoms from Genus *Chaetoceros*

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Abstract: The limited availability of phosphate, nitrogen and silicon in the growth media affects the growth, cellular processes, and metabolism of diatoms. Silicon deficiency primarily affects diatom morphology, while phosphate deficiency reduces the production of nucleic acids and phospholipids. Differences in pigment and protein composition are mainly due to nitrogen deficiency. In this study, *Chaetoceros socialis* and *Chaetoceros costatus* were cultured under phosphate, nitrogen, and silicon deprivation conditions. The diatom biomass was collected during the stationary growth phase and extracted with 70% ethanol under ultrasonication. The chemical profiles of the extracts were analyzed by high-performance liquid chromatography with high-resolution mass spectrometry with electrospray ionisation (UHPLC-ESI-HRMS), while the antioxidant capacity was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and oxygen radical absorbance capacity (ORAC) assays. Pigments, fatty acids, sterols, and derivatives were detected in both species. The total phenolic content in the extracts ranged from 46.25 ± 1.08 to 89.38 ± 6.21 mg of gallic acid equivalent (GAE)/L and from 29.58 ± 1.08 to 54.17 ± 1.18 mg GAE/L. for *C. costatus* and *C. socialis*, respectively. Antioxidant activity was higher in *C. costatus* extracts, especially those obtained from nitrogen-deprived media. The results of this study contribute to the existing knowledge and the ongoing efforts to overcome application and commercialization barriers of microalgae for wide-ranging potential in different industries.

Keywords: phytoplankton; DPPH; ORAC; UHPLC-ESI-HRMS; fatty acid amides; pigments

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1. Introduction

Within the marine ecosystem, microalgae constitute a significant group of photosynthetic microorganisms, playing a vital role in the marine environment. Marine microalgae produce about 50% of atmospheric oxygen and present the most important food source in marine ecosystems [1]. The most abundant group of marine microalgae are diatoms, which are responsible for over 40% of the total primary production of the oceans [2]. As diatoms are widely distributed in various marine environments, from polar to tropical regions, they are highly adaptable to different abiotic parameters. Temperature, salinity, pH, light intensity, CO_2 concentration and especially the availability of nutrients are parameters that have a great influence on the growth and production of the metabolites of diatoms [3–5].

Among the macronutrients that have the greatest influence on the growth and metabolism of diatoms, silicon (Si), nitrogen (N) and phosphorus (P) are of primary importance. Si plays a role in the marine environment by controlling primary productivity [6]. In diatom growth, silicon requirements are related to the process of building a cell wall (frustule) but are also indirectly involved and important for cellular metabolic processes such as DNA replication and cell division [7,8]. On the other hand, N is an essential nutrient required for the biosynthesis

of many molecules, including amino acids, nucleic acids, lipids, and some sugars, but most of the assimilated nitrogen is used for the synthesis of proteins and nucleic acids [7]. For the production of chemical energy in the form of NADPH and ATP, diatoms use P. Phosphorus is also an important part of diatom cell membranes, where it is found in the form of phospholipids and is a component of nucleic acids (DNA and RNA) [9].

A higher concentration of these macronutrients in the growing media usually leads to an increased growth rate, while their limitation or deficiency affects the synthesis of compounds such as phenols, pigments, and fatty acids [7,10]. Nutrient deprivation with N, P and Si could be a stress trigger needed to increase the metabolic production and accumulation of various bioactive compounds in diatom cells [9–12]. Previous studies have shown that the species *Chaetoceros muelleri* experiences the greatest physiological stress under nitrogen deprivation [3,13]. In addition to a higher proportion of lipids, a decrease in the content of *n*-3 polyunsaturated fatty acids (PUFA) was also observed, while the proportion of monounsaturated fatty acids (MUFA) increased in these conditions [3]. The same results of lipid increase were observed in the species *Phaeodactylum tricornutum*, where a decrease in phenolic compounds and pigments was also observed during cultivation under N-deprivation compared to standard culture conditions [10]. In diatoms, the nutrient deficiency of Si and P has the same effects on metabolic processes [11,14]. Inducing stress by complete nutrient deprivation acts in the same way as a limitation of particular key nutrients; however, the metabolic response is species-dependent [14].

Therefore, the aim of this study was to culture *Chaetoceros costatus* and *Chaetoceros socialis* in N-, P- and Si-deprived culture media and extract the collected biomass. Furthermore, the antioxidant properties and chemical profile of the extracts were compared to extracts from biomass cultured in a standard growth medium (F/2).

2. Results and Discussion

2.1. Total Phenolic Content & Antioxidant Potentials

The TPC of *C. socialis* and *C. costatus* in a standard F/2 medium was 89.38 ± 6.21 and 54.17 ± 1.18 mg of gallic acid equivalent (GAE)/L, respectively. The lowest TPC results were found in the Si-deprived medium, an almost 2-fold decrease compared to the control medium (Figure 1).

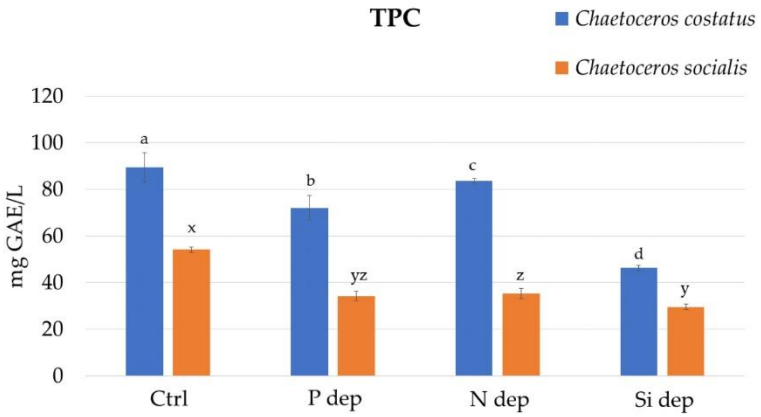

Figure 1. Total phenolic content (TPC) of the extracts from the biomass of *Chaetocerus costatus* and *Chaetocerus socialis* cultivated in standard F/2 medium (Ctrl) and under nutrient deprivation with phosphorus (P dep), nitrogen (N dep) and silica (Si dep). Letters a–d denote statistically significant differences ($p < 0.05$) between the extracts from *C. costatus* and letters x–z denote statistically significant differences ($p < 0.05$) between the extracts from *C. socialis*.

In the extracts of *C. costatus*, TPC was in range from 46.25 ± 1.08 to 89.38 ± 6.21 mg GAE/L, being the highest for diatoms cultured in the control (F/2) medium. The TPC of *C. socialis* was significantly lower in all media, ranging from 29.58 ± 1.08 to 54.17 ± 1.18 mg GAE/L.

The phenolic compounds in diatom cells serve as a protective mechanism against oxidative stress that can be triggered by abiotic parameters such as a nutrient deficiency. It is known that the contents of phenolic compounds vary between different diatom species, as do the mechanisms of adaptation to nutrient stress [15]. In a study on the diatom *Phaeodactylum tricornutum* exposed to nitrogen deprivation (N−) for 15 days, significant differences in total polyphenol content were found between cultures compared to standard culture conditions (N+) [10]. *Phaeodactylum tricornutum* produced a higher phenolic content in N-enriched medium (3.07 ± 0.17 mg GAE/g DW) compared to *P. tricornutum* grown under N-limitation (1.12 ± 0.00 mg GAE/g DW). The same was observed in the species *C. costatus* and *C. socialis* from this study, where the phenolic content was significantly lower in all experimental groups exposed to nutrient deficiency (P, N and Si) compared to the control group. Previously, a positive correlation between antioxidant activity and TPC was found in microalgae *Tetraselmis marina* isolated from the northern Adriatic Sea [16]. Interestingly, in nutrient-deprived culture conditions, both TPC and pigments may contribute significantly to antioxidant capacity [17].

A comprehensive insight into the antioxidant potential of diatom extracts is shown in Figure 2. All three methods demonstrated the higher antioxidant potential of *C. costatus*. When cultured in N-deprived medium, the antioxidant potential of the *C. costatus* extracts were higher for all assays. However, this trend cannot be confirmed for *C. socialis*, as no clear correlation between the antioxidant activity and the culture medium can be established. The highest potential for the inhibition of the DPPH radical (21.52 ± 4.35% of inhibition) was found for *C. costatus* extracts cultured in the N-deprived medium, while the extract of diatoms cultured in the P-deprived medium showed the lowest results. For this antioxidant method, *C. socialis* extracts followed the same trend but with a lower inhibition. The FRAP results for the extracts of *C. costatus* ranged from 205.13 ± 22.21 to 41.03 ± 4.44 µM Trolox equivalents (TE), with the highest results obtained for the N-deprived medium, followed by the P-deprived medium and control medium, and the lowest in the Si-deprived medium. For the *C. socialis* extracts, the highest FRAP result (94.87 ± 4.44 µM TE) was observed in the control medium. The FRAP results decreased when nutrient deprivation was tested following the Si > N > P trend.

The results of ORAC obtained for *C. costatus* extracts ranged from 2264.63 ± 170.02 to 1143.78 ± 73.18 µM TE. Only extracts of the N-deprived medium showed higher results than the control medium. The growth of diatoms in P-deprived and Si-deprived media resulted in lower peroxyl radical inhibition of extracts for this species. However, the opposite results were observed for *C. socialis* extracts, where the N-deprived medium showed the lowest ORAC results (864.67 ± 18.16 µM TE), while P-deprived and Si-deprived media showed higher results than the control medium, 1062.63 ± 60.64 and 1185.08 ± 59.42 µM TE, respectively.

In all three tests, the highest antioxidant activity during N deprivation was observed in the species *C. costatus*, but the same trend was not recorded in the species *C. socialis*. Therefore, the relationship between nutrient deprivation and antioxidant properties should be observed at the strain level, as differences were found within microalgal groups, but also at the genus level. In a study by Curcuraci et al. [10], the antioxidant properties of the diatom *P. tricornutum* cultivated in nutrient deprivation media were estimated using DPPH and FRAP assays. Both assays revealed a statistically significantly lower antioxidant capacity for *P. tricornutum* from a medium with N deprivation. Furthermore, a lower ability to scavenge DPPH radicals and a decrease in reducing power were observed for the green microalgae *Dunaliela salina* from an N-deprived medium [18]. On the other hand, Jeyakumar et al. [19] observed the highest DPPH scavenging activity in the haptophyte *Isochrysis* sp. under N-deficient conditions with an inhibition of 85%, while the nitrogen-rich and control medium showed a lower inhibition of 75% and 64%, respectively.

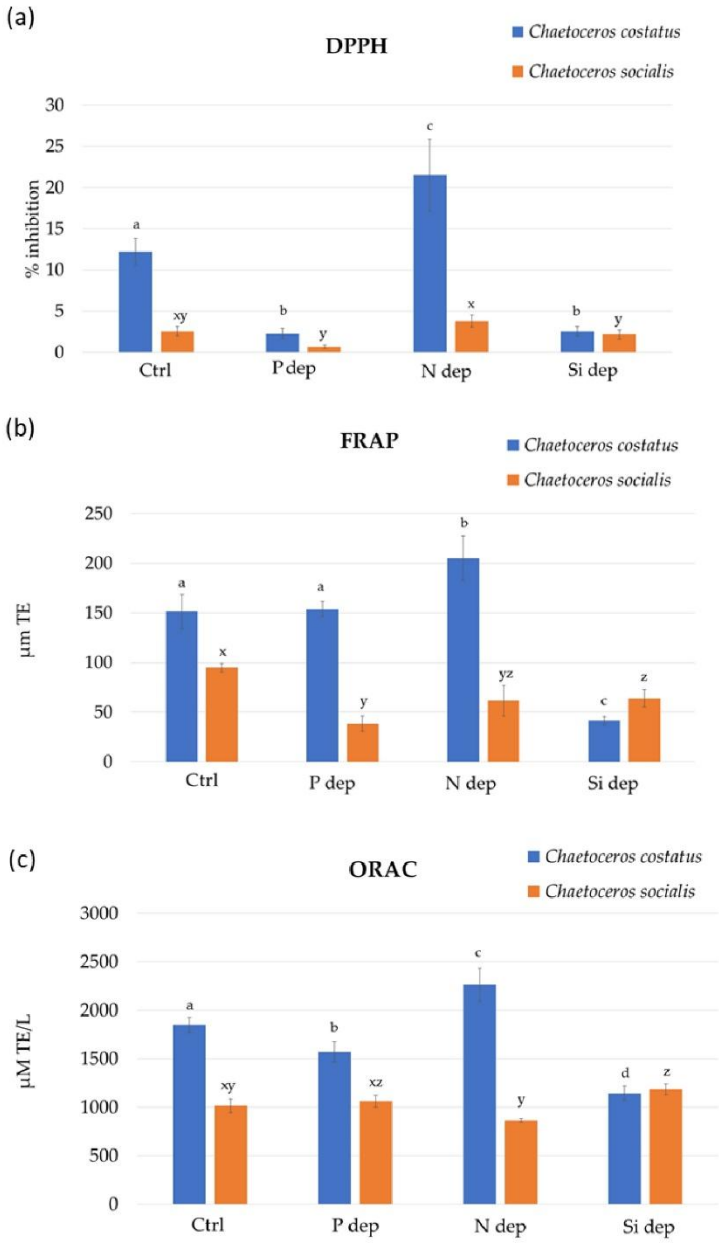

Figure 2. A 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability (DPPH) (**a**); ferric reducing/antioxidant power (FRAP) (**b**); and oxygen radical absorbance capacity (ORAC) (**c**) for the extracts of *Chaetocerus costatus* and *Chaetocerus socialis* biomass cultured in standard F/2 medium (Ctrl) and under nutrient deprivation with phosphorus (P dep), nitrogen (N dep), and silica (Si dep). Letters a–d denote statistically significant differences ($p < 0.05$) between the extracts from *C. costatus* and letters x–z denote statistically significant differences ($p < 0.05$) between the extracts from *C. socialis*.

2.2. Non-Target Screening of Non-Volatile Compounds in Ethanol Extract

The ethanolic extracts of the freeze-dried microalgae samples were analyzed by ultra-high-performance liquid chromatography-high-resolution mass spectrometry (UHPLC-ESI–HRMS). The compounds were identified based on the provided elemental composition in combination with MS/MS spectra with confidence levels 2 (probable structure) and 3 (possible structure) [20]. Out of the thirty-eight identified compounds, fourteen were pigments and derivatives, nineteen were fatty acid derivatives, and five were steroids and derivatives (Table 1).

The group of pigments and derivatives was the most diverse when comparing different growing conditions and microalgae species. Five chlorophyll *a* derivatives (pheophorbide *a* (no. 8), divinyl pheophytin *a* (no. 11), 15^1-hydroxy-lactone-pheophytin *a* (no. 12), 13^2-hydroxy-pheophytin *a* (no. 13) and pheophytin *a* (no. 14)); two chlorophyll *b* derivatives (pheophorbide *b* (no. 4) and pheophytin *b* (no. 10)); five xanthophylls and derivatives (no. 2–3, 5–7); one pheophytine derivative (no. 9) and monoterpene lactone (loliolide, no. 1) were detected (Table 1). Pheophytin *a* was the most abundant pigment derivative in all samples, except in the samples with Si-deprivation in both species. In the N-deprived samples of *C. costatus*, loliolide, pheophorbide *a*, pheophytin *b* and three chlorophyll *a* derivatives compounds no. 9, 12 and 13 were more abundant compared to the other samples. Also, in *C. costatus* extracts, a compound with a potential application in nutrition and pharmacy, fucoxanthin, was significantly higher in the P-deprived and N-deprived medium by 80% and 85%, respectively. The compound (3β)-3-HydroXystigmast-5-en-7-one, known for its antimalarial activity [21], was detected in *C. costatus* extracts cultured in the N-deprived medium. Similar results were detected for a pheophorbide in *C. socialis* extracts (higher values in P-deprived and N-deprived medium by 77% and 86%, respectively), a chlorophyll derivative known for its anti-cancer, antioxidant, immunostimulatory, neuroprotective and anti-inflammatory activity [22]. Collier and Grossman [23] found the degradation of chlorophyll as soon as nitrogen was removed from the medium when culturing cyanobacterium *Synechococcus* sp. It is very likely that these compounds contributed to the increased antioxidant activity in these samples, particularly in *C. costatus*. The monoterpene hydroxylactone loliolide, a photo-oxidative degradation product of carotenoids, such as fucoxanthin [24], is known as an antioxidant [25] and is widely distributed in macroalgae [26–30]. Menzel et al. [31] used it as a biomarker in haptophytes, diatoms, dinoflagellates, and eustigmatophytes while investigating the deposition of sapropel. Loliolide was detected in acetone and MeOH extracts of the Antarctic diatoms *Craspedostauros ineffabilis* and *C. zucchelli*, as well as in a supercritical CO_2 extract of the green microalga *Tetradesmus obliquus* [32]. In the N-deprived sample, it was 1.2 times more abundant than in the control sample, 1.8 times more than in the P-deprived sample and 5.0 times more than in the Si-deprived sample. Loliolide has shown neuroprotective and anti-inflammatory activities [33] as well as anti-apoptosis and anti-scratching activities in human skin [29]. Pheophytines are the simplest derivatives of chlorophylls, in which the Mg atom is removed from the porphyrin ring. Further degradation leads to pheophorbides and their derivatives such as compounds no. 9 and 11–13. Both pheophytines and pheophorbides have shown antioxidant activities [22,34,35]. Pheophorbid *a* has shown anticancer [22], antiviral [36,37], anti-inflammatory [38], and antiparasitic activities [39].

Table 1. Major non-volatile compounds in *Chaetoceros costatus* and *Chaetoceros socialis* ethanol extracts identified using high-performance liquid chromatography–high-resolution mass spectrometry with electrospray ionisation (UHPLC-ESI–HRMS). Ctrl—control sample; samples of biomass cultured under nutrient deprivation with phosphorus (P dep), nitrogen (N dep), and silica (Si dep).

No.	Compound Name	Mass	[M+H]+	Molecular Formula	t_R (min)	Mass Difference (ppm)	Peak Area (Arbitrary Units)							
							Chaetoceros costatus				*Chaetoceros socialis*			
							Ctrl	P dep	N dep	Si dep	Ctrl	P dep	N dep	Si dep
	Pigments and Derivatives													
1	Loliolide	196.110	197.11722	$C_{11}H_{16}O_3$	5.866	0.1	1.65×10^6	1.06×10^6	1.95×10^6	3.87×10^5	2.53×10^5	1.32×10^5	1.62×10^5	1.54×10^5
2	Apo-10-fucoxanthinal	424.261	425.26864	$C_{27}H_{36}O_4$	9.514	1	1.50×10^5	4.13×10^3	7.99×10^4	-	6.09×10^4	6.98×10^3	3.54×10^4	-
3	Halocynthiaxanthin acetate	640.413	641.42005	$C_{42}H_{56}O_4$	12.364	2	6.39×10^5	1.88×10^5	3.89×10^5	-	3.64×10^5	2.26×10^4	4.21×10^5	2.41×10^4
4	Pheophorbide *b*	606.248	607.25511	$C_{35}H_{34}N_4O_6$	12.371	1	6.33×10^4	4.83×10^4	5.82×10^4	7.13×10^3	1.43×10^4	6.17×10^3	9.87×10^3	4.85×10^2
5	Fucoxanthin	658.423	659.43062	$C_{42}H_{58}O_6$	12.385	2.1	1.18×10^5	9.73×10^4	7.69×10^4	-	6.90×10^5	4.34×10^4	7.97×10^5	6.02×10^5
6	Diatoxanthin	566.412	567.41966	$C_4 0H_{54}O_3$	12.772	0.1	4.21×10^4	3.90×10^4	1.22×10^4	1.86×10^4	6.43×10^4	3.52×10^4	3.69×10^4	1.92×10^4
7	Fucoxanthinol	616.413	617.42005	$C_4 0H_{56}O_5$	12.836	2.8	9.89×10^4	5.03×10^4	6.45×10^4	4.14×10^4	5.75×10^5	9.44×10^3	6.02×10^3	9.99×10^3
8	Pheophorbide *a*	592.269	593.27585	$C_{35}H_{36}N_4O_5$	13.125	1.3	5.18×10^6	1.26×10^6	5.75×10^6	2.78×10^4	2.50×10^5	3.91×10^4	1.24×10^5	4.15×10^3
9	3-[21-Methoxycarbonyl-4,8,13,18-tetramethyl-20-oxo-9,14-divinyl-3,4-didehydro-3-24,25-dihydrophorbinyl]propanoic acid	588.237	589.24455	$C_{35}H_{32}N_4O_5$	13.247	3	9.06×10^5	3.67×10^5	9.17×10^5	5.31×10^4	1.51×10^5	9.19×10^4	1.66×10^5	1.32×10^4
10	Pheophytin *b*	884.545	885.55246	$C_{55}H_{72}N_4O_6$	18.463	3.5	1.50×10^5	2.75×10^4	1.62×10^5	-	9.22×10^4	5.06×10^3	3.12×10^5	-
11	Divinyl pheophytin *a*	868.550	869.55755	$C_{55}H_{72}N_4O_5$	18.721	2.4	4.20×10^3	1.27×10^3	2.69×10^3	-	1.04×10^3	9.56×10^3	2.03×10^5	-
12	13^1-hydroxy-lactone-pheophytin *a*	902.556	903.56303	$C_{55}H_{74}N_4O_7$	18.842	0.8	9.45×10^5	5.63×10^5	1.20×10^6	2.67×10^5	4.66×10^5	5.32×10^4	4.43×10^5	2.13×10^5
13	13^2-hydroxy-pheophytin *a*	886.561	887.56811	$C_{55}H_{74}N_4O_6$	18.859	0.1	4.03×10^5	5.46×10^5	5.44×10^5	1.64×10^4	4.65×10^5	5.03×10^4	4.14×10^5	1.99×10^4
14	Pheophytin *a*	870.566	871.5732	$C_{55}H_{74}N_4O_5$	19.071	0.4	2.40×10^7	4.74×10^6	1.91×10^7	5.27×10^4	3.31×10^6	2.12×10^5	3.98×10^6	5.24×10^4
	Fatty Acid Derivatives													
15	Hexadecasphinganine	273.267	274.27406	$C_{16}H_{35}NO_2$	6.24	0.6	8.20×10^6	7.33×10^6	5.69×10^6	5.19×10^6	9.40×10^6	5.76×10^6	6.39×10^6	4.33×10^6
16	Myristamide (Tetradecanamide)	227.225	228.23219	$C_{14}H_{29}NO$	10.328	0.6	2.59×10^6	1.76×10^6	1.41×10^6	2.15×10^6	3.69×10^6	1.98×10^6	2.89×10^6	2.04×10^6
17	Monomyristin (2,3-Dihydroxypropyl tetradecanoate)	302.246	303.25299	$C_{17}H_{34}O_4$	10.641	6.4	7.80×10^4	8.87×10^4	1.86×10^5	8.32×10^4	6.21×10^4	2.08×10^5	2.09×10^5	1.53×10^5
18	Palmitoleamide (Hexadec-9-enamide)	253.241	254.24784	$C_{16}H_{31}NO$	10.743	0.2	6.10×10^6	4.33×10^6	3.30×10^6	4.44×10^6	9.16×10^6	4.91×10^6	6.28×10^6	4.43×10^6
19	Linoleamide (Octadeca-9,12-dienamide)	279.256	280.26349	$C_{18}H_{33}NO$	11.182	0.1	5.91×10^6	4.60×10^6	1.31×10^6	5.05×10^6	1.14×10^7	6.11×10^6	8.14×10^6	4.86×10^6
20	Palmitamide (Hexadecanamide)	255.256	256.26349	$C_{16}H_{33}NO$	11.437	0.2	1.48×10^7	1.22×10^7	9.33×10^6	1.11×10^7	2.29×10^7	1.30×10^7	1.54×10^7	1.17×10^7
21	Monopalmitin (2,3-Dihydroxypropyl hexadecanoate)	330.277	331.28429	$C_{19}H_{38}O_4$	11.71	0.1	3.62×10^6	3.49×10^6	3.98×10^6	2.64×10^6	4.36×10^6	3.88×10^6	4.49×10^6	2.70×10^6
22	Oleamide (Octadec-9-enamide)	281.272	282.27914	$C_{18}H_{35}NO$	11.813	0.3	1.06×10^8	8.81×10^7	6.67×10^7	7.65×10^7	9.55×10^7	5.47×10^7	1.03×10^7	7.53×10^7
23	Stearamide (Octadecanamide)	283.288	284.29479	$C_{18}H_{37}NO$	12.506	0.8	7.89×10^6	6.91×10^6	5.23×10^6	6.17×10^6	1.27×10^7	6.93×10^6	9.03×10^6	7.06×10^6

Table 1. *Cont.*

No.	Compound Name	Mass	[M+H]+	Molecular Formula	t_R (min)	Mass Difference (ppm)	Peak Area (Arbitrary Units)							
							Chaetoceros costatus				*Chaetoceros socialis*			
							Ctrl	P dep	N dep	Si dep	Ctrl	P dep	N dep	Si dep
24	Monostearin (2,3-Dihydroxypropyl octadecanoate)	358.308	359.31559	$C_{21}H_{42}O_4$	12.765	0.6	3.70×10^6	3.51×10^6	3.98×10^6	2.27×10^6	4.16×10^6	2.92×10^6	4.46×10^6	2.46×10^6
25	Gondamide (Icos-11-enamide)	309.303	310.31044	$C_{20}H_{39}NO$	12.796	1.7	1.29×10^6	1.01×10^6	7.99×10^5	1.03×10^6	2.29×10^6	1.07×10^6	1.61×10^6	1.09×10^6
26	Arachidonic acid (Icosa-5,8,11,14-tetraenoic acid)	304.240	305.24751	$C_{20}H_{32}O_2$	13.16	0.3	3.85×10^4	5.46×10^4	5.14×10^4	5.25×10^4	5.75×10^4	5.61×10^4	5.50×10^4	3.53×10^4
27	Erucamide (Docos-13-enamide)	337.334	338.34174	$C_{22}H_{43}NO$	13.787	0.6	2.00×10^6	1.47×10^6	1.30×10^6	9.99×10^5	2.09×10^6	1.16×10^6	1.80×10^6	1.01×10^6
28	1-(9-Octadecenoyl)-2-(9-pentadecenoyl)-glycero-3-phosphocholine	743.547	744.55378	$C_{41}H_{78}NO_8P$	15.863	2.8	7.52×10^4	1.74×10^4	4.53×10^4	3.51×10^4	8.82×10^4	6.16×10^4	8.76×10^4	5.54×10^4
29	1-(11,14-Eicosadienoyl)-2-heptadecanoyl-glycero-3-phosphoserine	801.552	802.55926	$C_{45}H_{80}NO_{10}P$	16.300	0.1	1.95×10^5	1.22×10^5	1.45×10^5	7.31×10^4	1.68×10^5	1.07×10^5	1.63×10^5	6.01×10^4
30	1-Octadecanoyl-2-(9,12-heptadecadienoyl)-glycero-3-phosphocholine	771.578	772.58508	$C_{43}H_{82}NO_8P$	16.403	2.6	7.63×10^4	1.31×10^4	3.56×10^4	5.58×10^4	9.23×10^4	8.82×10^4	8.22×10^4	7.74×10^4
31	1-(9-Octadecenoyl)-2-(9-nonadecenoyl)-glycero-3-phosphocholine	799.609	800.61638	$C_{45}H_{86}NO_8P$	16.737	1.5	7.04×10^4	3.50×10^4	3.33×10^4	2.29×10^4	6.11×10^4	4.75×10^4	5.33×10^4	1.31×10^4
32	Dipalmitin	568.507	569.51395	$C_{35}H_{68}O_5$	17.729	1.2	3.60×10^4	3.09×10^4	3.70×10^4	1.52×10^4	2.53×10^4	2.70×10^4	3.39×10^4	3.20×10^4
33	1-Octadecanoyl-2-hexadecanoyl-*sn*-glycerol	596.538	597.54525	$C_{37}H_{72}O_5$	18.276	1.6	2.30×10^4	1.76×10^4	2.54×10^4	8.29×10^3	1.96×10^4	1.59×10^4	2.09×10^4	1.75×10^4
	Steroids and Derivatives													
34	Chola-5,22-dien-3-ol	342.292	343.29954	$C_{24}H_{38}O$	7.426	4.8	1.30×10^5	1.14×10^5	1.36×10^5	1.37×10^5	1.01×10^5	8.97×10^4	1.54×10^5	1.60×10^5
35	Campesterol	400.371	401.37779	$C_{28}H_{48}O$	7.944	0.8	9.75×10^3	4.13×10^2	7.92×10^3	5.52×10^3	1.20×10^4	4.19×10^3	8.97×10^3	4.80×10^3
36	24-Hydroperoxy-24-vinyl-cholesterol	444.360	445.36762	$C_{29}H_{48}O_3$	14.914	0.2	3.95×10^4	2.26×10^4	4.35×10^4	2.08×10^4	7.14×10^3	1.45×10^4	9.19×10^3	1.14×10^4
37	(3β)-3-Hydroxystigmast-5-en-7-one	428.365	429.37271	$C_{29}H_{48}O_2$	15.434	3.8	1.70×10^3	1.01×10^3	1.79×10^3	6.52×10^4	3.07×10^4	-	3.02×10^4	3.43×10^4
38	Stigmastatriene	394.360	395.36723	$C_{29}H_{46}$	15.609	1.9	2.33×10^4	3.16×10^4	1.99×10^4	2.04×10^4	1.02×10^4	4.29×10^4	2.01×10^4	2.33×10^4

In the group of fatty acid derivatives, there were eight primary fatty acid amides (PFAAs), with oleamide (no. 22) being the most abundant (Table 1). This PFAA has already been found as the dominant compound in green and brown macroalgae [28,40,41] and in the diatom *Skeletonema grevillei* [42]. Two other C:18 PFAAa were detected, linoleamide (no. 19) and stearamide (no. 23). In addition, two C:16 PFAAs, palmitoleamide (no. 18) and palmitamide (no. 20), one C:14 PFAA, myristamide (no. 16), C:20 PFAA, gondamide (no. 25), and C:22 PFAA, erucamide (no. 27) were detected. The abundance of all PFAAs was the highest in the control sample and decreased significantly in the samples deprived of nutrients, especially in the samples deprived of N in both species. PFAAs are bioactive signaling molecules that have the ability to bind to the receptors of many drugs. In this way, they could influence locomotion, angiogenesis, and sleep in mammals [43]. They have numerous bioactive properties, such as anticancer, antimicrobial, anthelmintic and antidiabetic activities [44–47]. Oleamide is the most studied PFAA and has promising potential against Alzheimer's disease [43–45,48]. As secondary metabolites in algae, they probably play a role in defense against predators, as is the case in higher plants [44]. Four glycerophosphocholines (no. 28–31) were detected with the highest content in the control samples of both species. Three fatty acid esters (no. 17, 21, 24) and two diacylglycerols (no. 32–33) showed an increase in the N-deprived samples. A long-chain fatty acid C:20 (no. 26) and a C:16 sphingoid bases sphingolipid [49] hexadecasphinganine (no. 15), were also detected.

Five compounds (no. 33–38) were detected in the group of sterols and derivatives. Their contents varied from sample to sample and were not comparable between species. Studies have shown that microalgae can synthesize animal and plant sterols, with cholesterol, stigmasterol, ergosterol and champesterol being the most abundant [50]. In this study, champesterol (no. 35), deoxidised stigmasteol (stigmastatriene, no. 38) and three other derivatives (no. 34, 36, 37) were found. These compounds may increase antioxidant activity, as sterols are known to be antioxidant, anticarcinogenic, and anti-inflammatory compounds [51].

3. Materials and Methods

3.1. Experimental Design and Cultivation Conditions

The diatoms *C. costatus* (CIM935) and *C. socialis* (CIM929) were donated from the culture collection of the Center for Marine Research of the Ruđer Bošković Institute (Rovinj, Croatia). The strains were isolated from the northern Adriatic Sea.

The control groups (Ctrl) of both strains were cultivated in the standard F/2 medium, while the other three treatment groups were cultivated in phosphorus (P dep)-, nitrogen (N dep)- and silicon (Si dep)-deficient conditions (Table 2).

Table 2. The composition of a nutrient medium used for the cultivation of the strains *Chaetocerus costatus* and *Chaetocerus socialis* under nutrient deprivation with phosphorus (P dep), nitrogen (N dep), and silica (Si dep).

Treatment Group	Composition
Control group (Ctrl)	The F/2 medium was prepared according to the previously described recipe [52]
P dep	Based on F/2 medium without addition of $NaH_2PO_4 \cdot H_2O$
N dep	Based on F/2 medium without addition of $NaNO_3$
Si dep	Based on F/2 medium without addition of $Na_2SiO_3 \cdot 9H_2O$

During the cultivation, all groups were held at a temperature of 18 °C, at a light intensity of 2500 lux (Led GNC Minu Deep AM140, Sicce, Pozzoleone, Italy) and a 16:8 light:dark cycle. The diatoms were cultured in cell culture flasks, each containing 500 mL of the nutrient medium and 20 mL of the diatom inoculum (10^5 cells/mL). The cultivation of each treatment group was carried out in two replicates.

3.2. Harvesting and Extraction

The diatom biomass was harvested at the stationary growth phase by the filtration trough glass microfiber filters (Grade GF/F Whatman) at a pressure of 3.21 psi. The collected biomass was transferred to falcon tubes with cell scrapers and freeze-dried (FreeZone 2.5, Labconco, Kansas City, MO, USA) [53].

Ultrasound-assisted extraction (UAE) of the freeze-dried biomass was performed in an ultrasonic bath (DU-100 Digital ultrasonic cleaner, Giorgio Bormac, Carpi, Italy) with 70% ethanol at a frequency of 40 kHz and 50 °C for 1 h. The samples were centrifuged (Rotafix 32A, Hettich, Tuttlingen, Germany) for 5 min at room temperature and 3220× *g* (4000 rpm) and the obtained supernatants were filtered through a 0.45 µM mixed cellulose ester filter (LGG, Meckenheim, Germany) and dried by centrifugal evaporator (RC10-22, Jouan, Herblain, France).

3.3. Antioxidation Assays

The dried extracts of both diatom strains were dissolved in 70% ethanol at a concentration of 20 mg/mL prior to the analyses.

The Folin–Ciocalteu method was used to determine the total phenolic content (TPC) of the diatom extracts [54]. In summary, 25 µL of the *Chaetocerus* extracts and 1.5 mL of distilled water were combined with 25 µL of the Folin–Ciocalteu reagent. The reagent was added, and the mixtures were agitated and left for a one minute before adding 375 µL of 20% sodium carbonate solution and 475 µL of distilled water. Samples were kept in the dark at room temperature for two hours, and measurements were performed with a spectrophotometer (UV-1900i, Shimadzu, Tokyo, Japan) at an absorbance of 765 nm. The results were expressed in milligrams or gallic acid equivalents (GAE) per L of extract.

The ability to scavenge 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, ferric reducing/antioxidant power (FRAP), and the oxygen radical absorbance capacity (ORAC) were used to evaluate the antioxidant potential of *C. costatus* and *C. socialis* extracts.

The reducing activity was measured with FRAP [55]. The absorbance of 300 µL of FRAP reagent solution was measured at 592 nm using a plate reader (SynergyHTX Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA) in 96-well microplates. The change of absorbance was measured 4 min after adding 10 µL of the sample to the FRAP reagent. The absorbance of the FRAP reagent before the addition of the sample and four minutes afterward was compared with a value determined for the Trolox reference solution and expressed in µM TE.

The ability of the diatom extracts to scavenge DPPH radicals was also assessed [53]. Measurements were performed at 517 nm after adding of 290 µL of DPPH radical solution with an initial absorbance of 1.2 nm in the microplate wells. A plate reader was used to measure the decreased in absorbance one hour after adding 10 µL of the *Chaetocerus* extracts to the wells. The percentage of DPPH radical inhibition that the diatom extracts were able to inhibit (% inhibition) was used to quantify their antioxidant activity.

The ORAC assay was performed according to previously described protocols [56,57]. A volume of 25 µL of the diatom extracts was added to the wells of a microtiter plate containing 150 µL of 4.2 mM fluorescein (3′,6′-dihydroxyspiro[isobenzofuran-1(3H),9′-[9H] xanthan]-3-one). After thermostating at 37 °C for 30 min, 25 µL of AAPH (2,2′-azobis (2-amidinopropane) dihydrochloride) was added to the plates. Excitation and emission wavelength measurements were performed at 485 and 520 nm every minute for eighty minutes and the results were expressed in µM Trolox equivalents (µM TE). All mentioned assays were performed in triplicate.

3.4. Ultra-High-Performance Liquid Chromatography-High-Resolution Mass Spectrometry (UHPLC-ESI-HRMS) of Ethanol Extract

ExionLC AD UHPLC system (AB Sciex, Concord, ON, Canada) connected to a quadrupole time-of-flight (Q-TOF) mass spectrometer TripleTOF 6600+ (AB Sciex, Concord, ON, Canada) with a duospray ion source was used for the UHPLC-ESI-HRMS analyses. The Acquity UPLC

BEH Phenyl-Hexyl analytical column (Waters, Milford, MA, USA) 2.1 mm × 100 mm with a particle size of 1.7 µM was used for the chromatographic separation of the compounds. Water, as a mobile phase A, and acetonitrile, as a mobile phase B, both contained 0.1% formic acid. The flow rate of 0.4 mL/min and the oven temperature of 30 °C were constant throughout the analysis. Elution started at 2% B and was held for 0.6 min, followed by a linear B gradient to 100% until 18.5 min. From 18.5–25 min, elution was again isocratic at 100% B. Electrospray ionization was set in positive mode (ESI+) with collision-induced dissociation (CID) in information-dependent acquisition mode (IDA) for MS/MS mass spectra acquisition. A detailed description of the parameters can be found in our previous article [40]. The mass spectrometer data were processed using ACD/Spectrus Processor 2021.1.0 software (ACD/Labs, Toronto, ON, Canada). Based on the mass spectra and the reported elemental compositions of the compounds combined with the results of the search in the MassBank, Lipid Maps, ChemSpider and ChEBI databases, the identification of the compounds was proposed.

3.5. Statistical Analysis

Analyses of variance (one-way ANOVA followed by Fisher's least significant difference test) were used to express the statistical difference for the results of the TPC, FRAP, DPPH and ORAC assays between the results obtained for different culture media of each species [58]. The analyses were performed with Statgraphics Centurion-Ver. 16.1.11 (StatPoint Technologies, Inc., Warrenton, VA, USA).

4. Conclusions

Nutrient deprivation with phosphorus, nitrogen, and silicon in the species *C. costatus* and *C. socialis* resulted in a lower TPC. On the other hand, a significant increase in antioxidant capacity was observed during nitrogen deprivation in *C. costatus*, while the same trend was not observed in *C. socialis*. Among the identified compounds, pigments and derivatives, fatty acid derivatives as well as sterols and derivatives were detected in both species. Higher occurrences of pigment derivatives, loliolide, pheophorbide *a*, pheophytin *b*, and three chlorophyll *a* derivatives, which are possibly responsible for antioxidant activity, were observed in the N-deprived medium. Among the dominant compounds from the group of fatty acids, oleamide was detected and, in contrast to the pigments, deprivation with N led to a significant decrease in the content of PFAAs in both species. For this reason, the influence of single-nutrient deprivation on the chemical composition and antioxidant activity, even at the level of a single genus, is inconclusive. Certainly, future research should investigate cell growth rate and biomass yield in a nutrient-deprived medium and their correlation to specific compound contents, and whether the combination of nutrient deprivation with another stress trigger can further increase the synthesis of these bioactive compounds to further explore their overall bioactive potential.

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References

1. Rizwan, M.; Mujtaba, G.; Memon, S.A.; Lee, K.; Rashid, N. Exploring the potential of microalgae for new biotechnology applications and beyond: A review. *Renew. Sustain. Energy Rev.* **2018**, *92*, 394–404. [CrossRef]
2. Field, C.B.; Behrenfeld, M.J.; Randerson, J.T.; Falkowski, P. Primary production of the biosphere: Integrating terrestrial and oceanic components. *Science* **1998**, *281*, 237–240. [CrossRef] [PubMed]
3. Lin, Q.; Zhuo, W.H.; Wang, X.W.; Chen, C.P.; Gao, Y.H.; Liang, J.R. Effects of fundamental nutrient stresses on the lipid accumulation profiles in two diatom Species *Thalassiosira weissflogii* and *Chaetoceros muelleri*. *Bioprocess Biosyst. Eng.* **2018**, *41*, 1213–1224. [CrossRef] [PubMed]
4. Gatamaneni, B.L.; Orsat, V.; Lefsrud, M. Factors affecting growth of various microalgal species. *Environ. Eng. Sci.* **2018**, *35*, 1037–1048. [CrossRef]
5. Vello, V.; Phang, S.-M.; Poong, S.-W.; Lim, Y.-K.; Ng, F.-L.; Shanmugam, J.; Gopal, M. New Report of *Halamphora subtropica* (Bacillariophyta) from the Strait of Malacca and its growth and biochemical characterisation under nutrient deprivation. *Reg. Stud. Mar. Sci.* **2023**, *62*, 102947. [CrossRef]
6. Martin-Jézéquel, V.; Hildebrand, M.; Brzezinski, M.A. Silicon metabolism in diatoms: Implications for growth. *J. Phycol.* **2000**, *36*, 821–840. [CrossRef]
7. Orefice, I.; Musella, M.; Smerilli, A.; Sansone, C.; Chandrasekaran, R.; Corato, F.; Brunet, C. Role of nutrient concentrations and water movement on diatom's productivity in culture. *Sci. Rep.* **2019**, *9*, 1479. [CrossRef]
8. Shrestha, R.P.; Hildebrand, M. Evidence for a regulatory role of diatom silicon transporters in cellular silicon responses. *Eukaryot. Cell* **2015**, *14*, 29. [CrossRef]
9. Lovio-Fragoso, J.P.; de Jesús-Campos, D.; López-Elías, J.A.; Medina-Juárez, L.Á.; Fimbres-Olivarría, D.; Hayano-Kanashiro, C. Biochemical and molecular aspects of phosphorus limitation in diatoms and their relationship with biomolecule accumulation. *Biology* **2021**, *10*, 565. [CrossRef]
10. Curcuraci, E.; Manuguerra, S.; Messina, C.M.; Arena, R.; Renda, G.; Ioannou, T.; Amato, V.; Hellio, C.; Barba, F.J.; Santulli, A. Culture conditions affect antioxidant production, metabolism and related biomarkers of the microalgae *Phaeodactylum tricornutum*. *Antioxidants* **2022**, *11*, 411. [CrossRef]
11. Smith, S.R.; Glé, C.; Abbriano, R.M.; Traller, J.C.; Davis, A.; Trentacoste, E.; Vernet, M.; Allen, A.E.; Hildebrand, M. Transcript level coordination of carbon pathways during silicon starvation-induced lipid accumulation in the diatom *Thalassiosira pseudonana*. *N. Phytol.* **2016**, *210*, 890–904. [CrossRef]
12. Yu, S.J.; Shen, X.F.; Ge, H.Q.; Zheng, H.; Chu, F.F.; Hu, H.; Zeng, R.J. Role of sufficient phosphorus in biodiesel production from diatom *Phaeodactylum tricornutum*. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 6927–6934. [CrossRef] [PubMed]
13. Gao, Y.; Yang, M.; Wang, C. Nutrient deprivation enhances lipid content in marine microalgae. *Bioresour. Technol.* **2013**, *147*, 484–491. [CrossRef] [PubMed]
14. Lovio Fragoso, J.; Hayano Kanashiro, C.; Lopez Elias, J. Effect of different phosphorus concentrations on growth and biochemical composition of *Chaetoceros muelleri*. *Lat. Am. J. Aquat. Res.* **2019**, *47*, 361–366. [CrossRef]
15. Goiris, K.; Van Colen, W.; Wilches, I.; León-Tamariz, F.; De Cooman, L.; Muylaert, K. Impact of nutrient stress on antioxidant production in three species of microalgae. *Algal Res.* **2015**, *7*, 51–57. [CrossRef]
16. Trentin, R.; Custódio, L.; Rodrigues, M.J.; Moschin, E.; Sciuto, K.; da Silva, J.P.; Moro, I. Total phenolic levels, In vitro antioxidant properties, and fatty acid profile of two microalgae, *Tetraselmis marina* strain IMA043 and *Naviculoid* diatom strain IMA053, isolated from the North Adriatic Sea. *Mar. Drugs* **2022**, *20*, 207. [CrossRef]
17. Kuczynska, P.; Jemiola-Rzeminska, M.; Strzalka, K. Photosynthetic pigments in diatoms. *Mar. Drugs* **2015**, *13*, 5847–5881. [CrossRef]
18. Singh, P.; Baranwal, M.; Reddy, S.M. Antioxidant and cytotoxic activity of carotenes produced by *Dunaliella salina* under stress. *Pharm. Biol.* **2016**, *54*, 2269–2275. [CrossRef]
19. Jeyakumar, B.; Asha, D.; Varalakshmi, P.; Kathiresan, S. Nitrogen repletion favors cellular metabolism and improves eicosapentaenoic acid production in the marine microalga *Isochrysis* Sp. CASA CC 101. *Algal Res.* **2020**, *47*, 101877. [CrossRef]
20. Blaženović, I.; Kind, T.; Ji, J.; Fiehn, O. software tools and approaches for compound identification of LC-MS/MS data in metabolomics. *Metabolites* **2018**, *8*, 31. [CrossRef]
21. Indriani, I.; Aminah, N.S.; Puspaningsih, N.N.T. Antiplasmodial Activity of stigmastane steroids from *Dryobalanops oblongifolia* stem bark. *Open Chem.* **2020**, *18*, 259–264. [CrossRef]
22. Saide, A.; Lauritano, C.; Ianora, A. Pheophorbide a: State of the art. *Mar. Drugs* **2020**, *18*, 257. [CrossRef]
23. Collier, J.L.; Grossman, A.R. Chlorosis induced by nutrient deprivation in *Synechococcus* Sp. strain PCC 7942: Not all bleaching is the same. *J. Bacteriol.* **1992**, *174*, 4718–4726. [CrossRef]

24. Repeta, D.J. Carotenoid diagenesis in recent marine sediments: II. degradation of fucoxanthin to loliolide. *Geochim. Cosmochim. Acta* **1989**, *53*, 699–707. [CrossRef]
25. Percot, A.; Yalçin, A.; Aysel, V.; Erduğan, H.; Dural, B.; Güven, K.C. Loliolide in marine algae. *Nat. Prod. Res.* **2009**, *23*, 460–465. [CrossRef]
26. El Hattab, M.; Culioli, G.; Valls, R.; Richou, M.; Piovetti, L. Apo-fucoxanthinoids and loliolide from the brown alga *Cladostephus spongiosus* f. *verticillatus* (Heterokonta, Sphacelariales). *Biochem. Syst. Ecol.* **2008**, *36*, 447–451. [CrossRef]
27. Radman, S.; Cikoš, A.M.; Flanjak, I.; Babić, S.; Čižmek, L.; Šubarić, D.; Čož-Rakovac, R.; Jokić, S.; Jerković, I. Less polar compounds and targeted antioxidant potential (in vitro and in vivo) of *Codium adhaerens* c. Agardh 1822. *Pharmaceuticals* **2021**, *14*, 944. [CrossRef]
28. Radman, S.; Čižmek, L.; Babić, S.; Cikoš, A.M.; Čož-Rakovac, R.; Jokić, S.; Jerković, I. Bioprospecting of less-polar fractions of *Ericaria crinita* and *Ericaria amentacea*: Developmental toxicity and antioxidant activity. *Mar. Drugs* **2022**, *20*, 57. [CrossRef]
29. Park, S.H.; Kim, D.S.; Kim, S.; Lorz, L.R.; Choi, E.; Lim, H.Y.; Hossain, M.A.; Jang, S.G.; Choi, Y.I.; Park, K.J.; et al. Loliolide presents antiapoptosis and antiscratching effects in human keratinocytes. *Int. J. Mol. Sci.* **2019**, *20*, 651. [CrossRef]
30. Yang, X.; Kang, M.-C.; Lee, K.-W.; Kang, S.-M.; Lee, W.-W.; Jeon, Y.-J. Antioxidant activity and cell protective effect of loliolide isolated from *Sargassum ringgoldianum* Subsp. *coreanum*. *Algae* **2011**, *26*, 201–208. [CrossRef]
31. Menzel, D.; Van Bergen, P.F.; Schouten, S.; Sinninghe Damsté, J.S. Reconstruction of changes in export productivity during pliocene sapropel deposition: A biomarker approach. *Palaeogeogr. Palaeoclimatol. Palaeoecol.* **2003**, *190*, 273–287. [CrossRef]
32. Vladić, J.; Jerković, I.; Radman, S.; Jazić, J.M.; Ferreira, A.; Maletić, S.; Gouveia, L. Supercritical CO_2 extract from microalga *Tetradesmus obliquus*: The effect of high-pressure pre-treatment. *Molecules* **2022**, *27*, 3883. [CrossRef]
33. Silva, J.; Alves, C.; Martins, A.; Susano, P.; Simões, M.; Guedes, M.; Rehfeldt, S.; Pinteus, S.; Gaspar, H.; Rodrigues, A.; et al. Loliolide, a new therapeutic option for neurological diseases? In vitro neuroprotective and anti-inflammatory activities of a monoterpenoid lactone isolated from *Codium tomentosum*. *Int. J. Mol. Sci.* **2021**, *22*, 1888. [CrossRef] [PubMed]
34. Lanfer-Marquez, U.M.; Barros, R.M.C.; Sinnecker, P. Antioxidant activity of chlorophylls and their derivatives. *Food Res. Int.* **2005**, *38*, 885–891. [CrossRef]
35. Hsu, C.-Y.; Chao, P.-Y.; Hu, S.-P.; Yang, C.-M. The antioxidant and free radical scavenging activities of chlorophylls and pheophytins. *Food Nutr. Sci.* **2013**, *4*, 8A. [CrossRef]
36. Ohta, S.; Ono, F.; Shiomi, Y.; Nakao, T.; Aozasa, O.; Nagate, T.; Kitamura, K.; Yamaguchi, S.; Nishi, M.; Miyata, H. Anti-*Herpes simplex* virus substances produced by the marine green alga, *Dunaliella primolecta*. *J. Appl. Phycol.* **1998**, *10*, 349–356. [CrossRef]
37. Ratnoglik, S.L.; Aoki, C.; Sudarmono, P.; Komoto, M.; Deng, L.; Shoji, I.; Fuchino, H.; Kawahara, N.; Hotta, H. Antiviral activity of extracts from *Morinda citrifolia* leaves and chlorophyll catabolites, pheophorbide *a* and pyropheophorbide *a*, against Hepatitis C virus. *Microbiol. Immunol.* **2014**, *58*, 188–194. [CrossRef]
38. Lauritano, C.; Helland, K.; Riccio, G.; Andersen, J.H.; Ianora, A.; Hansen, E.H. Lysophosphatidylcholines and chlorophyll-derived molecules from the diatom *Cylindrotheca closterium* with anti-inflammatory activity. *Mar. Drugs* **2020**, *18*, 166. [CrossRef] [PubMed]
39. Miranda, N.; Volpato, H.; da Silva Rodrigues, J.H.; Caetano, W.; Ueda-Nakamura, T.; de Oliveira Silva, S.; Nakamura, C.V. The photodynamic action of pheophorbide a induces cell death through oxidative stress in *Leishmania amazonensis*. *J. Photochem. Photobiol. B Biol.* **2017**, *174*, 342–354. [CrossRef]
40. Radman, S.; Cikoš, A.M.; Babić, S.; Čižmek, L.; Čož-Rakovac, R.; Jokić, S.; Jerković, I. In vivo and in vitro antioxidant activity of less polar fractions of *Dasycladus vermicularis* (Scopoli) Krasser 1898 and the chemical composition of fractions and macroalga volatilome. *Pharmaceuticals* **2022**, *15*, 743. [CrossRef]
41. Radman, S.; Čagalj, M.; Šimat, V.; Jerković, I. Seasonal monitoring of volatiles and antioxidant activity of brown alga *Cladostephus spongiosus*. *Mar. Drugs* **2023**, *21*, 415. [CrossRef] [PubMed]
42. Frleta, R.; Popović, M.; Smital, T.; Šimat, V. Comparison of growth and chemical profile of diatom *Skeletonema grevillei* in bioreactor and incubation-shaking cabinet in two growth phases. *Mar. Drugs* **2022**, *20*, 697. [CrossRef] [PubMed]
43. Farrell, E.K.; Chen, Y.; Barazanji, M.; Jeffries, K.A.; Cameroamortegui, F.; Merkler, D.J. Primary fatty acid amide metabolism: Conversion of fatty acids and an ethanolamine in N 18TG 2 and SCP Cells. *J. Lipid Res.* **2012**, *53*, 247–256. [CrossRef] [PubMed]
44. Tanvir, R.; Javeed, A.; Rehman, Y. Fatty acids and their amide derivatives from endophytes: New therapeutic possibilities from a hidden source. *FEMS Microbiol. Lett.* **2018**, *365*, fny114. [CrossRef] [PubMed]
45. D'Oca, C.D.R.M.; Coelho, T.; Marinho, T.G.; Hack, C.R.L.; Da Costa Duarte, R.; Da Silva, P.A.; D'Oca, M.G.M. Synthesis and antituberculosis activity of new fatty acid amides. *Bioorganic Med. Chem. Lett.* **2010**, *20*, 5255–5257. [CrossRef] [PubMed]
46. Kabara, J.J.; Swieczkowski, D.M.; Conley, A.J.; Truant, J.P. Fatty acids and derivatives as antimicrobial agents. *Antimicrob. Agents Chemother.* **1972**, *2*, 23–28. [CrossRef]
47. Dembitsky, V.M. Microbiological aspects of unique, rare, and unusual fatty acids derived from natural amides and their pharmacological profile. *Microbiol. Res.* **2022**, *13*, 377–417. [CrossRef]
48. Ano, Y.; Ozawa, M.; Kutsukake, T.; Sugiyama, S.; Uchida, K.; Yoshida, A.; Nakayama, H. Preventive effects of a fermented dairy product against Alzheimer's disease and identification of a novel oleamide with enhanced microglial phagocytosis and anti-inflammatory activity. *PLoS ONE* **2015**, *10*, e0118512. [CrossRef]
49. Fahy, E.; Subramaniam, S.; Brown, H.A.; Glass, C.K.; Merrill, A.H.; Murphy, R.C.; Raetz, C.R.H.; Russell, D.W.; Seyama, Y.; Shaw, W.; et al. A comprehensive classification system for lipids. *J. Lipid Res.* **2005**, *46*, 839–861. [CrossRef]

50. Fagundes, M.B.; Vendruscolo, R.G.; Wagner, R. *Sterols from Microalgae*; Elsevier Inc.: Amsterdam, The Netherlands, 2020. [CrossRef]
51. Volkman, J.K. *The Physiology of Microalgae*; Borowitzka, M.A., Beardall, J., Raven, J.A., Eds.; Springer International Publishing: Cham, Switzerland, 2016. [CrossRef]
52. Andersen, R.A. *Algal Culturing Techniques*; Elsevier Academic Press: New York, NY, USA, 2005.
53. Frleta Matas, R.; Popović, M.; Čagalj, M.; Šimat, V. The marine diatom *Thalassiosira rotula*: Chemical profile and antioxidant activity of hydroalcoholic extracts. *Front. Mar. Sci.* **2023**, *10*, 1–9. [CrossRef]
54. Amerine, M.A.; Ough, C.S. *Methods Analysis of Musts and Wines*, 2nd ed.; Wiley: New York, NY, USA, 1980.
55. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef] [PubMed]
56. Prior, R.L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity ($ORAC_{FL}$)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **2003**, *51*, 3273–3279. [CrossRef] [PubMed]
57. Burčul, F.; Generalić Mekinić, I.; Radan, M.; Rollin, P.; Blažević, I. Isothiocyanates: Cholinesterase inhibiting, antioxidant, and anti-inflammatory activity. *J. Enzyme Inhib. Med. Chem.* **2018**, *33*, 577–582. [CrossRef] [PubMed]
58. Šimat, V.; Vlahović, J.; Soldo, B.; Generalić Mekinić, I.; Čagalj, M.; Hamed, I.; Skroza, D. Production and characterization of crude oils from seafood processing by-products. *Food Biosci.* **2020**, *33*, 100484. [CrossRef]

9. ŽIVOTOPIS

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Obrazovanje:

2015 - 2018 - Sveučilišna prvostupnica (baccalaurea) inženjerka morskog ribarstva, Sveučilišni odjel za studije mora, Sveučilište u Splitu

2018 - 2020 - magistra inženjerka morskog ribarstva, Sveučilišni odjel za studije mora, Sveučilište u Splitu,

2020 - danas – studentica poslijediplomskog/doktorskog studija Biofizika, Prirodoslovno-matematički fakultet, Sveučilište u splitu

Radno iskustvo:

2019-2020 – stručni suradnik na projektu Interreg „AdriAquaNet Enhancing Innovation and Sustainability in Adriatic Aquaculture“ (nutritivna analiza, identifikacija i kvatifikacija masnih kiselina GC-FID), Institut za oceanografiju i ribarstvo, Split

2020-2024 – asistent na projektu STIM-REI, Sveučilište u Splitu

Stručne edukacije:

2021 - Centar za istraživanje mora – Institut Ruđer Bošković, Rovinj

2022 - Biocentar, Zagreb

2022 - Institut za jadranske kulture i melioraciju krša, Split

Dodatne edukacije:

2019-AMARE-MED First advance course on quantitative methods for ecosystem approach to fisheries application

2021-SEA-EU DOC training event for doctoral students „Carrer develpoment for doctoral students“

2022.- SEA-EU Joint PhD Course „Paper Writing Academy“

Radionice:

2021-HACCAP radionica program pomoću subjektima u poslovanju s hranom

2023- Webinar-Foods „The Use of Biopreservation and Bioactive Components as a Sustainable Strategy for Food Safety and Shelf Life Prolongation“

Nagrade:

2019 - Rektorova Nagrada za izvrsnost, Sveučilište u Splitu

Sudjelovanje na projektima:

Godina	Naziv
2018-2023	STIM-REI (Research, Education, Innovation) (KK.01.1.1.01.0003) (voditeljica projekta: prof. Dr. Dr. h.c. Vlasta Bonačić-Kouteckỳ)
2020-2023	Bio-protective cultures and bioactive extracts as sustainable combined strategies to improve the shelf-life of perishable Mediterranean food (BioProMedFood ID. 1467) (Voditeljica projekta: prof. dr. sc. Vida Šimat)
2022-2023	Marine natural compounds: an untapped source of biomass for biotechnological applications in the human health sector (MarHealth) (Voditeljica projekta: prof. dr. sc. Claire Helio)
2022-2023	Inovativni, ekološki pristup uzgoju dagnje na konopima od recikliranih materijala uz eDNA barkodiranje i pasterizaciju konzumnih školjki s ciljem podizanja kvalitete i vrijednosti finalnog proizvoda te zaštita okoliša – „INNODAGNJA“ (Voditeljica projekta: izv. prof. dr. sc. Mirela Petrić)
2022-2025	Innovative sustainable organic sea fennel (*Crithmum maritimum* L.) – based cropping systems to boost agrobiodiversity, profitability, circularity, and resilience to climate changes in Mediterranean small farms (SEAFENNEL4MED) (Voditeljica projekta: Izv. prof. dr. sc. Ivana Generalić Mekinić)
2023-2026	Innovative sustainable solutions for ready-to-eat traditional Mediterranean products and non-conventional healthy foods (InnoSol4Med) (Voditeljica projekta: prof. dr. sc. Vida Šimat)

Popularizacija znanosti:

2018.- Sudjelovanje u pripremi i provedbi aktivnosti na Festivalu znanosti 2018.-Otvaranje 16. Festivala znanosti „Otkrića“

2018.-Organizacija i provedba aktivnosti-„Revolucionarna otkrića u ribarstvu“-16. Festival znanosti „Otkrića“

2021.-Organizacija i provedba aktivnosti na Festivalu znanosti 2021.-„Utjecaj bakterija na sigurnost hrane“

2022.- Organizacija i provedba aktivnosti na Festivalu znanosti 2022.- „Zdrave

boje za zdrav život“

2022. - Organizacija i provedba aktivnosti na Europskoj noći istraživača 2022. - „Biološki aktivne molekule nusproizvoda hrane“

2023. - Organizacija i provedba aktivnosti na Europskoj noći istraživača 2023. – „Biološki aktivne molekule iz nusproizvoda prehrambene industrije“

Ostalo:

2024. – Znanstveni odbor, „1st International Congress for Sustainable Ecosystems in the Mediterranean area“ STEcoMed 2024, održanog u Splitu 2. i 3 . 10. 2024.

Sudjelovanje na međunarodnim konferencijama:

1. Roberta Frleta Matas, Martina Čagalj, Katarina Jelušić, Sanja Radman, Vida Šimat „The chemical composition and antioxidant potential of microalgae *Chaetoceros costatus*“; 1st International Congress for Sustainable Ecosystems in the Mediterranean area, Split, Hrvatska, 2.-3.10.2024.
   **Nagrada*: 1. nagrada za najbolju poster prezentaciju
2. Roberta Frleta Matas, Marija Spajić, Živko Skračić, Vida Šimat, Martina Čagalj, Danijela Skroza “Vinification by-products preservatives of the future” 4th International ZORH conference of scientists, professionals, and students - “Environmental protection, sustainable production and example of best practice”, Split, Hrvatska, 20.-21.4.2023.
3. Vida Šimat, Martina Čagalj, Roberta Frleta, Ivan Šimat, Sonja Smole Možina, Danijela Skroza “Effect of natural extracts and pure compounds on the fish burgers’ quality parameters”; 10th International Congress of Food Technologists, Biotechnologists and Nutritionists, Zagreb, Hrvatska, 30.11.-2.12.2022.
4. Danijela Skroza, Martina Čagalj, Ivona Krivić, Roberta Frleta, Vida Šimat “Antimicrobial activity of by-product extracts in combination with pure compounds”; 10th International Congress of Food Technologists, Biotechnologists and Nutritionists, Zagreb, Hrvatska, 30.11.-2.12.2022.
5. Roberta Frleta, Vida Šimat, Martina Čagalj„Application of ultrasound-assisted extraction in combination with ethanol to improve antioxidant activity of diatom extracts “; The 4th International Congress on “Green Extraction of Natural Products” Poreč, Hrvatska, 27.-28.11.2022.
6. Roberta Frleta, Vida Šimat “Comparison of the growth of diatom *Skeletonema grevillei* in a bioreactor and an incubation-shaking cabinet” 11th Central European Congress on Food and Nutrition, Čatež na Savi, Slovenija, 27.-30.9.2022.

Popis objavljenih radova:

1. Šimat V, Skroza D., Frleta Matas R., Radelić D., Bogdanović T., Čagalj M. 2025. The Effect of Combined Extracts from By-Products, Seaweed, and Pure Phenolics on the Quality of Vacuum-Packed Fish Burgers.Applied Sciences, 15 (10), 5008. https://doi.org/10.3390/app15105508.
2. Frleta Matas, R., Čagalj, M., Jelušić, K., Radman, S., Šimat, V. 2025. The Effect of Solvent Choice on Antioxidant Potential and Chemical Composition of Extracts from Microalgae *Chaetocerus costatus*. Phycology, 5(8). https://doi.org/10.3390/phycology5010008

3. Marić, N., Radman, S., Skroza, D., Frleta Matas, R., Generalić Mekinić, I. 2024. Funkcionalni napitak na bazi ječmenog slada. Glasilo Future, 7, 1; 38-47; https://doi.org/10.32779/gf.7.1.4.
4. Frleta Matas, R., Radman, S., Čagalj, M., Šimat V. 2024. Influence of Nutrient Deprivation on the Antioxidant Capacity and Chemical Profile of Two Diatoms from Genus *Chaetoceros*. Marine Drugs, 22(96); https://doi.org/10.3390/md22020096
5. Tkaczewska, J., Kulawik, P., Jamróz, E., Čagalj, M., Frleta Matas, R., Šimat, V. 2023. Valorization of Prawn/Shrimp Shell Waste through the production of biologically active components for functional food purposes. Journal of the Science of Food and Agriculture; https://doi.org/10.1002/jsfa.12969
6. Frleta Matas, R., Popović, M., Čagalj, M., Šimat V. 2023. The marine diatom Thalassiosira rotula: chemical profile and antioxidant activity of hydroalcoholic extracts. Frontiers in Marine Science; 10. https://doi.org/10.3389/fmars.2023.1221417
7. Hamed, I., Moradi, M., Ezati, P., O'Higgins, L., Meléndez-Martínez J., A., Frleta, R., Šimat, V., McClements, D.J., Nordeng Jakobsen, A., Lerfall, J. 2023. Encapsulation of microalgal-based carotenoids: Recent advances in stability and food applications. Trends in Food Science & Technology; 138: p.(382-398). https://doi.org/10.1016/j.tifs.2023.06.027
8. Popović, M., Burčul, F., Veršić Bratinčević, M., Režić Mužinić, N., Skroza, D., Frleta Matas, R., Nazlić, M., Ninčević Runjić, T., Jukić Špika, M., Bego, A., Dunkić, V., Vitanović, E. 2023. In the Beginning Was the Bud: Phytochemicals from Olive (*Olea europaea* L.) Vegetative Buds and Their Biological Properties. Metabolites; 13(2); 237. https://doi.org/10.3390/metabo13020237
9. Režić Mužinić, N., Veršić Bratinčević, M., Grubić, M., Frleta Matas, R., Čagalj, M., Visković, T., Popović, M. 2023. Golden Chanterelle or a Gold Mine? Metabolites from Aqueous Extracts of Golden Chanterelle (Cantharellus cibarius) and Their Antioxidant and Cytotoxic Activities. Molecules; https://doi.org/10.3390/molecules28052110
10. Frleta, R., Popović, M., Smital, T., Šimat, V. 2022. Comparison of Growth and Chemical Profile of Diatom *Skeletonema grevillei* in Bioreactor and Incubation-Shaking Cabinet in Two Growth Phases. Marine Drugs; 28(5), 2110. https://doi.org/10.3390/md20110697
11. Skroza, D., Šimat, V., Vrdoljak, L., Jolić, N., Skelin, A., Čagalj, M., Frleta, R., Generalić Mekinić, I. 2022. Investigation of Antioxidant Synergisms and Antagonisms among Phenolic Acids in the Model Matrices Using FRAP and ORAC Methods. Antioxidants; 11(9), 1784. https://doi.org/10.3390/antiox11091784
12. Čagalj, M., Skroza, D., del Carmen Razola-Díaz, M., Verardo, V., Bassi, D., Frleta, R., Generalić Mekinić, I., Tabanelli, G., Šimat, V. 2022. Variations in the Composition, Antioxidant and Antimicrobial Activities of *Cystoseira compressa* during Seasonal Growth. Marine Drugs; 20(1), 64. https://doi.org/10.3390/md20010064

Popis objavljenih poglavlja u knjigama:

1. Vida Šimat, Roberta Frleta, Martina Čagalj, and Danijela Skroza 2023. Bioactive Compounds and Nutraceuticals from Dairy, Marine, and Nonconventional Sources; Technological and Analytical Aspects of BioactiveCompounds and Nutraceuticals from Marine Algae. Apple Academic Press

Područje interesa: uzgoj morskih mikroalgi, utjecaj uzgojnih uvjeta na morfologiju, prinos i udio bioaktivnih spojeva, određivanje bioaktivnog potencijala (antimikrobna i antioksidativna), identifikacija i kvantifikacija spojeva različitim kromatograskim metodama

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