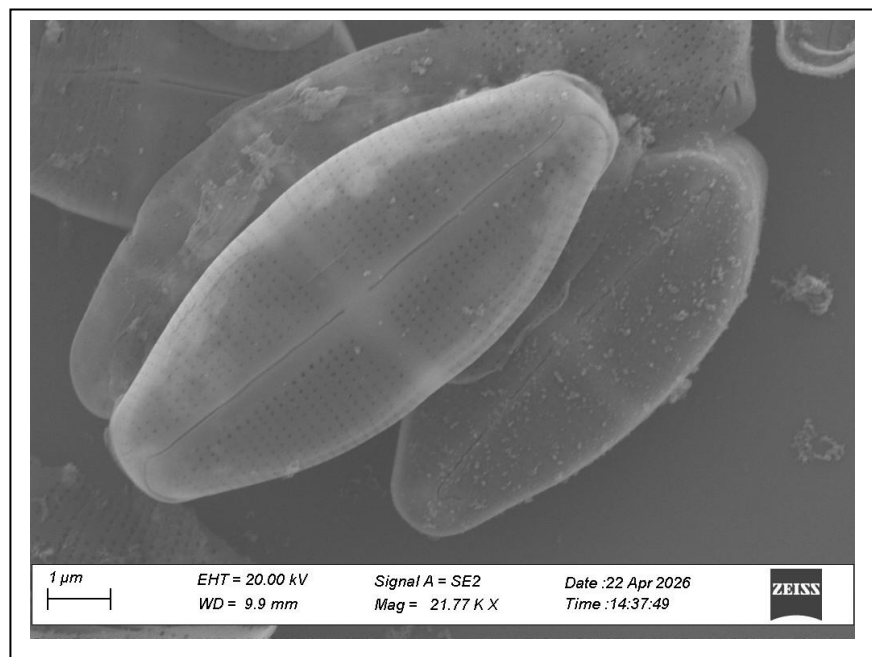


FATTY ACID YIELD AND COMPOSITION IN THE DIATOM *CRASPEDOSTAUROS* SP.

Effects of pre-treatment, preservation, and storage



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Abstract

Diatoms are unicellular microalgae that produce silicified cell walls, called frustules, a sustainable alternative to high-purity industrial silica, conventionally synthesized from mined quartz sand, the second most used non-renewable resource and primary source of industrial silica. Diatoms also accumulate substantial lipid fractions including polyunsaturated fatty acids (PUFAs) with nutraceutical value and triacylglycerols suitable for biodiesel. Co-producing both would improve resource efficiency and strengthen the economics of microalgal biorefining, yet the practical feasibility in industrial conditions remains largely unexplored. This study characterizes the fatty acid (FA) profile of *Craspedostauros* sp., a previously uncharacterized production strain, while evaluating the influence of pre-treatment method (untreated control, dilute acid at 1 % H₂SO₄, and probe ultrasonication), preservation strategy (freeze-drying and refrigerated wet storage), and storage duration (up to 4 months) on FA yield and composition. FAs were extracted using a modified Folch-based solvent method and evaluated against the traditional Folch method by gas chromatography-mass spectrometry (GC-MS). The two extraction methods were found to return similar average yields but with performance dependent on pre-treatment. Frustule integrity was assessed by scanning electron microscopy (SEM), showing no visible frustule breakage following dilute acid treatment in either fresh or freeze-dried biomass. Total extractable FA content ranged from 0.70 to 8.72 % dry weight (6.95 to 87.16 µg mg⁻¹ dry weight) across treatments, with a profile notably rich in the PUFAs EPA and ARA. The highest yield was recovered from freeze-dried biomass after 4 months of room-temperature storage following dilute-acid pre-treatment, indicating that FA content depleted over 3 months (90.6 % loss relative to immediate extraction in untreated biomass) can be substantially recovered. These findings provide the first baseline FA characterization for *Craspedostauros* sp. and identify practical pre-treatment and storage combinations relevant to frustule and lipid co-production at industrial scale.

Sammanfattning

Kiselalger är encelliga mikroalger som bildar skal av kiseldioxid, så kallade frustuler, ett hållbart alternativ till industriell kiseldioxid av hög renhetsgrad som konventionellt utvinns ur kvartssand, den näst mest använda icke-förnybara resursen. Kiselalger ackumulerar även betydande lipidfraktioner, däribland fleromättade fettsyror med hälsofrämjande egenskaper och triacylglyceroler lämpliga för biodiesel. Att utvinna båda fraktionerna skulle förbättra resurseffektiviteten och stärka lönsamheten i mikroalgbaserad bioraffinering, dock är de praktiska möjligheterna under industriella förhållanden till stor del outforskade. Denna studie karakteriserar fettsyraprofilen hos *Craspedostauros* sp., en tidigare icke-karakteriserad produktionsstam och utvärderar hur förbehandlingsmetoder (obehandlad kontroll, utspädd syra med 1 % H₂SO₄ och ultraljudsprob), konserveringsstrategier (frystorkning och kyld våtlagring) och lagringstider (upp till 4 månader) påverkar fettsyornas mängd och sammansättning. Fettsyrorna extraherades med en modifierad Folch-metod baserad på organiska lösningsmedel och utvärderades mot den traditionella Folch-metoden med hjälp av gaskromatografi-masspektrometri. De två extraktionsmetoderna resulterade i liknande genomsnittliga mängder men var beroende av förbehandlingen. Frustulernas integritet bedömdes med svepelektronmikroskopi och visade inga synliga skador efter förbehandling med utspädd syra i färsk eller frystorkad biomassa. Den totala extraherade fettsyramängden varierade mellan 0,70 och 8,72 % av torrsvikt (6,95 till 87,16 µg mg⁻¹ torrsvikt) beroende på behandling och profilen var särskilt rik på de fleromättade fettsyrorna EPA och ARA. Den största mängden erhöles från frystorkad biomassa som förbehandlats med utspädd syra efter 4 månaders lagring i rumstemperatur. Detta indikerar att fettsyror som bedömts försvunna efter 3 månader (90,6 % förlust jämfört med omedelbar extraktion i obehandlad biomassa) i stor utsträckning kan återfås. Dessa resultat utgör den första grundläggande fettsyrakarakteriseringen för *Craspedostauros* sp. och identifierar praktiska kombinationer av förbehandling och lagring som är relevanta för att utvinna både frustuler och lipider i industriell skala.

1. Introduction

1.1 Scientific Background

1.1.1 Redefining Resource Sustainability – Diatoms as Renewable Feedstocks

The global demand for sustainable, biobased raw materials is steadily increasing as society seeks viable alternatives to fossil-derived and resource-intensive materials (Ahmed et al., 2021).

Conventional renewable resources, including agricultural crops and forestry products, remain vital for sustaining the global food supply and providing key raw materials. However, their reliance on arable land, freshwater, and fertilizers intensifies competition within the growing food production and increases pressure on terrestrial ecosystems (Davis et al., 2024). It is therefore imperative to explore novel feedstocks that deliver high productivity while alleviating these ecological stressors.

A similar challenge can be observed in the case of silica production. Traditionally, high-purity silica is derived from geological sources such as quartz sand, the second most used non-renewable resource (Singh et al., 2025). Its extraction and processing involve mining, beneficiation, and energy-intensive purification, with substantial environmental drawbacks (Grbeš, 2016). Quantitative green chemistry metrics (DOZN 2.0) rank industrial silica production poorly in terms of energy consumption, waste generation, and use of hazardous reagents (Brambila et al., 2022). Moreover, high-purity industrial silica for technical applications such as photovoltaics, electronics and optics demands complex, resource-intensive processing such as acid leaching, ultrasonic-assisted purification, roasting, and quenching to control impurities like iron, aluminium, and sodium that degrade material performance (Long et al., 2024). These steps intensify energy and chemical use, generate additional waste and increase the environmental footprint of silica production (Brambila et al., 2022), underscoring the long-term challenges of conventional supply chains and the need to explore more sustainable alternatives (Karande et al., 2021).

Against this backdrop, microalgae, particularly diatoms, have emerged as promising candidates and present a compelling biological alternative. Diatoms can grow in freshwater, brackish, marine, wastewater, and effluent water from fish farms. Thereby avoiding competition for freshwater with arable agriculture (Davis et al., 2024). Their metabolic versatility enables high productivity under controlled cultivation, and they naturally accumulate substantial quantities of lipids and fatty acids, offering feedstocks for biofuels, nutraceuticals, specialty chemicals and other high value bioproducts (Wang & Seibert, 2017; Geng et al., 2025). Simultaneously, diatoms synthesize nanoporous cell walls of biogenic silica, called frustules, which encapsulate the organism (Tramontano et al., 2020). These frustules consist of naturally pure, non-contaminated silica that requires minimal post-processing to achieve high material quality. Practically, they represent a renewable, biologically fabricated alternative to conventionally mined synthetic silica. As such, this naturally nanoengineered material has inspired the use of frustules in nanotechnology and advanced material design (Rabiee et al., 2021), offering a sustainable pathway to high-purity silica production and reducing the ecological footprint associated with traditional mining and refining (Karande et al., 2021).

1.1.2 Diatoms – Biology, Geology and Classification

Diatoms belong to the phylum Bacillariophyta and are a highly diverse group of single-celled, photosynthetic eukaryotic microorganisms, characterized by their complex silica cell wall, the frustule. They exist as single cells or as colonies in various forms including chains, filaments, tubes, or star-like clusters (Round et al., 1990). They inhabit almost every aquatic environment, including polar ice-shelves, tropical oceans, estuaries, lakes, rivers as well as damp terrestrial habitats where they are found in the soil and on the moist surfaces of rocks and on some higher plants (Nichols et al., 1993; Johansen in Smol & Stoermer, 2010). They occupy niches as planktonic, benthic, attached to or living in symbiosis with other organisms (Simon et al., 2009). Due to this variety in habitat, they exhibit a remarkable range of sizes and morphologies. In fact, diatoms constitute the most abundant and diverse group of eukaryotic microalgae numbering in the tens of thousands of described species (Mann & Vanormelingen, 2013) and are among the most important primary producers in aquatic ecosystems. Diatoms' collective photosynthesis accounts for an estimated 40 % of the total oceanic primary production and roughly 20 % of total global primary productivity (Tréguer et al., 2018). They form the base of aquatic food webs and perform a significant role in the biogeochemical cycles of carbon and silicon (Round et al., 1990; Smol and Stoermer, 2010). In the carbon cycle, they contribute to the biological pump, exporting carbon to deep waters (Tréguer et al., 2018) and constitute the single largest biological sink for dissolved silica in the oceans (Tréguer et al., 2021). Diatoms are divided into two major groups, centric and pennate, based on the symmetry of the frustule. Centric diatoms are radially symmetric which possess disc-like or cylindrical forms (Figure 1, right), while pennate diatoms are bilaterally symmetric, exhibiting elongated boat-shaped forms that are mirrored across a central plane (Figure 1, left) (Round et al., 1990).

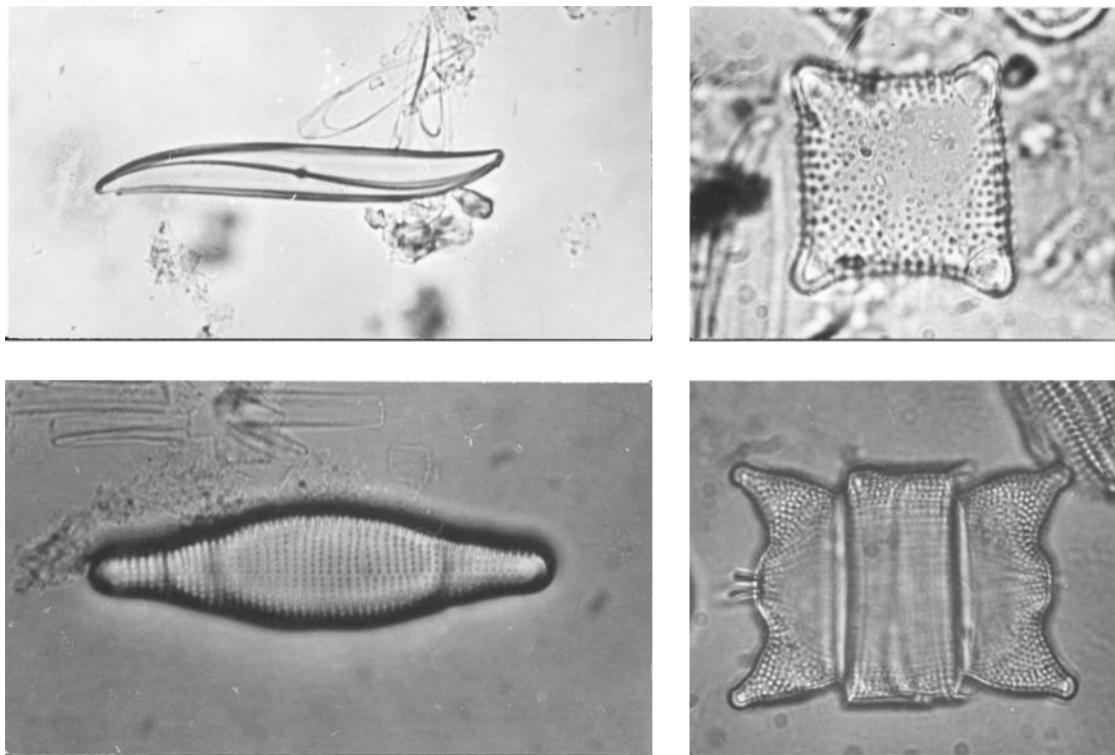


Figure 1. Light micrograph examples of pennate diatoms (left column) and centric diatoms (right column), reproduced from Edsbagge (1968).

1.1.3 *Craspedostauros* sp.

In this study, we focus on the species *Craspedostauros* sp. (Figure 2), a not yet identified species of the genus *Craspedostauros*. It is a relatively small genus of 19 taxonomically accepted species listed on AlgaeBase (Guiry & Guiry, 2022). *Craspedostauros* are bi-raphid pennate diatoms distinguished by a raphe on each valve, i.e. a slit or opening on the frustule that facilitates the secretion of mucilaginous material used for cell motility (Cox, 2006). *Craspedostauros* was formally separated from the genus *Stauroneis* by Eileen Cox in 1999. It is distinguished by a silica stauros, an internal transverse rib running across the centre of the valve (Cox, 1999).

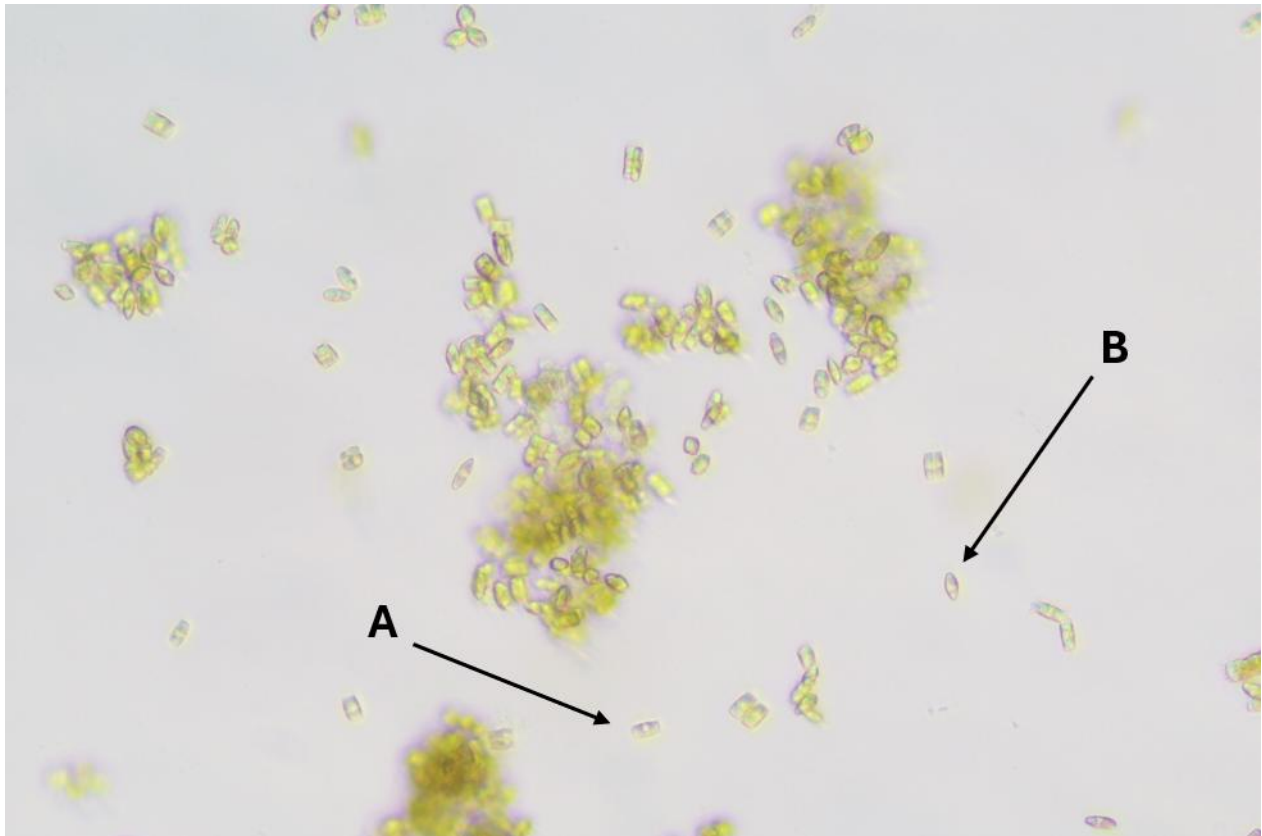


Figure 2. Inverted light microscope image of *Craspedostauros* sp. monoculture, prepared as a droplet on slide. Cells present as small, elongated yellow-green cells enclosed within their frustules. Both girdle view (rectangular profile, example A) and valve view (oval profile, example B) are discernible as solitary cells or aggregates.

Species of *Craspedostauros* occur mainly in marine environments and are often epizoic or epiphytic, growing attached to living substrates (Al-Handal et al., 2024). For example, recent work has found *Craspedostauros* species living on sea turtles and sea turtle-associated barnacles in the Mediterranean and South Atlantic, highlighting their adaptation attachment strategies (Majewska et al., 2020). However, free-living strains of *Craspedostauros* have also been isolated from plankton samples (Al-Handal et al., 2024).

1.1.4 Frustules – Biological Function and Applications

A defining feature of the diatoms is the frustule. The frustule is a rigid, intricately patterned cell wall made of biogenic hydrated amorphous silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$). Structurally it consists of two

overlapping valves, held together by girdle bands that encircle the cell perpendicular to the valves (Round et al., 1990). Silica deposition in diatoms is a biologically controlled process in which specialized proteins (*silaffins*) and polyamines catalyse the polymerization of dissolved silicic acid into amorphous silica. This occurs within intracellular compartments known as silica deposition vesicles (SDVs). These vesicles mediate the controlled formation of the frustule, which is subsequently exocytosed to encase the cell. The resulting architecture is highly porous and species-specific (Figure 3), ranging from simple symmetric shapes to elaborate ribbed and perforated patterns (Lechner & Becker, 2015).

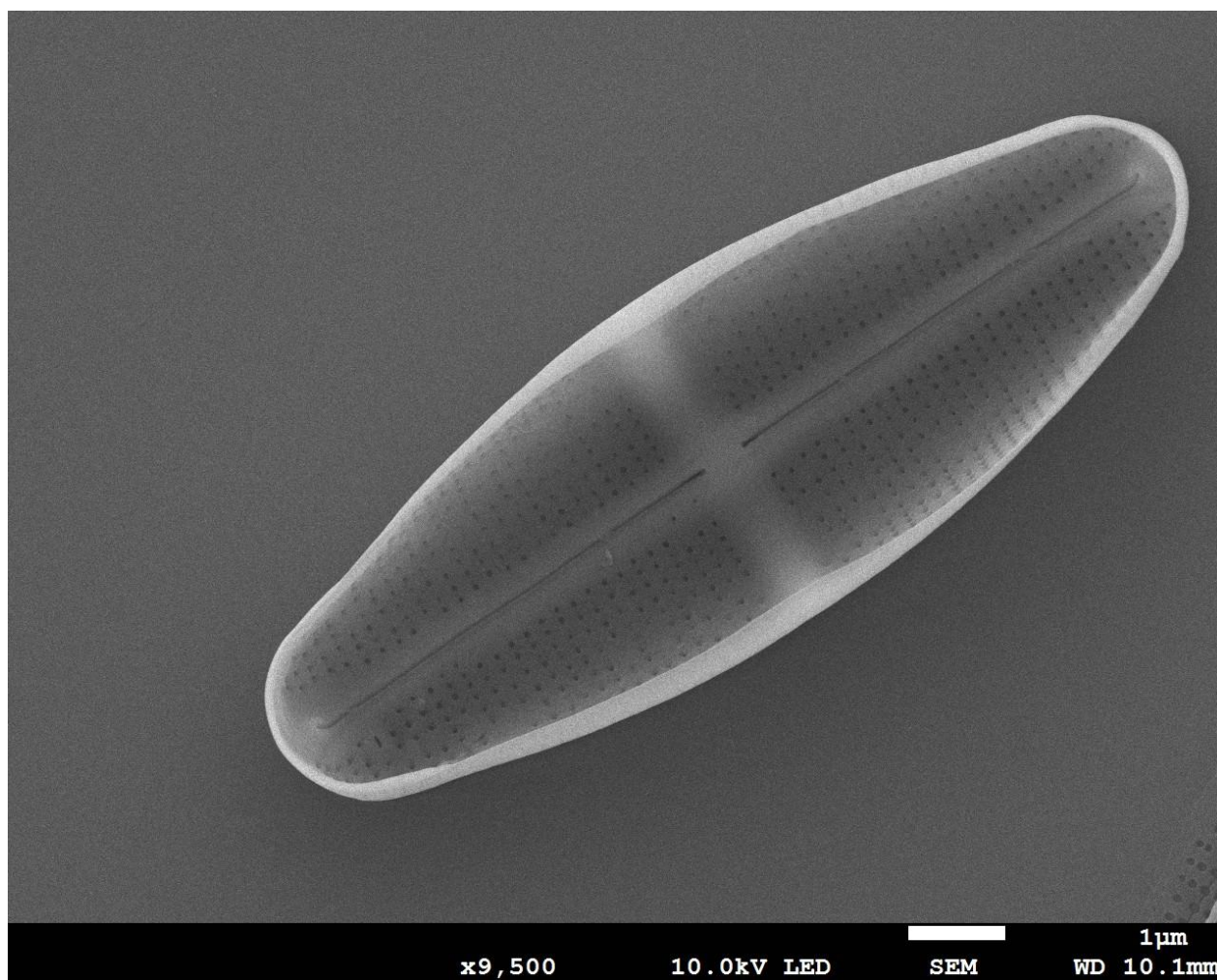


Figure 3. Scanning electron microscope (SEM) image of the interior of an individual *Craspedostauros* sp. valve.

The frustule's porous architecture occurs on the nano- and micrometre scale, featuring elements such as pores (areolae), slits, and intricate ornamentation (Tramontano et al., 2020). These structural features play essential roles in providing mechanical strength and possibly serve as a defence mechanism against grazers (Pančić et al., 2019). Furthermore, the pores form the only interface between the cell and the surrounding environment (Round et al., 1990) and are therefore central to the organism's biological processes and diffusive capabilities (Mitchell et al., 2013). They facilitate the selective transport of gases, nutrients, water, and metabolic waste, enabling the uptake of molecules such as CO₂ while excluding unwanted biological entities, including viruses. In addition, the pores modulate sunlight to optimize photosynthetic performance and provide

protection against harmful ultraviolet radiation (UVR, 280-400 nm) (De Tommasi et al., 2017; Aguirre et al., 2018; Yang et al., 2023).

These features make frustules attractive for diverse industrial applications, ranging from biosensors and drug delivery to advanced filtration systems and solar cells (Rabiee et al., 2021). Materials that exploit the frustule's structural properties can detect volatile compounds via photoluminescence, focus radiation, filter nanoparticles, and attenuate UVR (Rogato & De Tommasi, 2020). By incorporating diatom frustules into the production process of dye-sensitized solar cells, the result was a 35 % increase in efficiency (Bandara et al., 2020), and recently 101 % increased efficiency (Bandara et al., 2023).

1.1.5 Lipids – Composition, Biological Function and Use

Within the frustule lies the protoplasm, an organic matrix rich in proteins, carbohydrates, pigments, and lipids (Yi et al., 2017). Of these, pigments and lipids are of particular interest due to their biochemical diversity and economic potential and are therefore considered high-value compounds (Levasseur et al., 2020). While pigments typically account for less than 1 % of the cellular dry weight in diatoms (Hildebrand et al., 2012), lipids represent a substantially higher fraction, about 15–25 % of dry weight (Yi et al., 2017), although this is highly species and condition-dependent (Section 1.1.6).

Diatoms produce a wide variety of lipids that can be broadly classified into two groups with different biological functions, membrane lipids and storage lipids (Gurr et al., 2016). The fatty acid (FA) components of these lipids typically range from 14 to 22 carbons in chain length (Zulu et al., 2018), encompassing FAs that contain no double bonds (saturated fatty acids (SFAs)), one double bond (monounsaturated fatty acids (MUFAs)), or two or more double bonds (polyunsaturated fatty acids (PUFAs)) (Gurr et al., 2016). To describe the FAs, conventional FA nomenclature will be used alongside common FA names. FAs are denoted as C:XnY, where C represents the total number of carbon atoms in the chain, X indicates the number of double bonds, and Y denotes the position of the double bond closest to the methyl end of the chain. Throughout this thesis, the modern notation is used, however, older literature frequently employs the equivalent and interchangeable ω (omega) notation (Figure 4).

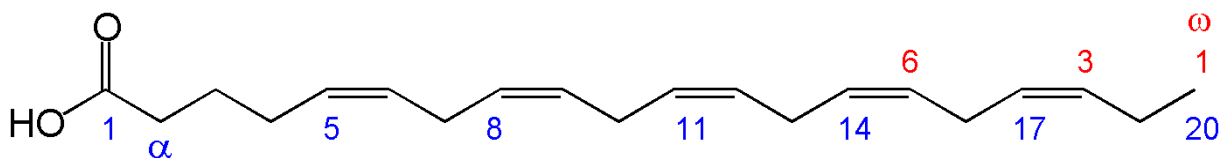


Figure 4. Chemical structure of eicosapentaenoic acid (EPA) denoted 20:5n3 or equivalently 20:5 ω 3 in omega notation. The three components of the shorthand are read as follows: number of carbons in the chain: 20, number of double bonds: 5, position of the double bond closest to the methyl end: 3. Figure adapted from Edgar181 (2007), public domain, via Wikimedia Commons. Retrieved from https://en.wikipedia.org/wiki/Eicosapentaenoic_acid

Common FAs in diatom lipids include large amounts of long-chain saturated fatty acids (LCSFAs C13:0 – C21:0) such as myristic acid (14:0) and palmitic acid (16:0), MUFAs such as palmitoleic acid (16:1n7), and the omega-3 PUFA eicosapentaenoic acid (EPA, 20:5n3, Figure 4) (Nichols et al., 1993; Torstensson et al., 2013; Zulu et al., 2018). Another omega-3 PUFA found in diatoms is docosahexaenoic acid (DHA, 22:6n3). Values across multiple species report the relative

abundance (% of total FA) of EPA and DHA fractions exhibit substantial variability, with EPA ranging from 12.30 to 23.66 % of total FAs and DHA from 2.85 to 19.20 % of total FAs (Zulu et al., 2018). This variability is attributed to species-specific and environmental influences which are discussed in Section 1.1.6.

The structural lipids include glycolipids and phospholipids which are integral components of the lipid bilayer that forms cellular and organelle membranes. These assemble into bilayer membranes that maintain cellular and chloroplast integrity and host the photosynthetic machinery. Some glycolipids are essential for photosynthesis and optimize the environment for complexes in the thylakoid membranes, the internal chloroplast membranes where the reaction occur (Gurr et al., 2016). These lipids also influence the organization of light-harvesting complexes and electron carriers, thereby directly impacting the photosynthetic efficiency (Kates & Volcani, 1966; Opute, 1974; Gurr et al., 2016).

Another important group of membrane lipids are the sterols. Although the chemical profile of sterols in diatoms is not yet fully characterized (Yi et al., 2017), they play a structural role in the cellular membrane by regulating its stability, permeability, and fluidity (Levasseur et al., 2020).

The storage lipids are primarily triacylglycerols (TAGs) which serve as energy-reserving molecules for carbon and energy. TAGs consist of three FAs (taking the places as R_1 , R_2 , and R_3 in Figure 5) esterified to a glycerol backbone (blue area in Figure 5) (Gurr et al., 2016). In diatoms these FAs are most often palmitic (16:0) and palmitoleic (16:1) acids (Zulu et al., 2018) with lower amounts of myristic (14:0) and eicosapentaenoic (20:5) acid (Abida et al., 2015). The TAGs are stored in cytoplasmic lipid droplets and are visible in many diatom cells (Zulu et al., 2018).

In addition to being found in esterified forms, any FA can in principle occur in non-esterified form as a free fatty acid (FFA). FFAs constitute a minor lipid class in cellular extracts (Christie & Han, 2010). Unlike the esterified lipids described above, FFAs are not linked to a glycerol backbone or other carrier molecule. Although typically present at low levels in intact cells, FFAs can accumulate as artefacts when lipases cleave ester bonds in complex lipids during cell damage or prolonged sample storage (Gurr et al., 2016; Christie & Han, 2010).

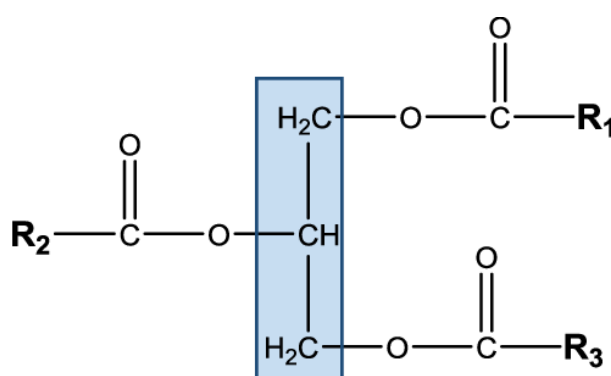


Figure 5. General structure of a TAG molecule. R_1 , R_2 , and R_3 represent different FAs. The blue area demarcates the glycerol backbone of the molecule. Reproduced from Berry et al. (2019).

1.1.6 Environmental Influences and Biotechnological Applications

It is of the utmost importance to note that FA compositions in diatoms are highly dynamic, varying markedly between species and being strongly influenced by environmental and culture conditions (Kates & Volcani, 1966; Volkman et al., 1989). Under optimal (species-dependent) growth, FAs are primarily synthesized into structural membrane lipids including glycolipids, which contain long-chain PUFAs (LC-PUFAs) such as EPA (20:5n3) and DHA (22:6n3). Under these conditions, membrane lipids constitute approximately 5–20 % of dry cell weight. Under

unfavourable conditions, lipid biosynthesis shifts towards TAG accumulation. Hu et al. (2008) reported average total lipid contents across multiple diatom species under normal and stress conditions increasing from 22.7 % to 44.6 % dry weight, with the increase consisting primarily of TAGs. These changes are governed by a complex interplay of biological growth factors including nutrient availability, PAR (photosynthetically active radiation, 400–700 nm), temperature, and CO₂ (Zulu et al., 2018).

Light intensity significantly affects both total lipid content and FA composition. Field and outdoor experiments on Arctic diatoms demonstrated by Leu et al. (2016) that elevated natural near-surface PAR reduces PUFA proportions and growth. In *P. tricornutum*, FA composition was unaffected across mid-range light intensities (60–750 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), whereas lower and higher irradiance were found to enhance TAG accumulation. Complete darkness increased the relative proportions of palmitic acid (16:0), palmitoleic acid (16:1), and EPA (20:5n3) alongside 2.3 times increase in total lipids, though at the expense of growth (Zulu et al., 2018).

Temperature is another key factor. *P. tricornutum* cultured at 10 °C promotes EPA (20:5n3) accumulation, whereas 20–25 °C shifts composition towards palmitic acid (16:0) and palmitoleic acid (16:1) (Zulu et al., 2018). In the polar diatom *N. lecointei*, higher relative PUFA proportions, notably EPA (20:5n3), hexadecatetraenoic acid (16:4n4) and hexadecatrienoic acid (16:3n4), were observed at 3 °C compared to -1.8 °C, though total FA content and relative MUFA proportions were higher at the lower temperature. Reduced salinity further increased FA content at -1.8 °C where salinity 10 reached a higher amount compared to salinity 20 and 35 (Torstensson et al., 2019). Increased dissolved CO₂ reduced total FA content in *N. lecointei* at -1.8 °C but had no effect near the species' optimal growth temperature (5 °C), suggesting temperature and salinity exert a stronger influence over lipid accumulation than CO₂ in this species (Torstensson et al., 2013; 2019).

Nutrient deprivation generates particularly noticeable metabolic changes. Nitrogen limitation in *P. tricornutum* promotes accumulation of 16:0 and 16:1 while reducing LC-PUFAs such as EPA (Zulu et al., 2018). Cui et al. (2023) reported total lipid and TAG content in *A. coffeaeformis* increased from 28.22 % to 44.05 % and from 10.41 % to 25.21 % after five days of N starvation. Phosphorus limitation provokes a milder but comparable shift, increasing TAG content by 60 % in *P. tricornutum* (Abida et al., 2015).

By deliberately selecting species and tuning growth conditions, it is possible to shift FA distributions between PUFA-rich membrane lipids and storage TAGs, a principle directly exploitable in biotechnological contexts (Zulu et al., 2018; Hu et al., 2008). Cultivation under conditions close to the species optimum favours polar lipid synthesis, enriching cells in LC-PUFAs, notably the omega-3s EPA (20:5n3) and DHA (22:6n3), and the omega-6 ARA (20:4n6) which in human health have documented roles in cardiovascular protection, anti-inflammatory responses, and neural development, with ARA being particularly critical for infant brain development (Yi et al., 2017; Nilsson et al., 2020). Conversely, imposing stress conditions shifts metabolism towards TAG accumulation, allowing the potential use of diatoms as third-generation biofuel feedstocks (Dhanker et al., 2024). Their TAG profiles, enriched in C14 and C16 FAs, yield biodiesel with lower viscosity and superior performance compared to that derived from green microalgae (Zulu et al., 2018).

1.2 Contextual Background

1.2.1 Swedish Algae Factory

One company that is already capitalizing on the unique properties of diatoms is Swedish Algae Factory AB (SAFAB), a Swedish biotech firm that cultivates specific diatom strains at its facility in Kungshamn. SAFAB operates as a part of a circular production system in which effluents from a land-based salmon farm serve as the growth medium while residual biomass is converted into biogas and fertilizer. The company markets purified frustules under the trade name Algica® which exhibits the functional properties mentioned before. Algica® has reportedly demonstrated applications in enhancing solar cell efficiency, providing UVR protection, and serving as a controlled-release material in wound care, antifouling, and personal care products (Swedish Algae Factory, n.d.).

By utilizing the naturally occurring nanoscale architecture of diatom frustules, SAFAB demonstrates the practical feasibility of converting biological silica into commercially relevant materials. This approach highlights the potential for controlled diatom cultivation to provide a renewable and sustainable source of advanced silica, reducing dependence on traditional mining and chemical processing while opening new avenues for bioproduct innovation. Furthermore, extracting additional high-value compounds from SAFAB's biomass could enhance resource efficiency and reduce waste. Efficient extraction of these compounds has the potential to substantially increase the economic value of SAFAB's biomass and improve the resource efficiency and sustainability of the cultivation system.

1.2.2 Study Rationale and Objectives

Despite growing interest in diatoms as sustainable sources of both biogenic silica and bioactive compounds (Dhanker et al., 2024), SAFAB does not currently extract lipids, FAs or other high-value compounds from diatom's biomass. Scaling extraction methods to industrial levels remains challenging and co-extraction processes require careful assessment for potential integration. Accurate assessments of yield and co-product value are needed to justify industrial development. The strategies that seek to sustainably utilize the entire cell, both frustules and lipids, face further challenges. The practicality of growing microalgal biomass industrially is that harvested material cannot always be processed immediately. Variability in growth rates, fixed downstream processing capacity, and logistical constraints mean that biomass is often held in interim storage prior to extraction. During this period, the biomass remains metabolically active. The choice of storage method, temperature, and duration all have measurable effects on lipid integrity that must be taken into consideration.

The *aim* of this thesis is therefore fourfold. First, to evaluate the solvent-based lipid extraction procedure from Wigert (2025) building on the Folch method. Second, to evaluate cell-lysis treatments as a possible means of improving extraction yield and its effect on structural integrity of the silica frustules. Third, to characterise the lipid and FA composition of the diatom production strain *Craspedostauros* sp. and fourth, investigate changes in FA composition under varying practical storage and preservation conditions. Together, these provide baseline data to inform future assessments of process feasibility, economic viability, and scalability. They address both the analytical and bioprocess dimensions of integrating diatom biomass into a dual yielding biorefinery framework. By addressing these aims, the study further contributes to broader goals of sustainable biomanufacturing: maximizing resource efficiency, minimizing energy use and

waste, and preserving the utility of co-products while advancing the potential of diatoms as renewable sources of high-value biomolecules for applications in material science, biofuels, nutraceuticals, and other bioproduct sectors.

1.3 Methodological Background

1.3.1 Analytical Workflow

To investigate the lipid class distribution and FA composition of the marine diatom *Craspedostauros* sp., this thesis applies an analytical workflow commonly used in lipid biochemistry and microalgal research. The study includes solvent-based lipid extraction to isolate total lipids, thin-layer chromatography (TLC) for separation of major lipid classes and gas chromatography (GC) for FA analysis. Together, these approaches enable characterisation of lipid composition at both the class and FA levels.

1.3.2 Storage and Preservation Methods

When storing the harvested biomass, endogenous lipases, intracellular lipolytic enzymes, hydrolyse membrane and storage lipids into FFAs and polar glycerol components, resulting in altered FA profiles (Ryckebosch et al., 2011). Ryckebosch et al. found that 48 h of wet storage at 4 °C caused pronounced lipolysis in *P. tricornutum*, resulting in more than 3 times as much FFA compared to freeze dried after the same time. Dalheim et al. (2021) made similar discoveries in *Porosira glacialis* where the percentage FFA of total FAs increased from 31 % after 24 h of wet storage at 4 °C to 66 % after 14 days. Temperature markedly accelerates this deterioration. The same biomass reached approximately 70 % FFA within 24 h at 20 °C. Moreover, PUFA content declined minutely at 4 °C, but showed a more pronounced loss at 20 °C (Dalheim et al., 2021). In contrast to wet biomass, freeze-dried samples exhibit lower levels of FFAs after 48 h at 4 °C, indicating reduced lipolytic activity immediately after processing. However, FFA content was shown to increase during long-term storage from 4 to 6 mg/g DW (independent of temperature: -20 °C, 4 °C and 20 °C) after 14 days with further increase after 35 days at 4 °C and 20 °C (Ryckebosch et al., 2011).

1.3.3 Solvent-Based Lipid Extraction

Solvent extractions were established as the gold standard for isolating lipids from biological samples in the 1950s and remain foundational in the field to the present day. The method was established by the works of Folch et al. (1957). They used a mixture of chloroform and methanol (2:1, v/v) that dissolves the lipophilic fractions in 20 times wet sample before adding 0.2 volume of water or an aqueous saline wash to a final ratio of 8:4:3, v/v/v, CHCl₃:MeOH:H₂O. This induced a biphasic separation where lipids accumulate into the lower chloroform phase (known as the organic) while more polar non-lipids remain in the upper methanol-aqueous phase.

Building on this, Bligh and Dyer (1959) adapted Folch's approach but adjusting the solvent ratios to reach a final system of ~1:1:0.9, v/v/v, CHCl₃:MeOH:H₂O, to accommodate samples with higher water content, and became widely favoured for dilute biological samples or wet biomasses. Both methods achieve favourable lipid yield extractions if applied properly, but differences in protocol: solvent ratios, biomass characteristics, ionic concentration, choice of ion,

acidification, can bias recovery of certain lipid classes by cater to specific sample types (Saini et al., 2021). Furthermore, many modern protocols adopt dichloromethane and methanol (2:1, v/v) as a safer solvent alternative, reducing hazardous exposure to chloroform. Dichloromethane (DCM) has been shown to be a suitable alternative to chloroform and demonstrated comparable total lipid recoveries, lipid class distributions, and FA profiles across a range of biological sample types (Cequier-Sánchez et al., 2008).

More recently, Wigert (2025) developed a modified extraction method in collaboration with Swedish Algae Factory, specifically adapted for frustule and lipid co-production from diatom biomass. The method adopts DCM:MeOH (2:1, v/v) as the extraction solvent and a 5 % NaCl aqueous wash to drive biphasic separation and was validated by TLC.

1.3.4 Lysis Methods

The rigidity of the diatom frustule presents a significant physical barrier to intracellular lipid extraction, making cell disruption a critical preparatory consideration (Hogan et al., 2021). Among the mechanical cell disruption methods available, ultrasonication is widely applied. The method relies on high-frequency acoustic waves to generate cavitation, a rapid formation and violent collapse of microbubbles. This produces localised extremes of temperature and pressure that fracture the cell wall and membrane. The method is valued for its relatively low operating temperatures compared to autoclaving or microwave treatment, and for requiring no addition of beads or reagents that must be removed downstream (Günerken et al., 2015). Ultrasonication can be applied via an ultrasonic bath, in which transducers at the base of a water-filled vessel transmit waves indirectly to the sample, or via a probe-based sonicator (also referred to as a sonic wand) in which a vibrating titanium tip is immersed directly into the sample (Figure 6),

concentrating acoustic energy at the point of contact and generating more intense localised cavitation (Günerken et al., 2015). The latter approach is particularly effective at achieving total cell lysis and has been demonstrated specifically for diatom frustules. Svenning et al. (2020) confirmed that probe sonication at 20 kHz for 10 minutes produced complete lysis of *Porosira glacialis* cells, whereas manual grinding, microwave treatment, lyophilisation, and Ultra-Turrax (a high-speed rotor-stator homogeniser) were all ineffective at disrupting the frustule under the same conditions. The efficacy of sonication is, however, species- and condition-dependent, and the energy input required for complete disruption can vary considerably (Halim et al., 2012).

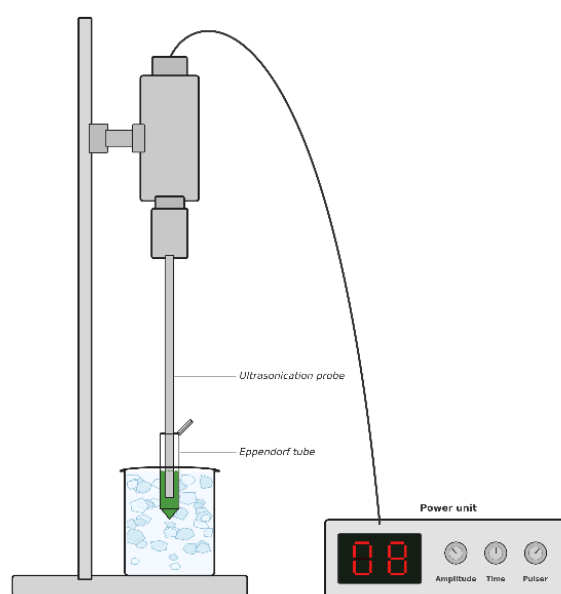


Figure 6. Schematic illustration of the ultrasonication cell disruption setup used for lipid extraction from *Craspedostauros* sp. The ultrasonication probe is mounted on a stand and connected to the power unit. Biomass in Eppendorf tube is placed in an ice bath during sonication to prevent thermal degradation of lipids. The power unit settings: amplitude, time, and pulser were adjusted according to the optimisation protocol. Figure created using Python (v3.10) with matplotlib.

Probe-based ultrasonication is therefore a common laboratory approach for achieving total cell lysis, valued for experiments requiring complete and quantitative release of intracellular contents, such as comprehensive lipid profiling or total biomass characterisation (Günerken et al., 2015).

However, in practice, approaches that improve extraction efficiency without destroying the frustule are often preferred. Among the chemical disruption strategies, dilute acid pre-treatment has attracted attention as a relatively low-cost and scalable alternative to mechanical and enzymatic methods (Günerken et al., 2015). Acids act by causing poration of the cell membrane and wall, degrading structural components such as proteins and polysaccharides, thereby allowing solvents to more readily access intracellular lipids (Günerken et al., 2015; Saini et al., 2021). Mineral acid treatments using hydrochloric acid (HCl) and sulphuric acid (H₂SO₄) have been applied to microalgal biomass with species- and target-dependent outcomes. Heat may be applied alongside the acid to accelerate these reactions. However, ester bonds within acyl lipids are themselves susceptible to acid-catalysed hydrolysis at elevated temperatures, which can compromise lipid integrity (Saini et al., 2021). For example, acid-catalysed hot-water extraction using H₂SO₄ has been found to favour the recovery of SFAs from the diatom *Phaeodactylum tricornutum*, while unsaturated FAs, including EPA and DHA, were more effectively captured with solvent extraction alone (Otero et al., 2017). H₂SO₄ has also been used to extract intracellular polysaccharides from the diatom *Odontella aurita* (Xia et al., 2014). This dual functionality of H₂SO₄ may be advantageous within biorefinery frameworks by enabling the recovery of multiple product classes from a single pre-treatment.

1.3.5 Thin-Layer Chromatography

TLC is a well-established and simple technique that remains widely applied in lipid analysis due to its simplicity, low cost, and broad applicability. Since its rapid adoption in the 1960s, TLC has been recognised as a quick method, requiring minimal preparation for separating and qualitative analysis of lipid classes (Wall, 2005). Although high-performance liquid chromatography (HPLC) and mass spectrometry-based approaches now dominate quantitative lipid analysis, TLC remains widely used for lipid class separation. Advances in commercial pre-coated and high-performance TLC plates have improved reproducibility and resolution (Christie, 2019). Combined with the ability to use selective staining reagents to directly visualise lipid classes and functional groups, these features make TLC particularly well suited for qualitative profiling and the comprehensive visual assessment of complex lipid mixtures, especially when class-specific detection is required (Wall, 2005; Christie, 2019).

In TLC, lipid extracts are applied near the base of a polar stationary phase, most commonly silica gel coated plate, and developed in a liquid organic solvent system that ascends the plate by capillary action. Lipid classes migrate according to their polarity and interactions with the stationary phase, enabling effective separation of neutral and polar lipids (Wall, 2005). Polar lipids, including phospholipids and glycolipids, interact strongly with the polar silica gel stationary phase through hydrogen bonding and electrostatic interactions, resulting in relatively slow migration during chromatographic development. In contrast, non-polar lipids such as TAGs, sterol esters, and hydrocarbons exhibit minimal affinity for the silica surface and are therefore more readily solubilised by the mobile phase, leading to greater migration distances toward the solvent front (Wall, 2005; Gurr et al., 2016). By selecting solvent systems with carefully adjusted polarity, the balance between retention and elution can be systematically controlled, allowing

lipid class separations to be positioned in regions of the plate that provide optimal resolution (Wall, 2005).

For example, TLC employing a non-polar mobile phase such as hexane/diethyl ether/acetic acid, promotes the migration of neutral lipid classes including TAGs and FFAs, resulting in their separation into distinct bands toward the upper region of the plate (Figure 7). Polar lipids interact more strongly with the silica gel stationary phase and are therefore retained at or near the origin of the sample spot (Kates, 1986). Conversely, TLC performed using a polar mobile phase will differentiate lipid classes within the polar fraction. By calculating the relative migration distance of each lipid class in relation to the mobile front, solvent system specific R_f -values can be determined. Comparison of these values against authenticated standards allows lipid classes to be distinguished and qualitatively identified (Wall, 2005).

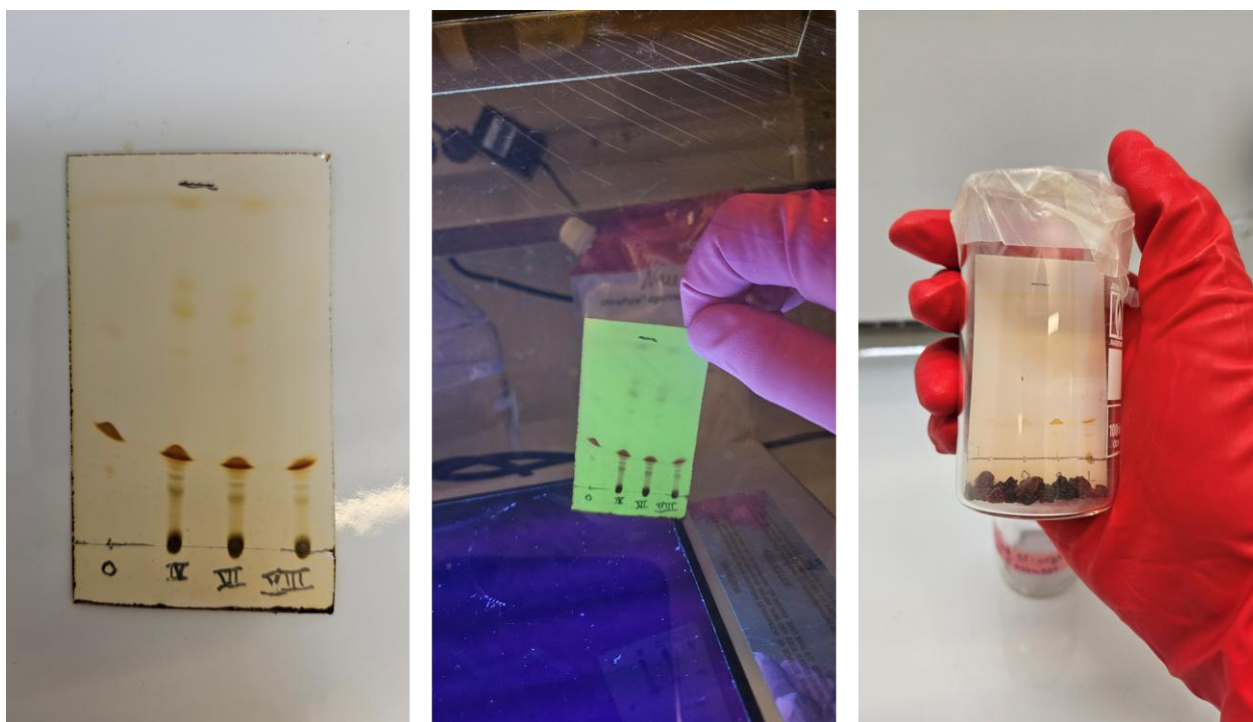


Figure 7. TLC plates from lipid class profiling. Left, developed plate after removal from the iodine chamber. Middle, the same plate visualised under UV radiation at 254 nm prior to iodine staining, where lipid bands appear as dark spots. Right, plate inside the sealed iodine chamber during staining, with iodine flakes dispersed in silica powder visible at the base.

Most lipids are not inherently visible on TLC plates and therefore require visualisation using chemical or physical detection methods. One such method is iodine vapour staining, a rapid and non-destructive technique commonly employed for the temporary visualisation of lipid classes. Iodine vapour interacts reversibly with unsaturated bonds in lipid molecules, producing a transient yellow to brown coloration that allows chromatographic bands to be readily located, marked, and subsequently recovered for further analysis (Wall, 2005).

1.3.6 Gas Chromatography and Transesterification

GC is a cornerstone technique for determining FA composition in lipid-rich organisms and has more than likely been the most important tool for facilitating the separation of lipids. Because FAs in biological samples typically are bound in complex lipids, they are not sufficiently volatile for GC analysis and must first be converted to fatty acid methyl esters (FAMES) (Christie & Han, 2010). The process of converting FAs to FAMES is achieved by transesterification, a reaction in which the organic group of an ester is replaced with the organic group of an alcohol which may be catalysed by either chemical reagents or biological catalysts such as enzymes. The conversion of FAs to methyl esters through reaction with methanol is a specific example of such a reaction. For example, in the case of TAGs, which consist of three fatty acyl chains esterified to a glycerol molecule (Gurr et al., 2016), transesterification with methanol yields three FAME molecules and glycerin, as illustrated stoichiometrically and schematically below (Figure 8. R₁–R₃ denote the original fatty acyl chains). Methanol reacts with the acyl group (R–CO–) of each ester bond, cleaving it from the glycerol backbone and yielding the corresponding FAME (R–COOCH₃) (Christie & Han, 2010).

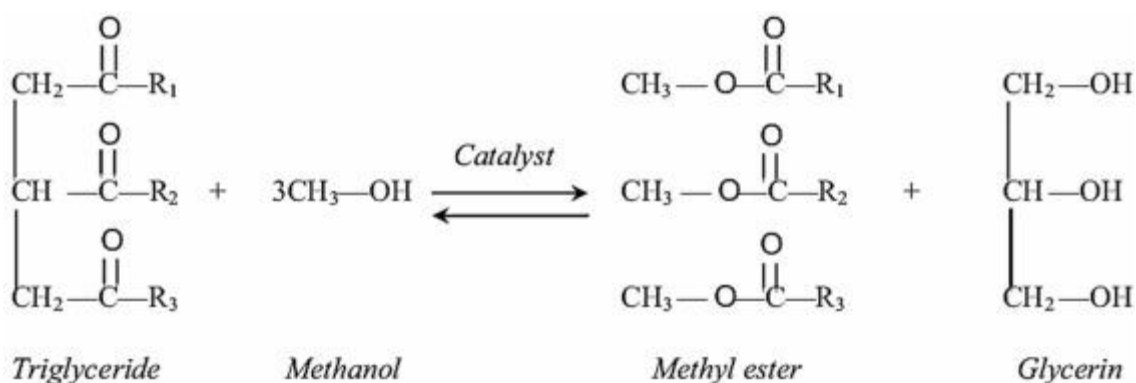
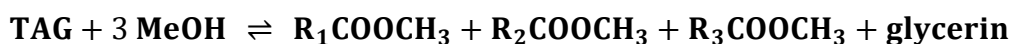


Figure 8. Acid-catalysed transesterification of a TAG. In the presence of an acid catalyst and MeOH, the three fatty acyl chains (R₁, R₂, R₃) are cleaved from the glycerol backbone and converted to their corresponding FAMES with glycerol as a co-product. Figure reproduced from Tazikheh et al. (2022).

Acid-catalysed transesterification yields high recoveries from both simple and complex lipid classes (Lepage & Roy, 1986), converting ester-bound FAs linked via ester bonds to the glycerol backbone in TAGs and phospholipids, as well as FFAs and amide-bound FAs in sphingolipids, into their corresponding methyl esters (Christie & Han, 2010; Lepage & Roy, 1986). Once the reaction is complete the resulting FAMES are sufficiently volatile at the temperatures employed during GC analysis (Gurr et al., 2016).

Following injection into the GC, the sample is rapidly vaporized in the heated inlet and transferred onto the analytical column, where it is continuously transported by an inert carrier gas while the temperature increases according to a programmed temperature regime. Separation is governed by their volatility and interactions with the stationary phase and is achieved as the temperature increase reduces the vaporized FAMES retention over time. As a result, individual FAMES exit the column (elute) sequentially at characteristic retention times, enabling their

subsequent identification. FAMES that interact more strongly with the stationary phase are retained for longer periods, whereas more volatile or weakly interacting compounds elute earlier. Generally, elution order of FAMES is governed by carbon chain length, degree of unsaturation and, for saturated isomers of identical chain length, the position of structural features along the carbon backbone (*n*-position), with shorter-chain, more unsaturated, and higher *n*-number isomers eluting earlier (Figure 9) (Christie & Han, 2010).

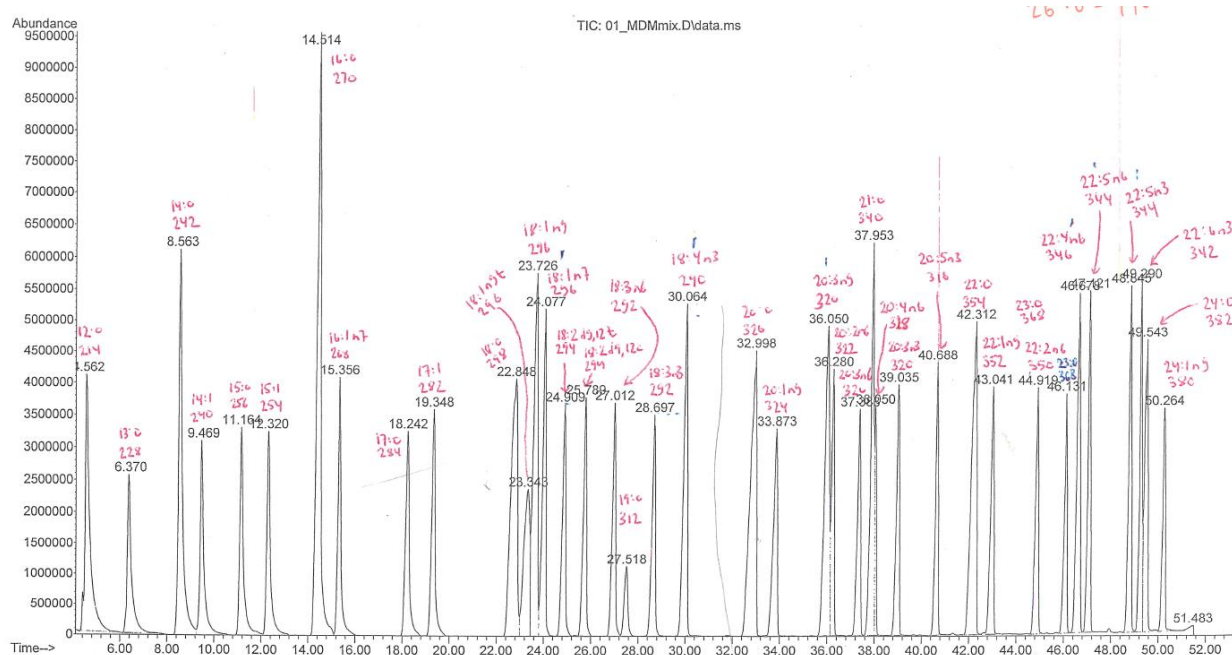


Figure 9. TIC of a FAME reference mixture analysed on the GC-MS used in this study. Peaks are annotated with FA shorthand notation (exemplified in Figure 4) and corresponding molecular ion mass. Retention time (min) is shown on the x-axis and detector response (abundance) on the y-axis.

While chromatographic separation defines when individual compounds elute, chemical identification requires additional structural information. For this purpose, the GC is coupled to a mass spectrometer (MS) (Christie & Han, 2010). After separation in the GC column, the eluate is drawn into the ion source in the MS. Once inside, the gaseous FAME molecules are ionized by electron ionization (EI). A filament emits a beam of high-energy electrons. When one strikes a FAME molecule, it can knock out a valence electron, thereby producing a positive ion (the molecular ion, M^+). The collision often imparts excess energy that causes the molecule to fragment, generating a collection of ions (Gross, 2017).

After ionization, the resulting ions are separated in the MS according to their mass-to-charge ratio (m/z) (Christie & Han, 2010). In GC-MS, this is commonly achieved using a quadrupole mass analyser (QMA). The QMA generates a dynamic electric field that selectively stabilizes the trajectories of ions with a specific m/z , allowing them to reach an electron multiplier detector, while ions with unstable trajectories are filtered out. By sequentially altering the selected m/z value across a predefined scan range, ions transmitted to the detector are converted into an

electrical signal, allowing ion intensity to be recorded at each m/z and producing a mass spectrum displaying relative ion abundances. The summed ion intensity across the scanned m/z range at each time point generates a total ion chromatogram (TIC), which reflects the chromatographic separation (Gross, 2017). This process yields characteristic EI mass spectra of associated FAMES, identification relies on interpreting these spectra (Christie & Han, 2010). In EI, FAMES produce a M^+ together with fragment ions arising from cleavage of the carbon chain. The relative intensity of the molecular ion varies between compounds and may range from clearly detectable to very weak or absent. When fragmentation dominates, fragment ions prevail and the molecular ion shows little or no signal, whereas greater molecular ion stability results in a more prominent M^+ peak (Christie & Han, 2010). This variability in molecular ion intensity, together with the overall fragmentation pattern, gives each compound a characteristic mass spectral fingerprint that supports compound identification (Figure 10) (Christie & Han, 2010; Gross, 2017).

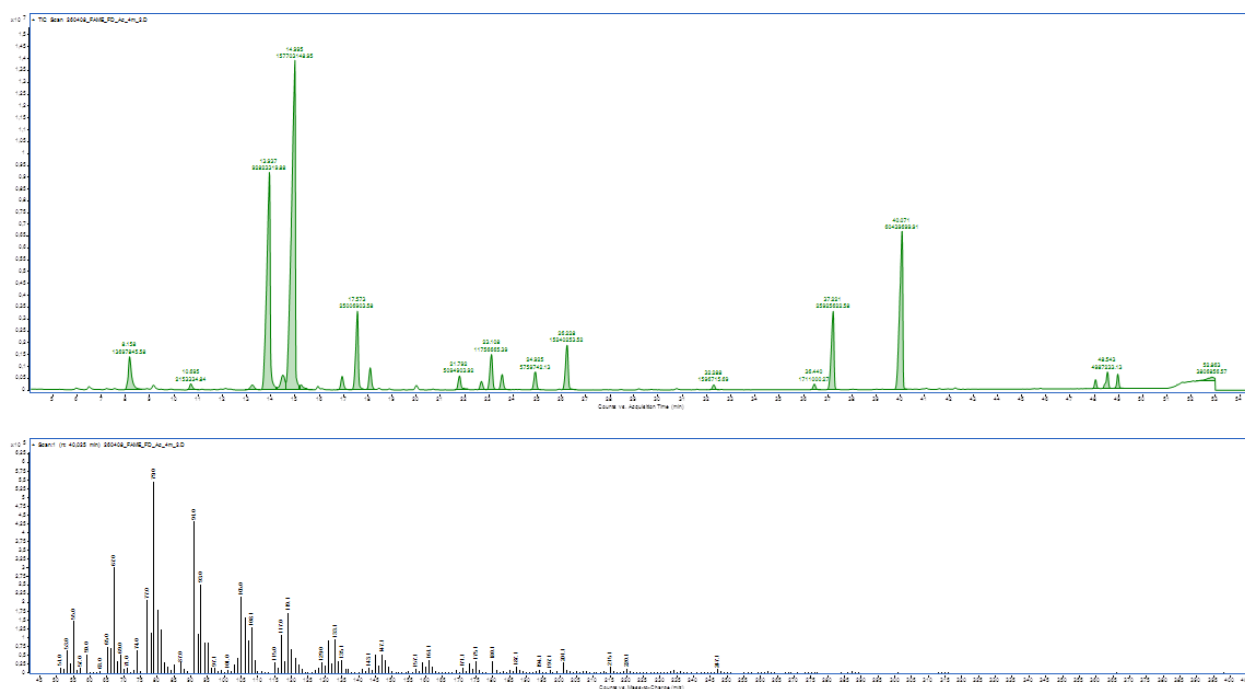


Figure 10. TIC obtained from GC–MS output for sample FD_Ac_4m. The upper panel shows retention time (min) on the x-axis and the summed ion intensity across the scanned m/z range at each time point on the y-axis. Each peak corresponds to a FAME. Below is the EI mass spectrum for the EPA peak, showing m/z on the x-axis against relative ion abundance (%) on the y-axis. The fragmentation pattern gives the compound its characteristic spectral fingerprint used for identification.

Using automated spectral matching software, each FAMES spectrum is compared with reference library entries, and the best match is determined based on the pattern and relative intensities of characteristic ions, a high match score provides confidence in the compound assignment (Christie & Han, 2010; Gross, 2017). Furthermore, the presence of specific fragment ions that are diagnostic for specific structural features can offer supplementary evidence (Christie & Han, 2010). Together, agreement between mass spectral library matching, diagnostic fragment ions, and retention behaviour provides a reliable basis for compound identification (Christie & Han, 2010; Gross, 2017). However, retention time is commonly only used as an additional confirmation, since absolute retention times are not definitive because they depend on the precise column condition at the time of analysis and are affected by variables including operating

temperature, column age, and the condition of the stationary phase. When available, analysis of authentic standards under identical conditions may be employed, as this allows retention times to be accurately referenced and detector response to be normalized. Depending on the sample, different types of standards may be employed, including commercial standard mixtures of accurately known composition. The use of appropriate standards, closely resembling the samples being analysed, is essential for ensuring reliable identification (Christie & Han, 2010).

Quantitative analysis can be achieved simultaneously through the addition of an accurately known amount of an internal standard, prior to transesterification and GC analysis (Christie & Han, 2010). An odd-chain FA such as C17:0 and C21:0 is commonly used for this purpose due to their rare natural occurrence in lipid compositions (Christie & Han, 2010; Gurr et al., 2016). The quantitative analysis is performed by integrating chromatographic peak areas and expressing the abundance of each FAME relative to the known amount of the internal standard, thereby enabling calculation of absolute FA content per sample (Christie & Han, 2010).

2. Materials and Methods

2.1 Experimental Design

The experimental work was conducted at Natrium, University of Gothenburg and follows the analytical workflow outlined in Section 1.3.1. The design addresses the four objectives stated in Section 1.2.2. Total lipids were isolated by solvent extraction, major lipid classes profiled by TLC, and FA composition determined by GC-MS after acid-catalysed transesterification. Frustule integrity was assessed by scanning electron microscopy (SEM). All treatments were performed in triplicate. Samples were coded by biomass state, lysis pre-treatment and storage duration. State was either fresh (LC, formerly live culture) or freeze-dried (FD). Pre-treatments were untreated control (non), probe ultrasonication (son) or dilute acid (Ac). Storage duration is given in days or months (0d, 1d, 3m or 4m). For example: FD_Ac_4m refers to freeze-dried, dilute-acid-treated biomass extracted after 4 months of storage.

2.2 Chemicals

Dichloromethane (DCM, $\geq 99\%$), methanol (MeOH, $\geq 99\%$), chloroform, heptane, n-hexane (99%), diethyl ether (99%), and glacial acetic acid (100%), sulfuric acid (H_2SO_4), sodium hydroxide (NaOH), sodium chloride ($\geq 99\%$), and hydrogen peroxide (30% w/w), iodine flakes and silica powder, polyethyleneimine (PEI, branched, average $M_n \sim 60,000$ by GPC, average $M_w \sim 750,000$ by LS, 0.1 wt% in H_2O), nitrogen ($\geq 99.999\%$), palmitic acid ($\geq 98\%$), oleic acid ($\geq 99\%$), and myristic acid ($\geq 99\%$), 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine (di-17:0-PC) in chloroform.

2.3 Biomass Sources and Cultivation

Initial protocol development was performed on a mixed-species diatom biomass supplied by Swedish Algae Factory AB (SAFAB, Kungshamn, Sweden) as centrifuged, frozen biomass. Once sufficient biomass had been cultivated in-house, all experimental work was transitioned to a monoculture of *Craspedostauros* sp.

The monoculture was maintained in culture flasks in a climate chamber under a 16 h light: 8 h dark regime at a constant 16 °C. PAR in the cultivation area was 38.25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ on average (range 31.13 - 44.83 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, LI-COR LI-1000 DataLogger). 50 ml of SAFAB's proprietary medium were added to each flask weekly after which the flask was closed, inverted and gently homogenised. The cap was subsequently rested loosely on the flask to allow gas exchange while preventing airborne contaminants. Culture development was monitored by inverted light microscopy (Nikon camera) and by pulse-amplitude modulated (PAM) fluorometry. Cultures were harvested once sufficient biomass had accumulated.

2.4 Harvesting and Dry-Weight Determination

Biomass was harvested by scraping the interior walls of each culture flask with a cell scraper and collecting the resulting suspension of cells in medium, where required, a small aliquot was reserved as inoculum for continued cultivation. The collected volume was concentrated by repeated centrifugation at $2000 \times g$ in 15 ml falcon tubes (5 min per cycle), supernatant was discarded and the pellets pooled into a single final tube. Biomass intended for freeze-drying was processed as described in Section 2.5, otherwise, the pooled pellet was resuspended in a small volume of medium and homogenised on a vortex mixer until the pellet was fully dispersed. Subsequently, 1 ml aliquots of the volume were transferred to pre-weighed Eppendorf tubes, one of which was reserved for dry-weight determination. To verify equal distribution, the tubes were centrifuged at $2000 \times g$ and visually inspected. Dry weight was determined gravimetrically, the reserved 1 ml aliquot was transferred to a pre-weighed crucible and dried in a microwave oven at 500 W for 1 min, and the dry mass was recorded and used to calculate absolute FA yields (Figure 11). Equal distribution of biomass across aliquots was assumed.



Figure 11. Gravimetric dry-weight determination of fresh biomass. Left: pre-weighed crucible containing a 1 ml aliquot of freshly harvested *Craspedostauros* sp. biomass suspension prior to drying. Right: the same crucible after microwave drying at 500 W for 1 min to constant mass.

2.5 Storage and Preservation Treatments

To assess the effects of post-harvest storage on lipid integrity, three storage regimes were applied. Fresh biomass was extracted immediately after harvest (day 0). Wet biomass was stored at 4 °C and extracted after 7 or 14 days. For freeze-dried biomass, concentrated biomass was held at -76 °C and then lyophilised using a VirTis 24DX48 GPF 25L XL-70 general-purpose freeze dryer

(SP Scientific, USA, see Appendix 2 for the full protocol). Freeze-dried material was then stored in sealed containers and extracted after 1 day, 3 months, or 4 months. Immediately prior to extraction, freeze-dried samples were rehydrated by resuspending 50 mg dry biomass in 950 μ l deionised water and briefly homogenised to restore a matrix accessible to solvent extraction.

2.6 Cell-Lysis Pre-Treatments

Three pre-treatment conditions were employed prior to solvent extraction: untreated control, probe ultrasonication and dilute acid. In the untreated control, biomass was transferred directly to extraction without mechanical or chemical disruption. For probe ultrasonication, biomass (1 g wet, or 50 mg dry resuspended in 950 μ l deionised water) was weighed into an Eppendorf tube and sonicated with an ultrasonic probe at 80 % amplitude for 2 sec on / 1 sec off pulses for 5 min, with the tube held in an ice bath. For dilute acid pre-treatment, biomass (1 g wet, or 50 mg dry resuspended in 950 μ l deionised water) was suspended in 10 ml of 1.1 % (v/v) H_2SO_4 in deionised water (final concentration $\sim$ 1 % v/v) and held at 65 $^\circ\text{C}$ for 30 min on a magnetic stirrer hotplate (IKA C-MAG HS 7) controlled by an external contact thermometer (IKA ETS-D5). Samples were cooled to room temperature before proceeding to lipid extraction.

2.7 Lipid Extraction

Total lipids were extracted using a modified Folch procedure in which DCM was substituted for chloroform. 1 gram of biomass (wet or rehydrated freeze-dried) was transferred to a 50 ml glass beaker and 20 ml of DCM:MeOH (2:1 v/v) was added using a volumetric flask. Two variants were compared: the standard Folch protocol (1957), in which the solvent (DCM:MeOH) was added alone, and the variant of Wigert (2025), in which 10 ml of deionised water was added together with the solvent (DCM:MeOH), difference visualised schematically in Figure 12. In both cases, the mixture was stirred at $\sim$ 800 rpm with a 2 cm magnetic stirrer bar for at least 60 min at room temperature.

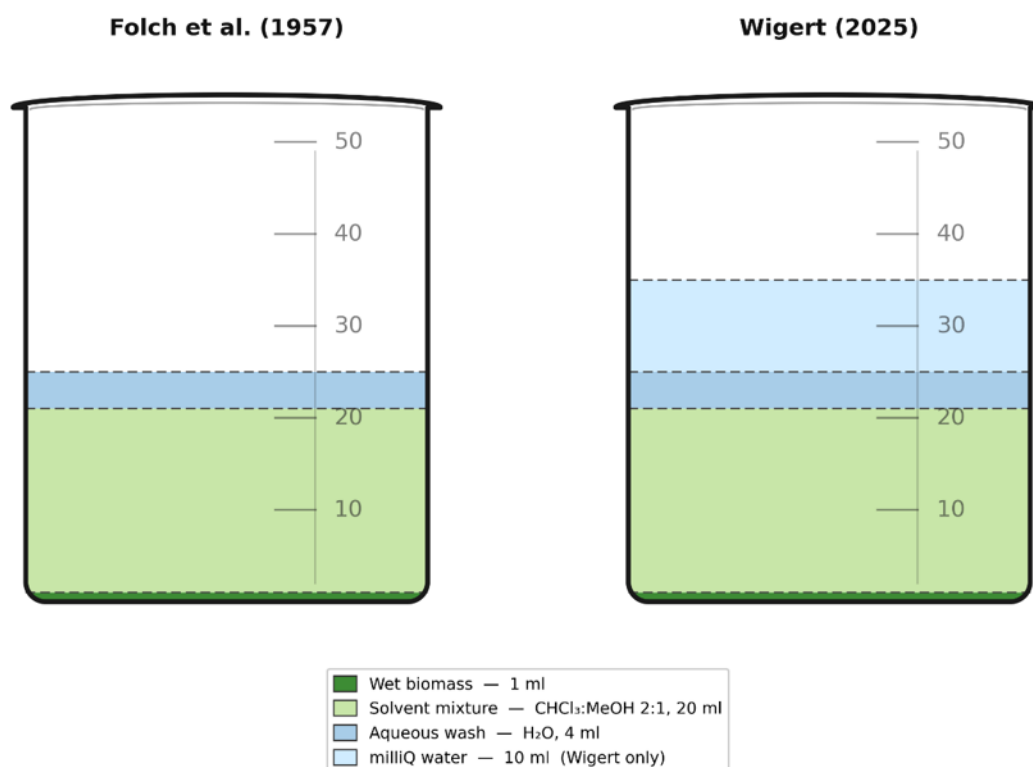


Figure 12. Schematic illustration of the solvent extraction protocols for total lipid isolation from wet microalgal biomass. Left: standard Folch et al. (1957) procedure. Right: variant after Wigert (2025).

In the standard Folch protocol, phase separation was induced after stirring by addition of 4 ml 5 % (w/w) NaCl to the mixture. The suspension was then transferred to a 15 ml falcon tube and centrifuged at $5000 \times g$ for 5 min to accelerate phase separation and pellet residual cell material (Figure 13, left, Folch variant after centrifugation). The upper aqueous phase was removed and discarded, and the lower organic phases was transferred to a scintillation vial, care was taken to not disturb the interfacial pellet which was reserved in-tube for SEM analysis (Section 2.11).

In Wigert's variant, the added water induced phase separation already during stirring. The suspension was divided between two 15 ml centrifuge tubes and centrifuged at $5000 \times g$ for 5 min, the upper aqueous phase removed and discarded, and the lower organic phases pooled in a 50 ml beaker. The wash of 4 ml 5 % (w/w) NaCl was then added to the recovered organic phase and stirred for 5 min at 600 rpm before the mixture was centrifuged again under identical conditions to drive a second separation (Figure 13, right, Wigert variant after the second centrifugation). As above, the upper aqueous phase was removed and discarded and the lower organic phases was transferred to a scintillation vial, pellet reserved for SEM analysis (Section 2.11). In both cases, the scintillation vial with the solvent was allowed to evaporate in a ventilated fume hood overnight. Dried lipid residues were stored at $-20\text{ }^{\circ}\text{C}$ until GC-MS analysis.

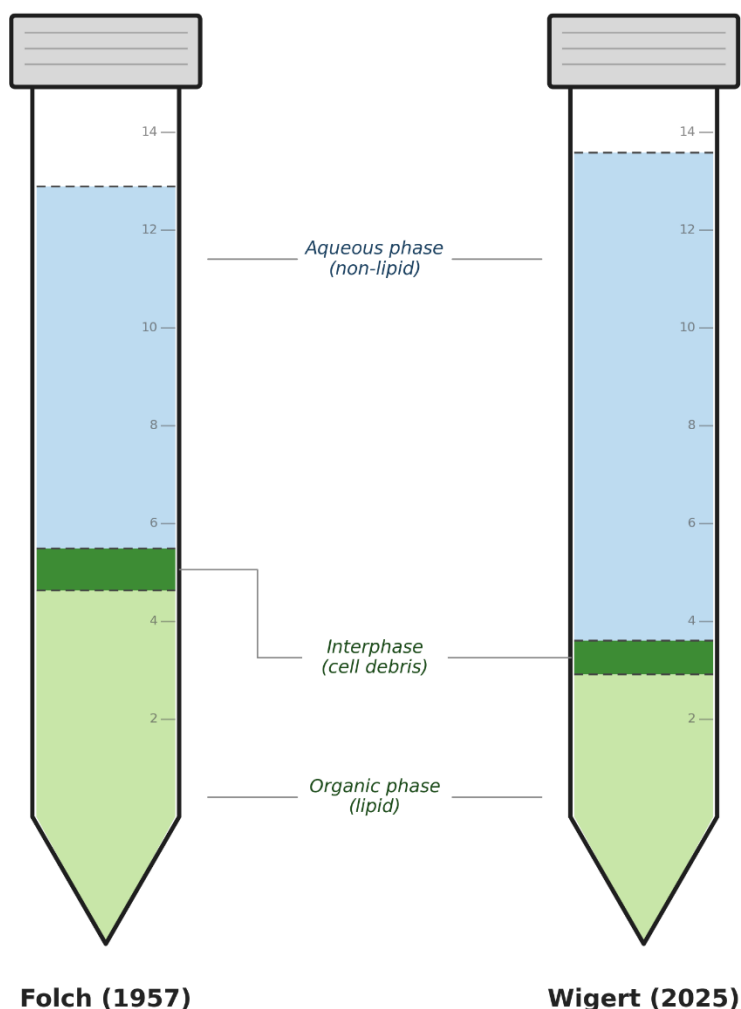


Figure 13. Schematic illustration of 15 ml centrifuge tubes following phase separation by centrifugation in the Folch et al. (1957) and Wigert (2025) extraction protocols. In both methods, centrifugation induces separation into a lower organic phase (DCM, containing the lipid fraction), an interphase retaining cell debris and an upper aqueous phase (MeOH/H₂O) containing non-lipid contaminants such as sugars, amino acids, and salts. Figure created using Python (v3.14.3) with matplotlib.

2.8 Lipid Class Profiling by Thin-Layer Chromatography

Lipid class composition was profiled by TLC on aluminium-backed silica gel F254 plates (20 × 20 cm, Merck) cut into 7 × 4 cm strips, with a baseline marked 1 cm from the bottom edge. Dried lipid extracts were redissolved in DCM. Aliquots of 2.5 µl of each sample and of reference standards (palmitic, oleic or myristic acid) were applied to the baseline with a micropipette and allowed to dry. Plates were developed in a sealed glass chamber containing 3 ml of mobile phase (hexane:diethyl ether:glacial acetic acid, 90:10:5 v/v/v) until the solvent front had risen to approximately 0.5 cm from the upper edge where the front was marked after which the plate was air dried.

Plates were first visualised under UV radiation at 254 nm and developed in a sealed iodine chamber containing iodine flakes dispersed in silica powder. Bands were marked and documented

photographically. Lipid classes were identified from R_f values relative to co-spotted reference standards, with neutral classes (TAGs and FFAs) migrating toward the solvent front and polar classes (phospholipids, glycolipids, sphingolipids and sterols) retained closer to the baseline (Figure 7).

2.9 Transesterification

Lipid extracts were converted to FAMES prior to GC-MS analysis by acid-catalysed transesterification. The dry lipid extract was redissolved in 1 ml chloroform. Subsequently, 50 µl aliquot was transferred to a screwcap Kimax tube together with 20 µg of internal standard (di-17:0-PC in chloroform) and the combined solvents were evaporated under a stream of nitrogen. The lipid residue was redissolved in ~1 ml of 2.5 % (w/v) H₂SO₄ in methanol and ~0.5 ml of heptane, each added with a Pasteur pipette. The tubes were capped and incubated in a heating block at 85 °C for 90 min. Once removed from the heating block, the tubes were cooled to room temperature and 1 ml of deionised water was added, the tubes were vortexed and the phases were allowed to separate (Figure 14, right). The upper heptane phase was transferred to a GC vial by Pasteur pipette and held pending GC-MS analysis.

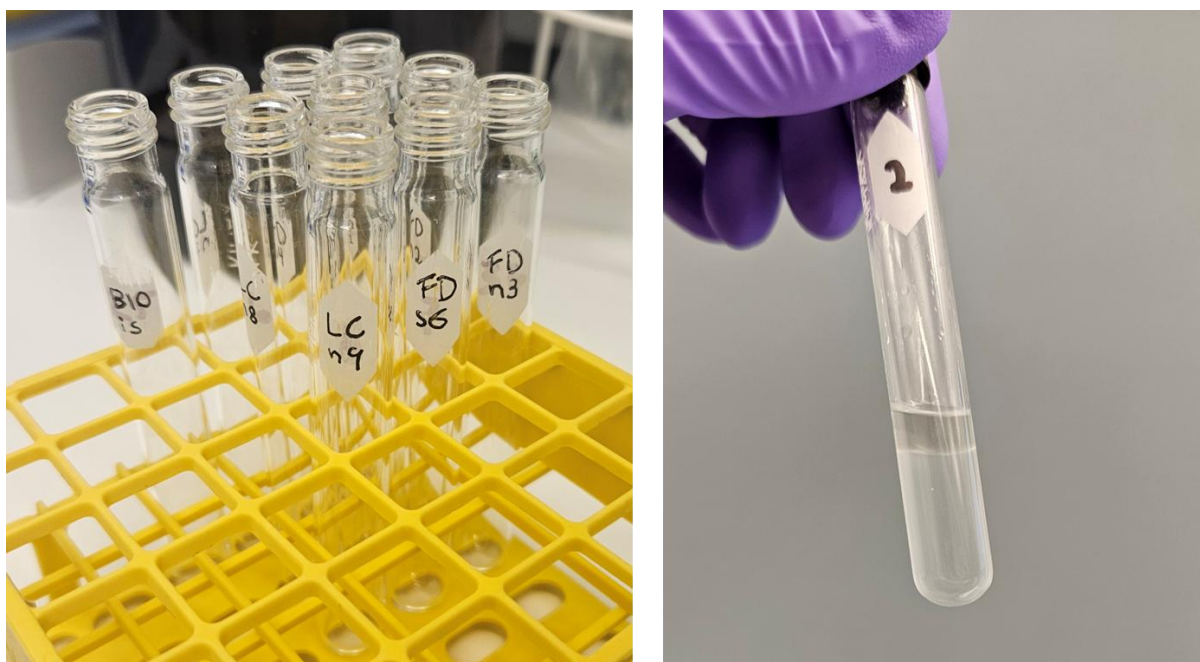


Figure 14. Left, Labelled GC vials prepared for sample loading, representing fresh biomass (LC) and freeze-dried biomass (FD) replicates alongside a blank (B). Right, Kimax tube following transesterification showing phase separation between the upper organic phase (heptane layer containing FAMES) and the lower aqueous phase.

2.10 Gas Chromatography

FAMES were separated by GC: One microlitre of each sample was injected in pulsed splitless mode (40 psi for 1 min) into an Agilent 7820 gas chromatograph and separated on a 30-meter DB-23 capillary column (0.25 mm i.d., Agilent Technologies) with a polar stationary phase and helium as carrier gas at a flow rate of 0.62 ml per min. The column temperature was maintained at 135 °C, increased at 1 °C/min to 150 °C, then at 2 °C/min to 175 °C, and finally at 70 °C/min

to 250 °C, where it was held for 2 min. The FAMES were detected by electron-ionization mass spectrometry on an Agilent 5975 mass selective detector (Agilent Technologies, Palo Alto, CA, USA) operated in scan mode (m/z 50–400) at 70 eV with the transfer line heated to 220 °C. Each sample yielded a TIC, from which FAMES were identified by mass-spectral library matching. TIC peak areas were integrated and individual FAs quantified relative to the internal standard.

2.11 Scanning Electron Microscopy of Frustules

To evaluate the effect of lysis pre-treatments on frustule integrity, biomass from each treatment was prepared for SEM. Residual organic material was removed by oxidative cleaning. 0.05 g of treated biomass was transferred to a 50 ml beaker with 5 ml deionised water and 15 ml 30 % H_2O_2 . The mixture was heated on an IKA C-MAG HS 7 hotplate (250 °C) until the volume had reduced to approximately 5 ml. The cleaned frustules were rinsed with MilliQ water and filtered through a track-etched polycarbonate membrane (3 μ m pore size) using a syringe. The filter was transferred to a 15 ml falcon tube containing ~10 ml MilliQ water and the frustules were released by stirring and brief vortexing. SEM stubs were prepared from silicon wafers glued to aluminium stubs and submerged in 0.1 wt% polyethyleneimine (PEI) in water for 10 min to promote frustule adhesion, excess PEI was rinsed off with deionised water and the stubs were air-dried. The frustule suspension was pipetted onto each stub (target 0.1 wt% frustules in water) and were allowed to settle for 10 min, after which, excess suspension was rinsed off with deionised water and air-dried. Stubs were sputter-coated with 4 nm gold in a Leica EM ACE600 coater (30 mA, 1×10^{-2} mbar, working distance 50 mm, tilt 0°) and imaged on a Zeiss Ultra 55 FEG SEM (Gemini column, Oxford X-max detector) using SmartSEM v6 with Zeiss Microscopy service pack 5.

2.12 Data Processing

The GC-MS output was processed and converted to mass-normalised FA concentrations (μ g FA mg^{-1} DW) using the nmol quantities determined from each GC-MS injection, the fraction of the total lipid extract transferred and the dry weight of the extracted biomass. The known mass of di-heptadecanoyl phosphatidylcholine (di-C17:0-PC, MW 762.1 $g\ mol^{-1}$) added as internal standard prior to transesterification yields two C17:0 methyl ester molecules upon transesterification, 20 μ g of di-C17:0-PC therefore corresponds to $(20 / 762.1 \times 1000) \times 2 = 52.5$ nmol of heptadecanoic acid methyl ester. The GC-MS software integrated raw detector signals into peak areas, and individual FAs were identified by retention time and MS fragmentation patterns. The nmol of each FA in the injection (n_{FA}) was then calculated as:

$$n_{FA} = \frac{A_{FA}}{A_{17:0}} \times 52.5$$

Where A_{FA} and $A_{17:0}$ are the peak areas of the respective FA and the internal standard. The ratio of these reflects the relative abundance of each FA, which when scaled by the known molar quantity of C17:0 (52.5 nmol) gives the nmol of that FA. Knowing the nmol of each FA in the injection, the dry weight of the extracted biomass (m_{DW}), the ratio of total solvent volume to the volume transferred to transesterification ($V_{solvent} / V_{extracted}$), and the molecular weight of each FA as its methyl ester (M_{FAME}), the mass-normalised FA concentration (μ g FA mg^{-1} DW) in the original biomass was calculated as:

$$\mu g \text{ FA } mg^{-1} DW = \frac{n_{FA} \times \frac{V_{solvent}}{V_{extracted}} \times M_{FAME} \times 10^{-3}}{m_{DW}}$$

These calculations were performed for each individual FA using Python (version 3.14.3) with numpy and pandas. The FAs were assigned to their respective class: SFA, MUFA, or PUFA and the resulting long-format dataset was exported as a single CSV file for downstream analysis. The resulting CSV file comprised all treatment groups with $n = 2\text{--}3$ replicates per group. Although all samples were prepared in triplicate, some extractions failed, and two individual replicate outliers were excluded from all plots and statistical testing.

2.13 Statistical Analysis

All statistical analyses and figures were generated using Python (version 3.14.3) with the following libraries: numpy and pandas for data handling, matplotlib for visualisation, scipy for Bray-Curtis distance computations and one-way ANOVA, and statsmodels for two-way ANOVA. For multivariate analyses, scikit-learn was used for principal component analysis (PCA) and non-metric multidimensional scaling (nMDS), and scikit-bio for dispersion testing (median-based PERMDISP).

Two-way ANOVA with interaction was applied to assess the effect of extraction method $\times$ pre-treatment (Folch vs. Wigert comparison), biomass state $\times$ pre-treatment (fresh vs. freeze-dried comparison), and pre-treatment $\times$ storage duration (freeze-dried storage comparison), with total FA yield as the response variable throughout. One-way ANOVA was applied for pre-treatment comparisons within each biomass state (fresh and freeze-dried) and for the fresh biomass wet-storage time series. Multivariate FA composition was assessed using PERMANOVA on Bray-Curtis dissimilarity matrices computed from untransformed $\mu g \text{ mg}^{-1} DW$ values (9999 permutations), with homogeneity of multivariate dispersions confirmed by PERMDISP prior to interpreting PERMANOVA results.

3. Results

This section presents the FA yield and composition results across all treatment conditions. Two replicates were excluded as outliers (Section 3.1). Folch-extracted samples are included only in Section 3.2, where the two extraction methods are compared. All remaining sections rely exclusively on Wigert-extracted, acid-catalysed FAMES. An overview of FA yields and composition is presented in Section 3.3, followed by a multivariate examination of the dataset in Section 3.4. Sections 3.5 to 3.7 present comparisons examining the effects of biomass state, pre-treatment method, and storage duration respectively. Finally, SEM images examining the impact of pre-treatment on frustule integrity are presented in Section 3.8.

3.1 Exclusion of Outliers

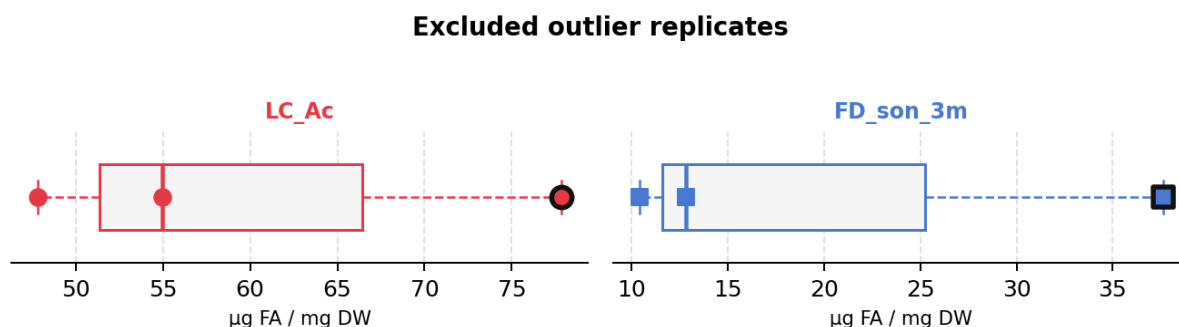


Figure 15. Total FA content per replicate for the two groups containing outlier replicates prior to exclusion. Each point represents one replicate ($\mu\text{g FA mg}^{-1}\text{ DW}$). The black-edged marker denotes the outlier replicate in each group that is subsequently excluded, no other replicates within either group were affected.

Early exploratory inspection of the dataset identified two replicates with values markedly above the range of their respective groups (Figure 15). In both cases the elevated total was confined to a single replicate. The LC_Ac replicate displayed a total FA yield more than 50 % above the group range, driven by an anomalously elevated 18:0 content. The FD_son_3m replicate was similarly elevated relative to its group, with disproportionately high 16:0 and 18:0 concentrations. Both replicates were excluded from all subsequent analyses as individual-replicate outliers, possible technical origins are discussed in Section 4.5.3.

3.2 Comparison of Wigert and Folch Extraction Methods

The Folch and Wigert extraction methods were compared on fresh biomass extracted immediately after harvest, with two out of three pre-treatment conditions, untreated control and probe ultrasonication (Figure 16).



Figure 16. Total FA and FA class composition for untreated control (non) and probe ultrasonicated (son) fresh biomass (LC) extracted with Wigert vs Folch (acid-catalysed, mean $\pm$ SE, $n = 3$ except the Folch LC_son_0d where $n = 2$). Hatched bars indicate standard error.

Table 1. Two-way ANOVA on total FA yield ($\mu\text{g mg}^{-1}$ DW), extraction method and pre-treatment as fixed factors.

Source of variation	df	F	p
extraction	1	0.90	0.373
pre-treatment	1	35.30	0.001
extraction $\times$ pre-treatment	1	20.15	0.003

A significant interaction was detected between extraction method and pre-treatment method (Table 1: $F = 20.15$, $p = 0.003$), indicating that the relative performance of the two extractions depended on whether the biomass had been sonicated or left untreated. For untreated biomass, Folch yielded more total FA than Wigert (51.50 vs $35.53 \mu\text{g mg}^{-1}$ DW), but for sonicated biomass, the outcome reversed and Wigert outperformed Folch (67.56 vs $54.47 \mu\text{g mg}^{-1}$ DW). Within-condition one-way ANOVAs confirmed that the untreated difference was significant ($F = 28.26$, $p = 0.006$), whereas the sonicated difference was not ($F = 4.36$, $p = 0.128$). The main effect of extraction was consequently non-significant ($F = 0.90$, $p = 0.373$), averaged across the two pre-treatments, the methods returned a near-equivalent yield (51.55 vs 52.99 , $\Delta 1.44 \mu\text{g mg}^{-1}$ DW), reflecting the cancellation of two opposing within-condition effects rather than genuine equivalence. Pre-treatment was the major effect ($F = 35.30$, $p < 0.001$): sonicated samples yielded more total FA than untreated samples under both extraction methods. However, the increase was significant for Wigert and numerical for Folch. At the individual-FA level, the same interaction pattern held across nearly all FAs. For untreated biomass, Folch recovered more of every FA

except 16:3, which was recovered in similar amounts by both methods. For sonicated biomass the pattern was reversed, Wigert recovered more of essentially every FA.

3.3 Overall Fatty Acid Yields and Composition

Total FA content spanned over an order of magnitude across treatments (Figure 17), ranging from 6.95 $\mu\text{g mg}^{-1}$ DW (FD_non_3m) to 87.16 $\mu\text{g mg}^{-1}$ DW (FD_Ac_4m). The highest yields came from freeze-dried biomass treated with dilute acid after 4 months of storage (FD_Ac_4m, 87.16 $\mu\text{g mg}^{-1}$ DW), followed by freeze-dried untreated biomass after lyophilisation (FD_non_1d, 74.13 $\mu\text{g mg}^{-1}$ DW) and fresh-biomass probe-ultrasonicated samples (LC_son_0d, 67.56 $\mu\text{g mg}^{-1}$ DW). At the bottom, freeze-dried untreated biomass stored for three months (FD_non_3m, 6.95 $\mu\text{g mg}^{-1}$ DW) yielded approximately one order of magnitude less FA than its 1-day counterpart.

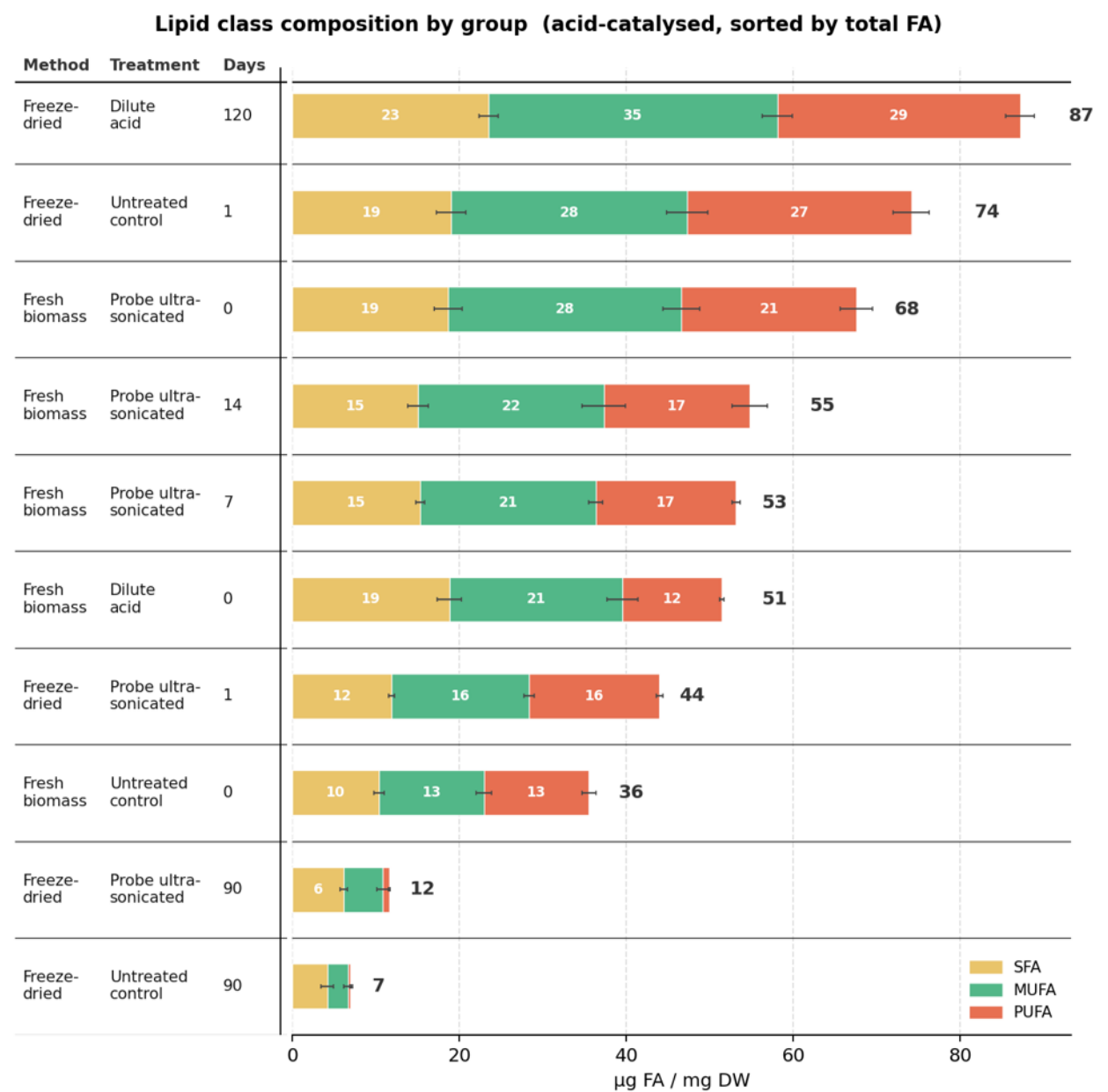


Figure 17. Total FA yield per treatment group separated by FA class (SFA, MUFA and PUFA, acid-catalysed FAMES, mean ± SE, n = 2–3). Treatments are arranged in ascending order of total FA. Within each stacked bar, error bars on the SFA, MUFA and PUFA segments represent the standard error of that class across replicates.

No single factor alone clearly governed the overall yield or FA class composition across pre-treatment conditions, extraction methods, or storage timepoints (Figure 17). FD_Ac_4m had the highest absolute MUFA content ($34.63 \mu\text{g mg}^{-1}$ DW), driven primarily by palmitoleic acid (16:1n7), as well as the highest absolute PUFA content ($29.05 \mu\text{g mg}^{-1}$ DW), with EPA (20:5n3) alone contributing $14.31 \mu\text{g mg}^{-1}$ DW, as well as the highest absolute SFA content ($23.48 \mu\text{g mg}^{-1}$ DW), with 16:0 as the dominant contributor at $18.44 \mu\text{g mg}^{-1}$ DW (Figure 17).

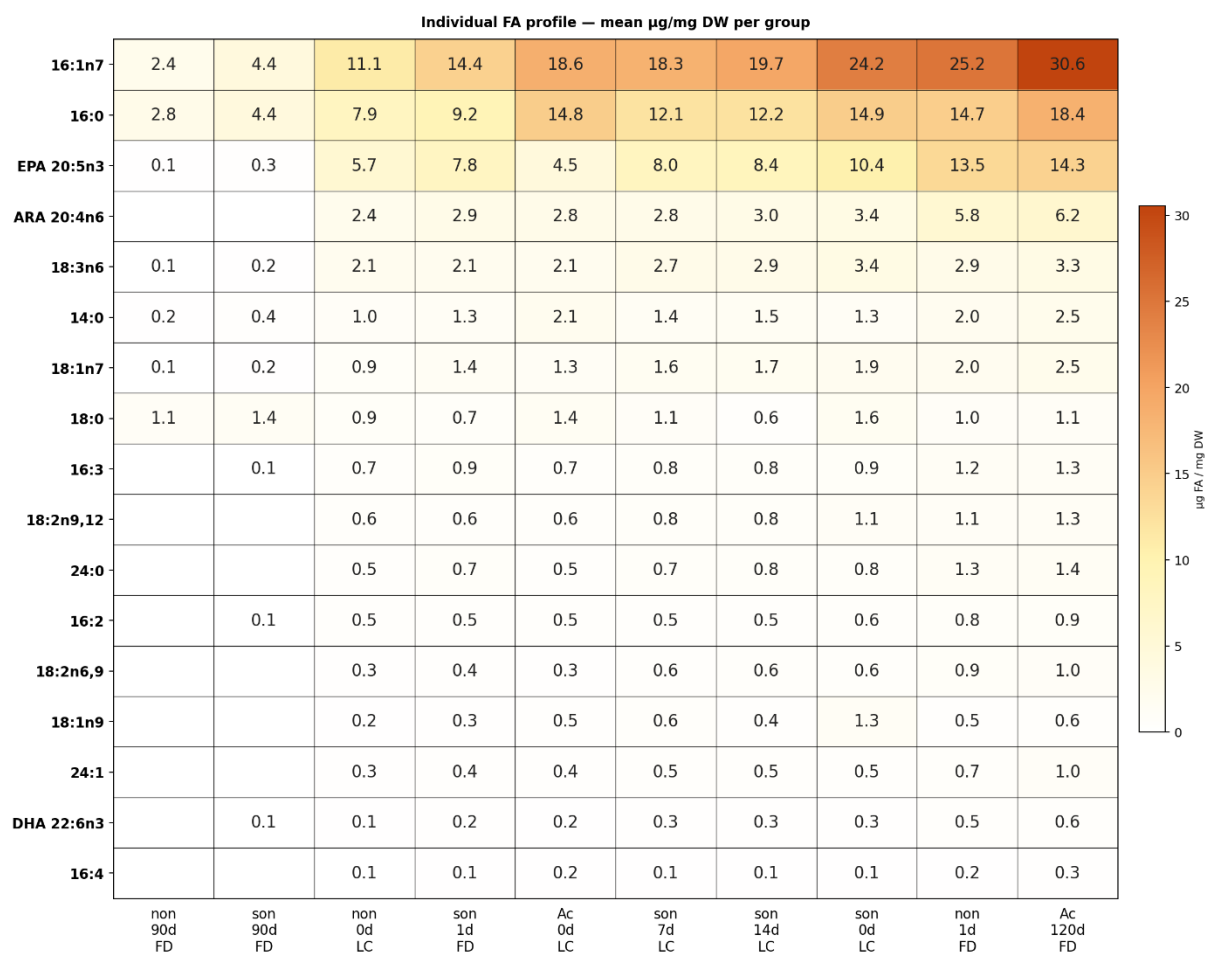


Figure 18. Heatmap with mean FA composition ($\mu\text{g FA mg}^{-1}$ DW) for each FA across all treatment groups. Rows represent individual FAs, ordered by mean concentration, columns represent treatment groups. Each cell colour encodes the mean concentration of one FA in one group, with intensity of shading corresponding to higher absolute content. Empty cells indicate values below the detection threshold (0.1). Acid-catalysed FAMES, $n = 2-3$ per group.

Across all treatment groups, the FAs fell into three groups separated by clear discontinuities in abundance (Figure 18). The top tier comprised three FAs at substantially higher mean concentrations than the rest of the profile: 16:1n7 (palmitoleic acid, mean: $16.88 \mu\text{g mg}^{-1}$ DW), 16:0 (palmitic acid, mean: $11.15 \mu\text{g mg}^{-1}$ DW), 20:5n3 (EPA, mean: $7.31 \mu\text{g mg}^{-1}$ DW). Together, these accounted for 72.7 % of the mean total FA across the dataset and the most abundant MUFA, SFA and PUFA, respectively. The midrange tier comprised two FAs with mean concentrations between 2 and $3 \mu\text{g mg}^{-1}$ DW and included 20:4n6 (ARA, 2.9) and 18:3n6 (2.2). The twelve remaining FAs: 14:0, 18:1n7, 18:0, 16:3, 18:2n9,12, 24:0, 16:2, 18:2n6,9, 18:1n9, 24:1, 22:6n3 (DHA) and 16:4 constituted a minor tier with mean concentrations below $1.40 \mu\text{g mg}^{-1}$ DW, several of which fell below the $0.1 \mu\text{g mg}^{-1}$ DW threshold.

3.4 Statistical Structure of the Dataset

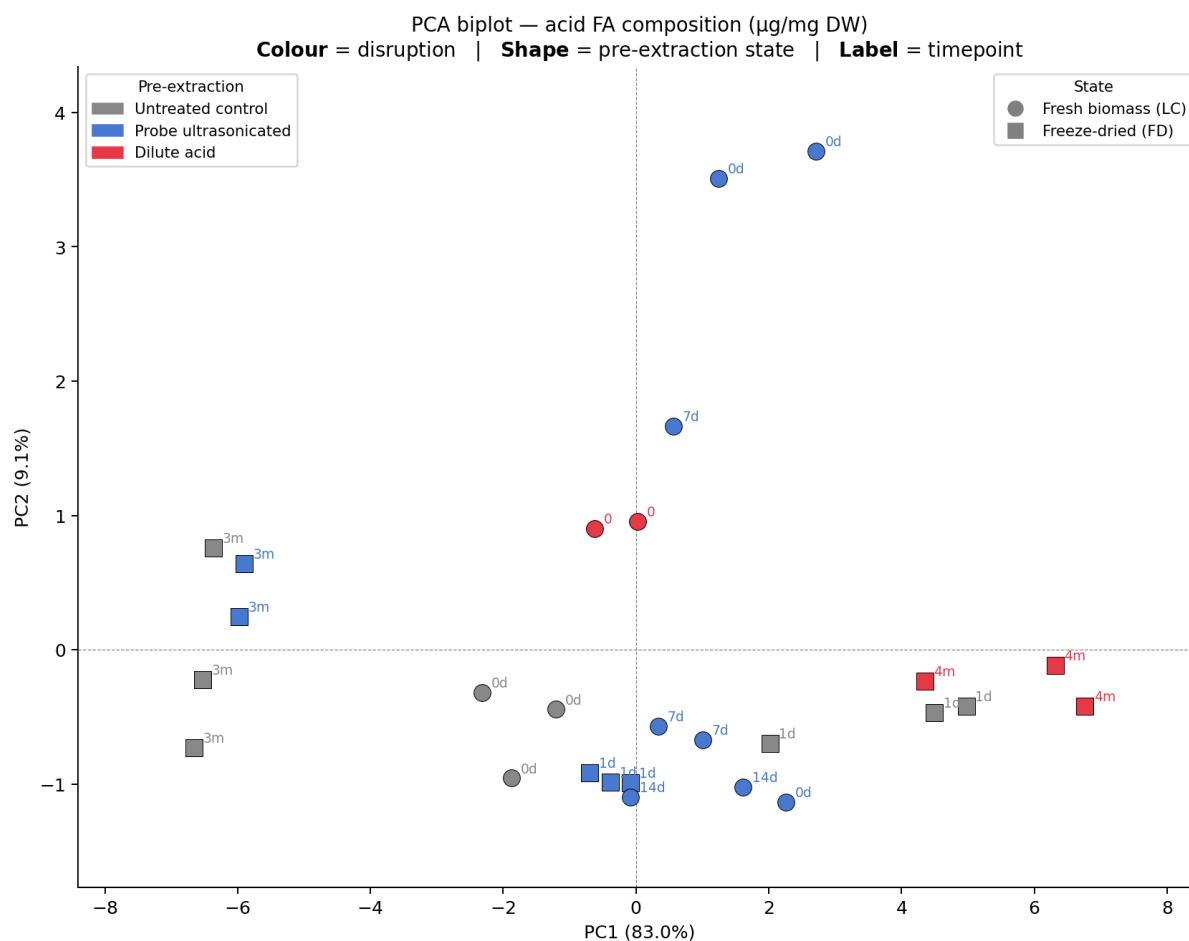


Figure 19. Principal component analysis (PCA) of acid-catalysed FA composition ($\mu\text{g FA mg}^{-1} \text{ DW}$) across storage conditions and pre-treatment methods. Each data point represents one biological replicate ($n = 2-3$). Colour indicates pre-treatment method applied prior to extraction: untreated control (grey), sonication (blue), or dilute acid pre-treatment (red). Marker shape indicates biomass state: fresh biomass (circle) or freeze-dried biomass (square). Point labels indicate storage duration at time of extraction.

PC1 and PC2 together explained 92.1 % of total variance (83.0 % and 9.1 %, respectively), indicating that the two-dimensional projection can be considered highly faithful to the underlying data (Figure 19). PC1 functioned primarily as a total FA abundance axis, samples were separated primarily by their overall FA content, higher PC1 scores correspond to FA-rich samples and lower scores to FA-depleted samples. The three-month freeze-dried groups (FD_non_3m and FD_son_3m) were positioned at the negative extreme, reflecting FA-depleted profiles, whereas FD_non_1d and FD_Ac_4m occupied the positive extreme. Notably, two FAs, 18:0 and 18:1n9, deviated from this pattern (Figure 20), indicating that their abundance varied largely independently of total FA content. PC2 was dominated by the same two FAs (18:0 and 18:1n9).

Fresh-biomass replicates clustered centrally along PC1, whereas freeze-dried replicates were distributed toward both extremes, reflecting the wider range of storage durations represented in the freeze-dried groups. Fresh-biomass samples appeared compositionally stable across the 0-to-14-day 4°C wet-storage period and largely insensitive to pre-treatment method, suggesting that the handling of fresh biomass introduces relatively little FA variability for this timeframe.

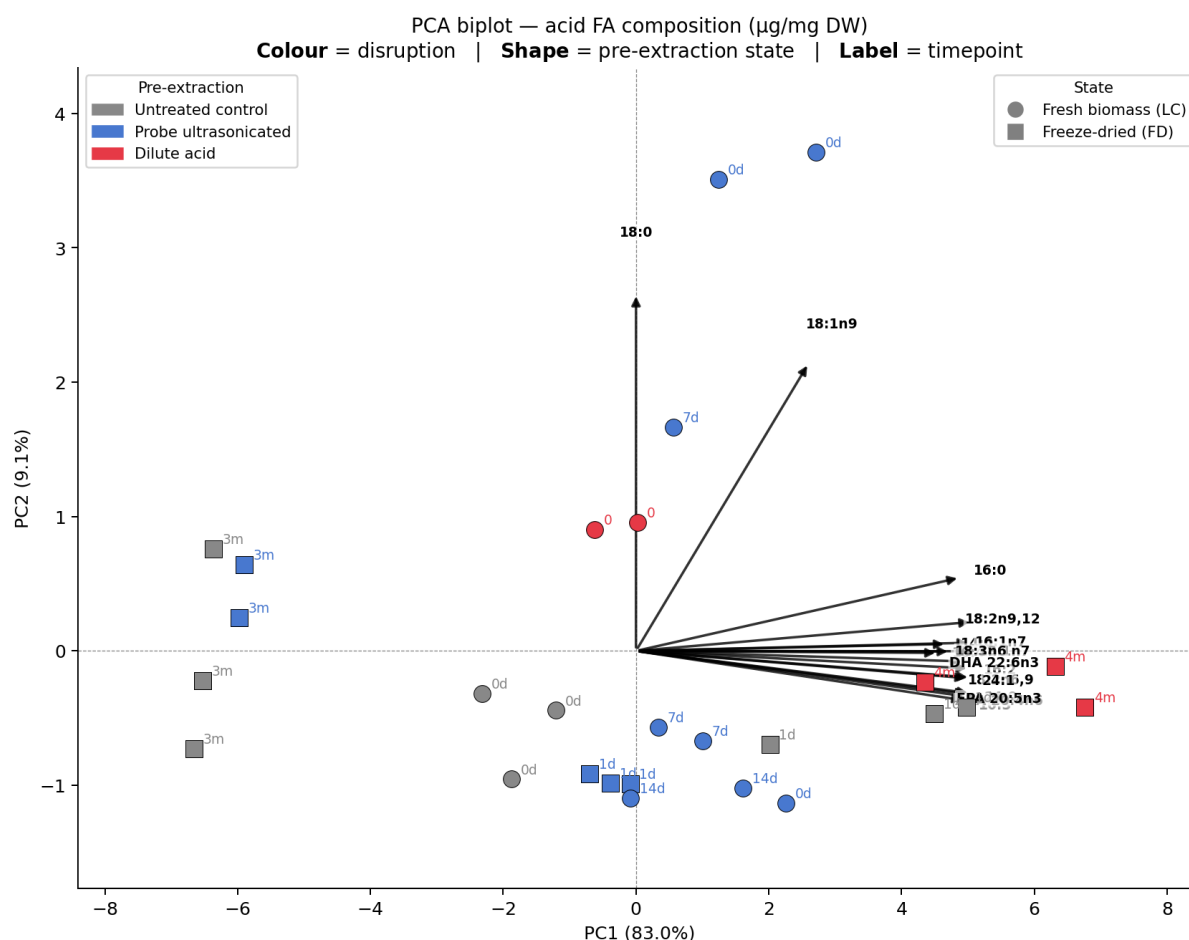


Figure 20. PCA of acid-catalysed FA composition (as Figure 19) with loading arrows included showing the direction and relative magnitude of each FAs contribution to PC1 and PC2. Arrow length is proportional to the strength of the loading. FAs loading strongly on PC1 (83.0 % of variance) point predominantly along the horizontal axis, while 18:0 and 18:1n9 deviate markedly toward PC2 (9.1 % of variance)

The two replicates of LC_son_0d were markedly displaced along PC2 relative to the remaining replicate of the same group and is best interpreted as within-group variability rather than a systematic treatment effect, given that PC2 accounts for only 9.1 % of total variance. Whether a systematic compositional trend exists across the wet-storage time series cannot be determined from the present data as the available replicates are insufficient to distinguish a genuine time effect from within-group variability along a minor principal component.

At three months of freeze-dried storage, the contrast between untreated control and sonicated biomass had narrowed considerably relative to the one-day timepoint, with FD_non_3m and FD_son_3m converging toward a common FA-depleted profile. This contrasts with the one-day timepoint, where the untreated control condition occupied a more positive PC1 position than the sonicated condition. FD_non_1d and FD_Ac_4m occupied similar ordination positions despite differing in storage duration and pre-treatment.

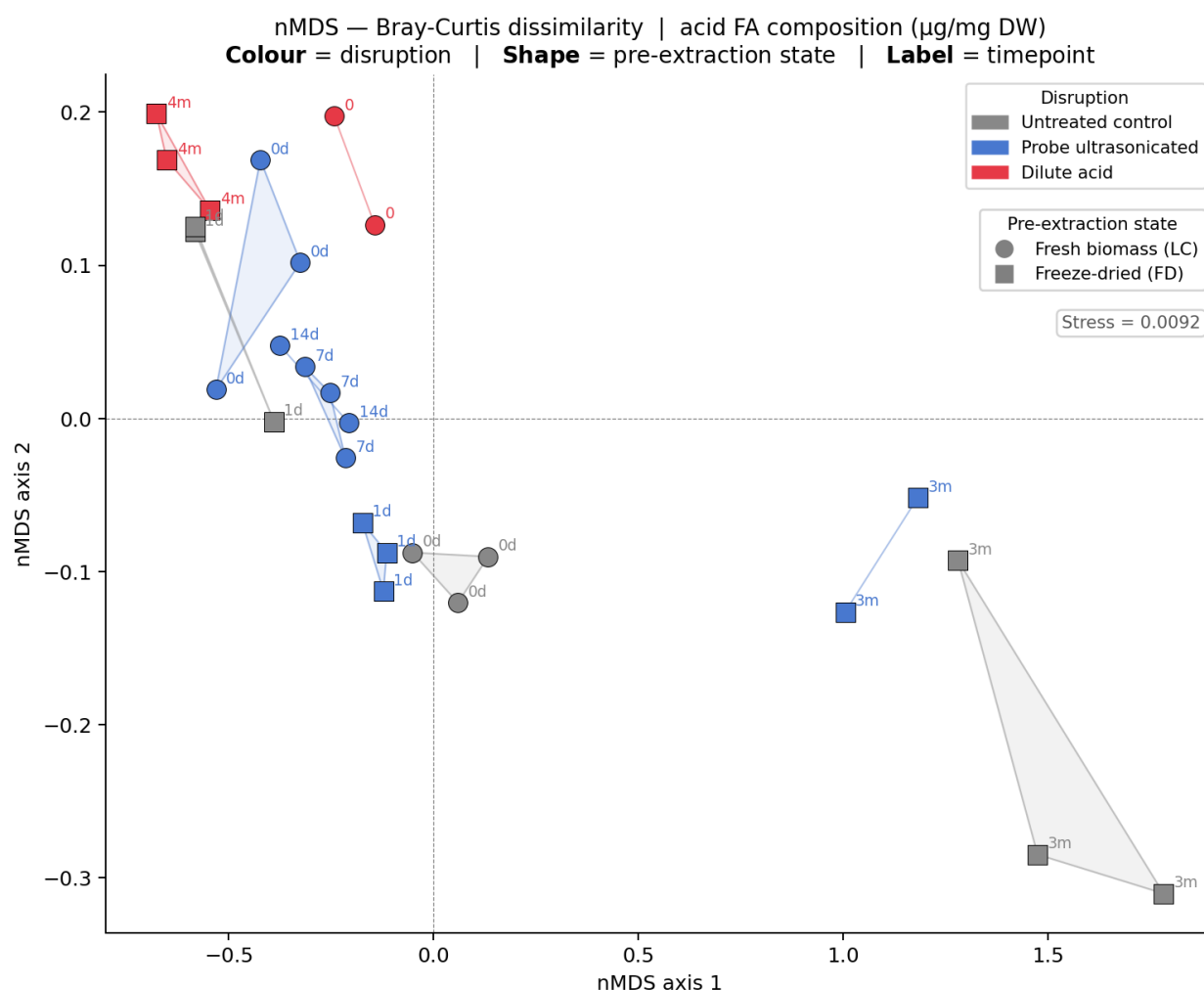


Figure 21. Non-metric multidimensional scaling (nMDS) ordination based on Bray–Curtis dissimilarity of acid-catalysed FA composition ($\mu\text{g FA mg}^{-1}\text{ DW}$) across all treatment groups. Each point represents one biological replicate ($n = 2\text{--}3$ per group). Colour indicates pre-treatment method applied prior to extraction: untreated control (grey), probe ultrasonication (blue), dilute acid (red). Marker shape indicates pre-extraction biomass state: fresh biomass (circle) or freeze-dried (square). Point labels indicate storage duration at the time of extraction. Convex hulls are drawn around groups with three replicates and groups with two replicates are connected by a line. Kruskal stress = 0.009, indicating an excellent fit of the two-dimensional representation to the underlying dissimilarity matrix.

The nMDS ordination on the Bray-Curtis dissimilarity matrix (Figure 21) reproduced the group separation observed in the PCA with a Kruskal stress value of 0.009 (well within the "excellent" range, < 0.05), indicating an excellent fit. The agreement between the two ordinations indicates that the observed group separation is not specific to the linear framework of PCA.

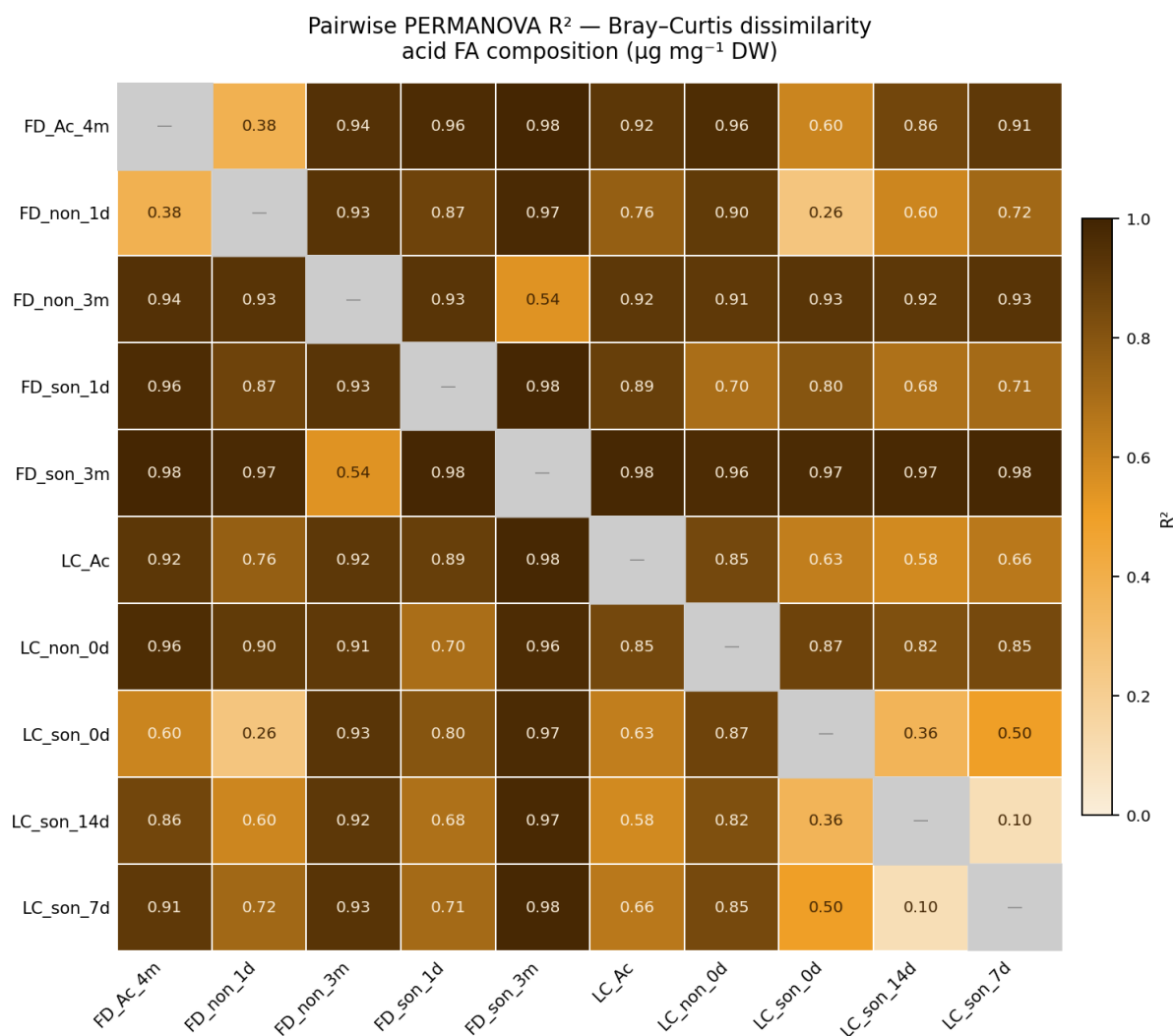


Figure 22. Pairwise PERMANOVA R^2 values based on Bray–Curtis dissimilarity of acid-catalysed FA composition ($\mu\text{g FA mg}^{-1}$ DW). R^2 represents the proportion of compositional variance explained by group identity for each pairwise comparison. Colour intensity reflects the magnitude of R^2 , from light (low compositional separation) to dark (high compositional separation). All pairwise p-values are at or near the permutation floor (~ 0.10) due to $n = 2\text{--}3$ replicates per group and are therefore not reported, R^2 is the sole informative metric.

Global PERMANOVA confirmed that group identity accounted for 95.6 % of total multivariate compositional variance (pseudo- $F = 40.61$, $p < 0.001$). PERMDISP was non-significant ($F = 0.81$, $p = 0.42$), indicating that the PERMANOVA result reflects genuine differences between groups rather than unequal within-group dispersion.

Given the small number of replicates per group ($n = 2\text{--}3$), pairwise PERMANOVA p-values are constrained at the permutation floor (0.10) and are therefore uninformative. Pairwise R^2 values are reported as the sole meaningful metric of compositional distance between group pairs (Figure 22). The greatest compositional separation was observed between FD_Ac_4m and FD_son_1d ($R^2 = 0.96$) and between FD_Ac_4m and LC_non_0d ($R^2 = 0.96$), while the lowest overall was between LC_son_7d and LC_son_14d ($R^2 = 0.10$). Within the fresh biomass sonicated wet-storage time series, compositional similarity was greater between adjacent later timepoints than between the start and middle of the series: LC_son_7d and LC_son_14d were highly similar ($R^2 = 0.10$), whereas LC_son_0d and LC_son_7d showed greater compositional distance ($R^2 = 0.50$).

The convergence of FD_non_3m and FD_son_3m ($R^2 = 0.54$) relative to their one-day counterparts is consistent with the pattern observed in the PCA. Notably, FD_non_1d and FD_Ac_4m showed a relatively low compositional separation ($R^2 = 0.38$) despite differing substantially in both storage duration and pre-treatment, corroborating the unexpected proximity of these two groups observed in the PCA.

3.5 Freeze-Dried vs Fresh Biomass

To isolate the effect of freeze-drying, fresh biomass and freeze-dried groups were compared after immediate extraction (LC, $t = 0$ d vs FD, $t = 1$ d, treated as equivalent timepoints since the only difference is the lyophilisation step itself). As dilute-acid pre-treatment was not applied to freeze-dried biomass at the immediate timepoint, the comparison between fresh and freeze-dried biomass is restricted to the untreated control and sonicated pre-extraction conditions.

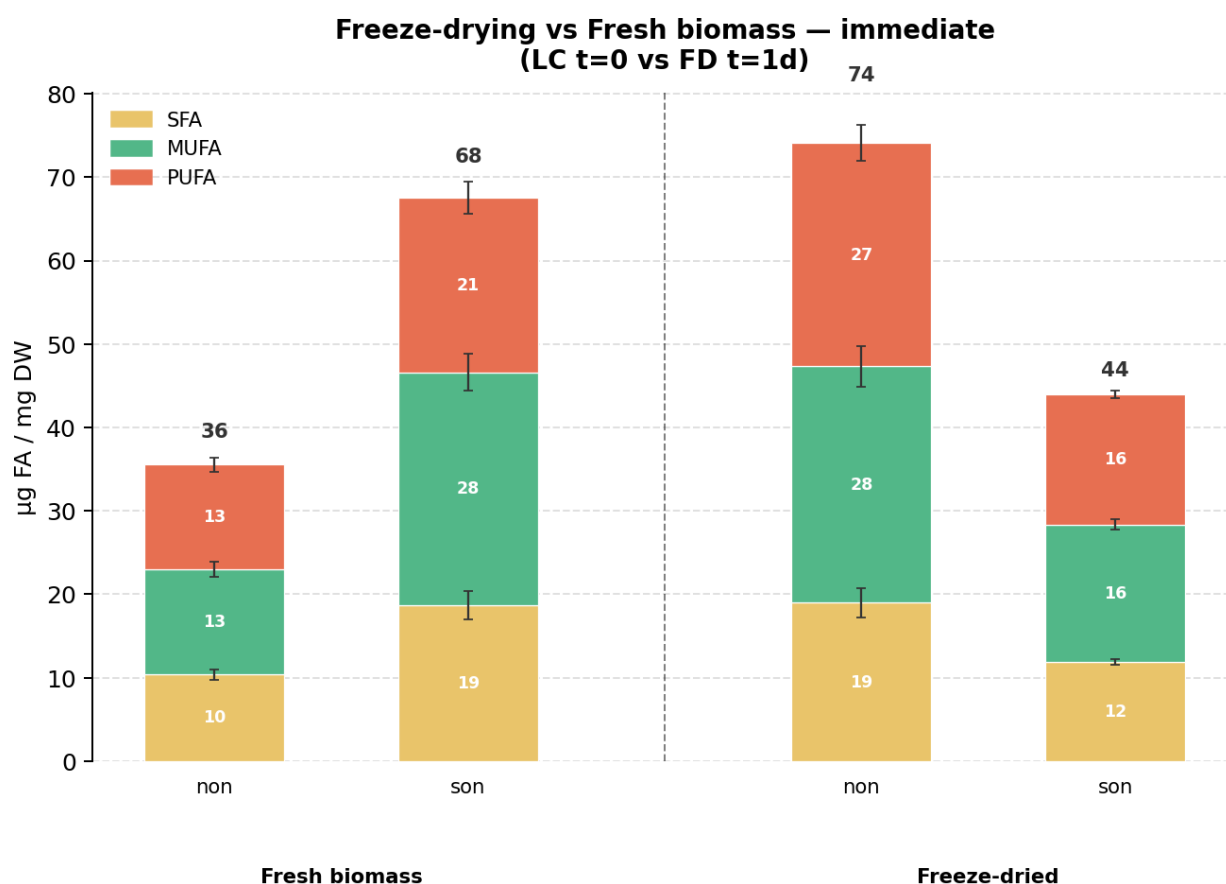


Figure 23. Total FA yield and FA-class composition (SFA, MUFA, PUFA, $\mu\text{g mg}^{-1}$ DW) for matched fresh (LC, $t = 0$) and freeze-dried (FD, $t = 1$ d) biomass under untreated control (non) and sonicated pre-treatments (son). Acid-catalysed FAMES, mean $\pm$ SE, $n = 3$ per group.

At the immediate timepoint, freeze-drying produced a divergent effect on total FA yield depending on the pre-treatment applied (Figure 23). In the absence of sonication, freeze-dried biomass yielded approximately twice the total FA of its fresh counterpart (74.13 vs $35.53 \mu\text{g mg}^{-1}$ DW), whereas under sonication the relationship reversed and fresh biomass yielded more than freeze-dried (67.56 vs $43.98 \mu\text{g mg}^{-1}$ DW). A two-way ANOVA on total FA confirmed a strong

interaction between pre-extraction state and pre-treatment (state $\times$ pre-treatment: $F = 65.60$, $p < 0.001$), while neither main effect was significant on its own (state: $F = 3.83$, $p = 0.09$, pre-treatment: $F = 0.06$, $p = 0.81$). This pattern indicates that the effect of freeze-drying on FA recovery is entirely conditional on whether ultrasonication is subsequently applied.

The relative proportions of SFA, MUFA and PUFA within each group varied across conditions. Two-way ANOVAs on the proportional composition of each FA class revealed that SFA proportions were unaffected by either factor or their interaction (all $p \geq 0.18$). The proportional MUFA content was significantly affected by pre-treatment ($F = 9.97$, $p = 0.013$) and by the state $\times$ pre-treatment interaction ($F = 18.17$, $p = 0.003$), but not by state alone. The proportional PUFA content showed significant main effects of both state ($F = 8.40$, $p = 0.020$) and pre-treatment ($F = 7.11$, $p = 0.029$), with a marginal interaction ($F = 4.10$, $p = 0.078$). Under sonication, freeze-dried biomass produced a proportionally MUFA-depleted and PUFA-enriched profile relative to fresh biomass. The interaction therefore acts predominantly on total FA recovery, the compositional shift between SFA, MUFA and PUFA is a secondary signal.

3.6 Cell-Lysis Pre-Treatments

To assess the effect of cell-lysis pre-treatment on FA yield and composition, untreated control, probe ultrasonication and dilute acid were compared separately for fresh and freeze-dried biomass. The fresh-biomass comparison was performed at $t = 0$ with all three pre-treatments matched in storage time. For the freeze-dried biomass, the untreated and sonicated groups were extracted after 3 months of storage, whereas the dilute-acid-treated group was extracted from the same freeze-dried material following an additional month of storage (4 months total).

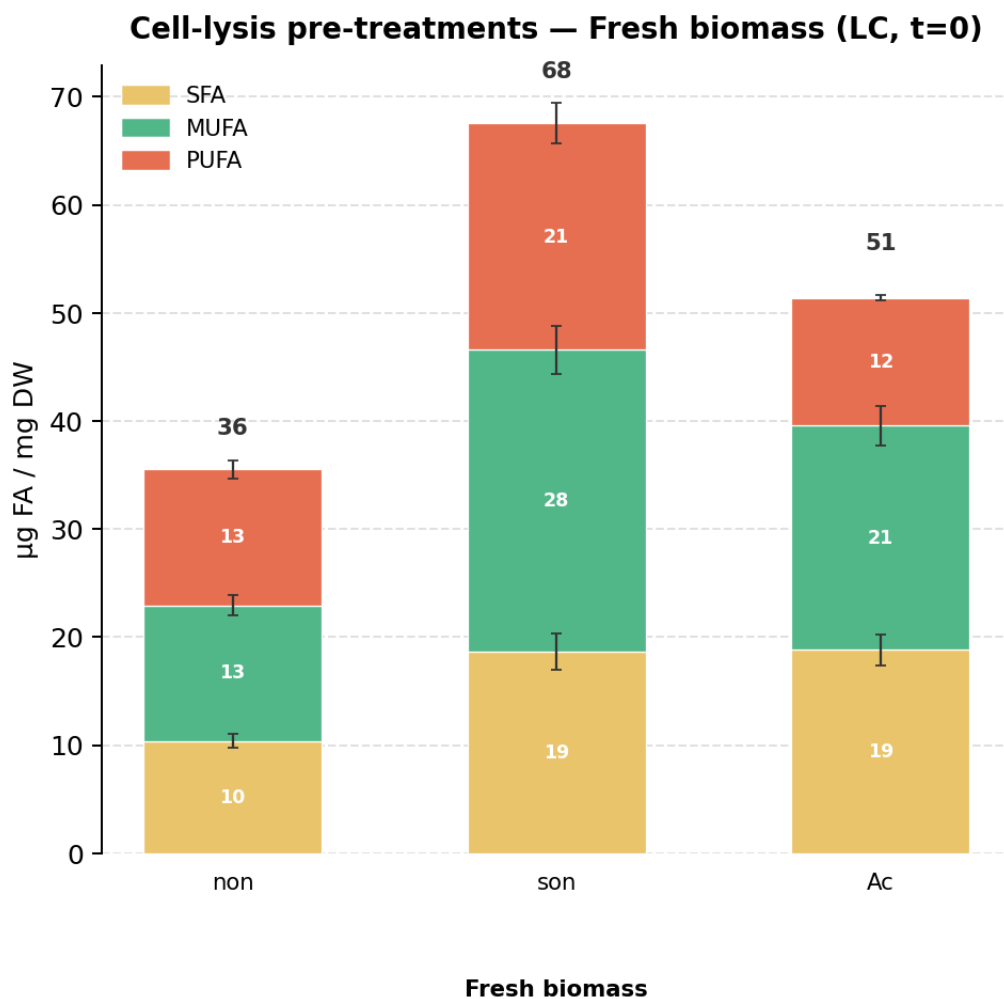


Figure 24. Total FA yield and FA-class composition ($\mu\text{g mg}^{-1}$ DW) for fresh biomass (LC, $t = 0$) under three cell-lysis pre-treatments: untreated control (non), probe ultrasonication (son) and dilute acid (Ac). Acid-catalysed FAMES, mean $\pm$ SE, $n = 3$ per group.

For fresh biomass extracted at $t = 0$, both sonication and dilute-acid pre-treatment increased total FA yield relative to the untreated control (67.56 and 51.40 vs $35.53 \mu\text{g mg}^{-1}$ DW, respectively, Figure 24). A one-way ANOVA confirmed a significant overall effect of pre-treatment on total FA ($F = 30.87$, $p = 0.002$). The FA-class composition differed markedly between the three pre-treatments. Dilute-acid pre-treatment and sonication produced similar SFA content (18.81 vs $18.66 \mu\text{g mg}^{-1}$ DW) both substantially higher than the untreated control ($10.40 \mu\text{g mg}^{-1}$ DW). Sonication produced the highest MUFA ($27.92 \mu\text{g mg}^{-1}$ DW) and PUFA content ($20.98 \mu\text{g mg}^{-1}$ DW). Notably, PUFA content was slightly lower in the dilute-acid group than in the untreated control (11.83 vs $12.57 \mu\text{g mg}^{-1}$ DW).

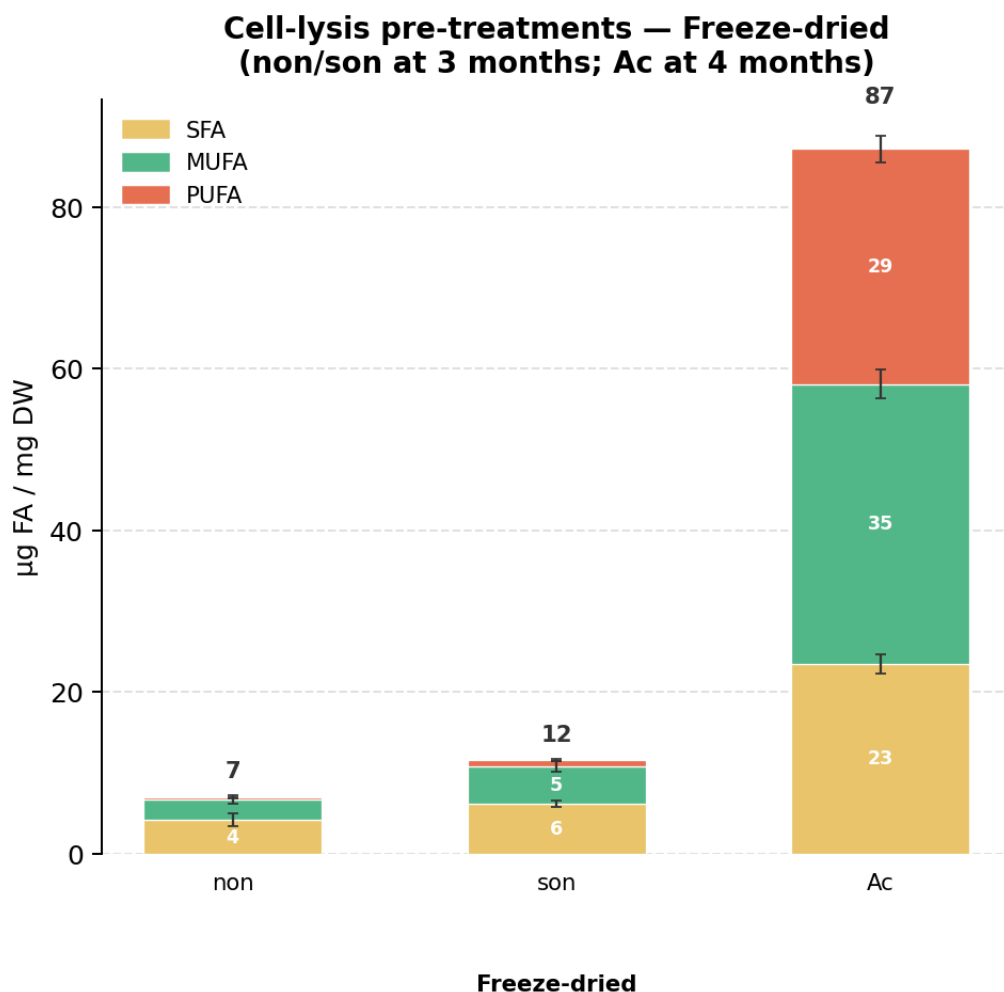


Figure 25. Total FA yield and FA-class composition ($\mu\text{g mg}^{-1}$ DW) for freeze-dried biomass under three cell-lysis pre-treatments. Untreated (non) and sonicated (son) samples were extracted after 3 months of storage, dilute-acid (Ac) samples after 4 months. Acid-catalysed FAMES, mean $\pm$ SE, $n = 3$ per group.

For freeze-dried biomass, the contrast between pre-treatments was substantially larger than for fresh biomass (Figure 25). Dilute-acid pre-treatment after 4 months of FD storage yielded $87.16 \mu\text{g mg}^{-1}$ DW, almost an eight-fold increase compared to the sonicated 3-month yield ($11.61 \mu\text{g mg}^{-1}$ DW) and approximately twelve-fold the untreated 3-month yield ($6.95 \mu\text{g mg}^{-1}$ DW). A one-way ANOVA confirmed a strong effect of pre-treatment ($F = 204.69$, $p < 0.001$).

3.7 Storage and Preservation Treatments

The effect of storage duration and preservation method on FA yield and composition was assessed across two biomass states: freeze-dried biomass stored in airtight containers at room temperature in the dark for one day or three months (biomass extracted from different batches, it is therefore not possible to fully separate batch effects from storage effects, discussed in Section 4.5.3), and fresh biomass maintained in wet storage at 4°C for up to 14 days. The wet-storage time series exists only for the sonicated condition and the results about wet-storage stability are therefore conditional on ultrasonication.

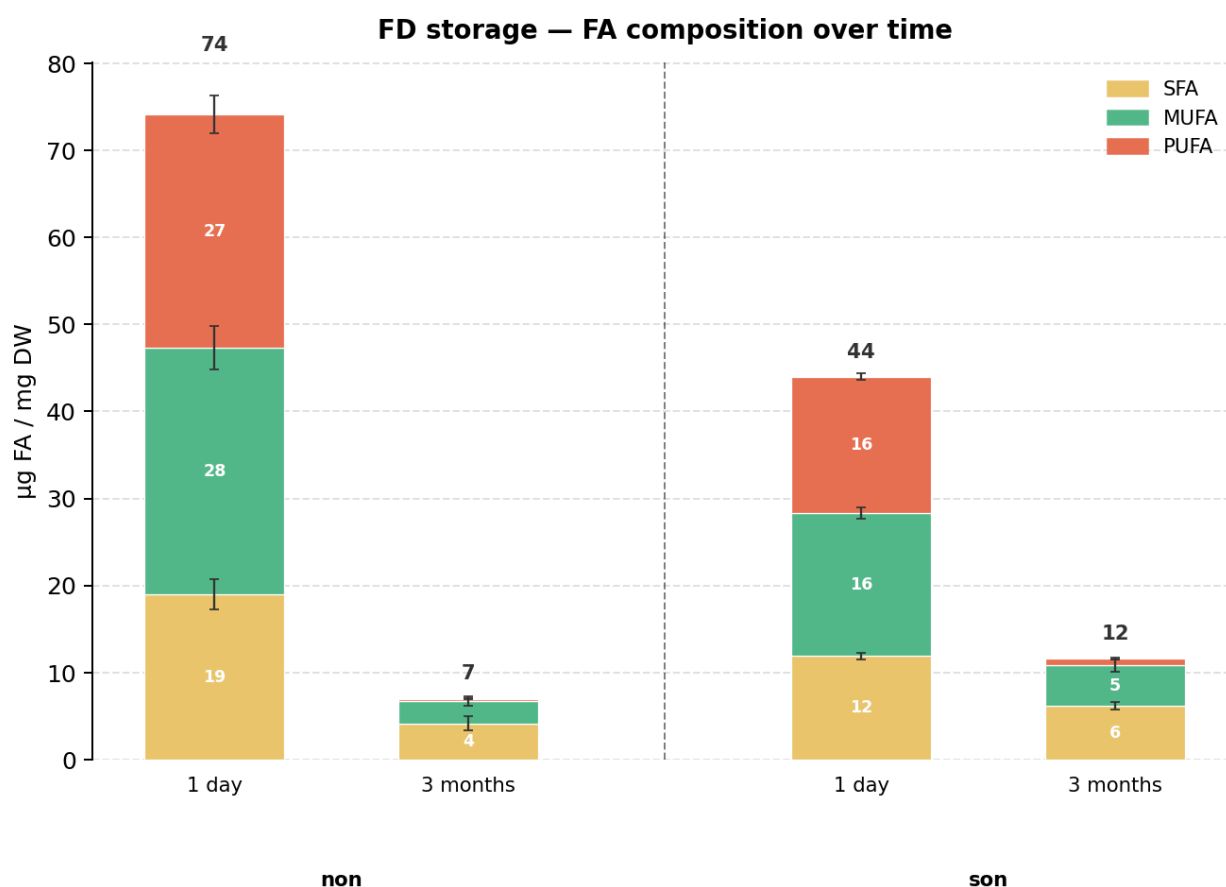


Figure 26. Total FA yield and FA-class composition ($\mu\text{g mg}^{-1}$ DW) for freeze-dried biomass across two storage timepoints (1 day and 3 months), shown separately for untreated control (non) and sonicated pre-treatment (son) conditions. Acid-catalysed FAMES, mean $\pm$ SE, $n = 3$ per group.

For freeze-dried biomass, total FA yield declined substantially over storage in both conditions, with the decline differing markedly between them (Figure 26). In the untreated control, total FA fell from 74.13 to $6.95 \mu\text{g mg}^{-1}$ DW between 1 day and 3 months, a 90.6 % reduction. In the sonicated condition, the decline was slightly less severe, from 43.98 to $11.61 \mu\text{g mg}^{-1}$ DW, a 73.6 % reduction.

A two-way ANOVA confirmed a strong main effect of storage time ($F = 188.00$, $p < 0.001$) and a significant pre-treatment $\times$ time interaction ($F = 21.03$, $p = 0.003$). The effect of pre-treatment was also significant ($F = 15.15$, $p = 0.006$). Averaged across timepoints, the untreated condition yielded more total FA ($40.54 \mu\text{g mg}^{-1}$ DW) than the sonicated condition ($27.80 \mu\text{g mg}^{-1}$ DW), driven largely by the difference at the 1-day timepoint. Notably, the pre-treatment-method ranking inverted between timepoints: at 1 day, the non-disrupted condition exceeded the sonicated condition (74.13 vs $43.98 \mu\text{g mg}^{-1}$ DW), whereas at 3 months the relationship reversed (6.95 vs $11.61 \mu\text{g mg}^{-1}$ DW).

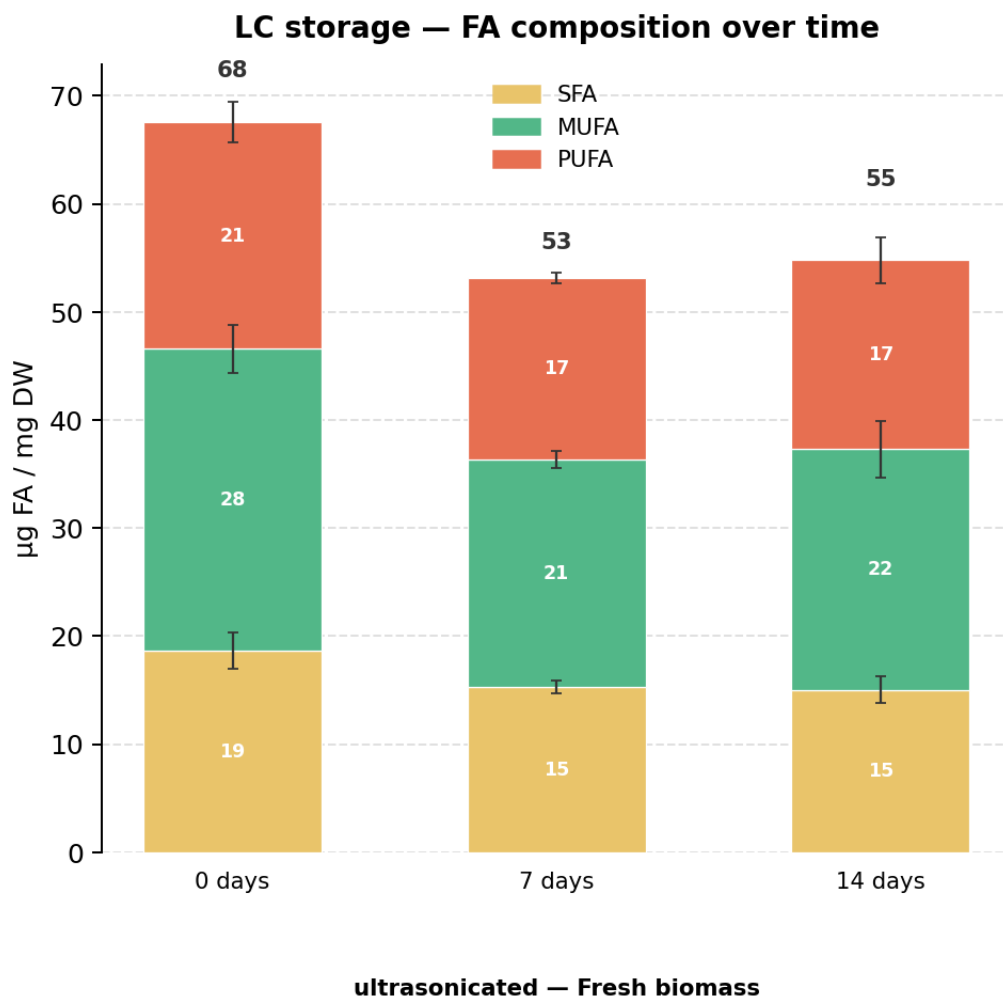


Figure 27. Total FA yield and FA-class composition ($\mu\text{g mg}^{-1}$ DW) for sonicated fresh biomass across the wet-storage time series at 4 °C (0, 7 and 14 days). Acid-catalysed FAMES, mean $\pm$ SE, $n = 3$ per group.

For sonicated fresh biomass, total FA yield showed a modest numerical decline over the 14-day wet-storage period at 4 °C (Figure 27), from $67.56 \mu\text{g mg}^{-1}$ DW at day 0 to $53.12 \mu\text{g mg}^{-1}$ DW at 7 days and $54.79 \mu\text{g mg}^{-1}$ DW at 14 days. No individual FA class exhibited a significant change in absolute yield over the storage period (SFA: $F = 2.60$, $p = 0.17$, MUFA: $F = 4.19$, $p = 0.09$, PUFA: $F = 2.25$, $p = 0.20$), and the proportional composition remained stable throughout (all $p \geq 0.50$). At the level of resolution afforded by the present sample size, neither the total nor the class-specific FA composition can be shown to change significantly over the wet-storage period.

3.8 SEM Imaging Results

SEM imaging was qualitatively analysed and used to assess the impact of pre-treatment and preservation on frustule integrity (Figure 28). The frustule halves were separated because of the SEM preparation step, assessment therefore focuses on the integrity of the individual halves rather than whether they remained joined. Freeze drying alone caused minimal additional breakage relative to the untreated fresh control (D vs A). Probe ultrasonication produced extensive frustule breakage on freeze-dried biomass (E), but on fresh biomass many frustules remained intact (B). Dilute acid produced little to no breakage in either biomass state (C and F).

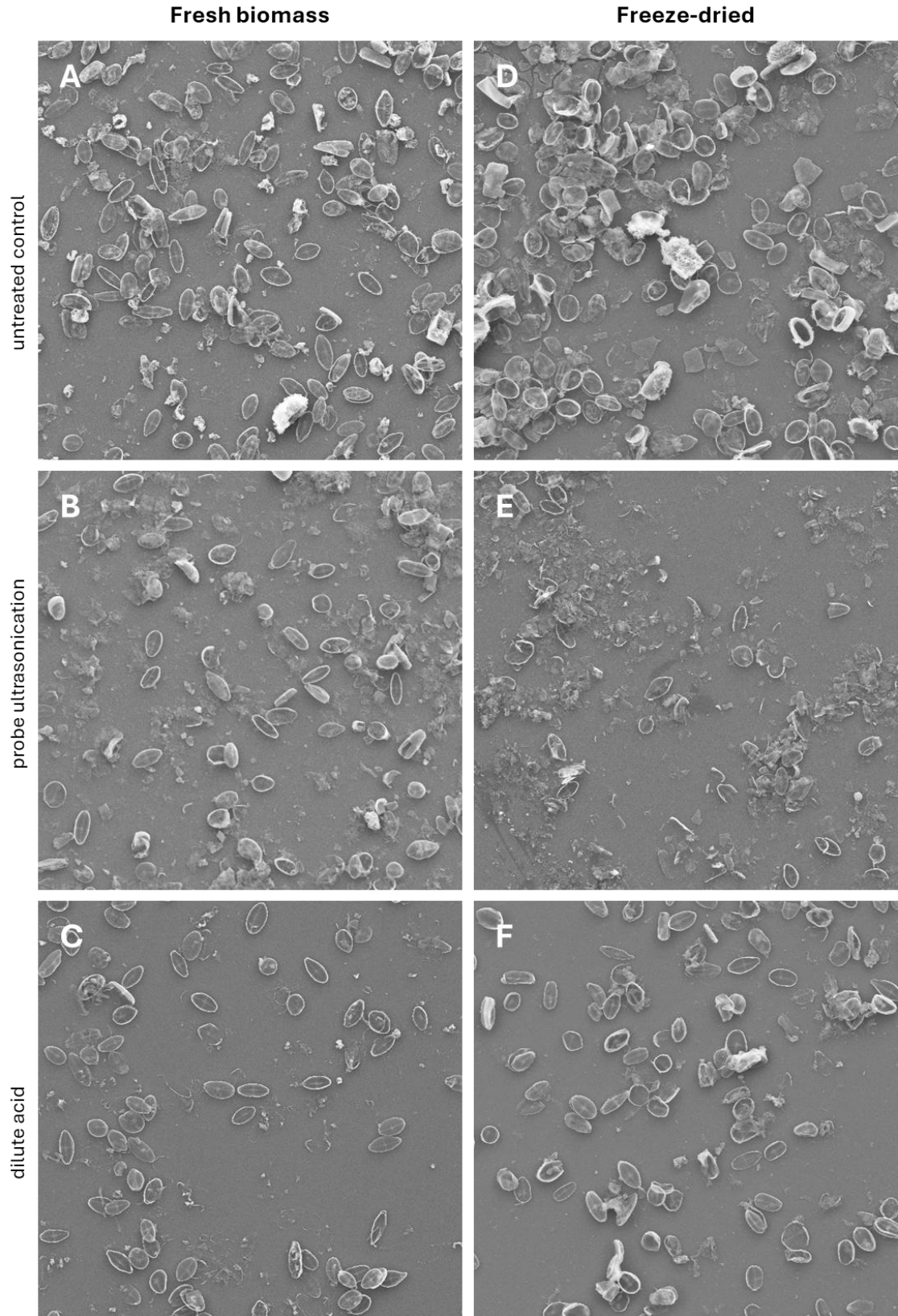


Figure 28. SEM images of *Craspedostauros* sp. biomass under the six pre-treatments and preservation combinations: (A) fresh biomass, untreated control (B) fresh biomass, probe ultrasonicated (C) fresh biomass, dilute acid pre-treatment (D) freeze-dried, untreated control (E) freeze-dried, probe ultrasonicated (F) freeze-dried, dilute acid pre-treatment.

4. Discussion

The discussion is structured around the four aims of the study: evaluation of the Wigert extraction procedure against the established Folch method, evaluation of cell-lysis pre-treatments, effects of storage and preservation conditions, and characterisation of the FA composition of the diatom *Craspedostauros* sp. The following chapter discusses the results, compares them to existing literature, addresses methodological considerations and outlines recommendations for future work building on these findings.

4.1 Comparing the Wigert Methodology to Folch

Wigert and Folch returned similar total FA yields when averaged across the two pre-treatment conditions (51.55 vs 52.99 $\mu\text{g mg}^{-1}$ DW), with no significant difference for any FA class in pooled one-way ANOVAs (Section 3.2). However, this apparent equivalence is misleading. The significant extraction $\times$ pre-treatment interaction (Table 1: $F = 20.15$, $p = 0.003$) indicates that the pattern of means reversed between untreated and sonicated conditions. Within-condition one-way ANOVAs revealed that on untreated biomass, Folch recovered significantly more total FA than Wigert (51.50 vs 35.53 $\mu\text{g mg}^{-1}$ DW, $F = 28.26$, $p = 0.006$). On sonicated biomass the ranking reversed, with Wigert recovering numerically more (67.56 vs 54.47 $\mu\text{g mg}^{-1}$ DW), though this did not reach significance ($F = 4.36$, $p = 0.128$), likely reflecting the reduced replication in the Folch sonicated group ($n = 2$). The similar pooled means are therefore an effect of averaging two opposing effects rather than evidence of equivalence. Wigert was nonetheless used throughout the present study, as evaluating it against the established Folch method was itself one of the stated aims. Since the GC-MS results were not available until late in the project, this comparison could only be made retrospectively, by which point all extractions had been completed.

The differences between Wigert and Folch are as described in Section 1.3.3 with the most important being the additional water. Wigert also deviates from Folch with a higher salt concentration (5 % vs 0.9 % NaCl) and the substitution of DCM for chloroform. The DCM substitution itself is not evaluated here, since it has previously been supported by Cequier-Sánchez et al. (2008), who reported comparable lipid recoveries, and Svenning et al. (2020), who showed that DCM:MeOH (2:1) outperformed non-polar solvents for the centric diatom *P. glacialis*. The remaining difference is therefore the effect of the added water on the biphasic system ratio. Comparing them places Wigert (8:4:9 DCM:MeOH:H₂O) as more aqueous than the original Folch (8:4:3 CHCl₃:MeOH:H₂O) and even more aqueous than Bligh & Dyer (~8:8:7.2 CHCl₃:MeOH:H₂O). In practice, Wigert follows the Bligh & Dyer method more closely than Folch by adding an aqueous phase to drive the biphasic split twice. According to Saini et al. (2021), this makes the protocol particularly suited to low-biomass feedstocks, where maximising phase separation effectively is critical to recovering sufficient lipid material for quantification. The larger aqueous fraction in the Wigert method also helps partitioning non-lipid contaminants such as proteins, carbohydrates and other hydrophilic contaminants into the aqueous phase, thereby improving the purity of the organic layer (Breil et al., 2017). The elevated NaCl concentration of 5 % in the aqueous wash (driving the second biphasic split) may further sharpen the organic aqueous interface and suppress emulsion during separation, though this effect has not been formally evaluated.

An additional observation worth noting is that the Wigert protocol with dilute-acid pre-treatment (51.40 $\mu\text{g mg}^{-1}$ DW) returned a comparable total FA yield to Folch with probe ultrasonication (54.47 $\mu\text{g mg}^{-1}$ DW), suggesting that dilute acid may achieve similar extraction to sonication without requiring cell disruption. However, this comparison carries two caveats: SEM imaging

indicated that probe ultrasonication did not achieve complete frustule disruption on fresh biomass and both groups had $n = 2$, so the result should be interpreted with caution. Broader validation of the Wigert protocol is therefore warranted, and the solvent ratios themselves may be worth revisiting. Wigert (8:4:9 DCM:MeOH:H₂O) is considerably more aqueous than both Folch (8:4:3) and Bligh and Dyer (~8:8:7.2), and Breil et al. (2017) demonstrated that increasing the water fraction in biphasic systems progressively reduces lipid yield. The higher water content in Wigert, while beneficial for phase separation and removal of non-lipid contaminants, may therefore come at a cost to extraction efficiency, particularly for untreated biomass where solvent access to the lipid pool is already limited. Systematically varying the water volume in the Wigert protocol could identify the optimal aqueous ratio, preserving the phase-separation advantages of the method while improving absolute yields.

4.2 Pre-Extraction Treatments: Cell Lysis

Two pre-treatments were evaluated for their effect on FA recovery and class composition: probe ultrasonication and dilute-acid pre-treatment. Ultrasonication was included as an established method for complete cell lysis, expected to provide an upper-bound benchmark for FA recovery against which untreated control and acid-treated samples could be compared. Dilute-acid pre-treatment was hypothesised to yield comparatively lower FA concentrations due to its less aggressive mode of cell disruption. This hypothesis was not supported by the results. Since GC data were not available until late in the study, it was not possible to adapt the experimental design in response to the emerging findings. While this represents a limitation in the development of the methodology, the findings nonetheless yield valuable insights that are discussed further below.

4.2.1 Probe Ultrasonication

Continuing the sonication effect described in Section 3.5, sonication of fresh biomass increased total FA yield from 35.53 to 67.56 $\mu\text{g mg}^{-1}$ DW relative to the untreated control and produced the highest absolute MUFA and PUFA contents among the three fresh-biomass pre-treatments in the dataset. This indicates that probe ultrasonication selectively enhanced recovery of unsaturated FAs without inducing detectable thermal degradation. This is consistent with the findings of Svenning et al. (2020) on *P. glacialis*, who compared probe ultrasonication to mechanical mixing and untreated control and found that despite sonication achieving complete cell lysis, FA composition was similar across all treatments, with no selective degradation of PUFAs by sonication.

Interestingly, the SEM imaging showed much less frustule breakage than expected. The probe ultrasonication time and intensity were methodologically developed specifically for *Craspedostauros* sp. by progressively increasing the duration until sufficient breakage was visible by inverted light microscopy. Possible reasons for the discrepancy between the light-microscopy observations during method development and the SEM observations are discussed in Section 4.5. However, the observation presents an important implication, the use of probe ultrasonication of *Craspedostauros* sp. increased FA recovery without comprehensively breaking the frustules. This differs from Svenning et al. (2020) who found that despite complete frustule disruption, sonication did not increase total lipid yield above mixing when polar solvents (DCM:MeOH) were used.

For freeze-dried samples, SEM revealed that probe ultrasonication completely disrupted the frustules and for the long-stored freeze-dried samples specifically, sonication increased the yield

from 6.95 to 11.61 $\mu\text{g mg}^{-1}$ DW (Figure 26), a smaller but qualitatively similar increase as observed in the fresh-biomass sonication treatment, although both values remain low. However, the reversed pattern was seen in the freeze-dried biomass extracted immediately after lyophilisation (Figure 23), where sonication decreased yield from 74.13 to 43.98 $\mu\text{g mg}^{-1}$ DW. A plausible interpretation is that freeze-drying itself improves solvent access through structural changes during ice-crystallisation and sublimation, reducing the need for active cell disruption. When sonication is then applied to freeze-dried biomass, the additional energy input may offer no extra benefit and could even cause yield losses, possibly through mechanical entrapment of lipids in the resulting pellet which sits between the aqueous and organic phases during extraction. For fresh biomass, where no pre-treatment has been applied, sonication remains necessary for the solvent to access the intracellular lipids. This aligns with Svenning et al. (2020), who concluded that for polar solvents (DCM:MeOH), the number of sequential extractions was the primary determinant of lipid yield in *P. glacialis*, rather than cell disruption.

4.2.2 Dilute Acid

The most striking single effect in the entire study was that of dilute-acid pre-treatment. For fresh biomass, dilute acid increased total FA yield from 35.53 to 51.40 $\mu\text{g mg}^{-1}$ DW relative to the untreated control (Figure 24), less than the yield produced by sonication (35.53 to 67.56 $\mu\text{g mg}^{-1}$ DW) but resulted in a different FA-class profile.

The acid-treated fresh biomass exhibited an SFA content of 18.81 $\mu\text{g mg}^{-1}$ DW. This was among the highest in the dataset (Figure 17), comparable to LC_son_0d (18.66) and FD_non_1d (18.99), exceeded only by FD_Ac_4m (23.48). PUFA content was slightly lower than in the untreated control (11.83 vs 12.57 $\mu\text{g mg}^{-1}$ DW, Δ 0.74), consistent with the known susceptibility of PUFAs to degradation under low pH conditions (Dalheim et al., 2021). The sonicated group recovered substantially more PUFA (20.98 $\mu\text{g mg}^{-1}$ DW), meaning the increase in total yield from dilute acid on fresh biomass was carried entirely by SFA and MUFA.

MUFA recovery showed a similar pattern as SFA: dilute acid (20.75 $\mu\text{g mg}^{-1}$ DW) recovered more than untreated control (12.56) without reaching the sonication value (27.92). Across all three treatments, 16:1n7 (palmitoleic acid) was the dominant MUFA, accounting for >85 % of the class. The differential gain between pre-treatments therefore reflects access to 16:1n7 rather than a compositional shift within the MUFA class. Together with the SFA result, this indicates that on fresh biomass, dilute-acid pre-treatment selectively enhances recovery of the more chemically robust FA classes (SFA, MUFA) while leaving PUFA recovery essentially unchanged.

This pattern is consistent with Otero et al. (2017), who found that acid-catalysed hot-water extraction (1 % H_2SO_4 , 100 °C, 30 min) substantially reduced MUFA and PUFA recovery in *P. tricornutum* while SFA levels remained comparable. It is also consistent with Hogan et al. (2021), who reported that 0.5 M HCl pre-treatment of the benthic diatom *Amphora* sp. markedly reduced the relative content of EPA from 53.9 % to 14.8 % while SFAs (myristic acid, 14:0) and MUFAs (palmitoleic 16:1 and oleic acid, 18:1) increased proportionally. In both cases, the elevated SFA proportion is therefore likely a consequence of relative PUFA loss rather than SFA enrichment, with acid-catalysed oxidation of double bonds producing degradation products such as aldehydes. Notably, Hogan et al. (2021) observed this reduction without a heating step (20 °C, 4 h), suggesting that acid alone, independent of elevated temperature, is sufficient to cause PUFA degradation, consistent with the known oxidative susceptibility of PUFAs under acidic conditions. It is also worth noting that the dilute acid extraction in Otero et al. (2017) was followed by extraction overnight with hexane.

The present design does not separate the chemical effect of the acid from the thermal effect of the heating step (65 °C, 30 min), so the SFA bias on fresh biomass cannot be unambiguously attributed to acid hydrolysis versus heat-induced PUFA loss. It should also be noted that the temperature and time of the present protocol are based on observations from Simon Nilsson who found 65 °C for 30 minutes to be favourable when extracting polysaccharides (S. Nilsson, personal communication). The observation that PUFA content in the present fresh-biomass dilute-acid treatment was preserved at the level of the untreated control, rather than suppressed as in Otero et al. (2017) or markedly reduced as in Hogan et al. (2021) may reflect the shorter extraction time or lowered temperature (or solvent in Otero et al., 2017). Since thermal degradation and acid-catalysed oxidation are both time- and temperature-dependent processes, and no study holds one variable constant while varying the other, isolating their respective contributions is not possible.

The most surprising result, however, was the effect of dilute-acid pre-treatment on long-stored freeze-dried biomass. After 4 months of room-temperature FD storage, dilute-acid pre-treatment gave the highest total FA yield in the entire dataset (87.16 $\mu\text{g mg}^{-1}$ DW, Figure 25), almost an eight-fold increase from the sonicated 3-month yield (11.61 $\mu\text{g mg}^{-1}$ DW) and approximately twelve-fold the untreated 3-month yield (6.95 $\mu\text{g mg}^{-1}$ DW). FD_Ac_4m had the highest absolute concentration in every FA class of any treatment: SFA 23.48 $\mu\text{g mg}^{-1}$ DW, MUFA 34.63, and PUFA 29.05. Within PUFA, EPA contributed 14.31 $\mu\text{g mg}^{-1}$ DW and ARA 6.19, both of which were the highest values in the dataset. The pattern therefore differs from the fresh-biomass dilute-acid result, rather than producing an SFA-skewed profile through PUFA loss, the 4-month FD acid treatment recovered substantially more of every FA, including PUFAs, suggesting an effect of accessing all FA classes equally rather than selectively.

The magnitude of this increase suggests that dilute-acid pre-treatment liberates FAs that have become inaccessible to solvent extraction during long-term room-temperature FD storage, possibly bound to oxidised cell-wall components, denatured proteins, or other matrix material that solvent alone cannot mobilise. The mechanism, whether thermal, acidic, or some combination, cannot be determined from the present data. Some caution is warranted given the extraordinary magnitude of this difference, however, the within-group standard error on FD_Ac_4m total FA is small relative to the group mean (mean = 87.16, SE = 4.59 $\mu\text{g mg}^{-1}$ DW, $n = 3$), supporting the result statistically.

The practical significance, however, is striking. Long-stored room-temperature freeze-dried biomass may be revivable with dilute-acid pre-treatment, recovering lipid pools that would otherwise be considered lost. The result also indicates that the effective storage window for freeze-dried biomass may be considerably longer than previously assumed, provided that downstream extraction includes a dilute-acid step. Notably, the long-stored freeze-dried biomass treated with dilute acid returned the highest absolute content of every FA in the dataset for 14 of the 17 quantified FAs (Figure 18). The three exceptions were 18:3n6 (3.26 vs 3.41 $\mu\text{g mg}^{-1}$ DW in LC_son_0d), 18:0 (1.13 vs 1.63), and 18:1n9 (0.59 vs 1.28), all three of which were marginally exceeded by LC_son_0d. This suggests that dilute-acid extraction of long-stored freeze-dried biomass may represent a most productive combination of preservation and extraction conditions.

For fresh biomass, dilute acid offers a non-destructive alternative to increase lipid recovery from untreated biomass, achieving the dual potential and satisfying one of the primary objectives of this study. For biofuel applications specifically, where SFAs are the preferred feedstock (Hu et al., 2008), dilute acid achieves SFA yields comparable to sonication without requiring cell disruption. Adding a freeze-drying step (and 4 months of storage) prior to dilute-acid extraction did return a moderately higher absolute SFA yield (23.48 versus 18.81 $\mu\text{g mg}^{-1}$ DW), but this difference must

be weighed against the considerable energy and cost associated with lyophilisation. However, it should be noted that the freeze-dried dilute-acid sample was stored for 4 months prior to extraction, whereas the fresh biomass was processed after 1 day. Whether freshly freeze-dried biomass subjected to dilute-acid extraction would yield substantially higher SFA remains a question of great interest since a substantially higher yield could justify the additional processing step.

However, if the target product instead was MUFA or PUFA, other treatments should be considered according to Otero et al. (2017), Hogan et al. (2021) and the result here of lower MUFA and PUFA yields of the fresh-biomass dilute-acid treatment relative to sonication. Yet, as discussed, the highest absolute MUFA and PUFA contents were recovered from long-stored freeze-dried biomass treated with dilute acid, complicating any straightforward conclusions. Testing dilute acid on freshly freeze-dried biomass would again be of interest to determine whether dilute acid is a viable option for MUFA and PUFA recovery despite the potential losses associated with this treatment.

4.3 Storage and Preservation Conditions

The pre-treatment effects discussed above cannot be cleanly separated from storage effects, since the long-stored FD samples behave very differently from the freshly processed FD samples. This section therefore considers storage and preservation independently.

4.3.1 Freeze-Dried

The most striking storage result was the loss of FA content during room-temperature FD storage. Untreated FD biomass lost 90.6 % of total FA between 1 day and 3 months (74.13 to 6.95 $\mu\text{g mg}^{-1}$ DW, Figure 26). Sonicated FD biomass declined substantially but less severely, by 73.6 % over the same period (43.98 to 11.61 $\mu\text{g mg}^{-1}$ DW). The same trend is noticeable in the PCA ranking on PC1 where at 1-day, untreated biomass scored higher than sonicated and at 3 months, the order was reversed. Furthermore, FD_non_3m and FD_son_3m converged on a common FA-depleted profile despite diverging at the 1-day timepoint, indicating that pre-treatment has a stronger effect on freshly freeze-dried biomass than on long-stored biomass, with the exception of the previously discussed dilute acid treatment (Section 4.2.2).

The unexpected proximity of FD_non_1d and FD_Ac_4m in the PCA (pairwise $R^2 = 0.38$) is also worth noting. These two groups gave the highest total FA yields in the dataset despite very different post-harvest handling. Whether this reflects genuine convergence in FA composition or biomass-composition variation between harvests cannot be determined here, since the 4-month and 1-day FD samples come from different harvests.

The class-level pattern in the FD storage data is consistent with oxidative degradation of unsaturated FAs. In untreated FD biomass, the PUFA fraction collapsed from 36.2 % after immediate extraction to 3.6 % after 3 months of storage, and the SFA fraction rose proportionally from 25.6 % to 60.2 %. In FD sonicated biomass, PUFA dropped from 35.6 % to 7.0 % and SFA rose from 27.1 % to 53.2 %. This is the expected pattern if oxidative cleavage of double bonds destroys PUFA and MUFA more readily than SFA: the resulting breakdown products (aldehydes, ketones, hydroperoxides, epoxides) are either volatile or are not detected as FAMES by GC, so the apparent SFA fraction remains comparatively intact.

Direct comparison with previous literature is limited because few studies have measured FA content in pure freeze-dried microalgal biomass after extended room-temperature storage. The 90.6 % loss of total FA observed here in *Craspedostauros* sp. freeze-dried biomass over the 3-month window is not unexpected, though it should be noted that the 1-day and 3-month samples originated from different harvests, meaning batch effects cannot be fully excluded as a contributing factor. Nonetheless, the magnitude of the decline extends the existing literature into a temporal and compositional space that has not previously been characterised.

Despite being the most gentle drying method, freeze-drying is an energy-intensive step requiring expensive equipment and associated high energy consumption, which restrict its use to applications where biochemical composition must be preserved (Ryckebosch et al., 2011). However, at the production scale Swedish Algae Factory operate at, freeze-drying is currently the most cost-effective drying option available, as alternatives such as spray-drying require large-scale industrial infrastructure with upfront costs not economically viable at the scale of smaller microalgae companies (Simon Nilsson, personal communication). A formal energy comparison between continuous cold storage and ambient storage of freeze-dried biomass with downstream dilute-acid revival would be valuable to determine whether the latter constitutes a genuinely lower-cost alternative, but this is beyond the scope of the present study.

4.3.2 Fresh (Wet) Storage at 4 °C

In contrast to freeze-dried storage, sonicated fresh biomass at 4 °C showed a numerical but non-significant decline in total FA yield over 14 days (67.56 to 54.79 $\mu\text{g mg}^{-1}$ DW, one-way ANOVA $F = 5.72$, $p = 0.051$, Figure 27). No FA class showed a significant change in absolute yield, and the proportional composition was stable throughout (all $p \geq 0.50$). The near-identical values at the two later timepoints are consistent with any yield loss occurring within the first 7 days and stabilising thereafter (Section 3.7). The present data therefore only show that total extractable FA and individual FA class remain stable from day 7 to 14. This indicates that no major oxidative degradation of MUFAs or PUFAs occurred during this interval, since such degradation would have manifested as a proportional increase in the SFA fraction, which was not observed.

Wet-biomass storage of *P. glacialis* has been characterised in detail by Dalheim et al. (2021), who showed both progressive lipolysis (FFA fraction increasing from 31 % after 24 h to 66 % after 14 days at 4 °C) and progressive PUFA loss, with degradation proceeding fastest at 20 °C. Their measurements were made by HPLC-ELSD (High-performance liquid chromatography with evaporative light scattering detection), which distinguishes between lipid classes such as FFA and esterified lipids. The acid-catalysed transesterification used in the present study converts both fractions to FAMES prior to GC analysis and is therefore insensitive to the redistribution Dalheim et al. detected. The present data show only that the total extractable FA pool remained stable across the storage window after the initial decline, not that lipolysis was absent.

Together, these findings suggest that refrigerated wet storage at 4 °C preserves FA composition adequately over a two-week period for sonicated *Craspedostauros* sp. biomass, with any yield loss appearing to be largely confined to the first week and not accompanied by detectable changes in FA class proportions. This is broadly consistent with previous observations that 4 °C substantially slows, but may not fully arrest, lipid degradation in wet-stored diatom biomass (Dalheim et al 2021).

4.4 Overall Fatty Acid Yields and Composition

The FA yields reported here were obtained as the sum of individual FAs quantified by GC of FAMES and are not gravimetric total-lipid yields. Several studies report higher numbers expressed as total lipid (e.g. Yang et al., 2017, Hu et al., 2008), but those values include every lipid that partitions into the organic phase: sterols, pigments, waxes, glycolipid headgroups, and the glycerol backbones of all glycolipids. Such values will therefore be systematically reported higher than the ones reported here and not directly comparable to the present FA mass values.

Studies reporting FA content per cell (Volkman et al., 1989, Torstensson et al., 2013, 2019) are excluded from yield comparison because cells were not counted in the present study since large-scale industrial handling of microalgal biomass is mass-based rather than cell-based. Studies reporting only relative compositions (mol% or wt%TFA e.g. Torstensson et al., 2019, Zulu et al., 2018) are likewise restricted to compositional comparison, once the total FA mass has been divided out, the information needed to recover absolute yield is lost. The yield comparison in this section is restricted to only Orcutt & Patterson (1975), they also report wt%TFA but they are the only ones that include a FA (% dry wt) mass anchor, allowing conversion to absolute yield ($\mu\text{g FA mg}^{-1}\text{ DW}$).

Three of the eighteen treatment combinations tested were selected for further discussion since these favourable conditions function as the most practically relevant downstream conditions, the remaining combinations did not reach yields that would be viable in practice. The three selected treatments and their per-FA yields, alongside the eight pennate diatoms reported by Orcutt & Patterson (1975), are summarised in Table 2.

4.4.1 *Craspedostauros* sp. Among Cultivated Pennate Diatoms

Table 2 reports the yields from the three chosen favourable treatments obtained in this study alongside those reported by Orcutt and Patterson (1975) on a common numerical basis ($\mu\text{g FA per mg dry biomass}$). Conversion of the Orcutt and Patterson (1975) values required combining two of their tables: their Table 2, which reports the relative composition of each FA as a percentage of total FAs (wt% TFA), and their Table 3, which reports total FA content as a percentage of dry weight (% DW total FA) determined gravimetrically. The mass anchor (% DW total FA) and relative composition (wt% TFA per FA) were combined as:

$$\mu\text{g FA mg}^{-1}\text{ DW} = \frac{\text{wt\% TFA}}{100} \times \% \text{ DW total FA} \times 10$$

Orcutt & Patterson did not report stearic acid (18:0), DHA (22:6) or 24:X and lumped 16:3 with 18:1 in a single column. These gaps are reflected as "-" or compound-column entries in Table 2. The eight pennate species included from Orcutt are *Nitzschia closterium*, *N. longissima*, *N. ovalis*, *N. frustulum*, *Amphora exigua*, *Amphora* sp., *P. tricornutum*, and *Fragilaria* sp.

Table 2. Total FA yield (μg per mg dry biomass) in three selected treatments of *Craspedostauros* sp., compared with values reported for cultivated marine pennate diatoms (Orcutt & Patterson, 1975). Values for this study are mean (standard error) and the ΣFA column is not the row sum across the displayed FA columns: only those FAs reported by Orcutt & Patterson (1975) are shown while ΣFA includes every FA quantified in this study (including 18:0, 22:6, 24:0 and others not in Orcutt's table). Trace values were carried over as "trace", "-" indicates the FA was not detected or not reported. Values for the Orcutt rows are converted from % DW to $\mu\text{g mg}^{-1}$ DW.

Source	Species	14:0	16:0	16:1	16:2	16:3+ 18:1	18:2	18:3	ARA 20:4	EPA 20:5	ΣFA
FD_Ac_4m	<i>Craspedostauros</i> sp.	2.49 (0.16)	18.44 (0.91)	30.55 (1.58)	0.89 (0.05)	4.36 (0.26)	2.31 (0.13)	3.26 (0.18)	6.19 (0.36)	14.31 (0.82)	87.16 (4.59)
FD_non_1d	<i>Craspedostauros</i> sp.	1.98 (0.15)	14.69 (1.43)	25.18 (2.17)	0.78 (0.07)	3.66 (0.33)	2.02 (0.18)	2.86 (0.23)	5.77 (0.48)	13.48 (1.06)	74.13 (6.40)
LC_Ac_0d	<i>Craspedostauros</i> sp.	2.08 (0.12)	14.80 (1.29)	18.64 (1.80)	0.55 (0.03)	2.43 (0.06)	0.94 (0.02)	2.11 (0.07)	2.77 (0.06)	4.46 (0.05)	51.40 (3.57)
O & P 1975	<i>N. closterium</i>	3.15	3.98	34.86	15.77	26.23	6.97	1.16	-	74.04	166
O & P 1975	<i>N. longissima</i>	6.02	33.02	36.21	0.34	2.58	1.38	0.52	2.58	3.1	86
O & P 1975	<i>N. ovalis</i>	3.07	14.5	25.17	0.99	3.19	2.03	-	3.19	5.51	58
O & P 1975	<i>N. frustulum</i>	3.9	25.8	107.25	3.9	3.3	-	-	trace	6	150
O & P 1975	<i>Amphora exigua</i>	2.27	12.05	19.66	1.01	2.56	0.67	-	-	3.74	42
O & P 1975	<i>Amphora</i> sp.	9.36	16.92	67.92	1.56	9	trace	trace	trace	15.24	120
O & P 1975	<i>P. tricornutum</i>	2.86	5.99	27.93	1.11	5.19	2.07	trace	trace	7.79	53
O & P 1975	<i>Fragilaria</i> sp.	22.32	16.62	34.2	0.95	1.43	7.6	-	-	12.35	95

Total FA yield across the three selected treatments ranged from 51.40 to 87.16 $\mu\text{g mg}^{-1}$ DW. This places *Craspedostauros* sp. in the lower-middle portion of the range reported by Orcutt and Patterson (1975) for cultivated marine pennate diatoms, which spanned 42 to 166 $\mu\text{g mg}^{-1}$ DW across their eight species with a group mean of 96.25 $\mu\text{g mg}^{-1}$ DW. The most favourable treatment (FD_Ac_4m, 87.16 $\mu\text{g mg}^{-1}$ DW) fell within 9.4 % of the group mean.

Orcutt & Patterson grew all their species in a chemically defined media, under defined illumination in batch cultures. Once dense cultures had developed, the cells were harvested and freeze-dried. Lipids were extracted by Soxhlet method, a precursor to Folch (1957) with the same

solvent and ratio (chloroform:methanol, 2:1 v/v) for 24 h, and gravimetric total lipid was determined after solvent evaporation. The lipid was then saponified and the released FAs converted to methyl esters for GC analysis. The main differences therefore are the 24-hour extraction time and the saponification (for full cultivation conditions, see Lewin 1966, cited in Orcutt & Patterson 1975). These could contribute to a systematic offset in absolute yield and should be acknowledged.

At the individual FA levels, the present treatments and the Orcutt & Patterson species share a common dominance of palmitoleic acid (16:1n7), palmitic acid (16:0), and EPA (20:5n3), consistent with the common diatom FA profile reported in the literature. Within the most favourable treatment (FD_Ac_4m), 16:1n7 was the most abundant FA at 30.55 $\mu\text{g mg}^{-1}$ DW, positioning it just below the median of the Orcutt & Patterson species distribution and 30.8 % below the group mean of 44.15 $\mu\text{g mg}^{-1}$ DW, though that mean is strongly influenced by *N. frustulum* (107.25) and *Amphora* sp. (67.92). For Palmitic acid (16:0) however, this treatment ranked third across the diatom species at 18.44 $\mu\text{g mg}^{-1}$ DW, behind *N. longissima* (33.02) and *N. frustulum* (25.80), while 14.5 % above the group mean of 16.11 $\mu\text{g mg}^{-1}$ DW. Of greater interest is the PUFA content of FD_Ac_4m. EPA levels at 14.31 $\mu\text{g mg}^{-1}$ DW places *Craspedostauros* sp. in the upper quartile, exceeded by *N. closterium* (74.04 $\mu\text{g mg}^{-1}$ DW) and comparable to *Amphora* sp. (15.24 $\mu\text{g mg}^{-1}$ DW). The Orcutt & Patterson EPA mean of 15.97 $\mu\text{g mg}^{-1}$ DW is strongly skewed by the exceptionally high *N. closterium* value, excluding it, position FD_Ac_4m at the top, 86.3 % above the mean of 7.68 $\mu\text{g mg}^{-1}$ DW. Two PUFAs in FD_Ac_4m exceeded all compared species, 18:3n6 and ARA. For 18:3n6, the treatment yielded 3.26 $\mu\text{g mg}^{-1}$ DW, more than twice the highest value reported by Orcutt & Patterson (*N. closterium*, 1.16 $\mu\text{g mg}^{-1}$ DW), with only trace amounts or no detection across the remaining species. ARA showed a similar pattern at 6.19 $\mu\text{g mg}^{-1}$ DW. Both FAs stand out as particularly distinguishing features of the FA profile of *Craspedostauros* sp.

It is important to remember that this is a secondary consideration for *Craspedostauros* sp., the primary application is frustule production, for which the species is already established in practice. FA yields reported therefore represent what is achievable under a co-production condition rather than targeted FA optimisation. Comparison with species cultivated specifically for FA production should therefore be interpreted with this context in mind. Taken together, the absolute yield and compositional profile support *Craspedostauros* sp. as a credible FA-producing pennate diatom alongside its primary use as a frustule producing species. With the most favourable treatment within 9.4 % of Orcutt & Patterson of mean yield and the standout features of high PUFA (EPA, ARA and 18:3n6) content positions *Craspedostauros* sp. as an especially promising PUFA-producing species.

4.5 Other Methodological Considerations

This section addresses methodological aspects of the study that do not fit in above but are relevant to the interpretation of the reported FA yields and composition. These include the dry-weight determination approach, the inclusion placement of the internal standard and the identification and exclusion of outlier replicates.

4.5.1 Suspected Contamination and Outlier Exclusion

Two replicates were excluded on the basis of abnormal FA concentrations that could not be attributed to a biological effect (Section 3.1): LC_Ac_3 and FD_son_3m_1. In the LC_Ac group,

the 18:0 concentration of the outlier replicate exceeded the other two replicates and the levels across the remaining treatment groups by more than an order of magnitude. A technical origin is therefore more plausible than a biological one. Stearic acid (18:0) is a well-documented contaminant introduced from disposable plastic labware, including pipette tips, plastic syringes, and syringe filter discs, where it is present as a leachable from the polymer matrix and from amide-based polymer lubricants (Cheng & Yu, 2020; Canez & Li, 2024). The elevated 18:0 in LC_Ac_3 was therefore treated as a contamination artefact and the replicate excluded.

The anomaly in FD_son_3m_1 has a different signature from that of LC_Ac_3. Rather than an isolated 18:0 spike, FD_son_3m_1 showed elevated concentrations across 16:0, 18:1, and 18:0 simultaneously, suggesting a broader sample-level disruption rather than a single contaminant source. While plastic-leachable contamination cannot be excluded as a contributing factor, the pattern is more consistent with a sample-level technical error, most plausibly contamination introduced during sample handling. The replicate was treated as a contamination artefact and the replicate excluded.

4.5.2 Statistical Power and Replicate Constraints

The replicate structure ($n = 2-3$ per group) is typical for a novel exploratory study of this kind, where the primary aim is to map treatment effects across a broad condition space rather than achieve high statistical power within any single comparison. It does however limit the ability to resolve within-condition variability and constrains pairwise PERMANOVA p-values to 0.10, leaving R^2 as the primary informative metric for pairwise comparisons.

4.5.3 Pellet-Associated Lipid Loss

The two-phase aliquoting approach assumes homogeneous distribution of lipids in the organic layer, which may not hold when emulsions or cell debris are present. The intermediate pellet that emerges in the middle of the layers may trap lipids. It is therefore not possible to exclude the possibility that the lower yield observed under some sonication conditions reflects lipid entrapment in the interfacial pellet rather than incomplete extraction. This uncertainty could be tested by re-extracting the pellet to determine whether additional lipids are recoverable.

4.5.4 Internal Standard Placement and Underrepresentation

The internal standard (di-17:0 PC) was added prior to transesterification. Reported FA concentrations are therefore corrected for losses during derivatisation and GC injection but not for losses during the extraction process (Christie & Han, 2010). Extraction related losses from incomplete partitioning, emulsion trapping, adsorption to cell debris, or physical transfer are not accounted for, and the absolute yields reported here should therefore be regarded as minimum estimates of the true FA content. Between-treatment comparisons are valid to the extent that extraction efficiency is reasonably consistent across conditions. The tight clustering of replicates within each treatment group (Figure 21) suggests this was likely the case, as consistent extraction efficiency is the most plausible explanation for the generally high within-group reproducibility observed.

4.5.5 Dry-Weight Determination, Oxidation Risk and Yield Underestimation

Drying is a critical step for lipid and FA analysis as the choice of method can significantly influence lipid class distribution, oxidation state, and consequently the resulting FAME profile. For the fresh biomass, dry weight was determined gravimetrically from parallel aliquots dried by microwave at 500 W, potentially introducing a source of inconsistency. The use of parallel aliquots rather than the sample reserved for extraction was necessary to avoid exposing it to the heat and oxidation conditions of the drying process, which would compromise the integrity of the lipids prior to extraction. However, any heterogeneity in biomass distribution between aliquots translates directly into the dry-weight estimate.

Oxidation, heat degradation, and volatilisation are factors that cannot be eliminated during dry-weight determination. Out of the conventional drying methods available, microwave drying was chosen for its speed, both methodologically, by shortening the overall protocol, and analytically, by minimising the sample's exposure to heat and oxygen. Following standard gravimetric practice, samples were dried to constant mass, confirmed by agreement between two successive readings, yielding a dry weight estimate as faithful as possible under the circumstances. This was the only feasible approach given that the limited biomass available was insufficient for direct weighing of the sample reserved for extraction. However, a more substantial oxidation risk in the overall workflow is the overnight evaporation of organic solvent. While unavoidable under the available equipment, this step is the most likely point of PUFA loss in the workflow. Oxidative loss at this stage is suspected, and PUFA yields and recovery could potentially be improved by drying under nitrogen or other inert-gas techniques.

4.6 Future Outlook

This study mapped the effects of cell disruption, pre-treatment, and storage conditions on extractable FA yield and composition in the pennate marine diatom *Craspedostauros* sp., identifying several treatment combinations of potential practical relevance, while also raising questions that warrant further investigation. Several extensions to the present work would clarify the most ambiguous findings and strengthen the practical applicability of these results.

The dilute-acid result for long-stored freeze-dried biomass is the single most striking finding in the dataset and warrants direct follow-up investigation. Would applying dilute acid immediately after freeze-drying further improve yield? If so, it could represent the optimal combination of pre-treatment and practical storage. The first priority would be to confirm reproducibility, after which a time-series experiment tracking FA yield from day 0 to, and possibly beyond, 4 months would establish whether the yield accumulates progressively with storage duration, appears only beyond a certain threshold, or is in fact independent of storage duration altogether. A heat-only control (65 °C, 30 min, no acid) would separate the chemical effect of the acid from the thermal effect, and a systematic experiment varying acid concentration, temperature, and duration independently would be required to fully resolve their relative contributions and identify the optimal combination, given that these parameters differ markedly across the protocols of Otero et al. (2017) and Hogan et al. (2021) and the present study.

Second, quantitative lipid-class profiling, such as HPLC-ELSD as used by Dalheim et al. (2021), would complement the FA-class data presented here and would clarify whether storage-related losses occur preferentially in particular lipid classes. The relevance of lipid-class profiling also depends on the target application. For biofuel production, the desired lipid profile is characterised not only by high total FA yield but specifically an abundance of TAGs containing medium- to short-chain FAs, as these are the most suitable feedstock for biodiesel. The present GC analysis

establishes the FA composition, covering half of the necessary characterisation, but cannot resolve lipid class distribution. Quantitative lipid-class profiling would therefore be a necessary next step in fully evaluating whether *Craspedostauros* sp. is a viable biofuel feedstock candidate.

Third, whether wet storage at 4 °C continues to follow the day 7 profile beyond 14 days should be tested at 21, 28 days and possibly beyond. This would resolve whether the present non-significance reflects true compositional stability, a logarithmic degradation pattern, or whether the present sample size simply lacked the power to detect a real shift. Establishing a wet-storage timeline would also be valuable in setting practical handling windows for industry, since 4 °C wet storage is considerably less energy-intensive than freeze-drying. However, the practical viability of wet storage as an alternative must ultimately be evaluated against a full cost-benefit analysis of the energy and infrastructure costs of continuous refrigeration against the yield gains achievable through freeze-drying combined with dilute-acid pre-treatment.

5. Conclusion

The Wigert and Folch methods returned similar total FA yields when averaged across pre-treatment conditions, but this masks a significant interaction: Folch recovered significantly more from untreated biomass, while Wigert recovered more from sonicated biomass. The methods are therefore not equivalent, the similar averages reflect cancellation of opposing effects and broader assessment of Wigert is warranted.

The single most striking finding of the study was that dilute acid treatment applied to freeze-dried biomass after 4 months of room-temperature storage recovered the highest total FA yield in the entire dataset, with the highest absolute content in every FA class. This represents a remarkable revival of the same batch: samples extracted from the freeze-dried material one month earlier, without acid treatment, had lost 90.6 % of total FA after 3 months of room-temperature storage.

Craspedostauros sp. showed ARA (20:4n6) and 18:3n6 concentrations exceeding all eight species reported by Orcutt & Patterson (1975). Total FA yields place the strain in the lower-middle range of cultivated marine pennate diatoms but the high PUFA content, EPA, ARA and 18:3n6, positions *Craspedostauros* sp. as a promising candidate for PUFA production.

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Appendix 1 – Popular Science Summary

Turning microalgae into a source for supplements, silica, and fuel

Imagine a single microscopic organism that could supply the omega-3 in your fish-oil capsule, the silica in your computer chip, and a share of biofuel for the truck that delivered them. That organism already exists and growing it does not require a single hectare of farmland. It is called a diatom: a single-celled microalga that produces both lipid-rich biomass and a cell wall made of silica.

Why diatoms?

Diatoms grow almost everywhere there is water: oceans, rivers, lakes, brackish ponds, and even the runoff from fish farms. Unlike conventional crops, they do not need arable land or freshwater to thrive. Growing diatoms therefore does not compete for the two resources farming depends on most. What makes them especially interesting is that each cell produces two entirely different valuable materials at once. Inside the cell, diatoms accumulate large quantities of lipids, including the omega-3 fatty acids found in fish-oil supplements and shorter-chain fats suitable for biodiesel. The cell wall is just as valuable. Every diatom surrounds itself with a rigid shell of silica, currently the second most used non-renewable resource on Earth. Producing it industrially from quartz requires energy-intensive mining, acid treatment, and high-temperature purification. Diatoms grow it in a biologically pure form that requires minimal post-processing to reach the quality standards required for electronics and solar panels.

The study organism: *Craspedostauros* sp.

With over 10 000 known diatom species, identifying the most productive and practically viable candidates requires systematic evaluation. Lipid yield, fatty acid composition, silica quality, and how well a strain tolerates cultivation and processing are all factors that matter. This study focuses on one such candidate: *Craspedostauros* sp., a pennate diatom and established production strain. The first goal was to characterise its fatty-acid composition. But knowing what a strain contains is only half the picture. In industrial processing, biomass is rarely processed the moment it is harvested. It may be stored, dried, and transported before extractions can be done, and each of those steps can degrade the very lipids we are trying to recover. So, the second, equally important question was a practical one: how well does the fatty-acid profile hold up through real-world handling?

Key findings

Under the most favourable conditions, fatty acids made up nearly 9 % of dry biomass by weight, confirming *Craspedostauros* sp. as a genuinely lipid-rich strain. The most abundant fatty acids were palmitoleic acid and palmitic acid, both of interest for biodiesel production. The species also stood out for its omega-3 EPA and omega-6 ARA accumulation. The latter, ARA, reached concentrations exceeding all other species examined, and has documented roles in both infant brain development and the immune system. The way biomass is stored, it turns out, can be the difference between a rich lipid extract and almost nothing at all. Freeze-dried biomass left at room temperature for three months lost up to 91 % of its fatty acids. Yet a simple dilute-acid wash before extraction was enough to revive it, recovering the highest fatty-acid yield in the entire study and suggesting that freeze-dried diatom biomass may remain viable for far longer than previously thought.

Appendix 2 – Freeze-Drying Protocol

Step	Temperature (°C)	Time (min)	Hold/ramp	Condenser (°C)	Pressure (mTorr)
Freeze	-18	0		-45	280
1	10	60	Hold		130
2	15	60	Hold		130
3	20	60	Hold		130
4	25	60	Hold		130
5	30	60	Hold		130
6	35	1200	Hold		130
7	35	1200	Hold		130
8	35	1200	Hold		130

Appendix 3 – AI Statement

During this project, Claude (Anthropic) has been used continuously alongside the process for the following purposes: First, to search for relevant literature and summarise papers to review their relevance. All papers that were found and used were stored locally, allowing the AI to find relevant passages on a given subject across several papers at once. The AI only pointed to existing text and generated nothing of its own. Therefore, the accuracy of all content rests solely on the source papers and is not fabricated by the AI. Second, to review the text and flag unclear, ambiguous, overly complicated, or bulky passages. All suggestions and comments were reviewed, fact-checked, and considered by me before I revised the passages. Third, to help write and debug Python code in Jupyter for data processing, statistical analysis, and figure generation (all plots and schematics). I reviewed, edited, and ran all code locally.