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Hypotonic osmotic shock treatment to enhance lipid and protein recoveries from concentrated saltwater *Nannochloropsis* slurries

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ABSTRACT

This study reports the use of hypotonic osmotic shock as a treatment step to enhance the recoveries of biofuel-convertible lipids and proteins from lipid-rich saltwater *Nannochloropsis gaditana* (*N. gaditana*) slurries (biomass content = ~140 mg biomass / g slurry, total lipid content = ~600 mg lipid /g biomass). The osmotic shock was induced through repeated washing of microalgal slurries with multiple batches of fresh water. Subjecting the slurries to 2 stages of freshwater washing resulted in a measurable damage to cell membranes (the uptake of membrane permeability marker increased by 6 folds), a partial loss of cell viability (only 64% of available cells were recoverable), and a minor release of free protein (~2 wt% of available protein) from the biomass into the interstitial space of the slurries. Hypotonic osmotic shock was revealed to be ineffective in rupturing *N. gaditana* slurries (only 13 ± 9% of available cells were ruptured after 2-stage washing) and, as such, had a limited prospect as a stand-alone cell disruption technology for the saltwater strain.

The washing treatment, however, was found to be able to weaken the structural integrity of *N. gaditana* slurries and enhance the performance of subsequent mechanical or chemical cell disruption technologies when installed as a preparatory step. Applying the washing treatment prior to high-pressure homogenisation (HPH) and low-solvent-to-biomass ratio hexane extraction (hexane : slurry = 1:1 w/w) for the recovery of biofuel-convertible lipids increased the extent of cell rupture from 28 ± 8 to 46 ± 19% of available cells and more than doubled neutral lipid yield from 25.1 ± 2.0 to 64.6 ± 4.9 wt% of available neutral lipid. Initial analysis revealed that the washing treatment had a minimal energy cost (~6% of the total energy expenditure of downstream processing) and that its integration into HPH + hexane lipid recovery led to a 2.5 fold increase in the energy output of the biomass. Partnering the washing treatment with NaOH hydrolysis increased protein yield from 6.7 ± 2.4 to 31.9 ± 10.7 wt% of available protein.

1. Introduction

In recent years, microalgae have been shown to be a highly promising feedstock for sustainable biofuels and high-value bioproducts (*e.g.* chlorophyll, β-carotene, ω-3 fatty acids and protein) because of their high areal productivity and non-requirement for agricultural resources (*e.g.* arable land and freshwater for marine microalgae) [1–7]. These products are intracellular in nature and can generally only be recovered after they have been liberated from the encapsulation of the cell walls [3,8–11]. The *Nannochloropsis* sp. is an industrially attractive genus of saltwater microalgal strains because of its 1) high growth rate, 2) contamination resistance, 3) high basal protein content (20–30 wt% of biomass), 4) high basal lipid content (10–30 wt% of biomass under nitrate replete condition) that includes an abundance of the commercially

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valuable ω-3 eicosapentaenoic acid fatty acid (3–5 wt% of biomass under nitrate replete condition) and 5) ability to accumulate biofuel-convertible neutral lipids as carbon reserve under nitrogen starvation (total lipid content previously reported to increase to ~ 60 wt% of the biomass) [12–17]. The saltwater strains, however, are highly resistant to cell rupture, having a mechanically rigid and structurally complex cell wall that is comprised of an outer algaenan layer and an inner layer made of cellulose (~75 wt% of cell wall) and protein (~6 wt% of cell wall) [18–20]. Algaenan is a cutan-like alipathic hydrocarbon able to withstand harsh oxidative treatments and resist acid/alkali hydrolysis [10,19].

Osmotic shock treatment achieves cell disruption by suspending cells in either a hypotonic solution (salt concentration of solution < salt concentration of cytoplasm) or hypertonic solution (salt concentration of solution > salt concentration of cytoplasm) and inducing osmotic gradient across the cell membrane [21]. For hypotonic treatment, the reverse osmotic gradient results in a net diffusion of water into the cells and leads to a build-up of internal pressure that ultimately bursts the cells. For hypertonic treatment, the forward osmotic gradient results in a net diffusion of water out of the cells and leads to a reduction of internal pressure and the collapse of the cellular structures [21]. Osmotic treatment works well with animal cells because they lack cell walls but has generally been shown to have limited effectiveness when applied to plant and other microbial cells protected by rigid cell walls [21]. For saltwater microalgal species, osmotic shock is generally performed with a hypotonic shift, induced by concentrating the culture and transferring (or resuspending) the resulting biomass to either a medium with lower salt concentration (<30 g salts / L medium) or fresh water. For freshwater microalgal species, on the other hand, osmotic shock is carried out with a hypertonic shift, triggered by adding solutes (such as NaCl and sorbitol) to the culture medium. [22,23].

Even though a few studies have described the use of osmotic shock as a mechanical cell disruption technology that can be combined with solvent extraction for enhanced lipid recovery from microalgae, they have generally limited the scope of their investigation to the induction of hypertonic osmotic shock on freshwater microalgae species (such as *Chlamydomonas reinhardtii*, *Botryococcus* sp., *Scenedesmus* sp.) [22,23]. These studies lacked scalability as they had used biomass which was either too expensive to process at an industrial scale (*e.g.* dilute microalgae suspension at ~1 wt% solid) [22] or too energy demanding to produce (*e.g.* freeze-dried biomass) [23]. The narrow focus of the studies on analysing lipid yield means that there is also currently a lack of understanding on the impact of osmotic shock on cell physiology and cell wall integrity.

In Yoo et al. [22], subjecting a relatively dilute suspension of *C. reinhdardtii* biomass to hypertonic osmotic shock with 60 g/L NaCl solution was shown to result in 2.5-fold increase in lipid yield. The treatment was found to be significantly more effective when applied on wall-less mutant strain than on wild-type strain, further underscoring the role that cell wall plays in erecting mass transfer barrier and hindering lipid accessibility. In Lee et al. [23], hypertonic osmotic shock (100 g NaCl / L suspension) was shown to be equally effective as other cell disruption techniques (such as microwave, bead beating, sonication and autoclave) in recovering lipids from freeze-dried *Chlorella vulgaris* and *Scenedesmus* biomass.

In the few cases where hypotonic shift has been successfully used as a strategy to release intracellular products from microalgae biomass, the treatment has been applied exclusively to fragile saltwater strains with no cell walls, such as *Dunaliella viridis* for the release of water-insoluble neutral lipids [24] and *Rhodomonas salina* for the release of water-soluble phycocyanin and phycoerythrin [25,26]. Since wall-less species are significantly more vulnerable to external rupture and membrane damage, their defence profiles cannot be used to represent those of thickly walled strains, such as species in the *Nannochloropsis* and *Chlorella* genus. In their study investigating the use of hypotonic osmotic shock as a low-cost strategy to induce milking from saltwater *D. viridis* culture (0.04–0.22 g biomass / L culture) in a semi-continuous system, Davis et al. [24] found that up to 62 wt% of accumulated lipids in the biomass could be successfully discharged into the bulk medium through the introduction of intermittent osmotic drift and the ensuing reversible membrane permeation. In their study comparing eight different methods of isolating phycoerythrin from *R. salina* cells, Thoisen et al. [26] found that direct extraction of the filtered wet biomass with 0.1 M phosphate buffer was sufficient to incur cell disruption and release most of the available pigments.

To the best of our knowledge, no study has yet investigated the use of hypotonic osmotic shock as a potential means to process and rupture thickly walled saltwater microalgae slurries (such as those belonging to the *Nannochloropsis* genus). This study therefore aims to examine the use of hypotonic osmotic stress as a means to weaken and rupture lipid-rich concentrated *Nannochloropsis gaditana* slurries (biomass content = 137.7 ± 12.6 mg biomass / g slurry, total lipid content = 607.0 ± 102.5 mg/g biomass) for biorefinery production of biodiesel and food products (protein). Hypotonic osmotic shock on the saltwater strain was induced by repeated washing of the slurry with batches of fresh water. The biomass concentration used in the study was similar to those encountered in industrial scale biorefinery systems. In the first part of the study, we characterised the effect of hypotonic osmotic shock on biomass composition, cell integrity, cell viability and examined the capacity of the treatment as a stand-alone technology for rupturing *N. gaditana* slurries. In the second part of the study, we examined the ability of the hypotonic osmotic shock to weaken cellular integrity and enhance the performance of other disruption technologies for the recoveries of intracellular products from *N. gaditana* slurries. The washing treatment was coupled with either high-pressure homogenisation and hexane extraction for the recovery of biofuel-convertible lipids or alkali hydrolysis for protein recovery. We also calculated the energy requirements for the osmotic shock treatment and evaluated the energy balance of integrating the treatment into a lipid recovery and biofuel conversion process.

2. Materials and methods

2.1. Strain, inoculum preparation and cultivation

N. gaditana strain (SAG 2.99) was acquired from the Culture Collection Centre at Georg-August-Universität Göttingen (SAG, Germany). The inoculum culture was grown with modified f medium in synthetic seawater (30 g/L of sea salts from Red Sea Coral Pro Salt, Red Sea, USA). The composition of the modified f nutrient composition was as follows: 0.2 g/L $NaNO_3$, 0.02 g/L $NaH_2PO_4{\cdot}2H_2O$, 0.009 g/L $FeC_6H_5O_7$, 0.009 g/L $C_6H_8O_7$, 0.005 mg/L $CuSO_4{\cdot}5H_2O$, 0.023 mg/L $ZnSO_4{\cdot}7H_2O$, 0.011 mg/L $CoCl_2{\cdot}6H_2O$, 0.2 mg/L $MnCl_2{\cdot}4H_2O$, 0.0084 mg/L $Na_2MoO_4{\cdot}2H_2O$ and 0.00065 mg/L H_2SeO_3, 0.00005 mg/L vitamin B12, 0.00005 mg/L biotin, 0.1 mg/L thiamin. This inoculum culture was inoculated into a modified 3f medium in a custom-made 25 L bubble-column photobioreactor (PBR).

N. gaditana culture in the PBR was cultivated at 21°C with permanent white illumination from external LED sources. The operating principles of our bubble-column PBR has previously been reported [27,28]. The illumination was increased stepwise every few days from 120 μmol m^{-2} s^{-1} on day 0 to 370 μmol m^{-2} s^{-1} on day 14 (Light intensity was measured on the surface of the reactor). The culture was aerated with CO_2-enriched air at an air flow rate of 0.15 vvm and a CO_2-to-air ratio of 1/100 v/v.

A 3f medium contained 3 times the nutrient concentrations of a modified f medium with the same sea salt concentration. We intentionally provided the PBR culture with additional nutrients (3f instead of f) to maximise biomass growth potential under continuous CO_2 supplementation. Each cultivation cycle lasted 12 to 18 days and there was a total of 7 cycles. The amount of inoculum culture added at the beginning of each cultivation cycle was calculated to give a starting

OD_{750} of 0.08 (equivalent to an initial biomass concentration of 0.029 g biomass / L culture).

2.2. Cultivation monitoring, harvest and dewatering

For each cultivation cycle, samples of the culture were collected almost daily from the PBR for biomass concentration and nitrate concentration measurements. The biomass concentration of the microalgal culture was determined via filtration and gravimetric measurement as previously described [29]. To determine the NO_3^- concentration in the medium, ~10 ml of the culture was filtered (0.22 μm) and the resulting supernatant subjected to colorimetric nitrate analysis in accordance to the reagent's instruction manual (Spectroquant Nitrate Test, Merck Millipore, USA). The absorbance of the solution was read at 497 nm and total NO_3^- in the medium was quantified against a linear calibration curve.

At the end of each cultivation cycle, the culture was completely harvested from the PBR and concentrated at 3000 g using a centrifuge with a swinging-bucket rotor. The resulting paste was re-diluted with supernatant obtained from centrifugation to produce ~480 g of concentrated slurry at a biomass concentration of 139.6 ± 12.6 mg biomass / g slurry. There was a total of 7 slurries, one from each cycle or harvest. The slurry was then used for the washing procedure in Section 2.3. For all experiments, biomass was concentrated, washed (Section 2.3), subjected to lipid recovery (Section 2.13) or protein recovery (Section 2.14) on the same day that they were harvested from the PBR.

2.3. Freshwater washing and induction of hypotonic osmotic shock

N. gaditana slurry was subjected to 2 stages of freshwater washing (refer to Fig. 1 for full schematics of the washing process). In brief, microalgal slurry (~270 g slurry) was centrifuged at 7698 g and 20°C for 10 min. The supernatant was carefully decanted, weighed and stored at −5°C. A batch of DI water was added to the microalgal pellet in order to reconstitute the slurry. The mixture of microalgal pellet and DI water was agitated, first with a spatula and then moderately with magnetic stirring, to ensure complete homogeneity before being subjected to another centrifugation step. Once partitioned, the supernatant was collected for weighing and stored −5°C and a second washing stage was repeated on the pellet.

As shown in Fig. 1, slurries generated throughout the washing steps were denoted in the order at which they were produced: untreated slurry, washed slurry1 and washed slurry 2. Supernatants and pellets obtained during the washing procedure were also denoted in the same way (Fig. 1): untreated supernatant, untreated pellet, washed supernatant 1, washed pellet 1, washed supernatant 2, washed pellet 2.

Table 1 provides the complete mass balance of all the streams in the washing treatment. The mass balance reports the average values from 6 biological repeats and has been normalised to 100 g of untreated slurry. The amount of water used for the first washing stage was set to be almost the same as the mass of the initial slurry (*i.e.* mass of wash water 1 = 86.7 g water / 100 g untreated slurry). For the second washing stage, we decided to reduce the overall water requirements of the washing treatment and deliberately set the amount of water used during the step to be roughly half the mass of slurry (*i.e.* mass of wash water 2 = 44.6 g water

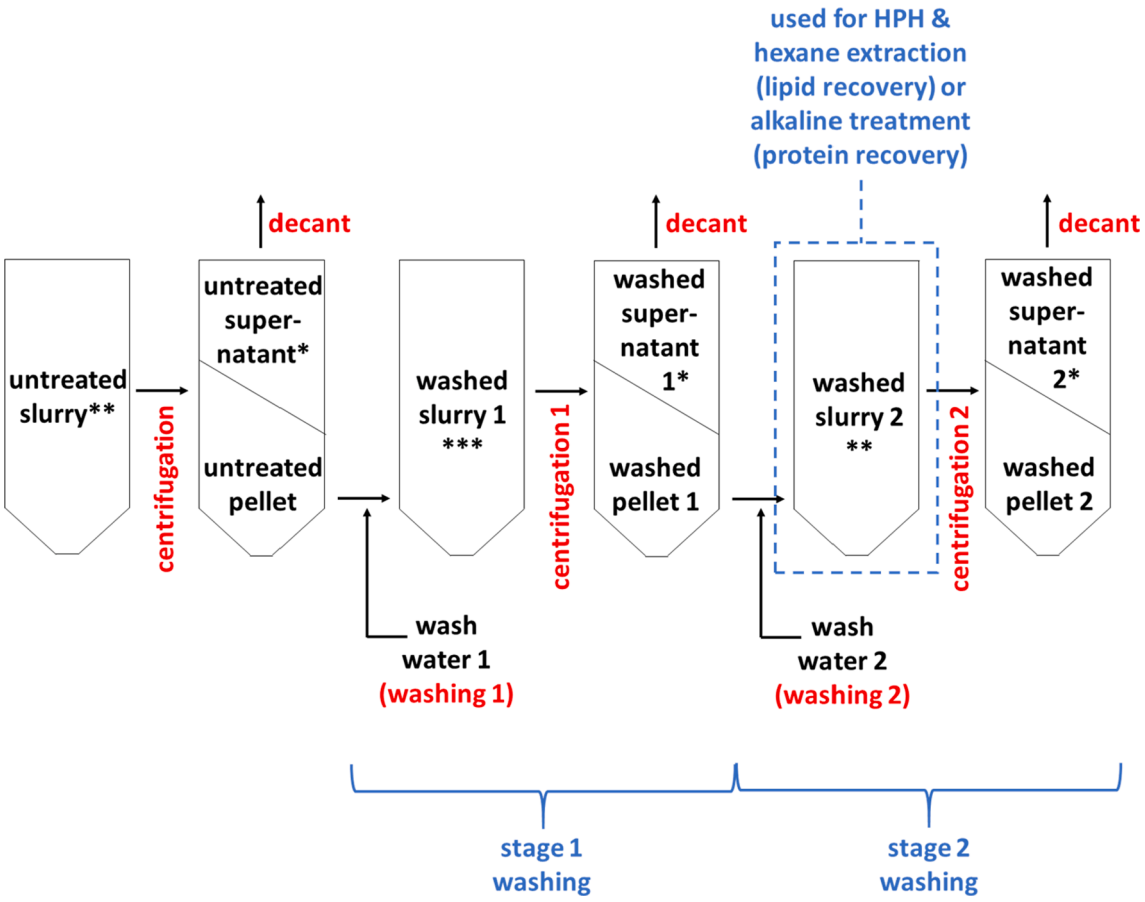

Fig. 1. Schematic of the steps involved in biomass freshwater washing and supernatant isolation. Complete mass balance of the washing steps can be viewed in Table 1. * subjected to sugar analysis, protein analysis, lipid analysis, biomass concentration measurement, salt measurement. ** subjected to sugar analysis, protein analysis, lipid analysis, conductivity measurement, biomass concentration measurement, salt measurement, membrane permeabilisation assessment, cell viability assessment. *** subjected to conductivity measurement, membrane permeabilisaton assessment, cell viability assessment.

/ 100 g untreated slurry).

Each slurry (untreated slurry, washed slurry 1 and washed slurry 2) was divided into two separate lots. The first lot was immediately processed for analyses that required fresh biomass (conductivity measurement in Section 2.7, membrane permeability and cell viability assessment in Section 2.11), while the second lot was stored (-5℃) for analyses that could be performed on thawed biomass at a later date (sugar analysis in Section 2.4, protein analysis in Section in 2.5, lipid analysis in Section 2.6 and salt concentration measurement in Section 2.10). Each supernatant (stored at −5℃) was subjected to sugar analysis, protein analysis, solid concentration and salt concentration measurement as outlined in Section 2.4, 2.5, 2.9 and 2.10 respectively. Each stream in Fig. 1 has been annotated with the specific set of analysis that it was subjected to. The same parameters were used for all centrifugation steps in the washing process (7698 g and 20℃).

Untreated slurry and washed slurry 2 were further subjected to either a lipid recovery process (with high-pressure homogenisation and hexane extraction: Section 2.13) or a protein recovery process (alkaline hydrolysis: in Section 2.14).

2.4. Sugar analysis of slurry and supernatant

2.4.1. Anthrone analysis

The total sugar content of microalgal slurry or supernatant was determined using an anthrone-sulphuric acid assay. Freshly prepared starch standard solutions (Merck 1.01257, Merck, USA) with a concentration ranging from 0.02 g/L to 0.4 g/L in DI water were processed at the same time with the samples. For the supernatant analysis, the samples were filtered (0.22 μm) to remove residual biomass and diluted in DI water (dilution factor ranging from 5 to 200x). For the biomass analysis, microalgal slurries were diluted in DI water to a biomass concentration ranging between 0.1 and 0.4 g biomass /L. An aliquot of the diluted supernatant or diluted slurry or standard solution (400 μl) was mixed with 800 μl of freshly prepared anthrone reagent (0.1% w/v in 95% sulphuric acid) in a 1.5 ml Eppendorf safe-lock tube through inversion. The mixed solution was first placed on ice (5 min), then incubated in a pre-heated thermo-incubator at 95℃ and 300 rpm for 16 min before being re-transferred to ice for cooling. Absorbance of the mixed solution was measured at 625 nm; the carbohydrate content for each sample solution was calculated using the standard curve generated during the analysis and corrected with the appropriate dilution factor.

2.4.2. Biomass hydrolysis and high-performance liquid chromatography (HPLC) analysis

For some experiments, the sugar content of the slurry was also verified using sulphuric acid / HPLC analysis. For this, freeze-dried biomass of the untreated or washed slurry was subjected to a two-stage acid treatment for complete polysaccharide hydrolysis as previously described [30]. The resulting hydrolysate was analysed for sugar monosaccharides with a reverse-phase HPLC equipped with a Metrosep Carb 2 column (Metrohm, Germany) and an amperometric detector as previously described [31]. Sugar monosaccharides (glucose and galactose) were identified by retention-time comparison with pure standards and quantified against calibration curves of standard solutions.

2.5. Protein analysis of slurry and supernatant

Protein in the microalgal slurry was made accessible for total analysis with a high-temperature alkaline hydrolysis. Microalgae slurry (130 mg) was added to 2 ml of 1 N NaOH solution, heated at 95℃ for 1 h, cooled, diluted with 2 ml of DI water and centrifuged to obtain a protein-rich serum. For the analysis of microalgal supernatant, the sample was filtered through a 0.22 μm syringe filter to remove any residual biomass and diluted 5x to make sure that its final absorbance fell within the range of prepared calibration curve. An aliquot (25 μl) of the hydrolysis serum or post-filtered supernatant was then subjected to a modified Lowry analysis as previously described for the determination of total protein content [18].

2.6. Lipid analysis of slurry

2.6.1. Total lipid extraction

The total lipid content of microalgal slurry was determined using a four-stage monophasic Bligh and Dyer extraction method as previously described [12,18]. Total lipid recovery was confirmed through complete bleaching of the cell debris after the final stage. The chloroform phases isolated from all four extraction stages were pooled together, filtered (0.2 μm nylon syringe filter) and dried under N_2 gas for gravimetric measurement. The dried lipid (40 mg lipid) was re-dissolved in chloroform/methanol solution (2:1 v/v) for lipid fractionation.

2.6.2. Solid phase extraction (SPE) for lipid fractionation

An aliquot (600–700 μl) of the lipid solution (containing 11.7 ± 3.2 mg lipid) was separated into its constituent fractions (neutral lipids, glycolipids and phospholipids) by sequential elution with different

Table 1
Complete composition of the streams (slurry, supernatant, pellet, wash water) involved in biomass freshwater washing treatment. The mass of each component (water, biomass, released sugar, released protein, salts) in every stream has been normalised to 100 g of untreated slurry. Biomass in supernatant denotes unsettled biomass.

Treatment step	Stream	Water (g)	Biomass (g)	Released sugar (g)	Released protein (g)	Salts (g)	Total mass of stream (g)
	Untreated slurry	83.82	13.77	0.00	0.00	2.42	100.00
Centrifugation	Untreated supernatant	51.54	0.13	0.02	0.01	1.15	52.84
Centrifugation	Untreated pellet	32.28	13.62	0.00	0.00	1.27	47.16
Washing 1	Wash water 1	86.74	0.00	0.00	0.00	0.00	86.74
Washing 1	Washed slurry 1	119.01	13.62	0.00	0.00	1.27	133.90
Centrifugation 1	Washed supernatant 1	72.12	0.21	0.12	0.03	0.81	73.28
Centrifugation 1	Washed pellet 1	46.90	13.26	0.00	0.00	0.46	60.62
Washing 2	Wash water 2	44.60	0.00	0.00	0.00	0.00	44.60
Washing 2	Washed slurry 2	91.50	13.26	0.00	0.00	0.46	105.22
Centrifugation 2	Washed supernatant 2	53.18	0.40	0.89	0.18	0.27	54.91
Centrifugation 2	Washed pellet 2	38.32	11.79	0.00	0.00	0.19	50.30
	Untreated + washed 1 + washed 2 supernatants		0.74	1.02	0.22	2.22	

solvent systems in a SampliQ pre-packed silica cartridge (Agilent Technologies, USA) as previously described [12,18].

2.7. Conductivity measurement of slurry

The conductivity σ_T (μS/cm) of the microalgae slurry was measured using a conductivity meter (CLM 381, Endress + Hauser, Switzerland). The adjusted conductivity at 25℃, σ_{25} (μS/cm), was calculated using Eq. 1:

$$\sigma_{25} = \sigma_T \frac{1}{1 + \alpha_{25}(T - 25)} \quad [1]$$

where T was the temperature the slurry during conductivity measurement (℃) and α_{25} was the temperature coefficient of variation at 25℃ (assumed to be 2.8%/°C based on the findings of our previous study [32]).

2.8. Biomass concentration of microalgal slurry

The biomass concentration of microalgal slurry was the sum of the sugar content, the protein content and the lipid content of the slurry.

2.9. Solid concentration measurement of microalgal supernatant

The solid content of microalgal supernatant was determined gravimetrically by oven drying 130 mg of the supernatant on a pre-weighed aluminium cap at 95℃ for 16 h.

The solid content of supernatant is defined as the total amount of non-water components found in the supernatant after centrifugation. This included both insoluble (*e.g.* suspended or unsettled biomass) and soluble (*e.g.* dissolved salts, released sugar and released protein) constituents. Unsettled biomass describes any intact cells and cell fragments that partitioned in the supernatant instead of the pellet after centrifugation. In order to determine the amount of unsettled biomass, microalgal supernatant was filtered through 0.22 μm syringe filter to remove residual biomass before being subjected to oven drying and gravimetric analysis. The difference in the solid contents of unfiltered supernatant and filtered supernatant measured the amount of unsettled biomass.

2.10. Salt concentration measurement of slurry and supernatant

The salt content of microalgal slurry or supernatant was determined gravimetrically by oven drying a known amount of slurry or supernatant at 650℃ on a pre-weighed porcelain crucible inside a high-temperature furnace for 20 h. After removal from the furnace, the crucible was left to cool to room temperature and re-weighed. The amount of ash left after the drying process was equal to the quantity of inorganic solid (*i.e* salts) present in the slurry or supernatant [32,33].

2.11. Assessment of membrane permeability and cell viability

Membrane permeability of the cells during freshwater washing was evaluated with nucleic acid staining followed by flow cytometer analysis. Microalgal slurry was diluted 1:1500 to reach an approximate biomass concentration of 0.1 g/L. The diluted suspension was stained with a Yo-Pro solution (final Yo-Pro concentration in the diluted suspension = 1 μM) and left for 10 min at room temperature in the dark. Flow cytometer measurement was conducted with Attune NxT (Thermo Fisher Scientific, USA) using 488 nm laser as excitation source. Emission fluorescence signal was collected with the green filter of the device (530/30 nm). For each sample, 30,000 cells were analysed.

Agar plating was used to assess cell viability before and after freshwater washing. The plates were prepared using modified f/2 medium complemented with 0.5% plate count agar (X930.1, Carl Roth, Germany). Microalgal slurry was diluted to reach 1000 cells/ml. The diluted suspension (100 μl) was gently spread across the agar plate using a glass spatula. Cell colonies were counted after leaving the plate for 14 days at 25℃ under low light intensity (50 μmol m^{-2} s^{-1}). The proportion of recoverable cells on agar plate or $cells_{viable}$ (% of cells) for each slurry was calculated with:

$$cells_{viable} = \frac{colonies_{visible}}{cells_{plated}} \cdot 100 \quad [2]$$

where $colonies_{visible}$ was the number of visible cell colonies counted on the agar plates at the end of the 2-week observation period (no. of cell colonies) and $cells_{plated}$ was the initial number of cells placed on the plate (no. of cells).

2.12. Mass balance of the washing treatment

The composition of every stream (biomass, salts, released sugar, released protein) involved in the freshwater washing treatment was compiled by either direct measurement of the stream using the procedures outlined in Section 2.4, 2.5, 2.6, 2.9, 2.10 or indirect evaluation using multicomponent mass balance of the adjacent streams. For the washing steps (Fig. 1), untreated pellet, washed slurry 1, washed pellet 1 and washed pellet 2 were not directly subjected to compositional analysis. The mass of individual component of these streams or $m_{component}$ (g) was therefore quantified through mass balance against nearby streams as follows:

$$m_{component}(\text{untreated pellet}) = m_{component}(\text{untreated slurry}) - m_{component}(\text{untreated supernatant}) \quad [3]$$

$$m_{component}(\text{washed slurry 1}) = m_{component}(\text{untreated pellet}) \quad [4]$$

$$m_{component}(\text{washed pellet 1}) = m_{component}(\text{washed slurry 1}) - m_{component}(\text{washed supernatant 1}) \quad [5]$$

$$m_{component}(\text{washed pellet 2}) = m_{component}(\text{washed slurry 2}) - m_{component}(\text{washed supernatant 2}) \quad [6]$$

The mass of water in each stream or m_{water} (g) was not directly measured and thereby calculated using:

$$m_{water} = m_{total} - -\Sigma m_{component} \quad [7]$$

where $\Sigma m_{component}$ (stream) was the sum of the mass of all non-water components in the respective stream (*e.g.* biomass, salts, released sugar and released protein).

The wash waters contained only water and no other components.

The mass of unsettled biomass in the supernatants (*e.g.* untreated supernatant, washed supernatant 1, washed supernatant 2) was calculated based on the difference in solid content between filtered and unfiltered supernatants as outlined in Section 2.9.

Table 1 provides the complete mass balance of all the streams in the freshwater washing treatment. For ease of discussion, all of the masses shown in the table have been normalised to 100 g of untreated slurry, instead of being reported as their actual values.

2.13. Coupling of washing treatment with high-pressure homogenisation and low-solvent-to-biomass-ratio hexane extraction for the recovery of biofuel-convertible lipids

Between 25 and 45 g of microalgal slurry (untreated slurry or washed slurry 2) from Section 2.3 was passed once through a high-pressure homogenisation (HPH) unit (Avestin EmulsiFlex-C3, Avestin, Canada) at 1000–1500 bar without any further dilution. After homogenisation, 4 g of the microalgal slurry was mixed with an equal mass of hexane (4 g), tumbled using a rotation wheel at room temperature (20°C) for 2 h and centrifuged at 7028 g and 20°C for 10 min. After carefully recovering the top hexane phase with a glass pipette, the remaining post-centrifugation layers were mixed using a spatula and the resulting biomass mixture was further subjected to another extraction stage. Hexane phases recovered from both extraction stages were pooled and dried under N_2 gas to enable gravimetric determination of the extracted lipid. The dried lipid extract was re-dissolved in chloroform/methanol solution (2:1 v/v) and subjected to SPE lipid fractionation as outlined in section 2.6.2. The total lipid content of the slurry was determined with Bligh and Dyer extraction as described in Section 2.6.1. Roughly 500 mg from each slurry (untreated slurry, homogenised slurry, washed slurry, homogenised washed slurry) was sampled prior to hexane extraction for cell rupture evaluation in Section 2.15.

2.14. Coupling of washing treatment with alkaline hydrolysis for protein recovery

Microalgal slurry from Section 2.3 (25 g of untreated slurry or washed slurry 2) was mixed with 3 – 9 g of 1 M sodium hydroxide solution (pH value of the slurry before NaOH addition = 6.0 ± 0.3, pH value of the slurry after NaOH addition = 12.3 ± 0.2). The mixture was incubated at 65°C for 2 h before being centrifuged at 7028 g and 20°C for 10 min. The resulting supernatant was carefully isolated with a glass pipette, weighed, filtered (0.22 μm) to remove any residual biomass and subjected to protein analysis with a Lowry assay as described in Section 2.5. Roughly 500 mg from each slurry (untreated slurry, alkali-treated slurry, washed slurry, alkali-treated washed slurry) was sampled for cell rupture evaluation in Section 2.15.

2.15. Image analysis for cell rupture evaluation

Microalgal slurries from Section 2.13 and 2.14 were diluted to an appropriate concentration for microscopic imaging (dilution ratio = 540 – 630x). An aliquot of the diluted slurry was placed on a standard Neubauer haemocytometer (10 μl) and left to settle for 15 min before being observed under the microscope (Axioplan 2 from Zeiss, Jena, Germany).

For each sample of diluted slurry, between 5 and 12 images of different 0.04 mm^2 haemocytometer grids were captured. The apparent number of intact cells in each grid was evaluated using an automated image algorithm (ImageJ Software, National Institutes of Health, USA) as previously described [18].

The extent of cell rupture (% of available cells) for a specific slurry was calculated as follows:

$$\text{The extent of cell rupture} = \frac{\overline{C}_{\text{untreated slurry}} - \overline{C}_{\text{specific slurry}}}{\overline{C}_{\text{untreated slurry}}} \cdot 100 \quad [8]$$

where $\overline{C}_{\text{untreated slurry}}$ was the average apparent number of intact cells for the untreated slurry and $\overline{C}_{\text{specific slurry}}$ was the average apparent number of intact cells for the specific slurry.

2.16. Energy analysis of washing treatment for lipid recovery and biofuel conversion

Energy assessment of coupling the washing treatment with a lipid recovery and biofuel production process described in section 2.14 (HPH + hexane extraction) were carried out at a demonstration-scale basis of 1000 kg of untreated slurry. Experimental results from the study were used to predict the lipid yield and composition, the biodiesel yield and the energy output of the process.

The energy output of the extracted lipid or E_{lipid} (MJ) was calculated as follows:

$$E_{lipid} = \left(Y_{neutral}\eta_{neutral} + Y_{polar}\eta_{polar}\right)\cdot\Delta H^0_{c\ biodiesel} \quad [9]$$

where $Y_{neutral}$ was the neutral lipid yield of the biomass processing system (kg neutral lipid), $\eta_{neutral}$ was the neutral lipid-to-biodiesel conversion efficiency of the lipid transesterification step (assumed to be 0.9 kg biodiesel / kg neutral lipid [34]), Y_{polar} was the polar lipid yield of the biomass processing system (kg polar lipid), η_{polar} was the polar lipid-to-biodiesel conversion efficiency of the lipid transesterification step (assumed to be 0.7 kg biodiesel / kg polar lipid), $\Delta H^0_{cbiodiesel}$ is the heat of combustion of biodiesel (37.5 $MJ/kg_{biodiesel}$) [34]. The polar lipid yield was the sum of glycolipid and phospholipid yields.

The washing treatment was comprised of 2 cycles of centrifugation and agitation. The energy cost of the washing treatment or $E_{washing}$ (MJ) was calculated as the sum of the energy requirements of its constituent steps: centrifugation, washing 1, centrifugation 1, washing 2 (refer to Fig. 1). The energy cost of each centrifugation step or $E_{centrifugation}$ (MJ) was estimated with:

$$E_{centrifugation} = \dot{E}_{centrifugation}\frac{m_{slurry}}{\rho_{slurry}} \quad [10]$$

where $\dot{E}_{centrifugation}$ was the specific energy cost of centrifugal separation (MJ/m^3 slurry), m_{slurry} was the mass of slurry being processed (kg slurry) and ρ_{slurry} was the density of slurry which was assumed to be the same with water (1000 kg slurry/m^3 slurry).

Based on our survey of existing literatures and manufacturer data, the amount of typical energy consumption for concentrating microalgae suspension using either a disc-stack or a decanter centrifuge at an industrial scale is estimated to range from 0.3 to 8 kWh/m^3 suspension [35–42]. Centrifugation has previously been used as either a primary or a secondary dewatering step to dewater microalgae suspension from a solid concentration of 0.04–13 wt% to 4 – 22.2 wt% [35,36,38–40,42]. First-principle derivation of centrifugation energy demand is beyond the scope of the study as this requires detailed design of the parameters and geometry of the envisaged demonstration-scale centrifuge and measurement of critical settling properties of the biomass. For our calculation, we have decided to use the halfway point of the range of centrifugal energy cost estimation found in the literatures (4.15 kWh / m^3 slurry or 14.9 MJ / m^3 slurry) as the value for $\dot{E}_{centrifugation}$.

The energy cost of each agitation step or $E_{agitation}$ (MJ) can be calculated with:

$$E_{agitation} = I_{mixing}t_{mixing}\frac{m_{slurry}}{\rho_{slurry}} \quad [11]$$

where I_{mixing} was the volumetric energy requirement for medium-intensity agitation of slurry suspension (MW / m^3 slurry), t_{mixing} was the duration of agitation (1800 s) and m_{slurry} was the mass of slurry being processed (kg slurry). The value for I_{mixing} was estimated to be 0.001 MW / m^3 based on Martin [34].

The energy cost of the lipid recovery system or $E_{recovery}$ (MJ) measured the amount of energy needed to process the slurries

(unwashed slurry or washed slurry 2) into biofuels. It was envisaged that the slurries would be subjected to a biofuel production process involving 7 distinct unit operation steps: a high-pressure homogenisation (HPH) step to disrupt the cells, a hexane lipid extraction step to recover lipids from the disrupted slurries, a phase separation (post-extraction centrifugation) step to partition and isolate the lipid-solvent phase, a second hexane lipid extraction step, a second phase separation step, a hexane evaporation step to evaporate hexane for recycling and a lipid transesterification step to convert the extracted lipid into biodiesel. $E_{recovery}$ was therefore defined as the sum of the energy requirements for all seven unit operations and can be estimated by using the following energy equation (derived from Martin [34]):

$$E_{recovery} = E_{HPH} + 2 \cdot E_{extraction} + 2 \cdot E_{phase} + E_{evaporation} + E_{trans} \quad [12]$$

where E_{HPH} was the energy cost of HPH (MJ), $E_{extraction}$ was the energy cost of lipid extraction with hexane (MJ), E_{phase} was the energy cost of phase separation (MJ), $E_{evaporation}$ was the energy cost of hexane evaporation (MJ) and E_{trans} was the energy cost of lipid transesterification (MJ).

$$E_{recovery} = \dot{E}_{HPH}\frac{m_{slurry}}{c_{slurry}} + 2 \cdot I_{mixing}\ t_{extraction}\left(\frac{m_{slurry}}{\rho_{slurry}} + \frac{m_{hex}}{\rho_{hex}}\right) + 2 \cdot \dot{E}_{centrifugation}\left(\frac{m_{slurry}}{\rho_{slurry}} + \frac{m_{hex}}{\rho_{hex}}\right) + (2\ \lambda_{hex}\ m_{hex}) \cdot \eta_{heat\ recovery} + \left(Y_{neutral}\eta_{neutral} + Y_{polar}\eta_{polar}\right) \cdot \Delta H^{0}_{c\ biodiesel} \cdot \dot{E}_{trans} \quad [13]$$

where $\dot{E}_{HPH}$ was the specific energy cost of HPH (0.25 kWh/kg biomass = 0.9 MJ/kg biomass for 1 pass based on [43]), c_{slurry} was the biomass concentration of the microalgal slurry (0.138 kg biomass / kg slurry), $t_{extraction}$ was the extraction time (2 h = 7200 s), m_{hex} was the amount of hexane used for each lipid extraction stage (1000 kg hexane), ρ_{hex} was the density of hexane (754 kg hexane / m^3 hexane), λ_{hex} was the latent heat of vaporisation of hexane (0.34 MJ / kg hexane), $\eta_{heat\ recovery}$ was the efficiency of recovering heat energy from solvent evaporation (assumed to be 70%), $\dot{E}_{trans}$ was the specific energy cost of lipid transesterification (MJ / MJ of biodiesel). Readers are referred to Martin for detailed understanding of the energy equations used for $E_{recovery}$ calculation [34]

The total estimated energy requirement for the entire downstream processing or $E_{downstream}$ (MJ) was calculated with:

$$E_{downstream} = E_{washing} + E_{recovery} \quad [14]$$

The net energy yield of biomass processing or E_{net} (MJ) was the difference between the energy output of the extracted lipid and the total estimated energy cost of downstream processing:

$$E_{net} = E_{lipid} - E_{downstream} \quad [15]$$

The total lipid energy available in the biomass or $E_{biomass\ lipid}$ (MJ) was calculated as follows:

$$E_{biomass\ lipid} = \left(L_{neutral}\eta_{neutral} + L_{polar}\eta_{polar}\right) \cdot \Delta H^{0}_{c\ biodiesel} \quad [16]$$

where $L_{neutral}$ was the neutral lipid content of the slurry (kg neutral lipid), L_{polar} was the polar lipid content of the slurry (kg polar lipid).

3. Results and discussions

3.1. Cultivation and biochemical composition

Fig. S1 shows the growth kinetics and nitrate consumption profile of *N. gaditana* culture grown autotrophically in our bubble-column PBR with continuous CO_2 supply. Biomass concentration in the culture continued to increase beyond the point of nitrate depletion (day 10) and reached approximately 4 g/L on day 18. The delay in the cessation of biomass growth after nitrate depletion is suggestive of a possible lag between the time when cells assimilated dissolved nitrates into the biomass and the time when they utilised the acquired intracellular nitrogen for nucleic acid formation and cell division.

The harvested slurries were found to be lipid-rich, with total lipid accounting for 607.0 ± 102.5 mg/g of the biomass and total sugar and protein making up only 249.9 ± 40.1 and 143.1 ± 34.6 mg/g of the biomass respectively. The highly oleaginous nature of the biomass is in agreement with previous studies [13,44] which reported significant lipid enrichment in *Nannochloropsis* biomass grown with continuous CO_2 supply [13].

SPE fractionation of total lipid extracts of the slurries established biofuel-convertible neutral lipid fraction (consisting primarily of triacylglycerol or TAG) to be the largest constituent of the biomass total lipid (neutral lipid content = 76.0 ± 16.1 wt% of total lipid, glycolipid content = 16.5 ± 13.8 wt% of total lipid, phospholipid content = 7.5 ± 2.3 wt% of total lipid). Based on this lipid composition, the total neutral lipid content of the biomass was calculated to be 461.4 ± 97.6 mg neutral lipid / g biomass. Lipid and fatty acid enrichment under improved CO_2 availability can be attributed to the intensification of carbon-fixing Calvin cycle activities in the chloroplasts and the ensuing increase in the formation of G3P (glyceraldehyde-3-phosphate) fatty-acid precursor [13].

A qualitative analysis of the culture medium for phosphates right before harvest revealed that the culture was phosphate depleted. The relatively high glycolipid-to-phospholipid ratio in the biomass lipid can therefore be attributed to P-limitation. A recent study by Muhlroth et al. [45] has shown that *Nannochloropsis* cells have a unique repertoire of genes that facilitate the degradation and recycling of phospholipids into other P-free lipid classes (such as glycolipids) under phosphate deprivation.

In our previous studies investigating cellular acclimation to nitrogen deprivation, *Nannochloropsis* cells were shown to have the ability to partition excess carbon fixed during photosynthesis to the biosynthesis of both storage lipids (*i.e.* triacylglcerols) and structural polysaccharides (*i.e.* cellulose in the cell wall) [29,46]. Halim et al. [29] showed that lipid-rich nitrogen-deprived cells possessed thicker cell walls and required greater mechanical force to rupture than protein-rich nitrogen-replete cells. The increased energy requirement associated with processing nitrogen-deplete biomass reduced the net energy gain from lipid recovery and can be counterproductive to the process of lipid accumulation via nitrogen deprivation. Even though we have not analysed the cell wall thickness of our nitrogen-deplete cells in this study, the fact that the harvested slurries had managed to store a substantial amount of lipid (~60 wt% of biomass) suggests that the cells would have also likely invested some of their photosynthetically fixed carbon into cellulose formation and cell wall thickening. The simultaneous increases in *Nannochloropsis* lipid content and cell wall thickness underscore the need for a cost-effective and energy-efficient treatment step able to weaken cell walls and increase accessibility of intracellular products.

3.2. Freshwater washing

In this section, we evaluated the effect of hypotonic osmotic shock on the processability of *N. gaditana* slurries, characterising the impact of freshwater washing treatment on the biochemical composition, cell membrane permeability, cell viability, cell rupture and settling propensities of the biomass.

3.2.1. Effect on biochemical composition of the biomass

Washing the slurry with fresh water removed salts from the slurry, drastically reducing the salt content of the slurry from 22.1 ± 1.6 mg salt / g slurry (untreated slurry) to 3.7 ± 0.7 mg salt / g slurry (washed slurry 2). In Fig. 2a, the change in the salt content of the slurry during the washing steps was confirmed through a reduction in the conductivity value of the slurry (from 24.3 ± 2.7 for the untreated slurry to 2.7 ± 0.6

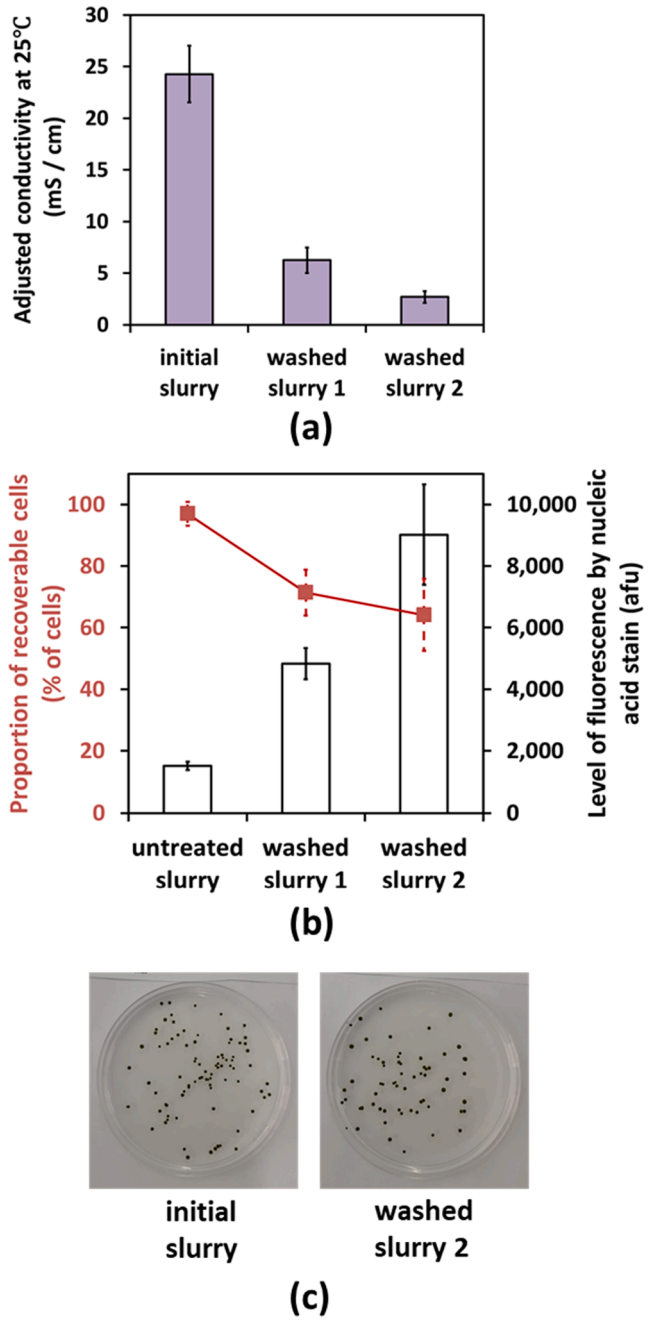

Fig. 2. (a) The effect of washing on conductivity of the slurry. Adjusted conductivity at 25°C was reported as mean ± stdev of 5 biological replicates (n = 5). (b) The effect of washing on cell viability and membrane permeability. The proportion of recoverable cells on agar plate was reported as mean ± stdev of 2 biological and 3 experimental replicates (n = 6). Level of fluorescence by nucleic acid stain was reported as mean ± stdev of 2 biological or n = 2. (c) Images of agar plates for recovery experiments after approximately 14 days of observation. For both slurries, no. of plated cells = 100 ± 9 cells, For untreated slurry, no of cell colonies (N) = 86, For washed slurry 2, N = 62.

mS/cm for the washed slurry 2). Conductivity measures the level of electrolytes (*i.e.* salts) in the solution.

3.2.2. Effect on cell membrane permeability and cell viability

Hypotonic osmotic shock appeared to have an adverse effect on both cell viability and membrane integrity. During agar plating, the ratio of recovered cell colonies to the number of cells initially placed on the plate provides a proxy measurement for the proportion of viable cells in a slurry. Fig. 2b and 2c showed the level of recoverable cells (and thus viable cells) to progressively decrease with the number of washing steps. This reduction in the level of cell viability was accompanied with an increase in the uptake of nucleic acid stain. YoPro is a green fluorescent dye which enters cells through damaged cell membranes. The steady increase in YoPro uptake suggested that the cell membrane was progressively degraded and became increasingly more permeable under osmotic stress. Such increase in membrane permeability validated the loss of cell viability. The two variables are intrinsically linked to each other as the membranes of cells that have lost their viability would also lose the capacity to regulate material transport. We did not, however, perform further surface characterisation or electron microscopic analyses to examine the type and extent of morphological and physiological damages on the cell wall/membrane complex.

3.2.3. Cell rupture

Hypotonic osmotic shock did not lead to extensive cell rupture in the *N. gaditana* slurries. Microscopic evaluation revealed that our freshwater washing treatment only managed to rupture ~13 ± 9% of available cells in the slurries after 2 stages of centrifugation and reconstitution with freshwater (*i.e.* the number of intact cells in the washed slurry 2 = 87% of the number of intact cells in the untreated slurry). The minimal cell rupture inflicted by freshwater washing in this study is not in agreement with previous results obtained by Lee et al. [23] which showed osmotic shock to be as effective as other mechanical treatments (such as bead beating and microwave processing) in preparing freshwater microalgae biomass for lipid extraction. This discrepancy can be attributed to the fact that saltwater species generally have thicker cell walls than freshwater species and are thus only moderately affected by an osmotic treatment. Additionally, since hypotonic osmotic shock is achieved through the reduction of salt concentration, its ability to produce osmotic gradient and thus pressure change on cell wall is capped to a maximum concentration gradient of 30 g salts /L medium (the difference in salt concentration between cytoplasm and fresh water). Hypertonic osmotic treatment, on the other hand, does not abide by such rules, since salts can always be added beyond saturation limit to create steeper concentration gradients at an elevated temperature. Yoo et al. [22] and Lee et al. [23] used medium with a maximum salt concentration of 200 and 100 g salt/ L medium respectively for their hypertonic osmotic stress treatment. We also note that Lee et al. [23] used freeze-dried biomass for their hypertonic treatment. Freeze drying removed water molecules from the cells through ice sublimation. The treatment led to forced water diffusion across cell membrane and likely resulted in cell membrane damage [1]. The increase in lipid yield observed in their study therefore measured the combined membrane damage inflicted from sequential freeze drying and osmotic treatments rather than the sole effect of membrane permeabilization from osmotic shock.

3.2.4. Effect on protein release, sugar release and biomass settlement

Fig. 3 presents the concentration of unsettled biomass, recovered salts, released (or free) sugar and released (or free) protein in every supernatant isolated from the washing treatment (untreated supernatant, washed supernatant 1, washed supernatant 2). Biomass content in the supernatant measured the amount of biomass that partitioned in the specific supernatant after centrifugation. Isolated supernatants from the washing step were thoroughly filtered (0.22 μm) to make sure that they were free of any contaminating biomass prior to being subjected to biochemical analysis. The sugar and protein contents of the supernatants therefore measured exclusively the levels of water-soluble free sugar and free protein that had been released from the biomass into the supernatants.

Subjecting the biomass to multiple washing steps triggered the release of free sugar and free protein into the supernatants (Fig. 3). The amount of sugar released into the supernatant can be observed to progressively increase with successive washing stages, from 0.3 ± 0.2 mg/g supernatant for the untreated supernatant to 16.2 ± 3.9 mg / g supernatant for the washed supernatant 2. The change in the release of free

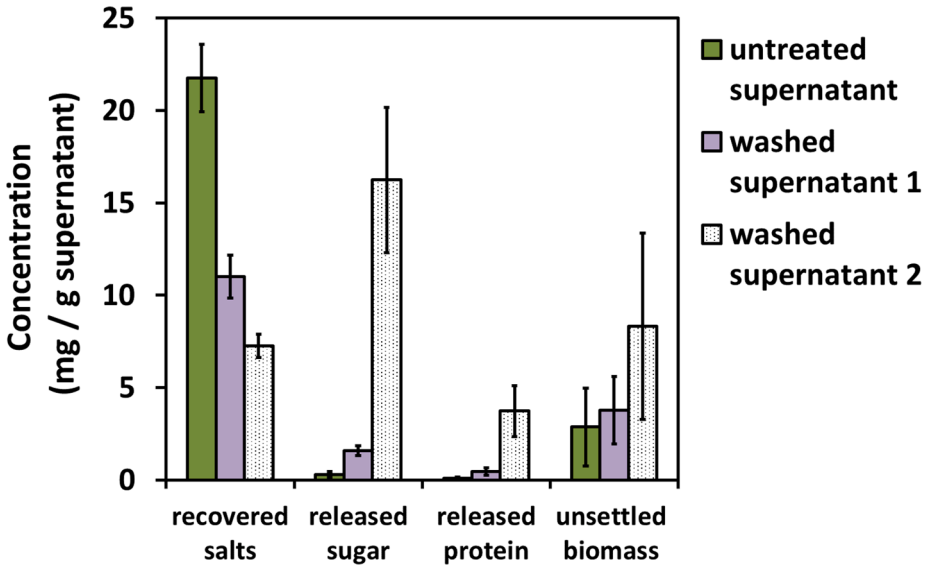

Fig. 3. The concentrations of unsettled biomass, recovered salts, released sugar and released protein in the supernatants isolated from the washing steps. For unsettled biomass: 4 biological and 1–3 experimental replicates (n = 4–8). For recovered salts: 5 biological and 1–3 experimental replicates (n = 5–12). For released sugar: 4 biological and 1–3 experimental replicates (n = 4–8). For released protein: 5 biological and 1–3 experimental replicates (n = 5–10).

protein also followed the same progressive pattern, with the content of supernatant protein increasing from 0.10 ± 0.06 mg/g supernatant for the untreated supernatant to 3.73 ± 1.37 mg / g supernatant for the washed supernatant 2. As discussed in the previous sections, hypotonic osmotic stress led to the permeabilization of cell membrane. The release of free protein and sugar can therefore be ascribed to the passive leakage/diffusion of intracellular products from the permeabilised cells down the solute concentration gradient into the surrounding medium.

Despite the increase in the levels of free sugar and free protein in the supernatant with the number of washing stages, the amounts of intracellular products released in these supernatants only represented relatively small fractions of the available sugar and protein in the slurry. Table 2 presents the amounts of components in each supernatant relative to the total available amount of the respective component in the preceding slurry (*i.e.* the amount of salt in the washed supernatant 1 has been expressed as a proportion of the total amount of salt present in the washed slurry 1). As shown in the table, the free protein released in the untreated supernatant, the washed supernatant 1 and the washed supernatant 2 only represented 0.3, 1.8 and 10.3 wt% of the available protein in the untreated slurry, the washed slurry 1 and the washed slurry 2 respectively. Coupling these results with the fact that the treatment only managed to rupture a small proportion of cells in the slurry (~13% of available cells), hypotonic osmotic shock appeared to be a relatively ineffective stand-alone disruption and processing step for the recovery of intracellular products from *N.gaditana* slurries. We note that leakage of intracellular product is restricted only to water-soluble components. No measurable lipid leakage to the supernatant was observed during any of the washing steps (results not shown).

As shown in Fig. 3, the washing treatment also led to an increase in the amount of unsettled biomass in the supernatants. The amount of unsettled biomass in the unwashed supernatant, the washed supernatant 1 and the washed supernatant 2 represented a loss of 1.1, 2,1 and 3.6 wt % of biomass from the slurries respectively. The increase in the levels of unsettled biomass in the supernatants with washing stages can potentially be attributed to the incremental loss of sugar and protein polymers from the cell structure and the consequential reduction in biomass density and settling propensity. Fig. S2 shows the physical appearance of post-centrifuged slurries from the freshwater washing steps. The figure confirms the incremental loss of biomass in the supernatants with the washed supernatant 2 adopting a cloudier and greener physical appearance than the untreated supernatant.

Table 2
The relative composition of untreated supernatant, washed supernatant 1 and washed supernatant 2 isolated from the washing steps. The recovered salts, released sugar and released protein contents have been presented as their relative amounts to the total available amount of the respective component in the preceding slurry. The unsettled biomass content has been presented as the amount of unsettled biomass relative to the total available biomass in the preceding slurry. 'Preceding slurry' denotes the immediate slurry that has been centrifuged to produce the specific supernatant (*e.g.* preceding slurry for washed supernatant 1 is washed slurry 1).

	Untreated supernatant	washed supernatant 1	washed supernatant 2
Recovered salts:			
Salt content relative to the mass of salt in the preceding slurry (wt% of salt in the slurry)	48.9 ± 4.3	63.9 ± 12.4	57.7 ± 7.3
Released (or free) sugar:			
Sugar content relative to the mass of sugar in the preceding slurry (wt% of sugar in the slurry)	0.6 ± 0.3	3.2 ± 2.6	28.6 ± 5.5
Released (or free) protein:			
Protein content relative to the mass of protein in the preceding slurry (wt% of protein in the slurry)	0.3 ± 0.2	1.8 ± 1.4	10.3 ± 3.6
Unsettled biomass:			
Biomass content relative to the mass of biomass in preceding slurry (wt% of biomass in the slurry)	1.1 ± 0.9	2.1 ± 1.2	3.6 ± 2.2

All results are reported as mean ± std. For unsettled biomass: 4 biological and 1–3 experimental replicates (n = 4–8).
For recovered salts: 5 biological and 1–3 experimental replicates (n = 5–12). For released sugar: 4 biological and 1–3 experimental replicates (n = 4–8). For released protein: 5 biological and 1–3 experimental replicates (n = 5–10).

3.2.5. *Mass balance of washing steps*

Table 1 displays the composition of every stream in the washing procedure. The mass of components in every stream has been normalised to 100 g of untreated slurry. Unlike Table 2 which summarised the amount of released protein, released sugar and unsettled biomass in

each supernatant relative to its previous slurry, the complete mass balance presented in Table 1 enabled the determination of global protein yield, sugar yield and biomass loss from the combination of all of the supernatants relative to the untreated slurry.

As shown in Table 1, if all of the free protein in all three supernatants were added together, the washing treatment can be seen to lead to the combined release of 0.22 g of protein from 13.77 g of biomass. This was equivalent to a total protein yield of 11.2 wt% of available protein in the untreated slurry. Such a low overall protein yield supported our findings in section 3.2.4 and confirmed the ineffectiveness of hypotonic shock as a stand-alone disruption technology for the recovery of intracellular products from *N. gaditana* slurries. This, however, did not preclude its potential as a pretreatment to a more vigorous mechanical or chemical disruption technology. The fact that the washing treatment substantially damaged the cell membranes (section 3.2.2 and Fig. 2b) opened up the possibility of applying the washing treatment as a preparatory method to enhance cell rupture and support subsequent product mass transfer of a primary disruption technology. In the next section (section 3.3 and 3.5), we investigated the partnership of the washing treatment with HPH and hexane extraction for lipid recovery as well as alkali hydrolysis for protein recovery.

Before coupling the washing treatment with other disruption technologies, we had to make a choice as to which biomass from the washing treatment would be subjected to further downstream processes (washed slurry 2 or washed pellet 2). Given that the most significant amount of unsettled biomass was found in washed supernatant 2, we believed that it was prudent to avoid the formation of this final supernatant to minimise biomass loss. Avoiding the formation of this final supernatant also had the added benefit of removing the last centrifugation stage and thus reducing the energy requirements for the washing treatment. For this reason, we decided to reduce the length of the washing treatment by removing the last centrifugation stage (and thus eliminating the formation of washed supernatant 2 and washed pellet 2) and to use washed slurry 2 when partnering the washing treatment with HPH and hexane extraction or alkali hydrolysis. With this decision, we reduced the total biomass loss during the washing treatment from 5.3 to 2.5% of the biomass originally present in the untreated slurry.

Table 3
The effect of washing treatment on the performance of HPH cell disruption and hexane lipid extraction for the recovery of biofuel-convertible lipids and on the performance of alkali (NaOH) hydrolysis for protein recovery. Lipid yield, neutral lipid yield and protein yield were evaluated based on the total available amounts of lipid, neutral lipid and protein in the respective slurry (*i.e.* lipid yield for washed slurry 2 was calculated to the total amount of biomass lipid in washed slurry 2). For extent of cell rupture (HPH): 4 biological and 5–12 microscope analytical replicates (n = 20–48). For lipid yield and neutral lipid yield: 3–4 biological and 1–2 experimental replicates (n = 5–6). For extent of cell rupture (alkali hydrolysis): 2–4 biological and 7 experimental replicates (n = 14–28). For protein yield: 3–6 biological and 1–2 experimental replicates (n = 5–9).

	Lipid recovery with HPH and hexane			Protein recovery with alkali hydrolysis	
	Extent of cell rupture (% of available cells)	Lipid yield (wt% of available lipid)	Neutral lipid yield (wt% of available neutral lipid)	Extent of cell rupture (% of available cells)	Protein yield (wt % of available protein)
Untreated slurry	28 ± 8	21.2 ± 1.7	25.1 ± 2.0	13 ± 6	6.7 ± 2.4
Washed slurry 2	46 ± 19	54.7 ± 4.1	64.6 ± 4.9	17 ± 6	31.9 ± 10.7

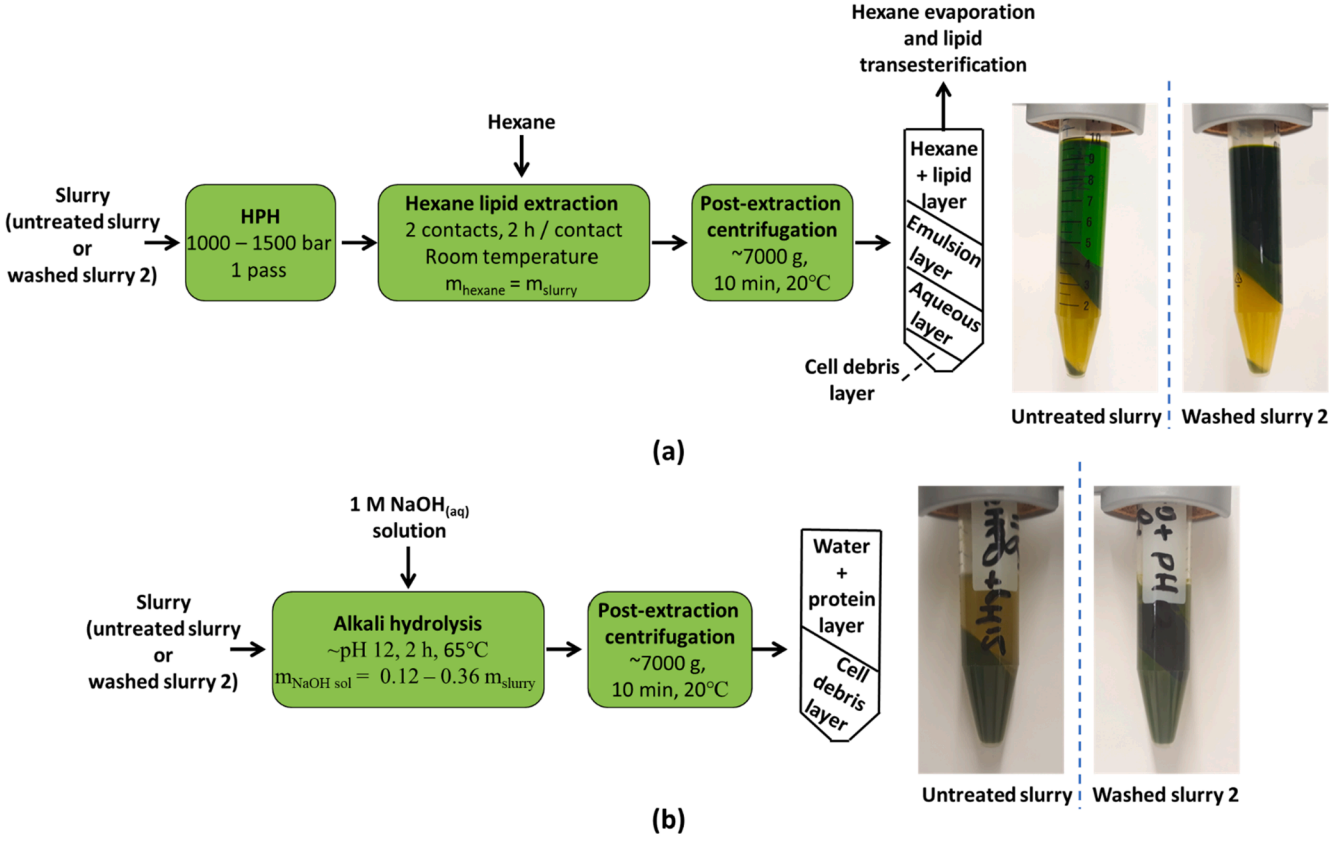

Fig. 4. (a) Schematic of biomass processing for biofuel production with HPH cell disruption and hexane lipid extraction. The separation of post-centrifuged extraction mixture into four distinct layers is illustrated. Refer to Halim et al. [7] for detailed understanding of the biphasic extraction layers. (b) Schematic of biomass processing for protein production with alkali (NaOH) hydrolysis.

3.3. Coupling freshwater washing with HPH and hexane extraction for the recovery of biofuel convertible lipids

In this section, we investigated the partnership of washing treatment with cell disruption by HPH and lipid extraction with hexane for the recovery of biofuel-convertible lipids from *N. gaditana* slurries (Fig. 4a). The HPH and hexane extraction system used in this section was designed in our previous study [7] with the aim to reach an optimal compromise between extraction yield, solvent volume and energy requirements associated for post-extraction solvent recycling. The water-immiscible lipid recovery system is not only selective to neutral lipid fractions rich in saturated and monounsaturated fatty acids desirable for biofuel conversion, it also has the added advantage of minimising solvent-to-slurry ratio and avoiding the use of an energy-intensive fractional distillation step that would otherwise be needed to recover water-miscible solvent from the post-extracted microalgal slurry.

Hypotonic osmotic shock was shown to be able to structurally weaken *N. gaditana* cells and render the slurry more vulnerable to subsequent mechanical rupture. As shown in Table 3, the washing treatment managed to significantly improve HPH performance, increasing cell disruption from 28% of available cells (untreated slurry) to 46% of available cells (washed slurry 2). The more prolific cell rupture translated to more rapid lipid mass transfer during subsequent hexane extraction and ultimately led to 2.5-fold increase in lipid yield (from 21.2 ± 1.7 wt% of available lipid for untreated slurry to 54.7 ± 4.1 wt% of available lipid for washed slurry 2). This increase in lipid yield was evidenced by the colour of partitioned hexane phases after the extraction. As can be observed in Fig. 4a, the hexane phase obtained from the washed slurry 2 was significantly darker in colour compared to that isolated from the untreated slurry.

As a highly non-polar solvent, hexane has a strong affinity towards biofuel-convertible neutral lipid fractions. SPE fractionation of lipid extracts obtained from our hexane extraction showed that neutral lipids accounted for 89.8 wt% of the extracts. This was higher than the neutral lipid proportion in the biomass total lipid (76.0 wt% of total lipid). Such selectivity enabled the HPH + hexane extraction system to recover 64.6 ± 4.9 wt% of available neutral lipids from washed slurry 2 (Table 3).

The centrifugation of hexane extraction mixture (hexane + microalgal slurry) resulted in the formation of four distinct layers with extracted lipid partitioning primarily in the top hexane phase and cell fragments, residual hexane, water, salts and other liberated products partitioning at varying levels in the other three water-rich phases (emulsion phase, aqueous phase and cell debris phase). We refer readers to our previous study for detailed understanding of post-centrifugation layers of the hexane-based lipid recovery system [7].

The increased lipid yield for osmotically treated biomass is in agreement with Yoo et al. [22] who reported significant increase in their lipid recovery after incorporating hypertonic osmotic shock (with NaCl or sorbitol as osmotic agents) in processing freshwater *C. reinhardtii* suspension. Their findings, however, are not directly comparable to ours as they have used vastly different parameters for their osmotic stress treatment and lipid extraction: a) freshwater species instead of saltwater species, b) relatively dilute suspensions (~1 wt% of biomass) instead of concentrated slurries (~14 wt% of biomass) as feedstock and c)

Table 4
Energy analysis of coupling the washing treatment with downstream process for lipid recovery and biofuel conversion.

Biofuel production with HPH + hexane lipid extraction system (per 1000 kg of untreated slurry)		Without washing treatment (untreated slurry)	With washing treatment (washed slurry 2)
Energy output	Lipid yield (kg lipid)	17.52	43.42
	Neutral lipid yield (kg neutral lipid)	15.73	39.00
	Polar lipid yield (kg polar lipid)	1.78	4.42
	Biodiesel yield (kg biodiesel)	15.41	38.19
	Lipid energy output, E_{lipid} (MJ)	577.78	1432.18
	Total lipid energy available in the biomass, $E_{biomass\ lipid}$ (MJ)	2634.58	2634.58
Energy cost of washing treatment	Energy cost of centrifugation, $E_{centrifugation}$ (MJ)	0.00	14.94
	Energy cost of agitation in washing 1, $E_{agitation}$ (MJ)	0.00	2.41
	Energy cost of centrifugation 1*, $E_{centrifugation}$ (MJ)	0.00	20.00
	Energy cost agitation in washing 2*, $E_{agitation}$ (MJ)	0.00	1.89
	$E_{washing}$ (MJ)	0.00	39.25
Energy cost of lipid recovery system	Energy cost of HPH, E_{HPH} (MJ)	124.20	130.68
	Energy cost of 2 extraction cycles, 2 $E_{extraction}$ (MJ)	36.42	38.32
	Energy cost of 2 cycles of phase separation, 2 E_{phase} (MJ)	75.57	79.51
	Energy cost of hexane evaporation, $E_{evaporation}$ (MJ)	204.00	214.64
	Energy cost of lipid transesterification, E_{trans} (MJ)	54.31	134.62
	$E_{recovery}$ (MJ)	494.50	597.78
Total energy cost of downstream processing, $E_{downstream}$ (MJ)		494.50	637.02
Net Energy yield of biomass processing, E_{net} (MJ)= E_{lipid} - $E_{downstream}$		83.29	795.15
$E_{lipid}/E_{biomass\ lipid}$ (%)		21.93	54.36
$E_{washing}/E_{downstream}$ (%)		N/A	6.16

*The washing steps altered the amount of water in the processed slurry (Table 1 for mass balance) which in turn changed the energy consumption for centrifugation and washing in subsequent cycles.
** Washed slurry 2 contained more water compared to untreated slurry and hence had a higher processing energy requirement for each step in the lipid recovery system (Table 1 for mass balance).

extraction solvent mixture comprising hexane and methanol aimed at all lipid fractions instead of just pure hexane aimed at the neutral lipid fractions.

3.4. Energy analysis of washing treatment for lipid recovery and biofuel conversion

Table 4 displays the results of our energy assessment comparing the hexane-based biofuel production system with and without the integration of the washing treatment. The calculations for the table were carried out based on 1000 kg of untreated slurry (this equates to 138 kg of biomass at the given biomass concentration). The biomass specifications (*e.g.* lipid content and composition) and washing and downstream parameters (*e.g.* amount of water added per kg of slurry for washing steps, centrifugation and agitation conditions, biomass concentration of the slurry, amount of hexane added per kg of slurry, lipid yield) used / obtained in this study were applied to the energy calculations. The instalment of the washing treatment prior to HPH + hexane extraction was predicted to increase the biodiesel yield and the lipid energy output (E_{lipid}) of the downstream processing system by 2.5 folds (from 578 MJ / 1000 kg slurry for system without washing to 1432 MJ / 1000 kg slurry for system with washing). The total amount of lipid energy available in the biomass was 2635 MJ / 1000 kg slurry.

On the other hand, the amount of energy needed to carry out the washing treatment (2 separate stages of centrifugation and agitation) or $E_{washing}$ was shown to be minimal at 39 MJ / 1000 kg slurry. This relatively low energy requirement meant that the washing treatment only accounted for 6% of the total energy expenditure for the entire chain of downstream processing ($E_{downstream}$ = 637 MJ / 1000 kg slurry). In fact, the energy demand for the washing treatment was dwarfed by the energy requirements for other steps in the downstream processing chain, in particular those allocated to HPH (131 MJ / 1000 kg slurry = 21% of $E_{downstream}$) and evaporation of hexane for solvent recycling (215 MJ / 1000 kg slurry or 34% of $E_{downstream}$).

Overall, the results of our energy assessment in Table 4 illustrated the ability of washing treatment to significantly enhance the energy balance of biomass processing. When all of the energy costs of downstream processing had been deducted from the energy output, the washed slurry 2 generated a positive net energy yield of 795 MJ / 1000 kg slurry (E_{net} is the difference between lipid energy output and the energy costs of downstream processing). This was ~ 9.5x the net energy yield from the unwashed slurry (83 MJ / 1000 kg slurry). Even when we substituted the highest reported rate of microalgal centrifugal energy consumption in the literature (8 kWh/m^3) into the calculations, the washing treatment was still shown to lead to ~ 9.2 -fold gain in the net energy yield of biomass processing (E_{net} without washing = 83 MJ / 1000 kg slurry, E_{net} with washing using maximum energy rating for centrifugation steps = 763 MJ / kg slurry).

Freshwater washing thus appeared to be a promising low-energy treatment that can be integrated into existing framework of hexane-based lipid recovery to maximise the energy return of downstream processing. The impact of the treatment on the overall energy balance of *N. gaditana* biofuel production, however, is difficult to assess as this is contingent on the energy footprints of the cultivation and primary dewatering technologies as well as the ability of the cultivation system in inducing biomass growth and lipid accumulation. We also note that the scope of our downstream energy analysis is currently limited to hexane-based lipid extraction. The effect of the washing treatment on the performance of other wet lipid extraction technologies (such as monophasic solvent extraction involving a polar/non-polar solvent mixture, pressurised solvent extraction and ionic liquid extraction) may not be the same with that observed for hexane extraction. A full-scale energy analysis of the entire *N. gaditana* cultivation and biofuel production system is beyond the scope of the study.

Since centrifugation has a high specific energy consumption (0.3–8 kWh/m^3) relative to other dewatering methods, its use as a primary harvesting method for dilute microalgal culture (0.5 – 4 g biomass / kg culture) is generally considered to be energetically unfavourable. However, we stress that we are not proposing the use of centrifugation as a primary dewatering method. The washing treatment should only be applied to the microalgal biomass in a slurry form right after primary dewatering of culture and before cell disruption. The use of centrifugation during the washing treatment was hence limited to the secondary dewatering of pre-concentrated slurries (102–138 g biomass / kg slurry) into paste (219 – 289 g biomass / kg paste). The treatment was able to maintain its low energy footprint by only processing slurries that had been pre-concentrated to ~1/150th the initial culture volume. The initial pre-concentration of dilute microalgal culture from cultivation into slurries can be carried out using primary dewatering methods known to have lower estimated energy demands, such as flocculation (0.002–0.1 kWh/m^3), filtration (0.2–6 kWh/m^3) or flocculation/filtration combination [38].

Table 5
Specific energy requirement for freshwater washing treatment relative to indicative specific energy requirements for other mechanical disruption technologies. The values for energy requirements for HPH, bead milling and microwave treatment are quoted from existing literatures [4,38,43,47]. Only values from studies that processed microalgal suspensions with biomass concentration at a slurry level (defined as greater than 35 g / kg slurry or 3.5 wt% solid) are included. *HPH value is reported for a single pass.

Mechanical cell disruption	Reported range of specific energy requirement (MJ / kg biomass)	Microalgae	Biomass concentration (g biomass / kg slurry)
Freshwater washing (as a pretreatment)	0.29	*Nannochloropsis* sp.	102–138
HPH*	0.73–0.9	*Chlorella* sp., *Chlorella vulgaris*	150–220
Bead or ball milling	1.67–36	*Chlorella* sp., *Chlorella vulgaris*	35–300
Microwave	9.61	*Scenedesmus* sp.	75

Table 5 evaluates the estimated energy requirements for the washing treatment relative to other known mechanical cell disruption technologies (such as HPH, bead milling and microwave treatment). The values quoted in Table 5 are indicative values obtained from literatures [4,38,43,47]. As can be observed from the Table, the amount of energy needed to implement the washing treatment ranged between 0.8 and 39.7% of the energy requirements for the partner disruption technology. The washing treatment's relatively small energy footprint meant that its inception would have minimal impact to the overall operational energy cost of the disruption technology.

3.5. Coupling freshwater washing with alkali hydrolysis for protein recovery

In this section, we have investigated the partnership of the washing treatment with alkali hydrolysis for the recovery of protein from *N. gaditana* slurries (Fig. 4b). In Table 3, even though the freshwater washing treatment did not appear to have a substantial impact on cell disruption by alkali hydrolysis, it was able to promote protein hydrolysis and resulted in a 4.5-fold increase in protein yield (from 6.7 ± 2.4 wt% of available protein for the untreated slurry to 31.9 ± 10.7 wt% of available protein for the washed slurry 2). Such findings suggested that the extent of cell disruption may not be an accurate indicator of the amount of protein that can be recovered from the biomass. This made sense as, unlike (neutral) lipids that form intracellular globules in the cytoplasm [1,14], protein is interwoven into organellar and membrane structures. Protein release therefore requires bond cleavage from the cell structures and is not directly correlated to the rupture (or physical destruction) of cells. In this case, hypotonic osmotic shock, by damaging the integrity of cell membrane and significantly reducing the ability of

the cells to regulate their active transport, was able to render the cells more susceptible to NaOH entry and cleavage and enhance the yield of protein extraction during subsequent alkali hydrolysis.

3.6. Perspective

The study demonstrated for the first time the ability of hypotonic osmotic shock to impair the cell membrane and weaken the structural integrity of concentrated *N. gaditana* slurries. Even though it did not exert sufficient physical damage to the cells to qualify as a stand-alone disruption technology, the osmotic treatment was able to weaken cellular defence and render them more susceptible to subsequent cell disruption. Incorporating hypotonic osmotic shock (induced through freshwater washing treatment) as a preparatory step to either HPH disruption or alkali hydrolysis improved the performance of the individual system and ultimately led to a multiple-fold increase in lipid or protein recovery.

Even though we have limited the investigation in this study to HPH and alkali disruption, we stipulate that hypotonic osmotic stress is a robust treatment step that can potentially be coupled with a wide range of cell disruption technologies (such as ultrasonication, bead milling, microwave, acid hydrolysis, enzymatic treatments) to augment the rupture performance of the selected partner technology and improve the efficiency of biorefinery product recoveries from concentrated *N. gaditana* or other saltwater microalgal slurries.

One of the main drawbacks of the washing treatment is its requirements for fresh water. From table 1, it can be observed that a total of 131.3 g of wash water was used to treat 100 g of untreated slurry for the 2 washing stages. This is quite contentious given the current global state of freshwater shortages. A more extensive optimisation of the water ratio as well as variables involved in the washing stages (such as agitation speed and duration) is needed in order to evaluate if the same extent of cell weakening can be achieved with lower wash water requirements. Additionally, there is also a possibility of using wastewater from other bioprocessing industries instead of fresh water for the treatment. The effects of using wastewater on the induction of cell weakening as well as the yield and purity of lipid or protein extracts will require further investigation, but the concept certainly fits within the framework of circular bioeconomy and the ongoing effort of the microalgae biotechnology field to recycle different wastewater streams from the dairy processing, brewery and biogas industries for microalgae cultivation and biorefinery systems. Any wastewater with an osmolarity lower than seawater (the medium of the slurry) has the potential to induce water potential gradient and generate osmotic shock.

It is also noted that the untreated supernatant and washed supernatant 1 can be pooled together for processing the free proteins that have been released into these supernatants into product development. Even though the amounts of free protein in these supernatants are low (a total of ~2.0 wt% of the amount of available protein in the untreated slurry), they can still contribute to the overall economics of the washing treatment and biomass processing.

4. Conclusions

This study reports the use of hypotonic osmotic shock as a treatment step to process highly lipid-rich saltwater *N. gaditana* slurries (137.7 ± 12.6 mg biomass / g slurry, 607.0 ± 102.5 mg lipid /g biomass). Subjecting the slurries to 2 stages of freshwater washing damaged the cell membranes (the level of dye uptake increased from 1,100 to 6,200 afu) and resulted in a limited release of free protein into the supernatants (~2 wt% of available protein). Hypotonic osmotic shock was found to be relatively ineffective in rupturing *N. gaditana* cells (only 13 ± 9% of available cells were ruptured after 2-stage washing) and thus had a limited prospect as a stand-alone cell disruption technology for the saltwater species.

The treatment, however, managed to physically weaken *N. gaditana* slurries and rendered the cells more susceptible to subsequent rupture. This facilitated the inception of the washing treatment as a preparatory step (or pretreatment) to other more vigorous mechanical and chemical cell disruption technologies. When coupled with HPH and hexane extraction for the recovery of biofuel convertible lipids, the washing treatment increased the extent of HPH cell rupture from 28 ± 8 to 46 ± 19% of available cells and more than doubled the hexane extraction's neutral lipid yield from 25.1 ± 2.0 to 64.6 ± 4.9 wt% of available neutral lipids. Our energy estimation of the osmotic shock treatment revealed that the energy demand associated with the washing stages was minimal (~6% of the total energy expenditure for downstream processing) and that integrating the treatment into an HPH + hexane lipid recovery system increased the net energy yield of the system by ~9.5x (net energy yield is the difference between lipid energy output and the total energy costs of downstream processing). When partnered with NaOH hydrolysis, the washing treatment was able to increase protein yield from 6.7 ± 2.4 to 31.9 ± 10.7 wt% of available protein. These findings demonstrated the promising nature of hypotonic osmotic shock as a biomass treatment step in a saltwater microalgae biorefinery system.

CRediT authorship contribution statement

Ronald Halim: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing. **Ioannis Papachristou:** Investigation, Writing - review & editing. **Christin Kubisch:** Investigation. **Natalja Nazarova:** Investigation. **Rüdiger Wüstner:** Investigation. **David Steinbach:** Validation, Writing - review & editing. **George Q. Chen:** Formal analysis, Writing - review & editing. **Huining Deng:** Formal analysis. **Wolfgang Frey:** Conceptualization, Resources. **Clemens Posten:** Conceptualization, Resources. **Aude Silve:** Conceptualization, Methodology, Resources, Project administration, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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