

# Phospholipids of New Zealand Edible Brown Algae

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**Abstract** Edible brown algae have attracted interest as a source of beneficial allenic carotenoid fucoxanthin, and glyco- and phospholipids enriched in polyunsaturated fatty acids. Unlike green algae, brown algae contain no or little phosphatidylserine, possessing an unusual aminophospholipid, phosphatidyl-*O*-[*N*-(2-hydroxyethyl) glycine], PHEG, instead. When our routinely used technique of <sup>31</sup>P-NMR analysis of phospholipids was applied to the samples of edible New Zealand brown algae, a number of signals corresponding to unidentified phosphorus-containing compounds were observed in total lipids. NI (negative ion) ESI QToF MS spectra confirmed the presence of more familiar phospholipids, and also suggested the presence of PHEG or its isomers. The structure of PHEG was confirmed by comparison with a synthetic standard. An unusual MS fragmentation pattern that was also observed prompted us to synthesise a number of possible candidates, and was found to follow that of phosphatidylhydroxyethyl methylcarbamate, likely an extraction artefact. An unexpected outcome was the finding of ceramidephosphoinositol that has not been reported previously as occurring in brown algae. An uncommon arsenic-containing phospholipid has also been observed and quantified, and its TLC behaviour studied, along with that of the newly synthesised lipids.

**Keywords** <sup>31</sup>P-NMR · Phospholipid · Algae · Ceramide phosphoinositol · Arsenosugar phospholipid · PHEG

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## Abbreviations

AsPL	Arsenophospholipid, 3-((4-((dimethylarsoryl)methyl)-3,5-dihydroxytetrahydrofuran-2-yl)oxy) phosphatidylglycerol
CPI	Ceramide phosphoinositol
DCL	Deacylated cardiolipin
DGTA	Diacylglyceryl hydroxymethyltrimethyl-β-alanine
DPNGE	Diphosphatidyl <i>N</i> -glycolylethanolamine
GPA	Glycerophosphate (deacylated PtdOH)
GPC	Glycerophosphocholine (deacylated PtdCho)
GPE	Glycerophosphoethanolamine (deacylated PtdEtn)
GPHEG	Glycerophospho- <i>O</i> -[ <i>N</i> -(2-hydroxyethyl) glycine], deacylated PHEG
GPI	Glycerophosphoinositol (deacylated PtdIns)
HEG	<i>N</i> -(2-Hydroxyethyl) glycine
N-CAPE	<i>N</i> -(1-Carboxy-3-aminopropyl-3)-1,2-diacyl- <i>sn</i> -3-glycerophosphorylethanolamine
NI ESI QToF MS	Negative ion electrospray ionisation quadrupole time-of-flight mass spectrometry
PECM	PtdEtn-carboxymethyl (also known as phosphatidylhydroxyethyl methylcarbamate)
PHEG	Phosphatidyl- <i>O</i> -[ <i>N</i> -(2-hydroxyethyl) glycine]
PiNGE	Phosphatidyl <i>iso-N</i> -glycolylethanolamine
PNGE	Phosphatidyl <i>N</i> -glycolylethanolamine

Ptd <sub>2</sub> Gro	Cardiolipin
PtdCho	Phosphatidylcholine
PtdEtn	Phosphatidylethanolamine
PtdGro	Phosphatidylglycerol
PtdIns	Phosphatidylinositol
PtdOH	Phosphatidic acid
PtdSer	Phosphatidylserine

## Introduction

Recent decades witnessed an ever-growing interest in marine algae as a component of a healthy diet. The species traditionally enjoyed by consumers in the Asian coastal areas are gradually finding their way into Western food markets in different forms, with the names like Wakame and Kombu becoming more and more familiar. Algae are a low fat source of minerals and fibre, vitamins and amino acids; they are good in removing heavy metals, and possess radio protective components; some species are rich in iodine [1]. Edible brown algae were recently hailed as a source of beneficial allenic carotenoids, like fucoxanthin (e.g. [2]). One more attractive feature of algae is that they contain glyco- and phospholipids enriched in polyunsaturated fatty acids. Some species contain elevated levels of betaine lipids—a group of polar lipids that do not contain phosphorus, but, nevertheless, may partially (and sometimes, completely) replace phospholipids in these organisms. For example, in order *Fucales* phosphatidylcholine, PtdCho, is mostly or even completely replaced with diacylglyceryl hydroxymethyltrimethyl- $\beta$ -alanine, DGTA, while for order *Laminariales*, where PtdCho might account for over 40% of total phospholipids, no DGTA was reported [1]. There was a controversy regarding the presence of phosphatidylserine, PtdSer, in brown algae: until early 1990s it was generally accepted that brown algae contain PtdSer as one of the major phospholipids, along with phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol. It is generally accepted now that brown algae contain little or no PtdSer (*ibid.* and references therein). In 1994 it has been confirmed that brown algae contain a different aminophospholipid instead, with a suggested structure of *N*-(1-carboxy-3-aminopropyl-3)-1,2-diacyl-*sn*-3-glycerophosphorylethanolamine, abbreviated as N-CAPE (Fig. 1) [3]. A year later a different structure of that lipid was published, namely that of phosphatidyl-*O*-[*N*-(2-hydroxyethyl) glycine], abbreviated as PHEG (Fig. 1) [4]. While the abbreviation PHEG is more commonly referred to in this century, N-CAPE is still occasionally mentioned (e.g. [1]).

When our routinely used technique of <sup>31</sup>P-NMR analysis of phospholipids was applied to the samples of some of New Zealand brown algae of a current or emerging

**Fig. 1** Phospholipids relevant to the current study: *N*-CAPE *N*-(1-carboxy-3-aminopropyl-3)-1,2-diacyl-*sn*-3-glycerophosphorylethanolamine, *PHEG* phosphatidyl-*O*-[*N*-(2-hydroxyethyl) glycine], *PNGE* phosphatidyl *N*-glycolylethanolamine, *PiNGE* phosphatidyl *iso-N*-glycolylethanolamine, *DPNGE* diphosphatidyl *N*-glycolylethanolamine, PtdSer methyl ester methyl ester of phosphatidylserine, *PECM* phosphatidylethanolamine-carboxymethyl, *AsPL* arsenophospholipid, 3-((4-((dimethylarsoryl)methyl)-3,5-dihydroxytetrahydrofuran-2-yl)oxy) phosphatidylglycerol, *CPI* ceramide phosphoinositol (sphinganine and sphingosine species). *R*<sub>1</sub> and *R*<sub>2</sub> depict various acyl groups, otherwise the major molecular species are presented

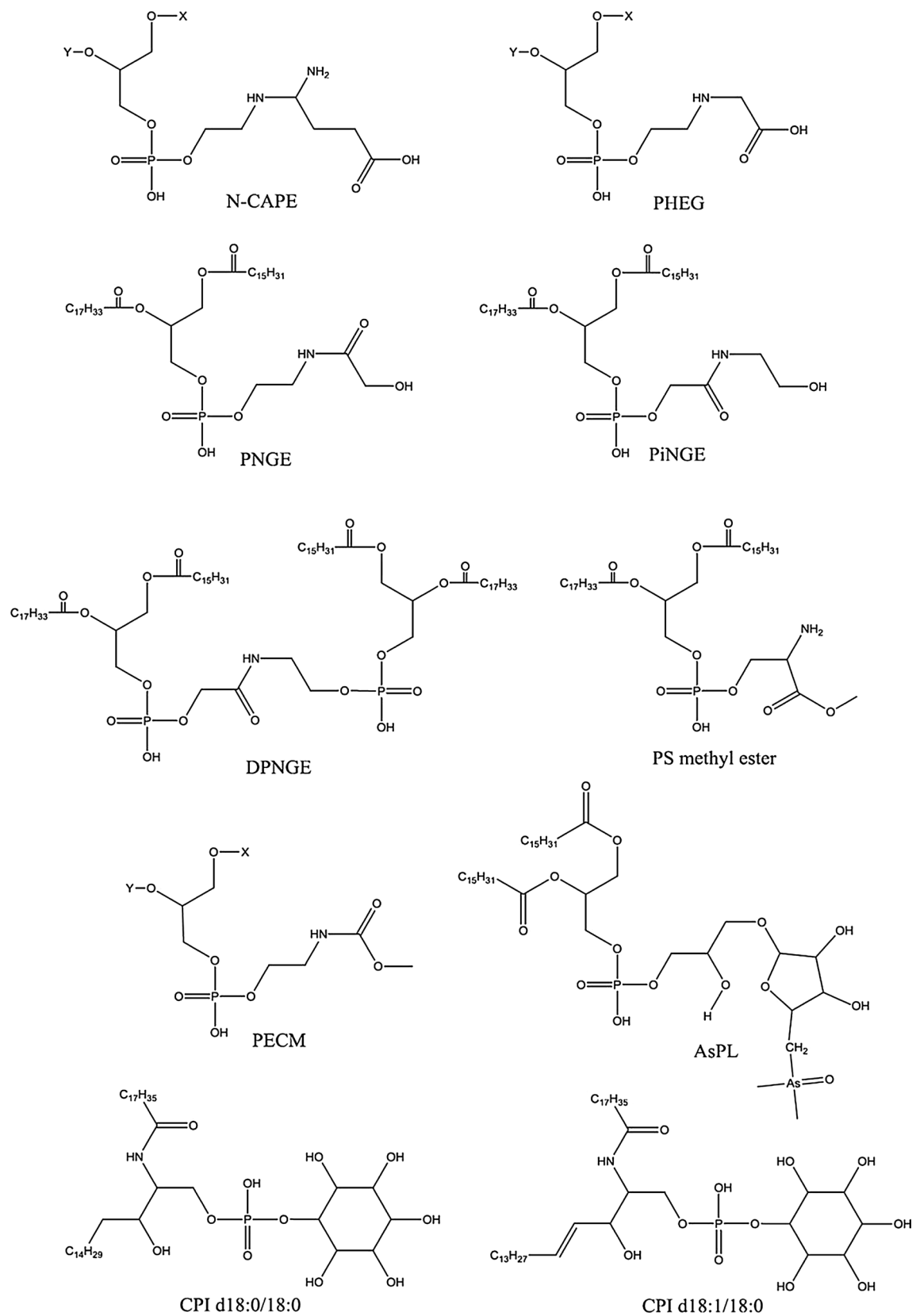
commercial importance, a number of signals corresponding to unidentified phosphorus-containing compounds were observed in total lipids. A combination of chemical and enzymatic synthesis and instrumental analytical approach was used to elucidate the structures of these compounds. The present publication reports our findings.

## Materials and Methods

Detached samples of *Ecklonia radiata* (common kelp) were collected in a tidal zone of Camp Bay (Eastbourne, Lower Hutt, New Zealand), and those of *Landsburgia quercifolia*, *Hormosira banksii* (Neptune's necklace), and *Scytothamnus australis*—in Lowry Bay (Eastbourne, Lower Hutt, New Zealand) on April 5th, 2015. A dietary supplement, Pacific Harvest Kelp Powder (dried *E. radiata*), was purchased from Commonsense Organics (Wellington, New Zealand). Detailed structure elucidation studies were performed on compounds from *E. radiata*.

Total lipids were extracted from samples using a method based on that of Bligh and Dyer [5]. Boiling water (500 ml) was added to the sample (100 g), boiled for 10 min to deactivate lipases, then the water drained off. The sample was chopped finely, methanol (200 ml) and chloroform (100 ml) were added and the sample placed in an ultrasonic bath for 10 min, then allowed to soak in the solvent overnight. The sample was sonicated for a further 30 min, solvent collected by filtration. Chloroform (100 ml) was added, and the sample sonicated for 30 min, then filtered, and the filtrates combined in a separating funnel. Aqueous KCl (0.88% w/w, 100 ml) was added, and the lower phase collected and dried by rotary evaporation.

Dried egg yolk powder (supplied by LM Wright, Dunedin, NZ) was enriched in phospholipids by Super Critical Fluid Extraction (SCFE) using CO<sub>2</sub> at 40 °C and a pressure of 500 bar to remove neutral lipids. The remaining phospholipid-enriched solid was then extracted by SCFE using CO<sub>2</sub> and 20% ethanol at 40 °C and 300 bar. The resulting phospholipid extract was washed and dried three times using chloroform to remove all traces of ethanol. The residue was 90% phospholipid by weight as determined by <sup>31</sup>P



NMR. Of these PtdCho accounted for 82.4%, PtdEtn-7.6%, lysoPtdCho-4%, SM-2.3%, and PtdIns-1.2%, with all other detectable phospholipids presented at less than 1% of total phospholipids by weight. When pure PtdCho was required (e.g. as the reference material for TLC), the PtdCho concentrate was purified by column chromatography on aluminium oxide with elution by chloroform–methanol (1:1, by v/v) and TLC control with Dragendorff reagent detection according to Barsukov *et al.* [6].

Phospholipase D from *Actinomadura* sp. was a gift from Dr. Shigeyuki Imamura (Imamura Enzyme Technologies Corporation, Shizuoka, Japan). Chloroform was of analytical grade from Fisher (USA). The rest of the solvents were of HPLC grade from Merck (Germany). Chemicals were of analytical grade. Calcium chloride was from Merck (Germany), the rest of the chemicals—from Sigma (USA).

### TLC

TLC plates, 10 × 10 cm glass-backed HPTLC Silica gel 60 plates (Merck, Germany) were activated at 110 °C for at least 90 min and left to cool to room temperature in a vacuum desiccator prior to sample loading. Phospholipids were analysed with 2D-TLC with chloroform–methanol-25% aqueous ammonia–benzene (65:30:6:10, by v/v/v/v) solvent system [7] in the 1st direction, and chloroform–methanol–glacial acetic acid–water (80:12:15:4, by v/v/v/v) system [8] in the 2nd direction. Detection—by phosphomolybdate spray prepared according to [9].

Elution of the spots from TLC plates was performed as follows: bands corresponding to individual phospholipids were scraped from the plate developed twice in 1st direction with chloroform–methanol-25% aqueous ammonia–benzene (65:30:6:10, by v/v/v/v) solvent system and eluted with chloroform–methanol (1:1, by v/v).

### NMR

<sup>31</sup>P-NMR analysis of both intact and deacylated phospholipids was performed as described in [10]. Lipid extract was dispersed in a sodium cholate detergent system (sodium cholate (10% w/w), EDTA (1% w/w), glyphosate (0.03% w/w internal standard) in 80:20 v/v H<sub>2</sub>O:D<sub>2</sub>O, pH 7.0, 700 μl) by ultrasonication. The solution was transferred to a 5-mm NMR tube and analysed on a two-channel Bruker Avance500 NMR. Quantification was achieved by comparison with the glyphosate internal standard and using an average fatty acid chain length of 18.0 for phospholipid class molecular weights (based on the fatty acid profiles of total lipid extracts of the four algae species). Deacylation of phospholipids was based on the method of Clark and Dawson [11]. The sample was dispersed in 0.5 ml ethanol and methylamine (40% wt in water, 3 ml), and heated at

55 °C in a capped test tube for 1 h. Solvent and reagent were removed under argon. Ethyl formate (150 μl) and ethanol (0.5 ml) were added to react with any residual methylamine, then removed under argon.

### HPLC–MS

An adaptation of the method described in [12] was used to perform LC ESI–MS and MS/MS experiments in negative mode with the use of a Shimadzu 8040-LCMS system. Data analysis was carried out using Shimadzu Labsolutions software. The MS settings employed for analysis were a nebulizer gas flow of 3 l/min, drying gas flow 15 l/min, heating block temperature 400 °C, DL temperature 250 °C, and collision energies in the range of 10–30 V for MS/MS experiments. Other MS settings were the defaults set during the instrument's tuning. Lipids were dissolved in chloroform prior to injection and injection volumes between 2 and 10 ml were used. Chromatography was carried out using a Waters BEH HILIC 2.1 × 150 mm 1.7 μm column with column oven temperature set at 30 °C. Elution solvents employed were acetonitrile (Solvent A) and aqueous 10 mM ammonium formate (Solvent B) with flow rate set at 0.25 ml/min. Initial solvent composition was set to 0% Solvent B. Following injection solvent composition was held at the initial composition for 2 min before being increased linearly to 8% B over 6 min, this was then increased to 30% B over 8 min, to 95% B over 9 min and held at this composition for 1 min. Solvent composition was then adjusted back to initial conditions over 1 min and the column allowed to equilibrate for 8 min before a subsequent injection was made.

### QToF MS

Direct infusion ESI–MS experiments were performed in positive and negative modes with the use of Waters Q-ToF Premier mass spectrometer working under MassLynx version 4.1 software. Calibration was performed with sodium formate solution. Lipids were submitted in the form of chloroform solutions, small amounts of which were transferred into the so called “standard solvent” (90% aq. methanol plus 0.05% formic acid) for direct infusion. When required, some dichloromethane could be added to get a clear solution for MS. The capillary voltage (1000–3000 V) and cone voltage (25–50 V) were optimized for each sample. Source temperature was 80 °C, desolvation temperature 180 °C, mass range 100–1200 Da. Typical run duration was between 0.5 and 1 min, with scan time 1 s, and interscan time 0.1 s. Cone gas flow was off, desolvation gas flow was 600 l/h. Collision energy in tandem MS experiments was within the 10–50 eV range.

## Synthesis of Substrates

*N*-(2-Hydroxyethyl) glycine, CAS Registry Number 5835-28-9, was synthesised according to [13]. *N*-Glycolylethanolamine (also known as 2-hydroxy-*N*-(2-hydroxyethyl)-acetamide), CAS Registry Number 3586-25-2, was prepared as described in [14]. Methyl (2-hydroxyethyl) carbamate, CAS Registry Number 13296-56-5, was synthesised with the use of an indium catalyst [15]. Methyl ester of serine, CAS Registry Number 2104-89-4, was prepared by stirring 116.0 mg of L-serine with 2.5 ml of 5% HCl in MeOH for 21 h at room temperature. The reaction was monitored by TLC on silica gel plates in *n*-BuOH-AcOH-H<sub>2</sub>O (3:1:1, by v/v/v) solvent system with detection by 0.2% ninhydrin in EtOH. The product was taken up in a stream of argon at +45 °C, and brought to a constant weight *in vacuo*, yielding 167.3 mg of colourless clear gum (97.6% of theoretical).

## Enzymatic Synthesis of Phospholipids

Briefly, egg yolk phosphatidylcholine was subjected to *Actinomadura* sp. phospholipase D treatment at +40 °C in acetate buffer containing calcium chloride at pH 6.8–7.2 (adjusted after substrate addition and prior to enzyme addition) for 72 h. The substrate was used as 10% (w/w) aqueous emulsions at substrate-to-donor molar ratio of 1:6. Enzyme concentration was 150 U/g substrate. The quantitative monitoring of enzymatic reactions was performed by <sup>31</sup>P NMR in a detergent system: an aliquot was phase

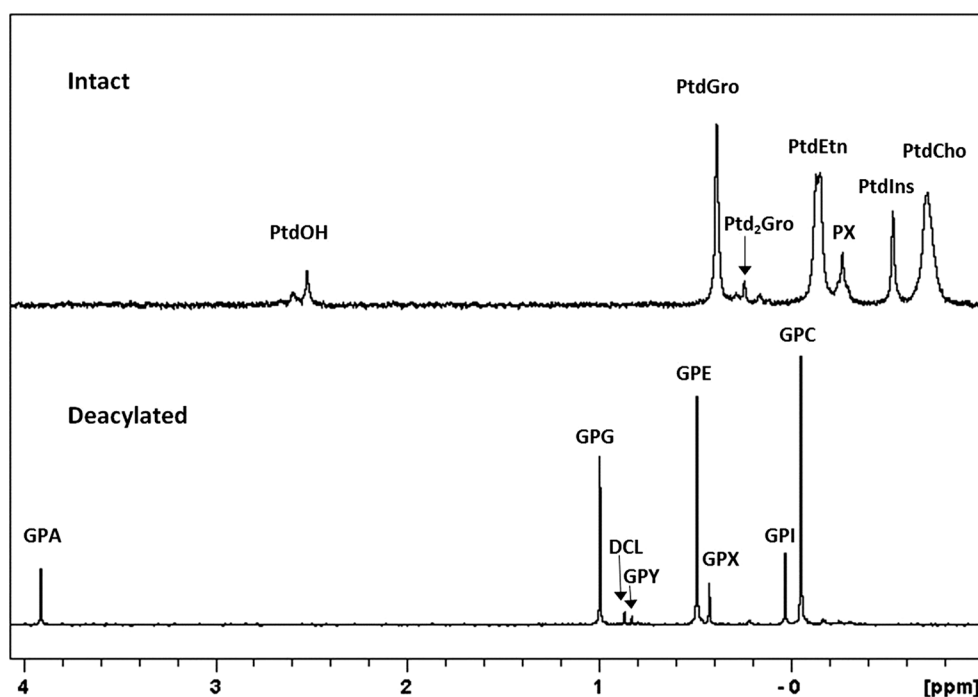
separated in chloroform–methanol–water (1:1:1, by v/v/v), and the bottom layer was used after a removal of the solvent *in vacuo*.

The structures of all the synthesised substrates and phospholipids were confirmed by <sup>1</sup>H NMR, <sup>13</sup>C NMR, COSY (where applicable), HSQC, PI (positive ion) and NI ESI QToF MS. GCMS of the TMS derivative was also employed to confirm the structure of methyl (2-hydroxyethyl) carbamate. The purity of all of the newly synthesised phospholipids, both intact and of their deacylated residues was determined by <sup>31</sup>P NMR.

## Results

A <sup>31</sup>P-NMR study of *E. radiata* (common kelp, also known as paddle weed) lipids showed the composition of intact phospholipids to be represented predominantly by PtdCho, PtdEtn, PtdGro, PtdIns, PtdOH and an unidentified peak PX (Fig. 2, upper spectrum). PtdCho and PtdEtn exhibited significant peak broadening due to a broad range of fatty acids present. Spectra of deacylated phospholipids are characterised by narrower signals due to the lack of interference from various fatty acid residues, which might be helpful if the intact lipids produce a complicated spectrum. The spectrum of the deacylated *E. radiata* lipids was simpler (Fig. 2, lower spectrum), with narrower peaks, and the same major phospholipid classes represented (as GPC, GPE, GPG, GPI, GPA) and the unidentified class (as GPX). The pH of the sodium cholate

**Fig. 2** <sup>31</sup>P-NMR spectra of intact *Ecklonia radiata* total lipids (*upper*), and after deacylation (*lower*), both recorded at pH 7. Abbreviations—see at the beginning of the paper. Chemical shifts relative to phosphoric acid. PX was identified as PHEG, GPX—as GPHEG and GPY—as deacylated AsPL



detergent was observed to significantly affect the chemical shift of GPX. At pH 7, the peak was upfield from GPE, at pH 8.1 the two peaks had the same chemical shift and were indistinguishable, at higher pH the GPX was downfield of GPE. The peak for GPX was observed in the deacylated lipids of all four brown algae species studied (Fig. 3).

NI ESI QToF MS experiments confirmed the presence of common phospholipids, as well as of some unidentified compounds of a higher molecular weight. To find out if GPX is related to PHEG we have synthesised the latter phospholipid by using the two-step approach: the first step was the synthesis of *N*-(2-hydroxyethyl) glycine, HEG, from ethanolamine and monochloroacetic acid according to Lowe and Vilaivan [13]. The second step was the synthesis of PHEG from HEG and egg yolk Ptd-Cho using a potent phospholipase D from *Actinomadura* sp.

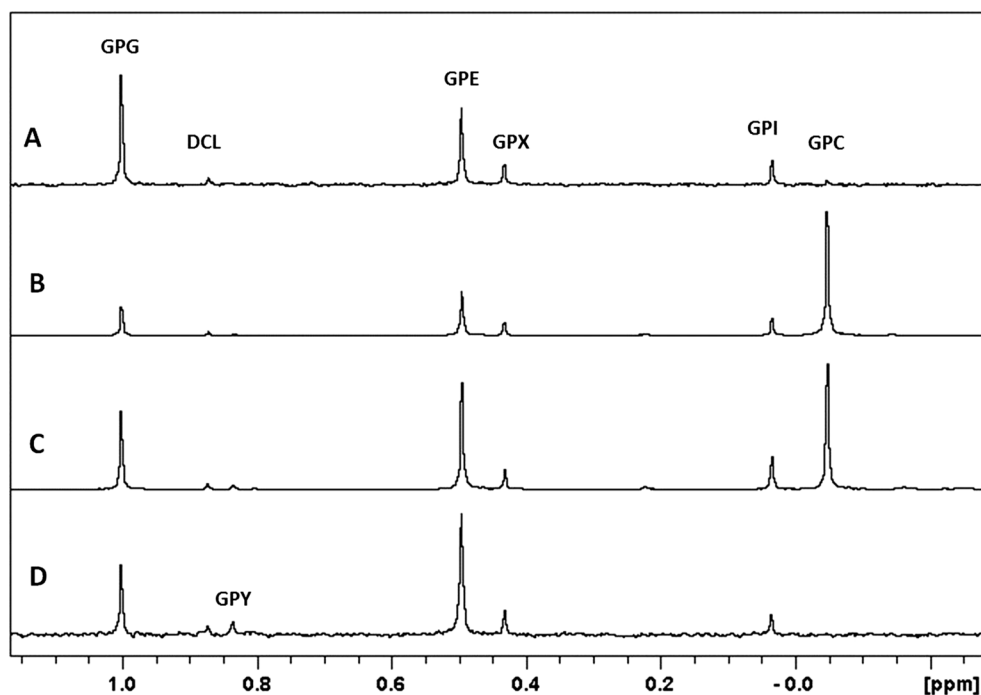
The structure of synthetic PHEG was confirmed by NI ESI QToF MS (Fig. 4a), with its fragmentation pattern matching that of PX isolated by preparative TLC from *E. radiata* (Fig. 4d). Since neither PC (20:4/20:4) or PE (20:4/20:4) were available to us, we could not synthesise PHEG (20:4/20:4) to study its fragmentation, having PHEG (16:0/18:1) employed as a reference instead. In  $^{31}\text{P}$ -NMR spectrum PHEG signal had the same chemical shift as PX. A comparison of  $^{31}\text{P}$ -NMR spectra of deacylated mixtures of synthetic PHEG with kelp lipids allowed us to confirm that GPX (Fig. 3) is identical to GPHEG (Fig. 5). PHEG accounted for 4.8–9.5% of total

phospholipids, or for 8.8–208.4 mg/kg in the samples studied (Table 1).

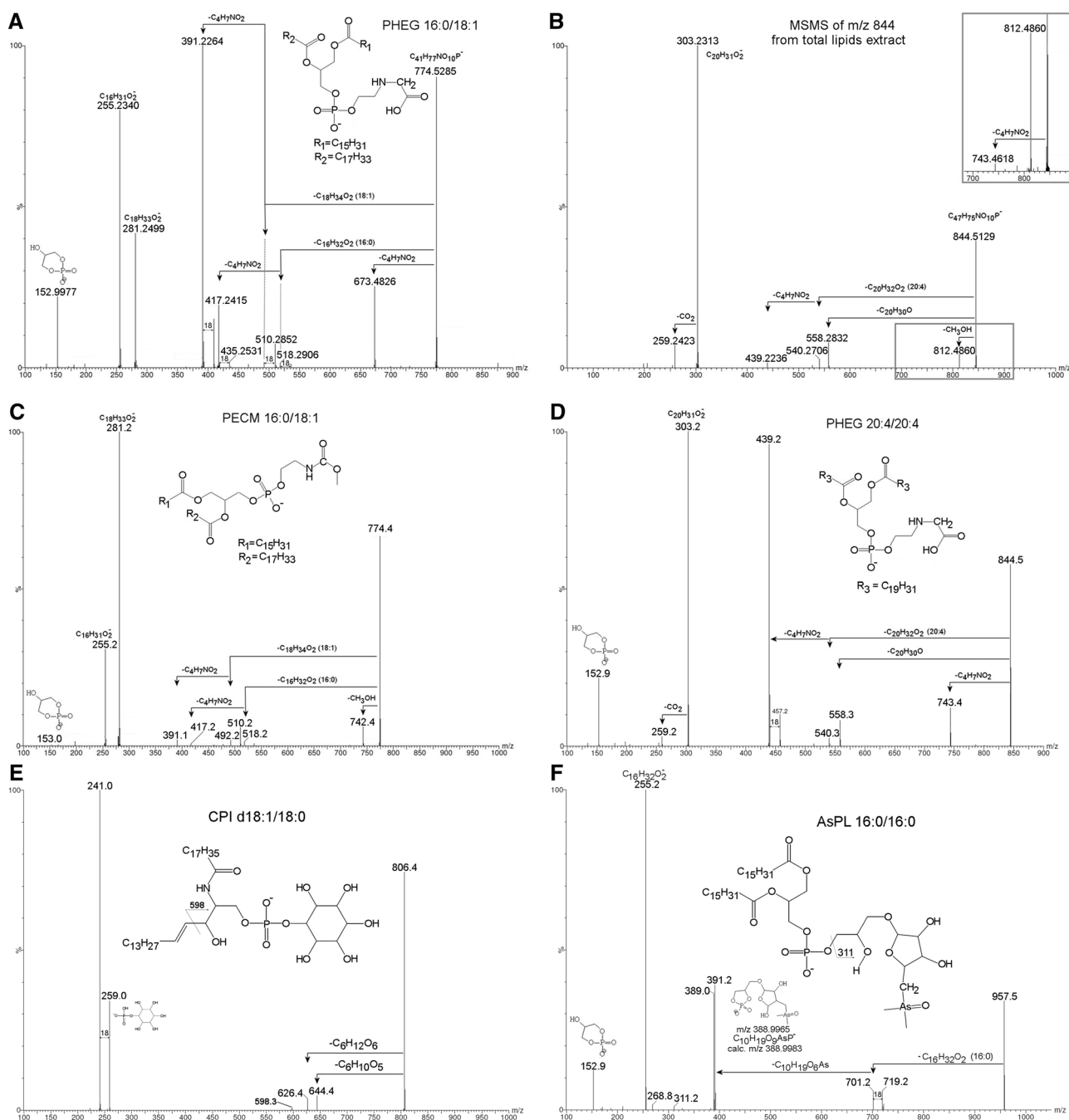
Interestingly, when the total lipids extract of *E. radiata* was subjected to mass-spectrometry, the parent ion at  $m/z$  844 in the NI ESI QToF MS MS spectrum of the diarachidonoyl species of kelp PHEG was also found to generate a low intensity M-32 daughter ion (Fig. 4b), which has been confirmed by accurate mass determination to result from the loss of methanol. Since no such feature has been observed in the spectrum of an authentic PHEG, as well as no other fatty residues but arachidonoyl were observed in that spectrum, we assumed that an isomer of PHEG is also present among kelp total lipids. In other words, there was one more lipid present, having a structure of a diarachidonoyl phosphatidic acid with a hydrogen atom in the phosphate group replaced with the  $\text{C}_4\text{H}_8\text{NO}_2$  moiety. To address this finding, and assuming that the compound in question may have a fragment of one of amino alcohols commonly found in algal phospholipids, either ethanolamine or serine, we have attempted to synthesise all of the relevant isomers.

The synthesis of two of the possible isomers of PHEG (Fig. 1), namely phosphatidyl *N*-glycolylethanolamine, PNGE, and phosphatidyl *iso-N*-glycolylethanolamine, PiNGE, required a three-stage approach. The first one, the synthesis of glycolic acid acetoneide, and the second one, that of *N*-glycolylethanolamine were performed according to Daryaee *et al.* [14]. The third stage included enzymatic synthesis from egg yolk PtdCho using phospholipase D as described above. None of these isomers

**Fig. 3**  $^{31}\text{P}$ -NMR spectra of deacylated algal lipids recorded at pH 7.0. A *L. quercifolia*, B *S. australis*, C *E. radiata*, D *H. banksii*. GPX and GPY were eventually identified as GPHEG, and deacylated AsPL, respectively







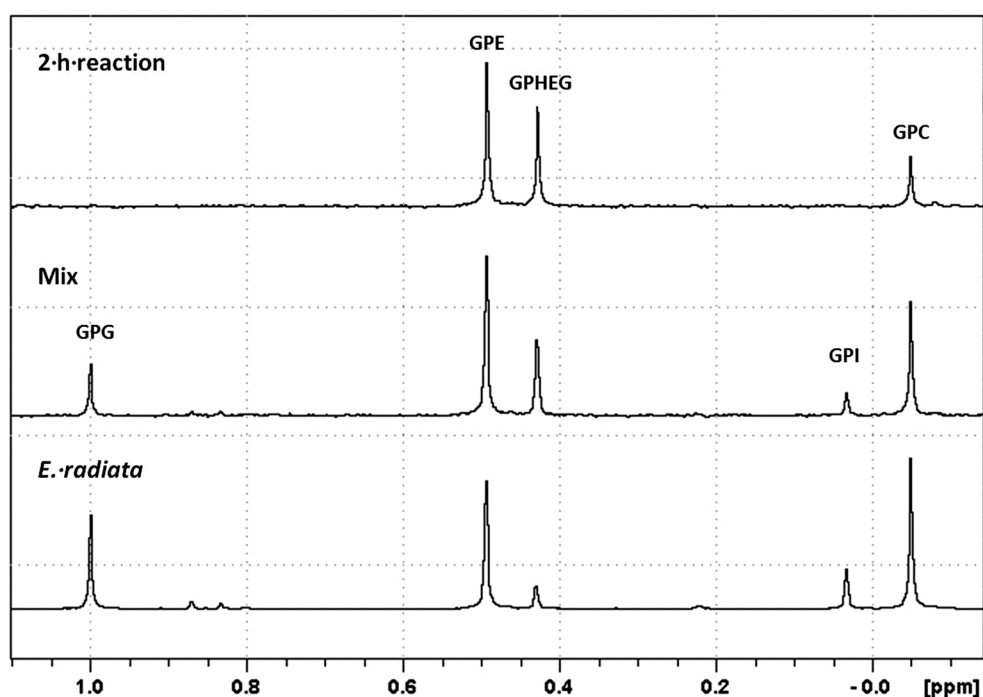
**Fig. 4** Mass-spectrometry of kelp lipids, and some relevant synthetic lipids. **A** NI ESI QToF MS of the major molecular species of synthetic PHEG. **B** Fragmentation of the molecular ion with  $m/z$  844 that corresponded to the diarachidonoyl species of kelp PHEG in NI ESI QToF MS has shown the loss of methanol, suggesting the presence of the polar head group isomer. **C** NI ESI QToF MS of synthetic PECM

shows the loss of methanol. **D** NI ESI QToF MS of kelp PHEG, isolated by preparative TLC: Fragmentation of the molecular ion with  $m/z$  844. **E** NI ESI QToF MS of kelp ceramidephosphoinositol: MSMS of the most prominent molecular ion, and the possible structure. **F** NI ESI QToF MS of kelp arsenophospholipid: MSMS of the most prominent molecular ion

produced an M-32 ion the NI ESI QToF MS experiment (data not shown). Interestingly, a side product, diphosphatidyl *N*-glycolylethanolamine, DPNGE, an analogue of cardiolipin, was also formed during enzymatic synthesis of PNGE and PiNGE (Fig. 1). Our attempt to

synthesise another possible isomer of PHEG, namely phosphatidylserine methyl ester, was unsuccessful: while we were able to prepare methyl ester of serine, an attempt to use it as a substrate for enzymatic synthesis of phosphatidylserine methyl ester resulted in a mixture of

**Fig. 5**  $^{31}\text{P}$ -NMR spectra of a mixture of deacylated synthetic PHEG with glycerophosphocholine and glycerophosphoethanolamine (*top lane*); deacylated kelp lipids (*bottom lane*); and a deacylated mixture of synthetic PHEG and kelp lipids (*middle lane*)



**Table 1** Phospholipid composition of the samples studied

	<i>Landsburgia quercifolia</i>	<i>Scyothamnus australis</i>	<i>Ecklonia radiata</i>	<i>Hormosira banksii</i>	Pacific Harvest sea kelp
CPI	0.4	0.8	2.2	n.d.	2.1
PtdCho	1.5	52.4	32.2	n.d.	32.8
PtdIns	10.0	8.7	8.9	7.6	11.5
PHEG	9.5	4.8	5.5	8.3	5.5
PtdEtn	29.9	17.9	25.9	46.9	17.7
AsPL	2.3	0.9	1.3	6.5	1.8
Ptd <sub>2</sub> Gro	2.4	1.3	1.4	3.7	1.8
PtdGro	39.9	11.0	17.6	22.0	16.0
PtdOH	4.0	2.4	5.0	5.0	10.8
Total PL wt% of total lipids	3.4	20.7	34.8	3.3	20.1
Total lipids, wt% of sample <sup>a</sup>	0.91	1.34	0.61	0.32	1.9
Total PL wt% of sample <sup>a</sup>	0.03	0.28	0.21	0.01	0.38
PHEG, mg/kg sample <sup>a</sup>	30.0	132.6	116.3	8.8	208.4
AsPL, mg/kg sample <sup>a</sup>	7.3	23.9	27.6	6.9	65.9

Minor phospholipids (<0.4% of total phospholipids) were not quantified

<sup>a</sup> Wet sample (algae), or powder (Pacific Harvest dietary supplement)

phosphatidylserine, phosphatidylmethanol, and phosphatidic acid formed, with no detectable levels of the target compound produced.

The remaining option was phosphatidylethanolamine methylcarbamate (Fig. 1, PtdEtn-MC, also known as PtdEtn-carboxymethyl, PECM). It was synthesised from methyl (2-hydroxyethyl) carbamate and egg yolk PtdCho using the same phospholipase D approach as described above. The substrate, methyl (2-hydroxyethyl) carbamate,

was synthesised from ethanolamine and methyl chloroformate with indium powder as a catalyst according to Kim and Jang [15]. Indeed, in NI ESI QToF MS experiment PECM produced M-32 ion (Fig. 4c). To further verify the identity of PECM (and PHEG) in kelp extracts, the Waters BEH HILIC column based HPLC–MS assay has been developed (see “Materials and Methods”). This method was applied to synthetic PECM, PHEG, and to *E. radiata* total lipids extract with the following results:



Synthetic PECM (16:0/18:1 molecular species): retention time 11.5 min, parent ion at  $m/z$  774, characteristic daughter ions at  $m/z$  742 (M-32 pattern, the loss of methanol),  $m/z$  281 (18:1), and  $m/z$  255 (16:0). Kelp total lipids extract: peak at 10.8 min, parent ion at  $m/z$  844, and characteristic daughter ions observed at  $m/z$  812 (M-32 pattern), and  $m/z$  303 (20:4), suggesting the presence of PECM 20:4/20:4 in the extract.

Synthetic PHEG (16:0/18:1 molecular species): retention time 14.3 min, parent ion at  $m/z$  774, characteristic daughter ions at  $m/z$  673 (M-101 pattern, the loss of  $C_4H_7NO_2$ ),  $m/z$  281 (18:1), and  $m/z$  255 (16:0). Kelp total lipids extract: peak at 13.9 min, parent ion at  $m/z$  844, and characteristic daughter ions observed at  $m/z$  743 (M-101 pattern), and  $m/z$  303 (20:4), suggesting the presence of PHEG 20:4/20:4 in the extract.

PECM was reported earlier as an artefact formed from PtdEtn during extraction of *Escherichia coli* with chloroform–methanol mixtures [16]. It is thus likely that PECM in our experiments was also an extraction artefact.

In the NI ESI QToF spectrum of intact kelp lipids the pattern of PHEG molecular ions was accompanied by a minor intensity pattern having the  $m/z$  that of PHEG + 58. While we could not positively identify the compound responsible, we noticed that the synthesis of the HEG substrate produced a minor by-product with an extra – $CH_2COOH$  moiety linked to the nitrogen atom. That compound did generate the molecular ion 58 Da above that of HEG in the NI ESI QToF experiment, hinting at a possible polar head structure of the M + 58 companion of PHEG.

### Other Phospholipids

An unexpected finding of ceramide phosphoinositol, CPI, was made initially by NI ESI QToF MS in the total lipids of *E. radiata*, and later confirmed by preparative isolation of the compound of interest, followed by NI ESI QToF MS and  $^{31}P$  NMR. CPI in kelp was represented mostly by saturated and monounsaturated species with 36 carbon atoms in the ceramide moiety. The presence of the same ion of a minor abundance at  $m/z$  598.3 in the MSMS spectra of two main molecular ions ( $m/z$  806.4 and 808.4) might correspond to the same  $C_{27}H_{53}NO_{11}P$  daughter ion formed via splitting the bond between C3 and C4 carbons of the sphingosine moiety, suggesting d18:1/18:0 and d18:0/18:0 being the main molecular species of CPI (Fig. 1). The NI ESI MS product-ion spectrum of the former molecular species in our experiment was identical to the one presented for the same species by Hsu *et al.* [17]. To the best of our knowledge of all macroalgae CPI was reported for red algae only ([1], and references therein). In the samples we were working with

CPI was always a minor lipid, possibly originating from epiphytes (Fig. 2, a small “blip” slightly upfield of GPC in the lower spectrum).

Another compound of interest was an arsenophospholipid (AsPL, Fig. 1), namely 3-((4-((dimethylarsoryl)methyl)-3,5-dihydroxytetrahydrofuran-2-yl)oxy) phosphatidylglycerol, often conveniently referred to as “arsenosugar phospholipid” that has been found in a number of species of brown algae (e.g. [18], and references therein). Indeed, we have observed the relevant molecular ions that corresponded predominantly to saturated molecular species of AsPL, with the dipalmitoyl-AsPL being the major molecular species, as determined by high accuracy MS measurements ( $m/z$  calculated for  $C_{45}H_{87}O_{14}PAs^-$  957.5049, observed 957.5040). The major molecular ion was accompanied by less prominent ones that corresponded to 20:0/16:0, and 18:0/16:0 species. Overall, fatty acids observed in AsPL by QToF MS ranged from 14 to 26 carbon atoms long, being predominantly saturated. Using preparative TLC we isolated AsPL from kelp *E. radiata* and determined its behaviour in  $^{31}P$ -NMR experiments. Deacylated AsPL possessed the same chemical shift as the signal labelled “GPY” (Fig. 2, lower spectrum, and Fig. 3).

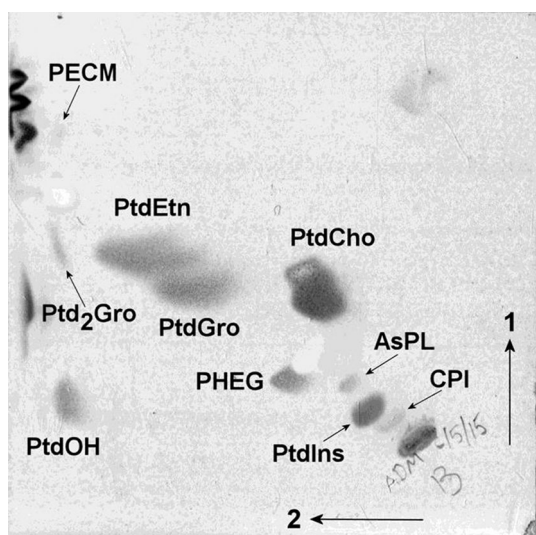
### Quantitative Analysis of Phospholipids in Selected New Zealand Brown Algae

The results of the quantitative analysis of the four species of New Zealand brown algae, and one kelp-derived dietary supplement that were studied in the current research are presented in Table 1.

Considering that all of the studied species are intended for human consumption in one form or another, the levels of PHEG and AsPL present in the species studied, especially—in *E. radiata* and *S. australis*, warrant the investigation of their biological activities. Levels of an artefact lipid PECM in all of the species studied were too low to observe a discernible signal in  $^{31}P$ -NMR spectra.

### TLC Behaviour of Novel Lipids

Since our laboratory commonly employs TLC for rapid qualitative analysis of complex lipids, it was of interest to observe the chromatographic behaviour of these newly identified compounds relative to common phospholipids (Fig. 6). The labels on the image were assigned after the mobilities of individual newly synthesised PHEG and PECM were determined by 1D-TLC relative to the known standards, and the identities of the labelled spots were confirmed by ESI QToF MS after eluting individual spots scraped off 2D TLC of *E. radiata* extract.



**Fig. 6** 2D TLC of kelp (*E. radiata*) phospholipids (see “Materials and Methods”) with phospholipids detected by the molybdate spray. White spots (one above Ptd<sub>2</sub>Gro, and another touching PHEG) are those of glycolipids. Non-labelled black and grey spots are those of pigments

## Discussion

Algae may no longer be considered as merely a local traditional food in some eastern cultures. Adding new species to diets warrants certain caution, since these might contain (or produce during processing) unfamiliar or overtly toxic products. Even traditionally enjoyed species might be dangerous if processed improperly: an example of fatal food poisonings in Japan linked to a consumption of prostaglandins formed by edible red alga [19] is a warning serious enough.

Commonly found in New Zealand brown algae *H. banksii*, and *E. radiata* are edible (e.g. [20, 21]), while *S. australis* is considered as a medicinal dietary supplement [22], and *L. quercifolia* is of interest as a functional [23] and cosmeceutical [24] ingredient, and thus are of a current or emerging commercial importance. The levels of PHEG and AsPL present in the species studied in the current research, especially—in *E. radiata* and *S. australis* warrant the studies of their biological activities.

Regarding individual phospholipids, the levels of PtdCho varied greatly in the species studied, from 0.0 to 52.4% of total phospholipids. The levels of PtdEtn and PtdGro were somewhat less variable between the species, 17.9–46.9% and 11.0–39.9%, respectively. The levels of PtdIns (7.6–10.0%), PtdOH (2.4–5.0%), and Ptd<sub>2</sub>Gro (1.3–3.7%) were more conservative. There was no visible link between the presence (or absence) of PtdCho, and the levels of PHEG (Table 1). PHEG content varied

from 30.0 mg/kg of wet alga in *H. banksii* to 132.6 mg/kg in *S. australis*, and arsenophospholipid—from 6.9 mg/kg of wet *L. quercifolia* to 27.6 mg/kg in *E. radiata*. Even higher levels of PHEG and arsenophospholipid were found in Pacific Harvest Sea Kelp dietary supplement—208.4 and 65.9 mg/kg, respectively.

While the lipid composition determined in the current study might not be representative in a quantitative sense of the brown algae species as a whole, and only describes the lipids observed in specific species collected in the specific environmental conditions and in a limited number of geographic locations, our findings confirm the qualitative diversity of the polar lipids classes in brown algae.

To summarise our observations: (1) the studied species of New Zealand edible brown algae contain uncommon phospholipids with bioactivities yet to be established; (2) <sup>31</sup>P NMR is a convenient way to qualitatively and quantitatively analyse kelp phospholipids (including arsenic-containing phospholipids); (3) novel phospholipids, including PHEG and a number of its isomers were synthesised, and their TLC, MS and NMR characteristics were determined.

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## Compliance with ethical standards

**Conflict of interest** Authors declare that there is no conflict of interest.

## References

- Khotimchenko SV (2003) Lipids of marine macrophytic algae and grasses: structure, distribution, analysis. Svetashev VI (ed) Vladivostok, Dalnauka, Russian Academy of Sciences. ISBN 5-8044-0347-8
- Kumar SR, Hosokawa M, Miyashita K (2013) Fucoxanthin: a marine carotenoid exerting anti-cancer effects by affecting multiple mechanisms. *Mar Drugs* 11:5130–5147. doi:10.3390/md11125130
- Schmid CE, Müller DG, Eichenberger W (1994) Isolation and characterization of a new phospholipid from brown algae. intracellular localization and site of biosynthesis. *J Plant Physiol* 143:570–574. doi:10.1016/S0176-1617(11)81826-24
- Eichenberger W, Bigler P, Gfeller H, Gribo C, Schmid CE (1995) Phosphatidyl-*O*-[*N*-(2-hydroxyethyl)glycine] (PHEG), a new glycerophospholipid from brown algae (*Phaeophyceae*). *J Plant Physiol* 146:398–404

5. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37:911–917
6. Barsukov LI, Batrakov SG, Bergelson LD, Dyatlovitskaya EV, Molotkovskiy JG, Prokazova NV (1980) In: Bergelson LD (ed) *Lipid biochemical preparations*. Elsevier/North-Holland Biomedical Press, Amsterdam-New York-Oxford
7. Vaskovsky VE, Terekhova TA (1979) HPTLC of phospholipid mixtures containing phosphatidylglycerol. *J High Resolut Chromatogr Chromatogr Commun* 2:671–672
8. Tindall BJ, Sikorski J, Smibert RM, Kreig NR (2007) Phenotypic characterization and the principles of comparative systematics. In: Reddy CA, Beveridge TJ, Breznak JA, Marzluf G, Schmidt TM, Snyder LR (eds) *Methods for general and molecular microbiology*, 3rd edn. ASM Press, Washington
9. Vaskovsky VE, Kostetsky EY, Vasendin IM (1975) A universal reagent for phospholipid analysis. *J Chromatogr* 114:129–141
10. Vyssotski M, MacKenzie A, Scott D (2009) TLC and <sup>31</sup>P-NMR analysis of low polarity phospholipids. *Lipids* 44:381–389
11. Clark NG, Dawson RMC (1981) Alkaline *O* → *N*-transacylation. a new method for the quantitative diacylation of phospholipids. *Biochem J* 195:301–306
12. Xiong Y, Zhao Y-Y, Goruk S, Oilund K, Field CJ, Jacobs RL, Curtis JM (2012) Validation of an LC–MS/MS method for the quantification of choline-related compounds and phospholipids in foods and tissues. *J Chromatogr B* 911:170–179. doi:10.1016/j.jchromb.2012.10.038
13. Lowe G, Vilaivan T (1997) Amino acids bearing nucleobases for the synthesis of novel peptide nucleic acids. *J Chem Soc Perkin Trans* 1:539–546
14. Daryae F, Kobarfard F, Khalaj A, Farnia P (2009) Synthesis and evaluation of *in vitro* anti-tuberculosis activity of *N*-substituted glycolamides. *Eur J Med Chem* 44:289–295
15. Kim J-G, Jang DO (2009) Indium-catalyzed reaction for the synthesis of carbamates and carbonates: selective protection of amino groups. *Tetrahedron Lett* 50:2688–2692
16. Garrett TA, Raetz CRH, Son JD, Richardson TD, Bartling C, Guan Z (2011) Non-enzymatically derived minor lipids found in *Escherichia coli* lipid extracts. *Biochim Biophys Acta Mol Cell Biol Lipids* 1811:827–837
17. Hsu F-F, Turk J, Zhang K, Beverly SM (2007) Characterization of inositol phosphorylceramides from *Leishmania major* by tandem mass spectrometry with electrospray ionization. *J Am Soc Mass Spectrom* 18:1591–1604
18. Raab A, Newcombe C, Pitton D, Ebel R, Feldmann J (2013) Comprehensive analysis of lipophilic arsenic species in a brown alga (*Saccharina latissima*). *Anal Chem* 85:2817–2824. doi:10.1021/ac303340t
19. Noguchi T, Matsui T, Miyazawa K, Asakawa M, Iijima N, Shida Y, Fuse M, Hosaka Y, Kirigaya C, Watabe K, Usui S, Fukagawa A (1994) Poisoning by the red alga ‘ogonori’ (*Gracilaria verrucosa*) on the Nojima Coast, Yokohama, Kanagawa Prefecture, Japan. *Toxicon* 32:1533–1538
20. Hutchinson A Yum Yum.. Edible New Zealand Plants! <http://www.campermate.co.nz/yum-yum-edible-new-zealand-plants>. Accessed Dec 2015
21. Smith JL, Summers G, Wong R (2010) Nutrient and heavy metal content of edible seaweeds in New Zealand. *N Z J Crop Horticult Sci* 38:19–28
22. Taylor S (2011) Marine medicinal foods: implications and applications, macro and microalgae. *Advances in food and nutrition research*, vol 64. Academic Press, San Diego, USA (ISBN 0123877008)
23. Dominguez H (2013) Functional ingredients from algae for foods and nutraceuticals. *Woodhead Publishing Series in Food Science, Technology and Nutrition*, Elsevier, Burlington, USA (ISBN 0857098683)
24. Kim S-K (2011) Marine cosmeceuticals: trends and prospects. CRC Press, Boca Raton, USA (ISBN 1439860297)