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Phospholipids in marine environments: a review

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Abstract

This paper provides a brief summary of environmental and ecological concepts, and analytical techniques for marine phospholipids (PL). These compounds have been recognized to be a major component of lipids which play a vital role as structural and functional components of biological membranes in all marine organisms from viruses to large animals. Major areas discussed are developed methods of PL analysis and their limitations and implications. Another important aspect of PL in marine ecosystems is also discussed here, that is, the role and dynamics of PL as a significant component of the nutrient phosphorus (P). However, information on PL as a P component in marine environments, such as their spatial and temporal distribution, their contribution to the total P pool, and their biological availability as the nutrient P source, is still limited. It is argued that this is due to the separation of marine PL studies into either "lipid studies" or "nutrient P studies". New techniques are reviewed that can be developed as a powerful tool to unite the two aspects of PL studies.

Keywords: Phospholipids; Phosphorus; Marine environment; Review

1. Introduction

Phosphorus (P) plays a significant role in various biological/biochemical processes in nature. All marine organisms, of course, require P to live. Therefore, it can be said that marine ecosystems rely upon the availability of P. Primary productivity in the ocean is limited by the availability of P, nitrogen (N) and iron as nutrients [1–5]. Recent works suggest that in selected oceanic environments, the central Atlantic Ocean, the North Atlantic, and the subtropical North Pacific, the ecosystems have undergone a regime shift from a predominantly N-controlled system to an ecosystem where production ultimately is controlled by the availability of P [6–8]. In addition to the role of P in global biogeochemical processes, there is another side to this element as an environmental factor on more regional and shorter time scales. The role of P as a pollutant is significant in marine environments. Human activities have lead to excess inputs of P and N to marine ecosystems. In particular, coastal areas exposed to large quantities of P associated with human waste have become eutrophic with the destruction of ecosystems and habitats in the area [9–11]. Marine environments and productivity are tightly linked with the amount and cycling of P on a wide scale in time and space. Thus, it is imperative to understand the cycling, e.g., the sources, pathways and fate, of P in marine ecosystems for predicting future change in global and regional environments.

Phosphorus is a component of many macromolecules involved in the metabolic processes of life, including adenosine triphosphate (ATP) in energy-consuming reactions in cells, ribonucleic acid and deoxyribonucleic acid (RNA and DNA) in the evolutionary development of life, and phospholipids (PL) in the formation of cell membranes and various organelles. Furthermore, there are various less common P compounds that are synthesized by some organisms. These P-containing organic compounds (organic P compounds) exist in various forms in marine environments and play potentially important roles in marine P cycling. In seawater, organic P exists in particulate and dissolved pools. Since soluble orthophosphate,

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the major component of dissolved inorganic P (DIP), is the preferred substrate of P for marine planktonic microorganisms, many previous studies have focused on the abundance and metabolic rates of DIP in seawaters [12–16]. The potential importance of organic P, however, also has been indicated in various marine environments. In oligotrophic oceanic regions as well as many coastal areas, concentrations of dissolved organic P (DOP) often exceed that of DIP [17–20]. Given the low DIP concentrations and the critical roles of P in supporting biological production, early studies indicated the potential importance of DOP as a nutrient source to primary producers in marine systems [21–24].

Particulate organic P (POP) includes P which is a component of living or dead marine phytoplankton, partly degraded phytoplankton cells, bacteria, zooplankton and larger multicellular organisms. Land-derived detrital materials also can be included in POP in estuarine and coastal marine environments [25,26]. POP must be an important component in P transport in marine environments. While dissolved matter export to the deep waters largely depends on the extent of downward diffusion and mixing, gravitational settling of particulate matter can export significant quantities of fresh organic materials produced in the euphotic zone to bottom sediments. Burial of settled POP as sedimentary organic P is one of the most important sinks of P from the ocean [27–29]. Despite the importance of organic P as the source and sink of biologically available P, investigation of the marine organic P cycle is complicated by the presence of numerous poorly identified reservoirs which precludes a straightforward determination of P inventories and fluxes. For better understanding of the P cycle in marine ecosystems, it is essential to examine the origin, distribution and fate of organic P compounds.

Among the various organic P pools that have been identified in marine environments, including nucleic acids, nucleotides, PL, sugar phosphate and phytic acids [30,31], this paper focuses on PL and their related compounds. PL are ubiquitous in nature as they are both structural and functional components of biological membranes in organisms from viruses to large marine animals. There are two significant roles of PL in marine ecosystems, namely, as a major component of lipids and a reservoir of organic P. As reviewed in this paper, because of the ubiquitous distribution in marine organisms and structural diversity of compound classes, studying PL as a lipid component has enabled evaluation of various microbiological parameters, including biomass, biodiversity, and energy flow in marine microbial ecosystems. On the other hand, the role and dynamics of PL as an organic P reservoir have not yet been well documented, and the quantitative significance, biological availability as a nutrient P source, and removal pathways from marine environments are poorly understood. The most notable distinguishing feature of PL compared with other organic P compounds is in their hydrophobic nature. Among the various characteristics of natural organic P compounds, hydrophobicity is presumed to be a key factor in determining the behavior of P in marine ecosystems. Hydrophobicity plays a role in determining potential organic P solubility in seawater, affinity to particulate matrices, and formation of some liposome-like structures. In addition, hydrophobic materials can act as a carrier of hydrophobic micropollutants, including halogenated hydrocarbons, fused-ring hydrocarbons, and pesticides in aquatic environments [32]. Because of the environmental importance of PL as nutrient P components and hydrophobic compound classes, it is important to understand the cycling of PL in marine environments. In this study, a review of the role, characteristics and analyses of marine PL is given.

2. Definition of phospholipids

The presence of P in lipids extracted with ethanol from brain tissue was first discovered in 1811 by Vauquelin. Similar substances isolated from brain tissues with hot alcohol were named matière blanche, cérébrote, acide cérébrique or oleophosphoric acid. Later, Gobley isolated a P-containing lipid from egg-yolk and brain tissues and named it lecithin (phosphatidylcholine) [33,34]. He showed that glycerophosphoric acid could be prepared from lecithin [35]. Thudichum made considerable progress with PL chemistry by isolating and characterizing many PL fractions from brain lipids. From his studies, Thudichum concluded that PL are “the center, life, and chemical soul of all bioplasm”. In 1927, three well-defined PL had been described: lecithin, cephalin (now phosphatidylethanolamine) and sphingomyelin. Later, several others were added to that list including phosphatidic acid [36], one acetal phosphatide (now plasmalogen) [37], phosphatidylinositol [38] and cardiolipin (diphosphatidylglycerol) [39]. The abbreviations of the representative PL compounds are shown in Fig. 1.

There is no general agreement on the best way to classify PL, but most classifications contain a category for glycerol-containing phospholipids (glycerophosphatides) and one for sphingolipids (sphingosyl phosphatides). In Fig. 1, the selected structures of representative compound classes with specific examples are illustrated. Glycerophospholipids are derivatives of *sn*-glycero-3-phosphoric acid that contains at least one *O*-acyl, or *O*-alkyl or *O*-alk-1′-enyl residue attached to the glycerol moiety and a polar head made of a nitrogenous base, a glycerol, or an inositol unit. This group of complex lipids is present in all cell membranes. Glycerophospholipids are derived from the simplest compound, PA, and may be classified into several sub-groups according to their molecular structure. For example, PC, PE and PS contains one nitrogenous base, PI inositol, and PG and DPG two glycerol molecules. Sphingosyl phosphatides are the group containing P and a long-chain fatty acid linked to the amino group of a long-chain base. Particularly representative of this group is SPM. Phosphonolipids are the analogues of PL, containing a direct C–P bond in their structure [40]. Phosphonolipids can be categorized into two major groups of glycerophosphonolipids and sphyngophosphonolipids.

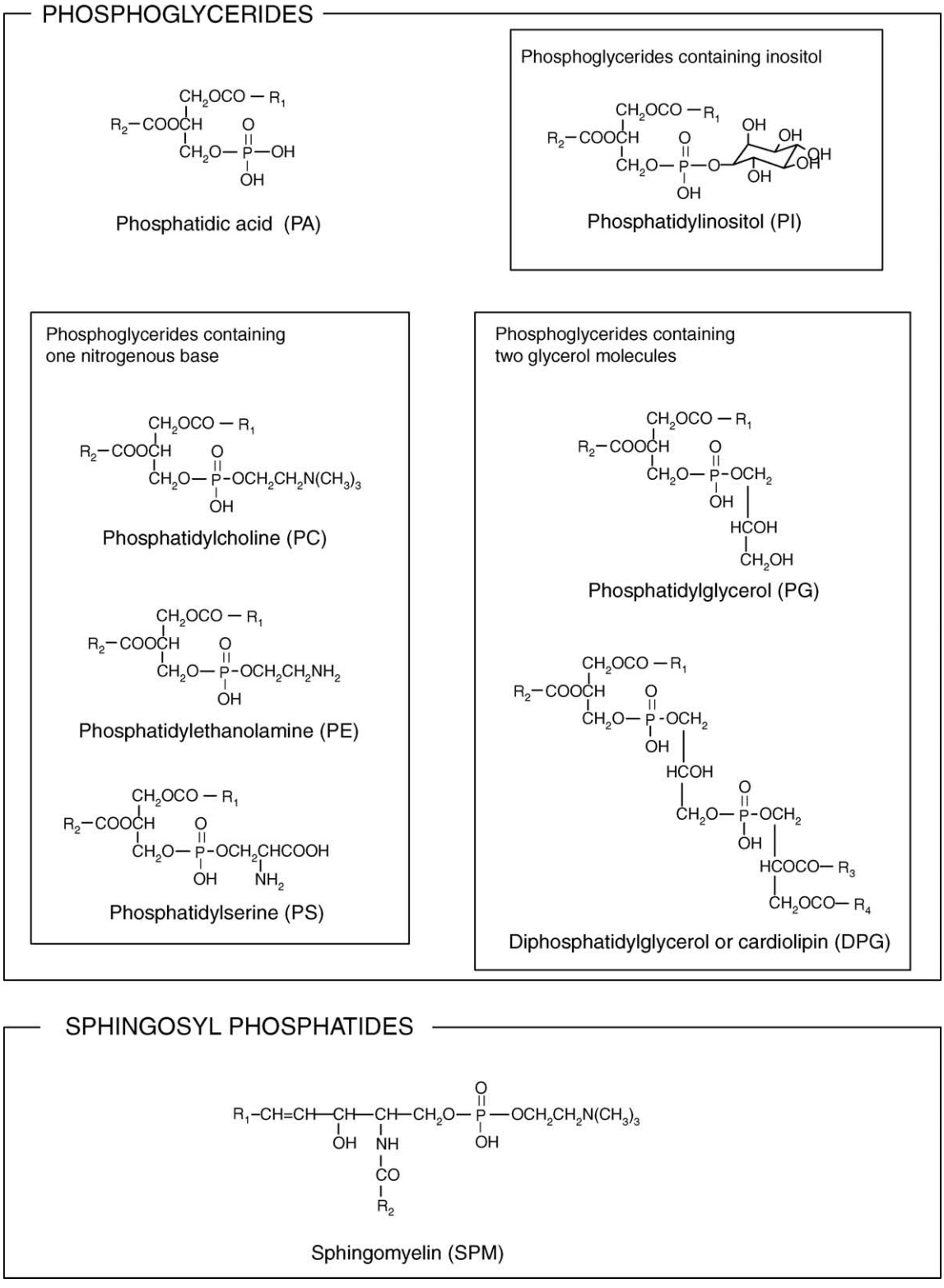

Fig. 1. Chemical structures of representative PL compounds with names and abbreviations.

As well as these PL (and phosphonolipids) compounds of biological origin, there are some hydrophobic compounds containing P which play significant roles as contaminants in marine environments. Organophosphorus pesticides and insecticides including malathion, fenitrothin, metamidophos and chlorpyrifos, have increased around the world replacing organochlorines in agricultural activities. As a consequence, they have been detected in ground and drinking water [41], natural surface waters [42], and marine organisms [43,44]. The bioaccumulation ability of some lipophilic organophosphorus compounds represents a potential risk for the marine organisms inhabiting vulnerable areas. However, no further discussion is given about these organophosphorus compounds in this study. Although they are significant disrupters of marine environments, they should be distinguished from the category of natural P components of biological origins which have an important role as nutrients. In this study, then, PL is defined as P-containing hydrophobic compounds of biological origin.

3. Extraction of phospholipids from marine samples

Schemes of marine PL analysis include extraction of hydrophobic components (lipids) from the samples, determination of P content, separation of lipid classes or compound classes, and further identification and determination of compounds (Fig. 2). First, the procedures and problems in PL extraction are discussed. The aim of extraction procedures is to recover PL from various marine samples including seawater, suspended particulate matter, sediments, and biological tissues. The additional aim is to separate PL from the other organic constituents (e.g., proteins, polysaccharides, amino acids, sugars) as well as P-containing non-lipid compounds including sugar phosphate, nucleotides, nucleic acids and phosphoproteins. For further isolation and identification of PL classes from the extracts, all PL must be protected against degradation through oxidation by solvent, oxygen, enzymes in combination with temperature and light during the extraction procedures and preservation.

Lipids are classified into two groups: the neutral and non-polar lipids (tri-, di- and mono-glycerides, and sterols), and the polar lipids (free fatty acids, sphingolipids and PL). Whereas the neutral and non-polar lipids dissolved well in apolar solvents, the polar lipids, in particular PL, only dissolve in relatively polar solvents. PL are similar to other lipids except that the first hydroxyl of the lipid triglycerides structure has a polar phosphate-containing group in place of the fatty acid (Fig. 1). This charge on the phosphate and amino groups in the structure of PL makes that portion of the molecule hydrophilic. The result is an amphiphilic molecule that contains hydrophobic hydrocarbon chains and hydrophilic groups in the molecular structure. This infers that a solvent mixture which is sufficiently polar to recover PL from sample matrix, but sufficiently non-polar to dissolved the neutral and non-polar lipids, is needed for efficient extraction of various lipids.

The greatest achievement in the extraction of lipids was made by Folch et al. [45], who developed a lipid extraction method from biological tissues using a solvent mixture of chloroform/methanol, followed by purification of the extracts with a KCl solution. The method of Bligh and Dyer [46] is a widely used simplification of Folch et al. (1957). For extraction of lipids from marine particulate samples including bio-

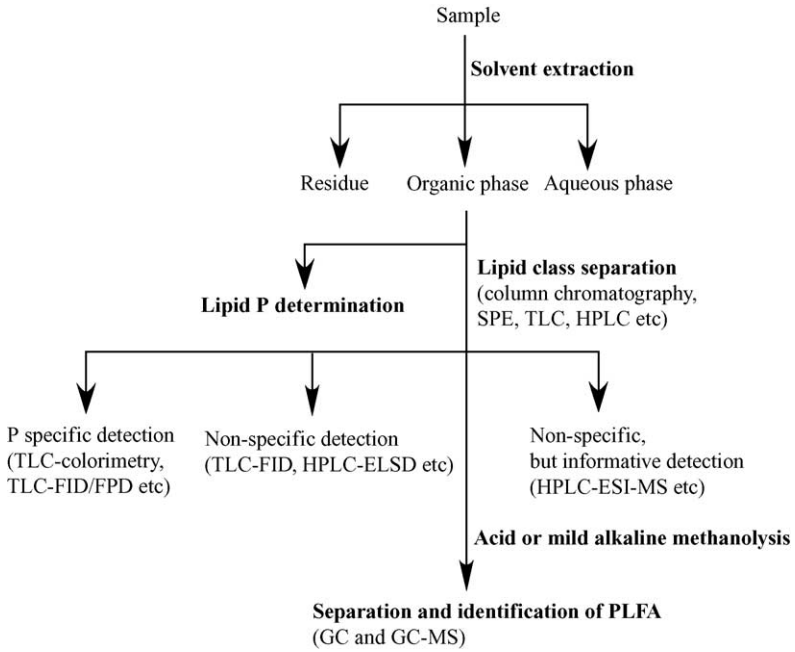

Fig. 2. Schemes for analytical procedures of marine PL.

Table 1
List of the solvent matrix utilized for extraction of PL and total lipids from a variety of marine samples

Solvent matrix	Sample	References
Chloroform	Seawater	[47–50]
Chloroform/methanol	Seawater	[51]
	Netted plankton	[52]
	Cultured ciliate	[53]
Chloroform/methanol/water	Invertebrate tissue	[54]
	Netted plankton	[55]
	Cultured bacteria	[56,57]
	Suspended particulate matter	[48,50,51,58]
	Settling particles	[55]
	Sediments	[59]
Dichloromethane	Seawater	[60–62]
	Cultured phytoplankton (diatom)	[61]
	Suspended particulate matter	[60,62]
Dichloromethane/methanol	Cultured phytoplankton (diatom)	[63]
	Sediments	[55,64]
Dichloromethane/methanol/water	Cultured bacteria	[65]
Diethyl ether	Mucilage aggregate in seawater	[66]

logical tissues, suspended particulate matter and sediments, most studies have used a variation of the protocol of Bligh and Dyer. There is, however, a great diversity of methodologies for extraction in terms of the combination and proportions of solvents (Table 1). The protocol of Bligh and Dyer is optimized for total lipid extraction, but not specialized for PL extraction. Evaluation and intercomparison studies of the Bligh and Dyer method have shown that the solvent composition alters the yields of extracted lipids, in particular PL [67–69]. The methanol content that did not vary in the Bligh and Dyer experiments affects the extraction efficiency of PL more than that of the other lipids.

For the extraction of dissolved PL from seawater samples, liquid–liquid extraction methods are generally used with chloroform or dichloromethane (Table 1). An extraction efficiency of less than 100% would be expected from seawater for dissolved PL because of their amphiphilic nature discussed above. It was reported that the extraction efficiency of PL from seawater was as low as 65% by dichloromethane extraction [60]. Suzumura and Ingall [51] examined the extraction efficiency in chloroform of PC, a commercially available phospholipid, added to seawater and found recoveries of 60%. They noted, therefore, that PL concentrations in seawater are underestimated due to the low extraction efficiency. Extraction at lower pH through the addition of acid to the sample seawaters might improve extraction efficiencies of PL by reducing the charge of the molecules [48,49,60]. However, there is no quantitative evaluation of the effect of reducing pH on extraction efficiencies of PL from seawater.

Suzumura and Ingall [50] determined the concentrations of hydrophobic P, but not PL, in seawater samples from the Pacific Ocean. Hydrophobic P was defined simply and operationally as the fraction of P that can be extracted with organic solvent (chloroform was used in this case). As described in Section 1, hydrophobicity is presumed to be a key factor in determining the behavior of P. However, there is no strict definition of “hydrophobic”. Generally, it is used to describe molecules or molecular groups that mix poorly with water. In terms of understanding the behaviors of P in seawater, operational definition using solvent extraction techniques is a simple and reasonable option for measurement of hydrophobic P concentrations. It can be assumed that various PL compounds exist in seawater that have variable extraction efficiencies with organic solvents due to the structural diversity of PL compounds (Fig. 1). Therefore, the measured values of hydrophobic P concentration may be a function of the composition of the hydrophobic P present and the variable extraction efficiencies associated with the compounds. Furthermore, extraction efficiencies of ambient hydrophobic P are likely to be altered with physicochemical forms of PL in seawater, including liposome-like macromolecules, micelles, and truly dissolved molecules. Thus, the measured hydrophobic P concentration does not equal PL concentration, even though PL are potentially significant compounds of hydrophobic P. As all extraction techniques in Table 1 are operationally defined methods, any deviation from the defined procedure can yield a different result. Further work should, therefore, focus on the comparability of the methods and the validity of normalizing PL data.

4. Separation and determination of extracted phospholipids

4.1. Determination of phosphorus in lipid fractions

The simplest way to determine PL is through direct measurement of total P in the extracted materials. This method does not need further separation of PL from other lipids which contain no P. Although the extraction techniques exhibited low efficiencies for PL, contamination of P by non-lipid compounds can be minimized [51]. A portion of the organic phase from solvent extraction can be digested to liberate orthophosphate from lipid P before measurement by

standard phosphomolybdenum blue methods [70,71]. Perchloric acid digestion is generally used for hydrolysis of lipid P extracted from marine samples [52,59]. A high temperature dry combustion method with addition of magnesium nitrate has been employed to hydrolyze phosphonolipids containing a C–P bond which is not as sensitive to hydrolysis as the usual C–O–P bond [50,51,72,73]. The detection limit of lipid P determination largely depends on the concentration factor from sample to the final solution. Very low values of 0.8 nM P and 0.04 nM P were reported as the detection limits for dissolved and particulate lipid P in seawater, respectively, due to a 20–500-fold concentration factor using 0.2–5 L of seawater samples [50]. However, the handling of a large volume of seawater is complicated in liquid–liquid extraction procedures. Tangential-flow ultrafiltration is a useful option for concentration and fractionation of the macromolecular colloidal fraction of lipids in seawater [49,74]. However, to date difficulties remain in concentrating the low molecular weight, truly dissolved fraction.

4.2. Chromatographic separation and quantification of phospholipids

4.2.1. Column chromatography and solid-phase extraction

Class separation of lipids leads to a broad diagnostic tool for biogeochemical and ecological studies of marine environments, including the evaluation of membrane components from living microbes, the determination of caloric capacity of organic matter evaluated from compounds linked to metabolic energy reserves, and determination of the extent of degradation of organic matter [75]. Chromatographic methods have been used widely for the isolation, separation, and quantification of PL along with other lipids including aliphatic hydrocarbons, polycyclic aromatic hydrocarbons, wax esters, sterol esters, glycerides, and free fatty acids. Silica gel column chromatography can divide lipids into neutral lipids, sugar lipids and PL using sequential elution with chloroform, acetone and methanol, respectively [76]. As argued above several different types of PL compound classes are present (Fig. 1), but this method can separate “total PL” from other lipids. Further fractionation of “total PL” into compound classes by silica gel column chromatography can then be achieved using mixtures of chloroform and methanol [77].

Traditional open column chromatography is time-consuming and requires relatively large quantities of solvents. Furthermore, open column chromatography usually involves a gradient elution to separate overlapping sample peaks. A rapid, efficient, high recovery method to separate PL involves solid-phase extraction (SPE) technology including pre-packed SPE cartridge and vacuum assisted elution. Silica gel (Si) and aminopropyl-bonded silica gel (NH_2) SPE cartridges have been used for the isolation of PL from other lipids and for the isolation of PL compound classes [78–80]. PC and PE can be separated from PI, PS and PA by NH_2-bonded SPE [81]. Silica gel SPE fractionated PL compounds into two groups of PE and PI, and PC, SM and lyso-phosphatidylcholine (lyso-PC) [82]. Suzuki et al. [83] evaluated five types of SPE cartridges for the isolation and quantification of PL, including NH_2, Si, cyanopropyl-bonded (CN), diol-bonded (2OH), and octadecyl-bonded (C_{18}). Among polar (CN, 2OH, Si), nonpolar (C_{18}) and ionic (NH_2) SPE, only NH_2-bonded SPE retained five PL compounds (PC, PE, PG, PS and DPG) efficiently from a hexane–chloroform–methanol matrix. Suzumura and Ingall [51] evaluated the separation and recoveries of seven analogues of natural PL by Si and NH_2-bonded SPE, including PC, PE, PI, PS, lyso-PC, PG and DPG. NH_2-bonded SPE exhibited low recoveries of PI, PS, PG and DPG, which is likely to be due to irreversible adsorption to the gel. In their study, two solvent systems for silica gel SPE were tested in which acetone and methanol/chloroform mixture were utilized as the washing solvents to remove non-PL compounds from the cartridge, respectively. Both solvent systems showed high recoveries of PL compounds (>98%). However, it has been demonstrated that acetone mobile polar lipids, non-PL, are significant components of marine lipids by silica gel thin-layer chromatography (TLC) [84]. Thus, a treatment that incorporates an acetone wash step can more effectively minimize the contamination of non-PL materials, that is acetone mobile polar lipids, for marine samples.

4.2.2. High performance liquid chromatography

With the advent of modern separation technology, high performance liquid chromatography (HPLC) has become a viable option for analysis of PL, with enhanced component resolution and analytical reproducibility. As reviewed by Abidi and Mounts [85], various combinations of HPLC columns and solvent systems, both normal- and reversed-phase HPLC, have been used for the separation of PL from other lipids as well as for the identification of individual PL compound classes. There are several detection techniques applicable for the detection of PL in HPLC analysis. Ultraviolet (UV) spectrophotometer has been a pragmatic choice of detector due to its relative convenience, simplicity, and low cost. Variations of UV adsorption with fatty acid composition, however, can incapacitate the direct estimation of PL by UV detector responses. Differential refractometric detection method is also widely used in HPLC. This method is not feasible with the use of gradient elution because of considerable fluctuation of refractive indices with changes in gradient mobile-phase conditions and other HPLC parameters.

Other detection methods of PL in HPLC involve direct measurement of total P and evaporative light-scatter detector (ELSD). Direct measurement of P has been accomplished using an automated total P analyzer coupled with HPLC system [86]. This method complements the UV detection for the direct quantification of molecular species, even in the case where no synthetic standard is available for calibration. Furthermore, direct measurement of P concentrations is essential in order to evaluate the abundance of lipid P to total organic P in the samples. ELSD has been introduced as a new

universal, versatile detection system for the analysis of various non-polar neutral lipids and polar lipids [87]. Norbäck et al. [88] tested ELSD coupled with polyvinyl alcohol-bonded stationary phase HPLC for lipid class separation as well as for the separation and quantification of individual PL compounds from the samples of marine particulate materials and cyanobacteria. It was found to be acceptable provided that the major lipid classes can be identified and quantified at a sensitivity high enough for natural samples containing low PL contents. Mass spectrometric detection methodology can provide valuable structural information about unknown PL compounds as well as the universal and sensitive detection capability. HPLC–ELSD and HPLC–electrospray ionization-mass spectrometry (HPLC–ESI-MS) have been adopted for structural characterization and quantification of intact PL in from bacterial membrane from marine sediments [64,65,89–91]. As described below, most studies of marine lipids have been based on different TLC techniques. Recently the use of HPLC instead of TLC has offered the possibility of full automation, higher separation capacity, and structural information of analytes for marine samples.

4.2.3. Thin-layer chromatography

TLC has been used widely for the separation and determination of PL in various biochemical studies [92–94]. PL developed on TLC plates can be visualized using non-specific reagents such as copper acetate–phosphoric acid [95], copper sulfate–phosphoric acid [96], and sulfuric acid–dichromate [97]. Furthermore, a number of P-specific detection methods have also been proposed [98–103]. Reliable computer-controlled densitometers are available from several manufactures. Densitometric determination coupled with P-specific visualization techniques is particularly useful in the identification of PL. Complex lipid components can be separated with two successive migrations in two orthogonal directions (two-dimensional TLC). The most efficient separation can be achieved using contrasting solvent systems, i.e., a neutral or basic solvent in the first direction followed by an acidic solvent in the second direction. Some individual compounds of PL have been successfully isolated by two-dimensional TLC [57,104], for example PE, PG and DPG were isolated from the marine bacterium *Pseudomonas nautical*. High-performance TLC with one-dimensional double-development technique also enables densitometric determination of all lipids in marine samples [105].

Even though these planar TLC techniques are reliable for PL separation and determination, the procedures are quite complicated. The Chromarod-Iatroscan system has been used in most marine lipid studies, and in this system lipid classes are separated on reusable quartz rods impregnated with silica and subsequently detected with a flame ionization detector (FID). The Chromarod-Iatroscan is based on two well-established analytical tools: TLC plus FID as used in gas chromatography (GC). Lipid extracts are spotted on the rods, developed by certain solvent system as planar TLC, dried, and set in a frame, and finally passed through the FID for detection. In various fields of environmental studies, the TLC–FID system allows for rapid fractionation of total lipids into classes and their quantification [106]. The applicability of the system has been examined for marine lipids with some modifications to improve the resolution and quantification of certain lipid classes [107–109]. The system is relatively straightforward and is workable under shipboard conditions [60].

The Chromarod-Iatroscan system has been applied to characterization of lipid classes in pure cultures of algae [63,84,110–112], bacteria [57,84,113–115], natural zooplankton [115], and marine mucilage [66]. This approach has also been used to examine the temporal and spatial distributions of lipids with respect to marine biological and geochemical processes [47–49,55,61,62,84,116–120]. Chromarod-Iatroscan is satisfactory for neutral lipids but the separation into compound classes of PL is relatively poor. Thus, most studies have reported "total PL". Gérin and Goutx [57], however, modified the TLC–FID method with a one-dimensional double-development technique to enable clear separation of DPG, PG, PE, and PC either in standard mixtures or a cultured marine bacterium.

In TLC–FID, measurement of PL concentrations is based on the FID response to the carbon atoms in the PL fraction isolated by TLC, and hence, direct measurement of P is not obtained. This is the critical disadvantage in the utilization of the TLC–FID system in PL studies as a marine organic P component. Flame photometric detection (FPD) is well known as a selective detection method for sulfur and P in GC analysis. Very recently, a new Chromarod-Iatroscan system of TLC–FID/FPD was developed. Fig. 3 shows the chromatograms of FID and FPD responses of human serum lipids, that demonstrates the utility of TLC–FID/FPD for the selective detection of P in PL compounds [121]. To date the TLC–FID/FPD system has not yet been applied to marine samples. This system has a great advantage in future studies of marine PL as lipids and an organic P component.

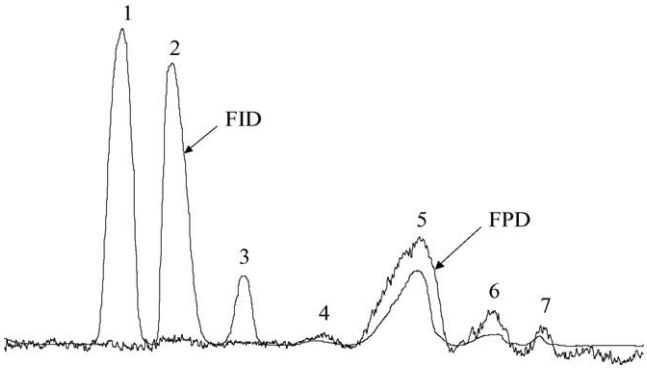

Fig. 3. Chromatogram of human serum lipids with Iatroscan TLC–FID/FPD system in the phosphorus mode. 1: cholesteryl ester; 2: triacylglycerol; 3: cholesterol; 4: PE; 5: PC; 6: SPM; 7: lyso-PC. Reprinted from Ogasawara et al. [121] with permission from Elsevier.

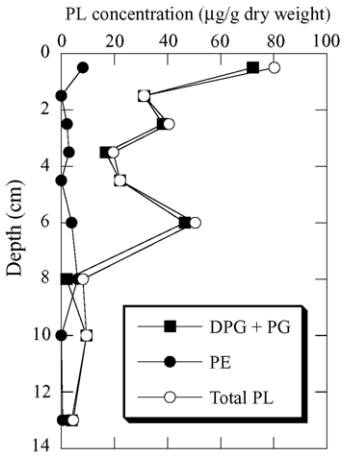

Fig. 4. Vertical profile of PL concentration in a sediment core taken from the subantarctic Indian Ocean. Redrawn from the data of Laureillard et al. [119].

4.3. Determination of phospholipids and phospholipid ester-linked fatty acids as biomarkers of aquatic microbial biomass and community structure

It has been shown that benthic bacterial biomass can account for a significant fraction of sedimentary organic matter [122–124]. The accurate measurement of biomass is a critical factor for understanding material transformation and fluxes, and the trophic interactions of bacteria in marine sediments. It has been proposed that the measurement of lipid P concentrations can give a reasonable estimate of the sedimentary microbial biomass [59,125]. The use of PL as a biomarker of bacterial biomass is based on the assumption that PL contained in cell membranes can rapidly decay after cell lysis [126]; consequently, the PL concentration reflects the abundance of living organisms. Fig. 4 shows the vertical profile of PL concentrations in a deep sea sediment core. The PL concentration rapidly decreases with depth indicating their active degradation in sediments. Dobbs and Findlay [125] compiled representative values for converting lipid P concentrations of carbon biomass for a variety of microbial organisms including bacteria, cyanobacteria, yeasts, diatoms and chlorophyta. Using the conversion of lipid P to bacterial biomass, the sedimentary bacterial abundance has been estimated to be $(0.6–2.7) \times 10^9$ cells per gram sediment, which were 2.7–31 times higher than those estimated by direct count using an epifluorescence microscopic (EFM) technique [127]. Given the inherent difficulties in using sediments for EFM techniques due to large grains and inactive bacteria, it is perhaps preferable to use techniques such as lipid P analysis [116,127].

Further developments in determining microbial community structures as well as biomass in marine environments have utilized the determination of fatty acids associated with PL (PL ester-linked fatty acids, PLFA). Certain PLFA are specific to certain microorganisms, and different microorganisms may have different PLFA compositions. Typical major fatty acids in diatoms, for example, are 14:0, 16:0, 16:1ω7, 20:5ω3 [128–130]. The non-methylene interrupted fatty acid (20:3ω3) was proposed as the biomarker of a marine flagellate [131]. Branched chain fatty acids (*iso* and *anteiso* C_{15}, *iso* and *anteiso* C_{17}), odd chain monoenoic acids (15:1, 17:1), cyclopropane fatty acids (C_{17} and C_{19}), and vaccenic acid (18:1ω7) are used as indicators of bacterial input to marine sediments [132–136]. Long chain saturated fatty acids (>C_{22}, up to C_{32}) also are derived from sedimentary bacteria [119,137]. Fatty acids are designated based on the number of carbons in the molecule, the number of double bonds, and the position of the first double bond from the "omega" or aliphatic end of the molecule.

It has been the practice to determine microbial community structure through the use of PLFA profiles [138,139]. The scheme for PLFA analysis in marine samples is included in Fig. 2. Briefly, the PL fraction extracted and isolated by the above mentioned methods is subjected to acid or mild alkaline methanolysis. The resulting fatty acid methyl esters are analyzed by GC and GC–mass spectrometry (GC–MS). Quantitative recovery (91–111%) in the methanolysis procedures, and high sensitivity and reproducibility in GC analysis of saturated, monoenoic, and branched fatty acids have been demonstrated [140]. PLFA analysis has been used to examine the depositional conditions and eutrophication of coastal marine sediments, based on the estimates of prokaryotic and microeukaryotic biomarkers [141–144]. PLFA analysis has also proven the presence of a large biomass, bioactivity and great biodiversity of deep-sea bacteria [145–149]. Recently, HPLC–ESI-MS techniques mentioned above, have been used as a tool to investigate the microbial biomass and community structure of deep sea environments [64,65,89,91]. By measuring the quantitative distribution of PL compound classes as well as PLFA composition, this advanced technique will be a dominant tool for biogeochemical studies of PL and PLFA in marine environments.

5. Presence and dynamics of phospholipids in marine ecosystems

5.1. Presence and abundance of phospholipids

In the first report on the distribution and dynamics of lipids in seawater, Jeffrey [150] separated and identified eight lipid classes, including PL, from seawater extracts using a combination of column chromatography, TLC, infrared analysis, GC and various chemical tests. Since that, PL have been determined in various marine samples and identified to be the major components of lipids. In Table 2 , values of PL concentrations reported in various marine samples are compiled. In seawater, a significant proportion of PL is present in a dissolved fraction as well as in the particulate fraction. Furthermore, in molecular size fractionation of PL in seawater, a higher concentration was found in low molecular weight "truly dissolved" fraction than in high molecular weight "colloidal" fraction [49] (Table 2). In aqueous matrices, high

Table 2
Selected marine PL concentrations, PL as percentage of total lipids, and list of identified PL compound classes reported from a variety of marine samples

Sample	Sample description	Concentration	% of total lipids	Identified compounds	References
Seawater (coastal)	Dissolved (<0.7 μm)	23.4–35.5 μg/L	34.3–52.3	PC, PE, DPG + PG	[48]
	Particulate (>0.7 μm)	3.7–40.3 μg/L	11.5–35.4	PC, PE, DPG + PG	
Seawater (coastal)	Colloidal (10 kDa ≪ 0.45 μm)	7.3–58.6 μg/L	3.3–19.0	N.A.	[49]
	Dissolved (<10 kDa)	66.0–322 μg/L	3.1–10.1	N.A.	
Seawater (coastal)	Dissolved (<1.0 μm)	39 ± 36 μg/L	17 ± 8	N.A.	[61]
	Particulate (>1.0 μm)	6.9 ± 3.1 μg/L	8 ± 3	N.A.	
Sediment (deep sea)	Top 15 cm	N.A.	36.9–79.7	PE, DPG + PG	[119]
Sediment (coastal)	Top 26 cm	N.A.	29.2–79.8	PE, DPG + PG	[120]
Phytoplankton	Ten species	0.08–48.2 pg/cell	3.6–52	DPG + PG	[84]
	Nitzschia pungens	N.A.	6.7–14	N.A.	[63]
Macrophyte	Fifteen green algae species	0.07–1.08 mg/g	3.8–27.8	PC, PE, PI, PS, PA	[151]
	Nineteen brown algae species	0.16–0.83 mg/g	2.9–19.7	PC, PE, PG, PI	[152]
Bacteria	Four species	N.A.	51–96	PE, DPG, PG	[84]
	Pseudomonas nautical	N.A.	88.1	PE, DPG, PG	[57]

N.A.: data are not available.

affinity to particles is the essential nature of hydrophobic materials, namely lipids. The high abundance of PL in the "truly dissolved" fraction of seawater emphasized the amphiphilic nature of PL. Dissolved and particulate PL are composed of PC, PE and DPG + PG (DPG and PG could not be separated) [48].

The proportion of PL in phytoplankton varies widely from 3.6 to 52% of the total lipids. It has been demonstrated that nutrient conditions affect the composition of cellular lipid composition in phytoplankton; diatoms grown under nutrient-replete conditions exhibited high proportions of PL [63]. PL make up a particularly significant proportion of the total lipids in bacteria (∼96%) [57,84] and sediments (∼80%) [119,120]. As described above, sedimentary PL can be mostly ascribed to living bacterial cells. This is supported by the consist composition of the major PL compounds, that is PE, DPG and PG, in bacteria and sediments (Table 2). PL in brown and green macrophytes contain PC, PE, PI, PS and PA [151,152].

A marine ciliated protozoan, *Parauronema acutum*, contains a broad range of compound classes of PL, including PE, PC, PI, PS, SPM, PA and lyso-PC in order of abundance [53]. Some of the lipids in the protozoan were identified to be phosphonolipids. Phosphonolipids have also been isolated from marine and estuarine invertebrates [54,153]. The chemical and biological stability of the C–P bond [40] infers that phosphonolipids are potentially an important class of organic P that is resistant to degradation and will accumulate in marine environments. Compounds containing a C–P bond have been shown to be widespread in seawater [154–156], sinking particles [157] and marine sediments [158]. Using tangential-flow ultrafiltration and ^{31}P nuclear magnetic resonance, C–P compounds were identified to be the dominant compound class of dissolved organic P in the colloidal fraction (1 kDa ≪ 0.1 μm) in the Pacific Ocean [154]. Even though certain C–P compounds are present in seawater, there is no direct evidence of the presence of dissolved phosphonolipids. Further research needs to identify the compound classes of C–P compounds as well as phosphonolipids in marine environments.

5.2. Dynamics and role of phospholipids in marine P cycling

Concentrations of lipid P in marine sediments have been summarized previously [125,159], and vary with a wide range from 0.1 to 464 nmol P/g (dry weight) among various depositional conditions. However, most of these studies provided data only for lipid P concentration, but neither total P nor total organic P concentrations in these samples. It is essential to examine the quantitative significance of lipid P as a function of total P (or total organic P) to obtain a better understanding of the importance of lipid P in marine P cycling. Although a few studies have reported lipid P concentrations as well as data on the proportion of lipid P in total organic P in seawater and plankton samples (Table 3), the information on the distribution and abundance of lipid P in marine environments is

Table 3
Selected lipid P concentrations and lipid P as percentage of total organic P in a variety of marine samples

Sample	Sample description	Concentration	% of total organic P	References
Seawater (coastal)	Dissolved (<0.7 μm)	0.7–6.0 nM P	0.1–0.9	[51]
	Particulate (>0.7 μm)	31–294 nM P	3.0–13.5	[51]
Seawater (coastal)	Particulate (>0.7 μm)	90–750 nM P	5.6–11.6	[160]
Seawater (pelagic)	Dissolved (<0.7 μm)	4.0–17.9 nM P	1.7–17.6	[50]
	Particulate (>0.7 μm)	0.05–1.72 nM P	0.8–34.4	[50]
Netted plankton (coastal)	Composite of zoo- and phytoplankton	27.9–67.6 μmol P/g	11.3–22.2	[52]
Zooplankton	*Calanus* sp.	N.A.	16.3–18.1	[161]

N.A.: data are not available.

still limited. The most comprehensive studies of the distribution and dynamics of PL in marine ecosystems to date were those conducted by C.C. Parrish and his colleagues in Canadian coastal areas, using a TLC–FID system [55,61,62,110]. In addition to these "lipid studies", Suzumura and Ingall have carried out "P studies": the distribution, dynamics and roles of PL (as hydrophobic P) in marine P cycling [50,51]. Based on the results of these studies, important roles of PL in marine ecosystems are suggested.

First, particulate PL is an important component of P in plankton-rich suspended particles. During a time-course observation in Bedford Basin, Canada, the particulate PL concentration was relatively low before and during the initial stages of the spring bloom, increasing at the height of bloom [61]. It was reported that much higher concentrations of particulate lipid P (107–294 nM) were observed in phytoplankton-rich Tokyo Bay, Japan, than in inorganic particle-rich Corpus Christi Bay, Texas, U.S.A. (31.4 nM) [51]. In the open ocean environments, particulate lipid P concentrations were high in the surface euphotic zone, rapidly decreasing with depth where chlorophyll *a* concentrations also decreased.

Second, a labile fraction of PL is rapidly remineralized and acts as an important bioavailable P source in oligotrophic marine environments. It was reported that in the open ocean environments DIP-depleted oligotrophic locations exhibited lower dissolved lipid P concentrations than those in the DIP-rich locations [50]. In the study of Parrish (1987) mentioned above, the dissolved PL concentration was high prior to a phytoplankton bloom, and decreased substantially during bloom. As argued, PL are the relatively significant components of organic P in plankton (11.3–22.2%, Table 3). The depletion of PL observed in the open ocean surface seawaters indicates that PL synthesized by plankton can be readily mineralized and utilized as a nutrient P sources in oligotrophic environments. The ephemeral life of particulate PL has been demonstrated also by a laboratory experiment using a coastal seawater sample containing freshly produced organic matter [50]. During the 166-day experiment, a large portion of particulate PL was removed within the initial 6 days. These results suggest that particulate PL produced through primary production is relatively enriched in labile components which can be rapidly decomposed. The labile fraction of PL thus may be an important pool of bioavailable P in oligotrophic environments.

Third, less reactive fractions of PL act as a P sink from marine ecosystems. It was demonstrated by field investigation and laboratory experiments that less reactive fractions of PL that withstand biological degradation in surface waters can be exported to and accumulated in deeper waters [50]. Fig. 5 shows the vertical profiles of dissolved and particulate lipid P as percentage of organic P (DOP and POP, respectively) in an oligotrophic open ocean location. The abundance of lipid P in organic P significantly increases in deep layers indicating the accumulation of a less reactive fraction of lipid P. Gravitational settling of PL-rich particles can export significant quantities of P as well as fresh organic materials from the surface euphotic zone to the deeper layer and bottom sediments. Relatively few studies have measured the flux of settling PL in marine environments [55].

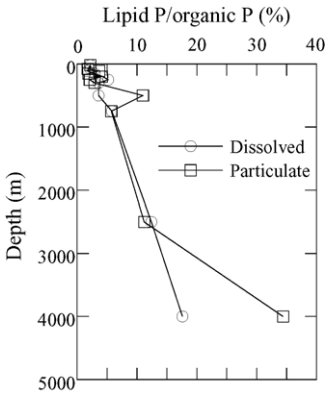

Fig. 5. Vertical profiles of dissolved and particulate lipid P as percentage of DOP and POP, respectively, in the oligotrophic North Pacific subtropical gyre. Redrawn from Suzumura and Ingall [50].

As an additional significant role of PL in marine ecosystems, it has been proposed that dissolved PL may enhance the preservation of other dissolved organic matter. Nagata and Kirchman [162] suggested that heterotrophic flagellates grazing on bacteria release PL-rich liposome-like macromolecules. They hypothesized that the liposome-like macromolecules are composed of undigested bacterial membrane components, which can render labile organic matter unavailable as a substrate for hydrolytic enzymes. It has been shown that encapsulation of labile proteins within liposomes reduced the degradation rate of the protein versus dissolved protein unassociated with liposomes [163,164]. In addition, it was found that ~70% DOP in the high molecular weight fraction (10 kDa $\ll$ 0.1 μm) of coastal seawater samples was resistant to remineralization by phosphohydrolytic enzymes [165]. Further characterization revealed that ~50% of the DOP fraction resistant to enzymatic remineralization was solvent extractable and that the aqueous fraction was then hydrolyzable with the enzymes. The result of enhanced hydrolyzability after extraction suggests that PL-rich liposomes can protect labile organic P compounds from enzymatic activities.

6. Summary and prospectus

It is clear that elucidating the cycling of nutrients and organic matter in marine systems is extremely important for gaining a better understanding of the current controls on eutrophication and organic pollution on a regional scale, and on primary productivity and carbon export on a global scale. As one of the major constituents of both nutrient P and organic matter, PL must be a key component in the controls of various processes in marine ecosystems. However, marine PL studies seem to be biased towards "lipid study"; there are rel-

atively few reports of PL as organic P components. This may be ascribed to the usefulness of the TLC–FID system, non P-specific detection. Although the P concentrations of the lipid fraction have been often reported in marine sediments, they were measured as a biomarker of microbial biomass rather than a component of organic P. Considering the potential importance of PL as a bioavailable P source and a P sink, more attention should be paid to the cycling of P through the dynamics and metabolisms of PL in marine environments.

Fig. 2 shows a schematic diagram of the PL analyses in marine samples. As argued, one of the potentially significant problems in this scheme is in non-quantitative recovery of PL during extraction procedures. Further developments of the extraction methods and the evaluation of the methods including intercomparison studies need to be carried out to aid better understanding and interpretation of the data obtained. However, based on the simple, operational definition of lipid P as P extractable with certain organic solvents, the problem of extraction efficiency may be reduced. Even though such simple measurement of lipid P concentration provides no further information on composition of PL compound classes, the abundance and contribution of hydrophobic P components can be examined, which exhibit distinguishable behavior and distribution from that of hydrophilic P components.

The recent developments in analytical techniques for the separation and detection of PL enable simultaneous determinations of lipid P and PL components using P specific detection including densitometric determination in planar-TLC with colorimetric P determination, Chromarod-Iatroscan TLC–FID/FPD system, and non-specific detection that provides molecular level information of analytes including HPLC–ESI-MS. In particular, TLC–FID/FPD system is an evidently useful, powerful tool that can unite with “lipid” and “organic P” studies, since the system is the most familiar and developed technique in marine PL studies. In addition to the use of these techniques to determine the abundance and forms of PL, it is essential that the various processes and pathways of transportation and transformation of PL in marine ecosystems are investigated. To gain a better understanding of the role and dynamics of marine PL, factors such as the presence and distribution of phospholipase enzymes, the role of PL compounds as P and C sources for primary and secondary production, and the flux of PL associated with settling marine particles from surface to deep oceans, all warrant investigation.

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