

Phytoplankton chlorophyll-breakdown pathway: Implication in ecosystem assessment

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Abstract

The chlorophyll-breakdown to pheophorbide is determined by biotic factors such as grazing (via chlorophyllide) or senescence (via pheophytin). So far, much of the information on chlorophyll-breakdown is available from sediments, but information from the water column is limited. This study addressed chlorophyll-breakdown-pathways (Chl-BP) on a seasonal basis from eight major ports (18-30 stations/port) representing freshwater, estuarine, and marine ecosystems. The distribution of chlorophyll and its breakdown fractions (pheophytin, pheophorbide) exhibited distinct spatial and seasonal variations. Fresh-water (except Haldia-port) and estuarine ports are characterized by high-biomass, high-pheophytin, and low-pheophorbide, whereas marine-ports by low-biomass (except Mangalore-port), low-pheophytin, and high-pheophorbide. Pheophytin and pheophorbide distribution were biomass independent and dependent, respectively. The pheophorbide: pheophytin ratio indicated a potential proxy for determining the dominant breakdown pathway, i.e., herbivory dominant (>1) or not dominant (<1). However, CHI-BP is taxa-specific and grazer's feeding habits. The ratios exhibited apparent differences between different ecosystems, i.e., the higher ratios in marine (up to 11.2) followed by estuarine (up to 0.9) and freshwater (up to 0.4; except Haldia) systems. The diatoms (preferred grazer diet) contribution to total phytoplankton was more in marine followed by estuarine and freshwater systems. The low and high ratios suggested the prevalence of chlorophyll-breakdown via senescence and grazing mode, respectively. We proposed that such scaling will have implications in the ballast water management – BWM (ballast tank conditions (eg. dark) during voyages, post-voyage discharge – including treated water using approved BWM systems, and the nature of ports, potential discharge point) and algal bloom research (e.g. understanding fate and in control measures).

Keywords: Phytoplankton-chlorophyll-breakdown-pathway; pheophytin; pheophorbide; freshwater; estuary; marine

Introduction

Phytoplankton, which are unicellular/filamentous and autotrophic micro-organisms, comprised of diverse algal groups (diatoms, dinoflagellates, phytoflagellates, coccolithophorids, red algae, green algae, and cyanobacteria) with sizes ranging from 0.2 microns to several millimeters. Phytoplankton are significant contributor to global carbon fluxes (Falkowski et al., 1998) and also influential determinants of tropical interaction in the marine ecosystem (Tester et al., 1995). However, in an aquatic ecosystem, phytoplankton undergoes several loss processes such as grazing, program cell death, viral lysis, sinking, etc.(e.g., Choi et al., 2017). In such cases, phytoplankton marker pigments (PMP), in particular, chlorophyll *a* (chl *a*) and its derivatives, which reflect the phytoplankton production and its pathway in the water column and sediments are considered as useful indicators. Chl *a* and pheopigments are commonly used as indicators of relatively fresh and degraded microalgal communities, respectively (Boon et al.,1998; Wieking and Kröncke, 2005; Pusceddu et al., 2009). Processes such as growth, grazing, cell sinking and senescence, photodegradation, fecal pellet sinking, physical mixing, and advective transport are known to affect chl *a* and pheopigments concentrations in the euphotic zone. However, different pathways of pigment degradation affect the integrity of chlorophyll, i.e., Mg-phorbins ring, the phytol chain, or both, and result in different degradation products such as pheophorbide (Mg-free and phytol-free chlorophyll), chlorophyllide (phytol-free chlorophyll), and pheophytin (Mg-free chlorophyll) (Fig. 1; Morata, 2011). The removal of the central magnesium atom and phytol chain from chl *a* are the early steps in chl *a* breakdown for all chlorophyll-containing organisms (Sato and Hama 2013; Hu et al., 2013; Kuai et al., 2018 and references therein). To the best of our knowledge, more in-depth information on chl *a* breakdown is available for leaf senescence in land plants in comparison to the situation for algae (Scheer 2012). Additionally, laboratory experiments on relationship between grazing pressure and pheopigment production (Klein et al., 1986; Penry and Frost, 1991; Gieskes et al., 1991; Head and Harris 1996; Penry et al., 1991; Pandolfini et al., 2000; Goericke et al., 2000) have shown that the presence of colourless products such as 132, 173–cyclopheophorbide *a* enol (CPP516), while pheopigments productions with grazers were recorded little to none. However, research in this area is in infancy. Nevertheless, the rates of these processes are of broad general interest, and knowledge of them is fundamental to understanding the causative factors underlying the dynamics of planktonic ecosystems.

Several studies have shown that pheophorbide and pheophytin as the primary chl *a* degradation products (CDP) from grazing in the water column (Carpenter et al., 1986; Spooner et al., 1994; Strom, 1993), sediment (Abele-Oeschger and Theede, 1991; Bianchi et al., 1988; Buffan-

Dubau et al., 1996; Cartaxana et al., 2003; Coelho et al., 2011), intertidal region (Brotas and Plante-Cuny 1998; Lucas and Holligan 1999; Cartaxana et al., 2003) and Ice algae (Moratta et al., 2011). At the same time, few reports also raised doubts over the use of pheopigments as a relevant indicator of herbivorous activities (Head et al., 1994; Villanueva and Hastings 2000; Ford and Honeywill 2002). Ford and Honeywill (2002) suggested that any use of pheophorbide as a marker pigment for grazing in surface intertidal sediments in the future should be tested (especially for biological reworking and deep oxygen diffusion) before use. To our knowledge, much of the information on CDP has come from sediments, but from the water column, information is limited.

Nevertheless, the presence of CDP in the water indicates the presence of zooplankton activity or cellular senescence (Bidigare et al., 1986; Coupel et al., 2015). Since there exists distinct spatial and temporal variability in biological production within or between ecosystems, it is possible that the chlorophyll breakdown pathway will also exhibit distinct variability. For example, chlorophyll breakdown into pheophorbide can be either via chlorophyllide or pheophytin. Laboratory experiments confirmed that under grazing pressure, chlorophyll breakdown pathway (i.e., via chlorophyllide or pheophytin) is taxa-specific and grazers feeding habitat (unpublished data). India has a vast coastline with different ecosystem types (e.g., freshwater, estuaries, coastal) and so with the environmental settings in each of the ecosystems. The influence of reversible monsoons (SWM – both east and west coast and NEM – east coast), high freshwater discharges on the east-coast compared to the west-coast, different coastal morphology, and variable anthropogenic pressures are some of the events determining the environment setting in a given ecosystem along the Indian coasts. Therefore, it has been hypothesized that the phytoplankton will have a specific distribution pattern for different ecosystems, and therefore indicating fate pathways. Evaluating biomass, composition, and CDP together will provide insights into the processes involved in growth and mortality. Given this, under the Ballast Water Management Program-India, the distribution patterns of the biomass and CDP from eight-port ecosystems (categorized into freshwater (FPE), estuarine (EPE) and marine (MPE) port ecosystems) located along east and west coast of India are discussed from the perspective of loss processes. Further, the field data generated will have implications for ecosystem assessment and management.

The discharge of water from ships' ballast water tanks is widely considered as the most critical vector for “bioinvasion” i.e. unintentional translocation of nonindigenous organisms from diverse taxonomic groups (such as microbes, flora, and fauna) across their bioregions. In ballast water management (BWM), we assume that the pheophorbide: pheophytin ratio will be useful for assessment of the (i) nature of the environment in the ballast water discharge point (e.g. ports), and

(ii) the ballast water sampling. Generally, ports are at the receiving end for ships ballast water, and therefore the port environment determines the fate of the introduced organisms. For instance, the introduced organisms survive/proliferate and do not under a conducive and non-conducive environment respectively. Irrespective of the climatic zones, the geographic location (riverine, estuarine, and marine) and the seasons (wet and dry seasons) determined the port environment and was evident in this study. Therefore, the biological production (e.g. plankton biomass and production) or occurrences of blooms vary between the ports and the seasons and so with its fate. Given, this the chlorophyll and its breakdown products (e.g. pheophytin, pheophorbide) and ratios can be useful ecological indicators for determining the fate of phytoplankton, including introduced viable phytoplankton and blooms,) during productive and non-productive conditions (Moratta et al., 2011; Grippo et al., 2009, Allison et al., 2013; Satish et al. submitted).

2. Materials and methods:

2.1 Study area and sample collection

Water samples (surface and sub-surface) were collected from 8 ports located at different locations along the east and west coast of India. The selected ports are listed among the major ports of India as they handled greater proportion of all cargo traffic. The Indian coast is influenced by monsoon reversals, a varying magnitude of freshwater influx (from major and minor rivers), and anthropogenic pressures (in particular ports). Therefore, the ports along the Indian coast represent different ecosystems. Among the eight ports, two (Kolkata and Haldia), one (Cochin), and five ports (Kandla, Mangalore, Paradip, Chennai, and Tuticorin) are identified as freshwater, estuarine and marine port ecosystems respectively. In each port, several samples were collected from 12 to 25 stations on different occasions representing different seasons (Fig. 2, Table 1). Surface and sub-surface (1m above the bottom) water samples were collected using Niskin bottles (5 L) along with the other environmental parameters such as temperature, salinity, dissolved oxygen, biological oxygen demand, phytoplankton pigments and inorganic nutrients (nitrate, nitrite, ammonia, phosphate, and silicate). The temperature was determined *in situ* using a multiparameter Sonde DS5X (Hydrolab). Salinity was measured with an Autosol (Guildline Autosol 8400B). Dissolved inorganic nutrients such as nitrate (NO₃), phosphate (PO₄), nitrite (NO₂), ammonium (NH₄), and silicate (SiO₄) were analyzed by SKALAR SANplus ANALYSER. Dissolved oxygen (DO) and biological oxygen demand (BOD) were analyzed following standard methods (Parsons et al., 1984). For the pigment analysis, 250 to 500 ml of water sample were passed through GF/F filter (27mm; pore size 0.7 mm) and stored in at -80° C until analysis using high-performance liquid chromatography (HPLC).

2.2. *Phytoplankton pigment analysis using HPLC*

The collected pigment samples were extracted by adding 3 ml of 90% acetone (V: V with the deionized water) to the frozen filter paper, which was sonicated using an ultrasonic probe (Labsonic U, B. Braun Biotech International, Leverkusen, Germany) for 10 sec at 20 kHz and kept on ice to prevent excessive. The extracts were then stored overnight at -20 °C for analysis using HPLC (Agilent Technology; Series 1200). Before analysis, all the extracts were filtered (Nylon filter membranes, 0.2 µm pore size) immediately before injection in the HPLC to remove cells and debris from sonicated filter paper. To prevent photolysis of the samples, extraction and injection procedures were performed under dim light. Pigment analysis was performed following the method of Van Heukelem (2002) with a slight modification. The pigment extracts were injected into the HPLC system equipped with an Agilent 1200 series pump; an Agilent diode array detector connected using a reverse-phase C8 Column maintained with 60°C (150 X 4.6 mm, 3.5 mm particle size) with 3 phase linear solvent gradient program at a flow rate of 1.1 ml/min. The excitation and elution of the extracted pigments were detected at 450 and 665 nm. The quantitative and qualitative analysis of about 20 pigments, including the chlorophyll degradation products was carried out using the commercially available pigment standards purchased from DHI Inc (Denmark) and Sigma–Aldrich (USA). However, for this study, only the total chlorophylls and two degradation pigments (pheophytin and pheophorbide) were considered. Chlorophyllide, another degradation pigment, was not detected in any of the samples analyzed. Additionally, following pigment ratios Chl *a*: Pheophytin, Chl *a*: Pheophorbide, pheophorbide: pheophytin for better interpretation. Further, the data on chlorophyll, pheopigments, and pheophorbide: pheophytin ratios were subjected to clustering (using Bray Curtis similarity and average group method) and non-metric multidimensional scaling (NMDS) to understand the ecosystem wise distribution. Before analysis, data were not transformed. Clustering and NMDS were performed using Primer version 6 software.

3. Results

3.1. Environmental parameters

The measured environmental parameters showed distinct seasonal variations in all the studied ports. However, the seasonal patterns varied according to the geographical location of the ports. Water temperatures in the ports are relatively warmer and cooler during southwest monsoon (SWM) and northeast (east coast) or post (west coast) monsoon, respectively. The averaged salinity variations were negligible (Kolkata: < 0.4 round the year and Haldia: 0.6 to 7.8), marginal (25 to

35) and large (5.1 to 26) in freshwater, marine and estuarine ports (Cochin) respectively. The low salinity observed in marine and estuarine ports were due to freshwater influx during monsoons. Interestingly, the freshwater and estuarine ports systems are nutrient (NO_3 , PO_4 , and SiO_4) rich compared to marine ports. However, the magnitude varies between the ports. For instance, among the nutrient-rich freshwater influenced ports the magnitude of nutrient concentrations was higher for Kolkata and Haldia ports compared to Cochin port (Table 2). Among the marine ports nutrients showed a distinct seasonal variability, but the seasonal patterns varied between the ports located on east (Tuticorin, Chennai, and Paradip) and west coast (Kandla and Mangalore) of India.

3.2. Phytoplankton biomass (chl *a*) and pheopigments (pheophytin and pheophorbide)

Results indicate that the chl *a* and degraded pigments showed significant seasonal variations in each of the ecosystems (except Haldia and Kandla due to the prevalence of low chl *a* in all the seasons). However, the seasonality pattern was different between the ecosystem, and this was true for chl *a* and pheopigments (Figs. 3 and 4). The detailed results for each of the ecosystems are provided below.

3.2.1. Biomass

Results revealed higher biomass in freshwater (except Haldia) and estuarine ports compared to marine ports. In each port (except Haldia and Kandla due to the prevalence of low chl *a* in all the seasons), biomass showed significant seasonal variations. However, the seasonality pattern for chl *a* was different between the port ecosystems (Figs. 3 and 4). Among freshwater port ecosystems, the highest (up to $40.8 \mu\text{g l}^{-1}$) and lowest (0.5 to $1.8 \mu\text{g l}^{-1}$) concentration of chl *a* are observed in Kolkata–port (including Hooghly riverine stations) and Haldia-port respectively. In Kolkata, high chl *a* concentration was observed in the stations located in Hooghly River (up to $40.8 \mu\text{g l}^{-1}$) compared to enclosed port stations (up to $20.3 \mu\text{g l}^{-1}$). In the estuarine port (Cochin) chl *a* remained high (11.97 to $18.37 \mu\text{g l}^{-1}$) round the year, and the high and low chl *a* was observed during SWM and PRM season respectively. Among marine ports, lowest ($<1 \mu\text{g l}^{-1}$) and highest (up to $25 \mu\text{g l}^{-1}$) chl *a* were observed in Kandla and Mangalore ports respectively, whereas, in the rest of the ports, chl *a* was variable, i.e., high in only one or two seasons. Clustering and non-metric MDS at 60% similarity also revealed two groups and three ungrouped individual ports. The ports characterized by low (Haldia and Kandla) and high biomass (Mangalore, Kolkata/Hooghly, and Cochin) for most of the year formed two groups (Fig. 7). In the remaining individual ports, high biomass was recorded once or twice during the observation (Fig. 7).

3.2.2. Pheophytin

Similar to chl *a*, higher pheophytin is observed in freshwater (except Haldia) and estuarine ports compared to marine ports. Among freshwater ports, high (up to 3.4 $\mu\text{g l}^{-1}$) and low ($< 0.1 \mu\text{g l}^{-1}$) concentrations were recorded in Kolkata (including riverine stations) and Haldia ports respectively during all the occasions. Interestingly, high pheophytin concentration (but lesser than freshwater) were observed most of the year in estuarine port (Cochin). Among the marine ports, pheophytin showed a distinct seasonal trend. The highest and lowest pheophytin was recorded in Mangalore (0.1 to 1.2 $\mu\text{g l}^{-1}$) and Kandla (0.03 to 0.13 $\mu\text{g l}^{-1}$) ports, respectively. In the remaining three ports pheophytin distribution was variable. Clustering and non-metric MDS at 60% similarity also revealed two groups and four ungrouped individual ports. The ports characterized by low (Haldia, Paradip, and Kandla) and relatively higher pheophytin (Mangalore, Kolkata, and Cochin) for most of the year formed two groups (Fig. 7). However, some stations located in Hooghly river (part of Kolkata sampling) recorded the highest pheophytin, whereas the remaining two individual ports recorded high pheophytin were recorded once or twice during the observation (Fig. 7).

3.2.3. Pheophorbide

Unlike chl *a* and pheophytin, the pheophorbide distribution was not same between the ports. On the contrary to high biomass Kolkata port, pheophorbide concentrations were low except during PM (0.1 to 2.0 $\mu\text{g l}^{-1}$), whereas in Haldia-port low pheophorbide concentrations (0.09 to 0.19 $\mu\text{g l}^{-1}$) were recorded throughout the year. Unlike freshwater systems, the estuarine-port (Cochin) recorded higher pheophorbide concentrations, and the maximum was during post-monsoon followed by southwest monsoon and premonsoon (Fig. 4). Among the marine ports, pheophorbide distribution showed a distinct seasonal trend. Unlike in freshwater and estuarine ecosystems, pheophorbide in marine ports exhibited a distinct relationship with chl *a*. Among ports, highest (up to 20.1 $\mu\text{g l}^{-1}$) and lowest ($< 0.2 \mu\text{g l}^{-1}$) pheophorbide concentrations were recorded in Mangalore and Kandla port, respectively. In the remaining marine ports, pheophorbide distribution showed distinct seasonality, but the seasonal patterns were different due to the geographic location of the ports. Clustering and non-metric MDS at 60% similarity also revealed two groups and three ungrouped individual ports. The ports characterized by low (Kolkata and Tuticorin) and relatively higher pheophorbide (Hooghly, Haldia, Kandla, and Paradip) for most of the year formed two groups (Fig. 7). However, Mangalore port followed by Cochin recorded the highest pheophorbide for most of the year, whereas the remaining Chennai port high pheophorbide concentration was recorded once during the observation (Fig. 7).

3.3. Pigment ratios

3.3.1. Chl *a* to pheopigments ratio

Generally, Chl *a*: pheopigments ratios were calculated to understand the freshness of organic matters from the overlying water column from different ecosystems. In this study, pigment ratio (Chl *a*: CDP, Chl *a*: pheophytin and Chl *a*: pheophorbide) showed a distinct spatial (between ecosystem types such as freshwater, estuary and marine ports) and seasonal variations (Fig. 5). High Chl *a*: CDP ratios and high- Chl *a*: pheophorbide ratios were observed in freshwater (except Haldia due to low biomass) and estuarine ports compare to marine ports. On the contrary, high Chl *a*: pheophytin ratios were mostly observed in marine ports (except Paradip due to low biomass) than freshwater ports (except Cochin and Haldia). Among the freshwater ports, high and low ratios were encountered in Kolkata and Haldia, respectively, whereas among the marine ports, high and low ratios were encountered in Mangalore and Paradip/Kandla, respectively (Figs. 5 and 7). The low ratios in freshwater and marine ports were mainly due to the prevalence of low chlorophyll.

3.3.2. Pheophorbide vs. Pheophytin

The ratios of pheophorbide to pheophytin were calculated to understand the ecological process of the different ecosystems, i.e., CCP/herbivory is dominant (>1) or not dominant (<1). Like Chl to CDP ratios, pheophorbide: pheophytin ratio also showed a distinct spatial and seasonal variation (Fig. 6). The average pheophorbide: pheophytin ratio ranged from a low of 0.2 in Kolkata to a maximum of 11.5 in Mangalore (Fig. 6). Pheophorbide: pheophytin ratios in freshwater (except Haldia) and estuarine ports were <1 , (i.e., ranges between 0.2 to 0.9) whereas in marine ports the average ratio was mostly >1 , i.e., ranges between 0.4 to 11.5 (Figs. 6 and 7). The low ratios in marine ports (especially in Chennai and Tuticorin) are observed only on a couple of occasions when the biomass is very low ($<1 \mu\text{g/l}$). Clustering and non-metric MDS at 60% similarity also revealed two groups and three ungrouped individual ports. The ports characterized by low, i.e., <1 (Kolkata, Hooghly, and Cochin) and relatively higher ratio, i.e., >1 (Haldia, Kandla, and Paradip) for the most of the year formed the two groups (Fig. 7). However, Mangalore port recorded with the highest ratio (up to 11.2) for the most of the year, whereas in the remaining Chennai and Tuticorin-port, high ratios (>1) were recorded only once during the observation (Fig. 7).

4. Discussion

In aquatic ecosystems, loss (mortality) processes (e.g., grazing, program cell death, viral lysis, sinking) determine the fate of phytoplankton and under such circumstances PMP can be used as a proxy to understand the potential chlorophyll breakdown (ChlB) pathways. In the ChlB pathway,

pheophorbide formation is an important step, formed either via chlorophyllide or pheophytin. Laboratory experiments reported that the initial ChlB-pathway induced by grazing and senescence are not the same, i.e., chlorophyll-chlorophyllide-pheophorbide (CCP) and chlorophyll-pheophytin-pheophorbide (CPP) in the former and latter case, respectively. Therefore documenting these different breakdown fractions will aid in determining the dominant ChlB pathway. Like plankton and environmental variables, the ChlB-pathway will also differ in different ecosystems (i.e., FPE, EPE, and MPE) and was documented in this study. The highest concentration of chl *a* and CDP were encountered in FPE (except Haldia with least values) and EPE compared to MPE (Figs. 3, 4, and 7), suggesting the existence of system-specific growth and loss process occurring at different time scales. Chl *a*: total pheopigments, an indicator for degradation of pigments (Boon and Duineveld 1996) or freshness of organic matter (Morata et al., 2011), did not reveal a clear pattern between port ecosystems as observed with the concentrations (Fig. 5). However, irrespective of the river influence, the systems with very low biomass ($<1 \mu\text{gm}^{-1}$) showed very low Chl *a*: CDP ratio suggesting the higher degradation activity (Fig. 5). Low and high ratios were observed in Cochin and Kolkata/Hooghly respectively. In the case of MPE, low and high ratios in Paradip and Mangalore respectively suggested the predominance of less or more actively growing biomass, respectively.

In this study, the pool of chlorophyll breakdown fractions is mainly contributed by pheophytin and pheophorbide, suggesting the prevalence of CCP and CPP pathways even when the chlorophyllide fraction was not detected. However, there exists considerable spatial and temporal variability in the chlorophyll breakdown pathways, as indicated by pheophytin and pheophorbide distribution. The presence of pheophytin (demetalated chlorophyll) and pheophorbide (demetalated and dephytalated chlorophyll) indicates chlorophyll has undergone a single-step and double-step breakdown reactions. Generally, pheophytin concentration is always higher in FPE and EPE compared to MPE (Figs. 4 and 7) due to the prevalence of high biomass as well as the contribution from the terrigenous influx. On the contrary, the distribution of pheophorbide concentrations was variable even with the high biomass in FPE and EPE, whereas in MPE, distribution showed high, low, and variable under high, low, and variable biomass, respectively (Figs. 4 and 7). In Cochin (EPE) and Mangalore ports (MPE) high pheophorbide concentrations were recorded, suggesting that these ecosystems are conducive for the chlorophyll to undergo double-step breakdown reactions. Overall pheophorbide concentrations showed independent and dependent on biomass in FPE/EPE and MPE, respectively (Fig. 5). Since chlorophyllide is not detected, the identification of chlorophyll breakdown pathway is not straightforward. The laboratory experiments suggested that

the chlorophyll breakdown to pheophorbide via CCP pathway is relatively faster compared to CPP pathway (unpublished data). In such circumstances, pheophorbide: pheophytin ratio will be a potential proxy to understand the dominant pathway or nature of mortality (an important ecological process) in different ecosystems. For instance, if the ratio is >1 , then the CCP pathway is more active than the CPP pathway and <1 then the CCP pathway is less active than CPP pathway. Both CCP and CPP pathways may be considered in equilibrium if the ratio is equal to 1. The laboratory grazing experiments using monocultures of phytoplankton belonging to different groups (diatoms, dinoflagellates, and green algae) and natural communities revealed that pheophorbide: pheophytin ratio can be proxy to determine herbivory is dominant (>1) or senescence is dominant (<1); Satish et al. submitted). These findings suggest that the additional information on the taxonomic composition of both phytoplankton and zooplankton along with the environmental settings (e.g., FPE, EPE, and MPE) will be useful to identify the cause for chlorophyll breakdown pathway. Given this, a schematic indicating the higher ratios for no-river influenced coastal systems (>1) followed by estuarine (0.5), and freshwater (0.2 to 0.4) systems is proposed (Fig. 8), which can aid in better ecological assessment and the details on the possible usefulness of the above insights is provided subsequently. For example the systems with low (<1) and high (>1) pheophorbide: pheophytin ratios suggested the dominance of cell senescence (suggesting CPP pathway due to high pheophytin concentrations) and cell destruction due to herbivory or grazing (suggesting CCP pathway due to high pheophorbide concentrations), respectively. Here the following scenarios: ballast water management (treatment and post-voyage discharge), the fate of algal bloom (the decline of bloom is due to herbivory or senescence or exhaustion of resource) and the ecological perspective are envisaged to elucidate the usefulness of pheophorbide: pheophytin ratio in the ecosystem assessment.

Generally, ports are at the receiving end for ship's ballast water discharge and therefore the information on the phytoplankton growth and loss according to the environmental conditions at discharge point will be valuable for ballast water management and is summarized in Table 3. The present study indicated that the pheophorbide: pheophytin ratio exhibited a distinct spatial (between/within ecosystems) and seasonal variability (Figs. 6 and 7). In FPE (except Haldia) and EPE, the ratio was <1 during all the seasons suggested that the CPP pathway is more active compared to CCP pathway (Fig. 6). In Kolkata and Hooghly systems, the pheophorbide concentration was minimal even when high biomass was present whereas, in the case of Cochin systems, the ratio was relatively higher as there was evidence of high pheophorbide production compared to freshwater ports during all the sampling occasion. Unfortunately, in Haldia, high ratios

(0.9 to 4.6) were observed despite the prevalence of low biomass on all the occasions (Fig. 6). One possible reason for such a different scenario is due to the prevalence of different phytoplankton composition. For instance, in Cochin and Kolkata/Hooghly, diatoms (63% - 78%) and non-diatoms (65% - 75%) dominated the phytoplankton community respectively. Generally, diatoms, which have high chlorophyllase activity (Jeffrey and Hallegraff 1987), are the preferred feed for grazers. Therefore, under such circumstances, the CCP pathway will be active and was the case in Cochin ecosystems along with CPP pathway. Even the laboratory experiments also indicated higher pheophorbide production for *Skeletonema* (diatom) than species belonging to other groups, including species (*Dunaliella*) with high chlorophyllase activity (unpublished data). It should also be noted that the chlorophyllase activity varied between the species of the same group (Jeffrey and Hallegraff, 1987). Therefore, the presence of low herbivore population/biomass or the non-preferred diet of the prevailing grazer in Kolkata/Hooghly cannot be ruled out. On the contrary, irrespective of the phytoplankton biomass, pheophorbide: pheophytin ratio in MPE ranged up to 11.2 (Figs. 6 and 7). This suggests that chlorophyll has undergone double-step chlorophyll breakdown reactions, unlike FPE, with one-step reactions. In MPE, the phytoplankton composition is dominated by diatoms round the year, and therefore, the observed high pheophorbide production via CCP pathway cannot be ruled out. In low (Kandla) and high (Mangalore) biomass systems, CCP pathway is active round the year. In the case of Paradip, Chennai and Tuticorin systems, biomass was variable and so with the ratios. A distinct seasonality in the pheophorbide production in diatom-dominated marine ports was observed, and this is because of the chlorophyll breakdown reactions of seasonal high biomass via CCP pathway, i.e., through herbivory. Nevertheless, this study suggests that the chlorophyll breakdown pathways exhibit apparent differences between the freshwater (enclosed; Kolkata port), riverine (Hooghly), estuarine (Cochin) and marine ecosystems, respectively. This generally means that the dominant loss factor for phytoplankton (including introduced species) will be cell senescence and herbivory in FPE (throughout the year) and MPE (due to seasonal diatom peaks) respectively, whereas both cell senescence and herbivory are responsible in EPE (due to prevalence of high biomass and freshwater influx). Given the above it is also envisaged that the time window of the conducive environmental condition for the growth of ballast introduced viable phytoplankton is lower for MPE (except Mangalore) and FPE (except diatoms and dinoflagellates) compared to that for EPE (Table 3). These findings suggest that the introduction of ballast water with viable phytoplankton during the growing season involves higher risk, and the risk level varied between the ports and season. Nevertheless, the study revealed grazing (in MPE and EPE) and senescence (in FPE and EPE) as the dominant loss factor for

phytoplankton. From the ballast water management perspective, the risk level involved in the studied ports is summarized for the reference (Table 3).

Further, most of the studied port ecosystems are prone to blooms and the knowledge on taxa, chlorophyll, and pheophorbide: pheophytin ratio will be useful to determine whether the decline of bloom is due to herbivory or other reasons (senescence, microbial degradation, resting stage formation). Here, the former scenario (mostly related to seasonal diatom blooms as noticed in MPE and EPE) signifies a good ecological state, and the latter (unusual or harmful algal blooms-HAB) is a matter of ecological and environmental concern. Some studies have also shown that the members of micro- and mesozooplankton don't prefer toxin-producing HAB species (e.g., Turner, 2010); however, such deterrent properties may also be highly specific to species and/or condition (e.g., Davis and Gobler, 2010; Stauffer et al., 2017) and was evident in the laboratory experiment. The grazing experiment with a bloom-forming diatom (*Skeletonema*) and dinoflagellate (*Amphidinium*) revealed higher (>1) and lower (<1) pheophorbide: pheophytin ratios respectively suggesting grazing induced mortality or growth control in the former but not in the latter (Sathish et al. unpublished data). The global increase in the occurrence of HABs continue to underscore the many remaining gaps in knowledge such as technological advances for early detection (Stauffer et al., 2019). Given this, the incorporation of the chlorophyll breakdown pathway indicator to determine bloom fate will be a step ahead in the ecological/environmental assessment and HAB research per se. For bloom events to occur, the growth rate must exceed losses, through biological and physical processes (Caron et al., 1989; Mitra and Flynn 2006; Tillmann 2004). Therefore biological control measures such as grazing and algicidal microbes are considered as promising techniques to control algal blooms (e.g., Secher 2009, Choi et al., 2017; Paerl et al., 2018). Given this, we assume that chlorophyll breakdown pathway will be a potential proxy for evaluating the efficacy of the bloom control techniques. Traditionally, in addition to grazing, the sinking of ungrazed phytoplankton or bloom to the bottom is considered as one of the major loss factor (Calbet and Landry, 2004; Choi et al., 2017). Thereby assessing the dynamics of PMP from the sediments and the overlying waters in a given ecosystem will improve the understanding of the linkages between the pelagic and benthic phytoplankton, which is an essential process in the functioning of the ecosystem. In view of this, it is assumed that chlorophyll: pheopigment and pheophorbide: pheophytin ratios together can be used as potential proxies to determine the fate of phytoplankton (in particular bloom) or pelagic primary production as the former is an indicator for actively growing microalgal community in the water column and freshness of organic input into the surface sediment (Moratta et al., 2011; Grippo et al., 2009; Allison et al., 2013] whereas the latter for

determining the factors (eg. herbivory or senescence) responsible for decline of phytoplankton population or bloom (Satish et al. submitted). However, specific studies in these directions will be a step ahead.

The International Convention for the Control and Management of Ships' Ballast Water and Sediments, 2004 (BWM Convention), entered into force globally on 8 September 2017. From this date of the BWM Convention, all ships must conform to the two ballast water management standards (D-1 and D-2). The D-1 standard requires ships to exchange their ballast water in open seas, away from coastal areas (i.e., ~200 nautical miles from land and in water at least 200 meters deep) whereas the D-2 standard specifies the maximum amount of viable organisms allowed to be discharged, including specified indicator microbes harmful to human health. According to the convention, BWM systems (e.g., use of filters and ultraviolet light or electrochlorination) have to be tested in a land-based facility and onboard ships to prove that they meet the performance standard set out in the treaty. Given this Ballast water sampling from the ships has started receiving the importance of proving that ships carrying ballast water comply with BWM standards (Gollasch and David 2017). Even port authorities may also sample ballast water for subsequent analyses to prove compliance with the ballast water performance standards. In view of this, analyzing the degraded pigments and their ratios along with the modern tools/methodologies for viable phytoplankton cells will be added advantage in the evaluation of the impact of (i) ballast tank conditions (eg. dark) during voyages, and (ii) treatment of the ships ballast water using approved BWM systems before discharge. During voyages, in ballast tanks, prolong darkness is the prominent stress factors, and this condition can have varying effects on the organisms. The laboratory experiments have shown that phytoplankton population decreased with increase in the length of the dark incubation (Carney et al., 2011; Desai et al., 2018; Patil and Anil, 2018), but the duration of dark survival or tolerance varies and is species-specific (Peters 1996; Paters and Thomas 1996; Unpublished data). The decline in the population could be due to senescence or microbial degradation and grazing or both. The sampling from the ballast tanks during voyages revealed different findings. In one study, decrease and increase in plankton (both phyto and zoo) abundance and bacteria was observed respectively till the end of the voyages (Desai et al., 2018) and in such condition senescence or microbial degradation will be dominant. Whereas in another study, sampling from ballast tanks observed a large fish and increase in copepod (a dominant zooplankton and a grazer) populations (Gollasch et al., 2000; Gollasch and David 2019) suggesting herbivory dominance cannot be ruled out. Moreover, most of the ballast water treatments involve killing or destruction of the cells, and in such cases, phytoplankton degradation process will be similar to that observed during herbivory.

The involvement of chlorophyllase activity in the conversion of chlorophyll into chlorophyllide is the reason, and this happens whenever there is cell destruction like chewing or grazing (Hu et al., 2013). Nevertheless, in each of the scenarios, the proposed pheophorbide: pheophytin ratio can be useful to determine the reason for phytoplankton population decline. Since the chl *a* breakdown pathway under grazing-pressure is taxon-specific and the grazer's feeding habits (Sathish et al. submitted), additional studies in this direction will be valuable in ecological surveillance and assessment.

Conclusion

This study concludes that the distribution of single (pheophytin) and double (pheophorbide) step chlorophyll-breakdown reaction fractions exhibit distinct spatial and seasonal variations. Pheophytin is higher in the FPE and EPE, but its distribution is not linked to biomass variations. Whereas pheophorbide is more in MPE followed by estuarine systems and its distribution could be linked with biomass distribution. Since chlorophyllide (single-step reaction of CCP pathway) was not detected, pheophorbide: pheophytin ratio can be a potential proxy for determining the nature of the breakdown pathway, i.e., CCP or herbivory dominant (>1) or not dominant (<1). The ratios exhibited clear differences between different ecosystems, i.e., the higher ratios in marine (up to 11.2) followed by estuarine (up to 0.94) and freshwater (0.2 to 0.4) port systems. This generally means that the dominant degradation pathway for phytoplankton (including introduced species) will be cell senescence and herbivory in FPE (throughout the year) and MPE (due to seasonal diatom peaks) respectively, whereas both cell senescence and herbivory are responsible in EPE (due to prevalence of high biomass and freshwater influx). Since laboratory experiments revealed that the chl *a* breakdown pathway under grazing-pressure is taxon-specific and the grazer's feeding habits (Sathish et al. submitted) additional studies in this direction will be valuable in ballast water management (treatment and post-voyage discharge), algal bloom research (eg. understanding fate and in control measures), and in the ecological surveillance. Also confirming the role of hydrolase enzymes and the sequence in the early as well as post-pheophorbide breakdown steps in different aquatic ecosystems will enhance the understanding of the chlorophyll breakdown pathways, which further alleviate the ecosystem assessment.

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Table 1 Details of sampling stations (numbers and location), sampling period, water temperature and salinity

Stations	No.Stations	Latitude	Longitude	Sampling period	Temperature	Salinity
Kochi	12	9.58	76.14	October 2011, May, August & November 2012	29.2 ± 1.2	17.9 ± 8.5
Kolkata	22	22.32	88.18	September 2013, Febraury 2014 Febraury & December 2015	25.5 ± 5.0	0.3 ± 0.02
Hooghly	8	22.33	88.17	September 2013, Febraury 2014 Febraury & December 2015	23.5 ± 5.7	0.3 ± 0.05
Haldia	22	22.07	88.07	September 2013 , Febraury & September 2014, April 2015	27.5 ± 0.7	4.4 ± 2.9
Mangalore	22	12.55	74.48	July & October 2012, January & April 2013	28.1 ± 1.8	35.2 ± 0.6
Kandla	26	22.50	70.07	October 2014, July & November 2015, Febraury 2016	26.5 ± 0.5	35.9 ± 2.7
Paradip	25	20.16	86.4	August & December 2014, May & August 2015	28.1 ± 0.3	30.1 ± 3.2
chennai	18	13.08	80.18	July & September 2012, January & March 2013	29.8 ± 3.2	32.1 ± 2.7
Tuticorin	25	8.45	17.13	July & October 2012, January & March 2013	28.8 ± 0.9	34.8 ± 1.2

Table 2 Environmental parameters from river influenced ports systems (RIPS) and no river influenced port systems (NRIPS). SWM – southwest monsoon, PM- post-monsoon, PrM – pre-monsoon, NEM – northeast monsoon, SIM – spring inter-monsoon, FIM – fall inter-monsoon, DO – dissolved oxygen (mg l^{-1}). The units for nutrients (ammonia, nitrate, nitrite, phosphate and silicate) are in μM .

Environmental parameters from RIPS								
Stations	Season	Salinity	DO	Phosphate	Silicate	Nitrate	Nitrite	Ammonia
Kochi	PM 1	22.6 ± 6.7	7.1 ± 1.0	3.2 ± 1.7	47.8 ± 9.9	4.0 ± 3.0	0.5 ± 0.5	24.9 ± 7.7
	PrM	20.5 ± 6.8	6.7 ± 1.1	3.8 ± 0.8	44.3 ± 9.3	5.9 ± 1.3	1.1 ± 0.3	42.6 ± 11.8
	SWM	5.3 ± 2.2	3.9 ± 1.6	3.5 ± 0.8	47.6 ± 18.1	10.2 ± 5.9	1.4 ± 0.8	26.2 ± 10.5
	PM2	23.1 ± 2.2	3.8 ± 1.4	2.6 ± 1.1	47.2 ± 16.7	3.4 ± 2.4	0.5 ± 0.2	20.5 ± 10.1
Kolkata	SWM	0.20 ± 0.05	6.2 ± 1.5	2.3 ± 1.4	66.9 ± 48.8	27 ± 12.2	1.6 ± 0.3	2.0 ± 1.0
	PrM 1	0.25 ± 0.05	8.8 ± 0.9	4.5 ± 0.7	76.4 ± 12.4	10.0 ± 6.6	4.3 ± 2.3	20.6 ± 7.8
	PrM 2	0.25 ± 0.05	8.2 ± 0.7	1.1 ± 1.2	61.6 ± 31.3	24.3 ± 7.0	1.3 ± 0.8	5.7 ± 3.5
	PM	0.28 ± 0.06	7.0 ± 1.8	6.7 ± 0.5	132.3 ± 26.1	116.5 ± 71.5	8.4 ± 3.6	3.7 ± 1.1
Hooghly	PM	0.3 ± 0	6.7 ± 0.8	5.1 ± 0.3	148.3 ± 3.1	38.2 ± 4.1	1.5 ± 0.4	2.5 ± 0.7
	PrM 1	0.3 ± 0	8.9 ± 0.6	4.8 ± 0.5	74.7 ± 1.8	7.6 ± 2.6	2.1 ± 1.7	22.3 ± 16.3
	PrM 2	0.3 ± 0	8.8 ± 0.3	5.5 ± 1.1	411.5 ± 76.1	28.1 ± 8.7	1.4 ± 0.8	5.6 ± 1.5
	SWM	0.25 ± 0.10	7.0 ± 1.5	10.7 ± 0.3	177.7 ± 13.0	51.5 ± 18.7	5.8 ± 1.8	4.2 ± 0.6
Haldia	PM	1.3 ± 0.5	6.0 ± 0.7	4.0 ± 1.5	114.7 ± 14.4	33.0 ± 10.2	1.1 ± 0.3	4.1 ± 1.6
	PrM 1	6.3 ± 0.9	8.2 ± 0.2	6.6 ± 1.1	79.7 ± 12.9	3.4 ± 1.1	1.6 ± 0.3	6.3 ± 1.4
	PrM 2	2.7 ± 0.6	5.3 ± 0.6	3.4 ± 0.7	152.5 ± 29.0	38.1 ± 5.0	3.0 ± 0.5	8.9 ± 3.8
	SWM	7.4 ± 0.5	5.7 ± 1.0	6.0 ± 1.3	142.9 ± 46.0	24.3 ± 10.7	0.8 ± 0.5	4.3 ± 1.4
Environmental parameter from NRIPS								
Stations	Season	Salinity	DO	Phosphate	Silicate	Nitrate	Nitrite	Ammonia
Kandla	PM 1	37.7 ± 0.3	5.0 ± 0.4	4.3 ± 1.4	54 ± 5.2	7.2 ± 2.0	4.4 ± 0.7	15.3 ± 3.0
	SWM	30.2 ± 10.2	6.2 ± 0.6	3.4 ± 0.7	30.9 ± 5.2	12.8 ± 5.6	7.0 ± 1.8	1.4 ± 0.5
	PM 2	35.8 ± 0.1	6.5 ± 0.6	3.0 ± 0.4	77.6 ± 9.8	15.9 ± 6.0	3.0 ± 0.5	4.9 ± 0.7
	PrM	39.8 ± 0.4	6.1 ± 0.7	5.3 ± 0.8	128.4 ± 9.7	14.6 ± 2.8	6.8 ± 1.8	1.2 ± 0.2
Mangalore	PM 1	35.9 ± 0.1	3.8 ± 1.2	2.2 ± 0.6	26.6 ± 15.5	8.5 ± 5.9	4.3 ± 3.2	39.9 ± 5.0
	PrM	35.7 ± 0.3	5.7 ± 1.9	1.8 ± 1.9	6.1 ± 5.7	5.2 ± 3.2	1.8 ± 0.7	25.3 ± 8.3
	SWM	34.1 ± 0.9	4.7 ± 1.6	2.7 ± 0.6	23.5 ± 8.4	0.7 ± 0.5	0.6 ± 0.2	29.4 ± 9.2
	PM2	34.8 ± 0.1	4.7 ± 0.9	2.3 ± 0.8	17.6 ± 5.6	3.9 ± 1.2	5.2 ± 1.2	18.8 ± 4.8
Paradip	SWM	26.3 ± 0.5	5.2 ± 0.4	1.9 ± 0.3	20.4 ± 5.0	4.9 ± 1.1	0.9 ± 0.2	25.0 ± 21.2
	NEM	31.0 ± 1.3	4.7 ± 1.0	1.8 ± 0.2	42.1 ± 15.0	4.9 ± 2.0	1.1 ± 0.2	12.5 ± 4.8
	SIM	33.8 ± 0.3	4.7 ± 0.4	2.5 ± 0.5	78.2 ± 13.7	8.7 ± 5.9	1.8 ± 0.2	5.0 ± 2.9
	FIM	29.6 ± 1.3	4.6 ± 0.6	0.2 ± 0.1	63.0 ± 38.9	4.5 ± 0.9	1.6 ± 0.7	3.7 ± 1.2
Chennai	SWM	34.4 ± 0.1	3.2 ± 0.7	5.1 ± 2.9	23.9 ± 6.3	13.4 ± 3.2	2.3 ± 0.5	24.8 ± 10.2
	FIM	29.1 ± 1.8	4.9 ± 0.4	3.1 ± 2.3	11.8 ± 3.1	8.0 ± 5.8	1.3 ± 1.1	51.3 ± 44.3
	NEM	30.4 ± 0.2	4.8 ± 0.5	1.8 ± 0.6	8.4 ± 2.4	9.1 ± 5.6	1.6 ± 0.9	27.2 ± 11.2
	SIM	34.3 ± 0.2	4.0 ± 0.8	2.6 ± 1.0	6.2 ± 2.1	6.5 ± 2.2	0.9 ± 0.3	12.9 ± 5.1
Tuticorin	SWM	35.4 ± 0.1	5.6 ± 0.4	1.2 ± 0.7	12.3 ± 4.6	1.1 ± 1.1	0.5 ± 0.5	12.9 ± 3.9
	FIM	36.2 ± 0.2	6.3 ± 0.4	1.8 ± 0.6	6.2 ± 1.8	4.4 ± 2.2	1.4 ± 0.7	21.5 ± 6.8
	NEM	34.0 ± 0.8	5.0 ± 0.7	1.9 ± 0.8	6.2 ± 1.8	11.0 ± 8.1	9.8 ± 7.5	23.2 ± 11.3
	SIM	33.5 ± 0.03	5.9 ± 0.4	1.8 ± 0.2	4.4 ± 0.7	5.3 ± 0.8	0.8 ± 0.2	12.3 ± 3.3

Table 3. Summary on the distribution of phytoplankton pigments, level of invasion risk and potential loss factor for different major port ecosystems along Indian coast.

Port location	Ecosystems	Biomass (Chlorophyll)	Dominant groups	Pheophytin (Phe)	Pheophorbide (Pheid)	Pheid:Phe	Seasonal	Phytoplankton loss processes	Invasion risk
Cochin	Estuarine	High	Diatoms, Chlorophytes	High	Variable	<1	Yes	Degradation & herbivory (biomass independent)	High
Kolkata	Freshwater	High	Chlorophytes					Degradation (biomass independent)	High*
Haldia	Freshwater	Low	Chlorophytes	Low	Low	>1	No	Herbivory	Low
Kandla	Marine	Low	Diatoms	Low	Low	>1	Yes	Herbivory (biomass dependent)	Low
Mangalore	Marine	High		High	High	Variable			High
Tuticorin	Marine	Variable	Diatoms	Variable	Variable	Variable	Variable		Variable
Chennai	Marine							Variable	
Paradip	Marine							Variable	

*Excluding diatoms and dinoflagellates

Figure Legends

Figure 1. Schematic illustrating the chlorophyll-breakdown pathway.

Figure 2. Geographical location of the eight ports (and the sampling stations within the harbor) in different ecosystems (blue–marine, green–freshwater and orange–estuary) along the Indian Coast.

Figure 3. The seasonal variations in the concentrations of chlorophyll and chlorophyll degraded pigments (CDP) from different port ecosystems (freshwater, estuarine and marine) located along the east and west coast of India. SWM – southwest monsoon; FIM – Fall inter-monsoon; NEM – northeast monsoon; SIM – spring intermonsoon; PrM 1 and PrM 2 – pre-monsoon of first and second sampling season, PM 1 and 2 – post-monsoon first and second sampling season.

Figure 4. The seasonal variations in the concentrations of pheophytin (Phe), pheophorbide (Pheid) and chlorophyll (Chl) from different port ecosystems (freshwater, estuarine and marine) located along the east and west coast of India. SWM – southwest monsoon; FIM – Fall inter-monsoon; NEM – northeast monsoon; SIM – spring intermonsoon; PrM 1 and PrM 2 – pre-monsoon of first and second sampling season, PM 1 and 2 – post-monsoon first and second sampling season.

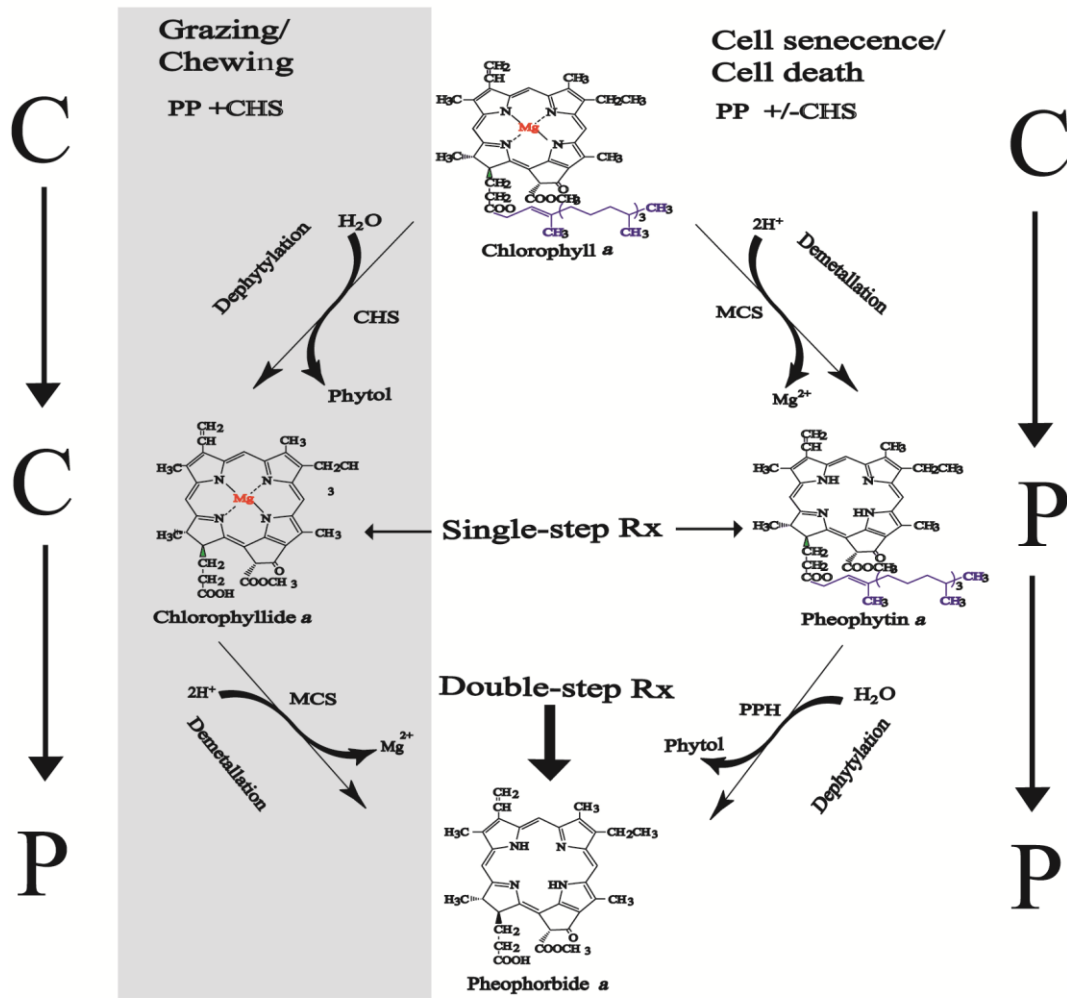
Figure 5. The seasonal variations in the pigment ratios (Chl:pheophorbide; Chl:pheophytin and Chl:CDP) from different port ecosystems (freshwater, estuarine and marine) located along the east and west coast of India. SWM – southwest monsoon; FIM – Fall inter-monsoon; NEM – north east monsoon; SIM – spring inter monsoon; PrM 1 and PrM 2 – pre-monsoon of first and second sampling season, PM 1 and 2 – post-monsoon first and second sampling season.

Figure 6. The seasonal variations in the pheophorbide : pheophytin ratios from different port ecosystems (freshwater, estuarine and marine) located along the east and west coast of India. SWM – southwest monsoon; FIM – Fall inter-monsoon; NEM – northeast monsoon; SIM – spring intermonsoon; PrM 1 and PrM 2 – pre-monsoon of first and second sampling season, PM 1 and 2 – post-monsoon first and second sampling season.

Figure 7. Non-metric multidimensional scaling (NMDS) ordination based on the Bray–Curtis similarity coefficient of (a) chlorophyll, (b) pheophytin, (c) pheophorbide and (d) pheophorbide:pheophytin ratio for the ports located in different ecosystems (marine, freshwater and estuarine). The grouping is based on the 60% similarity level of the cluster dendrograms constructed using the Bray–Curtis similarity coefficient and group average method. The text and symbols in blue, green and orange colour represents marine, freshwater and estuarine ecosystems respectively.

Figure 8. Schematic illustrating the chlorophyll-breakdown pathway in different ecosystems. CCP and CPP indicates chlorophyll-chlorophyllide-pheophorbide and chlorophyll-pheophytin-pheophorbide pathways respectively. Non-diatoms indicates that the phytoplankton is dominated by other groups such as chlorophytes, cyanobacteria, dinoflagellates. Pheid:Phe indicated phoephorbide:pheophytin ratio.

Chlorophyll breakdown pathway



PP +CHS - Phytoplankton with chlorophyllase activity
 PP +/-CHS - Phytoplankton with and without chlorophyllase activity
 CHS - Chlorophyllase, PPH - Pheophytinase, MCS - Magnesium chelating substances

Fig 1

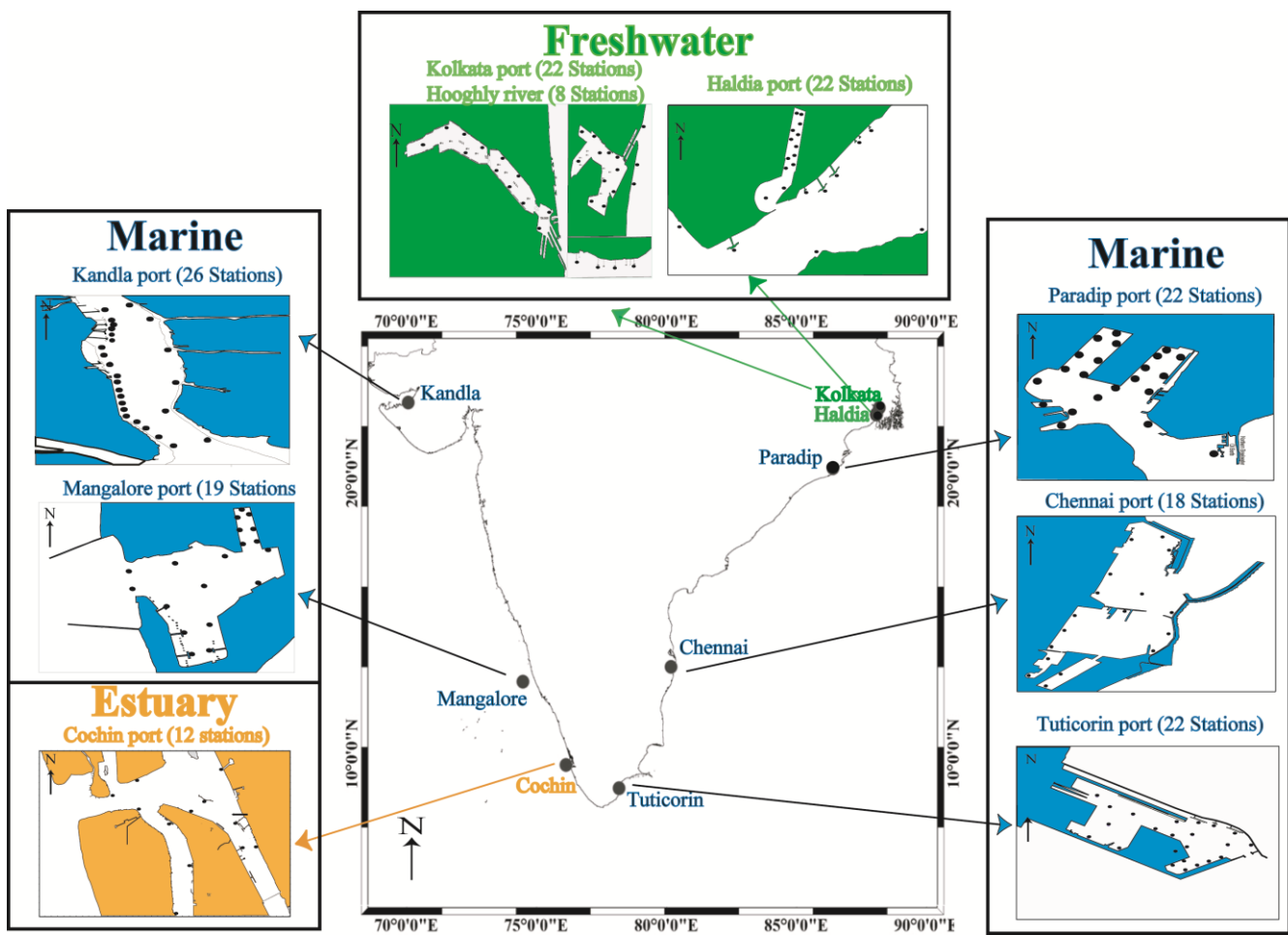


Fig 2

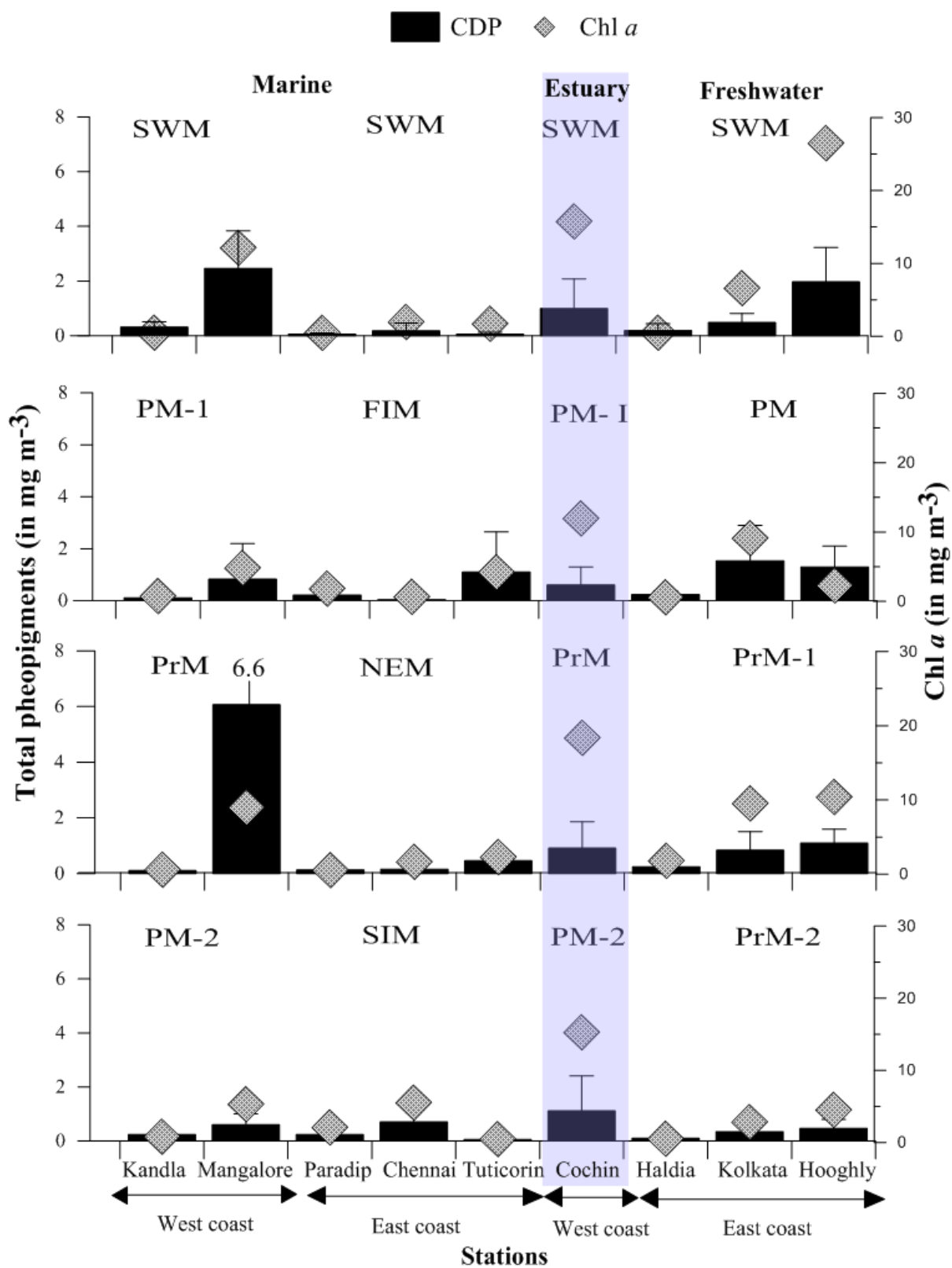


Fig 3

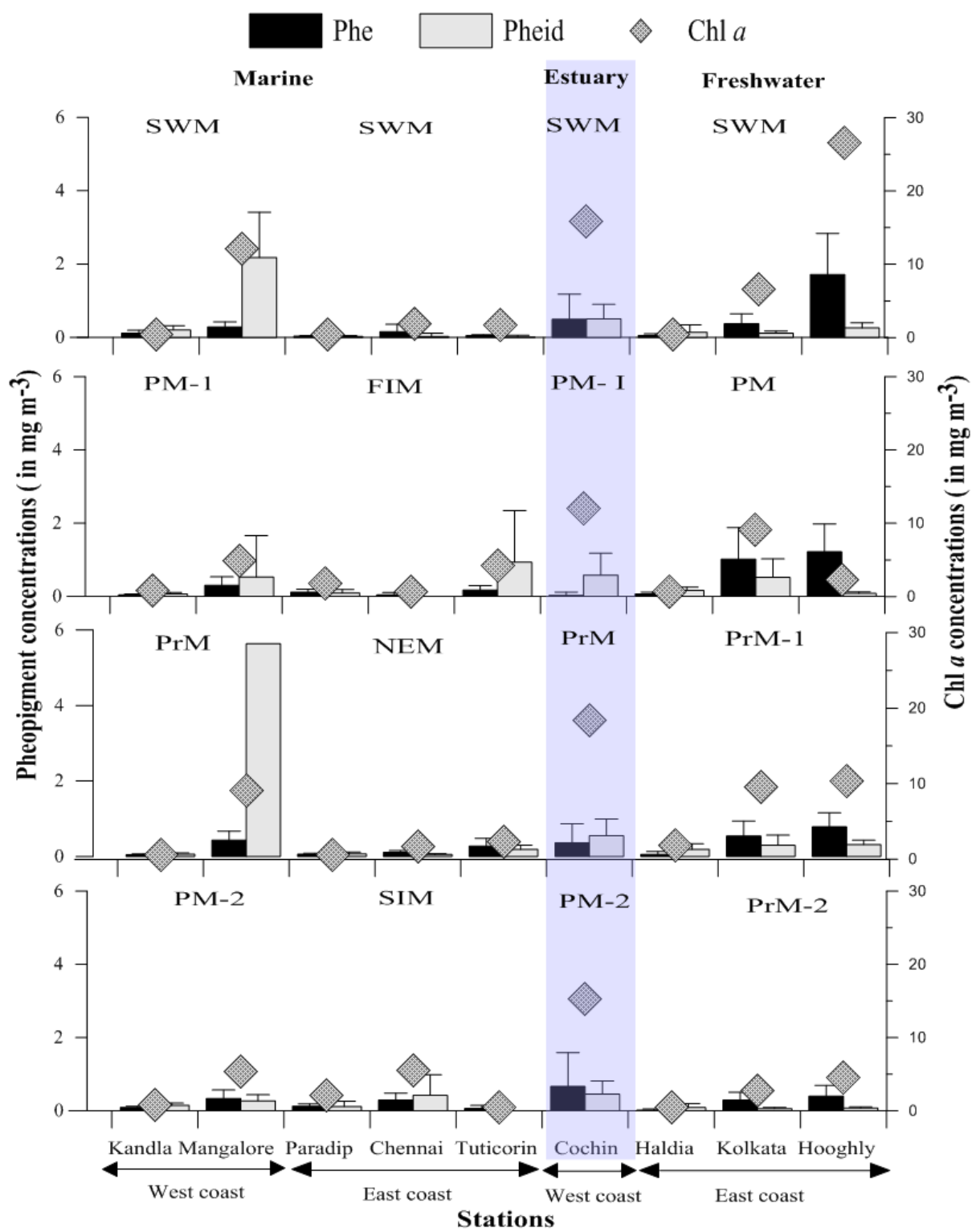


Fig 4

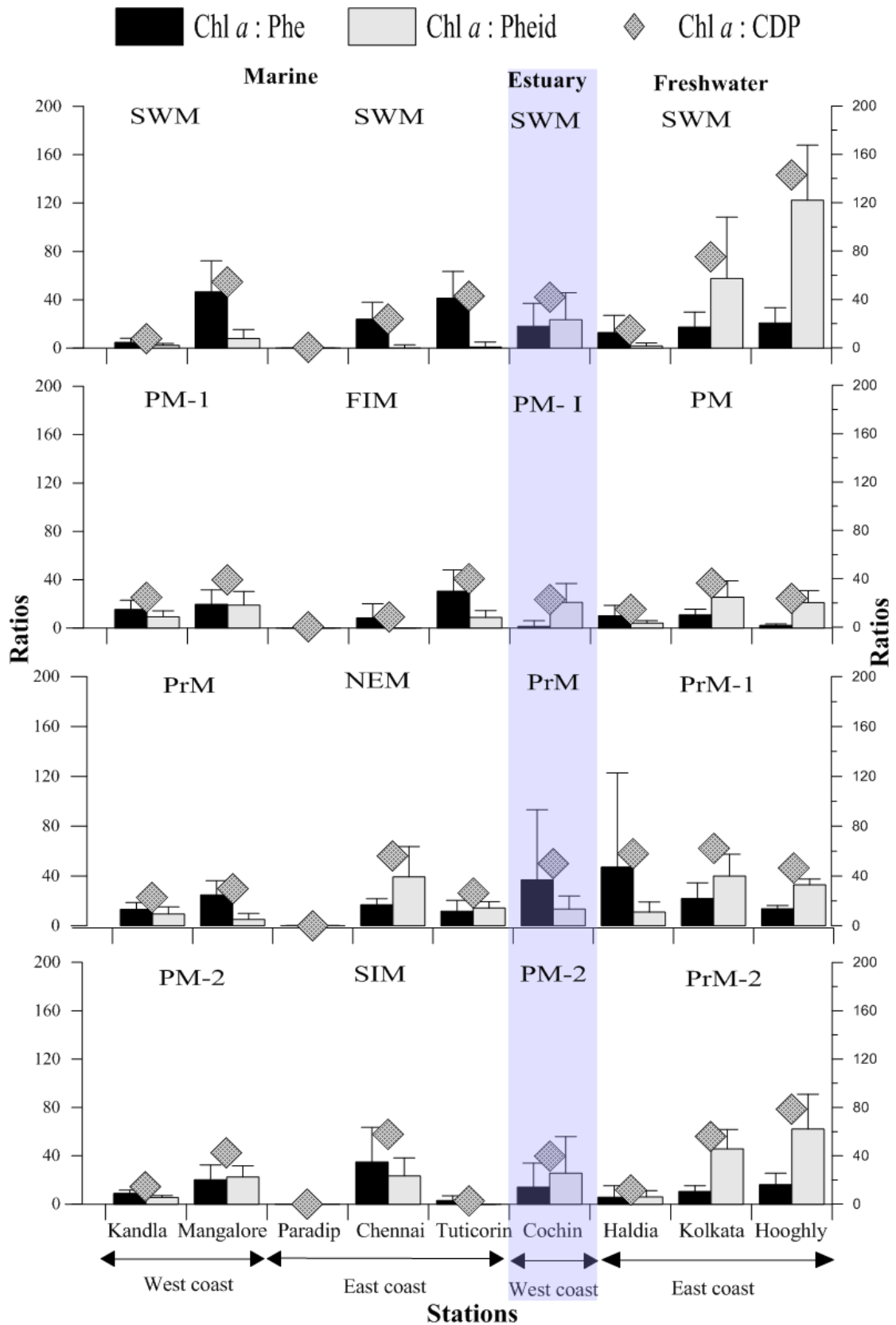


Fig 5

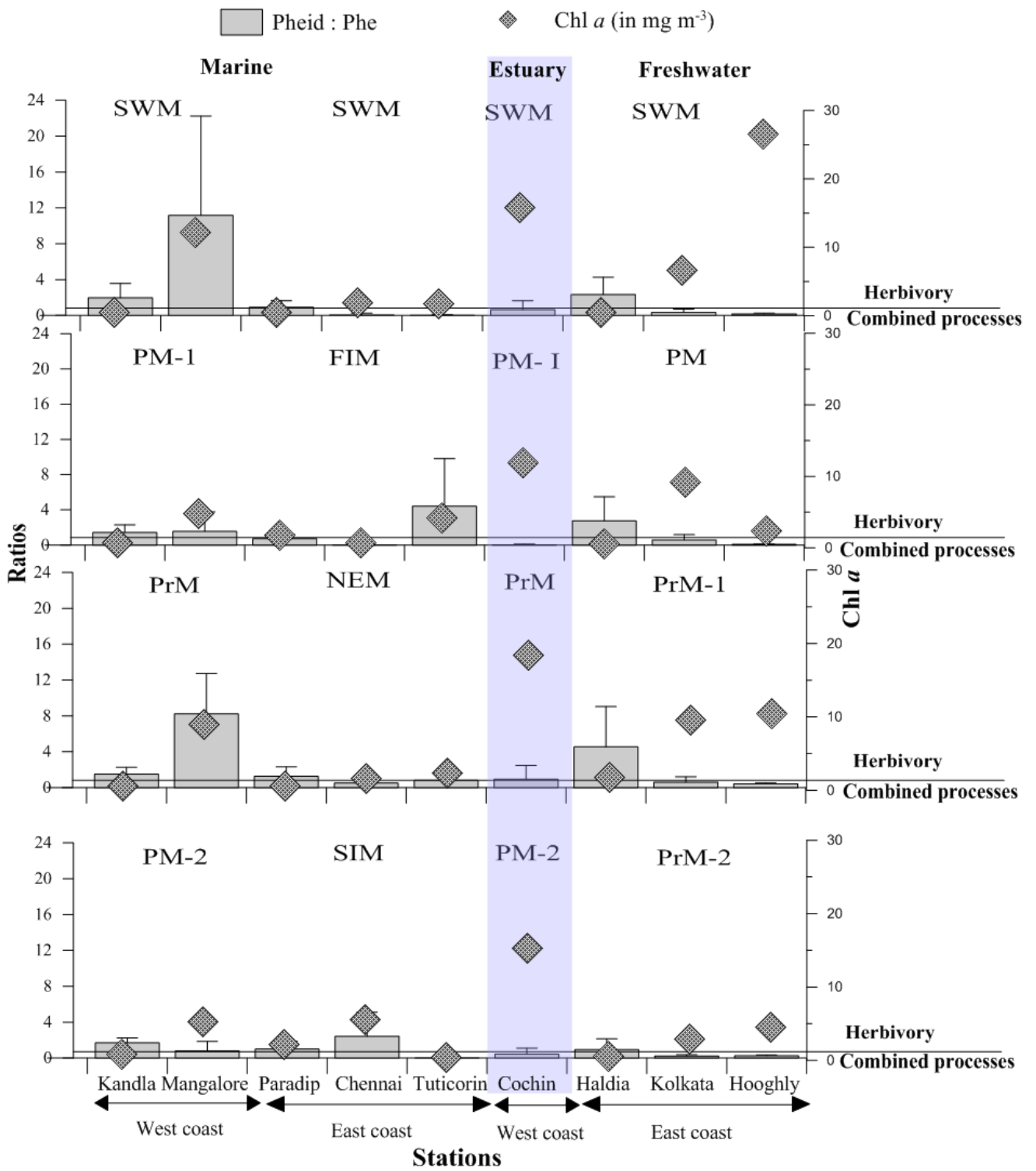


Fig 6

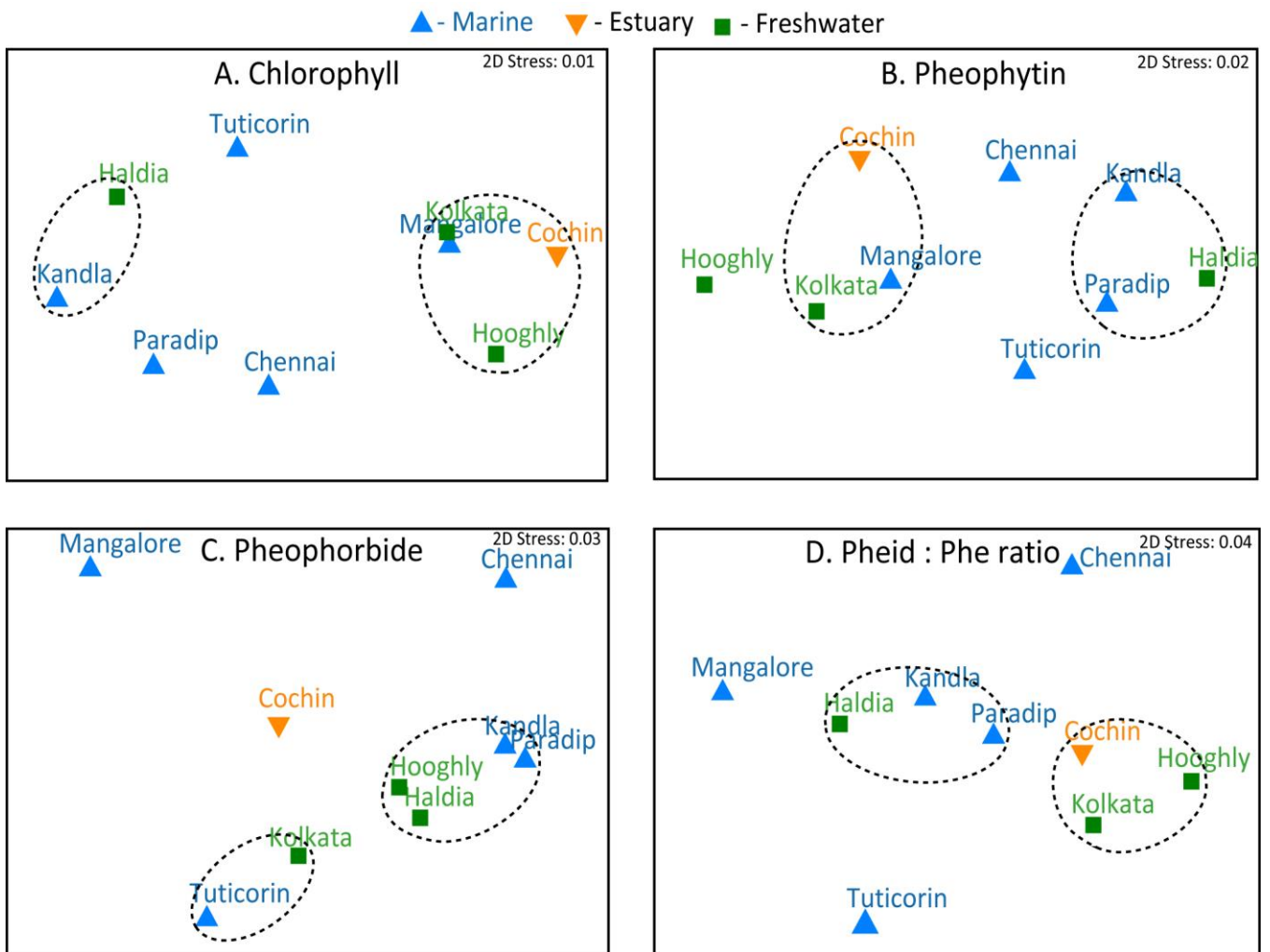


Fig 7

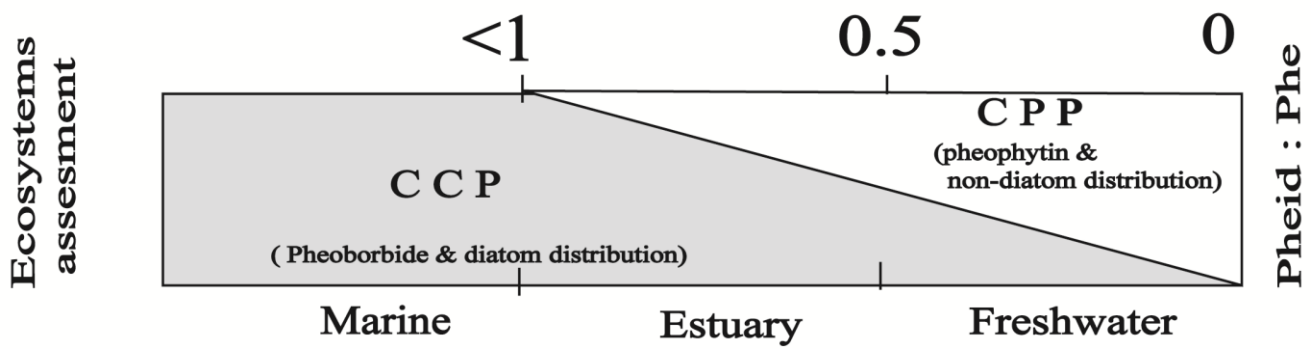


Fig 8