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RESEARCH PAPER

The response of *Prorocentrum sigmoides* and its associated culturable bacteria to metals and organic pollutants

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Abstract

This study investigates the effect of metals (cadmium, lead, mercury, and tellurium) and organic pollutants (benzene, diesel, lindane, and xylene) on a dinoflagellate—*Prorocentrum sigmoides* Böhm—and its associated culturable bacteria. Two bacterial cultures (*Bacillus subtilis* strain PD005 and *B. xiamensis* strain PD006) were isolated from *P. sigmoides* and characterized by scanning electron microscopy, 16S ribosomal RNA sequencing, biochemical analyses, and growth curve studies. This study points to a mutualistic relationship between *P. sigmoides* and its associated *Bacillus* isolates. *P. sigmoides* enhanced the growth of its associated *Bacillus* spp., through the secretion of extracellular exudates. In return, both *Bacillus* isolates contributed to the resistance of *P. sigmoides* to metals and organic pollutants. *P. sigmoides* and both *Bacillus* isolates exhibited concentration-dependent responses to metals and organic pollutants. An intriguing feature was the similar response of *P. sigmoides* and its associated *Bacillus* isolates to mercury and cadmium, indicating a co-selection of mercury and cadmium resistance. This provides support to the "dinoflagellate host-phycosphere bacteria" behaving as a single functional unit. However, the sensitivity profiles of *P. sigmoides* and its associated *Bacillus* isolates are different with respect to metals versus organic pollutants. These aspects need to be addressed in future studies to unravel the effect of metal and organic pollutants on dinoflagellates, an important component of the phytoplankton community, and to discern the influence of associated "phycosphere" bacteria on the response of dinoflagellates to pollutants.

KEYWORDS

associated bacteria, *Bacillus* spp., co-selection, metals, organic pollutants, *Prorocentrum sigmoides*

1 | INTRODUCTION

Marine environments are persistently plagued by pollutants, as a consequence of natural hazards, urbanization, and anthropogenic activities. Heavy metals, pesticides, hydrocarbons, organic pollutants, and so on, continuously find their way into the marine environment, through oil spills and release of effluents from industrial operations such as mining of ore, manufacturing of alkaline storage batteries, and production of agrochemicals [1]. The concentration of ubiquitous heavy metals such as Cd, Cr, Cu, Ni, Pb, and Zn in marine sediments varies from 0.09 to 88.6 μg/g [2]; they are usually complexed with inorganic or organic substances and persist in sediments. From the

environment, they enter the food web and biomagnify as they pass on through the food web, right upto the top of the food chain, resulting in physiological and metabolic imbalances [3].

The effects of pollutants on the eukaryotic, unicellular phytoplankton include changes in abundance, morphology, pigment composition, and biochemical activity; these are insidious and not readily noticeable. This has critical implications on the food web structure, as phytoplankton are the base of food webs in aquatic systems, and hence the entry point for biomagnification. The effect of toxicants on phytoplankton depend on the bioavailability and the partitioning/transport of the toxicant into the microbial cell. Metals, even at ppm and ppb levels, damage phytoplankton cells by causing lipid peroxidation [4], reduction of electron transport during photosynthesis, disruption of nutrient uptake processes, and inhibition of respiratory oxygen consumption [5]. Organic pollutants, depending on their partitioning into phytoplankton cells, also have similar deleterious effects on photosynthesis, electron transport, absorption of carbon dioxide and nutrients, replication, transcription, and translation processes [6].

The aim of the present study is to investigate the effect of metals and organic pollutants on dinoflagellates, a pivotal group of phytoplankton. Bacteria are intrinsically associated with dinoflagellates, in the "phycosphere" [7], either temporarily or persistently [8,9]. Their interactions may range from symbiotic (beneficial association), parasitic (antagonistic), commensal (neutral) to competitive (for limiting nutrients like phosphate) [10,11]. Generally, phytoplankton cells are known to release organic molecules (sugars, amino acids, lipids), which stimulate the growth of bacteria associated with them [12–15]. This is usually in return for vitamins (mainly vitamin B_{12}, B_1, and B_7), siderophores, and cytokinins [16,17]. Thus, we also wanted to investigate whether the dominant culturable bacteria associated with dinoflagellates could influence the response of its host to metals and organic pollutants. In addition, we were interested in discerning whether these associated bacteria had similar/different response profiles to metals and organic pollutants compared to their host.

2 | MATERIALS AND METHODS

2.1 | Experimental details

Prorocentrum sigmoides Böhm, a dinoflagellate culture isolated from the coastal waters of Goa, was used for this study. The dinoflagellate genus *Prorocentrum* is a diverse genus, encompassing more than 80 species [18]. Most of the members of this genus are marine, both benthic and planktonic [19]. *Prorocentrum* species prefer high temperature and low salinity for growth [20] and are ubiquitous in marine tropical areas as well as Arctic waters [21]. There are several species that are known to cause harmful algal blooms (HABs). However, *P. sigmoides* is not generally linked to red tides. To our knowledge, there is only a single report of a red tide attributed to *P. sigmoides* in Kochi, Japan, in 1998 [22].

A total of four metals (cadmium, lead, mercury, and tellurium) and four organic pollutants (benzene, diesel, lindane, and xylene) were selected to represent a wide variety of pollutants. The following metal salts were used: cadmium nitrate, lead acetate, mercuric acetate, and potassium tellurite. Among the organic pollutants, lindane is an isomer of hexachlorocyclohexane and is used as an agricultural insecticide [23]. Environmentally relevant concentrations (10, 25, and 50 μM) were selected for analysis.

2.2 | Culturing of *P. sigmoides*

P. sigmoides was grown in f/2 media [24] without silicate and with salinity adjusted to 32 psu, and was incubated under 8-h light:16-h dark conditions at room temperature (RT; 28 ± 2°C).

2.3 | Sensitivity of *P. sigmoides* to metals and organic pollutants

The sensitivity of *P. sigmoides* to metals and organic pollutants was determined using motility inhibition assays, carried out in 48 well microtitre plates, containing 0.8 ml of f/2 media containing different concentrations of metals and organic pollutants and 100 μl of *P. sigmoides* culture (corresponding to approximately 500 cells/ml). Appropriate controls (*P. sigmoides* control, metal control, organic pollutant control) were maintained. The microtitre plates were incubated under 8-h light:16-h dark conditions at RT (28 ± 2°C), for 5 days. Subsequently, the plates were observed for inhibition of motility and change in morphology, at ×100 and ×200 magnification using an inverted microscope. The number of healthy cells was enumerated, compared to *P. sigmoides* abundance in control, and expressed as percentage growth. Four replicates were maintained for each experimental treatment.

2.4 | Quantification of bacteria from *P. sigmoides*

The viable count of the bacteria associated with *P. sigmoides* culture was carried out on Zobell Marine Agar (ZMA) medium. The plates were incubated at RT (28 ± 2°C) for 48–72 h, and viable count was calculated based on the

number of distinct colonies observed. This analysis was carried out in triplicate.

2.5 | Isolation and identification of *P. sigmoides*-associated bacteria

Subsequent to the viable count analysis, morphologically distinct bacterial colonies from the ZMA plates were selected for purification by repeatedly streaking (three cycles) on ZMA plates and used for further characterization. The purified cultures were maintained on ZMA slants at 4°C. The *P. sigmoides*-associated bacterial isolates were identified based on 16S ribosomal RNA (rRNA) gene sequencing and biochemical tests. Polymerase chain reaction (PCR) amplification of 16S rDNA was done using the following eubacterial primers: 27 F: (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492 R: (5′-CGGTTACCTTGTTACGACTT-3′). 16S rDNA sequencing was done at Eurofins Genomics Pvt. Ltd., Bangalore. Gene sequence chromatographs were analyzed using the Chromas Lite software. 16S rDNA sequence was compared against the GenBank database using NCBI BLAST search [25]. The bacterial isolates were also characterized biochemically (Tables S1,S2), based on Bergey's Manual of Systematic Bacteriology [26].

2.6 | Characterization of *P. sigmoides*-associated bacteria

2.6.1 | Scanning electron microscopy (SEM) analysis

The bacterial isolates from *P. sigmoides* were inoculated in sterile nutrient broth flasks and incubated on a shaker (260 rpm) for 24 h at RT. The culture broth was centrifuged and pellet washed with phosphate buffer. The washed pellet was transferred onto a coverslip and used for SEM analysis following standard protocol. The coverslip containing the bacterial cells was immersed in 2% glutaraldehyde prepared in 50 mM phosphate-buffered saline (pH 7) and incubated overnight at RT. Subsequently, the fixed cells were washed using phosphate buffer and then dehydrated using increasing concentrations of acetone (30%, 50%, 70%, and 90%) for 10 min, followed by 100% acetone for 30 min. The smear was allowed to air dry, coated with gold using an autofine coater and visualized using SEM.

2.6.2 | Siderophore production

Siderophore production by *P. sigmoides*-associated bacteria was checked by spot inoculating on Chrome Azurol S agar plates and incubated at RT (28 ± 2°C) for 48 h [27].

2.6.3 | Growth curve of *P. sigmoides*-associated bacterial isolates

The growth curve of *P. sigmoides*-associated bacterial isolates was carried out in Minimal Salt Medium (MSM) with 0.2% glucose (5% inoculum). The MSM flasks were inoculated with fully grown bacterial isolates and incubated at RT (28 ± 2°C) on a shaker (260 rpm). The optical density (OD) at 600 nm was measured using a UV-visible spectrophotometer at 2- or 4-h intervals, until the stationary phase was obtained. This was done in duplicates.

2.6.4 | Influence of *P. sigmoides* cell-free exudate on growth of its associated bacterial isolates

Fully grown, exponential phase *P. sigmoides* culture was filtered through 0.22-μm filter paper. The filtrate (exudate) was added to MSM with 0.2% glucose (2% final concentration). Bacterial isolates, grown in MSM with 0.2% glucose, were inoculated in this medium, at the same concentration as above. Appropriate control (MSM with bacterial culture but without *P. sigmoides* exudate) was maintained. The flasks were incubated at RT (28 ± 2°C) on a shaker (260 rpm). OD at 600 nm was taken using a UV-visible spectrophotometer at 6, 12, and 24 h. Readings were compared between culture with dinoflagellate exudates and control.

2.7 | Influence of associated bacterial isolates on the sensitivity of *P. sigmoides* to metals and organic pollutants

The sensitivity of *P. sigmoides* to metals and organic pollutants in the presence of its associated bacterial isolates was analyzed to determine whether the associated bacterial isolates help *P. sigmoides* to tolerate metals and organic pollutants. For this experiment, only those metals and organic pollutants that inhibited motility or altered the morphology of *P. sigmoides* were chosen.

Prorocentrum sigmoides was treated with 10 μg/ml streptomycin to inhibit its associated bacteria, which was confirmed by the absence of colonies on ZMA spread plated with 100 μl of treated *P. sigmoides* culture. Streptomycin (10 μg/ml) was chosen based on prior standardization experiments with different concentrations (10, 25, and 50 μg/ml) of penicillin and streptomycin, individually and in combination, with the aim of inhibiting *P. sigmoides*-associated bacteria, without hampering the growth of *P. sigmoides* itself (data not shown). Subsequently, the sensitivity of the 10 μg/ml streptomycin-treated *P. sigmoides*

to metals and antibiotics was analyzed based on the methodology mentioned above in Section 2.3, the only change being the addition of 20 µl of bacterial isolates (corresponding to 10^6 cells/ml) to the different experimental isolates.

2.8 | Sensitivity of *P. sigmoides*-associated bacteria to metals and organic pollutants

The sensitivity of *P. sigmoides*-associated bacterial isolates to metals and organic pollutants was also assessed. This was done in MSM medium containing 0.2% glucose. The metals and organic pollutants and their concentrations used were the same as mentioned above in Section 2.2. The media was inoculated with exponentially growing bacterial cultures, incubated at RT (28 ± 2°C) on a shaker for 48 h, followed by measurement of the OD at 600 nm. Appropriate positive and negative controls were also maintained. Two replicates were maintained for this analysis.

2.9 | Statistical analyses

The variation in the sensitivity of *P. sigmoides* and its associated bacterial isolates to different metals and organic pollutants was assessed statistically. The sensitivity of *P. sigmoides* to metals and organic pollutants, in the presence of its associated bacterial isolates, was also subjected to statistical analysis. These analyses were performed using the STATISTICA 8 software. The first step consisted of checking for normality and homogeneity of variances using Shapiro–Wilk's and Levene's tests, respectively. The data (in terms of percentage compared to control) were not normal, even after arcsine transformation. Thus, the nonparametric Kruskal–Wallis test was used. A significance level of 0.05 was considered for the statistical analysis.

3 | RESULTS

3.1 | Sensitivity of *P. sigmoides* to metals and organic pollutants

P. sigmoides showed a statistically significant difference in sensitivity to the different metals tested (the Kruskal–Wallis test, Table 1), at all the three concentrations tested. It was most sensitive to mercury (2% surviving cells compared to control), with motility and growth being inhibited at all the three concentrations tested, followed by cadmium at 25 and 50 µM concentrations (Figure 1a). It showed a concentration-dependent response to all the metals. Statistically significant variation in the sensitivity of *P. sigmoides* to organic pollutants was observed only at 10-µM concentration (the Kruskal–Wallis test, Table 1), wherein *P. sigmoides* was most sensitive to xylene, followed by diesel, benzene, and lindane (Figure 1b). *Prorocentrum sigmoides* showed high sensitivity to organic pollutants at the higher concentrations (25 and 50 µM) (Figure 1b). Overall, *P. sigmoides* exhibited higher sensitivity to organic pollutants compared to metals (Figure 1).

3.2 | Isolation and identification of culturable bacteria from *P. sigmoides*

Actively growing *P. sigmoides* cultures supported culturable bacteria in the range of 10^3 to 10^4 CFU/ml (data not shown). Colonies appeared after a period of 48 h, indicating slow growth of these associated bacterial isolates. A total of three isolates, designated as PD005, PD006, and PD007, were isolated from *P. sigmoides*; only PD005 and PD006 were used for further study as PD007 lost viability after subculturing.

PD005 and PD006 were identified as *B. subtilis* and *B. xiamensis*, respectively, based on the 16S rRNA gene sequencing (GenBank accession nos. MG988381 MG988382 for *B. subtilis* and *B. xiamensis*, respectively).

TABLE 1 Results of the Kruskal–Wallis test to analyze the sensitivity of *Prorocentrum sigmoides* and its associated bacterial isolates to the different metals (cadmium, lead, mercury, tellurium) and organic pollutants (benzene, diesel, lindane, xylene)

		Concentration (µM)		
Test organism	**Analysis**	**10**	**25**	**50**
Prorocentrum sigmoides	Across metals	**.0235**	**.0047**	**.0044**
	Across organic pollutants	**.0035**	.8034	.6761
Bacillus subtilis strain PD005	Across metals	.0833	.0833	.0833
	Across organic pollutants	.129	.3208	.1038
Bacillus xiamensis strain PD006	Across metals	.0833	.0833	.0833
	Across organic pollutants	.0833	.0833	.0833

Note: Significant *p* values are marked in bold.

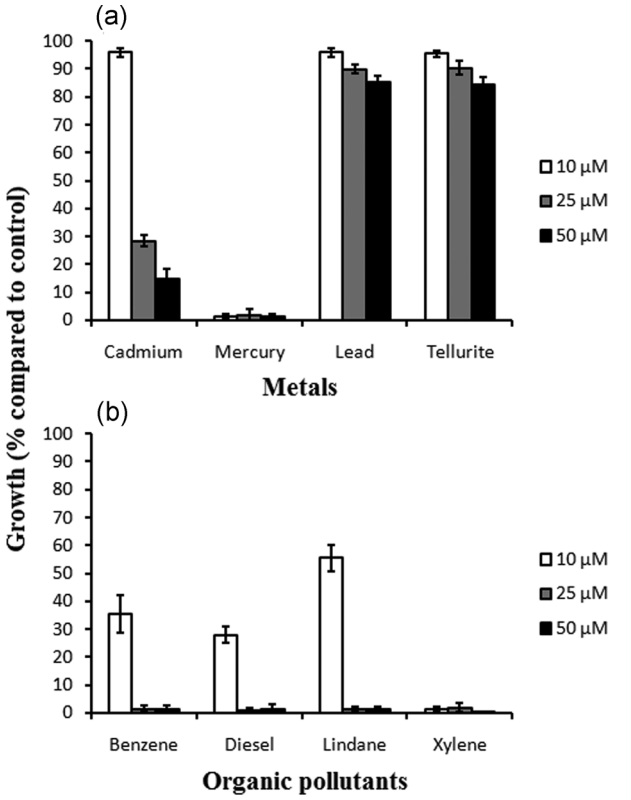

FIGURE 1 Percentage growth of *Prorocentrum sigmoides* (compared to control) in presence of different concentrations of (a) metals and (b) organic pollutants

They also differed in their biochemical characteristics (Tables S1,S2). SEM analysis indicated the rod-shaped morphology of both bacterial isolates, with *B. subtilis* strain PD005 cells joined end-to-end in the form of chains (Figure 2). *B. subtilis* strain PD005 was positive for siderophore production.

3.3 | Growth curve studies of *P. sigmoides*-associated bacterial isolates

Growth curve analysis indicated characteristic lag, log, and stationary phases for both cultures (Figure 3). Both *B. subtilis* strain PD005 and *B. xiamensis* strain PD006 showed an extended lag phase, upto the 12th hour in *B. subtilis* strain PD005 (Figure 3a) and upto the 28th hour in *B. xiamensis* strain PD006 (Figure 3b). The onset of the stationary phase also differed between both cultures, 26th hour in *B. subtilis* strain PD005 (Figure 3a) and 40th hour in *B. xiamensis* strain PD006 (Figure 3b). In addition, *B. subtilis* strain PD005 showed a faster growth rate and also reached much higher concentrations compared to *B. xiamensis* strain PD006 (Figure 3).

As growth of *P. sigmoides*-associated *B. subtilis* strain PD005 and *B. xiamensis* strain PD006 cultures in MSM is slow, an attempt was made to test whether *P. sigmoides* exudate enhances their growth. When grown in the presence of the host *P. sigmoides* exudate, growth of *B. subtilis* strain PD005 as well as *B. xiamensis* strain PD006 was enhanced, especially after 12 h, that is, the onset of the logarithmic phase (Figure 4a,b). The active compound(s) responsible is extracellular in nature, since 0.22-µm filtered cell suspension was used in these experiments.

3.4 | Influence of associated bacterial isolates on the sensitivity of *P. sigmoides* to metals and organic pollutants

Only those metals and organic pollutants to which *P. sigmoides* was sensitive were tested in these experiments, in the presence of *P. sigmoides*-associated *B. subtilis* strain PD005 and *B. xiamensis* strain PD006.

3.4.1 | Metals

In the presence of both *B. subtilis* strain PD005 and *B. xiamensis* strain PD006, the sensitivity profile of *P. sigmoides* with respect to mercury remained unchanged (Figure 5a). However, *P. sigmoides* showed reduced sensitivity (compared to control) to 25 and 50 µM cadmium, in the presence of both *Bacillus* isolates (Figure 5b). In fact, the sensitivity of *P. sigmoides* to 25 and 50 µM cadmium varied significantly between the treatments with and without the *Bacillus* isolates (Kruskal–Wallis test, Table 2).

3.4.2 | Organic pollutants

Both *B. subtilis* strain PD005 and *B. xiamensis* strain PD006 enhanced the tolerance of *P. sigmoides* to benzene and lindane, with one exception, 50 µM benzene, PrS + PSA (Figure 6a,b). The tolerance of *P. sigmoides* to diesel was enhanced only by *B. xiamensis* strain PD006 among the two *Bacillus* strains (Figure 6c). The sensitivity of *P. sigmoides* varied significantly across all the organic pollutants at all concentrations, except 50 µM diesel (Kruskal–Wallis test, Table 2). Differences in the stimulatory effect of *B. subtilis* strain PD005 and *B. xiamensis* strain PD006 on *P. sigmoides* was noticed in benzene and diesel treatments (Figure 6a,c). *P. sigmoides,* which was sensitive to 25 µM diesel under control conditions, showed growth in the presence of only *B. xiamensis* strain PD006 (Figure 6c).

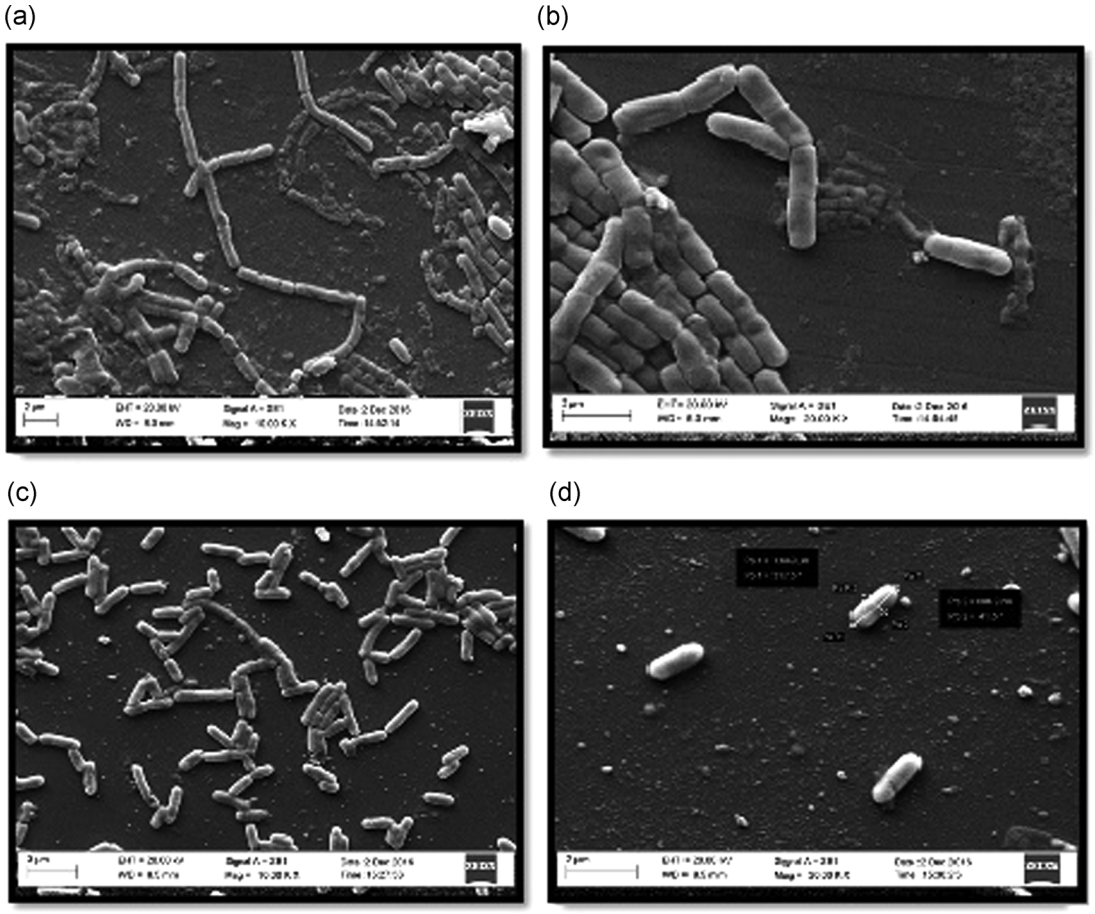

FIGURE 2 SEM images of bacteria associated with *Prorocentrum sigmoides* culture. (a,b) PSA, *Bacillus subtilis* strain PD005, and (c,d) PSB, *Bacillus xiamensis* strain PD006

3.5 | Sensitivity of *P. sigmoides*-associated cultural bacterial isolates to metals and organic pollutants

Both *B. subtilis* strain PD005 and *B. xiamensis* strain PD006 showed concentration-dependent inhibition (compared to control conditions) in response to the different metals and organic pollutants tested (Figures 7 and 8). The observations did not display significant variation across the metals organic pollutants at the different concentrations analyzed (the Kruskal–Wallis test, Table 1).

3.5.1 | Metals

Both *Bacillus* strains were most sensitive to mercury, followed by cadmium (Figure 7a,b). Differing trends were observed for lead and tellurite (Figure 7a,b).

3.5.2 | Organic pollutants

Both *Bacillus* strains showed different trends with respect to the different organic pollutants (Figure 8a,b). *B. subtilis* strain PD005 showed a similar extent of growth inhibition to all the four organic pollutants tested, at 10 and 25 μM concentrations (76–98% compared with control), and was most sensitive to xylene and diesel at 50 μg/ml (Figure 8a). *B. xiamensis* strain PD006 was most sensitive to xylene followed by benzene, diesel, and lindane (Figure 8a).

4 | DISCUSSION

Pollutants like pesticides, heavy metals, and organic pollutants have routinely been reported to affect a range of organisms in marine environments. More importantly, they tend to accumulate and biomagnify as they pass through the food web. As phytoplankton are the base of food webs in marine systems, their response to different pollutants is critical and determines both the fate of pollutants in the food web and their biomagnification potential. Another relevant point to be considered is that the response of phytoplankton to pollutants is not given the attention it deserves, since these effects are not readily visualized. Thus, this study

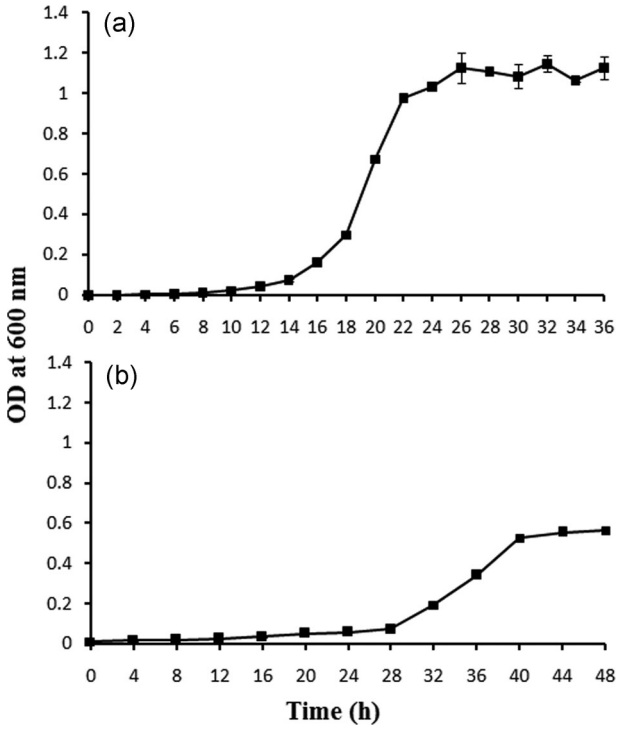

FIGURE 3 Growth curve profile of *Prorocentrum sigmoides*-associated bacteria. (a) *Bacillus subtilis* strain PD005, and (b) *Bacillus xiamensis* strain PD006

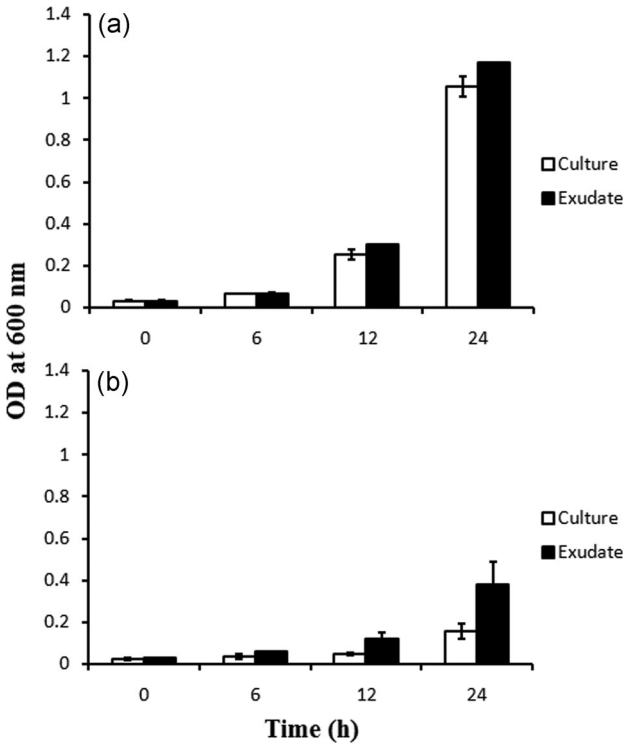

FIGURE 4 Growth curve profile of *Prorocentrum sigmoides*-associated bacteria. (a) *Bacillus subtilis* strain PD005, and (b) *Bacillus xiamensis* strain PD006, with and without *P. sigmoides* cell-free exudates

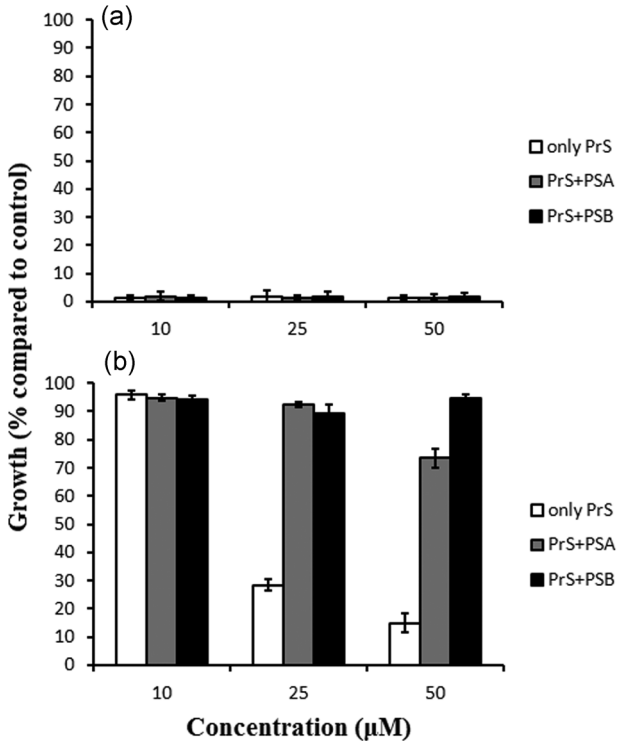

FIGURE 5 Percentage growth of *Prorocentrum sigmoides* (compared to control) in different concentrations of (a) mercury and (b) cadmium treatments, in presence of PSA (*Bacillus subtilis* strain PD005) and PSB (*Bacillus xiamensis* strain PD006)

focuses on the response of *P. sigmoides*, a dinoflagellate (important group of phytoplankton), isolated from the coastal waters of Goa, to different metals and organic pollutants.

The pollutants used in this study (cadmium, lead, mercury, tellurium, benzene, diesel, lindane, and xylene) represented the variety of metal and organic pollutants prevalent in the coastal waters of Goa, due to mining and shipping activities, discharge of sewage and industrial waste, and oil pollution. As early as the 1970s, Singbal et al. [28] reported 26–187 ng/l mercury in regions off

TABLE 2 Results of the Kruskal–Wallis test to analyze the variation in sensitivity of *Prorocentrum sigmoides* with and without its associated bacterial isolates, to cadmium, benzene, lindane, and diesel

	Concentration (µM)		
Pollutant type	**10**	**25**	**50**
Cadmium	.3059	**.0111**	**.0069**
Benzene	**.0189**	**.0067**	**.0179**
Lindane	**.008**	**.021**	**.0175**
Diesel	**.0148**	**.0179**	.9616

Note: Significant *p* values are marked in bold.

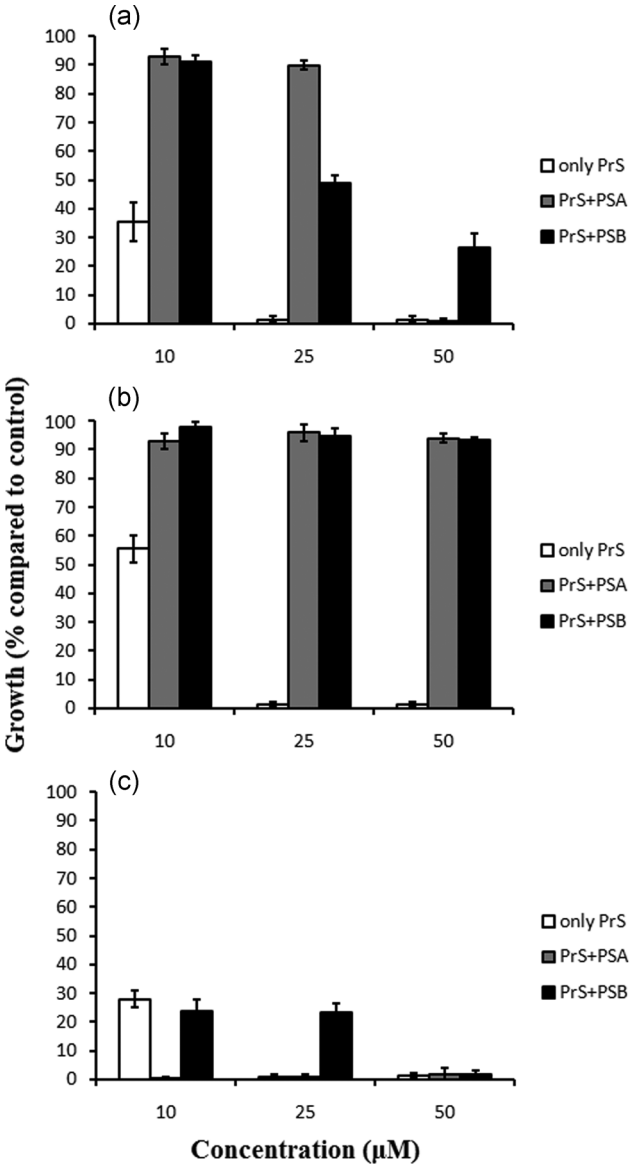

FIGURE 6 Percentage growth of *Prorocentrum sigmoides* (compared to control) in different concentrations of (a) benzene, (b) lindane and (c) diesel treatments, in presence of PSA (*Bacillus subtilis* strain PD005) and PSB (*Bacillus xiamensis* strain PD006)

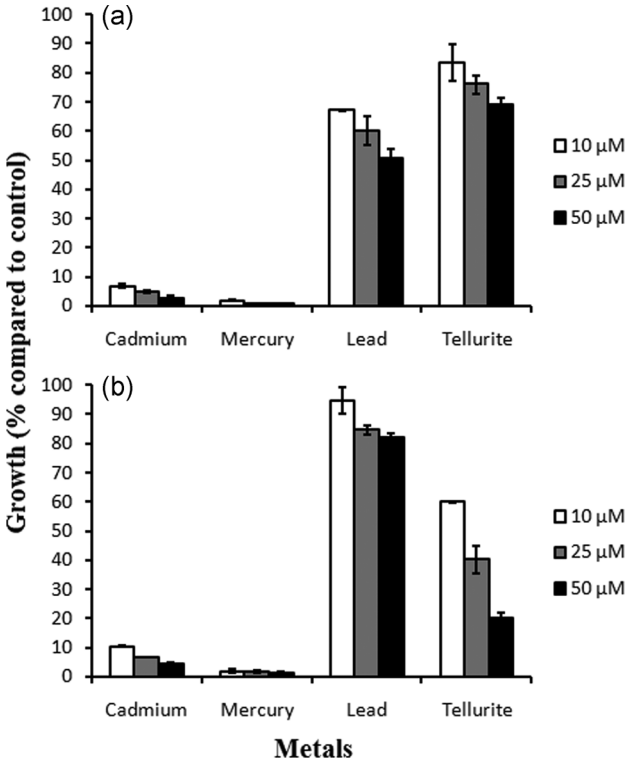

FIGURE 7 Percentage growth of (a) *Bacillus subtilis* strain PD005, and (b) *Bacillus xiamensis* strain PD006 in different concentrations of metals

Goa. Subsequently, after three decades, De et al. [29] observed 152–456 ng/l mercury in Mormugao, Goa. Lead concentrations ranging from 4.5–46.5 µg/g have been recorded in sediments of the Mandovi Estuary [30], whereas mangrove sediments along the Chapora and Mandovi Rivers revealed 7.9–50.5 µg/g of lead [31]. Since 2004, an increase in the concentration of metals in sediment cores of the Ribandar saltern, along the coast of Goa has been noticed [32]. Cadmium and lead have also been reported to bioaccumulate in the short-neck clam *Paphia malabarica*, from the Mandovi estuary, Goa, at levels of 3.8 and 30.3 µg/g dry weight, respectively [33]. Considering organic pollutants, Pasumarthi et al. [34] have highlighted the persistent oil pollution of the coastline of Goa. However, to our knowledge, there are no reports focusing on the concentration of specific organic pollutants in coastal waters of Goa, their effects on marine biota, and especially on phytoplankton.

The results of our study indicated that *P. sigmoides* exhibited a concentration-dependent response to all the metals with maximum sensitivity to mercury followed by cadmium at 25 and 50 µM concentrations. Our results are consistent with reports by Echeveste et al. [35] who reported mercury (Hg^{2+}) to be the most inhibitory to dinoflagellates from the Arctic and Antarctic phytoplankton communities. They studied the tolerance of polar phytoplankton to metals in Arctic and Antarctic oceans, based on changes in chlorophyll content and total biomass. Mercury was the most toxic metal in both the environments, whereas lead and cadmium were toxic only to Antarctic communities, indicating geographical differences in the sensitivity of dinoflagellates to lead and cadmium [35].

Overall, *P. sigmoides* exhibited higher sensitivity to organic pollutants compared to metals and was most sensitive to xylene, followed by diesel, benzene, and lindane. Compared to metals, studies on the effect of organic pollutants on dinoflagellates are scarce. A report by Leitão et al. [36] focused on the effect of Arochlor

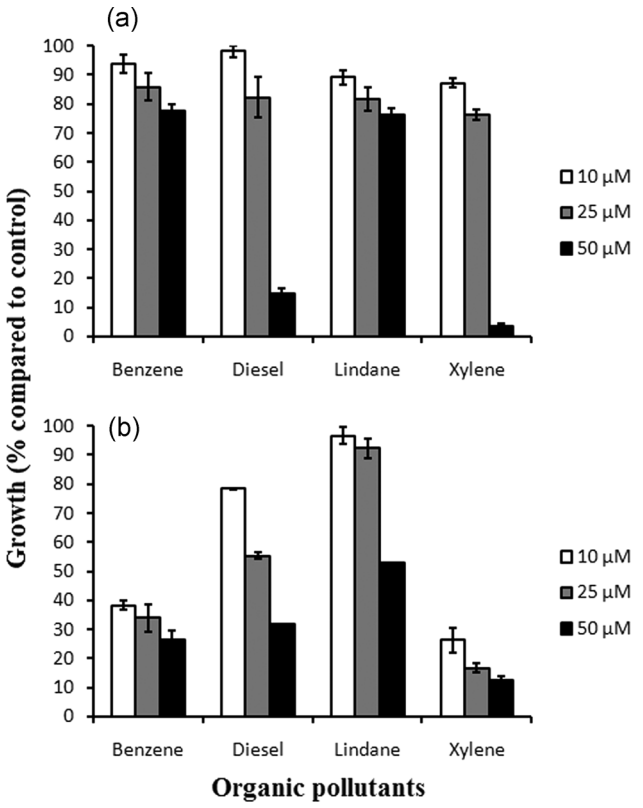

FIGURE 8 Percentage growth of (a) *Bacillus subtilis* strain PD005, and (b) *Bacillus xiamensis* strain PD006 in different concentrations of organic pollutants

1254, an organic pollutant on the dinoflagellate *Lingulodinium polyedrum*. At 120 ppb concentration of Arochlor, cells exhibited an increase in superoxide dismutase (SOD), ascorbate peroxidase activity and peridinin content, suggesting that the above adaptive strategies may be used by dinoflagellate *L. polyedrum* to overcome Arochlor 1254 stress. An increase in SOD activity has also been reported in *Gonyaulax polyedra* (a marine dinoflagellate) in response to metals such as mercury, cadmium, lead, and copper [4]. SOD functions as an antioxidant, to alleviate the oxidative stress caused by metal toxicity. SOD activity was dependent on time of exposure and concentration of metals. Heimann et al. [37] analyzed the effect of metals and organic contaminants on bioluminescence of the dinoflagellate *Pyrocystis lunula*. Bioluminescence was most sensitive to copper, cadmium, phenanthrene, lead, SDS, and nickel (4 h, IC_{50s} 0.96, 1.18, 1.64, 12.8, 15.6, and 73.1 µM, respectively), indicating no clear difference between the effect of metals and organic contaminants [37].

Interestingly, there are no reports on the effect of pollutants on dinoflagellates with respect to their origin, for example, temperate versus polar areas. This is crucial since polar and temperate areas differ not only in terms of temperature, but also the extent of exposure to pollutants. In a study on the toxicity of natural mixtures of organic pollutants to phytoplankton from temperate (Mediterranean Sea) and polar areas (Southern Ocean), Echeveste et al. [38] highlighted that exposure history may play a pivotal role in determining their sensitivity to organic pollutants. They further suggested that Antarctic communities, characterized by sporadic exposure to organic pollutants, have a weaker adaptive capacity compared to temperate environments (that have a higher level of exposure to organic pollutants), and thus, are more susceptible to organic pollutants. Another crucial aspect that must also be remembered is that different phytoplankton groups vary in their tolerance to pollutants. For example, dinoflagellates have a higher sensitivity to South Louisiana sweet crude (LSC) oil compared to diatoms [39]. Thus, understanding the response of dinoflagellates to different pollutants is pivotal to decipher their response to the changing environmental conditions accompanying future climate change.

The quantification of the culturable bacterial community and identification of the dominant bacterial isolates associated with dinoflagellates is the first step in understanding the role of associated bacteria. Though three bacterial cultures were isolated from *P. sigmoides*; only two cultures (PD005 and PD006) were used for further study as the third isolate (PD007) lost viability after subculturing. Such loss of viability of bacterial isolates is often a problem when culturing bacteria associated with phytoplankton cultures, as bacterial cultures require phytoplankton exudates for their optimal growth. In other words, they have mutualistic syntrophic relationships with phytoplankton [15]. Therefore, maintaining them as pure cultures, in the absence of the host phytoplankton, is a challenge. PD005 and PD006 were identified as *B. subtilis* and *B. xiamensis*, respectively. Among dinoflagellates, *Bacillus* has been reported earlier from *Alexandrium tamiyavanichii*, a harmful dinoflagellate [40]. Bacteria belonging to the *Roseobacter* clade (*α-Proteobacteria)* have been isolated from *Prorocentrum lima* cultures [41]. To our knowledge, our study is the first to report *Bacillus* spp. in *Prorocentrum* cultures.

Interestingly, both *Bacillus* strains exhibited slow growth, with logarithmic phases extending upto 48 h. However, their growth was stimulated by the host *P. sigmoides* cell-free exudates, implying that products secreted by *P. sigmoides* positively influenced the growth of both its associated bacterial strains (*B. subtilis* strain PD005 and *B. xiamensis* strain PD006). Phytoplankton cells are known to release organic molecules (sugars, amino acids, and lipids) which stimulate the growth of bacteria associated with them [12–15]. This is usually in return for vitamins, siderophores, and cytokinins provided by the associated bacteria [16,17]. This may also be the case in the present study.

One of the *Bacillus* cultures, *B. subtilis* strain PD005, was positive for siderophore production and showed a higher growth rate compared to *B. xiamensis* strain PD006. This could be beneficial to the host *P. sigmoides* in terms of the bacterial isolate enhancing the acquisition of iron by the host dinoflagellate, under iron-limiting conditions. This is especially important during the later stages of growth in dinoflagellate cultures, when nutrients tend to get depleted. Under such circumstances, siderophore production by associated bacteria can help the host dinoflagellate to sequester iron from the environment [13]. A similar strategy has been observed in *Marinobacter* sp., associated with the dinoflagellate, *Scrippsiella trochoidea* [42]. *Marinobacter* sp. produces vibrioferrin, a photolabile, dicitrate siderophore, that ensures a steady supply of soluble iron (III′) to the host dinoflagellate, presumably in exchange for organic carbon. Amin et al. [43] further state that considering that *Marinobacter* species are associated with different phytoplankton groups, including diatoms and dinoflagellates, the sharing of iron by *Marinobacter* sp. and its host phytoplankton appears to be a widespread strategy prevalent in marine environments. It is plausible that the production of siderophores by *B. subtilis* strain PD005 may play a similar beneficial role for *P. sigmoides.*

Our study indicated that both *Bacillus* strains had a protective effect on *P. sigmoides* with respect to sensitivity to cadmium, benzene, lindane and diesel (with a few exceptions). This could be attributed to the conversion of these toxic compounds to nonharmful forms by the *Bacillus* strains. In fact, this could be one of the additional benefits the associated bacterial community confers not only on their host dinoflagellates, but phytoplankton in general. Studies by Thomas and Robinson [44] and Wang et al. [45] support this hypothesis. The diatom, *Amphora coffeaeformis*, exhibited decreased sensitivity to copper and tributyltin in presence of extracts of associated bacteria compared to in their absence, possibly through production of a soluble (<0.2 μm) factor, which either enhanced the tolerance of *A. coffeaeformis* to copper and TBT or decreased their toxicity [44]. A similar study by Wang et al. [45] on the effect of glyophosate, a herbicide, on the bacterial community associated with *Prorocentrum donghaiense*, a HAB dinoflagellate, revealed that the bacterial community changed after addition of glyophosate. This change enabled *P. donghaiense* to not only tolerate glyophosate in the medium, but also grow on glyophosate as sole phosphorous source. These observations, along with those from our present study, provide support for the idea that associated bacterial communities enhance the ability of their host organisms to grow on metals and organic pollutants. This also serves to widen the range of substrates that the host dinoflagellate can utilize. Interestingly, these changes are inducible and thus play pivotal roles in shaping phytoplankton communities.

The differences in the stimulatory effect of *B. subtilis* strain PD005 and *B. xiamensis* strain PD006 on *P. sigmoides,* noticed in benzene and diesel treatments in our study, suggests that the interactions between *P. sigmoides* and its associated *Bacillus* strains exhibit a certain degree of specificity.

Considering the effect of metals on the associated bacterial isolates, both *Bacillus* strains were most sensitive to mercury, followed by cadmium, with differing trends observed for lead and tellurite. Previous studies on the sensitivity of bacteria to metals [46–48] have revealed similar trends. Baath [46] reported that bacteria were most sensitive to cadmium followed by copper and lead, whereas Sabry et al. [47] noticed that bacterial cultures were most sensitive to cadmium, mercury, cobalt, and zinc and resistant to lead, arsenate, copper, and nickel. These results were similar to those of our present study where bacterial cultures were found to be less sensitive to lead. Rodriguez-Rojas et al. [48] reported that bacteria can resist mercury through *mer* operon. To understand whether the mechanisms of mercury and tellurite resistance are linked, they checked whether mercury resistance can trigger *Pseudomonas* sp. to be concomitantly resistant to tellurite. In presence of mercury, *Pseudomonas* sp. showed enhance resistance to tellurite, indicating that resistance to mercury and tellurite could be linked [48]. This was not observed in the present study, indicating differences in the mechanisms of mercury and tellurite resistance in the *Bacillus* strains.

Both *Bacillus* strains showed varying sensitivity to the different organic pollutants. Lăzăroaie [49] reported that Gram-positive bacteria (*Mycobacterium* sp., *Oerskovia* sp., and *Corynebacterium* sp.) were more susceptible to organic pollutants such as benzene, toluene and ethyl benzene compared to Gram-negative bacteria (*Chryseomonas* sp., *Pseudomonas* sp., and *Burkholderia* sp.), due to their cell-wall structure. However, they may develop resistance to organic solvents either by forming endospores, expressing solvent-deactivating or emulsifying enzymes or induction of general stress regulon, leading to the production of general stress proteins [50]. Since the cultures in our study have been identified as *Bacillus* spp., and produce spores, it is possible that their spores may render them resistant to the effects of organic solvents, in a concentration-dependent manner.

Comparison of the sensitivity profiles of both *P. sigmoides* and its associated *Bacillus* isolates indicated a similarity with regard to mercury and cadmium among the metals, pointing to a co-selection of metal resistance

in both the host dinoflagellate (*P. sigmoides*) and its associated *Bacillus* strains. The similarity in sensitivity profiles of *P. sigmoides* and its associated bacteria to mercury and cadmium is interesting, given that both, the dinoflagellate and its associated bacteria, use different mechanisms to tolerate metals. *P. sigmoides* protects itself against metals by enlarging its cell size, thickening of the cell wall, sequestration of metals by ligands and their subsequent exclusion, and so forth. [51]. On the contrary, bacteria tolerate and resist heavy metals using diverse mechanisms which include extracellular and intracellular sequestration of metals through production of exopolysaccharides, efflux pumps for active transport of metals, presence of plasmids, and so forth. Horizontal gene transfer also contributes to the transfer of metal resistance genes in nature [52]. Considering this and the complexity of quorum sensing molecules involved in the interactions between dinoflagellates and their associated bacteria [53], it can be speculated that these two partners help each other to tolerate and, probably, even detoxify metals. Interestingly, a similar trend was not observed in case of organic pollutants, wherein the two *Bacillus* isolates exhibited higher resistance compared to *P. sigmoides*. This implies that the nature of the pollutant will determine whether the "dinoflagellate host-phycosphere bacteria" functions as a single unit.

In conclusion, this study points to a mutualistic relationship between *P. sigmoides* and its associated *Bacillus* isolates. *P. sigmoides* enhanced the growth of both *Bacillus* spp., through the secretion of extracellular exudates, whereas both *Bacillus* isolates contributed to the resistance of *P. sigmoides* to metals and organic pollutants. This study also highlighted the different sensitivity profiles of *P. sigmoides* and its associated *Bacillus* isolates with respect to metals versus organic pollutants. *P. sigmoides* (the host dinoflagellate) and its associated *Bacillus* isolates exhibited a co-selection of mercury and cadmium resistance whereas the trend was different for organic pollutants.

The above aspects need to be addressed in detail in future studies, to unravel the mechanisms involved in mediating the effects of metal and organic pollutants on dinoflagellates, and the contribution of their phycosphere bacteria to these responses. Another factor that needs to be considered is the mixotrophic mode of nutrition of *P. sigmoides*, and particularly, its influence on the response of *P. sigmoides* to metals and organic pollutants. Additionally, would its mixotrophic mode of nutrition influence its interactions with its associated bacteria, their community dynamics, and sensitivity to pollutants? Further studies focussing on these aspects are needed.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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