



OPEN Impact of multiple-factors on health and infections in marine mussels (*Perumytilus purpuratus*) inhabiting contaminated sites in the Humboldt Current System

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Marine organisms are increasingly exposed to a combination of environmental stressors. However, most studies focus on single factors, limiting our understanding of real-world ecological challenges. This study investigates the combined effects of metal pollution, parasites, pathogens, and environmental variables on the health of *Perumytilus purpuratus*, a mussel species inhabiting the coast of northern Chile. The upwelling system in this area, combined with low water turnover, creates a unique environment in which to study how multiple factors interact. Mussels were sampled from several sites affected by metal discharges. Analyses revealed that individuals from central and northern sites exhibited the highest levels of parasites, pathogens, and tissue lesions. These health impacts were strongly associated with elevated pH, salinity, cadmium and copper concentrations in the water. Findings emphasise the synergistic effects of chemical and abiotic factors, underscoring the importance of incorporating multiple factors interactions into monitoring programmes. Such an approach can enhance predictions of ecological responses, inform conservation efforts, and guide policies addressing global challenges like aquatic pollution. Our study provides critical insights into how combined factors threaten aquatic ecosystems, offering a framework for more comprehensive environmental assessment.

Keywords Bivalve molluscs, Humboldt Current System, Synergistic effects, Histopathology, Immune system, Marine ecotoxicology

Environmental changes are occurring worldwide as a result of a series of human-made stressors. Assessment of both individual and combined responses to these factors is critical to effective environmental monitoring¹. Scholars have acknowledged the importance of evaluating the effects of multiple factors, including those of chemical pollution, in aquatic systems in order to determine the real impact of environmental quality^{2,3}. Lima, et al.⁴ highlight the importance of evaluating and quantifying multiple factors to improve management actions and strategies. They identify gaps between the assessment of combined multiple stressors and the varying responses taken by governments and legislation across different countries. They also emphasise the need for a mutual collaboration to standardize proper methodologies in the field. For instance, spatial analysis tools are used to assess patterns in interplay of stress factors and their effects on ecosystem health⁵. Among the most serious chemical factors in marine environments are metals. These accumulate on the ocean floor due to human activities^{6,7}. Many industries are located along coastlines, accelerating pollution⁸.

Parasites are important natural stressors that have an impact on the development, reproduction, and lifespan of their host. Chemical stressors may potentially intensify the damaging effects of a parasite infection or, conversely, reduce infection rates by increasing in parasite vulnerability^{9–11}. Goutte and Molbert¹² emphasise that interactions between parasite and contaminant are complex and influenced by both biological and environmental factors. These dynamics have been studied extensively in fish and other aquatic organisms^{13–17}.

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However, research on the interplay between parasites, pathogens and metals in bivalves is scarce^{18–22}. Most existing studies have focused on the relationship between parasites, pathogens and abiotic factors such as temperature, salinity, and pH^{23–25}. However, few studies have explored the associations between parasites and pathogens in a context of abiotic and metal factors in molluscs.

Due to their sedentary habits and filter feeding behaviour, mussels are particularly vulnerable to the accumulation of toxic contaminants. As such, they are widely used in biological monitoring of metal pollution^{6,26–28}. Their open circulatory system relies on haemocytes – the primary defence against stressors such as parasites, pathogens and pollutants²⁹. Pollutants are known to induce immunomodulation in molluscs, thus altering immune parameters, which are considered indices of health^{30–32}. In addition, somatic health indices, reflecting an organism’s physiological activity under stress, provide insights into residual energy levels^{33,34}. Waller and Cope³⁵ assert that a holistic, standardised approach to monitoring of mussel health in wild populations is essential to detecting environmental factors and guiding restoration.

The Chilean coastline stretches some 4,700 km down the Pacific shore of South America. The northern city of Antofagasta is a focal point of port and mining activities – the main economic pillars of the region³⁶. San Jorge Bay, located in the northern Atacama Desert, is paradoxically one of the most productive fisheries in the south-eastern Pacific^{37,38} thanks to an upwelling to the south³⁹. However, low water turnover in the bay exacerbates pollutant retention, and historical mining and shipping activities have contributed to significant metal pollution^{38,40}. Although efforts have been made to understand the dynamics of water circulation systems and the chemicals that potentially have an impact on them, comprehensive data concerning these complex systems are unavailable either for the bay in question or anywhere else in Chile.

The purple marine mussel (*Perumytilus purpuratus*, Lamarck, 1819), distributed along the Pacific coast from northern Peru to southern Chile, has been successfully used as a sentinel species for evaluating pollution along the northern Chilean coast^{41–46}. The unique circulation patterns in San Jorge Bay contribute to the retention of mixtures of metals and nutrients³⁸. This makes it an ideal site in which to evaluate how multiple potential factors – including metals – influence interaction between parasites, pathogens, and the health indices of *P. purpuratus* as a model host.

In this study, we hypothesised that mussels inhabiting metal-contaminated sites exhibit higher levels of parasites and pathogens, along with impaired health indices. To test this hypothesis, we assessed parasite and pathogen loads, health indices, and metal concentrations in *P. purpuratus*. We also evaluated the metal content and environmental parameters of seawater to determine their association with mussel health. By examining these interactions, we aimed to provide a comprehensive understanding of how multiple factors impact marine organisms. This knowledge is essential to improving environmental monitoring programmes and informing conservation strategies in the face of increasing global challenges such as aquatic pollution.

Results

Biological characteristics: in vivo and histological surveys

Shell length (SL), total weight (TW), and soft tissue weight (STW) of mussels varied significantly between sites (Table 1). The largest mussels (SL) were recorded at Col and Chim, while the smallest were recorded at Pta. Mussels at Petr and Col had the greatest TW, and those at Pta had the lowest. Mussels from Petr had the highest condition index (CI), and THC levels were highest at Col and Petr. There was no variation in granulocytes and hyalinocytes between sites.

The histological survey revealed the presence of diverse parasites and pathogens. These include one resembling *Conchophthirus* sp. (Fig. 1a) and other unidentified ciliates (Fig. 1b). All of the latter were categorised as ‘ciliates in gills’, virus-like particles (VLPs), and intracellular microcolonies of bacteria named here as *rickettsia*-like organisms (RLOs), intracellular ciliates that resembled Rhynchodid-like Phyllopharyngea (Rhyn) in the digestive gland (Fig. 1c), and trematode metacercariae *Renicola* sp. in vivo in the mantle (Fig. 1d). We also recorded haemocytic infiltration (HI) in gills (Fig. 1e) and digestive gland (categorised as ‘in connective tissue’), along with brown cells (BC) in digestive gland (Fig. 1f).

	Len (Outside South)	Col (South)	Petr (Centre)	Chim (North)	Pta (Outside North)	A/K-W	p
SL (mm)	34.35 ± 2.12 ^b	35.47 ± 1.60 ^a	34.21 ± 2.24 ^b	35.60 ± 1.87 ^a	33.06 ± 2.74 ^c	F _{4,245} =13.63	0.001
TW (g)	6.24 ± 1.27 ^b	7.52 ± 1.40 ^a	7.54 ± 1.36 ^a	6.40 ± 1.14 ^b	5.37 ± 1.33 ^c	F _{4,245} =29.14	0.001
STW (g)	2.20 ± 1.23 ^b	2.43 ± 0.69	2.92 ± 0.78 ^a	2.12 ± 0.67 ^b	2.01 ± 0.94 ^b	H _{4,250} =43.45	0.001
CI	34.29 ± 9.94 ^b	32.10 ± 6.42 ^b	38.56 ± 6.23 ^a	33.15 ± 8.95 ^b	36.34 ± 9.59 ^b	H _{4,250} =22.79	0.001
THC*	6.20 ± 2.10 ^b	11.20 ± 6.12 ^a	10.60 ± 6.87 ^a	6.84 ± 4.94 ^b	7.40 ± 4.93 ^b	F _{4,245} =8.18	0.001
Gran	49.02 ± 9.94	48.98 ± 16.64	47.32 ± 18.94	51.04 ± 22.04	53.14 ± 18.49	F _{4,245} =0.80	0.52
Hyal	50.98 ± 9.94	51.02 ± 16.64	52.68 ± 18.94	48.96 ± 22.04	46.86 ± 18.49	F _{4,245} =0.80	0.52

Table 1. Site differences in biological characteristics (mean ± SD) of *P. purpuratus* sampled from San Jorge Bay, northern Chile. Different letters indicate significant differences between sites (*p* < 0.05). SL = shell length, TW = total weight, STW = soft tissue weight, CI = condition index, THC = total haemocyte count, Gran = granulocytes, Hyal = hyalinocytes. A/K-W = ANOVA/Kruskal Wallis tests. Asterisk indicates x10⁴ cells per ml^l of haemolymph. Sites within the bay: Col = Coloso, Petr = Petroleras, Chim = Chimba Beach. Sites outside the bay: Pta = Punta Itata Beach (north), Len = Lengudo Beach (south).

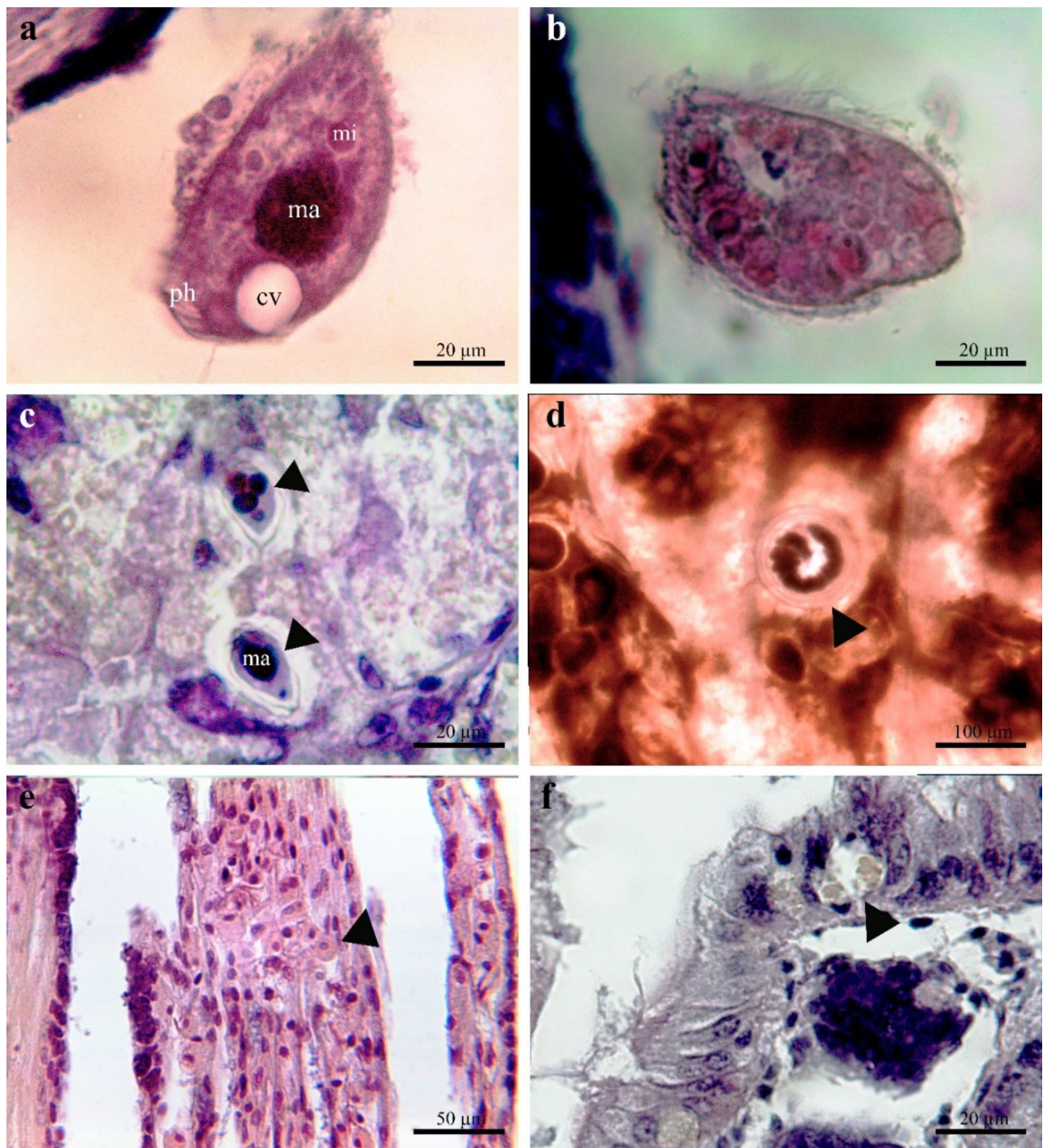


Fig. 1. Micrograph of parasites, pathogens and lesions found in *P. purpuratus* sampled from five study sites in San Jorge Bay, northern Chile. (a) ciliates resembling *Conchophthirus* sp. in gills; (b) unidentified ciliates in gills; (c) intracellular ciliate Rhynchodid-like *Phyllopharyngea* (arrows) in digestive gland; (d) trematode metacercariae *Renicola* sp. (arrow) in vivo in the mantle of *P. purpuratus*; (e) focal concentration of haemocytes between gill filaments (arrow); (f) brown cells (arrows) in the connective tissues. Ma = macronucleus, mi = micronucleus, cv = contractile vacuole, ph = cytopharynx.

Prevalence rates for parasites and pathogens were as follows: gill ciliates (53.2%) and VLPs (30%), RLOs (15.6%), Rhyn (9.2%), *Renicola* sp. in vivo (38.4%), infiltration of haemocytes (28.4%), and brown cells (58.4%). Results by site revealed significant variations in parasites and pathogens. Ciliates in gills were more prevalent in mussels from the centre of the bay (Petr [64%]) and less prevalent in mussels from outside the bay (Len [46%] and Pta [48%]) ($X^2 = 4.59$, $df = 4$, $p = 0.33$). There were no inter-site differences in intensity. Prevalence of RLOs was greatest in mussels from Chim (32%), with significant differences across sites ($G = 12.43$, $df = 4$,

Category	Key findings (Prevalence %)	Statistical results
Ciliates in Gills (Cil-Gill)	Highest prevalence at Petr (64%) and Chim (58%)	No significant difference ($p=0.07$)
Intracellular microcolonies of bacteria (RLOs)	Notable prevalence at Col and Chim (32%)	No significant difference ($p=0.87$)
Virus-like Particles (VLPs)	Higher prevalence at Petr and Chim (30%)	Significant difference ($p=0.001$)
Rhynchodid-like (Rhyn)	Highest prevalence at Petr (28%)	No significant difference ($p=0.27$)
<i>Renicola</i> sp. Metacercariae (Reni-Mnt)	Highest prevalence at Petr (66.3%)	No significant difference ($p=0.67$)
Brown Cells (BC-Ct)	Highest prevalence at Chim (54%) and Pta (48%)	No significant difference ($p=0.36$)
Haemocytic Infiltration (HI)	Highest prevalence at Pta (78%)	Significant difference ($p=0.001$)

Table 2. Summary: key results of parasite, pathogen, and lesion prevalence in *P. purpuratus* across sites, northern Chile.

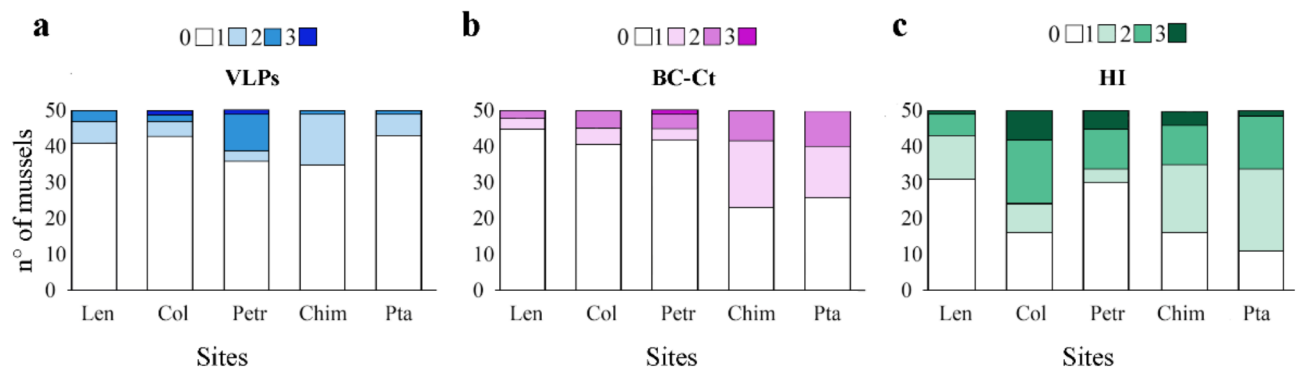


Fig. 2. Intensity and incidence of pathogens and lesions measured at the semi-quantitative scale in mussels from San Jorge Bay, northern Chile. Squares represent intensity of lesion/pathogen; white = no lesion, light colour = low, medium colour = moderate, dark colour = severe. VLPs = virus-like particles, BC-Ct = brown cells in the connective tissue, HI = haemocytic infiltration in the connective tissue.

$p=0.01$), but was generally consistent. VLP prevalence did not vary significantly between sites ($X^2=6.85$, $df=4$, $p=0.14$) (Table 2; Fig. 2b). However, intensity was greatest at Petr (moderate and severe intensity) and lowest at Pta (low intensity) (Fig. 2a). Prevalence of Rhyn varied between sites ($G=15.50$, $df=3$, $p=0.001$), and was greatest in mussels at Petr (28%), but was generally consistent. Trematode metacercariae was most prevalent in mussels from Petr (66.3%) and least prevalent in mussels from Pta (28%) ($X^2=20.3$, $df=4$, $p=0.001$) (Table 2), although variation in intensity was not significant. Prevalence of brown cells (BC) was greatest at Chim (54%) and least at Len (10%). There were no inter-site differences in brown cell intensity. Prevalence of haemocytic infiltration (HI) was lowest in mussels from Len (38%) and greatest in those from Col, Chim, and Pta (68%, 68% and 78%, respectively), ($X^2=38.61$, $df=4$, $p=0.001$) (Table 2). Intensity of HI also varied across sites at Len, presenting intensity ranging from low to moderate, and across Col, Chim, and Pta, ranging from moderate to severe (Table 2; Fig. 2c). More details are shown in Table S1.

Overall, total parasites and pathogens varied between sites. Prevalence was lowest at Pta (60%) and greatest at Petr (92%) and Chim (78%), ($X^2=15.73$, $df=4$, $p=0.001$). In contrast, total lesion prevalence was greatest at Chim (80%) and Pta (82%) ($X^2=30.3$, $df=4$, $p=0.001$) and lowest in mussels from Len (44%) and Petr (48%). Approximately 22% of mussels from Len showed no evidence of lesions, parasites, or pathogens ($X^2=6.7$, $df=4$, $p=0.15$).

Water quality parameters

Water temperature varied across sites, with the highest recorded at Chim (20.32 ± 0.28 °C) and the lowest at Col (16.12 ± 0.20 °C), $F_{4,70}=238.55$; $p=0.001$ (Fig. 3a). Salinity peaked at Petr (34.9 ppt), $F_{4,70}=255.64$; $p=0.003$ (Fig. 3b). Dissolved oxygen (DO) was highest at Chim (4.78 ± 0.01 mg L⁻¹) and lowest at Pta (4.76 ± 0.01 mg L⁻¹), $F_{4,70}=27.18$; $p=0.001$ (Fig. 3c). Levels of pH and chlorophyll *a* were both highest at Petr (8.24 ± 0.07 and 11.95 ± 6.98 µg L⁻¹, respectively), $F_{4,70}=21.08$; $p=0.001$ and $F_{4,70}=9.62$; $p=0.001$, respectively (Fig. 3d, e).

Metal concentrations

Water copper concentration significantly influenced site variability (Factor load=0.996 in PC1; PCA). Cu levels were highest at Pta, followed by Col. Pairwise comparisons revealed significant differences between Pta and all other sites, and between Col and other sites (except Len) (Table S2, Fig. S1). Data concerning metal concentrations in tissues are provided in the supplementary material (Fig. S2).

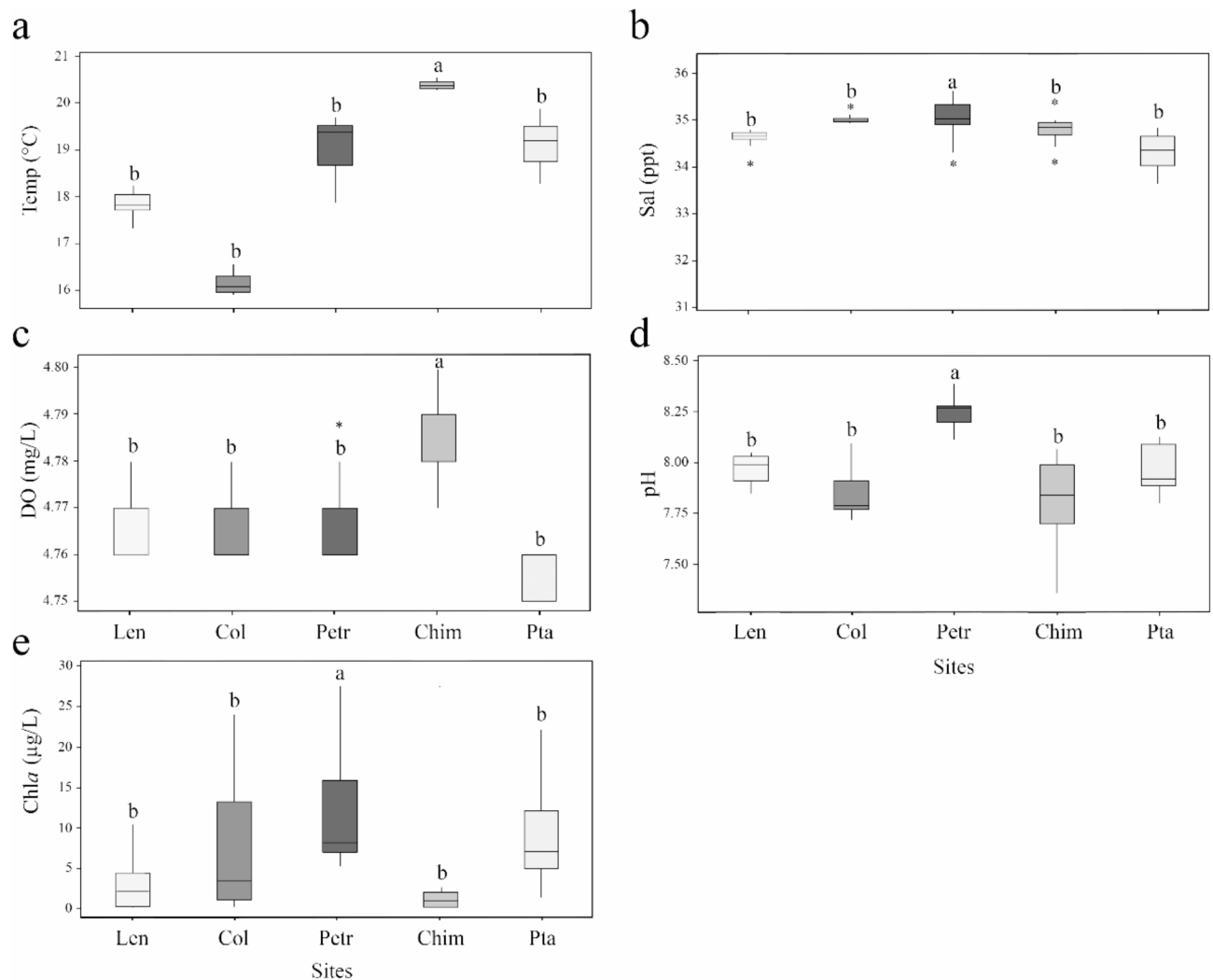


Fig. 3. Site differences in physicochemical parameters of seawater at San Jorge Bay, northern Chile, presented as a boxplot, $n = 15$ measurement points. Different letters indicate significant differences between sites ($p < 0.05$). (a) Temp = temperature, (b) Sal = salinity, (c) DO = dissolved oxygen, (d) pH, (e) Chla = Chlorophyll a. Sites within the bay: Col = Coloso, Petr = Petrolas, Chim = Chimba Beach. Sites outside the bay: Len = Lenguado Beach (south), Pta = Punta Itata Beach (north).

Multifactorial effects

In the CCA, physicochemical parameters and seawater metal concentrations explained 19.5% of the variation in parasites/pathogens harboured per mussel (eigenvalues 0.185 and 0.080 for axis 1 and 2, respectively) ($p < 0.01$). At Petr, there was a greater abundance of trematode *Renicola* sp., intracellular ciliate Rhynchodid-like Phyllopharyngea (Rhyn), ciliates in gills, and virus-like particles (VLPs) (see Fig. 5). These parasites and pathogens were positively correlated with pH and salinity. At Chim, intracellular microcolonies of bacteria (RLOs) were more abundant, which in turn correlated positively with high levels of Cd in seawater. At Pta, mussels presented a greater abundance of brown cells (BC) and haemocyte infiltration (HI), which were positively correlated with Cu (Fig. 4).

The first RDA revealed that physicochemical parameters, seawater metal concentrations, biological body conditions and health indices (size, weight, THC, DHC, CI) accounted for total variations of 10.5% (eigenvalues 0.082 and 0.023 for axis 1 and 2, respectively). At Petr, mussels recorded higher CI, THC, and hyalinocytes (and lower granulocytes). These, in turn, were positively correlated with salinity. Mussels at Col exhibited greater size and body weight (Fig. 5a). In the second RDA, metal concentrations in tissue explained 22.5% of variation in parasites, pathogens, and health indices (eigenvalues 0.16 and 0.06 for axis 1 and 2, respectively). *Renicola* sp., Rhyn, and VLPs predominated at Petr, although no significant correlations were observed between metals in tissue and the presence of parasites/pathogens and health indices (Fig. 5b).

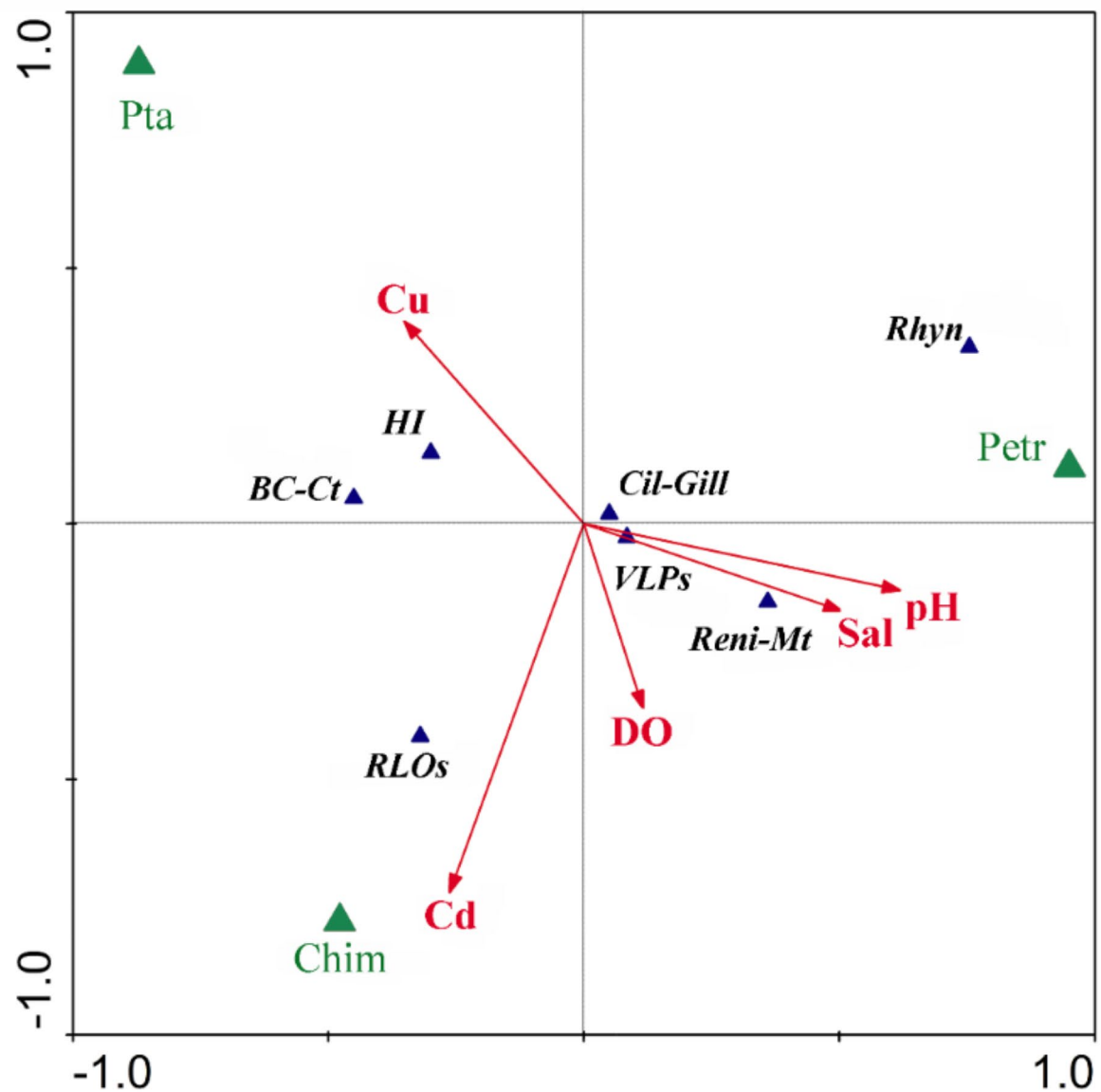


Fig. 4. Canonical correspondence analysis (CCA) showing associations between physicochemical parameters and metal concentrations in seawater with an abundance of pathogens, parasites and lesions in *P. purpuratus* at San Jorge Bay, Chile. Blue triangles are the abundance of parasites/pathogens/lesions. Green triangles are the sites. Red arrows are the physicochemical parameters and metals in the seawater. Cil-Gill = ciliates, RLOs = intracellular microcolonies of bacteria, VLPs = virus-like particles, Rhyn = intracellular ciliates, Reni-Mt = metacercariae *Renicola* sp., BC-Ct = brown cells, HI = haemocytic infiltration. Sal = salinity, DO = dissolved oxygen. Sites; Col = Coloso, Petr = Petroleras, Chim = Chimba, Pta = Punta Itata, Len = Lenguado.

Discussion

San Jorge Bay is subject to the influence of upwelling systems and effluents from domestic sewage and industrial activities. This study is the first to determine the existence of interactions between environmental factors (both chemical and abiotic) and both parasite/pathogen loads and health indices in the mussel *P. purpuratus*. We recorded a greater incidence of pathogens, parasites, and lesions in mussels collected at the central (Petr) and northern (Chim) sites within the bay and outside the bay to the north (Pta). At these three sites, we also identified the highest levels of pH, salinity, and concentrations of Cu and Cd in seawater. In addition, mussels from the Petr site exhibited high total haemocyte count (THC), a key immunological parameter, and high condition index (CI).

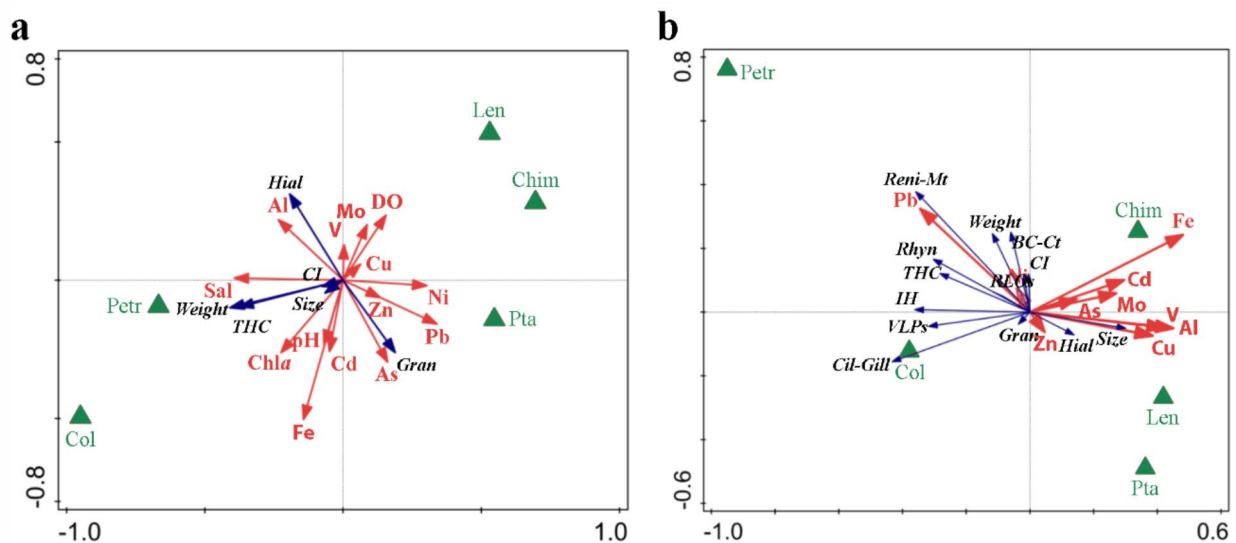


Fig. 5. RDA of environmental variables and metals on parasites, pathogens and lesions; and biological traits of *P. purpuratus* at San Jorge Bay, Chile. **(a)** RDA of environmental variables on parasites, pathogens, lesions and biological traits. **(b)** RDA of metal tissues on parasites, pathogens, lesions and biological traits. Blue arrows are the biological variables and abundance of parasite/pathogen/lesion. Red arrows are the environmental and metal tissues. Green triangles are the sites. Cil-Gill = ciliates, VLPs = virus-like, RLOs = intracellular microcolonies of bacteria, Reni-Mt = metacercariae *Renicola* sp., Rhyn = intracellular ciliates, BC-Ct = brown cells, HI = haemocytic infiltration, CI = condition index, THC = total haemocyte count, Gran = granulocytes, Hyal = hyalinocytes, Sal = salinity, DO = dissolved oxygen, Chla = Chlorophyll *a*, Sites: Col = Coloso, Petr = Petroleras, Chim = Chimba, Pta = Punta Itata, Len = Lenguado.

Assessment of the incidence of parasites and pathogens in the presence of a complex mixture of factors that includes chemicals is challenging. In the present study, the greatest prevalence of parasites and pathogens recorded in *P. purpuratus* was at the centre of the bay (Petr). Our CCA also revealed an association with pH and salinity, with the highest levels detected at the site being 8.2 and 34.9, respectively. These values are in line with maximum values previously reported by CEA⁴⁷. Khosravi, et al.²⁵ found that salinity was the most important factor at the cercariae stage, with the emergence of this trematode usually increasing two-fold with increased salinity. Koprivnikar, et al.⁴⁸ found that the interaction of pH and salinity showed more active behaviour in two different cercariae than a single factor tested. Results of these experiments also showed that the survival of one of them (*Euhaplorchis californiensis*) at salinity 35 and 40 ppt occurred only at pH 8.2 – similar to the pH and salinity reported in the present study at the Petr site. One important aspect mentioned by Byers⁴⁹ is that there are many examples of parasites responding distinctively according to their own optimal salinity. This applies not only to salinity but to other environmental factors. Perhaps the highest levels of pH and salinity recorded in the present study provide optimum conditions to certain parasites and pathogens at the Petr site. Hermosillo-Núñez, et al.⁵⁰ identified the Petr site as having been subject to a medium to high degree of disturbance, finding high mean values of carnivorous biomass. They suggested that a previous pollution incident that had reduced artisanal fishing activities may explain the high level of biodiversity at the site. Unfortunately, they did not evaluate the association between environmental variables and their biological results. In our study, we assessed these and found associations between high pH and salinity and higher parasite/pathogen loads in this species of mussel at the Petr site. Based on our findings, we could suggest that these environmental factors may be optimum for parasites and pathogens recorded at this site. However, further experiments to confirm this cause-effect response should be undertaken.

Intracellular microcolonies named *rickettsia*-like organisms (RLOs) – also reported as *chlamydiales* (CLOs) – are obligate intracellular Gram-negative bacteria^{51,52}. Although little is known about the pathogenicity of RLOs⁵³, some mortality of mussels by RLOs in farms has been reported (e.g. Zhu, et al.⁵⁴, Howells, et al.⁵⁵). In the present study, RLOs were associated with high levels of Cd in seawater in the north of the bay. These results are consistent with other studies in which pollution has been associated with the presence of these infections. Some studies have reported evidence of these intracellular bacteria in polluted areas. Khan, et al.⁵⁶, recorded high prevalence of RLOs in industrial areas with a presence of domestic effluents in the northern Arabian Sea. Likewise, Kim, et al.⁵⁷ reported an association of RLO infections with Cd and Ni in oysters. As such, it is suggested that the greater abundance of RLOs here may influence the high levels of this metal.

Another important physiological health index is the condition index (CI). It is used as an indication of nutritive status in marine bivalves, but it may also offer potential as an indicator of exposure to contaminants in mussels^{58,59}. Zeng and Yang⁶⁰ point out that CI is a non-specific biomarker, and that the physiological condition of bivalves may be influenced by multiple factors. In our study, mussels from the centre of the bay

(Petr) reported the highest CI values. Kanduč, et al.³⁴ found higher CI values in mussels collected from certain ports in comparison to mussels collected from farms. They found that the former presented higher quantities of metal(oids), suggesting that they have a strong defence mechanism with which to battle contaminants. We did not identify associations with any metals in our study, but we did find the highest values of chlorophyll *a* at the site. A number of authors have indicated that Chl *a* values are a measurement of the quantity of phytoplankton present in water, and phytoplankton are the main source of food for mussels. As such, higher Chl *a* values would increase CI in mussels^{61–63}, as was the case here. Thus, the high values of Chl *a* in Mytilidae from our site mean that the latter have plenty of food available to allow growth.

Immunological parameters are critical indicators of poor health and disease³⁵. Mussels lack the immunological memory that protects other organisms against stressors and helps to maintain their overall health²⁹. Carella, et al.⁶⁴ highlight that invertebrates – including bivalves – have a primitive innate immune system and that, although simple compared to that of vertebrates, is highly efficient in its response to infections. Our first RDA supported this, also revealing associations with higher percentages of hyalinocytes and lower percentages of granulocytes. The immune system can also be modulated in response to abiotic factors such as pathogens and parasites^{65–67}. In the mussel *Hyriopsis cumingii*, granulocytes have been shown to be more involved in the phagocytic process in bacterial cells than hyalinocytes⁶⁸. In a study by da Silva, et al.⁶⁹ involving the mussel *Perna perna*, a lower percentage of granulocytes was observed in mussels infected with trematodes compared to non-infected specimens. The authors suggest that this may be due to migration of haemocytes into the affected tissue. Similarly, Silva-Freire, et al.⁷⁰ report a loss of circulation of granulocytes in the oyster *Crassostrea gasar*, resulting from degranulation relating to the damaged tissue. Variations in cell types in infected molluscs may be explained by several processes, such as degradation and/or degranulation of granulocytes, and/or migration of haemocytes from the haemolymph into the connective tissues where the parasite is located⁷¹. Modulation of the immune system is reflected in the increased total haemocyte count and may be due to a variety of parasites and pathogens that are co-infecting mussels from the Petr site, while the lower abundance of granulocytes may be an indication of phagocytosis.

Interestingly, the highest prevalence of haemocytic infiltration in mussels was found at the Pta site. Our CCA showed an association between this lesion and the presence of brown cells in mussels, which correlated with high levels of Cu in seawater. Haemocytic infiltration is commonly considered a significant part of the inflammatory response that has been associated with serious histopathological injury occasioned by a number of factors such as toxicants, neoplasia and parasitosis⁷². On the other hand, brown cells contain lipogenic pigment that serves several response functions, including to aging⁷³, parasites⁷⁴, and pollution^{75,76}. While immune cell infiltration in connective tissue is a common lesion caused by different contaminants, it is also attributed to copper toxicity⁷⁷. Supporting this, Essawy, et al.⁷⁸ reported haemocytic infiltration in gills and large numbers of brown cells after exposing the marine mussel *Lithophaga lithophaga* to Cu for 28 days. Similarly, Nguyen, et al.⁷⁹ showed that Cu modulates the immune system of mussel *P. canaliculus* resulting, in increased haemocyte production. Our results therefore suggest that these non-specific lesions may reveal that mussels from the Pta site are responding primarily to this metal. However, further experimental studies are needed to confirm these mechanisms.

We have discussed the possible relationship between some parasites, pathogens, and lesions and environmental parameters and chemical pollution. The influence of multiple factors on the effects of parasites, pathogens and lesions on organisms is complex^{18,80}. Sauvé⁸¹ strongly encourages scientists to assess the toxicological effects of chemical mixtures. This can be achieved by providing a more comprehensive evaluation of chemical interactions and discovering ways to include toxicant interactions in environmental quality regulations. In our study, multivariate analyses have enhanced our understanding of the most significant environmental variables associated with biological variables, allowing for better comprehension of complex mixtures in the field. However, we must acknowledge that our results need to be complemented by experimental studies to establish cause and effects. Another important limitation of our study is that the sampling was conducted in a single season. We suggest that future studies assess multiple factors in different seasons in order to establish patterns or confirm whether our results are affected by seasonality. This would improve studies of this type. Our observation of the presence of parasites/pathogens indicated a relationship that is clearly not straightforward in field studies, as we found that the combination of factors – both anthropogenic and abiotic – influence the host's responses to these.

Finally, the incorporation of multiple factors into monitoring programmes is essential to prevent and protect our environment. Some authors have pointed out this should be done in order to understand the complexity of natural systems and their interactions^{4,82}, as this is crucial to predicting and managing ecological outcomes under anthropogenic factors such as marine pollution. Furthermore, knowledge of how multiple factors interact will make it possible to improve decisions in relation to policy on conservation, fisheries, climate change and ecology^{1,82–85}. The need to address the cumulative effects of environmental factors cannot be overstated. By understanding how multiple factors interact to influence organisms' health, policymakers and conservationists can develop more targeted and effective strategies for ecosystem management. Furthermore, integrating the present study into broader monitoring frameworks could provide a blueprint for addressing similar challenges in other coastal ecosystems worldwide.

Conclusion

This study highlights the complex interaction between environmental factors and biological responses in *Perumytilus purpuratus*. We observed that associations between parasites/pathogens and the environment do not always involve chemicals; they can also be affected by a number of abiotic factors such as pH and salinity. Furthermore, abiotic and chemical factors were suggested to induce immunomodulation in this mussel. Both types of factors should therefore be taken into account in any future monitoring studies. By identifying associations between immunological and physiological health indices and specific environmental parameters, it provides a basis for future studies on factor interactions. These insights are essential to informing interventions

and policies aimed at protecting our ecosystems like San Jorge Bay. Finally, our findings emphasise the importance of multi-dimensional approaches to studying and managing marine ecosystems. As pressures from human activity intensify, understanding the combined effects of factors will be critical to safeguarding the health of marine life and ecosystem.

Methods

Study area and sampling design

In November 2018, we collected 50 specimens of *P. purpuratus* for haemocyte, histology, and body size analysis. These specimens were selected randomly at low tide from five sites with varying contamination levels. Coloso (Col-South) (23°45'S; 70°27'W) is located near a copper mine loading terminus. Petroleras (Petr-Centre) (23°36'S; 70°23'W) is characterised by high concentrations of copper (Cu), lead (Pb), and zinc (Zn) in water and *P. purpuratus* tissue^{43,86}. Chimba Beach (Chim-North) (23°33'S; 70°24'W) is influenced by heavy fishing boat activity. We collected a further 50 specimens from two sites outside the bay. Lengudo Beach (Len; 23°46'S; 70°28'W) is situated 2.7 km south of Coloso⁸⁷. Punta Itata Beach (Pta; 22°55'S; 70°18'W) is located 85 km north of San Jorge Bay and 30 km from Mejillones⁸⁸ (Fig. 6).

We also collected an additional 15 mussels from each study site, along with water to measure metal concentration. Surface water samples were collected from twelve points at each site, following the protocol developed by Castro and Valdés⁸⁷. Water samples were transported in pre-treated polypropylene bottles with HNO₃ and preserved at 4° C. Live mussels were transported to the Laboratory of Marine Parasitology at the University of Antofagasta for pre-analysis processing.

Haemocyte analysis

Upon arrival at the laboratory, mussel haemolymph (100 µL) was collected from posterior adductor muscle sinuses using sterilised syringes, and diluted to a 1:1 ratio with cold anti-aggregate (modified Alsever's solution) containing EDTA, NaCl, glucose, and sodium citrate⁸⁹. Total haemocytes (THC) were counted under a light microscope at 400x magnification and expressed as mean number of cells mL⁻¹ of haemolymph. May-Grünwald-Giemsa stain was used to identify differential haemocyte counts (DHC) from 200 haemocytes⁹⁰. These were classified as granulocytes and hyalinocytes based on cytoplasmic granule presence⁹¹.

Condition Index (CI)

After haemolymph samples had been taken, total mussel weight (TW) and soft tissue weight (STW) were recorded using a digital balance (precision 0.001 g), and shell length (SL) was measured using a digital calliper (precision 0.01 mm). Condition index (CI) was calculated as wet animal mass (g) divided by total animal weight (g, including shell) multiplied by 100⁹².

Parasite and pathogen survey

Parasites and pathogens were assessed both in vivo and histologically. They were classified using the specialised literature^{93–96}. Taxonomical classification was carried out to the lowest possible level – in some cases, the genus level. In vivo examination consisted of inspection of the mantle tissue under stereomicroscope and calculation of parasite prevalence and mean intensity according to Bush, et al.⁹⁷. Prevalence was calculated as the number of mussels infected by a parasite species, divided by total number of mussels studied, multiplied by 100. Mean infection intensity was calculated as the mean of a parasite species in the group of mussels infected by that parasite. Abundance was the number of individuals of one particular parasite/pathogen species or lesion in/on a single host, regardless of whether or not the mussel was parasitised⁹⁷.

Histological analysis was conducted on digestive glands and gills, dissected and fixed in Davidson's for 24 h before transfer to 70% ethanol. Samples were dehydrated and embedded in Paraplast Plus, sectioned (5 µm) using a microtome (Minot Leica RM2125) and stained using Harris's haematoxylin and eosin. Prevalence of parasites, pathogens, and lesions was calculated as the number of mussels recorded with a particular parasite, pathogen, or lesion divided by the total mussels studied per site multiplied by 100⁹⁷. The intensity of counted parasites and pathogens per histology slide per organ per mussel was calculated according to Bush, et al.⁹⁷. Intensity of uncountable parasites/pathogens and lesions was categorised according to a semi-quantitative scale: 0 = none present, 1 = low (< 20% of tissue affected), 2 = moderate (20–50% of tissue affected), and 3 = severe (> 50% of tissue affected). Figures were on Montenegro, et al.⁹⁸ and showed intensity and incidence of parasite, pathogen, or lesion. Additionally, abundance was used for multivariate analyses. In the case of uncountable parasites, pathogens, or lesions, the same score described above was used for abundance.

Metal content analysis

Tissue and water samples were analysed for metal to determine concentration levels. Tissue samples (500–1,000 mg) were homogenised using a porcelain pestle and mortar. Digestion of the samples was with 10 mL of HNO₃ (Suprapur[®], Merck) in a microwave oven (Perkin-Elmer) following USEPA⁹⁹. Digested samples were diluted to a volume of 25 mL with deionised water using an Optima™ 8300 ICP-OES spectrometer. Concentrations of copper (Cu), arsenic (As), molybdenum (Mo), lead (Pb), nickel (Ni), zinc (Zn), vanadium (V), iron (Fe), and cadmium (Cd) were recorded.

Water samples were also processed using the USEPA¹⁰⁰ methodology. Tissue sample results were expressed as mg/kg wet-weight basis and water sample results as µg⁻¹L. The analytical procedure was validated with triplicates, blanks, and standard references of DORM-3 (dogfish), TORT-2 (lobsters hepatopancreas), and SLEW-4 and AQUA-1 (water). More details are shown in Tables S3, S4, and S5.



Fig. 6. Location of study sites in northern Chile. Sites within the bay: Col = Coloso, Petr = Petroleras, Chim = Chimba Beach. Sites outside the bay: Pta = Punta Itata (north), Len = Lenguado (south).

Water quality parameters

Hydrological parameters (temperature, salinity, dissolved oxygen, pH, and chlorophyll *a*) were evaluated at a depth of 0.5-m in 15 points at each site using a multiparameter hydrology meter (YSI Multiparameter, Xylem, USA). A boxplot was created using Minitab version 16 for Windows.

Statistical analyses

Normality and homogeneity requirements were tested prior to statistical analysis. One-way ANOVAs were conducted followed by Tukey's post-hoc tests to compare differences across sites between mussel shell length (SL), total weight (TW), soft tissue weight (STW), condition index (CI), total haemocyte count (THC), different haemocyte count (granulocytes and hyalinocytes), and intensity of parasites, pathogens and lesions. When assumptions of normality and homogeneity of variances were not met, Kruskal-Wallis tests with post-hoc Dunn

tests were conducted. Prevalence of parasites, pathogens, and lesions at each site was evaluated using the Chi Square test. These univariate analyses were performed in Statistica version 6.0 for Windows.

Principal component analysis (PCA) and Permutational multivariate analysis of variance (PERMANOVA) (Euclidean distances in both) was used to determine statistically significant differences in seawater metal concentration across sites. Canonical correspondence analysis (CCA) was used to evaluate the association of physicochemical parameters and metal concentrations in seawater with the abundance of lesions/parasites/pathogens found in mussels at each site^{101,102}.

A redundancy analysis (RDA) was conducted to evaluate the linear effects (log-transformed data) of the physicochemical parameters of seawater and metal concentrations at each site on mussel health indices (CI, THC, DHC, and size and weight). In a second RDA, we evaluated correlations between metals contained in body tissue (log-transformed data) and the abundance of lesions/parasites/pathogens and mussel health indices. Monte Carlo permutations (500 interactions) were used to test the significance of canonical axes. Variables with inflation factor > 5 were removed from the analysis¹⁰³. The analyses were conducted in CANOCO 5.0¹⁰⁴.

Data availability

Data is available from the corresponding author on request.

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Diana Montenegro: Conceptualization, Investigation, Methodology, Writing original draft and final draft. Maria Teresa Gonzalez: Statistical analyses, editing manuscript and final draft.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

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