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Effects of brine discharges on newly hatched larvae of the rocky-shore keystone gastropod *Concholepas concholepas*

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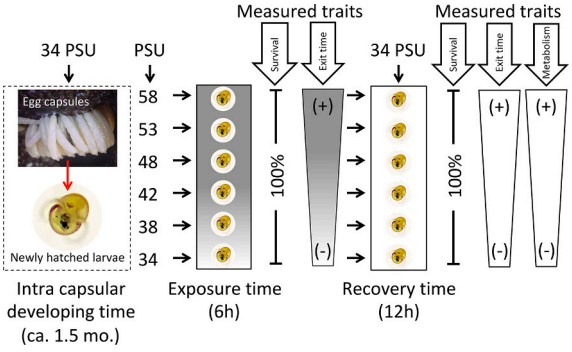
HIGHLIGHTS

- Undiluted brines do not kill newly hatched *Concholepas concholepas* larvae.
- After acute exposure, reduced larval mobility was observed above 42 PSU.
- After a recovery period, reduced larval mobility was observed above 48 PSU.
- After a recovery period, enhanced larval metabolism was observed above 38 PSU.

GRAPHICAL ABSTRACT



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ABSTRACT

In response to drought challenges in Chile, desalination plants are becoming crucial. Consequently, the implementation of more osmosis reverse desalination plants in central-northern Chile has been promoted, but the consequences of brine discharges on local organisms are largely unknown. The Chilean abalone (*Concholepas concholepas*) represent the most economically important marine resource exploited along the Chilean coast. In this study, newly hatched larvae of *C. concholepas* were used to investigate the effects of brine discharges of a reverse osmosis desalination plant on coastal organisms. Under laboratory conditions, larvae were exposed to various dilutions of a brine to assess the consequences on lethal (survival) and sublethal (swimming performance and metabolism) traits. The results indicated that brine discharges had no effects on larval survival. However, undiluted brines (ca. 56 PSU) and salinities above 38 PSU led to increased metabolic rates and a reduction in the swimming performance of the larvae. We suggest mitigating the negative effects of brines on newly hatched *C. concholepas* larvae by situating discharge points in highly hydrodynamic zones or as far as possible from

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mating and larval hatching areas. Additionally, we recommended using diffusers to enhance mixing and dilute brine to sub-lethal levels (below 48 PSU).

1. Introduction

Desalination plants enable the extraction of drinking (or fresh) water from seawater or brackish sources, thereby helping to meet the water demands of human consumption and industrial use, all without further depleting freshwater resources in water scare regions [1]. Therefore, desalination plants are a solution to the shortage of drinking water in many regions [2]. Regardless of whether a desalination plant is associated with the production of water for human consumption or industrial activities such as mining, an inevitable consequence of its operation is the discharge of hypersaline brines into surrounding coastal or estuarine environments, which may have no significant impacts or could lead to substantial environmental problems [3].

Over the past 12 years, Chile has been experiencing a prolonged period of drought, which is partially attributed to climate change [4,5], and has resulted in a current water crisis. Desalination plants are a good solution for addressing water scarcity in regions with limited freshwater resources [4]. In fact, this has encouraged the exploration of initiatives aimed at formulating a national desalination strategy, with the purpose of establishing guidelines and priorities for the use of seawater and the installation and operation of reverse osmosis (hereafter RO) desalination plants [6]. These efforts are intended to improve the quality of life for residents of coastal communities in north-central Chile by supplying water for human consumption, agriculture, as well as for various industrial uses, primarily in mining. In these regions the coastal area is characterized by a year-round upwelling activity and a near constant mean salinity field of 34 PSU. In fact, the seasonal variability does not exceed one salinity unit [7,8] with a minimum in summer and fall time in response to a lower presence of high-salinity subsurface water of equatorial origin exported to the surface by the upwelling dynamics [9].

Currently, in Chile, there are 38 operational RO desalination plants with various capacities, totalling 739,411 m^3d^{-1}. Among these operational plants, it is worth mentioning the small desalination plant designed for rural water consumption, capable of providing between 1 and 100 m^3d^{-1} for human use in these rural coastal areas. In this context, information regarding the potential consequences of brine discharges on local coastal organisms is largely needed. So far, the locally generated information is scarce and has been used to support negative effects of highly elevated salinities on coastal organisms such as macroalgae [10–13] and the marine angiosperm *Zostera chilensis* [14]. However, studies investigating the consequences of RO brine discharges on relevant traits of local invertebrate model species are still largely missing (but see [15]).

The muricid gastropod *Concholepas concholepas* is a key artisanal resource targeted by several thousand divers and intertidal food gatherers along the Chilean coast [16,17]. Benthic populations of this species are associated with subtidal and intertidal habitats [18] and their reproduction involves the production of egg capsules in both environments [19,20]. During the mating and egg capsule lying period, reproductive aggregation and egg capsules are registered up to a depth of 30 m [20]. The egg capsules remain cemented to intertidal and subtidal substratum during the intracapsular egg development for ca 1 to 2 months [19,20]. Then, after hatching, the veliger plankton-feeding larval stage remains in the plankton for ca. 3 months until reaching competence and settlement [21,22].

Although local hydrography might affect larvae distribution in *C. concholepas*, newly hatched larvae are commonly found either at the surface or depths of <10 m [23]. However, it has also been suggested that this species undergoes deeper larval development [24]. At the end of the larval period, competent larvae of *C. concholepas* become epineustonic found mainly at the sea surface [21–23]. This suggests that once hatching takes place near the seabed or they reaches deeper waters, newly hatched larval stages may come into contact with brine discharges from desalination plants located near the seabed. The long pelagic phases of these larvae makes *C. concholepas* an attractive biological model for testing lethal (survivorship) and sub-lethal (alteration in larval behaviour and metabolism) effects due to brine discharges into coastal waters. Finally, the main reason for choosing *C. concholepas* as a model species to assess the risk of brine discharges in the present study is the immense environmental concerns of the Chilean fishers and scientific community regarding the potential consequences on coastal organism and ecosystems of the future implementation of RO desalination plants as a solution to the increasing demand of water resources in times of water security [25].

Swimming performance and metabolism are integral to the survival, dispersal, growth, and overall success of marine invertebrate larvae in complex and dynamic oceanic environments. Swimming performance directly affects the larvae's ability to disperse, find suitable habitats, avoids predators, escape from unfavourable conditions and reach areas with better resources and safety [26,27]. Off north-central Chile, the larval distribution, dispersal, and abundance of *C. concholepas* along the shallow nearshore fringe (<5 km from shore) are influenced by tidal currents and year-round coastal upwelling activity [28], which is forced by the alternating periods of intensification and relaxation of the equatorward winds [29]. The associated coastal currents comprise a surface coastal jet flowing northward at mean speeds ranging between ca. 10–20 cms^{-1} and can reverse under southward wind stress forcing. The mean cross-shore circulation consists of an offshore flow (ca. 5–10 cms^{-1}) within the surface Ekman Layer and a weaker shoreward return flow in the subsurface, associated with the equator-ward alongshore pressure gradient. The latter geostrophic current feeds the coastal upwelling and their slow maximum positive vertical flow (ca. 0.1 cms^{-1}), which tilts the isotherms toward the surface along the coast during the upwelling episodes [30–34].

There is evidence indicating that the interaction of these coastal circulation features with *C. concholepas* veligers mostly occurs near the bottom during the first weeks. However, once the larvae become competent, they swim at velocities within the range of the vertical currents, toward the sea surface, feeding on plankton until they metamorphose on rocky intertidal and shallow subtidal bottoms [22,24,28]. Furthermore, the vertical positioning of competent larvae has also been proposed as a mechanism of nearshore larval retention during coastal upwelling conditions to avoid offshore advection by surface offshore Ekman current [35]. Therefore, given that these larval swimming traits are crucial factors in the subsequent successful settlement and establishment of the nearshore benthic population of *C. concholepas*, changes in salinity associated with brine discharges could potentially impact these events by affecting their larval performance.

Furthermore, metabolism is essential for invertebrate larvae as it regulates energy production, growth, development, maintenance, and a variety of physiological functions that contribute to their successful settlement and transition to adulthood [36]. On the other hand, ion transport, facilitated by the sodium pump (Na+, K + -ATPase), is one of the major energy-consuming processes [37,38]. It plays a crucial role in regulating ion gradients necessary for various essential physiological processes of ecological relevance, such as osmotic balance [39] and nutrient uptake [40].

In the marine realm, osmoconforming invertebrates are common, maintaining their internal fluids isotonic to the surrounding seawater. However, this strategy has limitations, requiring tolerance to fluctuations in seawater salinity [41]. Osmoconformers are typically found in environments with stable salinity, such as open ocean areas [42]. In

contrast, osmoregulators actively regulate internal fluid osmolarity to maintain a constant concentration, regardless of changes in surrounding salinity [41]. This ability allows osmoregulators to inhabit a broader range of aquatic environments, from freshwater to highly saline conditions [42]. Newly hatched larvae of a sub-littoral species would not normally encounter stable salinity levels as those of the open sea. However, in the southern waters of Chile, early larval stages of *C. concholepas* are typically found in areas with low salinities ranging from 5 to 30 PSU, and larval abundances tend to be low when salinity levels drop below 15–20 PSU [23]. Although in southern Chile newly hatched larvae of this specie may not encounter brine discharges, their presence in waters with this such a wide salinity range suggests that these larvae might have evolved mechanisms to cope with changes in salinity. This could involve passive adjustment in tissue fluids to maintain osmotic balance, allowing them to exhibit osmoconformity without incurring additional energetic costs. Alternatively, these larvae might have also evolved an alternative, energetically costly mechanism of osmoregulation. Therefore, in light of the existing evidence, it is not clear which mechanism is used by early larval stages of *C. concholepas* to successfully cope with changes in sea salinity.

By examining the behavioural and physiological components of fitness under controlled laboratory conditions, we can provide crucial insights into the dynamics of natural populations of a given species. Therefore, understanding the potential effects of brine discharges on local species as newly hatched *C. concholepas* veliger larvae (hereafter newly hatched larvae) could help guide recommendations to mitigate the consequences of brine discharges on marine organisms inhabiting coastal environments. We hypothesize that elevated salinities can have negative effects on newly hatched larvae survival and/or increase energy requirements and potentially posing limitation on their swimming performance. We also hypothesize that the effects of abnormally elevated salinities on metabolism and survival would provide insights into whether the larvae are actively incurring elevated energy costs to maintain homeostasis for successful acclimation (osmoregulators) or if they are either incurring low costs or not incurring such costs at all (osmoconformers). In this study, we examined the lethal (survival) and sub-lethal (swimming performance and metabolism) effects of acute and static exposures to various dilutions of a brine discharge on newly hatched veliger larvae under laboratory conditions to understand larval tolerances and potential damage.

2. Materials and methods

2.1. Collection of the study organisms

Mature egg capsules of *C. concholepas* were obtained from controlled spawning of brood specimens maintained in captivity in the laboratory at Coquimbo (23° 45′S; 70° 27′W), where all the experiments were conducted. Under the following conditions, running seawater at ca. 34 PSU and temperatures ranging from 15 to 17 °C, natural hatching of *C. concholepas* takes place after ca. 1.2 month (Manríquez unpublished data). Fully mature capsules of this species are easily identifiable by their brown colour and the presence of larval movement inside [20]. When mature egg capsules were identified in the rearing tank, they were carefully removed with a scalpel, cleaned with a soft paintbrush, and placed in 0.3 L plastic beakers filled with 0.45 μm filtered seawater (hereafter FSW). Special care was taken to ensure that only egg capsules laid by three different females were removed and used in the experiments. Under a stereomicroscope (Olympus SZ61), the egg capsules were placed in a glass Petri dish to assess the swimming activity of the contained veliger larvae, and the status of the vitelline plug at the tip of the egg capsules was examined. Only egg capsules bearing actively swimming larvae and with the vitelline plug showing clear signs of dissolution or opening were considered as a source of experimental larvae. One selected egg capsule assigned to each female was placed in a small glass Petri dish filled with FSW. Then, with the help of a fine dissection pin, the loose vitelline plug was carefully removed. Once the vitelline plug was removed, the very end of the egg capsule was cut to enhance larval hatching. The same procedure was conducted with a single egg capsule from each of the three females. The newly hatched larvae obtained from the three females were placed together in a large glass Petri dish filled with FSW, then poured into a container filled with FSW at normal salinity and gently mixed to ensure that larvae from different females will be evenly distributed across treatments. Then, larvae were assigned from this stock solution to the experimental salinities tests to evaluate their consequences on larval survival and swimming performance (first and second experiments) and larval metabolic rate (third experiment). In the three experiments, the newly hatched larvae were not feed with an external supply of microalgae. This is possible because they hatch with maternal provisioning that allows them to survive for ca. 1 week without an external food supply (PH Manríquez pers. obs).

2.2. Brine discharges collection and preparation of experimental dilutions

The saline brine was obtained from a rural seawater RO desalination plant located at La Higuera (29° 30′S; 71° 16′W), and then transported in borosilicate blue capped bottle to the laboratory at Coquimbo (29°57′ S; 71° 21′ W) where the experiments were conducted. This plant discharges the brine through a PVC pipeline not directly into the sea, but onto land composed of rocky boulders, located approximately 30 m above the highest sea level. The brine must percolate through the boulders and a sandy beach before reaching the sea. However, the brine was obtained at the discharge point and before its percolation through the boulders and sand was considered suitable for conducting the experiments. In the laboratory, before the beginning of the experiments, to remove bacteria and particulate material the brine was filtered at 0.2 μm and then maintained at the experimental temperature (16 °C) by partially submerging the bottles in a water bath with running water set at that temperature. The brine salinity was measured using a Hanna 98,194 multiparameter with a sensor Hanna HI 7698194, and the target salinities obtained by dilution with FSW (Table 1). The tests were implemented by preparing a dilution series of the brine to achieve the target salinities (58, 55, 53, 48, 42, 38, and 34 PSU), representing dilutions of 0 %, 5 %, 9 %, 17 %, 26 %, 34 %, and 41 % of the undiluted brine, respectively. These dilutions correspond to ca. 71 %, 60 %, 53 %, 40 %, 25 %, 12 %, and 0 % of excess salinity above average environmental salinity, respectively. Fresh seawater (FSW) was used for dilution. Moreover, two control salinities were used: normal FSW (34 PSU, hereafter 34 N), and reconstituted seawater (34 PSU, hereafter 34R) made by diluting brine with FSW to achieve the normal salinity. Also, before being used, all saline solutions were vigorously aerated. In addition to salinity, the pH of the experimental dilutions was measured using a Metrohm 913 pH meter. This measurement was conducted at the beginning of each experimental period for both the first and second experiments at each salinity treatment (see Table 1).

In the three experiments (below), we focus on the exposure effects to the brine plume in the proximity of the outfall in the short term, during which the newly hatched larvae were kept for 6 h (acute exposure period) at different salinities ranging from +4 to +24 PSU above their average environmental salinity. This simulates an extreme period of exposure to a hypersaline brine originating from a desalination plant. Until now, it is not clear how drifting organisms are affected by the brine plume [43], but their direct exposure time to the hypersaline area is expected to be relatively short. Indeed, several studies have measured the rapid and abrupt dilution of the saline plume just meters from the discharge point. For example, Sadhwani et al. [44] recorded a dilution from 75 to 38 PSU within 20 m from the brine outlet. Therefore, the acute exposure period of 6 h used in the present study is in the order of magnitude that newly hatched larvae might face in nature. This suggests a good connection between the study's timeframe and the potential real-world conditions in the vicinities of the brine discharge.

Table 1
Seawater salinity (PSU) and pH levels (mean ± SE) throughout the experimental treatment with newly hatched *Concholepas concholepas* veliger larvae during each experimental period for the first and second experiment at each salinity treatment. In each experiment, regardless of the experimental salinity during the exposure period (T6), one set of pH and salinity values is provided for T0 and T12-24. This is because the larvae were maintained in control seawater before (T0) and after (T12-24) exposure to the experimental salinities.

	First experiment					
	T0		T6		T12-T24	
Treatment	Salinity	pH	Salinity	pH	Salinity	pH
1. 58			58.47 (0.91)	7.76 (0.01)		
2. 55			55.12 (0.42)	7.74 (0.02)		
3. 53			52.98 (0.22)	7.73 (0.01)		
4. 48	34.72 (0.16)	7.76 (0.01)	48.34 (0.17)	7.75 (0.01)	34.85 (0.23)	7.74 (0.01)
5. 42			42.23 (0.23)	7.75 (0.01)		
6. 38			38.12 (0.18)	7.77 (0.01)		
7. 34N			34.93 (0.20)	7.75 (0.01)		
8. 34R			34.81 (0.35)	7.72 (0.01)		

	Second experiment					
	T0		T6		T12-T24	
Treatment	Salinity	pH	Salinity	pH	Salinity	pH
1. 58			58.47 (0.23)	7.74 (0.01)		
2. 55			55.34 (0.22)	7.75 (0.02)		
3. 53			53.18 (0.20)	7.74 (0.01)		
4. 48	34.48 (0.21)	7.75 (0.01)	48.32 (0.23)	7.74 (0.01)	34.59 (0.12)	7.75 (0.01)
5. 42			42.18 (0.12)	7.73 (0.01)		
6. 38			38.34 (0.19)	7.74 (0.01)		
7. 34N			34.11 (0.20)	7.74 (0.01)		
8. 34R			34.32 (0.35)	7.73 (0.01)		

	Third experiment					
	T0		T6		T12-T24	
Treatment	Salinity	pH	Salinity	pH	Salinity	pH
1. 58			58.34 (0.18)	7.73 (0.01)		
2. 55			55.33 (0.17)	7.74 (0.01)		
3. 53			53.19 (0.17)	7.72 (0.01)		
4. 48	34.69 (0.17)	7.74 (0.01)	48.43 (0.16)	7.75 (0.01)	34.56 (0.01)	7.75 (0.01)
5. 42			42.34 (0.09)	7.73 (0.01)		
6. 38			38.29 (0.12)	7.72 (0.01)		
7. 34N			34.23 (0.11)	7.75 (0.01)		
8. 34R			34.46 (0.21)	7.73 (0.01)		

2.3. Experiment 1. Effects on survival

This experiment consisted of 6 h acute static tests with naturally hatched larvae without an external larval food supply and with sporadic aeration. Static tests were chosen in order to minimize the potential negative effects on larval performance associated with water replacement. In this experiment, six groups of 10 newly hatched larvae each were assigned from the pool of larvae to each of the six wells of a multi-well plate, with a separate multi-well plate designated for each of the 6 salinity treatments and the 2 control conditions. In this experiment, the newly hatched larvae were assigned to two daily experimental salinities, covering a total of 8 experimental salinities over 4 consecutive days (Table 1). The water temperature was maintained at 16 °C ± 1 °C by placing the multi-well plates in a temperature test chamber. That temperature was chosen to emulate the temperature recorded from the moment when the capsule laying started in the brood stock tank until they were removed to begin the experiments.

At regular intervals of 1 h, each well was aerated for ca. 0.5 min, with air being released from a fine yellow pipette tip connected to an electric air pump through a silicone pipe. After 6 h, larval survival under each experimental condition (i.e., treatments and controls) was evaluated, and then the experimental seawater was carefully removed with the aid of a plastic pipette and replaced with aerated FSW at the control salinity and experimental temperature. Then the multi-well plates were returned to the temperature chambers, and survival was recorded after two consecutive recovery times (12 and 24 h). During the recovery, each well was aerated for ca. 0.5 min every 6–8 h. This experimental approach was used to emulate how the investigated larval traits (survival and swimming performance) in newly hatched larvae could be affected when released into the sea and subsequently encountering a water mass with abnormally high levels of salinity that then normalizes due to dilution. All observations were conducted under a stereomicroscope and the criterion for larval survival was the clear display of swimming activity or resting with evident signs of velar ciliary beating. Alternatively, non-swimming or resting newly hatched larvae with ciliary arrest were considered dead. This procedure has been previously used to evaluate the effects of secondary-treated kraft pulp mill effluents on survival of newly hatched larvae [45].

2.4. Experiment 2. Effects on survival and swimming performance

In this experiment, the same procedure and setting used in the previous experiment was used. Larval survival and swimming performance was evaluated before exposure to the experimental salinities (T0), after the 6 h of the acute exposure to the experimental salinities (T6) and then after a recovery period of 12 h and 24 h (T12 and T24) in FSW with control salinity. Larval survival was evaluated in the first 6 wells following the same procedure used in the first experiment. However, swimming performance was evaluated using larvae contained in the last 3 wells. To assess swimming performance for each treatment condition, 10 individual larvae were randomly selected and consecutively removed using a plastic pipette. They were then individually placed in separate clean small glass Petri dishes filled with FSW at the corresponding treatment salinity and experimental temperature. After 1 min of habituation, the Petri dish was carefully moved horizontally to position the larvae in the centre of the stereomicroscope field. Once the larva was placed in that position, we waited for the water to stop moving before analyzing its motion, and for this motion to cease moving the larvae. The magnification was set to maximum (×4.5), and the time taken by the larvae to exit the observation field was measured. Under that magnification, the linear distance from the centre to the edge of the stereomicroscope field was 2500 μm, which represents ca. 10 times the average size of the newly hatched larvae used in this experiment. Newly hatched larvae, under laboratory conditions, might display three swimming behavioural modes: directional swimming, hovering, and passive sinking. After hatching, hovering and upward swimming are the main

behaviours in these larvae, and when exposed to a direct light source, they exhibit photopositive directional swimming (Manríquez PH, per. obs.). In the experiment, to induce photopositive directional larval swimming, the upper light source of the stereomicroscope was directed toward one end of the Petri dish, while the underlying source was switched off. Moreover, to minimize movements along the vertical axis and maximize movements in the horizontal plane, the water level in the plates was not higher than 0.5 cm. Then, the exit times were recorded and considered as a proxy for larval swimming velocity or performance, with shorter and longer exit times indicating good and poor velocity/performances, respectively.

2.5. Experiment 3. Effects on the oxygen consumption (standard metabolic rate)

In this experiment, newly hatched larvae were obtained following the same experimental procedure described above. However, in this experiment, a new pool of larvae was obtained from egg capsules laid by the same females used in the first experiment. Larvae were assigned to 3 plastic container of 0.45 mL filled with FSW at the corresponding experimental salinities and with a continuous air supply. In this experiment, the newly hatched veliger larvae were assigned to the experimental salinities, covering a total of 8 experimental salinities (6 treatment and 2 control) (Table 1). After 6 h of an acute exposure to the experimental salinities, the water from each container was carefully removed using a silicone hose, the submerged end of which was equipped with a 50 μm sieve to prevent larval suction. Then the larval pool was assigned to a clean container that was refilled with air-saturated FSW at normal salinity and kept under those conditions for 12 h (recovery period). After the recovery period the oxygen consumption rate was measured in 5 groups of 60 randomly selected larvae from each experimental salinity level.

Only healthy-looking larvae displaying undamaged protoconchs and displaying swimming behaviour were selected for measuring the metabolic rate. In the measurements, metabolic chambers equipped with oxygen consumption measurement spots (PreSens) for non-invasive measurements. This chamber consisted of a 24-well plate of 200 μL mini chambers (Lolygo®System) each with integrated oxygen sensors and filled with FSW at the corresponding salinity. Then the metabolism was measured using a SDR SensorDish® Reader with a small 24-channel reader (PreSens). To control the temperature the complete SDR SensorDish® Reader was placed inside a temperature-controlled chamber, and the measurements were conducted in darkness. In our experiments the size of the glass chambers and the darkness inside the temperature-controlled chamber restricted active larvae displacement and allowed minimal functional activity. Therefore, any variation in oxygen consumption was attributable to changes in basal metabolism rather than to changes in large and active swimming displacement (i.e., standard metabolic rate sensu [46]). Measurements were conducted for at least 1 h, with the first 10 min eliminated to prevent possible effects of manipulation on the metabolic rate. During the measurements, oxygen levels were always above 70 % saturation. To calculate the metabolic rate, we measured the changes in the oxygen concentration (mg O_2) at the beginning (after adjusting for manipulation) and at the end of the analyzed period for each larval group. Then, these changes were expressed per larva and unit of time. The metabolic rates were corrected using the background respiration determined by measuring changes in oxygen levels in wells with FSW and without larvae.

3. Statistical analyses

The times required by the newly hatched larvae to leave the observation field (exit times) were compared using a two-way repeated measures analysis of variance (ANOVA), with salinity as the between-subjects factor and time as the within-subjects factor. The Sphericity assumption of the model was tested using the Mauchly test. If the assumption was rejected, the Greenhouse-Geisser and Huynh-Feldt corrections were considered. When significant interactions between salinity and time were observed, multiple comparisons were conducted using the Tukey test to detect specific differences among the experimental salinities. Metabolic rates were compared using a one-way ANOVA. Normality of residuals and homogeneity of variances were tested using Shapiro-Wilk and Levene's, respectively. All the analyses were performed using Rstudio software [47].

4. Results

In the three experiments of the present study, control (34 N and 34R) and target salinities were consistently similar throughout the experimental treatments (Table 1). Likewise, the achieved pH levels remained comparable throughout the experimental treatments (Table 1).

4.1. Experiment 1. Effects on survival

In this experiment, the experimental salinities did not affect the larval traits. Regardless of the experimental salinity, larval survival and the ability to exhibit larval swimming reached maximum values (100 %) at the end of the acute exposure period (6 h) and after the two recovery periods (12 and 24 h) in seawater with normal salinity (Table 2).

4.2. Experiment 2. Effects on survival and swimming performance

In this experiment, as in the previous one, regardless of the experimental salinity, larval survival and the ability to exhibit larval swimming also reached maximum values (100 %) at the end of the acute exposure period (6 h) and after the two recovery periods (12 and 24 h) in seawater with normal salinity (Table 2).

The swimming performance of the newly hatched larvae, evaluated as the time required to exit the observation field, measured before acute exposure to the experimental salinities, fluctuated around ca. 5 s (Fig. 1a). However, increments in the exit times, ranging from 26 % at 42 PSU to 44 % at 58 PSU in these times was recorded after the larvae were acutely exposed to the experimental salinities for 6 h (Fig. 1b). The same trend was observed once the larvae were maintained in natural FSW with control salinity for 12 h (Fig. 1c). The repeated measures analysis of variance, to assess differences in the exit time, did not detect differences in the exit time in the records taken at T0 ($F_{7,63} = 0.36$; $P = 0.92$). However, the same analysis detected a significant effect of the acute exposure to the experimental salinities (T6; $F_{7,63} = 4.32$; $P = 5.8 \times 10^{-4}$) and after the recovery period (12 h; $F_{7,63} = 6.52$; $P = 8.5 \times 10^{-6}$) on the exit time. The analysis of records taken after 6 h (T6) of acute exposure to experimental salinities using the Tukey HSD post hoc test revealed significant differences among the salinities. After 6 h of exposure to the experimental salinities, exit times were significantly longer in salinities equal to or >48 PSU compared to salinities lower than or equal to 38 PSU (Tukey's test $P < 0.05$). Additionally, the swimming times of larvae exposed to salinities greater than or equal to 42 PSU were not significantly different (Tukey's test $P > 0.05$). The analysis for exit times measured after 12 h of recovery (T12) in natural seawater with control salinity revealed significant differences among the salinities. Exit times were significantly longer in those larvae exposed to salinities above 48 PSU compared to salinities lower than or equal to 38 PSU (Tukey's test $P < 0.05$). Additionally, the exit times of larvae exposed to salinities greater than or equal to 42 PSU were not significantly different (Tukey's test $P > 0.05$).

4.3. Experiment 3. Effects on the oxygen consumption (standard metabolic rate)

The oxygen consumption of the newly hatched larvae was significantly affected by the experimental salinities to which they were acutely exposed for 6 h ($F_{7,40} = 7.435$; $P < 0.001$). Multiple comparisons

Table 2
Larval size (μm; mean ± SE) and survival (%) of newly hatched *Concholepas concholepas* veliger larvae at the end of each experimental period for the first and second experiment at each salinity treatment. T6 = at the end of the 6-hour acute exposure to experimental salinities; T12 and T24 = at the end of the 12-hour and 24-hour recovery periods in seawater with normal salinity, respectively. 34N and 34R represent natural seawater salinity (natural control) and seawater with reconstituted natural salinity (reconstituted control) obtained by diluting the brine with 0.45 μm filtered 34 N seawater, respectively. In each experiment, larval sizes (maximum shell length) were measured in a pool of 10 larvae per salinity.

Salinities (PSU)	First experiment				Second experiment				Third experiment
	Larval size	Survival (%)			Larval size	Survival (%)			Larval size
		T6	T12	T24		T6	T12	T24	
58	244.4 ± 0.2	100	100	100	244.2 ± 0.4	100	100	100	245.1 ± 0.4
55	244.5 ± 0.3	100	100	100	247.5 ± 0.2	100	100	100	246.8 ± 0.3
53	244.5 ± 0.2	100	100	100	246.3 ± 0.4	100	100	100	247.1 ± 0.4
48	244.6 ± 0.2	100	100	100	244.3 ± 0.3	100	100	100	244.5 ± 0.4
42	245.2 ± 0.3	100	100	100	246.1 ± 0.4	100	100	100	245.1 ± 0.4
38	245.3 ± 0.3	100	100	100	244.3 ± 0.4	100	100	100	246.0 ± 0.3
34N	244.8 ± 0.3	100	100	100	245.3 ± 0.3	100	100	100	244.3 ± 0.5
34R	244.3 ± 0.5	100	100	100	244.7 ± 0.4	100	100	100	246.2 ± 0.3

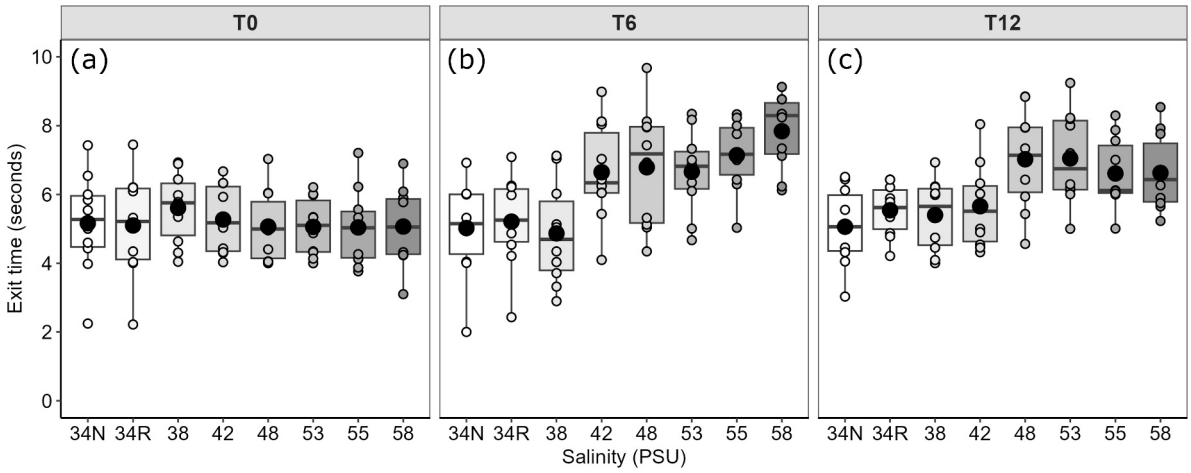

Fig. 1. Exit time displayed by newly hatched veliger larvae of *Concholepas concholepas* before being exposed for 6 h to different experimental salinities (a), at the end of this period (b), and after a 12-h recovery period (c) in normal seawater salinity (second experiment). The highest salinity corresponds to undiluted brine. The rest of the experimental salinities were obtained by diluting the brine with seawater to reach the normal seawater salinity at the time the experiments were conducted. 34 N and 34R represent natural seawater salinity (natural control) and seawater with reconstituted natural salinity (reconstituted control) obtained by diluting the brine with 0.45 μm filtered 34 N seawater, respectively. The box plots show the 25th and 75th percentiles (box), the median (solid line), the mean (large black dot) and the 10th and 90th percentiles (whiskers). The dot cloud above the boxplots represents all data points recorded for each treatment.

revealed that larvae exposed to all salinities equal to or >38 PSU had significantly higher oxygen consumption than those recorded at 34 PSU salinities (control seawater and similar salinity obtained through brine dilution, Tukey's test $P < 0.05$; Fig. 2).

5. Discussion

Based on the salinity effects associated with the RO brine discharge determined in this study, it is expected that these discharges should not have an impact on the survival of newly hatched *C. concholepas* larvae in the receiving coastal zone. This finding contrasts with a previous study in which low salinities (below or equal to 28 PSU) were lethal for encapsulated pre-hatching larvae of this species [48]. Differences in terms of resistance to changes in salinity can have their origin in various factors. In Gallardo's study, the encapsulated larvae were exposed for several weeks (chronic exposure) before hatching occurred, whereas only for 6 h (acute exposure) in our study. Furthermore, it is also possible that the mechanisms to cope with changes in salinities have not yet developed in pre-hatch larvae that rely on the properties of the capsules to face such changes in salinity. The lack of negative effects of hypersaline RO brines on newly hatched larvae is consistent with the reported absence of significant impacts of similar brines on surrounding coastal or estuarine environments [3].

The 6-hour exposure test to high salinities using newly hatched larvae seems to be a well-suited test for larval stages, as they are not likely to encounter such high salinities at the sea surface, only briefly near the discharge zone. Moreover, newly hatched larvae are expected to be present only briefly near the discharge zone while drifting away, and therefore they will not be chronically exposed to concentrated brines that flow on the seabed. Our study suggests that a 6-hour exposure test using newly hatched larvae cannot be considered a useful tool for the preliminary assessment of mortality linked to abnormally elevated salinities of a brine discharge. However, sub-lethal or other latent effects occurring later in larval development up to larval competence or settlement cannot be ruled out. Since newly hatched larvae are mobile and the highest salinity levels associated with brine discharges are concentrated at the discharge sites, long-term chronic exposures were not attempted in the present study.

Although we found no negative effects on survival, our results indicate that tests using behavioural and physiological traits of newly hatched larvae are suitable for detecting the sub-lethal effects of brine discharge. However, extended exit times in response to the high salinities found in the present study suggest a negative impact on swimming performance associated with these conditions. Newly hatched larvae, as well as the larvae of most marine invertebrates, exhibit photopositive behaviour as part of their natural behaviours and survival strategies

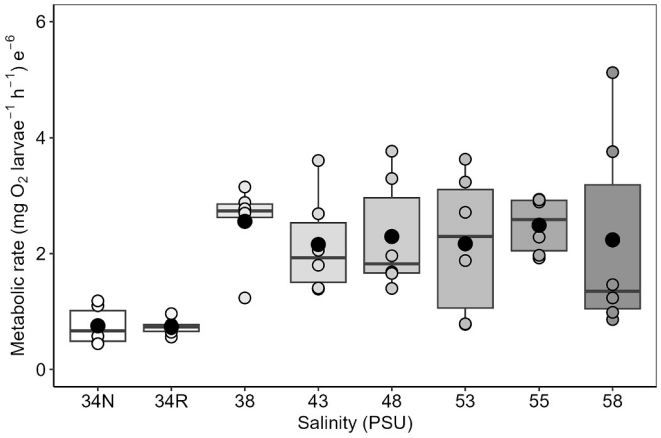

Fig. 2. Metabolic rate (oxygen consumption) of newly hatched *Concholepas concholepas* veliger larvae of measured after being exposed for 6 h to different experimental salinities and a subsequent 12-h recovery period in seawater with normal salinity (first experiment). The highest salinity corresponds to undiluted brine. The rest of the experimental salinities were obtained by diluting the brine with seawater to reach the normal seawater salinity at the time the experiments were conducted (34 N). Metabolic rates were measured in 4 groups of 60 larvae per salinity. For an explanation of the salinity levels and the information contained in the box plot, refer to the legend of Fig. 1.

[49]. This behaviour can help them reach the ocean's surface, where light is abundant. This surface movement can provide access to food sources, such as plankton and other small organisms that are attracted to light. Previous studies have reported reduction in swimming performance with decreasing salinity in copepods [50] and newly hatched larvae of shore crabs [51]. This suggests that changes in salinity (reductions or increments) are an important stressor that may constrain larval and plankton dispersal capacity in nature, potentially leading to lethal effects in the long term.

In our study, we measured 2-dimensional horizontal projections of swimming performance, which is a displacement behaviour that generally does not significantly affect larval distributions on large spatial scales, as most larvae cannot swim against ambient water currents [52]. In our study, swimming performance in terms of exit times was measured collapsing the 3-dimensional trajectories into ca. 2-dimensional projections. However, this experimental bias is frequent in behavioural studies of invertebrate larvae [53,54]. Average exit times measured in our study correspond to horizontal larval velocities ranging from ca. 0.04 cms^{-1} at elevated sanities to ca. 0.06 cms^{-1} at control salinities. Regardless of the salinity under which these swimming performances were measured, those values are only a 0.2 and 0.3 % of average horizontal current velocities (ca. 20 cms^{-1}) in near shore environments. This suggests that larval transport in near shore environments is mainly controlled primarily by local hydrodynamic conditions. However, the effects of changes in salinity on the horizontal larval swimming performance as those found in our study may also affect larval diel vertical positioning thereby affecting their feeding behaviours and surface-level drifting. Since larval displacement in the vertical plane might allow larvae to explore horizontal currents with different velocities and direction, future studies are needed to investigate how acute exposure to brine discharges might affect vertical larval swimming performance.

Considering that our study utilised very high salinities, reaching ca. 70 % of excess above background salinity levels, future studies will be necessary to investigate the consequences of more realistic salinities levels lower than 10 % of excess above background salinity levels. Newly hatched larvae require ca. 3 month to reach competence and settle [21]. Therefore, these studies should also consider acute exposure to brine discharges at different larval stages up to competence or alternatively a chronic exposure during the entire larval period will provide a more accurate evaluation of the potential effect of brine discharges on larvae of *C. concholepas*. It is worth noting that high salinities, as those described here as posing negative effects on newly hatched larvae, are only expected to occur in the near field region located in the vicinities of the discharge point. Indeed, Voutchkov [55] indicates that organisms moving in the seawater column and entering a perimeter of 1000 m from the plume source (far-field) do not exceed 140 min (2.3 h) of exposure to concentrations of ca. 38 PSU. On the other hand, at higher salinity levels, the modelled exposure is drastically reduced to 60, 30 and 10 min at 45, 50 and 55 PSU, respectively. These exposure times of marine organisms to the brine plumes are derived from computational models; however, they align with observational studies of the temporal and spatial evolution of brine discharges. In fact, several monitoring studies of brine discharges from RO plants have documented rapid dilution of the brine near the discharge point. For example, Talavera and Ruiz [56] measured the dilution of the discharge from the Maspalomas II Plant, located in the south of Gran Canaria (Canary Islands), through two outfalls positioned approximately 300 m from the coast and at a depth of ca. 7.5 m, with a brine volume of 17,000 m^{3}d^{-1} at 75.16 PSU. The study determined that within only 20 m from the outfalls, there was a marked dilution of the brine, resulting in a seafloor salinity of 38.44 PSU, returning to normal concentrations within a one-mile radius from the discharge point. It's important to note that measurements in this study were taken under reduced circulation conditions with minimal wind, waves, and tides.

Subsequent studies, such as that of Fernández-Torquemada et al. [57] in Alicante (Southeast Spain) for an RO plant with a discharge volume of 75,000 m^{3}d^{-1} at 68 PSU, determined similar levels of dilution in the immediate perimeter of the discharge but notably lower dilution in the distant perimeter. There were increases of 0.5 PSU above the average ambient salinity up to 4 km from the outfall. Additionally, this study demonstrated that salinity anomalies were not only confined to the seafloor in the far field but also exhibited seasonal vertical variations, with the highest salinity observed at the thermocline level during the summer months due to reduced vertical mixing and greater stratification. This resulted in lower seafloor temperatures compared to the discharge level. These variations reveal exposure to saline stress not only for benthic communities but also for drifting organisms such as the larvae analyzed in this study.

The extent and behaviour of the saline plume variations depend on the type of discharge and production level of the plant [58] and have been observed in coastal upwelling systems, similar to our study region off north-central Chile. Petersen et al. [59] identified saline anomalies of 2.7 units above the environmental range 600 m offshore from the Carlsbad desalination plant in California. Although these salinity anomalies do not exceed the threshold for the sub lethal effects observed in the study, they surpass the levels allowed by regulations (10 % above the ambient level), leading the authors to recommend a discharge design combining optimal diffusor systems and higher dilution of the brine prior to discharge. The latter mitigation measure has proven effective, as seen in Fernández-Torquemada et al. [57], where several plants that predilute the brine with seawater bypassing exhibit a sharp reduction in the area of influence. Specifically, for the Jávea desalination plant, a 4:1 dilution ratio was sufficient to ensure a reduction from 68 to 44 PSU. However, this solution entails high-energy consumption and economic cost, depending on the dilution ratio (see [60,61]). In our case, to reach the concentration without sublethal effects (i.e., < 38 PSU), a seawater-to-brine dilution ratio of 5.5:1 is needed. The negative effects on swimming performance found in our study at salinities above 48 PSU represent an increase in salinity of approximately 40 % above background levels. This is not relevant for most reverse osmosis discharges because brines are either dispersed through diffusers in deeper waters or diluted rapidly in coastal waters. Moreover, existing regulations prevent the discharge of highly concentrated brines into the marine environment, and measures are taken to reduce the salinity to below a 10 % excess level, or + 2 PSU above background levels [59].

A previous study with static and acute toxicity tests carried out with brine and shrimp artemia (*Artemia franciscana*) reported no effects on hatching rate of cysts and larval survival [62]. Similarly, a previous study on brine discharges found no effect on rotifer cysts or larvae but did inhibit bacterial bioluminescence [63]. Furthermore, the same study reported that the inhibition was attributed to the presence of residual chlorine in the brine [63]. This suggests that despite the fact that larvae are highly sensitive to environmental stressors, abnormally high salinities do not have lethal effects on them. In our study, the chemical commonly used during desalination processes such as biocides, coagulants, antiscalants, biofouling control additives, chemicals used for the removal of suspended solids and antifoaming, among others [64,65], were not measured and controlled. The RO desalination plant used in our study includes mainly three chemicals: sodium meta-bisulfite to prevent scaling and fouling, sodium hypochlorite, and 2-Phosphonobutane-1,2,4-tricarboxylic acid as an antiscalant. Since these additives are discharged into the sea and might have effect on coastal organism [66,67], their potential effect on newly hatched larvae and other organisms cannot be ruled out and should also be considered in future studies.

Significant increases in the oxygen consumption of newly hatched larvae exposed to salinities >38 PSU suggest that the larvae's cells are utilizing oxygen at a higher rate due to the altered salinity conditions. Our results do not allow us to identify the altered physiological mechanisms responsible for the reduced swimming performance. Regardless of the mechanism, our results support a potential link between metabolism and swimming performance. This heightened metabolic activity may indicate an increased demand for energy and resources to cope with a changing environment in terms of salinity, potentially leading to reduce energy availability for activities such as swimming, as the larvae prioritize energy conservation for maintenance. This suggests that, within a salinity range between normal (34 PSU) and elevated salinities up to 58 PSU, newly hatched larvae are osmoregulators capable of actively regulating their internal osmotic environment and surviving irrespective of the surrounding salinity for at least 6 h.

Osmoregulation involves a high-energy cost; therefore, extended exit times and significantly elevated metabolisms may indicate that newly hatched larvae have been exposed to salinity levels outside their optimal high tolerance range, where more energy is required to maintain homeostasis at the expense of energy available for swimming. In our study, we observed the negative effects of high salinities on larval exit times immediately after the end of the acute exposure period (T6) for larvae exposed to salinities higher than 38 PSU. Furthermore, these effects were evident again at the conclusion of the 12-hour recovery period (T12) for larvae that had been previously exposed to salinities exceeding 48 PSU. This suggests that at lower salinities—between the control level and below 48 PSU—larvae were able to regain their swimming performance once normal salinities were restored. However, the recovery of larval swimming performance once returned to control conditions (34 PSU) was not achieved when larvae were previously exposed to salinities higher than 48 PSU. This suggests that newly hatched larvae, in terms of swimming performance, are able to cope with short-term changes in salinities below 48 PSU once normal salinities are restored.

The capacity of the newly hatched larvae to recover their swimming performance is surprising since many molluscs and most invertebrates have a very limited capacity to regulate their internal osmotic concentration. However, an increase in exit time (reduction in swimming activity) above 48 PSU may represent an irreversible and negative impact of elevated salinities on swimming performance. Since we did not consider external food supply in our experiments, the salinity threshold above which negative effects on metabolism and swimming performance are observed may vary or even be absent. Our experiments were conducted using newly hatched planktotrophic larvae, which are typically designed to feed upon hatching [68]. Newly hatched larvae of *C. concholepas* can survive for a few days after hatching in the absence of external food (PH Manríquez unp. data). In our study, metabolism and swimming performance were measured after a 12-hour recovery period in larvae reared in FSW deprived of food, following the salinity stress period. Given that these measurements occurred 18 h after hatching and resulted in no mortalities under control conditions (34 PSU), it suggests that the larvae used in the experiments were energetically suitable for assessing the effects of the experimental salinities.

An increase in oxygen consumption in response to a reduction in salinity as part of osmoregulatory mechanisms has been described in coastal crabs [69,70]. Our experiments were carried out in the absence of external food; therefore, the increased energy losses at low salinity due to respiration could not be compensated for by an increase in the ingestion rate as has been described for estuarine larvae exposed to low salinities [71]. This can be an important aspect to be considered if maternal energy contributions to the newly hatched larvae are limited. The capacity to regulate osmolarity in newly hatched larvae can be an important trait evolved to cope with changes in salinity in rocky intertidal habitats exposed to freshwater events during rains or in the vicinity of river runoff characteristics typical of southern Chile, which is within the geographical distribution of *C. concholepas*.

The implications of the RO brine discharges on the fitness of *C. concholepas* still have to be assessed. However, it is worth mentioning that under our experimental condition's acute exposures (6 h) to elevated salinities did not affect survival, suggesting that the effects are not severe enough to have lethal effects, at least in laboratory conditions. Nonetheless, the reduced swimming activity (long exit times) and enhanced metabolic rates of newly hatched larvae could lead to more adverse consequences including, also mortality later in the larval development. The effects of biotic and abiotic stressors on animal behaviour have garnered considerable attention, as behaviour can mediate biological interactions and the impacts of climate change stressors or other global stressors such as light pollution which, in turn, affect the structure and functioning of ecosystems [72–77]. For instance, changes in larval swimming activity have been related to changes in food intake and survivorship due to exposure to predators and climate change stressors [78]. This suggests that stressors acting at the larval level could affect larval performance and, as a result, impact subsequent events. In our study, larvae with higher metabolic rates might have a greater energy demand, which makes them more active in searching for food. However, if larval swimming capacity is impaired by high salinities (resulting in longer exit times), these requirements may not be met, leading to additional and unknown hidden or latent effects.

A previous field study reported that salinity was not correlated with the abundance of newly hatched *C. conholepas* larvae, although they were more abundant when salinities exceeded ca. 15 PSU, and competent larvae were not observed when salinities were less than ca. 15 PSU [23]. Along with our results, this suggests that natural or anthropogenic changes in salinity can be important factors modulating patterns of *C. concholepas* larval distribution and abundance in nature. In terms of dispersion, the local impact of RO brine discharges on larval performance, including behaviour and metabolism, could potentially influence their vertical distribution and act as barriers to larval connectivity along the Chilean coast. Since brine discharges are denser than seawater, they tend to sink and spread on the sea bottom [67,79], primarily affecting benthic ecosystems rather than larvae swimming in pelagic ecosystems. Therefore, competent larvae of *C. concholepas*, actively searching for suitable benthic settlement habitats, as well as small post-settlement juveniles, are also susceptible to being affected by high salinities, just like newly hatched larvae.

6. Conclusion

Our main conclusion is that, after an acute and static exposure of 6 h to RO brines, newly hatched larvae of *C. concholepas* are not affected lethally by abnormal salinities associated with undiluted and diluted brine discharges. We also conclude that these larvae could serve as a suitable model for studying the sublethal effects of brine discharges from

desalination plants under laboratory and acute conditions. As a result, we have developed acute toxicity test using a native species found in the coastal area where operating or planned RO desalination plants currently discharge or are projected to discharge in the future. We conclude that, in nature, the negative effects of an acute and static exposure period (6 h) to brine on sub-lethal traits such as metabolism and larval performance of newly hatched larvae will occur primarily near the discharge points with high salinities (above 48 PSU). Our study was conducted under laboratory conditions with simulated hypersaline brine dilutions, utilizing newly hatched larvae of a gastropod species found exclusively along the Peruvian and Chilean coasts. While laboratory studies can offer valuable insights, they often oversimplify natural conditions and assume that the animal's responses in such controlled environments will mirror those in its natural habitat and can be extrapolated to other similar species. Therefore, further research is needed to assess the effects of exposure to abnormally high salinities associated with brine discharges on larvae of other species in a more realistic setting, such as by exposing larvae to brines collected directly at various distances from the discharge points.

Based on several monitoring and model studies of brine dilution, the negative effects of acute exposures to both undiluted and low-dilution brine on coastal organisms are expected to occur in close proximity to discharge points and for short durations [56–59,80–82]. Consequently, if the negative and sub-lethal effects identified in the present study are expected to occur in nature, they would primarily manifest in the near field. However, prolonged exposure to elevated metabolic rates and reduced larval performances under abnormal salinities associated with coastal brine discharges might expose them to low chances of survival. In conclusion, we recommend pre-dilution of undiluted brines before discharge into the sea to enhance the chances of reducing larval performance in terms of velocity and metabolism. Nevertheless, considering that pre-dilution with a new plant increases pumping costs [83] and the carbon footprint arising from energy consumption [84], we also advise the implementation of high and rapid mixing of brine with seawater using diffusers at discharge points into the sea to mitigate the negative effects of undiluted brines (see [85]). These proposed measures, pre-dilution and dilution at the sea level, align with findings from previous studies [86–89].

Although desalination through reverse osmosis seems to be a viable solution for water scarcity in arid zones such as central-northern Chile, other more ecologically friendly sustainability techniques can also be explored in the future. In this line, interfacial solar desalination, which relies on solar absorbers to capture solar energy for extracting water from seawater via solar evaporation [90], and sorption-based atmospheric water harvesters [91] are other techniques that can be explored to alleviate water scarcity in communities without incurring in large-scale infrastructure and reducing the potential effects on coastal ecosystem services.

CRediT authorship contribution statement

Patricio H. Manríquez: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Claudio P. González:** Writing – review & editing, Visualization, Investigation, Formal analysis. **Alejandro Abarca:** Investigation. **Katherine Jeno:** Investigation. **Viviana Jofré:** Investigation. **Orlando Astudillo:** Writing – review & editing, Writing – original draft, Validation, Resources, Investigation, Conceptualization. **Victor M. Aguilera:** Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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