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eDNA metabarcoding of marine invertebrate communities at RO desalination plant outfalls in Cyprus

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ABSTRACT

Seawater desalination has become an essential part of the drinking water supply of many arid countries in the world – including Cyprus and the wider Mediterranean – Middle Eastern region. In this study, the micro-invertebrate fauna near the outfall sites of 2 seawater desalination plants, Dhekelia and Larnaca, in Cyprus was assessed by microscopy-based classical taxonomy and eDNA metabarcoding. The results show impact of desalination brine discharge on marine benthic communities. The community structure and the impact of abiotic factors was more prominent in the sediments than the water, and the alpha diversity results from metabarcoding were consistent with the results from microscopy but differed taxonomically.

1. Introduction

As global freshwater demand rises and climate change reduces freshwater availability, especially in arid regions with a high and growing population such as the Mediterranean, Gulf Region and southern California, water security becomes paramount driving attention and investments into seawater desalination technologies. Desalination is a technology that has been used for several decades in countries that have long suffered water scarcity. However this comes with environmental impacts, which are not completely understood. Fundamentally, there are two types of technologies presently in large-scale use to remove salt from seawater or brackish water: (1) distillation and (2) seawater reverse osmosis (SWRO and RO, respectively, from here on). In 2020, 97.2 million m^3 of freshwater day^{-1} were produced by desalination plants, with an increasing trend (Eke et al., 2020).

Both types of desalination technologies may lead to site-dependent impacts on the marine environment around the inlet (entrainment of marine organisms, disturbance of sediment), as well as at the discharge (brine and chemicals discharge, disturbance of seabed) (Lattemann and Höpner, 2008). The negative impact of seawater-desalination technologies is derived mostly from the salt concentrate and chemical discharges, as well as greenhouse gas (GHG) emissions due to the high-energy demand for this process. In the case of distillation-based plants, negative impact may also be derived from thermal stress in the recipient waters with varying impacts on marine organisms across the food webs. Sensitivity at increased temperatures has been observed in red algae from the Arabian Gulf (Hasan et al., 2023), increased mortality of planktonic larvae, exacerbated by the impact of the chemical discharge (Miri and Chouikhi, 2005), with certain fish species being more vulnerable to salinity changes than algae, with possible effects on entire local food webs (Yoon and Park, 2011). Consequently, seawater-desalination impacts on ecosystems should be assessed and managed for each site (Lattemann and Höpner, 2008). Desalination technologies produce a hypersaline concentrate (brine) that can contain various

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chemicals used for process optimisation, as well as their by-products (Lattemann and Höpner, 2008; Panagopoulos and Haralambous, 2020). Brine consists of 57,400–70,488 mg L^{-1} total dissolved solids, with high levels of Cl^-, Na^+, SO_4^{2-}, Mg^{2+}, Ca^{2+} and HCO_3^- (concentrations in descending order, Panagopoulos and Haralambous, 2020), which means that brine is roughly 1½ to 2 times more saline than most types of seawater. This hyper-saline mixture of water and membrane-cleaning products (coagulants, antiscalants, anticorrosive chemicals, suspended solids, heavy metals) is discharged into the recipient waters. The impact of brine discharge on the marine ecosystem depends on the local geography and weather conditions (Argyrou, 2000). Brine from desalination plants is negatively buoyant due to its higher density compared to seawater, and consequently the impact would mainly be localised in the sediment and overlaying water (Lattemann and Höpner, 2008).

1.1. *Environmental impact of brine discharge*

The current scientific knowledge on the impact of desalination brine on marine life is limited. In the Mediterranean, there is particular concern about seagrass meadows, in which *Posidonia oceanica* is the keystone species – due to their multiple roles in coastal ecosystem functioning, coastal protection and stabilisation, as nurseries, and in capturing and storing carbon. It has been shown (Fernández-Torquemada and Sánchez-Lizaso, 2005; Gacia et al., 2007; Xevgenos et al., 2021) that increased salinity and documented eutrophication at brine discharges in the Mediterranean have deteriorated the health of *P. oceanica* meadows, which are extremely sensitive to salinity changes. A Spanish study (De-La-Ossa-Carretero et al., 2016a) highlighted the sensitivity of echinoderms to increased salinity, since echinoderms were absent at brine discharges in Alicante, Spain, except after installation of a discharge-pipe diffuser. This highlights the potential of echinoderms as bioindicators of brine pollution (Fernández-Torquemada et al., 2005). The diversity and abundance of amphipods have been compromised at brine-discharge sites in Spain (De-La-Ossa-Carretero et al., 2016b), with different species exhibiting different tolerance to hypersaline effluents, for example, polychaetes, segmented marine worms known to show high tolerance (Del-Pilar-Ruso et al., 2008). High salinity caused locally by brine discharge is an important environmental stressor, and ecosystems that are affected by such stressors are generally more susceptible to the introduction of non-indigenous species (NIS).

1.2. *Application of environmental DNA as a biomonitoring tool (eDNA)*

The use of conventional techniques for biomonitoring can be time-restrictive and costly, as well as invasive for the sampled environment. Analysis of environmental DNA (eDNA) is being explored as an alternative method to meet the limitations of conventional biomonitoring tools. eDNA can be defined as the DNA of all organisms present in an environmental sample, without pre-selection of organisms (Taberlet et al., 2012), where macrofauna shed eDNA through interaction with their environment (Pawlowski et al., 2020). eDNA metabarcoding is a molecular technique involving the extraction of eDNA from environmental samples, and the sequencing of a conserved region of the genome within the target taxa of interest, allowing simultaneous identification of DNA sequences within the selected barcode. The sequence data from metabarcoding is converted to species names through alignment with available sequence data from the expected target organisms present in the environment (Taberlet et al., 2012). Careful curation of this alignment is critical for evaluating eDNA metabarcoding data.

Anthropogenic impact on biodiversity becomes more prominent over the years, and the need to design efficient biodiversity-assessment methods and intelligent, long-term biomonitoring surveys is becoming increasingly urgent (Lopes et al., 2021). The increasing lack of taxonomic expertise also represents a driver towards more eDNA-based biodiversity monitoring enabling large-scale and standardised inventories of taxonomic diversity (Valentini et al., 2016). The implementation of the European Union Water Framework Directive (WFD 2000/60/EC) and the European Union Marine Strategy Framework Directive (MSFD 2008/56/EC) emphasise the urgent need for ecosystem management, as well as the development of efficient biomonitoring tools (Aylagas et al., 2018).

The use of eDNA is a promising tool for biomonitoring, including as a supplement to Environmental Impact Assessments, processes used globally for various industrial projects, as it allows for rapid and cost-efficient results, with little disturbance to the environment. The use of such molecular techniques is becoming increasingly common and may give us the ability to detect rare taxa, undetectable with conventional methods, to account for cryptic speciation, and for the early detection of Non-Indigenous Species (NIS) (Knudsen et al., 2022). While eDNA analysis is increasingly being applied to study environmental samples, the methodology still requires careful design and interpretation of results due to challenges relating to the dynamics and degradation of eDNA in the environment (Alberdi et al., 2018).

1.3. *Cyprus as a case study*

Cyprus was chosen as a case study due to its heavy reliance on desalination to meet its freshwater demand. This makes the marine environment of Cyprus particularly susceptible to environmental impacts from brine discharge. This impact is potentially amplified by the geographic location of the island in a hot, arid region and its susceptibility to Lessepsian migration – the movement of marine species of Indo-pacific origin in the Mediterranean basin via the opening of the Suez Canal – of halophilic organisms.

Cyprus has repeatedly suffered water scarcity problems over the years (Tsiourtis, 2004) due to lack of natural freshwater sources, a dry Mediterranean climate, as well as a large percentage of the arable land utilized for water-intensive agriculture (Giannakopoulos et al., 2010).

The shallow Cypriot benthos features extensive seagrass meadows (Kocak et al., 2002) of *P. oceanica* and *Cystoseira* forests as climax communities (Russo, 1997), with a mixture of sandy and rocky seabed. Close to industrialised zones, opportunistic seaweeds have replaced ecologically high-value *Cystoseiretum* climax communities (Kletou et al., 2018; UNEP/MAP-SPA/RAC, 2019). In total, the number of seaweed species and intraspecific taxa currently accepted is 313 (Tsiamis et al., 2014). Inshore marine life of Cyprus includes iconic species such as monk seals (Gucu et al., 2009; Marcou, 2015; Nicolaou et al., 2021), green turtles and loggerhead turtles (Casale, 2010; Duncan et al., 2018). Cyprus has numerous non-native and invasive species in its coastal sea, including the green seaweed *Caulerpa taxifolia* var. *distichophylla* (Aplikioti et al., 2016), the holothurian *Synaptula reciprocans* (Antoniadou and Vafidis, 2009), and, most recently, the fish *Parupeneus forsskali*, *Torquigener flavimaculosus*, *Sargocentron rubrum*, and the lionfish *Pterois miles.* Many of these originate from the Indo-Pacific, which have entered the Mediterranean via the Suez Canal as Lessepsian migrants.

Cyprus had four desalination systems installed in the years between 1997 and 2004 (Tsiourtis, 2004). The two desalination plants that are investigated in this project are located in Larnaca Bay (eastern region of Cyprus). They are comparable on the capacity of 60,000 m^3 day^{-1}, but the brine discharge at the Larnaca plant occurs through a single pipe, whereas at Dhekelia it is discharge via a multi-diffuser pipe (Argyrou, 2002).

A monitoring survey at Larnaca in August 2002 reported the presence of a highly turbid brine plume, reaching 200 m distance from discharge. A monitoring project (1997–2000) on the impact of brine discharge at Dhekelia showed the degradation of climax macroalgal communities of *Cystoseira barbata* and changes in macroinvertebrate assemblages at the outfalls (Argyrou, 1999).

The aim of this study is to assess the impact of brine discharge from desalination plants on marine invertebrate communities at two distinct locations: Larnaca and Dhekelia, situated in Cyprus. Our central hypothesis posits that impacted sites will exhibit reduced biodiversity, with the prevalence of taxa indicative of environmental disturbance, particularly polychaetes. Furthermore, we postulate that the magnitude of brine impact on marine communities will vary based on the distance to the discharge point and the type of discharge pipe (single or diffuser).

2. Materials and methods

2.1. Sample sites and collection

Preliminary studies and monitoring data show salinity levels returning to background levels at approximately 200 m from brine outfalls (Argyrou, 1999; Tsiourtis, 2004), with more pronounced impacts observed during summer months, attributed to water column stratification associated with calmer weather conditions. Key distinctions between the two SWRO plants located near Larnaca and Dhekelia in Cyprus are summarized in Table 1.

Hydromorphological analysis of currents in the Levantine Sea demonstrates that the mid-Mediterranean jet flows eastward within the eastern Levantine basin, passing along the eastern coast of Cyprus in a northeast direction (Zodiatis et al., 2005). Previous monitoring through diving surveys conducted by the government of Cyprus (Argyrou, 1999, 2002) had reported that the plume followed a north/northeast trajectory, with observable brine impact along this axis, and the direction as well as impact of the plume were later confirmed in a subsequent study (Xevgenos et al., 2021).

At the Larnaca site, the sampling transect protocol was based on the grid used in previous monitoring studies (Argyrou, 2002), which showed concentrated brine impact around the discharge point, with effects noted up to 50 m away (Tsiourtis, 2004; Xevgenos et al., 2021). Consequently, sampling stations were established at 0, 50, 100 and 200 m from the monitoring scheme, along with a reference point at approximately 1500 m from the origin (consistent with Xevgenos et al., 2021, Supporting Information S1 therein). Adjustments to station placement were made to ensure relatively constant bathymetry along the transect.

The sampling transect at the Dhekelia site was partly based on historical monitoring data and mirrored the gradient observed in Larnaca, where feasible. Sampling stations were positioned at 0, 50, 100 and 150 m from the multi-diffuser pipe discharging brine. A reference point was established 1500 m downstream (eastward along the coast), maintaining consistent bathymetry in an eastward direction along the transect. Four sampling surveys were performed in May 2022, August 2022, November 2022, and February 2023 to capture seasonal variations. Details regarding sampling stations for both sites are shown in Table 2 and Fig. 1.

At each sampling point, six litres of seawater were collected using a Van Dorn water sampler (Van Walt, The Netherlands). Water samples were filtered on the same day through a 0.22 μm nitrocellulose membrane filter using an oil-free vacuum pump (THOMAS, USA), with filters stored at −20 °C. Field and filter controls were included for each sampling day.

Sediments samples were collected from the undisturbed top layer (3–4 cm) of sediment in triplicate using a Van Veen grab (0.05 m^2), were homogenised in 50 mL Falcon tubes, and stored in 4 °C until DNA extraction, which occurred within two days of collection.

Table 1

Summary of the main differences between the two SWRO plants.

	Larnaca	Dhekelia
Instalment	May 2001	April 1997
Capacity (m^3 day^{-1})	60,000	60,000
Outfall pipe type	Single pipe	Multiple diffuser
Outfall pipe depth (m)	15	4
Outfall pipe distance from shore (m)	ca. 1000	ca. 250

Table 2

Coordinates and details of sampling stations used in this study.

Site	Sampling stations	Distance from discharge point	Longitude	Latitude	Depth (m)
Larnaca	L0	0 m north	33°39.264′ E	34°52.160′ N	13.0
	L50	50 m north	33°39.267′ E	34°52.187′ N	13.5
	L100	100 m north	33°39.270′ E	34°52.213′ N	13.5
	L200	200 m north	33°39.276′ E	34°52.267′ N	14.0
	LREF	1400 m south[b]	33°39.113′ E	34°51.393′ N	16.2
Dhekelia	D0	0 m east	33°45.599′ E	34°58.773′ N	3.5
	D50	50 m east	33°45.631′ E	34°58.770′ N	3.5
	D100	100 m east	33°45.663′ E	34°58.768′ N	3.0
	D150[a]	150 m east[a]	33°45.689′ E	34°58.785′ N	4.8
	DREF	1245 m east[b]	33°46.414′ E	34°58.726′ N	7.6

[a] Substrate 200 m east of the pipe at Dhekelia was rocky and sampling using a Van Veen grab was not possible. The sampling station was skewed closer to the pipe, to remain within the 200 m of brine impact reported in literature.

[b] Noted as 1500 from here onwards.

2.2. Measurement of environmental parameters

Temperature, pH, salinity, conductivity, and Oxidation-Reduction Potential (ORP) measurements of both surface (1 m below surface) and bottom water (1 m from seabed) were conducted on-board using the multiparameter instrument HANNA HI98194 (HANNA, UK). However, measurements were not taken at Dhekelia in November 2022, and Larnaca and Dhekelia in February 2023, due to unavailability of the instrument. pH, salinity (as Total Dissolved Solids) and conductivity measurements for samples collected during February 2023 from both sites, were alternatively performed at an external CYS-CYSAB accredited laboratory, following ISO/IEC 17025:2017 standards.

Seawater samples of 500 mL were collected from all sampling sites from both the surface and the overlaying water for analysis of orthophosphates, nitrates, nitrites, ammonium and dissolved silica. Samples were stored at −20 °C until analysis. Approximately 400 mL of top 3–4 cm sediment were collected in plastic jars for measurement of water-soluble Iron, Magnesium and Tin (except May 2022, where total Iron, Magnesium and Tin content was measured instead), and Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN). These measurements were performed at an external CYS-CYSAB accredited laboratory within three days of collection, following ISO/IEC 17025:2017 standards.

2.3. DNA extraction and metabarcoding

DNA extractions from water filters were performed using DNeasy Blood & Tissue DNA Extraction kit (Qiagen, USA), with modifications following the protocol described elsewhere (Holman et al., 2019). For sediment samples, the DNeasy PowerSoil Pro kit (Qiagen, USA) was utilized after prior homogenisations of the samples.

The mitochondrial Cytochrome Oxidase I gene was targeted using primers BF3/BR2 (Elbrecht et al., 2019), fused with Nanopore PCR barcoding overhangs. DNA concentration was measured using the Qubit dsDNA HS Assay kit (Invitrogen, USA). The PCR protocol consisted of 2 μL DNA template, 10 μL of primer mix (1 μM of each primer), 12.5 μL of PCR BIO Ultra Mix (PCR BIO Systems Ltd., UK), and 0.5 μL of Nuclease-

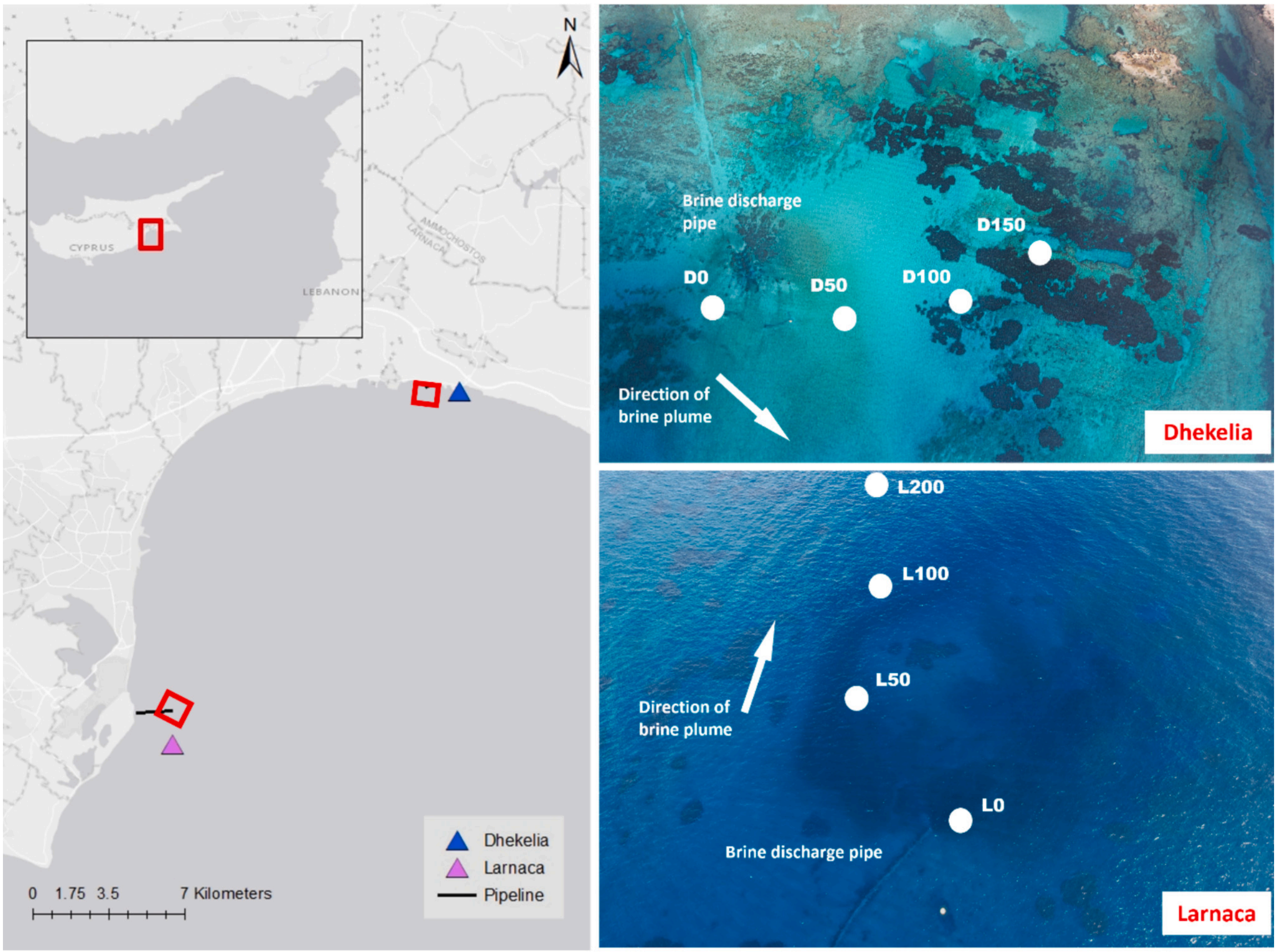

Fig. 1. Map of sampling locations at Larnaca and Dhekelia desalination plants. The locations of the aerial images to the right are shown in red frames on the left-side map (aerial images by Olivier Dargent, November 2023). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Free water (Invitrogen, USA). PCR conditions included an initial denaturation step at 95 °C for 2 min, followed by 35 cycles of 95 °C for 15 s, 50 °C for 15 s, 72 °C for 1 min, with a final extension at 72 °C for 5 min. Quality control of randomly selected PCR products was measured on TapeStation 2200 and D1000 ScreenTapes (Agilent, USA). Amplicons were purified with CleanNGS (CleanNA, USA), using a sample: bead ratio of 0.8, and their concentration was determined using the Qubit dsDNA HS Assay (Invitrogen, USA) on a TECAN Infinite F200 PRO plate reader (TECAN, Switzerland).

2.4. Sequencing, bioinformatics and statistics

Final libraries of the COI barcodes barcoded by PCR were prepared for sequencing according to the Nanopore protocol (PBAC96_9069_v109_revQ_14Aug2019) and samples were sequenced on an Mk1B MinION sequencer on a R9.4.1 flowcell for ~16 h (Oxford Nanopore Technologies). Raw sequencing data was generated using live basecalling while sequencing, using Guppy v6.5.7 with the R9.4.1 High Accuracy (HAc) model (Guppy is available only to ONT customers via their community site, https://community.nanoporetech.com). Demultiplexing and adapter removal was performed using Porechop v0.2.3 (https://github.com/rrwick/Porechop), with stringent quality filtering requirements; –discard_middle, –-require_two_barcodes and –barcode_threshold 85. The demultiplexed data were subjected to further quality control through NanoPlot v1.38 and quality filtered to the expected size range (read length 150–600 bp) with NanoFilt v2.6.0 (De Coster et al., 2018) with a minimum q-score of 10. Then, the reads were aligned using minimap2 v2.17 (Li, 2018) and polished using Racon v1.3.3 (Vaser et al., 2017), and subsequently clustered into Operational Taxonomic Units (OTUs) at 97 % sequence similarity using VSEARCH v2.13.4 (Rognes et al., 2016). Taxonomy was assigned using AMPtk v1.3.0 (Palmer et al., 2018) using the latest COI reference database. Additional manual curation was performed using BLAST (blastn suite), using standard databases, but excluding Models (XM/XP) and uncultured/environmental sample sequences from the search set, and optimised for highly similar sequences (megablast) with at least 51 % query cover and 89 % above sequence identity (Zhang et al., 2000). Taxon nomenclature was adapted to World Register of Marine Species up to 17 January 2024 (WoRMS Editorial Board, 2024). Taxa not belonging to the target phylum Animalia were removed prior to analysis.

Alpha diversity, the diversity within each sample, was measured as Shannon-Wiener H' index, Simpson D' index, Chao1 estimator and OTU richness. Group comparisons and post-hoc tests were calculated using Kruskal-Wallis and Dunn's tests. Statistical analysis and data visualization were performed in RStudio v2022.12.0 + 353 with R v4.3.0 (R Core Team, 2023) using ampvis2 (Andersen et al., 2018).

3. Results

3.1. Physicochemical parameters

3.1.1. Water

Salinity measurements in water samples collected from Larnaca remained consistent along the sampling transect, with salinity levels at the outfalls (40.9 ± 3.5 g L^{-1}) similar to those at the upstream reference point (40.0 ± 1.8 g L^{-1}). Marginally increased salinities were recorded in the bottom water samples compared to the surface water samples (39.5 ± 1.6 and 39.4 ± 2.6 g L^{-1} at 0 m and 1500 m, respectively), with no significant differences ($P > 0.05$) observed across sampling locations. Similar outcomes were observed in salinity measurements across the seasons (data not shown). Measurements of temperature, pH, ORP and conductivity showed little variation along the sampling transect. At the Dhekelia site, although no statistically significant differences were found among the parameters across stations and seasons (data not shown), it is noteworthy that the salinity values were elevated at and near the outfall sites, ranging from 43.4 g L^{-1} in the bottom water to 34.5 g L^{-1} at the bottom water of the reference point. Temperature means fluctuated between 0 m and 1500 m locations, with no significant differences ($P > 0.05$).

3.1.2. Sediment

Iron, magnesium, Total Kjeldahl Nitrogen (TKN) and Total Organic Carbon (TOC) exhibited higher concentrations in the sediment at the Larnaca outfalls, compared to the regional reference point. Conversely, tin concentrations showed an inverse relationship, increasing along the sampling transect. Specifically, iron levels peaked at 50 m from the outfall, significantly decreasing to regional background levels at 200 m ($P < 0.05$, Table 4). TKN and TOC levels were notably higher at the outfalls, with statistical significance ($P < 0.01$).

At Dhekelia, TOC levels in the sediment were higher at 100 and 150 m from the pipe. Similarly to Larnaca, iron levels were elevated at the outfalls compared to the reference point ($P < 0.01$) (Tables 3 and 7).

Table 4
Mean (±SD) physicochemical parameters of sediment at 0 to 1500 m distances from the brine discharge pipe. Kruskal-Wallis tests for differences of parameters were performed among sampling stations and all statistically significant ($P < 0.05$) relationships were tested post-hoc using Dunn's tests. Statistical significance from post-hoc Dunn's tests shown as; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Distance from pipe (m)	0	50	100	150	200	1500
Larnaca	Sediment					
Iron (mg kg^{-1})	3.3 ± 1.2	4.3 ± 1.7 *	2.8 ± 1.2	NA	2.1 ± 1.5 *	2.4 ± 1.1
Magnesium (mg kg^{-1})	299.5 ± 204.6	265.8 ± 201.0	222.4 ± 147.5	NA	217.2 ± 156.1	204.5 ± 151.5
Tin (mg kg^{-1})	0.0	0.0	0.0	NA	0.8 ± 1.4	0.5 ± 0.8
Total Kjeldahl Nitrogen (%)	0.2 ± 0.1 ***	0.1	0.1 ***	NA	0.1 ***	0.1 ± 0.0
Total Organic Carbon (%)	13.5 ± 9.0 ***	3.8 ± 0.4 **	3.9 ± 0.9 ***	NA	3.8 ± 0.6 **	3.4 ± 0.1 ***
Dhekelia						
Iron (mg kg^{-1})	0.9 ± 0.2 **	0.8 ± 0.2	0.9 ± 0.1	1.1 ± 0.4	NA	0.5 ± 0.1 ***
Magnesium (mg kg^{-1})	233.3 ± 200.1	255.0 ± 228.3	279.6 ± 192.7	277.7 ± 204.1	NA	379.1 ± 127.9
Tin (mg kg^{-1})	0.0 ± 0.0	0.4 ± 0.6	1.1 ± 1.9	0.7 ± 1.1	NA	0.0 ± 0.0
Total Kjeldahl Nitrogen (%)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NA	0.1 ± 0.0
Total Organic Carbon (%)	2.5 ± 0.5 *	2.4 ± 0.3 *	2.7 ± 0.4	3.4 ± 0.5 *	NA	3.2 ± 0.9

Table 3
Mean (±SD) physicochemical parameters of water at 0 to 1500 m distances from the brine discharge pipe at Larnaca and Dhekelia Desalination Plants. Kruskal-Wallis tests for differences of parameters among sampling stations were all statistically non-significant ($P > 0.05$).

Distance from discharge pipe (m)	0	1500	0	50	100	150	200	1500
	Surface water		Bottom Water					
Larnaca								
Temperature (°C)	23.9 ± 4.73	23.91 ± 4.82	23.8 ± 4.75	23.84 ± 4.71	23.88 ± 4.78	NA	22.64 ± 4.34	23.73 ± 4.96
pH	8.1 ± 0.1	8.2 ± 0.1	8.1 ± 0.1	8.2 ± 0.0	8.2 ± 0.1	NA	8.2 ± 0.0	8.2 ± 0.0
Oxidation-Reduction Potential (mV)	145.05 ± 135.69	229.75 ± 48.01	242	167.3 ± 66.33	245.3	NA	215.05 ± 68.09	247.7 ± 23.05
Conductivity (mS cm^{-1})	56.04 ± 8.15	53.11 ± 1.17	57.78 ± 8.85	58.6 ± 10.45	54.98 ± 2.16	NA	54.2 ± 3.17	53.58 ± 1.24
Salinity (g L^{-1})	39.5 ± 1.6	39.4 ± 2.6	40.9 ± 3.5	41.7 ± 4.1	39.0 ± 3.1	NA	40.4 ± 0.9	40.0 ± 1.8
Orthophosphates (μg L^{-1})	1.5 ± 3.0	0.0 ± 0.0	0.3 ± 0.5	0.8 ± 1.0	0.0 ± 0.0	NA	0.0 ± 0.0	0.0 ± 0.0
Nitrates (μg L^{-1})	23.5 ± 21.8	26.3 ± 13.5	15.5 ± 8.2	34.5 ± 17.6	38.5 ± 39.8	NA	16.6 ± 7.9	20.0 ± 6.1
Nitrites (μg L^{-1})	2.5 ± 2.4	3.5 ± 3.1	1.8 ± 1.7	2.0 ± 0.8	3.3 ± 1.7	NA	1.8 ± 1.1	2.5 ± 1.7
Ammonium (μg L^{-1})	1.0 ± 2.0	1.0 ± 2.0	1.0 ± 2.0	27.3 ± 51.2	1.8 ± 3.5	NA	0.4 ± 0.5	0.5 ± 1.0
Silica (mg L^{-1})	NA	NA	1.0 ± 0.0	1.0 ± 0.0	NA	NA	NA	1.3 ± 0.6
Dhekelia								
Temperature (°C)	26.0 ± 4.9	25.2 ± 5.5	25.0 ± 5.9	25.1 ± 6.2	25.0 ± 6.1	25.1 ± 6.2	NA	29.1
pH	8.2 ± 0.0	8.2 ± 0.1	8.2 ± 0.0	8.2 ± 0.1	8.2 ± 0.1	8.2 ± 0.0	NA	8.2 ± 0.1
Oxidation-Reduction Potential (mV)	173.1	240.3	180	240.7	190.3	226.3	NA	238.4
Conductivity (mS cm^{-1})	57.4 ± 4.8	55.8 ± 4.6	68.3 ± 14.3	61.3 ± 12.7	55.0 ± 2.9	52.6 ± 3.1	NA	51.0 ± 13.8
Salinity (g L^{-1})	40.1 ± 2.4	39.5 ± 2.1	43.4 ± 3.1	43.4 ± 3.6	38.8 ± 3.9	41.0 ± 0.2	NA	34.5 ± 4.0
Orthophosphates (μg L^{-1})	0.8 ± 1.0	0.5 ± 0.6	0.5 ± 1.0	0.3 ± 0.5	0.5 ± 1.0	0.8 ± 1.0	NA	0.7 ± 1.2
Nitrates (μg L^{-1})	40.0 ± 39.4	18.3 ± 3.6	30.8 ± 16.8	21.8 ± 4.5	22.0 ± 2.4	17.5 ± 10.5	NA	18.7 ± 4.2
Nitrites (μg L^{-1})	2.5 ± 1.9	3.3 ± 2.2	3.0 ± 2.4	2.8 ± 1.7	2.3 ± 1.9	4.5 ± 1.7	NA	3.3 ± 2.5
Ammonium (μg L^{-1})	1.5 ± 2.4	0.5 ± 0.6	0.5 ± 1.0	2.0 ± 2.4	2.3 ± 3.9	2.8 ± 3.0	NA	2.0 ± 2.0
Silica (mg L^{-1})	NA	NA	1.0 ± 0.0	1.3 ± 0.6	NA	NA	NA	1.0 ± 0.0

Table 5

Mean (±SD) of alpha diversity indices in surface and bottom water samples from the sampling sites of Larnaca and Dhekelia and their respective reference points. Kruskal-Wallis tests for differences of parameters among sampling stations were all statistically non-significant ($P > 0.05$).

Distance from pipe (m)	0	1500	0	50	100	150	200	1500
Sample type	Surface water		Bottom Water					
Larnaca								
OTU richness	28.0 ± 9.6	27.8 ± 12.7	32.5 ± 7.1	47.8 ± 19.8	37.8 ± 7.2	NA	30.4 ± 16.1	37.5 ± 11.5
Shannon-Wiener H' index	3.0 ± 0.3	2.9 ± 0.3	3.0 ± 0.3	3.2 ± 0.6	3.2 ± 0.2	NA	2.5 ± 0.7	3.1 ± 0.5
Simpson D' index	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	NA	0.8 ± 0.1	0.9 ± 0.1
Chao1	43.0 ± 18.8	61.1 ± 44.1	90.4 ± 58.3	70.6 ± 30.0	54.0 ± 4.1	NA	46.4 ± 26.1	60.0 ± 22.0
Dhekelia								
OTU richness	37.0 ± 8.0	38.5 ± 15.9	50.3 ± 19.7	46.5 ± 21.6	34.5 ± 12.8	32.0 ± 6.8	NA	39.0 ± 8.5
Shannon-Wiener H' index	3.1 ± 0.2	3.2 ± 0.2	3.3 ± 0.1	3.1 ± 0.6	3.0 ± 0.2	3.0 ± 0.1	NA	3.3 ± 0.1
Simpson D' index	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.0	NA	1.0 ± 0.0
Chao1	59.2 ± 17.2	72.5 ± 17.7	81.6 ± 30.9	92.9 ± 33.9	59.1 ± 21.4	62.7 ± 13.3	NA	63.6 ± 20.6

Table 6

Mean (±SD) of alpha diversity indices from metabarcoding and from microscopic identification in sediment samples from the sampling sites of Larnaca and Dhekelia and their respective reference points. Kruskal-Wallis tests for differences of parameters among sampling stations were all statistically non-significant ($P > 0.05$).

Distance from pipe (m)	0	50	100	150	200	REF
alpha diversity from eDNA metabarcoding						
Larnaca	Sediment					
OTU richness	30.6 ± 33.4	36.6 ± 32.6	35.4 ± 44.8	NA	45.2 ± 44.4	19.2 ± 24.3
Shannon-Wiener H' index	1.9 ± 0.8	2.6 ± 0.7	1.9 ± 0.7	NA	1.9 ± 0.8	1.7 ± 1.1
Simpson D' index	0.7 ± 0.3	0.9 ± 0.1	0.7 ± 0.2	NA	0.7 ± 0.2	0.7 ± 0.3
Chao1	49.2 ± 48.3	53.9 ± 53.9	49.4 ± 60.4	NA	62.2 ± 59.3	31.0 ± 41.6
Dhekelia						
OTU richness	4.6 ± 4.8	4.9 ± 3.4	18.7 ± 42.9	6.3 ± 4.0	NA	4.0 ± 3.6
Shannon-Wiener H' index	0.9 ± 0.8	1.2 ± 0.6	0.9 ± 1.0	1.4 ± 0.7	NA	0.9 ± 0.8
Simpson D' index	0.4 ± 0.4	0.6 ± 0.2	0.4 ± 0.4	0.7 ± 0.2	NA	0.4 ± 0.4
Chao1	7.7 ± 9.8	7.6 ± 6.4	36.7 ± 85.5	8.3 ± 6.7	NA	5.3 ± 5.0
alpha diversity from microscopic identification						
Larnaca	Sediment					
Species richness	23.4 ± 10.0	24.7 ± 7.8	22.7 ± 11.4	NA	17.0 ± 8.1	11.6 ± 5.9
Shannon-Wiener H' index	2.2 ± 0.5	2.5 ± 0.4	2.5 ± 0.5	NA	2.4 ± 0.4	1.9 ± 0.6
Simpson D' index	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	NA	0.9 ± 0.0	0.8 ± 0.1
Chao1	33.8 ± 16.5	36.0 ± 12.5	34.2 ± 18.1	NA	25.9 ± 15.3	19.7 ± 16.7
Dhekelia	Sediment					
Species richness	2.0 ± 2.1	3.2 ± 1.9	3.8 ± 1.4	5.3 ± 2.3	NA	4.2 ± 2.6
Shannon-Wiener H' index	0.6 ± 0.6	0.9 ± 0.6	1.2 ± 0.4	1.4 ± 0.5	NA	1.2 ± 0.5
Simpson D' index	0.7 ± 0.3	0.5 ± 0.3	0.6 ± 0.2	0.7 ± 0.2	NA	0.7 ± 0.2
Chao1	3.5 ± 5.4	4.2 ± 2.6	6.1 ± 3.7	10.0 ± 7.4	NA	6.2 ± 5.8

3.1.3. Sequencing data

A total of 264,749 high-quality reads were generated across 194 samples. After stringent data filtering and curation, a total of 40,730 reads and 584 OTUs were kept for alpha and beta diversity analysis, with an overall 78.1 % identified at family level, 69.0 % at genus level and 62.8 % at species level.

More than half (265 or 53.7 %) of all OTUs were found only in the sediment samples, 105 or 39.2 % were found in water samples, where 9 were found only in the surface water samples, and 78 OTUs (43.6 %) were found in all sample types. Larnaca samples had 210 unique OTUs and Dhekelia had 50 unique OTUs.

3.1.4. Species diversity

Alpha diversity in water samples from both locations tended to increase at 50 m from the pipe outlet before decreasing with distance from the pipe. The water column at Larnaca and Dhekelia showed increased Shannon-Wiener and Simpson index values in deeper waters compared to surface water samples, although the Chao1 estimator showed higher values in surface waters than in bottom waters at both locations (Table 5). No significant differences in alpha diversity were found among water samples along the sampling gradient at Larnaca and Dhekelia and their respective reference points ($P > 0.05$).

At Larnaca, OTU richness and Chao1 mean values were higher in the bottom waters compared to surface waters across all seasons, with an overall decrease of mean values of all four alpha diversity measures from spring to summer, and an increase from summer to autumn. At Dhekelia, OTU richness mean values were higher in the bottom waters compared to surface waters across spring, autumn and winter. The Chao1 mean values had a similar tendency except for winter samples, with an overall decrease of mean values of richness, Shannon-Wiener and Chao1 indices from spring to summer, followed by an increase to winter (Data not shown).

Alpha diversity for sediment samples followed a similar trend at both locations. At Larnaca, the Shannon-Wiener index was the highest at 50 m from the pipe and the lowest at the reference point. At Dhekelia, the highest was at 150 m from the pipe, and the lowest was at the outfalls and at the reference point (Table 6).

In the sediment samples from Larnaca, alpha diversity indices increased at 50 m, comparable to the water samples, with a sudden increase in OTU richness at 200 m from the outfalls pipe.

Table 7
Presence/absence of the ten most abundant genera from the eDNA and their comparison to microscopy samples for each location and sample type. From the sums of all OTU read counts aggregated at Genus level and number of individuals from microscopy: – = No detection, * = Low abundance, ** = Medium abundance, *** = High abundance.

		eDNA				Microscopy	
		Larnaca		Dhekelia		Larnaca	Dhekelia
Phylum	Genus	Sediment	Water	Sediment	Water	Sediment	
Annelida	*Aphelochaeta*	***	–	**	–	*	*
	Euchone	***	*	*	*	–	–
	Diplocirrus	**	*	*	*	*	–
	Aricidea	**	*	*	*	*	*
	Leiochone	*	**	*	**	*	–
	Scoloplos	*	**	*	*	–	*
	Limnodriloides	*	–	*	*	*	*
	Capitella	*	–	*	*	*	*
	Heteromastus	*	*	–	–	*	–
	Lysidice	**	–	*	*	*	–
	Melinna	***	*	**	*	*	*
	Saccocirrus	–	–	–	–	–	***
Arthropoda	*Oithona*	–	**	–	**	–	–
	Cletodes	**	*	*	*	–	–
	Acartia	–	*	–	*	–	–
	Mecynocera	*	*	*	*	–	–
	Centropages	–	*	–	*	–	–
	Diogenes	–	–	–	–	*	**
	Bathyporeia	–	–	–	–	*	*
	Centraloecetes	–	–	–	–	*	*
	Anapagurus	–	–	–	–	–	*
	Ampelisca	–	–	–	–	***	*
Mollusca	*Turbonilla*	**	*	*	*	–	–
	Bittium	**	**	–	**	–	–
	Creseis	*	**	–	*	–	–
	Steromphala	–	*	–	*	–	–
	Jujubinus	*	*	*	*	–	–
	Thracia	–	–	–	–	*	**
	Philine	–	–	–	–	*	–
	Nucula	–	–	–	–	*	–
	Smaragdia	–	–	–	–	*	–
	Loripes	–	–	–	–	–	*
Echinodermata	*Asterina*	**	**	*	**	–	–
(only identified to family level with microscopy)	*Paxillosida*	–	***	–	**	–	–
	Echinocardium	–	**	–	**	–	–
	Astropecten	–	*	–	**	–	–
	Acrocnida	*	–	–	–	–	–
	Amphiuridae	–	–	–	–	*	*
Cnidaria	*Bougainvillia*	***	***	*	***	–	–
(only identified with eDNA)	*Isozoanthus*	–	*	–	**	–	–
	Meira	–	*	–	*	–	–
	Liriope	–	**	–	*	–	–
	Edwardsia	–	–	–	*	–	–
	Aurelia	–	*	*	*	–	–
	Laviactis	*	–	*	–	–	–
Porifera	*Polymastia*	*	***	*	***	–	–
(only identified with eDNA)	*Cribrochalina*	**	–	**	–	–	–
	Mycale	*	*	*	**	–	–
	Cliona	*	–	*	–	–	–
	Haliclona	*	*	–	**	–	–
Chordata	*Ascidia*	*	***	–	**	–	–
(only identified to family level with microscopy)	Muraenidae	–	–	–	–	*	*
Nemertea	*Ototyphlonemertes*	**	*	*	*	–	–
(only identified to phylum level with microscopy)	*Cephalothrix*	*	**	*	**	–	–
	Cerebratulus	*	*	–	*	–	–
	Paradrepanophorus	*	*	*	–	–	–
	Tubulanus	–	*	–	–	–	–

In the sediment samples from Dhekelia this sudden increase pattern was observed at 100 and at 150 m, respectively, notably for OTU richness and Chao1 estimator. For both locations, their reference point's diversity indices had decreased values (Table 6).

At Larnaca, across the seasons, there was a considerable decrease in OTU richness, Shannon-Wiener and Chao1 indices mean values from spring all the way to winter. At Dhekelia, the decreasing trend was observed from spring to autumn, with an increase in mean values from autumn to winter for all alpha diversity measures (Data not shown).

Principal Component Analysis (PCA) of water samples demonstrated strong seasonality for both Larnaca and Dhekelia, with distinct communities in each season. The effect of seasonality was not as prominent in the sediment samples compared to the water samples, as the communities appear to overlap (Fig. 2).

Redundancy analyses constrained to the distance of the sampling locations from the discharge point were carried out for both sites. At Larnaca, it was found that invertebrate communities in the water samples were distinct among the four locations (the reference point for this

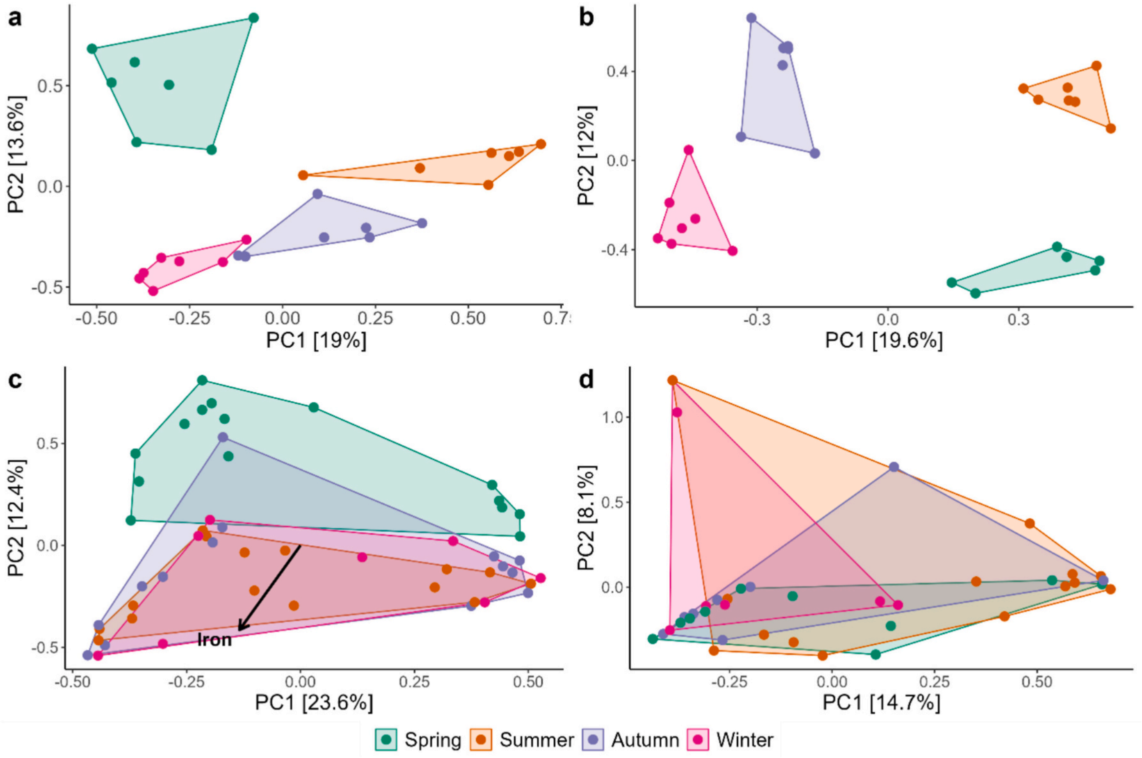

Fig. 2. Principal Component Analysis of OTUs in samples from a) surface and bottom waters of Larnaca, b) surface and bottom waters of Dhekelia, c) sediments of Larnaca and d) sediments of Dhekelia, with significant correlations to the environmental parameters showing on the plot (envfit, $P < 0.05$).

site is located upstream, therefore was not included in the constrained analysis), whereas in the sediment samples there was overlap of the communities around the outfalls (0 m), as well as at 50 m and 200 m (Fig. 3).

At Dhekelia, separated communities were observed in both water and sediment samples (Fig. 3). Invertebrate communities around the outfalls were distinct and different from those further away from the pipe. An overlap of the community composition at the points at 100 and 150 m could also be related to the similar rocky environment, as opposed to the rest of the sampling points that were taken from mainly sandy bottom (Fig. 1).

3.1.5. *Description of communities and comparison to morphological data*

Within the water and sediment samples from Larnaca, half of 534 OTUs were from the phylum of Annelida, and almost all were from the class Polychaeta. Within the Polychaeta, the dominating families were Sabellidae (higher numbers at outfalls), Maldanidae (44 OTUs, of which 41 *Leiochone leiopygos*, predominantly found in water samples), Trichobranchidae (higher numbers near outfalls, consisting of *Trichobranchus glacialis*), Cirratulidae (more abundant near outfalls and only one of 30 identified to genus level), Capitellidae (higher numbers at the discharge pipe), Flabelligeridae (12 of *Diplocirrus glaucus* and one of *D. seisuiae*). The majority of Arthropoda consisted of OTUs from Copepoda from the families of Clausocalanidae, Paracalanidae, in the water samples and Cletodidae, Cyclopinidae in the sediment samples, identified to species level. Two shrimps were observed within Arthropoda, *Artemia franciscana* and *Lucifer* sp. (only found at the reference sampling point). A smaller number of Mollusca families were observed, where Trochidae and Cerithiidae were the most dominant and found in the water samples. Echinodermata represented a lower amount of taxa observed, where four out of five families were found in water samples and described to species level. Members of the Family Asterinidae were more abundant, and Cnidaria had seven families with half the taxa from the genus *Bougainvillia*, found both in sediment and water samples. Five families were recorded for the Nemertea and six families for the Porifera, respectively.

Contrary to the samples from Larnaca, at Dhekelia only 134 out of 347 OTUs were from Annelida and 120 from Polychaeta. Maldanidae, Trichobranchidae and Cirratulidae were the most dominant families, represented by the same species as Larnaca. Of the 133 OTUs of Arthropoda, 78 were Copepoda, and *Armatoglyptes habei*, a species of the class Thecostrata, which was not observed in Larnaca. They also included seven families of Cnidaria, four families of Echinodermata, four families of Nemertea and seven families of Porifera.

At a family level, Ampharetidae, Capitellidae, Cirratulidae, Flabelligeridae, Maldanidae Orbiniidae, Paraonidae, Sabellidae were found in all sampling locations at Larnaca. At Dhekelia, Capitellidae and Flabelligeridae were found only at 0 or 50 m.

Within the dataset derived from the microscopic identification of sediment samples, Larnaca had higher species richness, Shannon-Wiener index and Chao1 estimator mean values at the sampling points near the outfalls (0–200) compared to the reference point. At Dhekelia, by contrast, the same index mean values were lower at 0–150 m from the pipe, compared to the reference point (Table 6). Additionally, and comparably to the results from metabarcoding, species richness and Chao1 estimator for Larnaca sediments were higher in spring samples, with a decreasing pattern to autumn, compared to winter. This was not the case for the Dhekelia sediment samples, as the same indices increased in the spring, reaching the highest numbers in the summer and decreasing during winter (Data not shown).

At the family level, the metabarcoding and microscopy datasets shared 18 out of 129 families observed in total, with two of them present only in Dhekelia. The majority of these families were Annelida, as shown in Fig. 4.

Within these families, 16 genera were shared out of 195, and ten species out of 244, all from the class of Polychaeta. Polychaeta (Annelida) were the most abundant phylum in spring in both datasets from microscopy and eDNA.

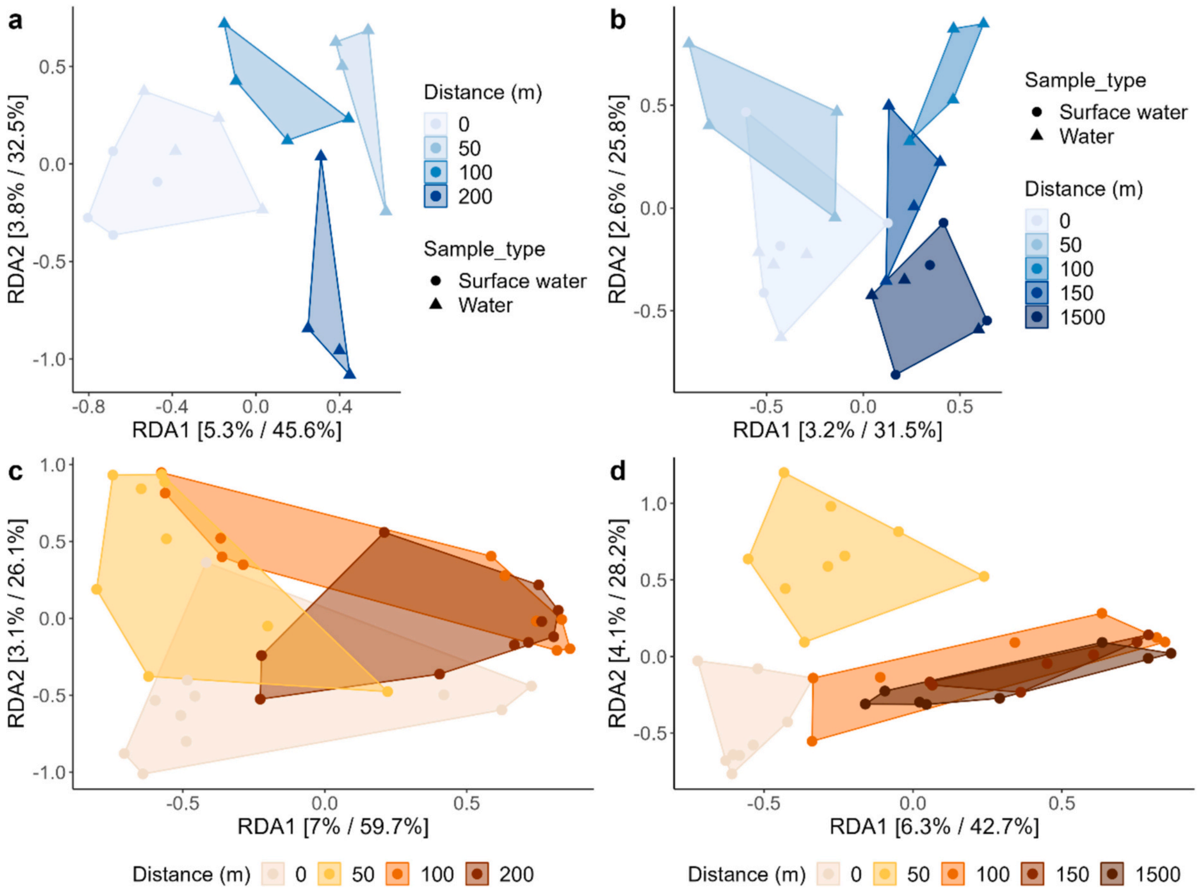

Fig. 3. Redundancy Analysis constrained to the distance from the brine discharge pipe of A) surface and bottom waters of Larnaca, B) surface and bottom waters of Dhekelia, C) sediments of Larnaca and D) sediments of Dhekelia. Correlations to the environmental parameters were fitted, but none of them were statistically significant (envfit, $P > 0.05$).

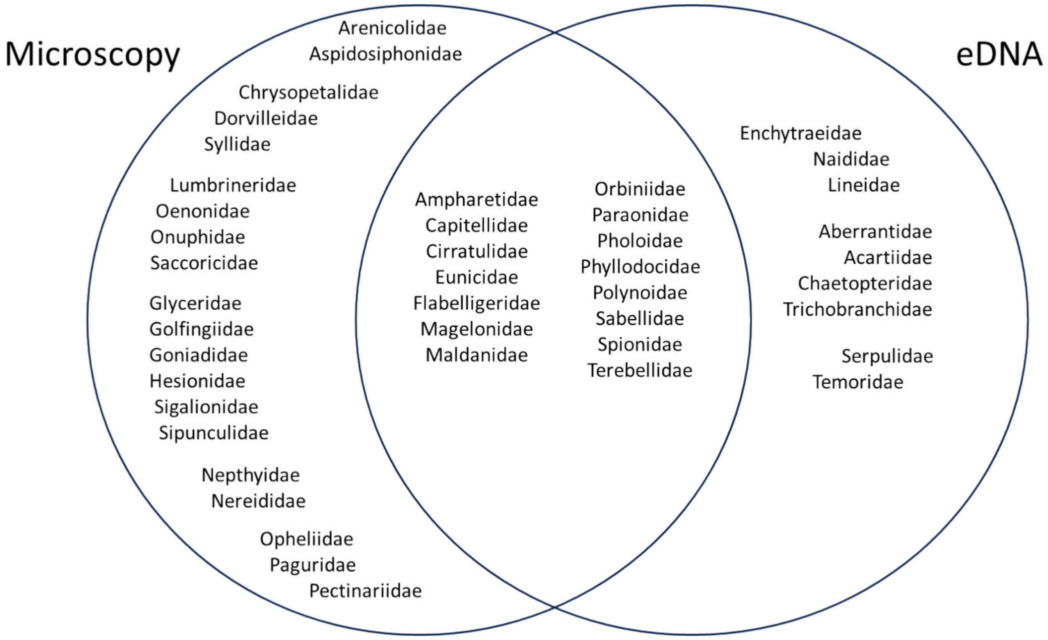

Fig. 4. Venn diagram of families within the phylum of Annelida, detected by eDNA and identified by microscopy.

Cnidaria and Porifera were only detected with eDNA and Nemertea and Echinodermata were only identified to phylum level with microscopy, but were detected to species level with eDNA (Table 6). Annelida was detected at a sufficient level from both microscopy and eDNA, and although there was not a lot of overlap at genus level, this was more prominent at family level. Arthropoda detected with eDNA were primarily Copepoda, whereas the microscopy resulted in more varied classes within the phylum. Nemertea was only identified to phylum level with microscopy, whereas eDNA identified Nemertea down to genus and species level.

4. Discussion

This study presents the results of a seasonal monitoring survey conducted at the outfalls and reference points of two SWRO desalination plants in Cyprus. Previous research at the Larnaca study site demonstrated a clear impact of the brine on seagrass (*P. oceanica)* meadows (Xevgenos et al., 2021). This study further advances the current knowledge around the impact of SWRO brine discharge on marine benthic communities in Cyprus, integrating eDNA metabarcoding with existing monitoring techniques, providing a new aspect in biodiversity assessment. Increased diversity was observed at and near the brine outfalls (up to 100 m) of both the Larnaca and Dhekelia desalination plants. However, the physicochemical parameters of water and sediment did not show a direct, significant impact on the communities. At Larnaca, diversity was mainly represented by polychaete families Capitellidae and Cirratulidae, known to be associated with disturbed environments. Communities at and near the outfalls of the Dhekelia plant were more taxonomically diverse.

4.1. Physicochemical parameters

Salinity was elevated at the outfalls compared to the reference points and bottom water, with an increase near the outfalls observed for Larnaca, potentially due to localised hydrodynamic conditions leading the brine dispersal away from the immediate discharge location. This highlights the importance of precise mapping of the brine plume in monitoring studies, especially in cases where impact is localised. Elevated salinities were expected at and near the outfalls, as brine from RO desalination, being denser than seawater, creates a stratification within the water column. This stratification of the water column occurs naturally during the warmer months in the Levantine Sea, and with higher salinities observed at the surface (Hannides et al., 2015). However, this was only the case for the samples furthest away from the pipe (controls), further confirming the brine-induced stratification at the two desalination plants. Brine lowers dissolved oxygen content, and it can create stratification in the water column, leading to anoxic conditions in the porewater and sediment directly below the brine, as well as hindering of nutrient flux from the surface to the bottom (Grossowicz et al., 2019). The discharge pipe of Larnaca is set at 13 m, compared to Dhekelia, which is at 3.5 m depth, and the dispersion of brine at the two desalination plants is extensive and its impact on seagrass meadows is evident. The substratum at the Larnaca outfall is fine sand, while the reference point as well as the sediments at Dhekelia are coarse sand, allowing for greater porewater movement. At the outfalls of both sites, the iron-rich "red brine" was observed when diving, which can increase water turbidity, and bury sessile organisms. Water iron levels at the Larnaca outfall were significantly higher compared to background levels. The chemicals used during the desalination process include ferric salts or hydroxide as a coagulant, also present in the RO membrane backwashing system, which is washed out in the rejected brine, potentially contributing to elevated iron levels at and near the Larnaca outfalls, such as previously observed in Saudi Arabia (Saeed et al., 2019). At Larnaca, TOC was considerably more elevated (almost four times) near the discharge pipe, and is potentially associated with the chemicals used during the desalination process, which in turn favours oxygen depletion and alteration of benthic communities (Lattemann and Höpner, 2008; Roberts et al., 2010).

4.2. eDNA results

The eDNA metabarcoding revealed significant variations in biodiversity at the two desalination plants. Shannon-Wiener index of surface and bottom water samples of Larnaca was higher near the outfalls than the samples at the reference point. Analysis of beta diversity showed a distinct separation of water samples, with no overlap between samples from the outfalls and the rest of the sampling points. This could be an indication of organisms showing opportunistic presence at the outfalls, attributed to the creation of new microhabitats on and around the discharge pipe structure. Apart from the presence of the pipe structure itself, organisms attached on the structure could be contributing to the creation of a microhabitat, acting as an artificial reef. This has been supported by similar studies performed on other types of manmade structures, such as oil platforms (Cordier et al., 2019; Mauffrey et al., 2021).

Shannon-Wiener index was the highest at 50 m from the pipe at Larnaca, with an overall decreasing trend, indicating greater diversity of species closer to the outfalls. Sediment communities showed little overlap between 0 and 50 m and no overlap between 0 and 100 m, making the communities along the sampling gradient distinct from each other. Since the metabarcoding results are not quantitative, this result is probably explained by the remaining DNA-traits from the original ecosystem still detectable, while a different brine-tolerant ecosystem was expanding close to the discharge point, potentially affected by the brine-related nutrient influx, such as the very high TOC levels observed at the outfalls. Polychaeta dominate the assemblages at and near the outfalls at Larnaca. At Dhekelia, sediment samples from 50 and 150 m distance from the pipe had the highest Shannon-Wiener index, however, the Chao1 index was comparatively very low, attributed to the dominance of polychaetes *Aphelochaeta, Melinna palmata* and *Trichobranchus glacialis*. Additionally, assemblages at the outfalls and at 50 m from the outfalls of Dhekelia had communities distinct from the rest.

4.3. Impact on specific taxa

Previous studies at desalination plant outfalls found that increased salinity levels negatively impacted benthic polychaetes, with a decrease in their abundance and richness, however, such disturbed environments can also favour stress-tolerant taxa, such as Cirratulidae and Capitellidae, both heavily present at outfalls in the present study, detected both by eDNA and microscopy. Polychaetes play a key role in benthic nutrient recycling and sediment bioturbation, thus affecting organic matter remineralisation and sedimentation (Sarda et al., 2009) and have been used as ecosystem health assessment indicators, due to the existence of tolerant and sensitive taxa within the class (Giangrande et al., 2005). The most recent Polychaeta checklist for Cyprus showed increased relative numbers of polychaetes recorded in Larnaca, mainly of Indo-Pacific origin, compared to the rest of the island (Rousou et al., 2023), with 6.1 % of overall numbers of recorded polychaetes being Non-Indigenous species (NIS) associated with desalination. On a family level, both morphological and molecular approaches confirmed the diversity of polychaetes, which was the most abundant class in both datasets. Families Eunicidae, Capitellidae, Cirratulidae, Spionidae, all indicative of opportunistic behaviour due to organic enrichment (Giangrande et al., 2005), have been detected at and near the outfalls of Larnaca by both methods. Cirratulidae and Spionidae were among the opportunistic families observed at the outfalls of two desalination plants in Algeria (Belatoui et al., 2017), where an overall decrease in abundance and diversity of polychaetes was also observed. However, the capacity of the two plants studied was more than triple that of either of Larnaca or Dhekelia desalination plants, with brine discharge at 245,000 m^3 day^{-1}. Given an approximate conversion rate of 40 % of brine from the desalination of 60,000 m^3 day^{-1} for each of the two plants studied here (Xevgenos et al., 2021), 90,000 m^3 day^{-1} of brine is expected to be discharged at Larnaca and Dhekelia. This number is almost ten times lower than the brine expelled from the two aforementioned desalination plants in Algeria, where the impact is expected to be much more acute, despite the presence of multi-diffuser pipes. The impact observed at Larnaca, which employs a single discharge pipe, is indeed more prominent than at Dhekelia, however, it is not possible to attribute this solely to the difference in outfall design. Some studies (De-La-Ossa-Carretero et al., 2016a; Petersen et al., 2018) have been conducted at desalination plant outfalls prior to and after the installation of a multidiffuser pipe, showing that benthic communities recovered after the switch, therefore it is possible to achieve ecosystem recovery via better design of the outlet. The two sites exhibited distinct differences in the impact of brine discharge on benthic communities.

4.4. Comparison with other studies

The observed impacts are consistent with previous studies on the impacts of desalination brine (e.g., Lattemann and Höpner, 2008; Roberts et al., 2010), which noted significantly altered benthic communities with increased chemical concentrations. An Environmental Impact Assessment of a desalination plant in Australia determined the impact to extend to 100 m from the discharge pipe, while incorporating a limit of 1 g L^{-1} of natural salinity variation, due to seasonality (Clark et al., 2018). At Larnaca, average salinity varies the most between 50 and 100 m from the outfalls, however at Dhekelia this fluctuation is much more prominent, with 4.5 g L^{-1} difference from 50 to 100 m. Assuming that marine organisms in the Mediterranean show increased tolerance to natural salinity fluctuation, it is evident that salinity does not play the only role in the formation of communities at outfalls. Instead, the impact is derived from the quality of the brine itself. A study at a smaller-scale RO desalination plant in Spain showed no significant variations in the benthic communities, even though salinity reached a maximum of 60 psu at the outfalls, however, the presence of more fish species was observed on the outfall structure, which served as an artificial reef (Raventos et al., 2006).

4.5. Methodological considerations

eDNA metabarcoding and microscopy provided complementary insights into the biodiversity patterns and community structures. Overall, the two methods approach showed more overlap on family level than species level. Cnidaria and Porifera were only detected with eDNA, but Arthropoda was better described with microscopy. Annelida showed little overlap, but both methods effectively described certain groups of taxa (Arthropoda, Nemertea) with high resolution.

A major challenge is the incomplete taxonomic coverage of currently available molecular reference databases, which is particularly important for Cyprus given its geographic location on a direct migration route for NIS via the Suez Canal (Baeta et al., 2021). The shortage of DNA barcode sequences from relevant taxa has previously been highlighted as an issue for Mediterranean biodiversity studies using eDNA (Bartolo et al., 2020). In this context, we were not able to find sequences that could correspond to the recently-described amphipod *Pontocrates marmario*, found in marine sediments at the Dhekelia outfall within the framework of this study (Garcia Gómez et al., 2024). The potential use of multiple primer sets to target more than one gene could be implemented in future studies to overcome taxonomic bias. The same applies for 2 other crustacean species, *Apseudopsis larnacensis* and *A. salinus* (Tanaidacea), which were described as new species following their finding in sediment samples at the outfall of the Larnaca desalination plant within the framework of this study (Stępień et al., 2024), which could not be detected among the eDNA sequences either.

4.6. Environmental and ecological implications

This study underscores the impact of desalination brine discharge on marine benthic communities, particularly in terms of community structure and diversity.

Cyprus is subject to the Lessepsian migration of a large number of taxa of Indo-Pacific origin via the Suez Canal. Because of this, its marine ecosystem is susceptible to changes in trophic levels of the food web, due to the arrival of opportunistic predators, with the ability to displace local fauna (Fanelli et al., 2015). Additionally, a wide range of organisms – especially alien species - may temporarily adapt to unusual salinity conditions, as shown previously for alien fish species and the sea cucumber *Synaptula reciprocans* (Xevgenos et al., 2021) and it is reasonable to assume that this also applies for microinvertebrates. However, extreme and unfavourable conditions can affect the abundance of fauna and flora. On some occasions, changes in the ecosystem may attract other unusual species already present in the region, yet in minor abundances, under normal conditions.

Reduced oxygen levels at brine discharge points, as well as the deposition of particles from membrane backwash, such as in the case of Larnaca, can lead to anoxic conditions, and increased residence time of pollutants. This may have a long-term effect on the sensitive habitats present in the area, such as seagrass meadows, which play a vital role in the food web (Fourqurean et al., 2012).

4.7. Management and mitigation recommendations

Effective management of brine discharge impacts requires detailed Environmental Impact Assessments and careful consideration of site-specific factors. Rapid mixing of brine is crucial to avoid long-term stratification, necessitating rigorous Phase I studies and long-term monitoring schemes. In this context, various studies have shown that the installation of a multi-diffuser discharge pipe facilitates the mixing of brine (Belatoui et al., 2017; Clark et al., 2018; Torquemada et al., 2009).

Future research should focus on the effectiveness of different mitigation strategies and the potential for recovery and, where appropriate and feasible, targeted restoration of impacted benthic communities. Integrating molecular techniques, classical morphology-based taxonomy and environmental parameters will provide a more comprehensive understanding of the ecological impacts. This work is better supported by analysis at family-level, however building case-specific reference databases and validating primers at the genus and the species level are essential steps towards better management of the sensitive communities in the marine environment of Cyprus.

5. Conclusion

This study has shown the significant impact of desalination brine discharge on marine benthic communities in Cyprus, particularly in terms of community structure and diversity. Seasonal and spatial variations following a gradient from the discharge points were revealed using eDNA metabarcoding. The eDNA results were affected by abiotic factors, such as nutrient levels and local topography. Metabarcoding indicated that the brine discharge introduced alterations in the taxonomic composition of the ecosystem. Surprisingly, metabarcoding showed increased diversity closer to the discharge point. This is probably explained by remaining DNA-traits from the original ecosystem still detectable, while the brine-tolerant ecosystem was expanding close to the discharge point. Salinity measurements were consistent along the sampling transect, for both Larnaca and Dhekelia, and did not show major seasonal or spatial fluctuations, which implies that other abiotic factors contribute to the variations observed. eDNA-metabarcoding successfully captured the local biodiversity at family level. However, the available DNA databases do not yet contain sufficient sequence information from relevant Mediterranean taxa to enable species identification of the identified OTUs.

CRediT authorship contribution statement

K. Grammatiki: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Data curation. **N. de Jonge:** Writing – review & editing, Supervision, Resources, Methodology, Data curation. **J.L. Nielsen:** Writing – review & editing, Supervision, Resources, Conceptualization. **S.C. García-Gomez:** Writing – review & editing, Investigation. **E. Avramidi:** Writing – review & editing, Methodology, Investigation, Data curation. **M.M. Lymperaki:** Writing – review & editing, Investigation. **M. Marcou:** Investigation. **G. Ioannou:** Investigation. **M. Papatheodoulou:** Investigation. **O. Dargent:** Writing – review & editing, Investigation. **D. Xevgenos:** Writing – review & editing, Funding acquisition, Conceptualization. **M. Hesselsøe:** Writing – review & editing, Resources, Conceptualization. **F.C. Küpper:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

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.

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Data availability

The raw sequencing data generated for this study will be made available at the European Nucleotide Archive under project number PRJEB79159 upon publication.

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