DOI:10.53539/squjs.Splisspp69-81

Sultan Qaboos University Journal for Science

Journal page: www.squ.edu.om/index.php/squjs/index

The Continued Presence of Antibiotic-resistant Bacteria and Heavy Metals at one of the World's Most Important Green Turtles' Nesting Location, Ras al-Jinz Reserve, Oman

Al-Amrani, S.A.[1], Al-Musharafi, S.K.[2], Mahmoud, I.Y.[1†], Al-Bahry, S.N.[1], Leduc A.O.H.C.[1*]

[1*]Department of Biology, Sultan Qaboos University, Al Khoud 123, Sultanate of Oman
[2]University of Technology and Applied Sciences SUR, Sur 411, Sultanate of Oman
†The author is deceased

*Email address: a.leduc@squ.edu.om

ARTICLE HISTORY

Received 26 March 2024
Received revised 10 June 2024
Accepted 10 June 2024

ABSTRACT: Environmental contamination of marine habitats is a global concern, especially in areas that are crucial for endangered species' life cycles. To gain a comprehensive understanding of environmental contamination, analyzing various biotic and abiotic components of ecosystems is necessary. Situated in the Arabian Peninsula, Ras al-Jinz Reserve serves as an invaluable nesting site for green sea turtles (*Chelonia mydas*). There, the prevalence of antibiotic-resistant microbial strains and heavy metals was assessed by collecting samples from diverse components of this ecosystem, including sand from turtle nests, dead turtle hatchlings, fresh turtle eggs, oviductal fluids from nestling turtles, turtle carapace smears, live crabs, and seawater. Microbial fauna was identified using a MALDI-TOF Biotyper. The concentration of heavy metals was measured by an inductively-coupled plasma mass spectrometry. Among the isolated bacterial strains, 70 heterotrophic species belonging to eleven genera were identified, with over 46% of isolates belonging to the *Pseudomonas* species. More than 85% of isolates displayed antibiotic resistance but had negligible resistance to chloride. Of the 23 heavy metals assessed, Titanium and, in lesser measure Vanadium, were found in the highest concentrations, reaching up to 12 µg g^{-1}. While such concentrations might not be immediately alarming, they are significant enough to warrant attention due to the potential health and environmental impacts. These results obtained over a decade after the last monitoring in the Reserve provide compelling evidence of the persistent presence of antibiotic-resistant bacteria. Additionally, the study confirms the prevalence of heavy metals, the sources of which remain unknown but are likely from anthropogenic sources. It is crucial to implement measures that mitigate sea turtles' exposure to contaminants in Ras al-Jinz Reserve. Regular monitoring efforts are required to assess the emergence of discernible contamination trends in this critically important area.
Keywords: Chelonian conservation; Environmental contamination assessment; Microbial contamination; Biological reserve; Coastal pollution.

استمرار وجود البكتيريا المقاومة للمضادات الحيوية والمعادن الثقيلة في أحد أهم مواقع تعشيش السلاحف الخضراء في العالم، محمية رأس الجنز، عمان

العمراني، س.أ.، المشرفي، س.ك.، محمود، إ.ي.، البحري، س.ن.، ليدوك، أ.أ.ه.س

الملخص: يشكل التلوث البيئي للموائل البحرية مصدر قلق عالمي، وخاصة في المناطق ذات الأهمية لدورات حياة الأنواع المهددة بالانقراض. للحصول على فهم شامل للتلوث البيئي، من الضروري تحليل المكونات الحيوية وغير الحيوية المختلفة للنظم البيئية. تقع محمية رأس الجنز في شبه الجزيرة العربية، وهي بمثابة موقع تعشيش لا يقدر بثمن للسلاحف البحرية الخضراء (Chelonia mydas). هناك، تم تقييم مدى انتشار السلالات الميكروبية المقاومة للمضادات الحيوية والمعادن الثقيلة من خلال جمع عينات من مكونات متنوعة لهذا النظام البيئي، بما في ذلك الرمال من أعشاش السلاحف، وصغار السلاحف الميتة، وبيض السلاحف الطازجة، وسوائل المبيض من السلاحف المعششة، ومسحات درع السلاحف، والكائنات الحية. السرطانات، ومياه البحر. تم التعرف على الحيوانات الميكروبية باستخدام MALDI-TOF Biotyper. تم قياس تركيز المعادن الثقيلة بواسطة مطياف الكتلة البلازمية المقترنة حثياً. من بين السلالات البكتيرية المعزولة، تم تحديد 70 نوعًا غيري التغذية تنتمي إلى أحد عشر جنسًا، وأكثر من 46% من العزلات تنتمي إلى فصيلة الزائفة. أظهر أكثر من 85% من العزلات بعض مستويات مقاومة المضادات الحيوية ولكن كانت لديها مقاومة ضئيلة للكلوريد. ومن بين المعادن الثقيلة الـ 23 التي تم تقييمها، تم العثور على التيتانيوم، وبدرجة أقل الفاناديوم، بأعلى التركيزات، حيث وصلت إلى 12 ميكروجرام لكل جرام. توفر هذه النتائج التي تم الحصول عليها بعد مرور عقد من المراقبة الأخيرة في المحمية دليلاً دامغًا على الوجود المستمر للبكتيريا المقاومة للمضادات الحيوية. بالإضافة إلى ذلك، تؤكد الدراسة انتشار المعادن الثقيلة التي لا تزال مصادرها مجهولة. ومن الضروري تنفيذ التدابير التي تخفف من تعرض السلاحف البحرية للملوثات في محمية رأس الجنز. ويلزم بذل جهود رصد منتظمة لتقييم ظهور اتجاهات التلوث الواضحة في هذه المنطقة البالغة الأهمية.

الكلمات المفتاحية: الحفاظ على السلاحف البحرية، تقييم التلوث البيئي، التلوث الميكروبي، المحمية البيولوجية، التلوث الساحلي.

1. Introduction

Coastal areas are of high importance to humanity because of the benefits they provide, including transportation, food resources and biodiversity. However, owing to tremendous human growth, coastal areas are now facing an unprecedented environmental crisis [1,2]. Specifically, pollution via chemical and bacteriological agents has raised attention, given the detrimental effects they may impose on biota. The confluence of bacterial resistance and the accumulation of heavy metals is rapidly transforming coastal regions into ecological hotspots of contamination [3].

Located in the Sultanate of Oman, Ras al-Jinz Reserve is a recognized sanctuary for the nesting of sea turtles. Yearly, ~20,000 – 25,000 green sea turtles (*Chelonia mydas*) are estimated to lay eggs in this reserve [4], highlighting this location as a significant conservation hotspot. Over a decade ago, concerns emerged regarding the levels of contamination that could threaten the ecosystem of this reserve [5,6]. Specifically, freshly laid eggs from green turtles at the reserve were found to harbor several elements, including some heavy metals, such as Zn, Cu and Mn in diverse concentrations [5]. Furthermore, bacterial contamination, including antibiotic-resistant strains, was also found, specifically, in the oviductal fluids of green sea turtles laying eggs in this reserve [6]. Together, these findings raised concern about the contamination in this otherwise pristine ecosystem.

Considering the crucial role of the Raz al-Jinz Reserve for sea turtle conservation, these findings led to the undertaking of more thorough monitoring programs in the following decade. However, details and conclusions of the research are still pending release, which prevents sharing important information that might be required for the development of strategic conservation planning. Sustained monitoring and the accessibility of data are indispensable for identifying the requisite measures for targeted environmental management in contaminated areas, as emphasized by prior research [7–9]. Consequently, our report presents the findings derived from the most thorough monitoring efforts on chemical contamination to date within Ras al-Jinz Reserve. These results were obtained over a decade after the initial assessments, providing an opportunity for meaningful comparisons to be made.

The simultaneous influx of heavy metals and bacterial contamination may impose severe health challenges on marine organisms [10]. For instance, heavy metals, such as lead, cadmium, and mercury can cause a range of harmful effects such as carcinogenesis, malformations, and damage to the nervous and reproductive systems [11]. Addressing these contamination issues is not only crucial for sea turtles, but also for maintaining the ecological balance of coastal regions. Here, our goal is to highlight potential trends in contamination, from the original monitoring efforts of chemical contaminants conducted in the reserve [5,6,12,13], and compare these results with those obtained a decade later, i.e., 2015. These monitoring efforts encompassed assessments of heavy metal and bacterial contamination, (including antibiotic resistance) within the reserve during the year 2015. Together, these findings will establish a foundation for comparative analyses with contemporary monitoring efforts in the Reserve.

2. Materials and Methods

Study area

Ras al-Jinz Reverse (coordinates: 22°25′24.5″N, 59°50′18.8″E) is the easternmost point of the Arabian Peninsula, which is located where the Sea of Oman and the Arabian Sea intersect (Figure 1). This location is widely recognized for its significance as a nesting ground for the green turtle (*C. mydas*). The Reserve consists of around 20 beaches of varying lengths (i.e., 50 - 5300 m). The shore is primarily protected by rocky hills, with the waves forming sand mounts that may reach heights of 2-3 meters. The majority of these protected beaches are thought to be suitable nesting places for green turtles. At this location, despite some uncertainties in the exact number, the annual presence of nesting females is estimated at 20,000 – 25,000, which underscores the immense significance of Ras al-Jinz for the species' global conservation efforts [14].

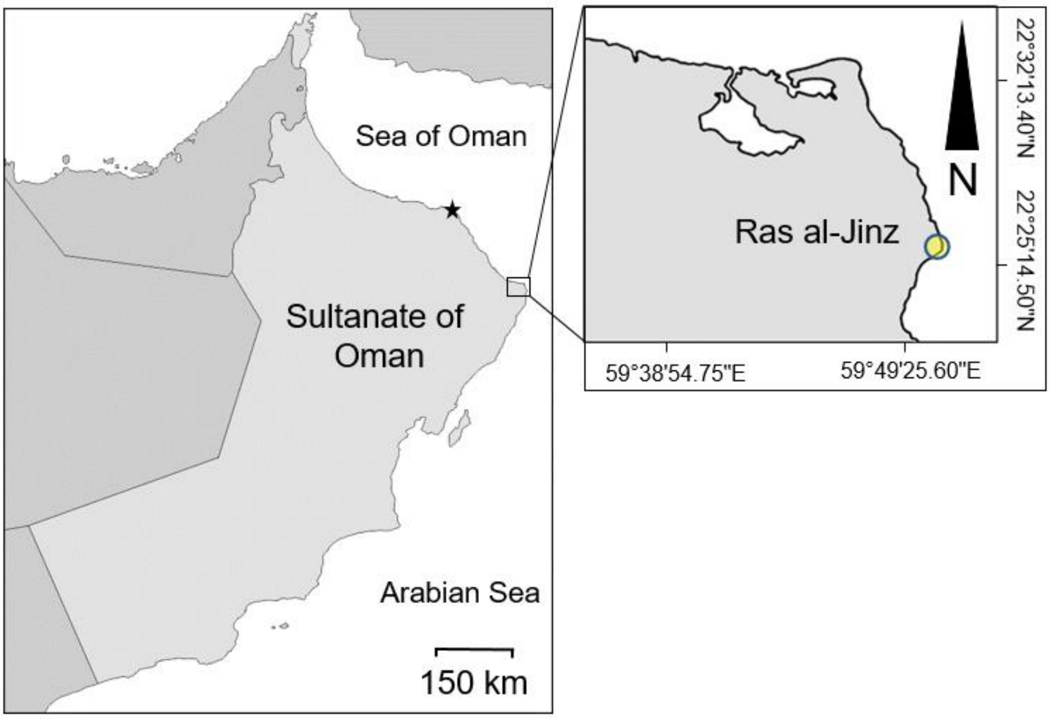


Figure 1. Ras al-Jinz Reserve is situated within Oman, as shown in the inset map on the right. The reserve is highlighted by a yellow dot and is located at the confluence of the Sea of Oman and the Arabian Sea. The star on the map denotes the capital of the country, Muscat.

Field sampling

During the 2015 nesting seasons (August - October), 10 samples were collected (N = 10) in each of seven components of Ras al-Jinz's habitat. These components included *i*) recently laid green turtles' eggs, *ii*) oviductal fluid, *iii*) sand from turtles' nests, *iv*) carapace shell scrubs, *v*) live crabs, *vi*) recently-dead hatchlings, and *vii*) seawater.

To measure the prevalence of bacteria, one egg from each of 10 nests was collected during the oviposition process. Furthermore, the oviductal fluid secreted by nesting turtles during oviposition was collected by placing a sterile opened plastic bag under the turtle's cloaca (after having disinfected the surrounding cloacal area), allowing aseptically collecting this fluid. Last, eggshells and sand from turtle nests were each collected and individually placed in sterile plastic bags. To measure potential concentrations of heavy metals, dead hatchlings, eggshells and sand from nests were each collected and individually placed in sterile plastic bags in addition to live crabs. Carapace scrubs were also conducted by scraping ~1 cm^2 of carapace. Eggshells, sand, dead hatchlings and crabs were treated with ~6 mL of nitric acid for subsequent heavy metal analyses (described below). Last, 50 mL of seawater from the intertidal zone at different beaches was collected and kept in six sterile containers.

All samples were placed in an ice chest and transported to the laboratory facilities of Sultan Qaboos University. The samples were stored in a cool box to minimize microbial metabolism. Within 24 hours, the bacterial contents of all collected samples were analyzed in the lab. Out of the 10 samples taken for each of the six aforementioned environmental components, half were analyzed for microbial activity while the other half was frozen for subsequent physicochemical analyses.

Microbiological analyses, enrichment, and isolation

The samples collected were swabbed, following which they were inoculated aseptically in nutrient broth (NB) and biosalinite broth (BSB). A portion of the inoculates were incubated at 37 °C for 18-24 h (following procedures described in [6,12,15]).

Considering the recently laid eggs, the microbial fauna of distinct egg components was sampled. Thus, each egg was separated into three portions, namely, the egg yolk, albumen and eggshell, which was done by using sterilized scissors and forceps (following [6,12,15]). Briefly, eggshells were placed in deionized water to remove loosely adherent contaminants and washed with 70% ethanol. The separated egg components were inoculated in NB and BSB and then incubated at 37 °C for 18- 24 h, allowing for microbial enrichment. A portion of the egg yolk and albumen were kept in test tubes for subsequent physicochemical analyses. All the other collected samples (i.e., oviductal fluid, sand from turtles' nests, carapace shell scrubs, live crabs, recently dead hatchlings and seawater) were processed as described above for the microbiological analysis of the sampled eggs. After 24 hours of incubation, these samples were streaked on differential media, specifically, eosin methylene blue (EMB) agar and *Salmonella Shigella* (SS) agar, and were further incubated at 37°C for 18-24 h. This was done to isolate target microbial communities.

Bacterial identification and biotyping

The suspected target bacterial colonies on EMB and SS agar were collected, and the pure cultures (i.e., those displaying strain-typical colony shapes) were streaked on nutrient agar. These pure culture colonies were identified by MALD- TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) mass spectrometry biotyper [16]. MALD-TOF biotyper provides specific identification of microorganisms based on the abundance of strain-specific proteins in microorganisms. These colonies were then transferred by sterile toothpick onto spots of the stainless-steel plate thereafter used for biotyping. They were then covered with 1 µl HCCA Matrix and let to dry for one minute before conducting tests for antibiotic susceptibility (detailed below).

Antibiotic susceptibility

In total, 70 bacterial isolates were tested for their susceptibility to antibiotics, using the disk diffusion method [17] by following the guidelines of the National Committee for Clinical Laboratory Standards described in [18] (Table 1). The lawn cultures with antibiotic disks were incubated on Diagnostic Sensitivity Test (DST) agar (Oxoid) at 37ºC for 24 hours and the inhibition zones were measured. In total, 15 antibiotics were used, specifically, Amikacin (Ak; 30 µg), Ampicillin (Amp; 10 µg), Carbencillin (Cn; 100 µg), Cephotaxin (Ctx; 30 µg), Chloramphenicol (C; 30 µg), Gentamycin (Gm; 10 µg), Kanamycin (K; 30 µg), Minocylin (Min; 30 µg), Nalidixic acid (Na; 30 µg), Neomycin (N; 30 µg), Sulphamethyoxazole (Smx; 30 µg), Streptomycin (S; 10 µg). Tetracyclin (Te; 30 µg), Tobramycin (Tob; 10 µg), and Trimethoprim (Tmp; 5 µg). After 24 hours of incubation, the inhibition zone diameters were measured to determine potential antibiotic resistance, following [18] (Table 1). *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used as control for the disk diffusion assay.

Chlorine exposures to antibiotic-resistant bacterial isolates

Of the antibiotic-resistant bacterial isolates found, it was tested whether those strains were also resistant to the presence of chlorine. Chlorination is commonly used in sewage treatments and chlorine-resistant strains can find their way into marine ecosystems [19]. The minimal inhibitory concentration of the antibiotic-resistant isolates to chlorine exposures was tested. For this, 96-microtiter well plates were used, for each of seven chlorine concentrations: 10 µl ml^{-1}, 20 µl ml^{-1}, 30 µl ml^{-1}, 40 µl ml^{-1}, 50 µl ml^{-1} 60 µl ml^{-1}, and 70 µl ml^{-1}. The resulting solution was then mixed with equal cell counts of microbial cultures grown in Luria Bertani brought to a final volume of 100 µl per well. The samples were prepared in triplicates and incubated at 37°C. After every two hours of incubation, three different spectrometric readings were taken using a 96 well-plate spectrometer. Using a two-way ANOVA, we tested for any statistically significant differences in bacterial resistance to the different concentrations of chlorine. In case of a statistically significant difference, we conducted a Least Significant Difference (LSD) post-hoc test.

Table 1. Antibiotic resistance and inhibition zone standards, follow the guidelines of the National Committee for Clinical Laboratory Standards of 1997, following [18].

Drug	Disk Potency (mg)	Inhibition Zone (mm)		
		Resistant	Intermediate	Sensitive
Amikacin (Ak)	30	<14	15-16	>17
Ampicillin (Amp)	10	<11	12-13	>14
Carbenicillin (Cn)	100	<17	18 - 22	>23
Chloamphenicol (C)	30	<12	13-17	>18
Gentamycin (Gm)	10	<12	13 - 18	>15
Kanamycin (K)	30	<13	14-17	>18
Minocylin (Min)	30	<14	15-18	>19
Nalidixic Acid (Na)	30	<13	14-18	>19
Neomycin (N)	30	<12	13-16	>18
Sμlphamethoxazole (Smx)	30	<12	13-16	>17
Streptomycin (S)	10	<11	12-14	>15
Tetracycline (Te)	30	<14	15-18	>19
Tobramycin (Tob)	10	<12	13-14	>15
Trimethoprim (Tmp)	5	<10	11-15	>16

Analyses of heavy metals content in the samples

To determine the concentration of heavy metals in samples (i.e., carapace smears, eggshells, albumen, yolk, dead hatchlings, crabs, sand, and seawater), 6 mL of nitric acid, and 50 mL ultra-pure water were added to 1 g of each of these samples. Then, samples were boiled for 15 min, until their complete digestion by the acid. Samples were kept at 4°C before determining the element contents. Previous studies conducted in Oman reported the presence of trace elements (i.e., heavy metals) in turtle eggs [20]. Thereby, the potential presence of heavy metals and other environmental elements was analyzed, which was done by using an inductively coupled plasma-mass spectrometer with an Aurora M90 ICP-MS (Bruker, USA). All samples were dissolved in liquid form prior to the analysis. The elements analyzed included the following: niobium (NB), titanium (Ti), zirconium (Zr), yttrium (Y), gold (Au), manganese (Mn), tin (Sn), scandium (Sc), phosphorus (P), gallium (Ga), bismuth (Bi), iridium (Ir), zinc (Zn), sulphur (S), beryllium (Be), aluminum (Al), boron (B), iron (Fe), magnesium (Mg), silver (Ag), arsenic (As), barium (Ba), cadmium (Cd), cobalt (Co), chromium (Cr), cesium (Cs), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb), antimony (Sb), selenium (Se), strontium (Sr), thallium (Tl), and vanadium (V).

Results

Identification of Bacteria

In total, 70 bacteria species were isolated and identified, which belonged to 11 genera. Our analyses revealed that all samples contained at least one bacterial strain. The frequency distribution of bacterial isolates, representing at least 1% in occurrence across all samples, was as follows: *Pseudomonas* spp. (46.8%), *Proteus* spp. (18.2%), *Citrobacter* spp. (16.9%), *Salmonella* spp. (6.5%), *Enterobacter* spp. (3.9%), *Stenotrophonas* spp. (1.3%), *Shewanella* spp. (1.3%), *Providencia* spp. (1.3%), *Ochrobactrium* spp. (1.3%), *Morganella* spp. (1.3%), and *Achromobacter* spp. (1.3%) (Figure 2). While *Pseudomonas* spp. were found to be the dominant strain, most of the other isolates were opportunistic and frank pathogens, e.g., *Salmonella*. Of the components sampled, eggs were the most frequently contaminated by these isolates, which was followed by oviductal fluids, and sand from turtles' nests. Among the egg components, the eggshells were the most likely to be contaminated, followed by albumen, and then the yolk (Figure 3).

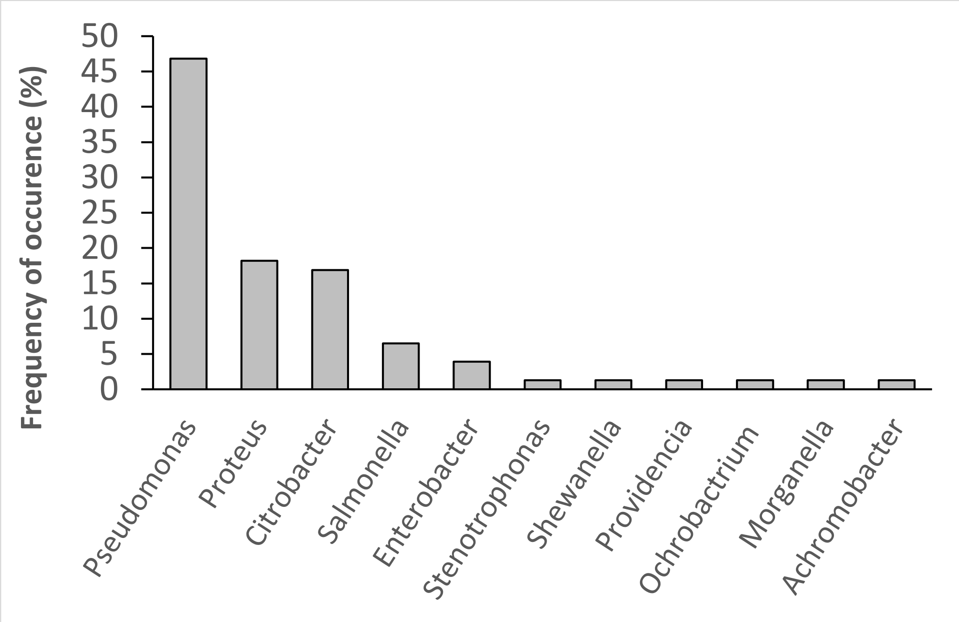


Figure 2. The frequency of occurrence (%) for each of the main 11 bacterial isolates (i.e., those with an occurrence of at least 1%) in the sampled components of Ras al-Jinz Reserve, with *Pseudomonas* as the most prevalent.

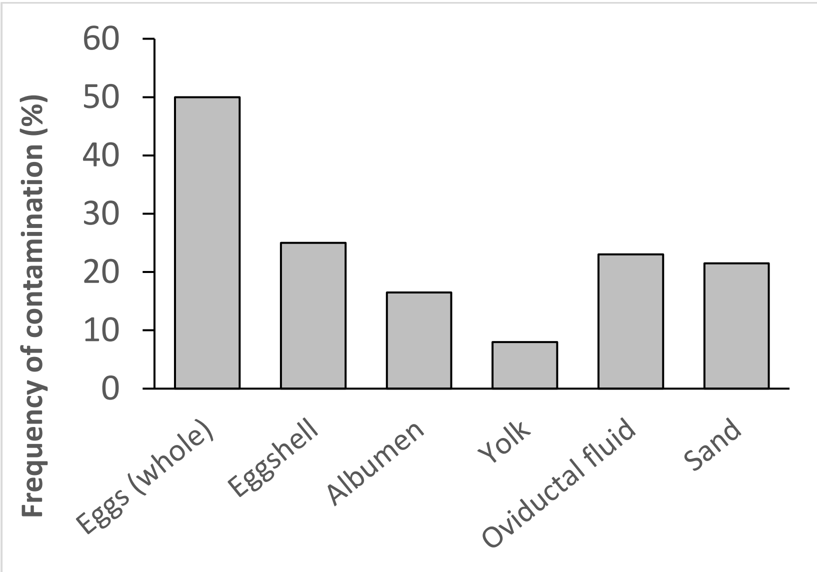


Figure 3. Frequency of bacterial contamination (%) in sampled eggs, eggshell, albumen oviductal fluid and sand from turtles' nests.

Antibiotic susceptibility

A total of seventy bacterial isolates were examined for their susceptibility to fifteen antibiotics (Figure 4). Some of these isolates demonstrated resistance to varying numbers of antibiotics, specifically, seven (16.7%), eight (18.3%), and nine (21.7%) antibiotics. Out of 60 antibiotic-resistant bacteria found, 83.3% were resistant to ampicillin, followed by cephotaxin (80%), trimethoprim (66.7%) and gentamycin (56.7%) (Figure 4). Conversely, the lowest bacterial resistance was found to be toward amikacin (1.7%) and minocycline (1.7%) (Figure 4).

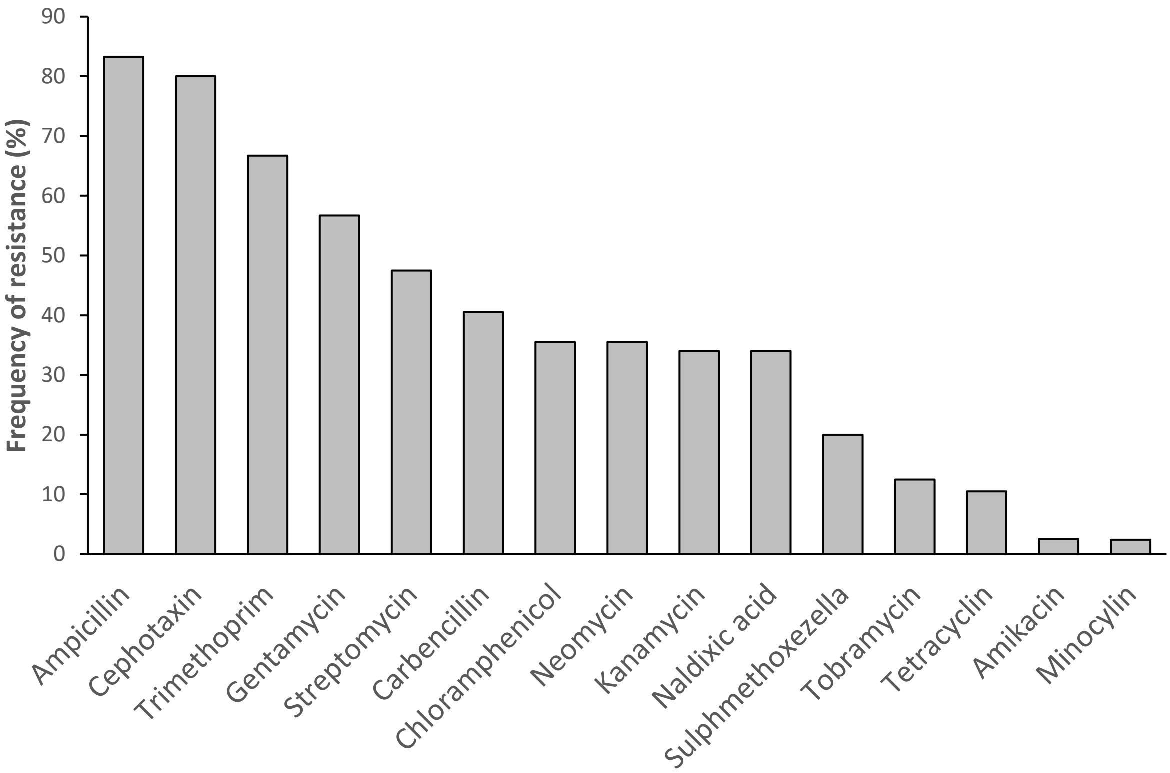
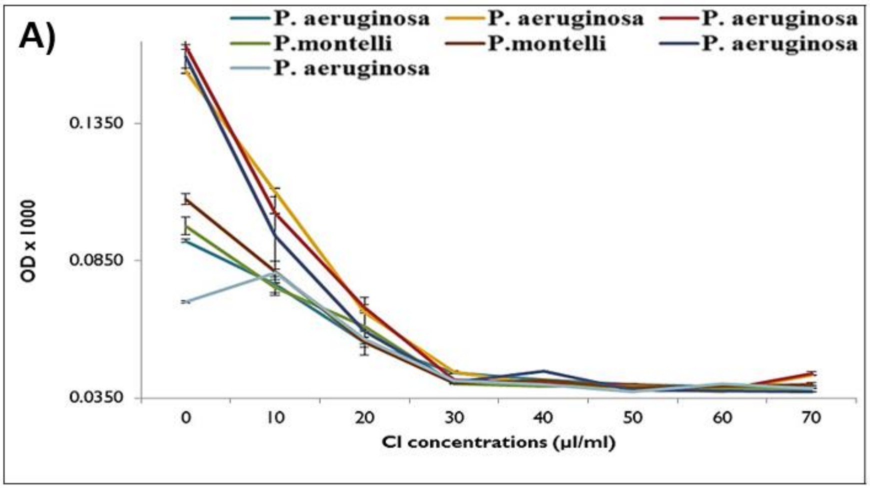


Figure 4. Frequencies (%) of bacterial isolates that showed resistance to each of fifteen antibiotics. More than 80% of the bacterial isolates were resistant to Ampicillin and Cephotaxin.

Chlorine exposures to antibiotic-resistant isolates

The antibiotic resistance observed in the most resistant isolates might have resulted from the chlorine present in tertiary sewage effluents and/or from ship ballast water [21]. Furthermore, migratory sea turtles could get contaminated by pollutants through their migration routes. Thus, the seven most resistant isolates were tested against seven chlorine concentrations, as detailed above. The microbial density (measured in optical density, OD) after six hours ranged between 6×10^5 - 6.9×10^5 cells per ml (Figure 5A). For any of the studied strains, we found no difference in OD, considering the chlorine concentrations of 30 µl ml^{-1} and above ($P > 0.05$) (Figure 5A). Seven *Pseudomonas* isolates were resistant to concentrations of chlorine ranging from 10-20 µl ml^{-1} (Figure 5B). However, these seven isolates ceased to grow when the concentration of chlorine reached 30 µl ml^{-1} (Figure 5B). Statistically, no difference in cell growth could be observed among the seven isolates tested in the presence of chlorine (two-way ANOVA, $P > 0.05$). Nonetheless, we observed a significant difference in OD between the different concentrations of chlorine, with the LSD test revealing a significant difference between the concentrations of 10 µl ml^{-1} and 20 µl ml^{-1} and all other concentrations ($P < 0.05$).



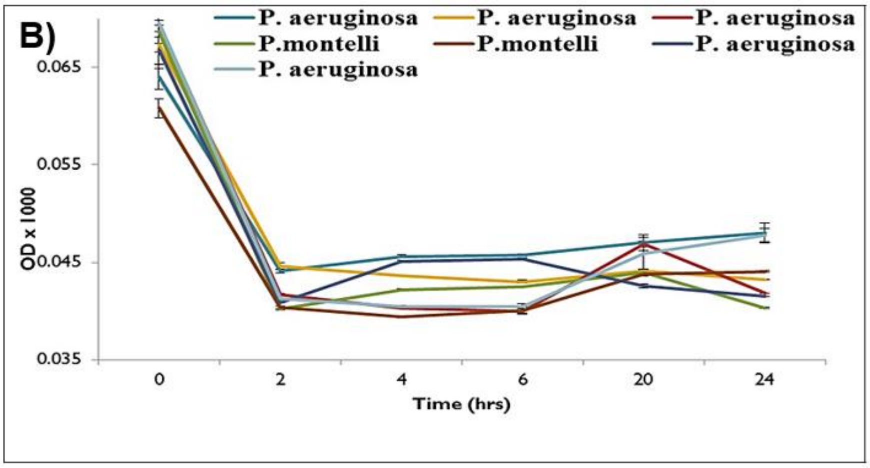


Figure 5. Bacterial resistance presented in terms of mean optical density (OD) (± SE) to different chlorine concentrations after six hours A), and the mean OD (± SE) after a total time of 24 hours B). Each line color represents a specific bacterial strain.

Environmental concentration levels of various elements, including heavy metals

The frequency of occurrence of the various studied elements, including some heavy metals in all the 10 sampled components of Ras al-Jinz's ecosystem varied substantially. For example, B and Ti were found to reach nearly 90% of all sampled components of the study. By contrast, Sb and Hg were rarely found in this study, with slightly less than 10% occurrence (Figure 6).

The sampled components of Ras al-Jinz Reserve showed a range of concentrations of diverse heavy metals (Figure 7). For instance, overall, Ti, V, and Cr emerged as the most prevalent heavy metals found in Ras al-Jinz Reserve (Figure 7). In the components associated with nesting turtles (e.g., eggshell, albumen, yolk, oviductal fluid and carapace), the concentrations of several environmental elements varied measurably. For example, in eggshells the concentration of Cr was highest, followed by V, P, and Mn, with Ti only minimally detected. However, in the albumen, Ti was in the highest concentration followed by V, Au, Cr and B, with no other environmental elements detected. Similarly, Ti was in the highest concentration (second highest heavy metal concentration of the entire study) in the yolk followed by Ba, Cr and Pb, with no other heavy metals detected. In contrast, in the oviductal fluids, we found the presence of 15 environmental elements, including some heavy metals, at very low concentrations, of which B was the most abundant followed by Fe and Zn. In the carapace, seven heavy metals were detected, with Ti, V and Zn as the dominant ones, and others present in negligible concentrations.

Environmental contaminations of diverse elements, including some heavy metals, were also detected in the other sampled components (e.g., dead hatchlings, live crabs, sand from nests and seawater). In dead hatchlings, five heavy metals were most abundant, with Ti in the highest heavy metal concentration of the entire study. Heavy metals found in live crabs showed similar patterns of concentration, with Ti as the dominant one. In sand taken from nests, Ti was also dominant but Cr and Pb were also detected. Seawater was characterized by low concentrations of heavy metals; while Ti, Cr and Pb were undetected, seven other heavy metals were found.

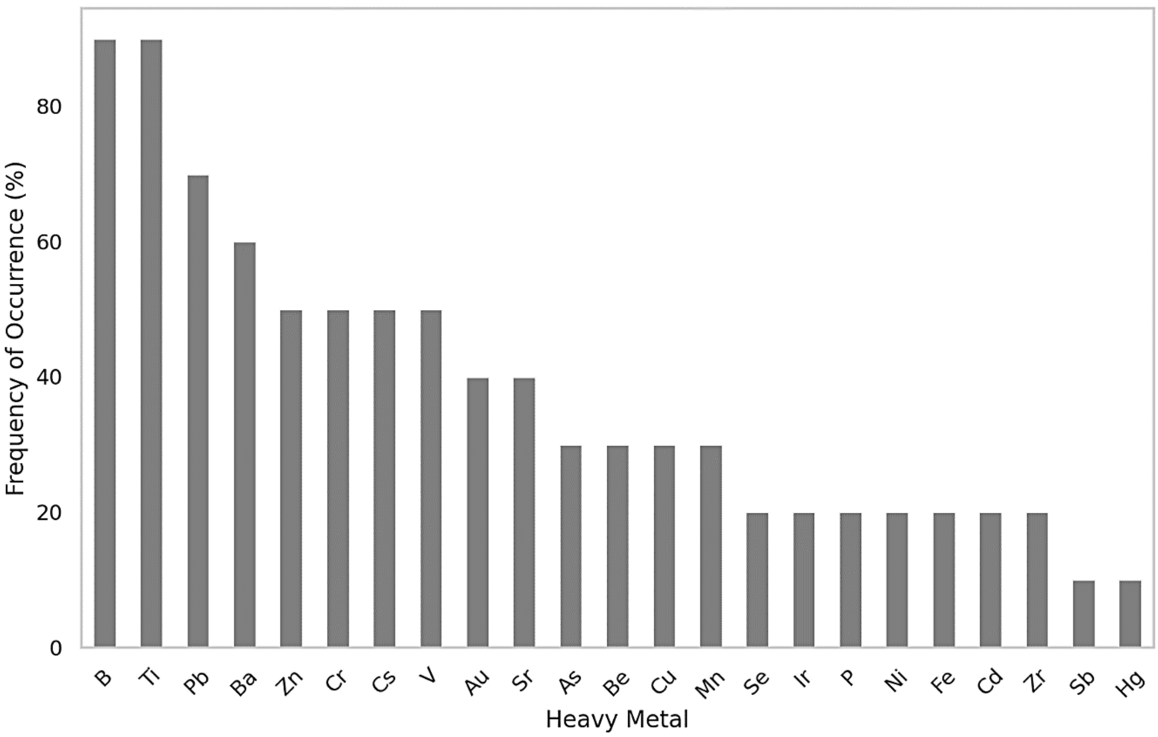


Figure 6. Frequency of occurrence (%) of each environmental element including some heavy metals in each of the 10 components sampled from Ras al-Jinz's ecosystem. Elements considered heavy metals include Ti, Pb, Ba, Zn, Cr, Cs, V, Au, As, Be, Cu, Mn, Ni, Fe, Cd, Sb, and Hg.

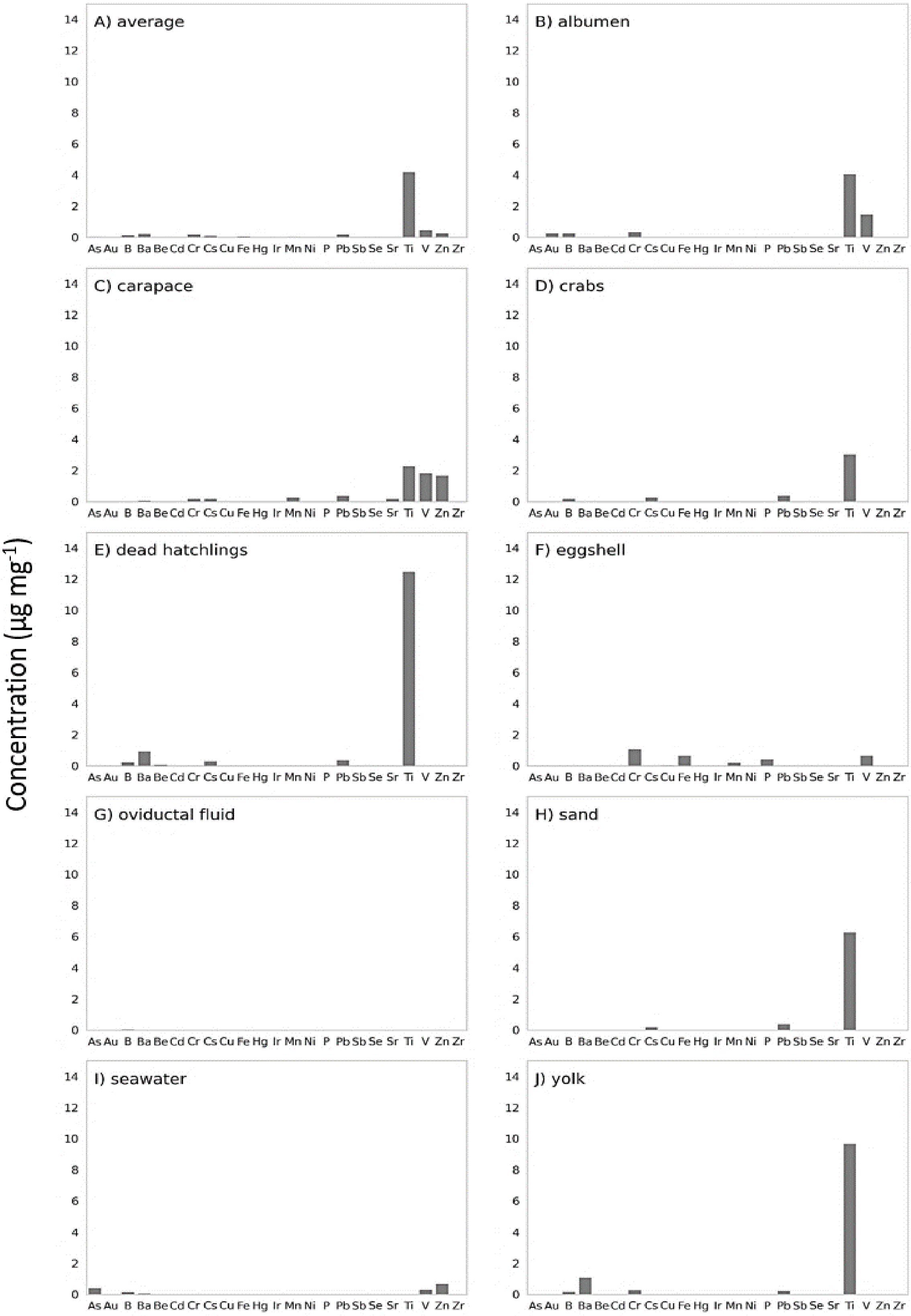


Figure 7. The concentration (µg mg^{-1}) of environmental elements, including several heavy metals, in different components of the Ras al-Jinz Reserve. The concentrations of these measured environmental elements are displayed in each respective panel: the average of all components (A), albumen (B), carapace (C), crabs (D), dead hatchlings (E), eggshells (F), oviductal fluid (G), sand from nests (H), seawater (I), and yolk (J). Elements considered heavy metals include Ti, Pb, Ba, Zn, Cr, Cs, V, Au, As, Be, Cu, Mn, Ni, Fe, Cd, Sb, and Hg.

Discussion

To date, the present report constitutes the most comprehensive monitoring effort focussed on chemical contamination from heavy metals and bacterial strains, including some exhibiting antibiotic resistance, within Ras al-Jinz Reserve. In 2015, this monitoring effort encompassed seven distinct components of the Ras al-Jinz Reserve's ecosystem to assess heavy metal and bacterial contamination. It occurred more than a decade after the preceding monitoring endeavors. These prior assessments were conducted in 2003-2004 for heavy metal contamination [5] and bacterial contamination [6,22,23]. The results obtained in the current report support the notion that chemical contamination in the reserve is ongoing and follows trends previously detected. Below, our results are compared against those of previous findings for heavy metal and bacterial contamination.

In their study, [5] highlighted the presence of 12 heavy metal species in Ras al-Jinz Reserve, specifically, Zn, Cu, Mn, Cd, V, Se, Hg, Cr, Pd, Ni, and Co, here presented in decreasing concentration. These heavy metals were found in green sea turtles' eggs and hatchlings' tissues, up to concentrations of 23.15 $\mu g\ g^{-1}$ (i.e., Zn in liver tissues). While such a concentration might not be immediately alarming, it is significant enough to warrant attention due to the potential health and environmental impacts [24,25]. Sampling conducted in 2015 confirmed the presence of 23 heavy metal species in the Reserve, including those found during the 2003-2004 sampling. When comparing a component of the ecosystem that was sampled in both studies, specifically sea turtles' egg yolk, in 2015, titanium (Ti) was found in the highest concentration, followed by vanadium (V). Regarding prevalence, boron (B) and Ti exhibited the highest percentage of occurrence. By contrast, zinc (Zn) was present in negligible concentrations. This result contrasts with the findings obtained a decade prior, whereby Zn was found in the highest concentration, which underscores the inherent variability in most environmental contamination research, highlighting the dynamic nature of pollutant distribution over time [26]. The 2015 sampling revealed the highest concentration of Zn to occur on sea turtles' carapaces, which was a component that had not been sampled in the 2003-2004 campaigns. Nonetheless, the concentration of chromium (Cr) was comparable among the sampling campaigns (i.e., 0.05 vs. 0.07 $\mu g\ g^{-1}$). Collectively, these findings confirm the persistence of heavy metal contamination within the Reserve, following more than a decade between sampling efforts.

Sea turtle eggs and oviductal fluids sampled in 2003 were shown to harbor at least 10 genera of bacteria, with *Pseudomonas* spp. emerging as the most prominent genus [6,20,23]. Furthermore, over 40% of the eggs were shown to be contaminated with up to 11 genera of bacteria. Resistance to ampicillin was highest among the sampled bacterial strains, with nearly 25% of *Pseudomonas aeruginosa* showing resistance. Results from 2015 confirmed the previous assessment, whereby *Pseudomonas* spp. also emerged as the most abundant strain, and 70 heterotrophic species belonging to eleven genera were identified. The broader sampling effort conducted on Ras al-Jinz's ecosystem in 2015 revealed that 45% of the sampled components were contaminated by *Pseudomonas* spp. Whereas in 2003 less than 25% of the sampled components were contaminated by *Pseudomonas* spp., the broader scale sampling of 2015 revealed that over 45% of the sampled components had *Pseudomonas* spp. contamination.

In terms of bacterial resistance, as in the 2003 results, in 2015, resistance to ampicillin remained the highest, with a slight (i.e., ~5%) increase in resistance compared to the 2003 findings. The congruence of the findings of 2003 and 2015 in terms of the breadth of bacterial contamination and antibiotic resistance underscores the continued presence of this chemical stressor in the Reserve. This highlights the imperative need for comprehensive and ongoing monitoring efforts moving forward.

Contamination in this reserve not only threatens the hatching success of turtle eggs but also endangers the overall health of turtle populations. Although bacterial contamination was detected, its precise origin remains unidentified, potentially stemming from either natural bacterial flora in the sand or anthropogenic sources introduced by human activities in and around the reserve. Additionally, the presence of heavy metals in different components of Ras al-Jinz habitat, including hatchlings, and turtles' egg yolks is cause for concern given metals may detrimentally impact turtles' health, influencing their reproductive success and overall survival rates [25,27]. Heavy metals like Titanium and Vanadium, observed in various marine components, pose a threat to marine organisms, potentially impacting their health and reproductive capabilities [24]. These contaminants, originating from both natural geological processes and human activities such as industrial waste, highlight the vulnerability of marine ecosystems to pollution. Heavy metals may enter marine ecosystems through industrial effluents, agricultural runoffs, and other human-made pollutants [28]. Antibiotic-resistant bacteria further increase the potential risk to marine fauna and the need for investigating their sources and transmission pathways. Treated wastewater and runoff to the environment, as well as ballast water from cruise ships, were reported to dissipate pollutants and antibiotic-resistant bacteria into the environment [15,29]. Hence these are two potential sources of environmental contamination in Ras al-Jinz Reserve.

The presence of these contaminants in such a critical turtle nesting site underscores the pressing need for regular monitoring to ensure the safety of marine life at Ras al-Jinz Reserve. Sustained exposure to chemical contamination poses a significant threat to the long-term survival and well-being of green turtles. This species, classified as endangered by the IUCN, requires sustained conservation efforts, particularly in their nesting range, if the safeguard of their future is sought. This situation also raises concerns for public health, considering the potential for these contaminants to enter the human food chain. The study emphasizes the necessity for ongoing research to monitor and understand the impact of these environmental threats, which is crucial for developing effective conservation strategies.

In light of these findings, it is imperative to develop and implement comprehensive mitigation strategies. Efforts should focus on pollution control, habitat protection, and the formulation of policies that address the root causes of environmental degradation in marine habitats. Collaborative approaches involving local communities, environmental agencies, and international bodies (e.g., UNEP, Sea Turtle Conservancy) are essential to preserve the ecological integrity of Ras al-Jinz Reserve. It is also essential that effective treatment plans are in place (i.e., in Ras al Hadd) while ensuring that ships do not empty their ballast water in territorial waters. Future research should aim at providing a deeper understanding of the impact of pollutants on marine life, guiding conservation efforts and policy-making to safeguard these critical ecosystems for future generations.

Conclusions

The continued presence of antibiotic-resistant bacteria and heavy metals in Ras al-Jinz Reserve, a crucial nesting site for green turtles, underscores the ongoing environmental challenges facing this ecosystem. Over a decade of monitoring reveals persistent contamination, with heavy metals like titanium and vanadium, and a significant proportion of bacterial isolates showing antibiotic resistance. These findings highlight the need for comprehensive pollution control measures, regular monitoring, and international collaboration to mitigate these threats. Ensuring the health of marine life and the ecological integrity of Ras al-Jinz Reserve is imperative for the conservation of endangered green turtles and the broader marine environment

Conflict of interest

The authors declare no conflict of interest

Acknowledgements

The authors are thankful to the Environmental Authorities of Oman and the management of Ras al-Jinz Reserve for granting us access and logistical support. This scientific contribution is dedicated to the memory of Professor Ibrahim Y Mahmoud, for which this work represents its last contribution to sea turtle research.

References

1. Small, C. and Nicholls, RJ. A global analysis of human settlement in coastal zones. *Journal of Coastal Research 2003*.
2. Halpern, BS., Walbridge, S., Selkoe, KA., Kappel, CV., Micheli, F. and D'Agrosa, C., *et al*. A global map of human impact on marine ecosystems. Science 2008, **319**, 948-52.
3. Griffin, DW., Banks, K., Gregg, K., Shedler, S. and Walker, BK. Antibiotic resistance in marine microbial communities proximal to a Florida sewage outfall system. Antibiotics 2020, **9**,118. https://doi.org/10.3390/antibiotics9030118.
4. UNESCO. Ras al Had Turtle Reserve and the heritage site of Ras al Jinz 2013. https://whc.unesco.org/en/tentativelists/5840/(accessed January 22, 2024).
5. Al-Rawahy, SH., AlKindi, AY., Elshafie, A., Ibrahim, M., Al-Bahry, SN. and Siyabi, SSA., *et al*. Accumulation of metals in the egg yolk and liver of hatching of green turtles *Chelonia mydas* at Ras Al Hadd, Sultanate of Oman. Journal of Biological Sciences 2007, **7**, 925-30.
6. Al-Bahry, S., Mahmoud, I., Elshafie, A., Al-Harthy, A., Al-Ghafri, S. and Al-Amri, I., *et al*. Bacterial flora and antibiotic resistance from eggs of green turtles *Chelonia mydas*: An indication of polluted effluents. Mar Pollut Bull 2009, **58**, 720-5. https://doi.org/10.1016/j.marpolbul.2008.12.018.
7. Hooper, MJ., Glomb, SJ., Harper, DD., Hoelzle, TB., McIntosh, LM. and Mulligan, DR. Integrated risk and recovery monitoring of ecosystem restorations on contaminated sites. Integr Environ Assess Manag 2016, **12**, 284-95. https://doi.org/10.1002/ieam.1731.
8. Burger, J. Incorporating ecology and ecological risk into long-term stewardship on contaminated sites. Remediat J 2002,**13**,107-19. https://doi.org/10.1002/rem.10058.
9. Denham, ME., Amidon, MB., Wainwright, HM., Dafflon, B., Ajo-Franklin, J. and Eddy-Dilek, CA. Improving long-term monitoring of contaminated groundwater at sites where attenuation-based remedies are deployed. Environ Manag 2020, **66**,1142-61. https://doi.org/10.1007/s00267-020-01376-4.
10. Elgendy, MY., Ali, SE., Abbas, WT., Algammal, AM. and Abdelsalam, M. The role of marine pollution on the emergence of fish bacterial diseases. Chemosphere 2023, **344**,140366. https://doi.org/10.1016/j.chemosphere.2023.140366.
11. Negahdari, S., Sabaghan, M., Pirhadi, M., Alikord, M., Sadighara, P. and Darvishi, M., *et al*. Potential harmful effects of heavy metals as a toxic and carcinogenic agent in marine food: An overview. Egypt J Vet Sci 2021, **52**, 379-85. https://doi.org/10.21608/ejvs.2021.83716.1245.
12. Al-Bahry, SN., Mahmoud, IY., Al-Zadjali, M., Elshafie, A., Al-Harthy, A. and Al-Alawi, W. Antibiotic resistant bacteria as bio-indicator of polluted effluent in the green turtles, *Chelonia mydas* in Oman. Mar Environ Res 2011, **71**,139-44. https://doi.org/10.1016/j.marenvres.2010.12.005.
13. Al-Bahry, SN., Mahmoud, IY. and Al-Musharafi, SK. Antibiotic resistant bacteria used as bioindicators of environmental pollution produced by tertiary treated sewage effluent. Wit Trans Ecol Envir 2012, 313-21. https://doi.org/10.2495/wp120271.
14. MECA. 5th national report to the convention on biological diversity (CBD) 2014. Sultanate of Oman, Ministry of Environment and Climate Affairs; 2014.
15. Al-Bahry, SN., Mahmoud, IY., Al-Khaifi, A., Elshafie, AE. and Al-Harthy, A. Viability of multiple antibiotic resistant bacteria in distribution lines of treated sewage

effluent used for irrigation. Water Sci Technol 2009, **60**, 2939-48. https://doi.org/10.2166/wst.2009.687.

16. Sogawa, K., Watanabe, M., Sato, K., Segawa, S., Ishii, C. and Miyabe, A., *et al*. Use of the MALDI BioTyper system with MALDI–TOF mass spectrometry for rapid identification of microorganisms. Anal Bioanal Chem 2011,400,1905. https://doi.org/10.1007/s00216-011-4877-7.

17. Bauer, AW., Kirby, WMM. and Sherris, JC., *et al*. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology* 1966, **45**, 493.

18. Kiehlbauch, JA., Hannett, GE., Salfinger, M., Archinal, W., Monserrat, C. and Carlyn, C. Use of the national committee for clinical laboratory standards guidelines for disk diffusion susceptibility testing in New York State laboratories. J Clin Microbiol 2000, **38**, 3341-8. https://doi.org/10.1128/jcm.38.9.3341-3348.2000.

19. Sorgen, A., Johnson, J., Lambirth, K., Clinton, SM., Redmond, M. and Fodor, A., *et al*. Characterization of environmental and cultivable antibiotic-resistant microbial communities associated with wastewater treatment. Antibiotics 2021,**10**, 352. https://doi.org/10.3390/antibiotics10040352.

20. Al-Musharafi, SK., Mahmoud, IY. and Al-Bahry, SN. Heavy metals and antibiotic resistant bacteria in green turtles are indicators of environmental pollution. International Journal of Biological, Food, Veterinary and Agricultural Engineering 2015, **9**, 354-9.

21. Liu, M., Zheng, L., Zhu, L., Lu, G., Guo, H. and Guan, J., *et al*. Characteristics of Carbapenem-resistant *Klebsiella pneumoniae* in sewage from a tertiary hospital in Jilin Province, China. PLOS ONE 2023, **18**, e0285730. https://doi.org/10.1371/journal.pone.0285730.

22. Al-Bahry, SN., Mahmoud, IY., Paulson, JR., Al-Musharafi SK. Antibiotic resistant bacteria in terrestrial and aquatic environments: A review. Int Arabic J Antimicrob Agents 2015;4. https://doi.org/10.3823/754.

23. Al-Bahry, SN., Al-Zadjali, MA., Mahmoud, IY., Elshafie AE. Biomonitoring marine habitats in reference to antibiotic resistant bacteria and ampicillin resistance determinants from oviductal fluid of the nesting green sea turtle, *Chelonia mydas*. Chemosphere 2012, **87**, 1308-15. https://doi.org/10.1016/j.chemosphere.2012.01.051.

24. Rehman, K., Fatima, F., Waheed, I. and Akash, MSH. Prevalence of exposure of heavy metals and their impact on health consequences. *Journal of Cellular Biochemistry* 2018, **119**, 157-84. https://doi.org/10.1002/jcb.26234.

25. Ross, DAN., Guzmán, HM., Potvin, C. and Hinsberg, VJ van. A review of toxic metal contamination in marine turtle tissues and its implications for human health. *Regional Studies in Marine Science* 2017, **15**, 1-9. https://doi.org/10.1016/j.rsma.2017.06.003.

26. Armiento, G., Barsanti, M., Caprioli, R., Chiavarini, S., Conte, F. and Crovato, C., *et al*. Heavy metal background levels and pollution temporal trend assessment within the marine sediments facing a brown field area (Gulf of Pozzuoli, Southern Italy). *Environmental Monitoring Assessment* 2022, **194**, 814. https://doi.org/10.1007/s10661-022-10480-3.

27. Gardner, SC., Fitzgerald, SL., Vargas, BA. and Rodríguez, LM. Heavy metal accumulation in four species of sea turtles from the Baja California peninsula, Mexico. Biometals 2006, **19**, 91-9. https://doi.org/10.1007/s10534-005-8660-0.

28. Gavhane, SK., Sapkale, JB., Susware, NK. and Sapkale, SJ. Impact of heavy metals in riverine and estuarine environment: A review. *Research Journal of Chemistry and Environment* 2021, **25**, 226-33.

29. Brinkmeyer R. Diversity of bacteria in ships ballast water as revealed by next generation DNA sequencing. *Marine Pollution Bulletin* 2016, **107**, 277-85. https://doi.org/10.1016/j.marpolbul.2016.03.058.