Heavy Metals and Antibiotic Resistant Bacteria in Green Turtles are Indicators of Environmental Pollution

S. K. Al-Musharafi, I. Y. Mahmoud, S. N. Al-Bahry

***Abstract*—**Freshly laid eggs from green turtles, *Chelonia mydas*, were randomly collected from Ras Al-Hadd Reserve, Oman. Eggshells taken from eggs and sand collected from the body chamber were analyzed for eight heavy metals (Al, Br, Cd, Co, Cu, Fe, S, and Zn) using inductively coupled plasma mass spectrometry (ICP). Heavy metal concentrations varied significantly ($P<0.05$) between nest sand and eggshells. Zn values were significantly higher than the other heavy metals. A total of 60 heterotrophic bacteria belong to eight genera were isolated from fresh egg contents (albumen and yolk). Resistance of the isolates to Ak = amikacin, Ak = amikacin, Amp= ampicillin, Gm= gentamycin, Cn = chloramphenicol, Min = minocycline, N = Neomycin, S= streptomycin, Smx = sulphamethoxazole, Tmp = trimethoprim, Tob = tobramycin was tested. More than 40% of the isolates were multiple resistant to 2-10 antibiotics. Most of the resistant strains were also resistant to Zn. The value of these findings may indicate that the origin of pollution is of human contaminated effluents.

***Keywords*—**Antibiotic resistance, bacteria, environment, heavy metals, sea turtles.

I. Introduction

On their migratory routes, sea turtles are exposed to various pollutants, including heavy metals and antibiotic resistant bacteria. Such pollutants can find their way into the digestive tract, and consequently contaminate the reproductive organs, such as the uterus and ovary where the eggs are developed [1]-[6]. Most of pollutants find their way into the developing eggs and consequently interfering with the embryonic development. Generally, these pollutants can be used as bio-indicator of contaminated effluents. For example, antibiotic resistant bacteria were use as bio-indicators of polluted effluents in turtles [2], [7].

Marine animals such as sea turtles and fish are subjected to pollutants and multiple antibiotic resistant bacteria (MARB) which is mostly originated in sewage water that enter the marine habitats. Sewage used for irrigation, land applied wastewater sludge, municipal and industrial waste can easily contaminate the marine environment [2]-[3], [7]-[11]. Certain heavy metals, such as Cu and Zn are essential for metabolic activity; however, they become toxic when they are at higher levels [4]. On the other hand, accumulation of non-essential heavy metals is toxic to organisms. Such heavy metals are indicators of environmental pollution [12]. In Oman, heavy metal accumulation was reported in terrestrial and marine habitats [2]-[5], [7]-[9].

Multiple antibiotic resistant bacteria and chemical pollutants originated in contaminated effluents are frequently discharged into the aquatic environment [1], [7], [13]-[17]. MARB resist chlorination used in the tertiary treatment process and can infect fish and marine turtles [1]-[3], [7]

Heavy metal tolerance is linked to resistance of antibiotics, thus heavy metals selects MARB in contaminated habitats [18], [19]. Some heavy metals in pig manure increased microbial resistance to antibiotics [20]. It was reported that sludge dumped on habitats can be a major source of heavy metal contamination some of which end up in the marine environment [21]. Thus, contamination of heavy metal can be used for bio-monitoring environmental pollution.

In turtles, heavy metals from the digestive tract can find their way to the eggshell during formation of the eggs [22], [23]. In another study, an increased accumulation of heavy metal contamination in turtle eggs at Ras Al-Had Reserve was reported [2], [4]. Microbial and heavy metal from polluted industrial sewage may contaminate the turtle feeding grounds. Also other studies in Oman reported MARB and heavy metal contamination in fish from a nearby sewage dumping site [7], [9].

The objectives of this study are to determine heavy metal levels in sand and eggshell as well MARB contamination in albumen and yolk. This study is crucial and may be used as a conservation indicator for the survival of marine turtles in this region.

II. Materials and Methods

A. Study Site

Ras Al-Hadd Reserve, Oman is located on the Gulf of Oman in the Arabian Sea between 22^{o}32’ N – 59^{o}45’ E and 22^{o}14’ N – 59^{o}48’ E.

B. Sample Collection and Preparation

A total of 20 sand samples from nest body-chambers were collected at random sites from each nest between June and September. Samples were collected at a depth of 30 – 80 cm. A total of 200 g of sand were collected from each nest, dried and ground to powder. Half gram of powder from each sample was acid digested in a tube containing HNO_3, HCl and H_2O_2, [24]. Three freshly laid eggs from each nest were collected for 60. Egg contents (eggshell, albumen and yolk) were separated. Each eggshell was dried, digested and grounded into powder.

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Digested eggshell samples were analyzed for eight heavy metals (Al, Br, Cd, Co, Cu, Fe, S, and Zn) using atomic absorption spectrophotometry (AAS) with electrothermic atomization in graphite furnace.

Albumen and yolk were used to isolate heterotrophic bacteria and the samples were inoculated in enrichment media. The samples were incubated at 37°C for 24 hr and then transferred to selective media [1], [7]. The isolates were tested for their antibiotic resistance using disk diffusion method and their resistance patterns were recoded. The following antibiotics were used Ak, Amp, Gm, Cn, Min, N, S, Smx, Tmp, Tob [1], [7], [25].

III. Results

There were significant differences in heavy metals concentrations between sand and eggshells. The highest levels in sand and eggshells were Zn followed by Al. In addition, Cu, Fe and S were significantly higher in sand over the eggshell (Fig. 1).

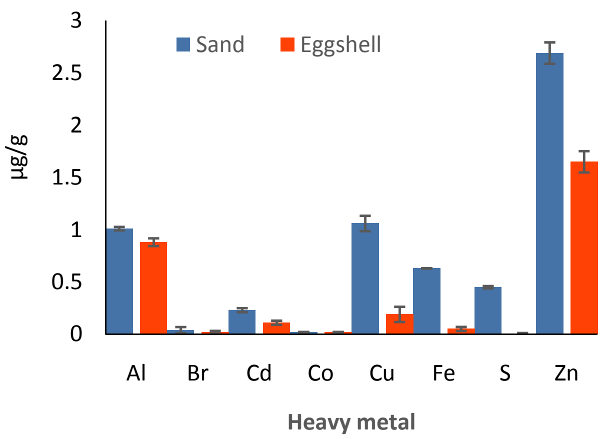

Fig. 1 Heavy metal concentrations in sand body-chamber and fresh eggshells

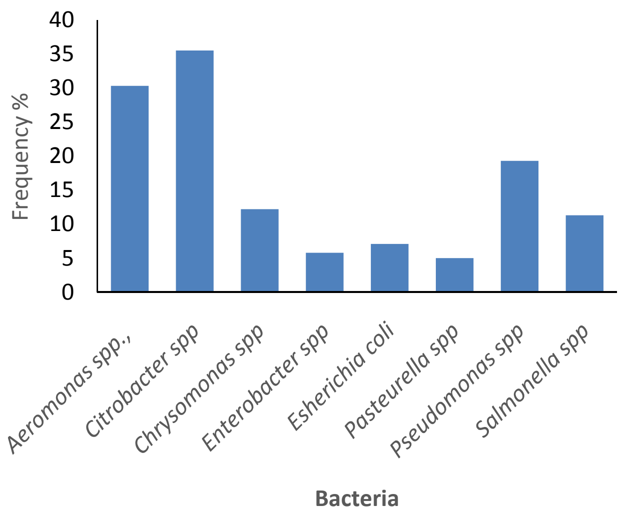

Fig. 2 Percentage of bacteria isolation in albumen and yolk

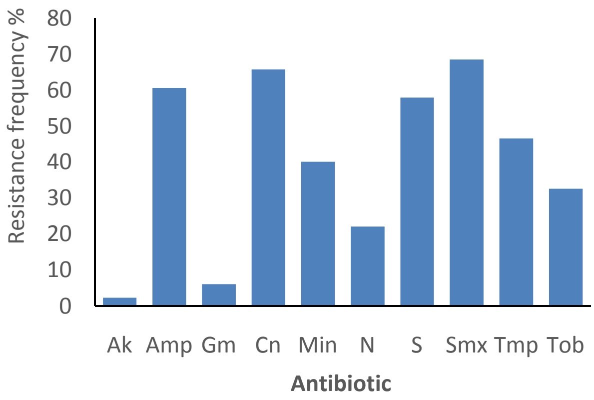

Fig. 3 Percentage of antibiotic resistant bacteria in albumen and yolk

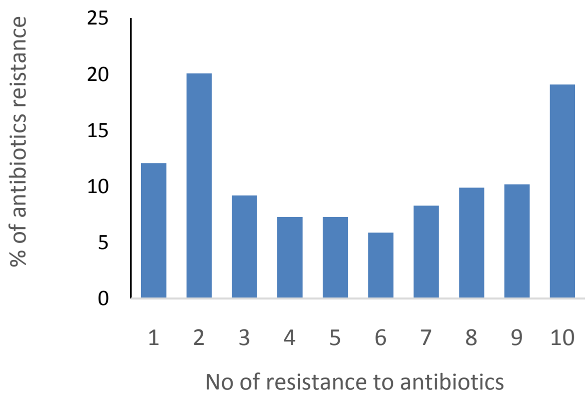

Fig. 4 Percentage of multiple resistance to antibiotics

The most frequent bacterial isolate in albumen and yolk was C*itrobacter* spp. followed by *Aeromonas* spp. and *Pseudomonas* spp. (Fig. 2).

The majority of the isolates were resistant to Smx followed by Cn, Amp and S. Only few isolates were resistant to Ak (Fig. 3). Most of the isolates were resistant to 2 and 10 antibiotics (Fig. 4).

IV. Discussion

In this study, contamination of heavy metal in sand-nest may be derived from the nesting turtles. Heavy metal deposition from industrial activities may lead to heavy metals accumulation in the feeding habitats. Biomonitoring of heavy metal contamination in coastal areas is important for assessing marine pollution. Rapid development of industrial sectors near the coastal cities of Oman, population growth and contaminated effluent discharges probably caused increase heavy metal contamination [2], [4], [9], [21], [26]-[29]. Rapid accumulation of heavy metals in marine habitats received more attention in recent years [30]. Increased usage of heavy metals in modern industries, anthropogenic metal from mining, intensive aquaculture activities, treated and untreated municipal wastewater from urban developments and agriculture led to a significant increase in contamination levels in marine ecosystem [9], [21], [27]-[29]. Approximately 8 million tons of Cu is globally produced for industrial application, such as chemical catalyst, electrical equipment, antifouling agents and wood preservative inevitably transferred to marine environment [4]. Burning of fossil fuels, run-off industrial effluents, use of electronic devices and batteries may also contribute to other heavy metal pollution such as Al, Fe and Zn. Continuous exposure of organisms, such as sea turtles, to heavy metals resulted in chronic intoxication which may be fatal [4], [31], [32].

In addition to heavy metals, several studies related to microbial antibiotic resistance were published in Oman revealed that sewage contaminated effluents contribute to environmental pollution [1], [2], [7], [8], [13], [17], [26]. Treated sewage effluents from industrial sources have higher heavy metal concentrations [4], [8], [21], [28], [29]. Also, bacterial isolates from sewage recycled water were resistant to several antibiotics. The resistant microbes to antibiotic isolated from sewage utilize heavy metals for their growth. These include *Aeromonas* spp., *Enterobacter* spp., *Pseudomonas* spp., and *Salmonella* spp. [1], [2], [7], [8], [13], [17], [26].

Chlorine is the most common disinfectant for bacteria and viruses used in tertiary treatment sewage effluent [33], [34]. However, it was reported that MARB resist chlorine activity and remain viable even at high concentrations [3], [8], [13], [35]. Bacterial population and re-growth increase significantly in distribution lines used for irrigation further from the point of disinfection with the significant loss of chlorine potency at the end of the distribution lines. The microbes were resistant to many antibiotics [1], [2], [7], [8], [13], [17], [26], [34].

Coliforms and fecal coliforms are standard indicators of enteric microbial pathogens in recycled water [36], [37]. In Oman, *E. coli* in many treated waste effluents exceeded the permitted standards of treated sewage effluents and were resistant to many antibiotics [1], [36]. The presence of pathogenic and non-pathogenic MARB in recycled water is environmental and public health concerns due to the potential transmittance of resistant determinants to pathogens [1].

Globally, MARB from different species were isolated from wild animals and sewage was found to have serious impact on terrestrial and aquatic ecosystems. [7], [13], [15], [16]. In parts of the world, including Oman, MARB were isolated from turtles, fish and fresh water habitats. This is a clear indication of antimicrobial drugs overuse and that the environment is used as a dumping ground of antimicrobial drugs and pharmaceutical compounds [1], [2], [7], [8], [13], [17], [26]. In Oman, MARB isolated from fish feeding near the treated effluents dumping site is a clear evidence how microbes from human activity can cause infection not only to fish, but also to sea turtles [1], [6], [7]. Resistant bacteria in cloacal fluid and eggs isolated from green and loggerhead turtles suggesting that the turtles were infected during their migration routes or in contaminated feeding habitats [1], [6], [13]. Therefore, MARB isolated from aquatic habitats were used as bio-indicators of pollution as well as to monitor degree of pollution in the turtles' feeding grounds [1], [3], [7], [17].

It was concluded that contaminated sewage effluent continuously causes an increase heavy metal pollution and dissemination of antimicrobial resistance genes to marine habitats. Stringent rules and regulations must be put in effect to prevent further environmental deterioration.

References

[1] SN Al-Bahry, IY Mahmoud, AE Elshafie, A Al-Harthy, S Al-Ghafri, I Al-Amri and AY Alkindi. Bacterial flora and antibiotic resistance from eggs of green turtles *Chelonia mydas*: an indication of polluted effluents. *Marine Poll Bull*. 41, pp. 214-221. 2009a

[2] SN Al-Bahry, Mahmoud, S Al-Rawahi and J Paulson. Egg contamination as an indicator of environmental health. *In*: Impact of Egg Contamination on Environmental Health. Nova Science Publisher Inc. New York. 2011a.

[3] SN Al-Bahry, MA Al-Zadjali, IY Mahmoud and AE Elshafie. 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*. 87, pp. 1308-1315. 2012.

[4] SK Al-Musharafi. Analysis of heavy metal in eggshells of green turtles, *Chelonia mydas*, by scanning electron microscopy and x-ray microanalysis. In: Biotechnology and Conservation of Species from Arid Regions. Nova Science Publisher, Inc. New York, USA. p.p. 138-144. 2014.

[5] SH Al-Rawahy, AY Al Kindi, A Elshafie, IY Mahmoud IY, S.N. Al-Bahry, S.S. Al-Siyabi, M.H. Mansour and A.A. Al-Kiyumi, Accumulation of metals in the egg yolk and liver of hatchling of green turtles *Chelonia mydas* at Ras Al Hadd, Sultanate of Oman. ANSI: *J. Biol. Sci*. 7, pp. 925-930. 2007.

[6] M Foti, C. Giacopello, T Bottari, V Fisichella, D Rinaldo and C Mamminac. Antibiotic resistance of Gram negatives isolates from loggerhead sea turtles (*Caretta caretta*) in the central Mediterranean Sea. *Mar. Pollut. Bull*. 58, pp. 1363-1366. 2009.

[7] SN Al-Bahry, IY Mahmoud, KI Al-Belushi, AE Elshafie, A Al-Harthy and CK Bakheit. Coastal sewage discharge and its impact on fish with reference to antibiotic resistant enteric bacteria and enteric pathogens as bio-indicators of pollution. *Chemosphere*. 77, p.p. 1534-1539. 2009c

[8] SN Al-Bahry, IY Mahmoud, JR Paulson and SK Al- Musharafi. Antibiotic resistant bacteria in terrestrial and aquatic environments: A review. IAJAA. 4, p.p. 1-11. 2015.

[9] SK, Al-Musharafi Mahmoud IY and Al-Bahry SN. 2013b. Contamination of marine fish by heavy metals from coastal sewage treated effluent runoff. The Third Asian Conference on Sustainability, Energy and the Environment. Osaka, Japan. IAFOR. Pp. 100-107. 2013

[10] Shahidul, and M Tanaka, Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. *Marine Poll Bull*. 48, pp. 624-649, 2004.

[11] FX Han, A Banin, WL Kingery, GB Triplett, LX, SJ Zhou Zheng and WX Ding. New approach to studies of heavy metal redistribution in soil. *Adv Env Res*. 8, pp. 113- 120. 2003.

[12] J Kojadinovic, M Potier, CM Le, RP Cosson, and P Bustamante. Bioaccumulation of trace elements in pelagic fish from the Western Indian Ocean. *Environ. Pollut*. 146, pp. 548-566. 2007.

[13] SN Al-Bahry, IY Mahmoud, A. Al-Khaifi, AE Elshafie and A. Al-Harthy. Viability of multiple antibiotic resistant bacteria in distribution lines of treated sewage effluent used for irrigation. *Water Sci Technol*. 60, pp. 2939-2948. 2009b.

[14] S Kim and DS Aga. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J Toxicol Environ Health B Crit Rev.* 10, pp.559-573. 2007.

[15] Kümmerer K. Antibiotics in the aquatic environment. A review. Part I. *Chemosphere* 75, 417-434. 2009a.

[16] Kümmerer K. 2009b. Antibiotics in the aquatic environment. A review. Part II. *Chemospher*e, 75, 435-441. 2009a.

[17] IY Mahmoud, SN Al-Bahry and SK Al-Musharafi. Fresh water habitat pollution by treated sewage effluent in relation to multiple-antibiotic-resistant bacteria. *APCBEES Procedia*. 5, pp. 363-367. 2013.

[18] OL Akinbowale, H Peng, P Grant and MD Barton. Antibiotic and heavy metal resistance in motile aeromonads and pseudomonads from rainbow

trout (*Oncorhynchus mykiss*) farms in Australia. *Int J Antimicrob Ag*. 30, pp. 177-182. 2007.

[19] L Bass, CA Liebert, MD Lee, AO Summers, DG White, SG Thayer and JJ Maurer. Incidence and characterization of integrons, genetic elements mediating multiple-drug resistance in avian *Escherichia coli*. *Antimicrob. Agents Chemother*. 43, pp. 2925-2929. 1999.

[20] CS Hölzel, C Müller, KS Harms, S Mikolajewski, S Schäfer, K Schwaiger and J Bauer. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environ Res*. 11, pp. 21-27. 2012.

[21] SK Al-Musharafi, IY Mahmoud and SN Al-Bahry. Environmental contamination by industrial effluents and sludge relative to heavy metals. *GEP* 2, pp. 14-18. 2014a.

[22] JW, Edwards, KS Edyvane, VA Boxall, M Hamann and KL Soole, Metal levels in seston and marine fish flesh near industrial and metropolitan centres in South Australia. *Marine Poll Bull*. 42, pp. 389-396. 2001.

[23] J Burger and M Gochfeld, Cadmium and lead in common terns (Aves: *Sterna hirundo*): relationship between levels in parents and eggs. *Environ. Monit. Assess*. 16, pp. 253-258. 1991.

[24] LA Grabowski, JLJ Houpis, WI Woods, and KA Johson, Seasonal bioavailability of sediemt-associated heavy metals along the Mississippi river floodplain. *Chemosphere*, 25, pp. 643-651. 2001.

[25] CLSI, Clinical Laboratory Standard Institution, Performance Standards for Antimicrobial Susceptibility Testing; 17th Informational Supplement. CLSI M100-S17. CLSI, Wayne, PA. 2007.

[26] SN Al-Bahry, IY Mahmoud, SK Al-Musharafi, IS Al-Gharaibi, NK Al-Harthy, HA Al-Zadjali, Microbial and chemical pollution of water-wells relative to sewage effluents in Oman. *IJSEE*. 1, pp. 35-56. 2014a.

[27] SK Al-Musharafi, IY Mahmoud and SN Al-Bahry. Heavy metal contamination from treated sewage effluents. 11th International Conference on Modelling, Monitoring and Management of Water Pollution. WIT Transactions on Ecology and The Environment, New Forest. UK. Vol. 164, pp. 381-389. 2012.

[28] SK Al-Musharafi, IY Mahmoud and SN Al-Bahry. Heavy metal pollution from treated sewage effluent. *Procedia APCBEES*. 5, 344-348. 2013a.

[29] SK, Al-Musharafi, IY, Mahmoud and SN Al-Bahry.. Environmental hazards and pollution from liquid waste lagoons. *IPCBEE* 69, pp. 1-5. 2014b.

[30] CJ Henry, KF Beal, RB Bury and R Goggans. Organochloride pesticieds, PCB, trace elements and metals in western pond turtle eggs for Oregon. *Northwest Sci*., 77, 46-53. 2003.

[31] ER Harper, JA St Leger, JA Westberg, L Mazzaro, T Schmitt, TH Reidarson, M Tucker, DH Cross and B Puschner. Tissue heavy metal concentrations of stranded California sea lions (*Zalophus californianus*) in Southern California. *Environ. Pollut.* 147, pp. 677-682. 2007.

[32] MM Storelli, G Barone, A Storelli and GO Marcotrigiano. Total and subcellular distribution of trace elements (Cd, Cu and Zn) in the liver and kidney of green turtles (*Chelonia mydas*) from the Mediterranean Sea. *Chemosphere*. 70, p. 908-913. 2008.

[33] Berger, MW LeChevallier, DJ Reasoner. Control of biofilm growth in drinking water distribution systems. United States Environmental Protection Agency, Office of Research and Development, Washington D.C. 1992.

[34] JA Tree, RM Adams and DN Less. Chlorination of indicator bacteria and viruses in primary sewage effluent*. J. Appl. Microbiol.* 69, pp. 2038-2043. 2003.

[35] R Shrivastava, RK Upreti, SR Jain, KN Prasad, PK Seth and UC Chaturvedi. Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant Pseudomonas aeruginosa. *Ecotoxicol Environ Safety*. 58, pp. 277-283. 2004.

[36] Omani Standards (OS) 52/1 for Wastewater reuse and discharge. Ministry of Environment Decree No 52/1-standards for the minimization of pollution to air, water and soil. Ministry of Environment. Muscat, Oman. 1996.

[37] World Health Organization (WHO). A compendium of standards for wastewater reuse in the Eastern Mediterranean Region. Regional Office for the Eastern Mediterranean. Regional Centre for Environmental Health Activities. 2005.