ANTIBIOTIC RESISTANCE OF METAL TOLERANT BACTERIA ISOLATED FROM SOIL IN JUJA, KENYA

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Abstract
Environmental pollution and increased levels of metal concentrations may influence the composition of bacterial populations. This study aimed to determine the relationship between antibiotic resistance and metal tolerance of bacteria isolated from five soils samples collected from Juja. Bacteria were isolated by culturing in metal enriched nutrient broth and a total of 41 isolates obtained. The isolates were identified by morphology and biochemical tests. Antimicrobial resistance patterns were determined using single disc diffusion method. Tolerance to heavy metal was determined by culturing bacteria in nutrient broth containing varying concentrations of Zinc, Mercury and Cadmium. The isolates showed 100% resistance to Augmentin and Cefuroxime (n=41), followed by Ampicillin 92.68% and Cotrimoxazole 68.29%. Sensitivity to Gentamicin was 95.12%, Norfloxacin 90.24%, and Nalidixic acid 68.3%. All the isolates where multi drug resistant (MDR) with 16 (39.02%) being resistant to four antibiotics. Many of the isolates were Zinc resistant (final concentration of 7.5g/l) but sensitive to mercury (50mg/l) and Cadmium (2.8g/l). Tolerance was highest in P. aeruginosa for Cadmium at 2.0g/l, E. coli for Zinc at 5.54g/l and *E. coli* and *S. aureus* for Mercury at 40mg/l. A direct correlation was established with correlation co-efficient (r) for Cadmium +0.97, and Mercury + 0.95 and Zinc + 0.91. This study demonstrated a correlation between metal tolerance and antibiotic resistance in soil bacteria from Juja. Association of metal tolerance and antibiotic resistance is of medical concern as resistance could be transferred to pathogenic bacteria.

Key words: Metal, metal tolerance, antibiotic resistance

1.0 Introduction

Antibiotics constitute the most relevant medical invention that has reduced human morbidity and mortality. The number of antibiotic resistant bacteria isolates is on the rise a fact attributed to intensive use and misuse of drugs. With the rate of development of new antibiotics being lower than the rate of resistance development, medical procedures such as organ transplant and general surgery may be unviable in the future due to untreatable bacterial infections (Carlet *et al.,* 2012). The versatility with which bacteria adapt to their environment and exchange DNA as a result of both evolutionary and adaptive stress poses multiple challenges to the medicine.

Mechanisms involved in development of antibiotic resistance include mutation and horizontal gene transfer. Genes coding for resistance can be transferred between bacteria in a horizontal fashion by conjugation, transduction, or transformation. The rate of resistance development has necessitated the WHO to declare the emergence of antibiotic resistance as a complex challenge driven by interconnected factors (WHO 2012) hence the need to analyze and understand other factors that potentially contribute to evolution and spread of resistance such as heavy metals.

Heavy metals from industries and agriculture create selective pressure on the resident bacterial communities. Some heavy metals are beneficial in cell physiological functioning such as chromium in the synthesis of cytochrome while others are toxic with no metabolic functions. The impact of these heavy metals is dependent on the environmental conditions that influence the concentration and bioavailability. According to Lawrence (2000) discussion on the Selfish Operon Theory clustering of genes on a plasmid, if both genes clustered are useful to the organism and beneficial to the survival of that organism and its species, the genes are more likely to be transferred in a single genetic event.

Studies have highlighted soil and water as recipient and reservoir environments for antibiotic and metal tolerance genes (Wright 2010). Bacterial communities in these environs are exposed to both heavy metal and antibiotic stress. The selective process is based on coupling of both mechanisms, either physiologically of genetically. Physiologically these mechanisms include multidrug efflux pumps and rapid extrusion mechanisms for toxins such

as heavy metals. Genetically they involve genes located close to each other or on the same mobile genetic element (Chapman 2003). These genes code for proteins such as B-lactamase enzyme that degrade B-lactams.

Heavy metals also act as gene transcription inducers with increase in the levels of expression of co-regulation genes responsible for antibiotic resistance (Baker-Austion *et al.,* 2006). An example is the soxS protein in *E. coli* whose translation is up regulated due to oxidative stress caused by heavy metals such as Cu2+, it enhances tolerance to antibiotics such as tetracycline and novobiocin. These interactions between antibiotic resistance and heavy metal tolerance necessitate the need to practically analyze the level of correlation between the two factors

2.0 Materials and Methods

2.1 Study Site and Sampling Points

The study was carried out in Juja area with five sampling sites: Muchatha market, Gachororo market, between Tuck-shop and Hall Six, JKUAT main gate opposite Jojawa and Juja–Thika stage adjacent to Barclays bank.

2.2 Collection of Samples

Soil samples were collected in clean sterile bags, marked according to point of collection and taken to the laboratory for processing.

2.3 Enrichment of Metal Resistant Bacteria

Metal resistant bacteria were enriched using nutrient broth containing a range of metal salts at varying concentrations. Nutrient broth volumes of 100ml was dispensed in 250ml conical flasks containing; Zinc Chloride at 0.13 6g/l, 1.36 g/l, 2.72 g/l and 5.54 g/l. Cadmium Chloride at 0.182 g/l, 0.364 g/l, 0.728 g/l, 1.456 g/l. Mercury Chloride at 5 mg/l, 10 mg/l, 20 mg/l, 40mg/l. 5g of the soil was added to each flask and incubated at 20^{o} C while shaking at 120 revolutions per minute. On the third day, sub-culturing was done into the same metal enriched media. This process was repeated for a third time and subsequently streaked on nutrient agar and incubated for three to five days at 20 ^{o} C

2.4 Pure Culture

By means of a transfer loop, a portion of the mixed culture was streaked across the surface of nutrient agar. During streaking the bacteria, colonies 'thinned out' that enabled growth of individual colonies. The isolates were re-streaked until only pure isolates had been acquired.

2.5 Effect of Increasing Metal Concentration

Pure culture bacterial isolates were grown on nutrient broth containing the respective metal salts overnight and then inoculated into media containing following metal concentration: Zinc Chloride; 0.136 g/l to 10 g/l. Cadmium Chloride; 0.182 g/l to 2 g/l. Mercury Chloride; 10 mg/l to 100 mg/l. Optical density at 600nm was immediately recorded after inoculation and then incubated at 20^{o} C at 120 revolutions per minute. Optical density was recorded daily for 10 days or until declining growth was observed.

2.6 Characterization of Bacteria

Pure isolates were observed and data recorded regarding the form, elevation, margin, and optical feature of colony of different bacteria. Cellular morphology of cells was observed after gram staining under microscope (100x). Gram staining was done to differentiate Gram-positive from Gram-negative bacteria with confirmation via string test. Gram positive bacteria retained the primary stain crystal violet hence appeared dark purple while Gram negative bacteria were stained pink by safranin the counter stain when observed under oil immersion.

2.7 Biochemical Tests

Catalase, citrate utilization test, hydrogen sulphide production, methyl red test; Voges-proskauer, Indole test, Nitrate utilization test and Triple Sugar Iron test biochemical tests were performed.

2.8 Identification of Bacteria

Identification of the isolates was done by comparing characterization results with descriptions in Bergeys Manual of Determinative Bacteriology and The Prokaryotes.

2.9 Antibiotic Susceptibility Tests

Disc diffusion method was used to test susceptibility to eight antibiotics used to treat bacterial infection in humans. Ampicillin, Augmentin, Cefuroxime, Nitrofurantoin, Nalidixic acid, Norfloxacin, Gentamicin and Cotrimoxazole. Plates were incubated at 37^{o} C for 24 hours after which diameter of zones of inhibition was measured and interpreted according to Clinical laboratory Standards Institute (CLSI, 2007) guidelines.

2.10 Data Analysis

Data was analyzed using Microsoft Excel 2010 to generate descriptive statistics involving the use of frequencies, totals, percentages and charts. To determine correlation, Pearson's correlation co-efficient (r) was used. The results were interpreted as strong positive relationship (r > +0.5 to +1.0), strong negative relationship (r > -0.5 to -1.0) and weak negative or positive relationship (+0.5 > r < -0.5).

3.0 Results

3.1 Isolation and Identification of Heavy Metal Resistant Bacteria

After heavy metal enrichment for the three metals under study (Cadmium, Mercury and Zinc), a total of 41 identified heavy metal resistant bacteria were isolated. The five sample sites showed similar isolate species but with varying resistance levels depicted by high number of isolates (Table 1).

Table 1: Isolated species from Juja

Bacteria	Isolates	Percentage (%)
Escherichia coli	12	29.27
Staphylococcus aureus	10	24.39
Klebsiella pneumoniae	3	7.32
Pseudomonas aureginosa	4	9.76
Proteus mirabilis	4	9.76
Citrobacter freundii	3	7.32
Serratia marscens	3	7.32

The isolates varied according to point of isolation with Gachororo market having the highest number with 11 isolates and the least at JKUAT main gate with 5 isolates (Table 2).

Table 2: Variation of species isolated from Juja according to sampling site

Sample Site	*E.coli*	*S. aureus*	*P. aureginosa*	*P. mirabilis*	*C. freundii*	*S. marscens*	*K. pneumoniae*
Muchatha market	4	3	0	2	0	0	1
Gachororo market	4	4	0	1	2	0	0
Jkuat tuck shop	1	0	2	1	1	0	2
Jkuat main gate	1	2	1	0	0	1	0
Juja-Thika stage	2	1	1	0	0	2	0
TOTAL	12	10	4	4	3	3	3

Morphological appearance of the colonies on nutrient agar was observed. Gram staining test and string test, were performed and results recorded. The 41 isolates showed different levels of tolerance to heavy metals with Mercury

being the most tolerated heavy metal (41.46%) and Zinc and Cadmium both at 29.27% tolerated, based on the number of isolated colonies from each metal enriched sample.

Table 3: Antimicrobial susceptibility patterns of metal tolerant isolates from Juja

Antibiotic	Abb	Number of isolates			
		R	S(+)	SS(++)	SSS(+++)
Augmentin	AUG	41	nil	nil	nil
Cefuroxime	CEF	41	nil	nil	nil
Nitrofurantoin	NITr	19	10	8	4
Nalidixic acid	NAL	13	7	9	12
Norfloxacin	NOR	4	10	14	13
Ampicillin	AMP	38	1	2	nil
Gentamicin	GEN	2	8	15	16
Cotrimoxazole	COT	28	3	4	6

KEY: R-Resistant, S-Sensitive, SS-Moderately sensitive, SSS-Very sensitive.

3.2 Profiles of Resistance to Antibiotics

Out of the 41 isolates, each showed resistance to one or more antibiotics. In this study the isolated population exhibited higher resistance to Augmentin and Cefuroxime 41 isolates, (100%), Ampicillin (92.68%) and Cotrimoxazole (68.29%). High sensitivity was exhibited by 38 isolates to Gentamicin (an amyl glycoside) (95.12%), 37 of the isolates to Norfloxacin accounting for 90.24%, and Nalidixic acid, 28 sensitive isolates (68.29%) (all quinolones).

3.3 Multiple Antibiotic Resistance

With the current health sector threat of multi-drug and extensive drug resistance, sensitivity pattern and resistance pattern among the isolates was compared by use of a graph of number of antibiotics against number of isolates resistant. Most of the isolates in this study were found to be resistant to more than three antibiotics with 16 isolates (39.02%) being resistant to 4 antibiotics and 13 isolates (31.70%) to 5 antibiotics. A combination of isolates resistant to both 4 and 5 antibiotics results in 29 isolates accounting for 70.73% of the total number of isolates Figure 2.

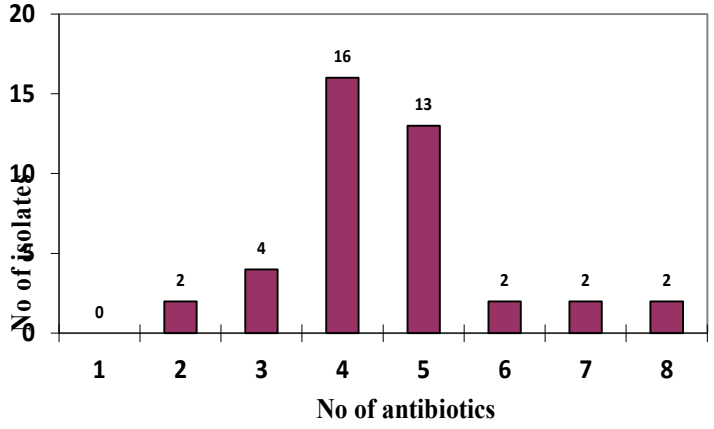

Figure 2: Multiple antibiotic resistance of bacteria isolates from Juja

2.4 Effect of Increasing Metal Concentration

This was determined by culturing the isolates in increasing concentrations of heavy metal. A representative for each genus was used. Optical density was measured at 600 nm after inoculation and for five continuous days or until growth started to decline. Due to their high occurrence *E. coli* and *S. aureus* were subjected to the three metals while all other isolates in the respective metal of isolation.

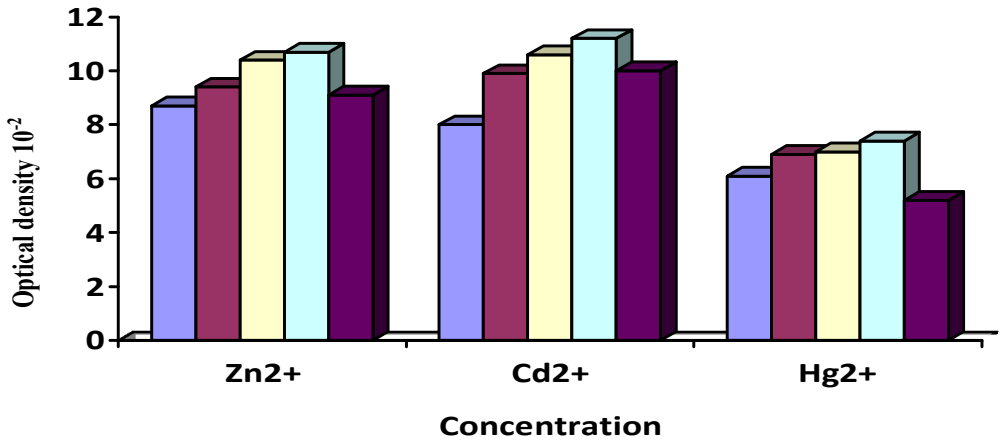

Figure 3: Metal tolerance for E. coli

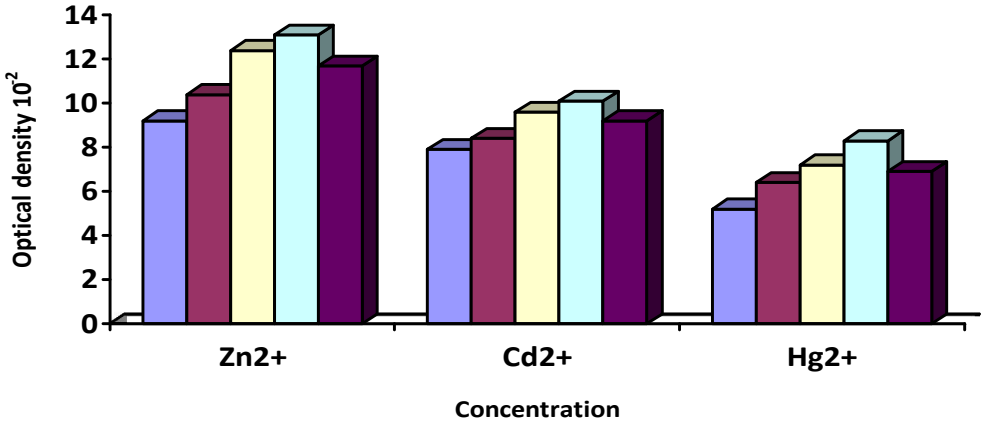

Figure 4: Metal tolerance for S. aureus

Co-relation between metal tolerance and antibiotic resistance

The transfer of resistance genes together with metal tolerance genes necessitates analysis for the relationship between metal and antibiotic resistance. In this study, high resistance to specific antibiotics was exhibited with 100% gentamicin and norfloxacin resistant isolates tolerant to zinc. Though there was an overall sensitivity of the isolates to nalidixic acid, most of the resistant isolates were mercury tolerant at 84.61%.

Table 4: Correlation between antibiotic resistance and metal tolerance

		Zinc			Cadmium			Mercury		
Antibiotic	T No.	No.	%	T %	No.	%	T %	No.	%	T %
Augmentin	41	12	29.27	29.27	12	29.27	29.27	17	41.46	41.46
Cefuroxime	41	12	29.27	29.27	12	29.27	29.27	17	41.46	41.46
Nitrofurantoin	19	6	31.58	14.63	4	21.05	9.76	8	42.10	19.51
Nalidixic acid	13	2	15.38	4.88	0	0.00	0.00	11	84.61	26.83
Norfloxacin	4	4	100	9.76	0	0.00	0.00	0	0.00	0.00
Ampicillin	38	8	21.05	19.51	12	31.58	29.27	17	44.74	41.46
Gentamicin	2	2	100	4.88	0	0.00	0.00	0	0.00	0.00
Cotrimoxazole	28	10	35.71	24.39	6	21.43	14.63	11	39.29	26.83
Average			**45.28**	**17.07**		**16.57**	**14.05**		**36.71**	**24.69**

T No., Total number of isolates resistant to the particular antibiotic; No., number of isolates resistant to metal and antibiotic; %, percent of total number of isolates resistant to the particular antibiotic and metal; T %, per cent of total number of isolates (41).

Table 4 results show varying levels of co-resistance to antibiotic and tolerance to metal with maximum at 100% (Zinc/Norfloxacin and Gentamicin) and minimum at 15.38% (Zinc/Nalidixic acid). None of the isolates in this study were Cadmium/Nalidixic acid and Norfloxacin, mercury/Norfloxacin resistant. The average value for a zinc tolerant isolate being antibiotic resistant was 17.07% with antibiotic resistant isolates have a 45.28%, average that they will be metal tolerant. This accounts for a change of 28.21 percentage points. Cadmium has a 2.57% change from 14.05% to 16.57% while Mercury has a 12.02% change from 24.69% to 36.71%.

3.5 Correlation Co-efficient Analysis (Pearson's)

Pearson's correlation co-efficient indicates that there was a strong positive correlation between metal tolerance and antibiotic resistant in this study with correlation co-efficient (r) for Cadmium being the highest at r = + 0.97, Mercury at r = + 0.95 and the minimum being Zinc at r = + 0.91. These correlation co-efficient values for this study are; -0.03 for Cadmium, -0.05 for Mercury and -0.09 for Zinc close to perfect positive correlation.

4.0 Discussion

In this study a total of 41 isolates across eight genera were isolated. *Escherichia coli 29*.27%, the lowest Bacillus subtilis 4.8%. The high occurrence of E. coli according to research by Rama *et al.* (2005) indicates that gastrointestinal tract provides the reservoir from which this bacterium can be introduced into the environment due to low hygiene and waste disposal which is the case in Juja area. Poor waste disposal and drainage in sample collection sites especially Gachororo and Muchatha market could play a role in the high number of *E.coli* isolates.

The isolates were highly resistant to drugs such as Cefuroxime and Augmentin (100%), but susceptible to second line drugs such as Norfloxacin 90.24% and Nalidixic acid 68.29% both quinolones. Juja has a high number of drug stores chemists that increases the level of drugs acquired without prescription with both easily accessible from these drug stores hence the high resistance can partly be attributed to drug misuse. Augmentin mode of action is the inhibition of cross links of peptidoglycan in the cell wall biosynthesis pathway hence acts as a transition

inhibitor resulting in bacterial cell death (modification of the cell wall to reduce permeability of heavy metals increases resistance since a reduction in permeability reduces antibiotic absorbance).

The high sensitivity to Nalidixic acid and Norfloxacin can be due to the un-availability of these drugs easily limiting their misuse. Multiple antibiotic resistance was exhibited with 16 isolates resistant to 4 antibiotics and 13 isolates to 5 antibiotics. High sensitivity was exhibited to Gentamicin an aminoglycoside (95.12%) which acts by binding to the bacterial 30S ribosomal subunit inhibiting translocation or by binding to p10 in the 30S ribosome complex and the mRNA codon is misread and the wrong amino acids are incorporated into protein. Resistance to Gentamicin especially in S aureus is mediated by a transposon carried gene found in large staphylococcal multi-resistance plasmids. In P. aureginosa resistance is exhibited due to transport or membrane impermeability and it results in cross-resistance to all aminoglycosides with levels of resistance being seen as moderate (Mingeot *et al*., 1999).

Metal tolerance varied according to species and the point of isolation of isolate with isolates from Juja – Thika stage adjacent to Barclays Bank exhibiting high tolerance and those from JKUAT tuck-shop area showing lowest tolerance. Main source of metal pollution increase is the booming construction activities that utilize high volumes of metal components such as zinc from iron sheets, cadmium from steel, plastics and paint and Mercury from switches, rectifiers and fluorescent lighting. Disposal of damaged fluorescent lighting bulbs is a major source of concern in relation to mercury tolerance. Species tolerance was highest in *P. aureginosa* for cadmium at 2.0Vg/l, E. coli for Zinc at 5.54g/l and *E. coli* and *S. aureus* for Mercury at 40mg/l. The high tolerance from Juja-Thika stage can be attributed to continuous exposure to metals from vehicles.

Bacteria have devised specific mechanisms to tolerate different heavy metals. Ability of bacteria to tolerate mercury is dependent on mercury tolerance determinants, Mer. In gram-negative bacteria such as E.coli, the periplasmic H_g2+ -binding protein MerP binds this cation as the first step of detoxification. Transports it into the cytoplasm where it is reduced by protein related to glutathione reductase and effluxed out of the cell. It is also based on unique peculiarities of Mercury- redox potential, vapor pressure, melting/boiling point of metallic Mercury, which is extraordinarily low for a metal. (Melting point -38.87^o C, boiling point 356.58^o C). This enables living cells to reduce Hg to the metal and this metal does not remain inside the cell with the potential of being oxidized but it leaves the cell by passive diffusion (Silver and Phung 1996).

Two systems are used for zinc detoxification in bacteria, P-type efflux ATPase's and RND-driven transporter. While P-type ATPases transport Zinc only across the cytoplasmic membrane, the RND-systems are hypothesized to efflux across the complete cell wall of gram-negative bacteria. This process is energy dependent (Nies and Silver, 1995).

Resistance to cadmium is based on cadmium efflux and enzymatic activity of the bacteria. In gram-negative bacteria, cadmium is detoxified by Resistant, Nodulation, Cell division (RND) driven system (Nies 1999). RND proteins mainly found in gram-negative bacteria play a role in export/efflux of Zn2+ and Cd2+. RND-driven transporter protein families are involved in multi-drug resistance with ability to detoxify or breakdown beta-lactamase inhibitors.

In gram-positive bacteria an example of cadmium exporting P type ATPase is cadA pump found in *Staphylococcus aureus*. It is a single polypeptide chain that forms the trans-membrane channel of the transporter and the ATP-binding and hydrolysis site. E. coli has four distinguished chromosomal genes of P type ATPase. This provides it with ability to tolerate metals and hydrolyze different antibiotics hence a higher degree of antibiotic resistance as exhibited in this study (i.e. one of the Zinc tolerant E. coli isolate was resistant to all the eight tested antibiotics.

These metal specific mechanisms are complexed with other non-specific mechanisms such as binding with bacterial cell envelope, metal reduction and metal efflux. These mechanisms are mainly encoded in plasmid genes facilitating their transfer from one cell to another. The type of organic constituent and presence of negatively charged ions like chloride in the media also influence tolerance. This can explain low tolerance to Zinc (29.27%) due to its increased toxicity in media containing sodium chloride such as nutrient broth, caused by formation of soluble zinc-chloro complex which increases the availability of the cation to the cell (Bezverbnaya *et al*., 2005).

Positive correlations were observed between antibiotic and mercury r = +0.95, cadmium r = +0.97 and zinc r = +0.91. Previous studies have demonstrated the role of plasmids in conferring resistance to both antibiotics and metals (Baker-Austion *et al.,* 2006) but researchers have emphasized that complicated sets of relationships exist between the host cell and the plasmid with respect to metal tolerance and antibiotic resistance.

The exponential rise in antibiotic resistance has great implications on public health with the health risk further stressed by the occurrence of a high frequency of strains that are typically resistant to more than one antibiotic and metal tolerant. Under the experimental conditions of this study 16 isolates were resistant to 4 antibiotics with 13 isolates being resistant to 5 antibiotics.

Heavy metal pollution has continually selected for tolerant organisms that has consequently resulted in increased levels of antibiotic resistance. Bacteria species and concentration of heavy metal influence the level of tolerance to both with bacteria being able to share these mechanisms. This correlation necessitates analysis and understanding adaptive stress contributing to drug resistance and mechanisms to combat them such as pollution management.

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