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Artículo original

Horizontal transfer of heavy metal and antibiotic-resistance markers between indigenous bacteria, colonizing mercury contaminated tailing ponds in southern Venezuela, and human pathogens

Wileidy Andrea Gómez Calderón, María Mercedes Ball Vargas, Wilmar Alirio Botello Suárez, Luis Andrés Yarzábal Rodríguez*

Laboratorio de Microbiología Molecular y Biotecnología, Facultad de Ciencias, Universidad de Los Andes. Mérida. Venezuela.

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***Abstract*:** Bacteria colonizing heavily polluted tailing ponds in Southern Venezuela exhibit multiple resistances against mercurial compounds and antibiotics. The corresponding genetic determinants, mainly acquired through horizontal gene transfer, might also be transferred to pathogenic bacteria, an issue which represents an important risk to public health. In this work we show that indigenous, mercury-resistant bacterial strains isolated from a model tailing pond, located in El Callao (Bolivar State, Venezuela) and exhibiting a high concentration of soluble Hg, were able to transfer *in vitro* both heavy metal- and antibiotic resistance markers to potential human- and animal- pathogens (*i.e. Escherichia coli* and *Pseudomonas aeruginosa*). The frequencies of transfer ranged between $1.2x10^{-6}$ and $5.5x10^{-7}$ transconjugants per recipient. Transconjugants were also detected in the field, in model biofilms previously grown in natural sponges (*Luffa cylindrica*) and submersed in the ponds, at frequencies ranging from $1x10^{-4}$ to $5x10^{-3}$ transconjugants per recipient. These results are of particular relevance from the public health viewpoint, especially in light of the potential risk of horizontal flow of antibiotic resistance genes between indigenous bacteria and potential human pathogens.

***Keywords*:** mercury resistance, antibiotic resistance, bacteria, horizontal gene transfer.

Transferencia horizontal de marcadores de resistencia a metales pesados y antibióticos entre bacterias indígenas, que colonizan lagunas de cola contaminadas con mercurio en el sur de Venezuela, y especies patógenas para el ser humano

***Resumen*:** Las bacterias que colonizan lagunas de cola altamente contaminadas en el sur de Venezuela, presentan resistencia a compuestos mercuriales y múltiples antibióticos. Los determinantes genéticos responsables de estas resistencias, adquiridos principalmente a través de transferencia horizontal de genes, pueden ser transferidos a bacterias patógenas. En este trabajo mostramos que cepas bacterianas indígenas, resistentes al mercurio y aisladas a partir de una laguna de cola modelo, localizada en El Callao (Estado Bolívar, Venezuela) conteniendo una alta concentración de Hg soluble, fueron capaces de transferir *in vitro* marcadores de resistencia a metales y antibióticos a cepas potencialmente patógenas para el hombre y animales (ej. *Escherichia coli* y *Pseudomonas aeruginosa*). Las frecuencias de transferencia variaron entre $1,2x10^{-6}$ y $5,5x10^{-7}$ transconjugantes por receptora. Los transconjugantes también fueron detectados en el campo, utilizando un modelo de biopelículas desarrollado en esponjas naturales (*Luffa cylindrica*) sumergidas en lagunas contaminadas, con frecuencias que variaron entre $1x10^{-4}$ y $5x10^{-3}$ transconjugantes por receptora. Estos resultados presentan una relevancia particular desde el punto de vista de salud pública, especialmente en vista del riesgo potencial de transferencia horizontal de genes de resistencia a antibióticos entre las bacterias indígenas y bacterias potencialmente patógenas para el hombre.

***Palabras clave*:** Resistencia al mercurio, resistencia a antibióticos, bacteria, transferencia horizontal de genes.

* Correspondencia:
E-mail: yluis@ula.ve

Introduction

In the last two decades, increasing small-scale mining activities in Venezuela led to a dramatic and widespread contamination of the Orinoco river basin, because of the enormous amount of mercury that has been (and continues

to be) released in small rivers and tailing ponds [1-5].

As a result of the presence of mercurial compounds, bacteria colonizing polluted environments often acquire Hg-resistance genes, organized in the so-called mer operon which includes merA, the gene encoding a mercuric reductase able to reduce Hg^{2+} to Hg^0, less toxic and volatile [6-8]. Very often the *mer* operon is located in conjugative plasmids and, therefore, Hg-resistance can be transferred to sensitive bacteria by conjugation, irrespective of the phylogenetic distances that separate donors and recipients [9,10]. By this way, bacteria can also acquire genes conferring resistance to multiple antibiotics and other heavy metals, physically linked to the *mer* operon in the same mobile genetic element [6,9]. Therefore, the combined expression of multiple antibiotic resistance (MAR) and resistance towards heavy-metals may be the result of natural selection as a consequence of the presence of Hg in the environment [11]. In other words, exposure to toxic metals may select for bacterial strains resistant to antibiotics and *vice versa*, as shown by Stepanauskas et al. [12] and others [8,13].

Plasmid-mediated conjugation is considered to be an important mechanism driving gene flow in soil and aquatic environments [14]. However, while genetic transfer by conjugation between laboratory strains of bacteria has been studied extensively *in vitro*, the same cannot be said for studies in the field.

Considering that people inhabiting El Callao's area are in frequent contact with highly-polluted tailing-ponds during normal everyday activities such as bathing, swimming and/or washing, and that these polluted waters are periodically discharged to the rivers which supply water to the population of El Callao and nearby communities [15], we were concerned about the potential development of bacterial pathogens with multiple-resistances to both heavy-metals and antibiotics as a result of Hg-driven horizontal gene transfer (HGT). Therefore, in the present work we evaluated the horizontal spreading of resistance determinants to Hg, several antibiotics and other heavy-metals from indigenous Hg^R bacteria to potentially pathogenic recipient bacterial species, both *in vitro* and in the field.

Materials and methods

Bacterial strains: Hg-resistant (Hg^R), indigenous bacterial strains used in this study were previously isolated by us from a small tailing pond located at a gold-processing center (namely "La Esquina Caliente", see below) near El Callao (Bolivar State, Venezuela) (latitude: 7 21' 00'', longitude: -61 49' 00') as reported in Ball et al. [1]. *Bacillus cereus* 5 (*mer*, Hg^R), *B. cereus* ATTC 14579 (Hg^S), *Pseudomonas stutzeri* OX (*mer*, Hg^R) and *P. stutzeri* OX 1 (*mer*$^-$, Hg^S) were used as reference strains for determination of either Hg-resistance or Hg-sensitivity [16].

Indigenous Hg^R strains were used as donors in mating experiments with the following recipient strains: *Escherichia coli* (prototroph, derivative of *E. coli* PA601), *Pseudomonas aeruginosa* PAO38 and *Pseudomonas fluorescens* ATCC 13.525. Spontaneous rifampicin (Rif) resistant mutants of each one of these strains, all of which are Hg^S and are closely related to human pathogens, were obtained by growth at 30 °C on selective Luria-Bertani agar (LB-agar) supplemented with 100 mg/L of Rif. Resistance to Rif was chosen as a selection marker for the conjugation experiments because it is a very stable chromosomal mutation, although rare in nature (*i.e.* unlikely to be exhibited by indigenous Hg^R strains).

Growth media and culture conditions: Hg^R strains were routinely grown on Luria-Bertani broth (LB-broth) (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7.2-7.4) supplemented with $HgCl_2$ (50-100 mg/L). Donor-, recipient- and transconjugant-strains were grown at 30 °C on LB broth supplemented with either the respective antibiotics or heavy metals at appropriate concentrations (see below).

Characterization of Hg^R isolates for conjugation experiments:

Antibiotic and heavy-metal resistance: Antibiotic resistance was tested on LB-agar amended with the following antibiotics: ampicillin (Ap, 40 mg/L), streptomycin (St, 30 mg/L), kanamycin (Km, 30 mg/L), chloramphenicol (Cm, 30 mg/L) and tetracyclin (Tc, 30 mg/L). Plates were incubated at 30 °C during 24 h. The susceptibility test was also performed per triplicate into 96-multiwell plates containing 200 µL of LB-broth supplemented with: 12.5, 25, 50, 100 and 200 mg/L of Ap, Cm, Tc and St. Each well was inoculated with 5 µL of bacteria (either donors, recipients or transconjugants), previously grown on LB-broth, and the plates were incubated for 12 h at 30 °C. The lowest concentration of antibiotic that caused no visible growth was considered as the minimal inhibitory concentration (MIC) [17].

Heavy metal resistance was determined using 96-well plates in a similar way, *i.e.* by inoculating each bacterial strain into 200 µL of LB-broth supplemented with the following: 12.5, 25, 50, 100 and 200 µM Hg^{2+}; 0.012, 0.025, 0.05, 0.1 and 0.2 µM Ag^+; 12.5, 25, 50, 100 and 200 µM Cu^{2+}. We also determined the resistance to MeHg at 2.5, 6.25, 12.5, 50 and 100 µM. Plates were incubated at 30 °C for 24 h to determine the MIC for each metal. Similar assays were carried out to evaluate the co-transfer of Hg- and antibiotic-resistance genes in transconjugants.

Plasmidic profiles: The selected Hg^R donor's strains and the putative transconjugants were screened for the presence of large (potentially conjugative) plasmids, as described in Ball et al. [1].

Molecular identification of Hg^R donor isolates: The gene-encoding 16S rRNA was PCR-amplified from selected strains using bacterial universal primers fD1 and rD1 [18], as described in Pérez et al. [19]. The PCR products were purified from agarose gels with the PCR Clean-up Gel Extraction Kit (Macherey-Nagel, Germany) and sequenced at Macrogen Inc. (Seoul, South Korea). The nucleotide

sequences were compared to sequences deposited in the GenBak using the BlastN program [20], and the closest match of known phylogenetic affiliation was used to assign the isolated strains to specific taxonomic groups. Species identification was improved by performing several classical microbiological and biochemical tests [21].

PCR amplification of the merA gene: The gene encoding mercury reductase (*merA*) was PCR-amplified using primers A1 and A5 [22]. The reaction conditions were the following: 95 °C for 5 min, then 35 cycles at 95 °C for 30 seg, 50 °C for 1 min and 72 °C for 1 min, plus a final extension step at 72 °C for 7 min. Amplification products were electrophoresed in a 0.8% agarose gel [23] and visualized under UV-light following ethidium bromide staining.

Patch conjugation experiments: The protocol described by Summers [24] was followed to perform these experiments. In brief: both $Hg^{R}Rif^{S}$ (donor strains) and $Hg^{S}Rif^{R}$ (recipient strains) were grown overnight in LB-broth containing either 50 mg/L $HgCl_2$ or 100 mg/L Rif at 30 °C. The next morning cultures were diluted 1:10 in fresh LB-broth and incubated for a further 6 h. Donors and recipients were mixed in a 1:1 proportion (v/v) and 10 µL aliquots of each mixture were spotted on top of LB-agar and incubated for 16 h at 30 °C. The mixed growth (*patch*) was scraped-off the agar's surface, suspended in 500 µL of sterile saline solution and thoroughly vortexed. The resulting suspension was serially diluted and plated on selective media (LB agar containing 100 mg/L Hg and 100 mg/L Rif) to enumerate transconjugants. Similarly, 100 µL of the last 3 dilutions were placed on LB-agar supplemented with either 100 mg/L $HgCl_2$ or 100 mg/L Rif to enumerate donor and recipient cells respectively. The frequency of gene transfer was calculated as the ratio: number of transconjugants/number of recipients. Conjugations were performed at least twice for each cross.

"In field" conjugation experiments: Natural sponges (*Luffa cylindrica*, see Discussion section) were tested as solid supports for the formation of a bacterial biofilm *in vitro*. The sponges were washed thoroughly with distilled water, cut into small slices of the same size and sterilized by autoclave. The slices were then submerged overnight in liquid cultures of *P. fluorescens* in LB-broth to allow growth of biofilms, rinsed vigorously several times with sterile broth and placed in the tailing pond at different locations: in the water column, near aquatic plants and on top of sediments. Every 24 h three sponges per location were collected and sacrificed (*i.e.* mechanically disrupted by maceration with a glass rod and vortexed) to dislodge the maximum number of bacteria, and the suspension were serially diluted. The total number of donors, recipients and transconjugants were determined by plating 100 µL of each dilution into selective media and incubating at 30 ºC. The weight of each sponge slice was measured.

Analytical techniques: Soluble Hg(II) was measured from water samples, per triplicate, as described before [1].

Results

Site description: In the area surrounding El Callao, dozens of processing centers can be found. One of these centers, "La Esquina Caliente", was chosen to perform the experiments described in the present article. In this center, tailings are dumped into a pond which consists of two reservoirs connected by a surface channel (Figure 1). Hg-containing water and solid particles, originated from four mills, are discharged in the first pond where sedimentation occurs. Sediment-free water flows through the aforementioned channel to the second pond (characterized by an exuberant growth of aquatic plants, Figure 1). Water is pumped back to the mills and the cycle begins again. Therefore, mercury accumulates gradually in the water and sediments of both reservoirs. To compensate for losses due to evaporation, water is frequently pumped into the pond.

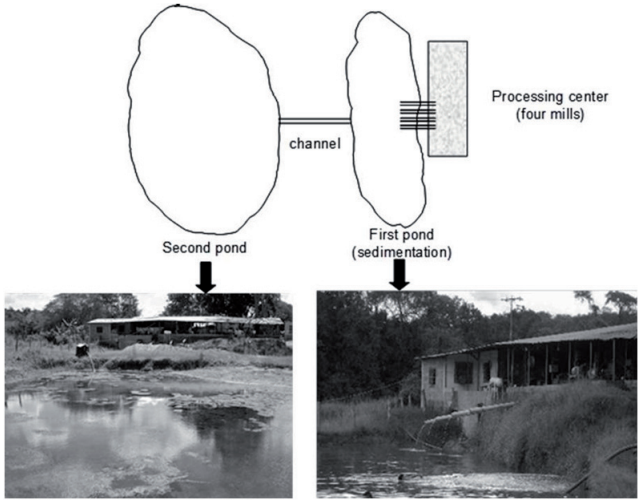

Figure 1. "La Esquina Caliente" processing center and tailing ponds. Notice the discharge of tailings from four mills into the first sedimentation pond and its use for bathing and swimming.

The Hg concentration determined from water samples harvested in the second reservoir, ranged between 1.35 and 2.19 (µg/L), *i.e.* well above the normal values reported for non polluted water (< 0.2 µg/L). The water pH and temperature were 8.62 and 28 °C, respectively at the time of sampling. The density of cultivable bacteria (heterotrophic) ranged between 1.3 and $5.4x10^5$ CFU/mL, depending on the specific site being monitored. The percentage of bacteria able to grow in the presence of 100 mg/L $HgCl_2$ attained 7.7% of the total cultivable fraction; on the other hand, almost 92% of the cultivable heterotrophs grew in the presence of 10 mg/L $HgCl_2$. This value represents twice the maximum concentration at which Hg^{S} reference strains (*B. cereus* ATCC 14579 y *P. stutzeri* OX 1) were able to grow. No Rif^{R} bacteria were detected in these samples.

Characterization of indigenous Hg^{R} isolates: Among the

hundred colonies grown on Hg-supplemented LB-plates, twelve were selected to be further characterized in order to perform conjugation studies, based on: a) a significant phenotypic difference between the colonies and, b) the presence of potentially conjugative plasmids (>30 Kb), as revealed by agarose gel electrophoresis of plasmid preparations. As shown in Table 1, resistance to one or several antibiotics and/or different heavy metals was present among these strains. The Hg^R isolates were also shown to be resistant to 5 mg/L Me-Hg. All Hg^R strains were positive for the presence of orthologs of the *merA* gene. Only eight isolates could be identified by performing 16S rRNA sequencing and analysis (Table 1). The molecular identification was improved by performing several classical microbiological and biochemical tests (not shown).

Patch conjugation experiments: In preliminary patch conjugation experiments, all twelve indigenous Hg^R strains were shown to be able to transfer Hg-resistance markers to *E. coli* (Rif^R), while 8/12 isolates transferred the Hg resistance to *P. aeruginosa* (Rif^R). From these, we choose five strains for further conjugation assays. As can be seen in Table 2, Hg-resistance markers were transferred from these strains to *E. coli* and *P. aeruginosa* at similar frequencies (*i.e.* between $1.2x10^{-6}$ and $5.5x10^{-7}$ Rif^R-Hg^R transconjugants per recipient). The MICs exhibited by Rif^R-Hg^R transconjugants for Hg and other heavy metals were more elevated than those exhibited by the corresponding recipient strains (=basal resistance). The same was also true in the case of antibiotic resistance (Table 2). Some transconjugants were also shown to acquire resistance to the antibiotics tested (Table 2). In some cases, transconjugants exhibited the same plasmidic profile than the donor strain (not shown), indicating that the resistance markers were possibly acquired through plasmid-mediated HGT (*i.e.* by conjugation).

Field conjugation assays (biofilm mode): During preliminary experiments under laboratory conditions, we confirmed that the natural sponge (*L. cylindrica*) permitted the development of recipient strain's biofilms and that transfer of resistance markers from selected donors (*i.e.* Hg^R strains described above) to recipient strains (*E. coli*, *P. aeruginosa* and *P. fluorecens*) occurred with frequencies of ~10^{-6} transconjugants per recipient (data not shown). For *in field* conjugation experiments, we decided to use *P. fluorescens* as

Table 1. Characterization of indigenous Hg^R strains isolated from "La Esquina Caliente" tailing pond.

Isolate	Antibiotic resistance	Heavy metal resistance	Most closely related organism (% identity)	GenBank Accession Number
PM005	Cm, Tc, Ap	Hg, MeHg, Ag, Cu	*Enterobacter* sp. (99)	KF040456
PM013	Cm ,St ,Tc, Ap	Hg, MeHg, Ag, Cu	*Pseudomonas putida* (99)	KF040457
PM021	Cm, St, Tc, Ap	Hg, MeHg, Cu	*Pseudomonas plecoglossicida* (99)	KF040458
PM030	Cm, Tc, Ap	Hg, MeHg	*Acinetobacter junii* (100)	KF040459
PM035	Cm, St, Tc, Ap	Hg, MeHg, Cu	NI	-
PM037	Cm, Ap	Hg, MeHg	*Enterobacter cloacae* (99)	KF040460
PM039	Cm, Tc, Ap	Hg, MeHg	*Acinetobacter junii* (99)	KF040461
PM040	Cm, Ap	Hg, MeHg	*Enterobacter hormaechei* (98)	KF040462
PM043	Cm, Ap	Hg, MeHg	*Enterobacter cloacae* (94)	KF040463
PM044	Cm, St, Tc, Ap	Hg, Cu, MeHg	NI	-
PM051	Cm, St, Tc, Ap, K	Hg, MeHg	NI	-
PM053	Cm, St, Tc, Ap	Hg, MeHg	NI	-

Cm: chloramphenicol; Ap: ampicillin; St: streptomycin; Tc: tetracycline; Km: kanamycin; Hg: ionic mercury; MeHg: methyl-mercury; Ag: silver; Cu: copper; NI: not identified.

Table 2. HGT of heavy metal and antibiotic-resistance genes from Hg^R indigenous bacteria to potentially pathogenic bacteria.

Mating strains (Hg^R donor x Rif^R recipient)	Hg^R-Rif^R transconjugants per recipient	Antibiotic resistance*	Heavy metal resistance*
E. coli (recipient strain)	--	Cm (25), Tc (3.12), Ap (6.25)	Hg (12.5), Ag (0.05), Cu (5), MeHg (25)
PM005 x *E. coli*	$3,6x10^{-7}$	Cm (200), Tc (12.5), Ap (200)	Hg (50), Ag (0.1), Cu (10)
PM013 x *E. coli*	$3,6x10^{-7}$	Cm (200), Tc (25), Ap (200)	Hg (50), Cu (10), MeHg (50)
PM021 x *E. coli*	$5,5x10^{-7}$	Cm (200), Tc (25), Ap (200)	Hg (50), Cu (10), MeHg (50)
PM030 x *E. coli*	$1,5x10^{-6}$	Cm (200), Tc (6.25), Ap (12.5)	Hg (50)
PM035 x *E. coli*	$2,7x10^{-7}$	Cm (200), Tc (12.5), Ap (100)	Hg (100), Cu (10)
P. aeruginosa (recipient strain)	--	--	Hg (12.5), MeHg (25)
PM013 x *P. aeruginosa*	$1,2x10^{-6}$	ND	Hg (50), MeHg (50)
PM035 x *P. aeruginosa*	$1,6x10^{-6}$	ND	Hg (50)

C: chloramphenicol; A: ampicillin; T: tetracycline; Cu: copper; MeHg: methyl-mercury. *The numbers between parentheses are the MICs for each compound, expressed in µg/mL (antibiotics), µM (heavy metals) and mM (in the case of Ag) determined as described in Materials and Methods. ND: not determined.

recipient, considering its lesser pathogenicity and ubiquity in natural environments. The biofilm-containing sponges were therefore submersed in the water, in close contact with aquatic plants and sediments. After 24 h, 10^{-4} transconjugants (Hg^R, Rif^R) per recipient were detected in the bacterial biofilm developed in sponges placed in the proximity of aquatic plants and sediments. Since no indigenous bacteria were found to be Rif^R, all the colonies able to grow in the selective media accounted for real transconjugants. After 96 h, recipient cells remained viable but their number per unit of weight (of sponge) decreased in one order of magnitude; after this period of time we detected ~10^{-3} transconjugants per recipient.

Discussion

In the present work we have shown that indigenous bacteria thriving in heavily polluted tailing ponds are able to transfer both heavy metal and antibiotic-resistance markers to pathogen-related bacterial species, most probably by HGT. We also showed that HGT can indeed occur under natural conditions in these ponds, *i.e.* in the presence of high concentrations of toxic metals and in the absence of any exogenous source of carbon and/or energy.

To date, only a few reports have been published concerning the effect of Hg^{2+} and MeHg pollution on microbial communities colonizing aquatic systems at the Orinoco basin (southern region of Venezuela) [1,4,15,25]. In a previous work, we showed that the fraction of bacterial isolates resistant to elevated concentrations of $HgCl_2$ was high in tailings ponds near El Callao and that multi resistance up to five antibiotics and four heavy metals was exhibited by some of these bacterial strains [1]. In the present work, we add additional information showing that genes conferring resistance towards Hg were transferred *in vitro* from indigenous Hg^R bacteria to *E. coli* and *P. aeruginosa* most probably by conjugation, since many Hg^R isolates harbored high molecular weight plasmids. Besides Hg-resistance, the transconjugants did also acquire resistance to a) other heavy-metals (Cu, Ag) and, b) several antibiotics. These results are in agreement with the idea that microbial exposure to one toxicant could result in an indirect selection for bacteria with resistance to multiple, chemically unrelated toxicants/antibiotics, possibly as a result of horizontal transfer of mobile genetic elements [2,5,26,27]. Indeed, it is well known that multiple genes encoding for metal and antibiotic resistance are frequently found on the same plasmids and/or transposons, conferring co-resistance [24]. Our assumption received further support by the fact that the transconjugants exhibited similar plasmid profiles to those shown by the corresponding donors.

Our main concern at the beginning of this study was that indigenous bacteria colonizing polluted tailing ponds would be able to rapidly transfer their resistance markers to exogenously introduced, potentially pathogenic bacteria, as recently suggested by Skurnik et al. [28]. Since the usual mode of growth of many bacterial populations in their natural environment is as biofilm [29], and considering that *in vitro* (patch mode) experiments might not reflect natural conditions, it seemed very important to us to study HGT in the field. For this, we used a highly porous, natural solid support to develop a biofilm of recipient bacteria on it. Transconjugants with the expected phenotype (Hg^RRif^R) were detected as soon as 24 h after introducing the recipient' strains biofilm developed inside the sponges, with frequencies ranging between 10^{-3} and 10^{-4} transconjugants per recipient, which falls in the range of previous reports [14,30]. This also showed that transfer of Hg resistance markers may occur quickly under natural conditions, *i.e.* in less than 24 h. The results obtained here confirm that the experimental approach followed by us, a natural vegetable sponge as a support for biofilm formation, can be used to monitor in the field the horizontal transfer of genetic markers in aquatic environments. Furthermore, recipient bacteria survived for at least 96 h when they were exposed to the tailing pond water, thus confirming that exogenous bacteria are not only able to survive for several days remaining viable and cultivable, but that they may acquire multiple resistances very quickly. This poses serious concerns regarding public health, owing to the potential appearance and co-selection of pathogenic bacteria with multiple antimicrobial resistances. Therefore, the human population in these areas is exposed not only to the intoxication with soluble forms of Hg, but to the possible infection with multi-resistant pathogenic bacteria which may arise by horizontal transfer of resistance determinants in these ponds.

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